TRPC1 Is Required for Functional Store-operated Ca\(^{2+}\) Channels

ROLE OF ACIDIC AMINO ACID RESIDUES IN THE S5-S6 REGION*

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The exact role of TRPC1 in store-operated calcium influx channel (SOCC) function is not known. We have examined the effect of overexpression of full-length TRPC1, depletion of endogenous TRPC1, and expression of TRPC1 in which the proposed pore region (S5-S6, amino acids (aa) 557–620) was deleted or modified by site-directed mutagenesis on thapsigargin- and carbachol-stimulated SOCC activity in HSG cells. TRPC1 overexpression induced channel activity that was indistinguishable from the endogenous SOCC activity. Transfection with antisense hTRPC1 decreased SOCC activity although characteristics of SOCC-mediated current, I\(_{\text{SOCC}}\), were not altered. Expression of TRPC1\(\Delta\)567–793, but not TRPC1\(\Delta\)664–793, induced a similar decrease in SOCC activity. Furthermore, TRPC1\(\Delta\)567–793 was co-immunoprecipitated with endogenous TRPC1. Simultaneous substitutions of seven acidic aa in the S5-S6 region (Asp \(\rightarrow\) Asn and Glu \(\rightarrow\) Gln) decreased SOCC-mediated Ca\(^{2+}\), but not Na\(^{+}\), current and induced a left shift in \(E_{\text{rev}}\). Similar effects were induced by E576K or D581K, but not D581N or E615K, substitution. Furthermore, expressed TRPC1 proteins interacted with each other. Together, these data demonstrate that TRPC1 is required for generation of functional SOCC in HSG cells. We suggest that TRPC1 monomers co-assemble to form SOCC and that specific acidic aa residues in the proposed pore region of TRPC1 contribute to Ca\(^{2+}\) influx.

Activation of cell surface receptors, which are coupled to inositol lipid signaling, results in phosphatidylinositol bisphosphatase (PIP\(_2\)) hydrolysis, generation of diacylglycerol and inositol 1,4,5-trisphosphate, release of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores, and activation of plasma membrane Ca\(^{2+}\) influx channels that appear to be activated as a result of PIP2 hydrolysis, here we have measured SOCC activity in control HSG cells and in cells overexpressing either TRPC1 or TRPC1 with mutations in the proposed pore region. The data demonstrate that TRPC1 is required for generation of functional SOCC in HSG cells and that specific acidic aa residues in the proposed pore region of TRPC1 contribute to Ca\(^{2+}\) influx.

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1 The abbreviations used are: PIP\(_2\), phosphatidylinositol bisphosphate; GFP, green fluorescent protein; SOCC, store-operated calcium influx channel; aa, amino acids; IP, immunoprecipitation; HA, hemagglutinin; Tg, thapsigargin; CCh, carbachol; CRAC, Ca\(^{2+}\) release activated Ca\(^{2+}\) channel; TRP, transient receptor potential protein; TRPC, TRP canonical family; TRP1, TRP vanilloid family; TRPM, TRP melastatin family.

2 The exact role of TRPC1 in store-operated calcium influx channel (SOCC) function is not known. We have examined the effect of overexpression of full-length TRPC1, depletion of endogenous TRPC1, and expression of TRPC1 in which the proposed pore region (S5-S6, amino acids (aa) 557–620) was deleted or modified by site-directed mutagenesis on thapsigargin- and carbachol-stimulated SOCC activity in HSG cells. TRPC1 overexpression induced channel activity that was indistinguishable from the endogenous SOCC activity. Transfection with antisense hTRPC1 decreased SOCC activity although characteristics of SOCC-mediated current, I\(_{\text{SOCC}}\), were not altered. Expression of TRPC1\(\Delta\)567–793, but not TRPC1\(\Delta\)664–793, induced a similar decrease in SOCC activity. Furthermore, TRPC1\(\Delta\)567–793 was co-immunoprecipitated with endogenous TRPC1. Simultaneous substitutions of seven acidic aa in the S5-S6 region (Asp \(\rightarrow\) Asn and Glu \(\rightarrow\) Gln) decreased SOCC-mediated Ca\(^{2+}\), but not Na\(^{+}\), current and induced a left shift in \(E_{\text{rev}}\). Similar effects were induced by E576K or D581K, but not D581N or E615K, substitution. Furthermore, expressed TRPC1 proteins interacted with each other. Together, these data demonstrate that TRPC1 is required for generation of functional SOCC in HSG cells. We suggest that TRPC1 monomers co-assemble to form SOCC and that specific acidic aa residues in the proposed pore region of TRPC1 contribute to Ca\(^{2+}\) influx.

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EXPERIMENTAL PROCEDURES

HSG Cell Culture, Transfection, and Crude Membrane Preparation—
HSG cells were cultured and stably transfected as described before (17, 18, 23). Before transient transfection, cells were detached, diluted to desired concentration, and allowed to attach to tissue culture plates overnight and then co-transfected with pcDNA3.1 encoding the required TRPC1 (1 μg) and pcDNA3.1 encoding GFP (0.5 μg) or with the GFP plasmid alone. Cells were cultured on coverslips or tissue culture plates for 24 h, and GFP-positive cells were selected for activity measurements.

Site-directed Mutagenesis—The expression plasmid pcDNA3.1 containing the TRPC1 gene was used as a template for PCR. Oligonucleotides were synthesized as required for the mutagenesis, and PCR was performed using the QuickChange Mutagenesis kit (Stratagene). Following PCR, the parental DNA template was removed by Dpn1 endonuclease digestion, and the remaining PCR product was transformed into Escherichia coli XLI-Blue cells. Colonies were selected, and each mutation was confirmed by sequencing.

Electrophysiology—Cell-attached patch clamp measurements were performed as described earlier (24). Pipette solution contained 100 mM Na/HEPES, 1 mM MgCl2, 2 mM CaCl2 (pH 7.2) (HCl). Standard bath solution containing (mM) 145 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES (pH 7.4) with NaOH was replaced by a high KCl solution containing (mM) 145 KCl, 5 NaCl, 1 MgCl2, 2 CaCl2, 10 HEPES (pH 7.4) with KOH after the seal was formed. Liquid junction potentials (8.0, 4.3, and 1.7 mV) with Ca2+, Na+, and K+, respectively) were calculated using pClamp7 software. Currents were recorded using an Axopatch 200A amplifier and digitized with Digidata 1200 (Axon Instruments) at a rate of 10 kHz with filtering at 1 kHz. P-Clamp 7 (Axon instrument) and Origin 6 (Microcal) were used for data analyses. The open probability of the channel (NP) was defined as the ratio of open channel area to the total area in an all-point amplitude histogram. The mean unitary amplitude of the single channel currents were determined from all-point amplitude histogram fit to a sum of Gaussian distributions. Mean open times were calculated by maximum likelihood estimation using pClamp7 software.

The store-operated inward Ca2+ current (I_{store}) was measured using the whole cell patch clamp technique as described before (23, 25) with some modifications. The external solution contained 135 mM sodium glutamate, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 10 mM HEPES (pH 7.2) (NaOH). The pipette solution contained 135 mM cesium glutamate, 3 mM MgCl2, 3 mM ATP (Mg), 10 mM HEPES (pH 7.2) (CsOH), 10 mM EGTA, and 3.5 mM CaCl2 (final [Ca2+] = 100 μM). Cells were held at 0 mV and subjected to voltage ramps (-100 to +100 mV at 2 mV/ms). Cells were stimulated by perfusion with Tg- or CCh-containing medium. Data are presented as mean ± S.E. of responding cells. Statistical analyses of the data were performed using Student’s t test with appropriate p values considered significant.

Immunoprecipitation and Western Blotting—Crude membranes were prepared as described previously (26). Protein concentration was determined by using the Bio-Rad protein assay (microassay procedure). Membrane solubilization and immunoprecipitation were performed as described previously (26, 27). Immunocomplexes were pulled down with protein A beads (anti-HA was used at 1:100 dilution for IP). Proteins were released with SDS buffer and visualized by the ECL reaction after Western blotting. Anti-HA (Roche Molecular Biochemicals, 3F10) and anti-TRPC1 (16) were used at 1:1000 dilution.

Confocal Microscopy—HSG cells expressing TRPC1 Δ567–793 were cultured on coverslips for 24 h. Cells were fixed, permeabilized, and treated with the HA antibody at 1:100 dilution for 1 h. Cells were then washed and probed with rhodamine-linked secondary antibody as described above (16). Confocal images were collected using an MRC 1024 krypton/argon laser scanning confocal equipped with a Nikon Optiphot II photomicroscope.

[Ca2+]i Measurements—Fura2 fluorescence in single cells (cultured overnight on glass bottom microwell dishes, MatTek Corp.) was measured as described earlier (23) by using an SLM 8000/DMX 100 spectrofluorimeter or Polychrome IV (TILL Photonics) attached to an inverted Nikon Diaphot microscope with a Fluor 40× oil-immersion objective. Images were acquired using an enhanced CCD camera (CCD-72, DAGE-MTI) and either Image-1 or Metafluor software (Universal Imaging Corporation, PA). In cells that were transiently transfected, fura2 fluorescence was measured in GFP-positive cells. Analog plots of the fluorescence ratio (340/380) are shown, and peak values were used for analysis.

RESULTS

SOCC Activity in HSG Cells Expressing TRPC1—SOCC activity was measured in control HSG cells and TRPC1-overexpressing HSG cells (TRPC1-cells) by using cell-attached patch clamp technique as we have described previously (24). Tg and CCh induced burst-like (Fig. 1, A and C) and flickery (E and G) SOCC activities, respectively, in both sets of cells (membrane potential was held at -40 mV). This difference in the kinetics is due to modulation of the channel by the [Ca2+]i in the internal store and in the subplasma membrane region near the channel (24). Both CCh- and Tg-induced SOCC activities displayed unitary current amplitudes of about -0.85 pA, with occasional overlapping channel openings corresponding to multiples of the first level (i.e. about -1.7 pA, Fig. 1, B, D, F, and H), although SOCC activity in TRPC1-cells was higher than in control cells. The number of events in the major current level was significantly increased from 1115 ± 145 (n = 9) to 2109 ± 237 (n = 12) and 1278 ± 141 (n = 11) to 2318 ± 265 (n = 11) with Tg and CCh, respectively (12 s recording; n = number of cells, p < 0.05 in both cases). Average NP values in TRPC1-cells 0.43 ± 0.045 with CCh and 0.33 ± 0.036 with Tg were significantly higher than the values in control cells 0.26 ± 0.019 and 0.12 ± 0.015, respectively (p < 0.05 in both cases, n is given above). The mean open times of SOCC (calculated from the activity at the major current level) with CCh or Tg were 5.5 ± 0.4 ms or 11.6 ± 2.1 ms, respectively, in TRPC1-cells and

FIG. 1. Expression of TRPC1 in HSG cells increases the endogenous SOCC activity. Tg- or CCh-activated single channel activities were recorded in control HSG cells (A and E) and cells expressing TRPC1 (C and G) using the cell-attached patch clamp method. The tracings show currents obtained at -40 mV (data were filtered at 200 Hz). The corresponding all-point histograms are shown in B, D, F, and H. Channel activity was monitored between -80 and +80 mV to obtain the I-V relationships shown in I and J (n = 7–9 cells in each case).
The I-V relationships obtained with 2 mM Ca\(^{2+}\) + 100 mM Na\(^+\) (Fig. 1, I and J, values were obtained from the major current peak), or 2 mM Ca\(^{2+}\) + 100 mM N-methyl-d-glucamine (data not shown), in the pipette solution were similar in control and TRPC1-cells stimulated either with CCh or Tg. In all cases, the current displayed inward rectification and reversed at about +20 mV. The slope conductance of SOCC with Ca\(^{2+}\) was 20 pS, which was similar in CCh- or Tg-stimulated TRPC1-cells and control cells. Furthermore, as in control cells, SOCC in TRPC1-cells was also blocked by Gd\(^{3+}\) and control cells. Additionally, as in control cells, SOCC in 20 pS, which was similar in CCh- or Tg-stimulated TRPC1-cells (current recordings not shown). Note that the Ca\(^{2+}\) currents as well as the slope conductances (44 pS with Na\(^+\) and 15 pS with Ba\(^{2+}\)) were also similar in control and TRPC1-cells (current recordings not shown). Note that the Ca\(^{2+}\) conductance of SOCC determined here is close to that estimated earlier for TRPC1 by noise analysis, 16 pS (28). In aggregate, the data for TRPC1 by noise analysis, 16 pS (28). In aggregate, the data

**Deletion of the TRPC1 Pore Region Decreased SOCC Activity**—TRPC proteins have been suggested to have six transmembrane domains. The aa sequence spanning the region between S5 and S6 includes a seventh hydrophobic domain and has been proposed to form the pore of the channel (3, 4), although the exact topology of TRPC1 has not yet been confirmed. To elucidate the role of TRPC1 in SOCC function, we have truncated TRPC1 after S5 (aa 567–793) thus deleting the proposed pore region, S6, and the C terminus. Stable expression of TRPC1567–793 exerted an inhibition of endogenous SOCC activity. 8/9 cells (89%) failed to display any channel activity in control cells (current traces not shown, data are summarized in Fig. 5). These data demonstrate that endogenous TRPC1 is required for the Tg- and CCh-stimulated SOCC activity and SOCE in HSG cells.

**Effect of Mutating Acidic Amino Acid Residues in the Pore Region of TRPC1 on SOCE**—To demonstrate directly the role of the TRPC1 pore region in SOCC function, we made Asp-pore (Fig. 2A) and Asn-pore (Fig. 2B) cells were held at either +40 or −40 mV). 1/9 cells (11%) displayed normal SOCC activity with both Tg and CCh. The frequency of detection of channel activity was significantly reduced (p < 0.01, χ² test) as compared with that in TRPC1-cells (23/31, 74%) and control cells (21/29, 72%). In agreement with these single channel measurements, Tg- or CCh-stimulated Ca\(^{2+}\) influx (Fig. 2, C–F) in cells expressing TRPC1567–793 was significantly reduced (>70%) as compared with that in control cells and in cells expressing TRPC1 (p < 0.05, see figure legend for the average fluorescence values). Consistent with our earlier reports (18, 23) and in contrast to the effect of TRPC1567–793 on SOCE, expression of TRPC1664–793 induced an increase in SOCE (Fig. 2, E and F). Thus, the region aa 567–664 is important for SOCE.

Fig. 2G shows that the levels of TRPC1, TRPC1664–793, and TRPC1567–793 were similar. Thus, the observed differences in SOCE in cells expressing these proteins are not due to differences in the levels of expression. Furthermore, deletion of the aa 567–793 of TRPC1 did not affect its localization to the plasma membrane (Fig. 2H). Importantly, TRPC1567–793 was immunoprecipitated with endogenous TRPC1 (Fig. 2I). Together, these data suggest that expressed TRPC1 proteins associate with the endogenous TRPC1, a component of the endogenous SOCC in these cells.

**Role of TRPC1 in SOCE**

3.8 ± 0.4 ms or 6.5 ± 1.2 ms in control cells.

The I-V characteristics of SOCC-mediated Na\(^+\) and Ba\(^{2+}\) currents as well as the slope conductances (44 pS with Na\(^+\) and 15 pS with Ba\(^{2+}\)) were also similar in control and TRPC1-cells (current recordings not shown). Note that the Ca\(^{2+}\) conductance of SOCC determined here is close to that estimated earlier for TRPC1 by noise analysis, 16 pS (28). In aggregate, the data for TRPC1 by noise analysis, 16 pS (28). In aggregate, the data
Because TRPC1Δ567–793 interacts with endogenous TRPC1 (Fig. 2F) and Mut-pore interacts with FLAG-tagged TRPC1, it is likely that Mut-pore can interact with the endogenous TRPC1.

To analyze further the effect of Mut-pore on CCh- and Tg-stimulated SOCC activity, we performed whole cell patch clamp experiments. Cells were held at 0 mV and subjected to voltage ramps every second. The traces shown in Fig. 4, A and D, represent CCh- and Tg-stimulated currents that have been reconstructed from the current amplitudes at −40 mV obtained from voltage ramps (−100 to +100 mV at 2 mV/ms). The individual ramps obtained at time “T” (before stimulation of cell) are shown in Fig. 4B and at “II” (30 s after stimulation of cell) are shown in Fig. 4, C and E. At time I, i.e., before addition of CCh or Tg to the bath, a small non-selective basal current was detected, which was not changed by expression of TRPC1 or Mut-pore. Also note that there was no change in the current for up to 10 min of recording, unless cells were stimulated (data not shown). Both CCh and Tg stimulated $I_{\text{SOCC}}$, a store-operated current that we have described previously (23, 25). Under the experimental conditions used in this study, $I_{\text{SOCC}}$ displayed inward rectification and reversed at about +10 mV (black trace in Fig. 4, C and E), the basal currents have been subtracted from each trace, also see Fig. 5 for additional data). The inward currents are primarily carried by Ca$^{2+}$ because replacement of Na$^+$ in the medium with N-methyl-d-glucamine did not alter this current (trace not shown). In TRPC1-cells the amplitude of the inward current was significantly increased (11.7 ± 0.9 pA in control versus 17.1 ± 1.4 pA in TRPC1-cells at −40 mV, red trace in Fig. 4, C and E). However, the characteristics of $I_{\text{SOC}}$ were not altered. Importantly, in Mut-pore-expressing cells the amplitude of $I_{\text{SOC}}$ was significantly lower than that in control cells expressing TRPC1 (green trace in Fig. 4, C and E, also see Fig. 5F for average values and statistical analysis), suggesting a decrease in Ca$^{2+}$ selectivity of the channel. Consistent with the single channel measurements, the effects induced by antisense TRPC1 on $I_{\text{SOC}}$ were different from those seen with Mut-pore; the amplitude of $I_{\text{SOC}}$ was significantly decreased, but the reversal potential was not significantly changed (see Fig. 5, G and H).

We also measured $I_{\text{SOC}}$ in the absence of external Ca$^{2+}$, in which case the inward current is carried by Na$^+$ (Fig. 4F). Leak current measured prior to stimulation of cells, trace not shown, has been subtracted). In control HSG cells both CCh (data not shown) and Tg (Fig. 4F) activated an inwardly rectifying current that reversed at 0 mV (black trace, 9.2 ± 1.0 pA at −40 mV). This current was significantly ($p < 0.05$) increased in TRPC1-cells (Fig. 4, F, red trace, 16.5 ± 1.2 pA at −40 mV). Note that TRPC1 expression induced the same increase (1.5–2.0-fold) in the Ca$^{2+}$ and Na$^+$ currents, which is consistent with the 1.5–2.0-fold increase in SOCE detected by fura2 fluorescence measurements. In contrast to the Ca$^{2+}$ current in Mut-pore-cells, which was 67% lower than in TRPC1-cells and 52% lower than in control cells, the Na$^+$ current was 17% less than that in TRPC1-cells (green trace, Fig. 4F) and significantly larger than that in control cells. The inset in Fig. 4F shows Mut-pore-mediated Ca$^{2+}$ and Na$^+$ currents relative to that seen in TRPC1-cells. Thus, mutation of the seven acidic aa residues in the TRPC1 pore region induced a much larger decrease of SOCC-mediated Ca$^{2+}$ influx than SOCC-mediated Na$^+$ influx. These data strongly suggest that these mutations do not result in an overall decrease in channel activity.

Identification of Specific Amino Acid Residues in the TRPC1 Pore Region That Are Involved in SOCC Function—Fig. 6A

***Pore Region That Are Involved in SOCC Function***

**A** TRPC1 Antibodies used for IP and Western blotting are indicated in the figures. B, C, D, and E, fura2 fluorescence changes induced by Tg and CCh. Time scale and $[\text{Ca}^{2+}]_{\text{c}}$ are indicated in the figures. $F$, average SOCC, Mut-pore value depends on the level of expression relative to that of full-length TRPC1. ***Pore Region That Are Involved in SOCC Function***

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shows the alignment of aa residues in the pore region of TRPCs 1, 4, and 5. We made individual substitutions of two residues E576K and E615K, which flank the proposed hydrophilic region (shaded region) and are conserved in TRPC1, -4, and -5. Asp-581, which is present only in TRPC1 and is closest to the pore, was also substituted with Lys or Asn. Fura2 fluorescence and whole cell patch clamp measurements were performed with cells expressing these mutant TRPC1 proteins (note that both stable and transient expression of these TRPC1 mutants yielded similar results). Fig. 5B summarizes the Ca^{2+} influx data. E576K and D581K induced dominant suppression of SOCE, similar to that seen by Mut-pore expression. Although E615K and D581N (data not shown) induced lower Tg-stimulated Ca^{2+} influx as compared with TRPC1-cells, it was not lower than that in control HSG cells. Fig. 5C shows that the expression levels of these mutant TRPC1 proteins were similar and do not account for their distinct effects on SOCE. Current-voltage ramps of I_{SOCE} generated by expression of these mutants are shown in Fig. 5, D–F. Averages of the reversal potentials and current densities in these cells are shown in Fig. 5, G and H. Importantly, there was maximum left shift of the E_{rev} (shown by the arrows in D–F) in D581K cells, from +10.8 ± 1.3 and +10.2 ± 0.9 mV in TRPC1-cells and control HSG cells, respectively to -12.2 ± 2.5 mV. E_{rev} was also left-shifted in E576K cells but not to the same extent as in D581K cells. E615K did not induce any shift in E_{rev}. Consistent with the Ca^{2+} measurements, the Ca^{2+} currents generated by Tg in D581K and E576K were significantly lower than that in control cells (see Fig. 5F). D581K induced the largest decrease in I_{SOCE}: I_{SOCE} in E615K, although lower than in TRPC1 cells, was similar to that in control HSG cells. These data demonstrate that two acidic amino acid residues in the TRPC1 pore region, Glu-576 and Asp-581, are involved in Ca^{2+} influx via SOCC. However, other amino acids in TRPC1 (which were not tested in this study) might also have a role in Ca^{2+} influx. We have not presently examined changes in the permeability of monovalent cations such as Cs+ (which is present in the intracellular solution) in cells expressing D581K, which might account for the negative reversal potential.

**DISCUSSION**

Previous studies (17–23) from our laboratory, and others, provide convincing evidence that TRPC1 has a critical role in SOCE. Based on the presently available data two possible roles can be assigned to TRPC1: (i) it is a regulatory subunit of SOCC that is required for its activation or assembly, and (ii) it is a component of the SOCC pore that directly contributes to SOCC-mediated Ca^{2+} influx. The data presented above suggest that TRPC1 is a functional component of an SOCC in HSG cells that contributes to Ca^{2+} influx in response to stimulation by either agonist or Tg. We have shown that the characteristics of SOCC activity generated by expression of TRPC1 in HSG cells are similar to the endogenous SOCC activity in these cells. Consistent with this, expression of TRPC1 in HSG cells induces an increase in the amplitude of the SOCC-mediated current, I_{SOCE}, without altering its characteristics (23) (Fig. 4). We have also shown here and previously (17) that endogenous TRPC1 in HSG cells is essential for SOCC activity. Tg- or Cch-stimulated SOCC activity, I_{SOCE}, and Ca^{2+} influx, as well as levels of endogenous TRPC1 in HSG cells are decreased by 80% or more in cells transfected with antisense-TRPC1. More significant is our finding that acidic amino acid residues in the proposed pore region of TRPC1 contribute to SOCE. We have shown that expression of TRPC1 with simultaneous mutations (Asp→Asn and Glu→Gln) of seven acidic aa residues in the proposed pore region suppressed endogenous SOCC activity in more than 80% of the cells. In the few cells expressing this mutant where channel activity was detected, there was an apparent decrease in the Ca^{2+} conductance. Importantly, the amplitude of I_{SOCE} in these cells was decreased, and there was a left shift in the...
reversal potential, suggesting a decrease in the Ca$^{2+}$ permeability of the channel. Interestingly, the Na$^+$ current mediated by Mut-pore was similar to that seen in TRPC1-cells. Thus, mutations in the acidic aa residues in the pore region do not appear to decrease SOCC activity per se. We have also demonstrated that two of these seven acidic amino acid residues, Glu-576 and Asp-581, are involved in SOCC-mediated Ca$^{2+}$ influx. Substitution of either residue, E576K or D581K, induced a suppression of Tg-stimulated Ca$^{2+}$ influx, expressed relative to the value in control cells. * indicates values significantly different from all other values; values marked ** but not from each other. C, Western blot of crude membranes isolated from TRPC1-, E576K-, D581K-, and E615K-transfected cells. Protein levels were detected using anti-HA antibody. D–F, voltage ramp data from cells expressing E576K, D581K, and E615K mutations, respectively. The arrows in each case indicates the $E_{rev}$, $G$, average of the reversal potentials. ** indicates values significantly different from the unmarked values. Unmarked values are not significantly different from each other. H, Tg-induced whole cell currents at $-40$ mV, shown as current densities. * indicates values that are significantly different from other values in the figures but not from each other. ** indicates values that are significantly different from unmarked values and values marked *. (p < 0.05 was considered significant, n = 4–6 cells in each case.)

role of TRPC1 in SOCE

Previous studies have shown that Asp $\rightarrow$ Asn substitution in the pore region of TRPV4 decreased Ca$^{2+}$ influx, although Asp $\rightarrow$ Lys substitutions completely blocked channel function (29). Substitution of conserved hydrophobic residues (LFW) in the TRPC6 pore region eliminated TRPC6-generated channel activity in HEK293 cells (30). However, neither of these channels appear to be involved in SOCE. While this manuscript was in preparation, it was reported (31) that expression of TRPV6 with substitution of FEL to AAA in the proposed pore region induced suppression of endogenous $I_{CRAC}$ in Jurkat cells. However, it is not clear whether the endogenous TRPV6 in Jurkat cells is required for $I_{CRAC}$. Furthermore, TRPV6 displayed both store-operated and store-independent activities. The data we have presented above are significant because they demonstrate the following: (i) endogenous TRPC1 in HSG cells is required for the endogenous SOCE detected in these cells; (ii) expressed TRPC1 is regulated by store depletion and is not spontaneously active; and (iii) acidic aa residues in the pore region of TRPC1 contribute to SOCE.

Although presently we do not have evidence for homo-multimerization of endogenous TRPC1 in HSG cells, we have shown that the expressed mutant TRPC1 is immunoprecipitated with endogenous TRPC1. This result has major implications because it suggests that TRPC1 monomers interact with each other to form SOCC. Our data are consistent with reports suggesting that Drosophila TRP and TRPC1/a homomultimerize via N-terminal interactions (32, 33). We suggest that endogenous or exogenously expressed TRPC1 monomers associate with each other to form functional SOCCs. Thus, when full-length TRPC1 is expressed channel activity is increased. When mutant TRPC1s are overexpressed relative to the endogenous protein, the probability of mutant TRPC1 monomers associating with each other or with endogenous TRPC1 monomers is relatively high. As a result, aberrant SOCCs are formed which have decreased permeability for calcium. In contrast, in cells transfected with antisense TRPC1 or expressing TRPC1Δ567–793, where either depletion of TRPC1 or the pore region is deleted, respectively, there is a reduction in the total number of functional channels rather than a change in the channel properties. However, other proteins, including other TRPCs, might be co-assembled with endogenous TRPC1 and required for functional SOCC in HSG cells. Studies using heterologous expression have shown that TRPC1 interacts with TRPC3 (33, 34), TRPC6 (30), and TRPC4 and TRPC5 (35). The latter study also showed that endogenous heteromers of either TRPC1 and TRPC4 or TRPC1 and TRPC5 were co-immunoprecipitated from rat brain. TRPC1 expression altered the currents generated when either TRPC5 or TRPC4 was expressed alone, and furthermore, these currents were not activated by store depletion. Although it is unclear why TRPC1 forms different types of channels in different cells, it is important to note that the characteristics of the channel formed by heteromeric TRPC1 channels (35) appear to be considerably different from those of $I_{SOCE}$ measured in HSG cells. Further studies will be required to determine which TRPC proteins are endogenously expressed in HSG cells and interact with endogenous TRPC1.

In conclusion, the data presented here suggest that TRPC1 is a component of the functional (pore-forming) unit of SOCC in HSG cells. However, these data do not rule out the possibility that SOCC might be a heteromer of TRPC1 with other TRPCs (5, 30, 33–35) or with other as yet unknown protein(s). We have
shown earlier that in HSG cells TRPC1, like the Drosophila TRP (14, 36), is assembled in a supramolecular protein complex with key proteins involved in the Ca\(^{2+}\) signaling cascade that leads to SOCC activation (26). We suggest that SOCC activity in any cell type will depend not only on the proteins that constitute its pore-forming unit but also other regulatory proteins that might affect its function, assembly, or localization. It will be important to determine whether differences in the molecular composition of the channel per se, or its regulation, account for the large variation in the characteristics of SOCCs seen in different cell types.

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