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Genetic evidence against involvement of TRPC proteins in SOCE, ROCE, and CRAC channel function

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Using genetically engineered mice and cell lines derived from genetically engineered mice we show that depletion of ER delimited Ca^{2+} stores activates heteromeric Ca^{2+} entry (SOCE) channels formed obligatorily, but not exclusively by Orai1 molecules. Comparison of Orai-dependent Ca^{2+} entries revealed Orai1 to be dominant when compared to Orai2 and Orai3. Unexpectedly, we found that store-depletion-activated Ca^{2+} entry does not depend obligatorily on functionally intact TRPC molecules, as SOCE monitored with the Fura2 Ca^{2+} reporter dye is unaffected in cells in which all seven TRPC coding genes have been structurally and functionally inactivated. Unexpectedly as well, we found that TRPC-independent Gq-coupled receptor-operated Ca^{2+} entry (ROCE) also depends on Orai1. Biophysical measurements of Ca^{2+} release activated Ca^{2+} currents (Icrac) are likewise unaffected by ablation of all seven TRPC genes. We refer to mice and cells carrying the seven-fold disruption of TRPC genes as TRPC heptaKO mice and cells. TRPC heptaKO mice are fertile allowing the creation of a new homozygous inbred strain.

TRPC | SOCE | ROCE | Orai

Significance

TRPCs are cation channels that permeate $Ca^{2+}$to varying degrees. In response to G proteins of the Gq/11 or Gi/o groups activated by GPCRs, TRPCs potentiate Orai-based Ca^{2+}entry that serves to replenish emptied reservoirs and to regulate among other gene expression, apoptosis, and neuro- and endocrine secretions. Their participation in Ca^{2+}entry mediated by Orai-based channels has been postulated but remains controversial mainly because Orai-based channel activity has never been tested in the absence of TRPCs—all vertebrate cells express at least two, most 3 TRPCs. Here, by structurally inactivating all 7 TRPC genes, we report that Ca^{2+}release activated Ca^{2+}entry, be it triggered by receptors, ROCE, or by mere store depletion, SOCE, are unaffected.

Cell stimulation with agonists that lead to activation of phospholipase C (PLC) β or γ and generation of inositol trisphosphate (IP3) results in activation of the Inositol trisphosphate receptor (IP3R) located in the endoplasmic reticulum (ER) membrane and in release of ER Ca^{2+} into the cytoplasm. The emptying of the ER Ca^{2+} reservoir causes the ER membrane-resident single-pass STIM molecules (STIM1 and STIM2) *to* change their cytoplasmic C termini to expose an Orai activating region (SOAR: Stim-Orai-Activating Region) and the assembly at the plasma membrane of a hexameric Orai channel (1) which is Ca^{2+} permeant and allows Ca^{2+} to enter the cell to regulate cell functions and to replenish the ER reservoir, pumped by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA pumps). Ca^{2+} entry resulting from assembly of the Orai-based channel is referred to as SOCE, for Store Operated Ca^{2+} Entry. Expression of Orai1 molecules in cells with stable expression of excess TRPC molecules results in augmented SOCE. This led us to propose a model in which SOCE channels are heteromultimers of Orai and TRPC molecules (2–5) reviewed in refs. 2–5. Support for the participation of TRP molecules in store-depletion-activated Ca^{2+} entry came from studies by Albert and colleagues (6) who reported that in cell-attached membrane patches of mesenteric artery myocytes in which passive store depletion with BAPTA or cyclopiazonic acid (inhibitor of SERCA pumps) leads to activation of a 2.2 picoSiemen cation channel. This channel was absent in cells from TRPC1$^{-/-}$ mice. Moreover, these channels were shown to be heteromultimers formed of TRPC1 and TRPC5 proteins and were immuno-neutralized by anti-TRPC1 and anti-TRPC5 peptide antibodies added to mesenteric artery inside-out membrane patches of cells in which the 2.2 pS store-depletion-activated channel had been activated under the cell-attached configuration prior to patch excision (7). Using TRPC-specific peptide antibodies has shown the existence of channels formed of 2 and 3 different TRPC subunits, the identity of which varied with the tissue tested.

The results presented below show that Orai1 molecules are obligatory for store depletion promoted Ca^{2+} entry and that TRPC molecules are not essential for store-depletion-activated Ca^{2+} entry as SOCE is unaffected in cells from mice in which all TRPC genes have been inactivated.

Materials and Methods

The highest commercially available grade of chemicals was used throughout. Most were purchased from Sigma Aldrich. Fura2 and Fura2.AM were from Molecular Probes. Sources of other biochemicals and kits are

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The authors declare no competing interest.

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named throughout the text. cDNAs were either cloned in-house, obtained from the investigators that had originally cloned them, or were procured from Addgene (Addgene.org). For expression purposes, cDNAs coding for TRPCs (human or mouse), human STIMs, M5 mAChR, and V1aR receptors were in pcDNA3 (Invitrogen); human Orai cDNAs were cloned into pCMV-myc (Clontech) with the cMyc tag at the N termini of the open reading frames (ORFs).

Solutions. The standard extracellular Ringer's solution used for electrophysiological experiments contained (in mM): 130 NaCl, 4.5 KCl, 20 $CaCl_2$, 10 tetraethylammonium chloride, 10 D-glucose, and 5 HEPES, pH 7.4 with NaOH. The divalent-free Ringer's solution contained (in mM): 150 NaCl, 10 HEDTA, 1 EDTA, 10 tetraethylammonium chloride, and 5 HEPES, pH 7.4. The internal pipette solution contained (in mM): 135 Cs aspartate, 8 $MgCl_2$, 8 Cs-BAPTA, and 10 HEPES, pH 7.2 adjusted with CsOH. Stock solutions of 2-Aminoethoxy diphenyl borate (2-APB, Sigma) and Thapsigargin (TG, LC Biochemicals) were prepared by dissolving reagents in DMSO, and stored at −20 °C. TG was diluted from a 1 mM stock in DMSO to a final concentration of 1 to 2 μM. For final use, these reagents were prepared immediately before use. Solutions were applied to the cells using a multibarrel local perfusion pipette with a common delivery port.

Patch-Clamp Experiments. were conducted in the standard whole-cell recording configuration at room temperature using an Axopatch 200B amplifier (Molecular Devices) interfaced to an ITC-18 input/output board (Instrutech). Routines developed by Dr. R. S. Lewis (Stanford) on the Igor Pro software (Wavemetrics) and modified by M. Prakriya were employed for stimulation, data acquisition, and analysis. Recording electrodes were pulled from borosilicate glass pipettes (Sutter Instrument) coated with Sylgard and fire-polished to a final resistance of 4 to 5 MΩ. The holding potential was +30 mV. Two types of stimuli were usually employed. The standard voltage stimulus consisted of a 100-ms step to −100 mV followed by a 100-ms ramp from −100 mV to +100 mV applied at 1 s intervals or a 300-ms step to −100 mV applied every 3 s. CRAC current (ICRAC) was typically activated by passive depletion of ER Ca^{2+} stores by Thapsigargin. All currents were acquired at 5 kHz and low-pass filtered with a 1 kHz Bessel filter built into the amplifier. All data were corrected for leak currents collected in 100 μM $LaCl_3$. The standard extracellular Ringer's solution used for electrophysiological experiments contained (in mM): 130 NaCl, 4.5 KCl, 20 $CaCl_2$, 10 tetraethylammonium chloride, 10 D-glucose, and 5 HEPES, pH 7.4 adjusted with NaOH. The divalent-free solution Ringer's solution contained (in mM): 150 NaCl, 10 HEDTA, 1 EDTA, 10 tetraethylammonium chloride, and 5 HEPES, pH 7.4. The internal pipette solution contained (in mM): 135 Cs aspartate, 8 MgCl2, 8 Cs-BAPTA, and 10 HEPES, pH 7.2 with CsOH. Stock solutions of 2-Aminoethoxy diphenyl borate (2-APB, Sigma) and Thapsigargin (Tg, LC Biochemicals) were prepared by dissolving reagents in DMSO, and stored at −20 °C. Thapsigargin was diluted from a 1 mM stock in DMSO to a final concentration of 1 μM. For final administration, these reagents were prepared immediately before use. Solutions were applied to the cells using a multibarrel local perfusion pipette with a common delivery port.

Mice. Except the mice used for electrophysiology experiments, mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited, specific pathogen-free vivarium of the Comparative Medicine Branch of the NIEHS. All procedures and crosses to which the mice were subjected were approved by NIEHS's Animal Care and Use Committee (ACUC). Wild type (WT) mouse strains were either 129SvEv, C57Bl/6J, or intercross breeds between 129SvEv and C57Bl/6J. $Trpc2^{-/-}$ mice and $Trpc4^{-/-}$ mice were backcrossed for more than five generations to C57Bl6J or C57Bl/6N, and then crossed into 129SvEv strains carrying the disrupted alleles with combinations of the Trpc1, Trpc3, Trpc5, Trpc6, and Trpc7 genes. The creation of the individual disrupted Trpc alleles and their validation have previously been described (8); Trpc2 (9); Trpc3, (10); Trpc4 (11); Trpc5 (12); Trpc6 (13); Trpc7 (14). When bred to homozygosity, all the disrupted genes have caused distinct phenotypes, proving that they had indeed been structurally (assessed with RT-PCR tests) and functionally disrupted [assessed by appearance of distinct phenotypes (cf. Table 1 in ref. 2)]. Mice homozygous for the floxed Stim1 gene (15) were kindly provided by Masatsugu Oh-hora (Graduate School, Tokyo Medical and Dental University). The disruption of the Orai1 gene by a promoter trap procedure was reported in ref. 16. Mice used to analyze ion channel characteristics (TRPC HeptaKO mice and wildtype controls) at Northwestern University's vivarium were cared for in accordance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals. Animals were group-housed in a sterile ventilated facility, under standard housing conditions (12/12 h light/dark cycle with lights on at 7 am, temperature 20 to 22 °C with ad libitum access to water and food. Male and female mice were used in approximately equal numbers. All research protocols were approved by the Northwestern University Institutional Animal Care and Use Committee. Animals 7 to 9 wk old were used for CD4 T cell isolations.

Plasmids and Transfections. For electrophysiology, MEF cells were plated at the time of cell passage and adhered to poly-D-lysine coated 35 mm dishes and grown in a medium containing High glucose DMEM (Gibco 10569010, Thermo Scientific),10% fetal bovine serum (HyClone), 2 mM glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. CFP-Orai1 was purchased from GeneCopoeia, CFP-Orai2 was a kind gift of Dr. S. Feske (New York University), and YFP-Orai3 was a kind gift of Dr. C. Romanin, (Johannes Kepler University, Linz). mCherry-STIM1 was a kind gift of Dr. R.S. Lewis (Stanford University, Stanford, CA). The indicated Orai1 constructs were transfected into Orai1KO MEF cells together with STIM1 (100 ng Orai and 500 ng STIM1 cDNA per coverslip) and currents were recorded 24 h later. All transfections were done using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Thermo Fisher Scientific).

TRPC Genotyping.

DNA isolation. Genomic DNA was isolated from mouse tail biopsies by the magnetic bead-based DNadvance method (Beckman Coulter, Brea, CA), as per the manufacturer's instructions. Liquid handling steps were performed on a Beckman FX robot fitted with an *AP*-96 pipetting head (Beckman Coulter, Brea, CA). DNA was eluted with H_2O and was split into duplicate plates for genotyping in independent laboratories.

Genotyping PCR analyses. Two sets of genotyping PCRs were used and an additional set to quantitate mRNA levels by qPCR. One, developed in the Molecular Genetics Core Facility at NIEHS, uses a 96-well plate format and robotic pipetting of reagents. This method uses as the identifying readout, the melting temperature (TM) of WT and mutant PCR fragments in a multiplexed amplification monitored by SYBR green fluorescence changes recorded in either a BioRad iCycler or an IdahoTechnologies Light Scanner (Biofire Diagnostics, Inc, Salt Lake City, UT). Trios of PCR primers (com, wt, and mut) were designed to amplify WT and mutant amplicons with differing melting temperatures in a single reaction. For three Trpc alleles, Trpc3, Trpc5, and Trpc7, loxP sites had been inserted into the mouse genomes creating conditional knock-out alleles, which therefore existed as floxed alleles–Trpc3flx, Trpc5flx, and Trpc7flx–and were analyzed with primer sets denoted as flxC3, flxC5, and flxC7, respectively. The floxed Trpc alleles served as precursors for the creation of global KO alleles (10, 12, 14). The primer sets used to identify the deleted (KO) alleles derived from floxed alleles were denoted as koC3, koC5, and koC5 primers. Two PCRs were required for the identification of WT (wt), floxed (flx), and knock-out (KO or ko) alleles of the Trpc5, Trpc3, and Trpc7 genes. One reaction tested for the global disruption (ko reaction); the other tested for the presence of the floxed allele (flx reaction).

The common [com], wt, and mutant [mut] primer trios were (TM):

Trpc1:
C1_com 5′-GATACTGTGTACCTAACATCAACCATGGT (58.0 °C)
C1_wt 5′-CTTTACTTGGCAACCTTTGCCCTCA (59.8 °C)
C1_mut 5′-GGGCTCGAGTTACCTGCCATAT (58.8 °C)
Trpc2
C2_com 5′-CATTCAGGGTAGGTCTTTCCTCCTCAGTA (60.4 °C)
C2_wt 5′-CTGCAGTGTGCCCAGCGATT (60.9 °C)
C2_ mut 5′-CCCTACCCGGTAGAATTGACCTGCAG (62.4 °C)
Trpc3
koC3_com 5′-CAAAGGAGGAAGCTATGGAATGGACA (58.6 °C)
koC3_wt 5′-CACTCTGATCTGACTATGTTCTTTAGGCAAA (58.1 °C)
koC3_mut 5′-GCGAATTCTGCAGATGTACGGTC (58.1 °C)
flxC3_com 5′-CTTTGTGTGTTTTGTCTGCATGTGTCTGT (59.9 °C)
flxC3_wt 5′-GCTTACAACCATGTGTAACTCCAGACCT (60.1 °C)
flxC3_mut 5′-GCGAATTCTGCAGATGTACGGTC (58.1 °C)
Trpc4
C4_com 5′-GTGCTCTGAACCCACGGGA (59.9 °C)
C4_wt 5′-AGAGATCAGGCGGAGGGAA (57.8 °C)
C4_mut 5′-CCTGCAGGCATGCAAGCTTCA (61.0 °C)

Trpc5
koC3_com 5′-CTTACCAATGGCTACAGCAGGGAAA (59.1 °C)
koC3_wt 5′-CTTGACCATGGCCACATAGGTACCAA (60.6 °C)
koC3_mut 5′-CAGTGCTTAAACATCTATAACATTCCTTCGAAGA (58.3 °C)
flxC3_com 5′-GTCTTACCCTGAGTCTAGAAGCTAACAGT (58.7 °C)
flxC3_wt 5′-ATGACAGAGATGTTTCAGTAAAGCTATGTGG (58.3 °C)
flxC3_mut 5′-GAGATGTTTCAGTAAAGCCGTACGGT (58.7 °C)
Trpc6
C6_wt-F 5′-CTAAACAGTGTTGCATAAGTGCTGAAAACA (58.1 °C)
C6_wt-R 5′-GATAGGTTCTGGGGACTGAACTAGAAGA (58.7 °C)
C6_ mut-F 5′-ACGAGACTAGTGAGACGTGCTACTT (58.8 °C)
C6_mut-R 5′-GGGTTTAATGTCTGTATCACTAAAGCCTC (57.1 °C)
Trpc7
koC7_com 5′-GCAATAGAAACTTAACTTAGGCACTACCCA (58.3 °C)
koC7_wt 5′-GATTGGAAGGGTGGAAGCCGCG (63.0 °C)
koC7_mut 5′-GTTTGCCTGGGAAACGTGTTGA (58.5 °C)
flxC7_com 5′-GCAATAGAAACTTAACTTAGGCACTACCCA (58.3 °C)
flxC7_wt 5′-GGGTTTGCCTGGGAAACGTAC (58.7 °C)
flxC7_mut 5′-GTTTGCCTGGGAAACGTGTTGA (58.5 °C)

Genotyping PCRs were performed in final volumes of 25 μL on 10 to 30 ng gDNA and 0.8 mM dNTPs, 0.025 U/μL of Platinum Taq (Invitrogen) and 1× SYBR Green PCR buffer. A 2X SYBR Green PCR buffer was prepared as follows: 100 mM KCl, 40 mM Tris-HCl pH 8.3, 0.02 mM EDTA, 4 mM $MgCl_2$, 1:2,500 dilution of 10,000× SYBR Green I gel stain (Invitrogen, cat#S-7563), 0.6 mg/mL acetylated BSA, 0.08% Tween-20, 0.08% Gelatin). Primer concentrations were at 0.5 μM each, except for Trpc3 for which the wt and com primers were at 4 μM, and for Trpc6 for which the wt set of primers C6_wt-F and C6_wt-R) were at 1 μM each. [It should be noted that the insertion of the LoxP sites into the genomes introduced up to 135nt of plasmid-derived flanking sequences creating partial repeats (sequences available upon request); likewise, Trpc1, Trpc2, Trpc4, and Trpc6 were disrupted by insertion of the same PGK-Neo cassette. These repeats had to be taken in consideration when the seven Trpc KO alleles were in the genome of a single mouse]. The second set of PCRs, used independently to assess the genotype of mice, analyzed the reaction products by classical 1.2% to 1.5% agarose gel electrophoresis using different primer sets, of which those for Trpc4 amplified WT and mutant (MUT) alleles simultaneously, whereas the other primer sets amplified selectively the WT or the MUT alleles. The primers were the following:

Trpc1
C1Ex8-F 5′-GGGATGATTT GGTCAGACAT TAAG
C1Int8-R 5′-GTGTACCTAA CATCAACCAT GGTAC
PGKProm-R1 5′-TGGATGTGGA ATGTGTGCGA GGC
Cycling program: 95 °C 2 min; 2. 38 cycles: 95 °C 30 s, 60 °C 30 s, 72 °C 30 s; and 3. 72 °C 7 min.
Trpc2
C2KO-F 5′-CTGTAGCCATCTTCAGACACACC
C2206-R1 5′- ACGAGACTAG TGAGACGTGC TACTTCC
C2Int-F 5′-ATGACGGGTTTATGGCTCAG
C2Int-R 5′-GATCCCCTGG AATTGGAGTT
Cycling program: 1. 95 °C 15 min; 2. 40 cycles: 94 °C 15 s, 52 °C 30 s, 72 °C 60 s; and 3. 72 °C 7 min.
Trpc3
C3Lox-F 5′-GCTATGATTAATAGCTCATACCAAGAGATCG
C3Lox-R 5′-GGTGGAGGTAACACACAGCTAAGCC
C3Lox-F2 5′GAATCCACCTGCTTACAACCATGTG
Cycling program: 1. 95 °C 15 min; 2. 45 cycles: 94 °C 30 s, 58 °C 30 s, 68 °C 60 s; and 3. 72 °C 7 min.
Trpc4
C4KO15-F 5′-ACAGTGCTCTGAACCCACGG
C4KO40-R 5′-CTCGCACCGG ATGCCTTTGC
C4NeopA-R 5′- GCCTGCTCTT TACTGAAGGCTCT
Cycling program: 1. 94 °C 90 s; 2. 10 cycles: 94 °C 30 s, 65 °C 30 s, 72 °C 30 s; 3. 26 cycles: 94 °C 30 s, 60 °C 30 s, 72 °C 30 s; and 4. 72 °C 5 min.
Trpc5
C5Lox-F 5′-GGCGCAGAAAGAGTTTATGGGGA
C5Lox-R 5′-GGATGTTGGC TCTGTGAAAC AATGACTC
C5Lox-F2 5′-AGCTAACAGTATCCCTAAGTGATCC
Cycling program: 1. 93 °C 15 min; 2. 35 cycles: 94 °C 30 s, 58 °C 30 s, 72 °C 120 s; and 3. 72 °C 10 min.
Trpc6
C6204 (Exon7)-F 5′-CAGATCATCTCTGAAGGTCTTTATGC
C6205 (Exon7)-R 5′-TGTGAATGCT TCATTCTGTT TTGCGCC
C6207 (Intron7)-F 5′-GGGTTTAATG TCTGTATCAC TAAA GCC TCC
C6206 (PGK)-R ACGAGACTAG TGAGACGTGC TACTTCC
Cycling program: 1. 94 °C 4 min; 2. 35 cycles: 94 °C 60 s, 60 °C 90 s, 72 °C 150 s; and 3.72 °C 10 min.
Trpc7
C7Lox-F 5′-GTAGTATCAGGAAGCTCGCGGCTCT
C7Lox-R 5′-GCTTTGTTTCACAGACCCACAGTCTAGC
C7Lox-R2 5′-CCTTCCTCTCTAGGTTCCTTCTAG
Cycling program: 1. 95 °C 15 min; 2. 35 cycles: 94 °C 30 s, 58 °C 30 s, 68 °C 60 s; and 3. 72 °C 7 min.

PCRs testing for gDNAs were performed on approximately 100 ng gDNA using reagents and enzymes from commercial PCR kits purchased from Qiagen (HotStarTaq PCR System cat#203445), Invitrogen (Platinum Taq DNA Polymerase System, cat#10966-018), and Roche (Expand High Fidelity PCR System, cat #04-738-250-001), using 0.4 μM of each PCR primer, supplemented to give 5% DMSO and 2.5 to 3.0 mM $MgCl_2$. Fig. 1 depicts the results of genotyping mouse 4537-7 using electrophoretic mobility to differentiate the amplicons from each other with the above primers. Wilt type (+/+) and heterozygous (+/−) gDNAs were used as controls.

T cell culture. Mouse CD4+ T cells were purified from splenocytes using the MagniSort Mouse CD4+ T cell enrichment kit (Invitrogen, MS22-7762-74) as demonstrated by Erdogmus et al. (17). Briefly, CD4+ T cells were stimulated in flat bottom 12-well plates (1×10^6 cells/mL per well) with 1 μg/mL plate-bound

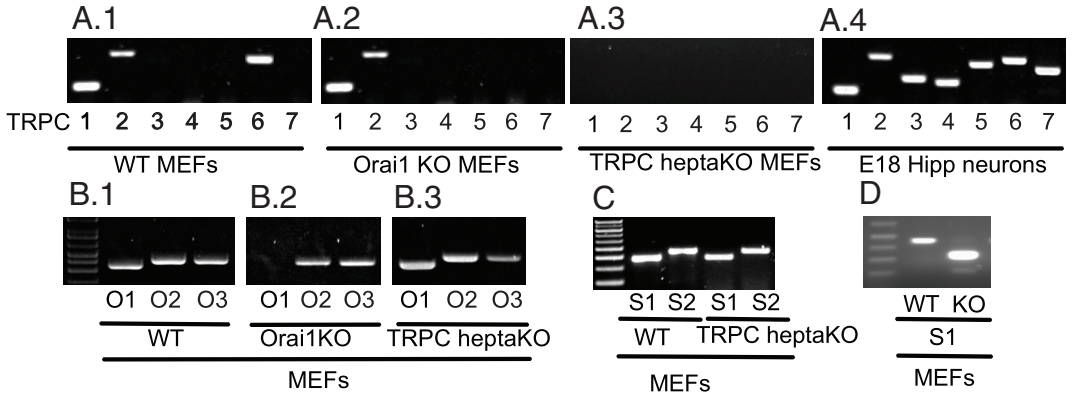

Fig. 1. Expression of full complements of three Orai and two STIM genes but only limited expression of TRPC genes in MEF cells. (*A*1) WT MEF cells only express TRPC1, -2, and -6, of which (*A*2) only TRPC1 and -2 are present in Orai1 KO MEF cells. (*A*3) MEF cells derived from TRPC heptaKO mice lack all seven TRPC mRNAs. (*A*4) Positive control RT-PCR analysis of RNA derived from E18 mouse hippocampal neurons in which all seven TRPC genes are expressed. (*B*1–*B*3) Orai1, -2, and -3 are expressed in wt and TRPC heptaKO MEF cells. (*B*2) Orai1 mRNA is absent in Orai1 KO MEF cells. (*C*) STIM 1 and STIM2 are present in wt and heptaKO MEF cells. (*D*) Confirmation of gene disruption in STIM1 KO MEF cells. Primer sequences and RNA isolation procedures are described in *Materials and Methods*.

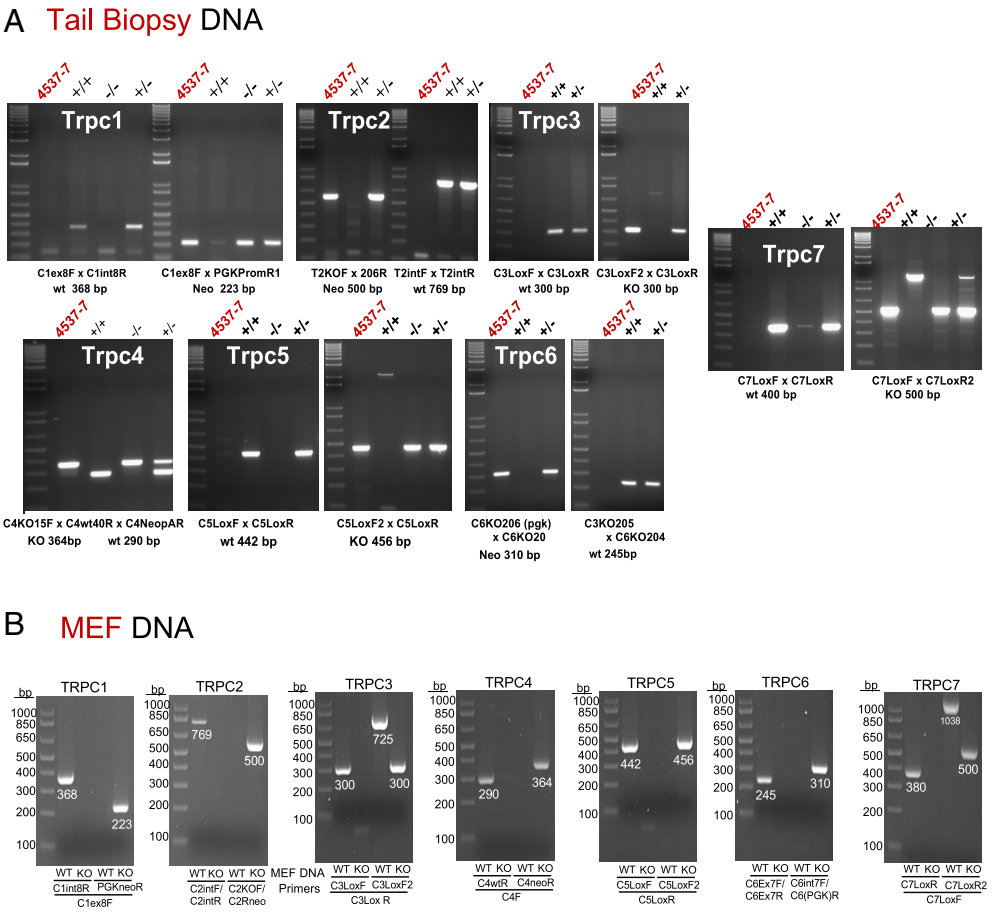

Fig. 2. (*A*) PCR analysis of the sevenfold, total Trpc KO genotype of female mouse tag #4537-7. Reactions were in 25 or 50 μL final volume. 10 μL of the each reaction was used for the electrophoreses shown. For primer set composition and cycling programs, see *Materials and Methods*. The gDNAs analyzed in each lane (4537-7, +/+, +/−) are depicted above the photographs of the electrophoretic runs, the primer combinations, and expected amplicon sizes are depicted below. M: 1 kb DNA ladder (Invitrogen) low to high, in bp: 100, 200, 300, 400, 500, 650, 850, 1,000, 1,650, 2,000 was used to calibrate the size of the reaction products. (*B*) Preservation of the TRPC heptaKO genotype in MEF cells from the established TRPC heptaKO mouse strain.

anti-CD3 (Bio X cell, clone 2C11, 14-0031-85) and 1 μg/mL anti-CD28 antibodies (Bio X cell, clone 37.5, BE0015-1) in complete RPMI medium (Corning, 10-040-CV) containing 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% β-mercaptoethanol. After 3 d of stimulation, T cells were detached and transferred to a new plate with fresh complete RPMI medium containing 20 IU/mL rh-IL-2 (Peprotech, 200-02) and 2.5 ng/mL IL-7 (Peprotech, 217-17). For patch-clamp experiments, cells were plated onto poly-D-lysine (Thermo Fisher Scientific)–coated coverslips immediately before the experiments.

Isolation and differentiation of bone marrow–derived macrophages (BMDM). BMDM were isolated according to ref. 18. Briefly, femurs from healthy 12-wk-old mice were used to isolate BMDM. Both femurs were aseptically removed and placed in a Petri dish filled with sterile PBS. Before transferring the bones to a Petri dish filled with DMEM + 10% FBS (Hyclone), each femur was placed on a sterile gauze pad where the rest of the muscle and connective tissue surrounding the bone was removed. Next, each femur was held with forceps, and the upper and lower parts of the femurs were cut off with scissors. The marrow was flushed out from the bone

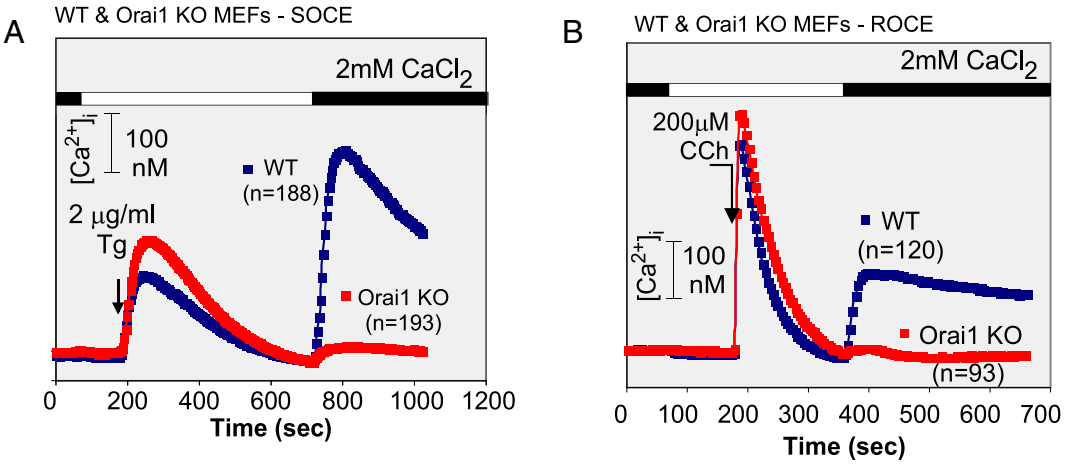

Fig. 3. Orai1-Deficient MEFs Exhibit Essentially no SOCE or ROCE. (*A*) SOCE promoted store depletion by inhibition of SERCA pumps with Thapsigargin (Tg) In Orai1 KO MEF cells. (*B*) ROCE triggered by Carbachol (CCh) induced activation of the transfected Gq- coupled M5 muscarinic receptor in Orai1 KO MEF cells.

by inserting a 25-gauge needle attached to a 20 mL syringe filled with PBS into the femur. PBS was passed through the bone and cells were collected in a 50 mL Falcon tube by centrifuging at 400×*g* for 5 min at 4 °C. The marrow cell pellet was resuspended in DMEM + 10% FBS, plated at 5×10^6 cells in 10 mL of DMEM + 10% FBS. The next morning, an additional 10 mL. of complete medium supplemented with 10 ng/mL of recombinant M-CSF (macrophage colony-stimulating factor). On the 4th day of culture, 10 mL of medium was removed and 10 mL of fresh complete medium supplemented with 10 ng/mL of recombinant M-CSF was added. On day 7, the cells were harvested and used in experiments.

Mouse embryonic fibroblasts (MEFs). Primary embryonic fibroblasts were prepared from 14.5 d embryos as described in ref. 19; SV40-transformed MEFs were derived from passage 3 to 5 primary MEFs as described in ref. 20. MEFs were grown at 37 °C in humidified 5%CO_2 incubators, passaged by trypsinization (0.05% in Dulbecco's PBS without $CaCl_2$ or $MgCl_2$ for 3 min), followed by 1:10 dilution in complete DMEM with 10% FBS (fetal bovine serum–Hyclone or Natocore–DMEM/10%FBS) and concentration by centrifugation. The cells were resuspended in complete DMEM/10% FBS supplemented with penicillin 100 units/mL and streptomycin 100 mg/mL and reseeded at 5- to 10-fold lower cell density.

HEK293 cells. were obtained from the American Tissue Culture Collection and used between passage 5 and 20, and were cultured as described above for MEFs.

Orai1 KO MEFs. Embryos homozygous for loss of Orai1 (Orai1$^{-/-}$) were obtained from a cross of heterozygous parents. SV40-transformed MEFs were prepared from 14.5-d embryos. Genotyping indicated which were Orai1$^{-/-}$ (mut). MEFs from two Orai1$^{-/-}$ embryos were used to isolate two clonal cell lines by limiting dilution. The genotyping reaction analyzed wt and mut amplicons in the same incubation. The genotyping primers were the following:

mO1wt-F 5′-TCACGCTTGCTCTCCTCATC
mO1mut-F 5′-TAAGGGCGACACGGAAATG
mO1com-R 5′-AGGTTGTGGACGTTGCTCAC

The cycling program was 1. 95 °C, 2 min; 2. 35 cycles :95 °C, 30 s; 60 °C, 30 s; 72 °C, 45 s; 3. 72 °C, 10 min; 4. 4 °C hold. HotStarTaq System reagents and enzyme were purchased as a kit from Qiagen (cat # 203445) Amplicon sizes were wt: 488 bp, mut (KO): 300 bp; 50 to 100 ng gDNA from tail biopsies were used as template.

Stim1 KO MEFs. SV4-transformed conditional Stim1 KO mice (Stim1flx/flx MEFs, passage 10 after transfection of the complete SV40 genome) were used to obtain Stim1$^{-/-}$ MEFs by lambda phage cre recombinase-mediated excision of the floxed segment. Briefly, 5×10^4 Sim1flx/flx MEFs were suspended in 2 mL DMEM/10%FBS, seeded into a well of a 6-well plate, grown overnight at 37 °C in a humidified 5%CO_2 incubator, and infected with lentivirus particles driving the expression of lambda phage cre recombinase and a puromycin selection marker, both encoded in Addgene's plasmid 17408 (Addgene.org). Selection of puromycin-resistant MEFs was begun 1 d after infection by addition of 1 μg/mL puromycin (Sigma-Aldrich). Eight surviving colonies were picked 2 wk later, amplified, and genotyped. Two had successfully excised the floxed segments of the Stim1 gene of both chromosomes. These cells were expanded and frozen for later use. They are referred to as Stim1 KO MEFs. The genotyping primers, used at a final concentration of 1.0 μM, were as follows:

mS1flx 5′-CGATGGTCTCACGGTCTCTAGTTTC
mS1ko 5′-AACGTCTTGCAGTTGCTGTAGGC
mS1com 5′-GGCTCTGCTGACCTGGAACTATAGTG

The cycling program, PCR reagents, and enzyme were as for murine Orai1 genotyping. The amplicon sizes were flx 410 bp, ko 550 bp.

Lentivirus particles were prepared by the NIEHS Viral Vector Core using packaging and VSV-G envelope proteins encoded in plasmids psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259), respectively. For cryopreservation, 10^6 primary and various SV40-transformed MEFs were pelleted and resuspended in 1.0 ml of freezing medium (70% DMEM, 20% FBS, 10% DMSO), placed into a Styrofoam box at −80 °C and allowed to freeze over night, after which they were transferred to liquid N_2. To retrieve, 1.0 mL of ice-cold complete DMEM/10%FBS supplemented with antibiotics penicillin and streptomycin was added to the frozen cells and the suspension was transferred to a 6 cm Petri dish and placed at 37 °C into a humidified 5%CO_2 incubator for 2 d, after which they were trypsinized and passaged for use.

Transfections. Freshly trypsinized MEFs or HEK293 cells, 8×10^4 cells suspended in 2 mL DMEM/10%FBS were seeded into wells of 6-well plates and grown overnight at 37 °C in humidified 5%CO_2 incubators. Transfections were then performed by addition of 100 μL of a mixture of 2 μg DNA and 6 μL Lipofectamine 2000 (Invitrogen) for MEFs or 1.5 μg DNA and 3 μL Lipofectamine 2000 for HEK293 cells. Transfection mixtures were prepared as recommended by the manufacturer by adding the Lipofectamine 2000 to 50 μL of GIBCO's Opti-MEM I in a 0.5-mL Eppendorf tube, separately diluting the DNAs to be transfected into 50 μL of Opti-MEM I in another 0.5-mL Eppendorf tube and allowing the tubes to stand at room temperature for 40 min. The two 50 μL aliquots were then combined, allowed to stand another 5 min and carefully distributed into the wells containing the cells to be transfected growing under 2 mL DMEM/10% FBS, followed by an overnight incubation at 37 °C in 5%CO_2 incubators. The cells were recovered by trypsinization and replated onto coverslips for imaging the next day. Cell recovery from the 6-well plates was achieved by first rinsing with Dulbecco's PBS and addition of 500 μL 0.05% trypsin in Dulbecco's PBS for 3 min, followed by addition of 4.5 mL DMEM/10% FBS and repeated up and down pipetting with a plastic pipette. After transfer of these cell suspensions into centrifugation tubes, the cells were pelleted, resuspended in 5 mL of fresh DMEM/10%FBS, and their concentration adjusted to give 3×10^4 (MEFs) or 1×10^5 (HEK cells) per 150 μL. In preparation for imaging, 150 mL of cell suspensions were placed onto round 18 mm coverslips (Warner Instruments cat# 64-0384–warneronline.com) in wells of a 12-well plate, allowed to settle for 1 h, overlaid with 850 μL DMEM/10%FBS, and incubated overnight at 37 °C in a humidified 5%CO_2 incubator.

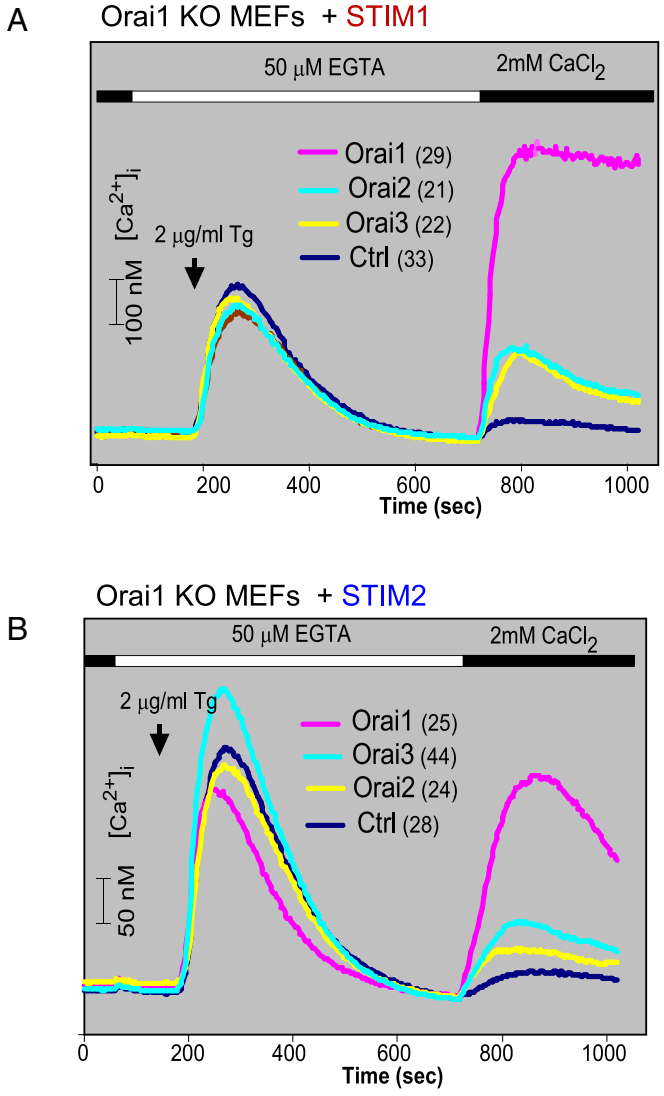

Fig. 4. All Orai genes are able to Form CRAC channels. Coexpression of transfected Orai with (*A*) STIM1 and (*B*) STIM2 cDNAs, in Orai1 KO MEF cells reveals the ability to form CRAC channels by all Orai proteins, but at differing efficiencies.

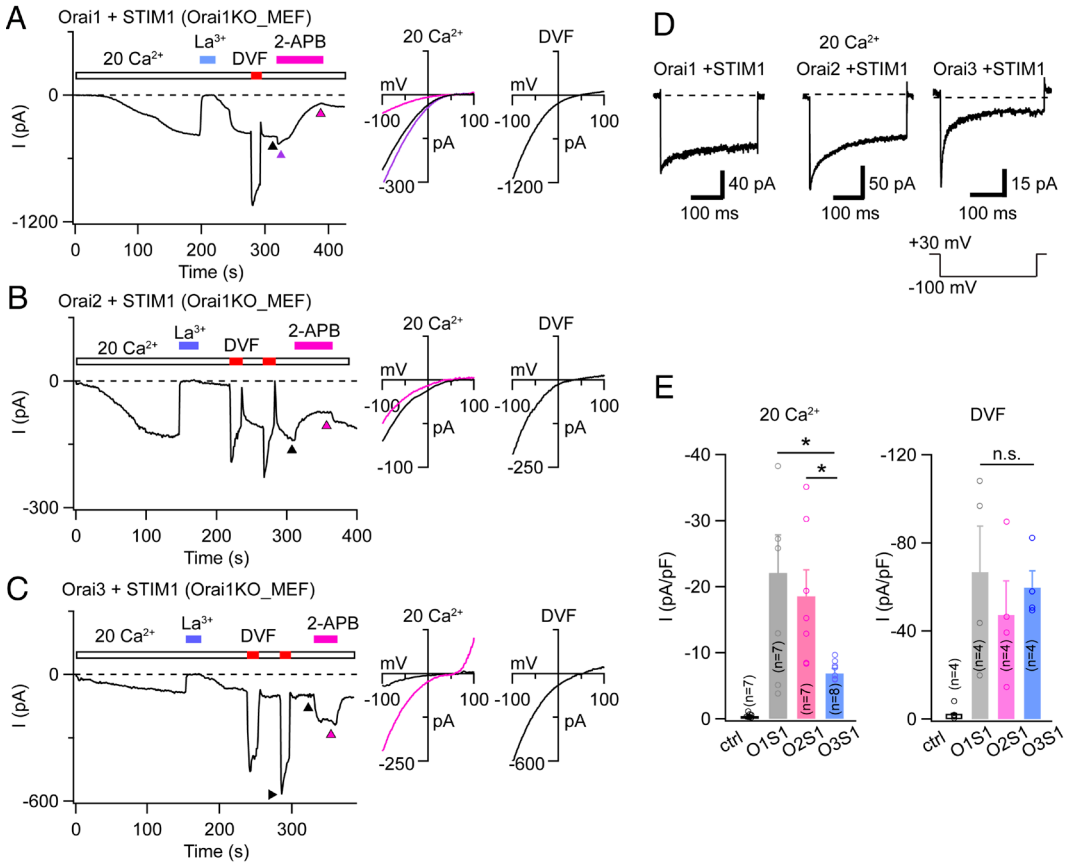

Fig. 5. Properties of CRAC currents arising from expression of different Orai isoforms in Orai1 KO MEFs. (*A–C*) Leak-corrected CRAC currents arising from overexpression of Orai1 (*A*), Orai2 (*B*), or Orai3 (*C*) in Orai1 KO MEFs. The membrane potential was stepped to −100 mV for 100 ms followed by a ramp from −100 to +100 mV for 100 ms. The traces plot the peak Orai current during the −100 mV voltage step over time. Orai1 KO MEF cells were transfected with CFP-Orai1, CFP-Orai2, or YFP-Orai3 together with mCherry STIM1. I_{CRAC} was activated by 8 mM BAPTA in the pipette. The standard extracellular Ringer solution (20 mM Ca^{2+}) was periodically switched with a Na^+-based DVF solution at the indicated time points, revealing the conduction of Na^+ currents in the absence of extracellular divalent ions. Leak-subtraction was performed by subtracting the current in the presence of 100 μM $LaCl_3$. 50 μM 2-aminoethoxydiphenyl borate (2-APB) was applied at the indicated time points. For Orai1, administration of 2-APB evokes a biphasic response with initial potentiation followed by strong inhibition. Orai2 currents are only modestly inhibited by 2-APB. By contrast, Orai3 channels are strongly activated by 2-APB, resulting in large nonselective Orai3 currents. The current–voltage (I–V) relationship of the Orai currents in 20 mM Ca^{2+} and DVF solutions obtained at the time point indicated by the arrowhead are shown in the *Right* graphs. (*D*) Calcium-dependent inactivation (CDI) of Orai currents overexpressed in Orai1 KO MEFs. The traces show currents arising from the indicated Orai isoform during 300-ms hyperpolarizing steps to −100 mV in 20 mM $Ca^{2+}{}_o$ Ringer's solution. The internal solution contained 8 mM BAPTA. (*E*) Summary graphs of current densities of I_{CRAC} in the presence of 20 mM $Ca^{2+}{}_o$ and DVF solutions in Orai1 KO MEF cells (ctrl), and in Orai1 KO MEFs overexpressing Orai1, Orai2, or Orai3 together with STIM1. These current densities are (in 20 mM $Ca^{2+}{}_o$): −0.42 ± 0.13 pA/pF (n = 7) (Orai1 KO MEFs, Ctrl), −22.1 ± 5.8 pA/pF (n = 7) (Orai1+STIM1), −18.6 ± 4.0 pA/pF (n = 7) (Orai2+STIM1) and −6.8 ± 0.8 pA/pF (n = 8) (Orai3+STIM1) (**P* <0.05). In DVF solution, the current densities are −2.5 ± 1.0 pA/pF (Ctrl, n = 7), −66.7 ± 21.0 pA/pF (Orai1 + STIM1, n = 4), −47.3 ± 15.5 pA/pF (Orai2 +STIM1, n = 4), and −59.8 ± 7.6 pA/pF (Orai3 + STIM1, n = 4), respectively (**P* >0.05). Values are mean ± SEM.

Unless indicated otherwise transfection mixtures contained 0.1 μg pEYFP-N1 (Clontech) to aid in selecting the cells that had taken up DNA, which for analysis purposes were designated as cells expressing the other cDNAs in the mixture. If one of the cDNAs was tagged with a yellow fluorescent protein (e.g. STIM1 and STIM2 cDNAs) the pEYFP was omitted.

Ratiometric imaging using fura2 and video microscopy. Transfected cells were plated on polylysine-coated 18 mm round coverslips as described above. On the day of imaging Fura 2.AM was added to the medium to give 0.5 μM and incubations were continued for another 40 min either at room temperature (MEFs) or at 37 °C in a 5%CO_2 incubator. The cells were then rinsed twice at room temperature with HPSS buffer with 2 mM $CaCl_2$ (HPSS buffer: 116 mM NaCl, 5.4 mM KCl, 1.13 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES pH 7.4). The coverslips with the Fura2 loaded cells were then placed into circular open-bottom chambers (Warner Instruments cat# 64-0368) and mounted onto the stage of an inverted Nikon TS 100 microscope fitted with an InCyt_Im2 imaging system (Intracellular.com). Intracellular calcium changes were followed using a 20× plan fluor (NA = 0.5; WD = 2.1 mm) objective. The InCyt_Im2 ratiometric acquisition and analysis software was used to record intracellular Ca^{2+} ($[Ca^{2+}]i$) changes in individual cells. Experiments were at room temperature; Fura2 was excited alternatingly at 340 and 380 nm and emitted fluorescence was recorded at 510 nm. All reagents added to cells were dissolved in HPSS with 10 mM glucose.

SOCE and ROCE experiments. Unless otherwise denoted, for SOCE (Store Operated Calcium Entry) experiments, cells on the coverslips were exposed sequentially to 2 mM $CaCl_2$ for 2 min, 0.05 mM EGTA (2 min), 0.05 mM EGTA plus 2 μM thapsigargin (7 to 10 min) and 2 mM $CaCl_2$ until the end of the experiment. SOCE is Ca^{2+} entering upon addition of Ca^{2+} to the thapsigargin-treated cells in which endogenous Ca^{2+} stores have been depleted. For ROCE (Receptor Operated Calcium Entry) experiments, the transfection mixtures contained 0.1 μg of either M5 muscarinic receptor-plasmid (M5R) cDNA in pcDNA3 (21), 100 μM carbachol (CCh) was substituted for thapsigargin and readdition of $CaCl_2$ was in the continuous presence of 100 μM CCh. Alternatively, an arginine vasopressin 1a receptor-carrying plasmid (V1aR) was used and 100 ng/mL AVP was substituted for CCh. Other additions are denoted in the figures and legends to figures. All additions were in HPSS buffer.

Isolation of RNA from tissues. Mice were euthanized in CO_2 chambers and their tissues rapidly dissected, rinsed with ice-cold Dulbecco's PBS, placed into ice-cold 15-mL Corex tubes with prealiquoted RLT solution supplied in RNAeasy Kits (Qiagen cat #74106) and homogenized with the aid of a Polytron homogenizer

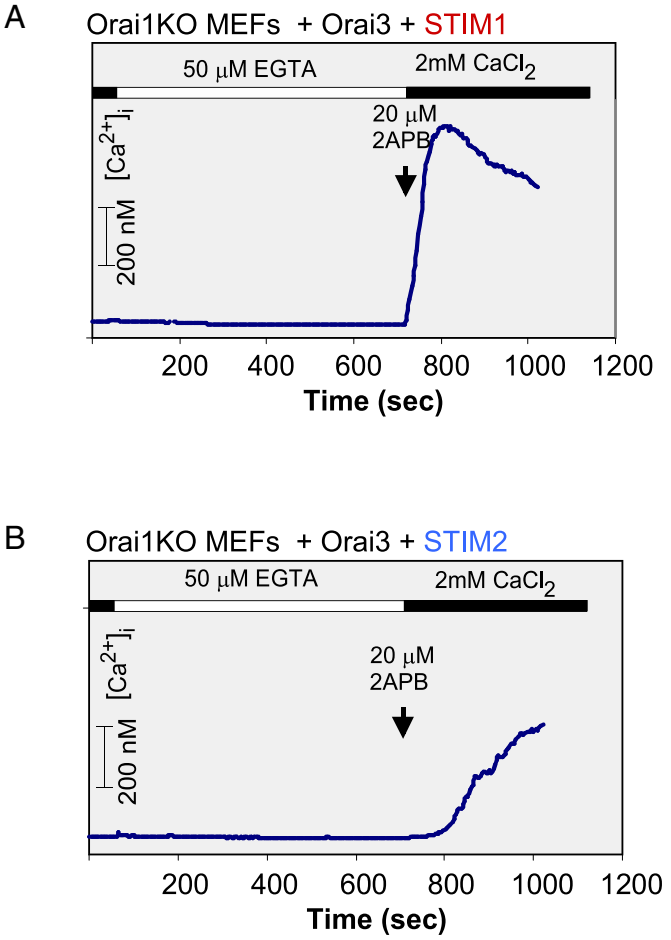

Fig. 6. Unactivated STIM proteins affect the kinetics and extent of activation of Orai3 by 2-APB. Tg-activated SOCE in Orai1 KO MEF cells cotransfected with Orai3 and (*A*) STIM1 or (*B*) STIM2 cDNAs.

fitted with a PT-10 generator. Total RNA was isolated following RNAeasy Kit instructions which include on-column DNAse treatment.

Isolation of RNA from cells in culture. 10 mL of freshly trypsinized and washed cells were resuspended in DMEM/10%FBS at 5×10^5 cells/mL and seeded into 10 cm Petri dishes, grown to 80% confluency, rinsed twice with ice-cold Dulbecco's PBS without Mg^{2+} or Ca^{2+}, and lysed by addition of 700 μL RTL lysis buffer. The lysate was pushed by centrifugation through the porous plaque of a Qiashredder tube and applied to Qiagen micosilica, treated with DNAse, and eluted following instructions of the RNAeasy kit. RNA was eluted with RNAse free (DEPC-treated) H_2O and quantified by spectrophotometry using a Nanodrop spectrophotometer set to RNA reading. Array-based RNA expression profiles were obtained with Affimetrix arrays and performed by the NIEHS MicroArray Core Laboratory.

RT-PCR analyses. Preparation of first-strand cDNAs (reverse transcripts) was with 1 to 2 μg total RNA isolated as above using reagents from the Superscript First Strand cDNA Synthesis System kit (Invitrogen Cat #11904-018) following the instructions provided with the kit. For control purposes, the process was repeated omitting addition of the reverse transcriptase (no reverse transcriptase controls). PCRs were in a final volume of 25 μL using 2 μL of first-strand cDNA and Roche Expand High Fidelity PCR System reagents with 0.2 mM dNTPs supplemented to give 5% DMSO and 2.4 mM $MgCl_2$. PCR primers were used at 0.3 μM each. Cycling program: 1. 94 °C, 5 min; 2. 94 °C, 30 s; 65 °C, 60 s; 72 °C, 120 s; 3. 72 °C, 10 min.

PCRs testing for gDNAs were performed on approximately 100 ng gDNA using reagents and enzymes from commercial PCR kits purchased from Qiagen (HotStarTaq PCR System cat#203445), Invitrogen (Platinum Taq DNA Polymerase System, cat#10966-018), and Roche (Expand High Fidelity PCR System, cat#04-738-250-001), using 0.4 μM of each PCR primer, supplemented to give 5% DMSO and 2.5 to 3.0 mM $MgCl_2$ (Fig. 1)

RNA analyses. Gene expression: 35-cycle RT-PCR primer compositions.

A. Mouse Trpc expression. Mus Trpc1, amplicon; 265 bp
mC1Ex8F 5′-CTCAAAGTGGTGGCTCACAACAAG
mC1Ex10R 5′-CATAGAGCTGAGTCAGTCCAATCG
Mus Trpc2, amplicon: 670 bp
mC2Ex13F 5′-GGCTGTCAACTACAACCAGAAACAG
mC2Ex17R 5′-AGCTGGCAGAATATATGCCAGTCG
Mus Trpc3, amplicon: 395 bp
mC3Ex7F 5′-GGACTGTCTGAAGTGACTTCTGTTG
mC3Ex9R 5′-CTCGAGTTAGACTGTGTGAAGAGG
Mus Trpc4, amplicon: 349 bp
mC4Ex4F 5′-GTGGAGTGGATGATATTACCGTGG
mC4Ex6R 5′-TCAGGATGTCCAGAAGCATCCTTC
Mus Trpc5, amplicon: 526 bp
mC5Ex3F 5′-TATCATGACCTGGCCAAGCTGAAG
mC5Ex5R 5′-TGACATAGGCCACGATCTTCAAGG
Mus Trpc6, amplicon: 639 bp
mC6Ex7F 5′-CCTACTGATCCTCAGATCATCTCTG
mC6Ex9R 5′-CTCTGCATCTTCTTGGAAGCCTTG
Mus Trpc7, amplicon: 501 bp
mC7Ex5F 5′-GACGGAGATGCTCATCATGAAGTG
mC7Ex7R 5′-CGGTAGTAGGAGTACAGGTTGAAC
In some instances, a second set of PCR primers was substituted for the above RT PCR primers:
Trpc1, amplicon: 107 bp
C1RT-F 5′-CTCAGCCTTGTGGAGGTGGAA
C1RT-R 5′-GTTCACGAGAATTCCGGGCTTGT
Trpc2, amplicon: 194 bp

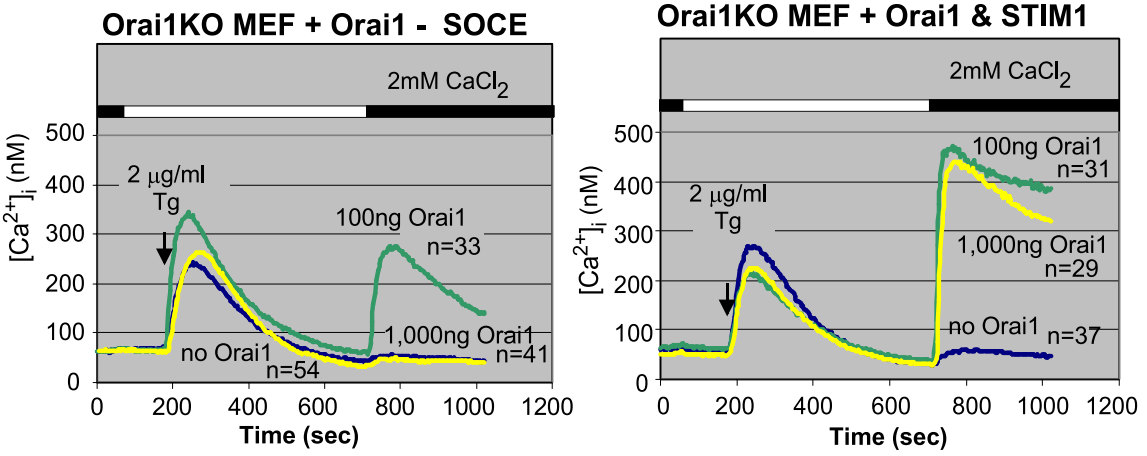

Fig. 7. Dual action of Exogenous Orai1 on SOCE in Orai1 KO MEF cells. (*A*) Low levels. of Orai1 reconstitutes SOCE, whereas high levels inhibit SOCE. (*B*) Scavenging excess Orai1 with STIM1 uncovers the reconstituting action of Orai1.

C2RT-F 5′-CCTTCCTCTGTGGCCTCAACA
C2RT-R 5′-CATGACGATGGTAAAGATGCCGTACA
Trpc3, amplicon: 299 bp
C3RT-F 5′-GGTGCACCTGCTGCTACTCAA
C3RT-R 5′-CCAGCACACCCACTACGAAGT
Trpc4, amplicon: 393 bp
C4RT-F 5′-CAGAGACCGCATCCCACTGA
C4RT-R 5′-GGTGTGATGTCCGGAGTGAATTCA
Trpc5, amplicon: 512 bp
C5RT-F 5′-CATGGCCCAGCTGTACTACAAGAA
C5RT-R 5′-GCCTTGGGATAGTGACCCTCTTT
Trpc6, amplicon: 609 bp
C6RT-F 5′-GCCAATGAGCACTTGGAAATCACAGA
C6RT-R 5′-GGC CTC CAC TTC CTC TGT GT
Trpc7, amplicon: 721 bp
C7RT-F 5′-CTGGAGGAATCCAAGACCCTCAAT
C7RT-R 5′-GGTCGGACCAGACTTGCAAGT
Cycling program: 1. 95 °C,120 s; 2. 35 cycles: 95 °C, 30 s, 62 °C, 30 s, 72 °C,45 s; 3. 72 °C, 10 min.

Fig. 1 depicts the genotyping results of using electrophoretic mobility to differentiate the amplicons from each other with the above primers. Wilt type (+/+) and heterozygous (+/−) gDNAs were used as controls.

PCRs testing for gDNAs were performed on approximately 100 ng gDNA using reagents and enzymes from commercial PCR kits purchased from Qiagen (HotStarTaq PCR System cat#203445), Invitrogen (Platinum Taq DNA Polymerase System, cat#10966-018), and Roche (Expand High Fidelity PCR System, cat#04-738-250-001), using 0.4 μM of each PCR primer, supplemented to give 5% DMSO and 2.5 to 3.0 mM $MgCl_2$.

B. Mouse Orai Expression. Mus Orai1: Amplicon: 335 bp
mO1F 5′-CTACTTAAGC CGCGCCAAGC TCAAAGCTTC CAGC
mO1R 5′-CCTCTGCTAG GAAAAGCAGC GTCCCGATGA CCG
Mus Orai2: Amplicon: 397 bp
mO2F 5′-CAGCACTTGCATCCTGCCCAACGTGGAAGCG
mO2R 5′-GCTCCGCTCGTGCCCATCCAGCTGCACCTTGAGC
Mus Orai3: Amplicon: 390 bp
mO3F 5′-GGAGAACGATCATGAATACCCACCAGGCCTGC
mO3R 5-GTAATAGAAGCAGAGGATGGTGTGAGGTTAGAAGC
C. Mouse STIM expression. Mus STIM1 amplicon: 384 bp
mS1F 5′-GAAGCTCTCAATGCCATGCCTTCCAATGGCAGC
mS1R 5′- CTTCTTCCTGCCTGGACTGGAGTCTGTCTC C
Mus STIM2 amplicon: 450 bp
mS2F 5′-GTCTGTGTCAAGTTGCCCTGCTCTGTATCGGAACG
mS2R 5′-CGAGGCTTCCTGAACTGGCTTGCTATCATTGC

Validation of PCRs. For all genotyping methods, as well as all RT-PCR analyses, the identity of the amplicons was verified by sequencing in both directions. To this end, the amplicons were adsorbed onto microsilica membranes of Qiaquick Spin Columns using reagents from the Qiaquick PCR Purification Kit (cat#28104), following the kit's instructions, and eluted with EB buffer (10 mM Tris-HCl, pH 8.5). The eluted amplicons (~400 ng) were then cycle-sequenced in NIEHS's DNA Sequencing Core Laboratory using the corresponding PCR primers as sequencing primers.

Results and Discussion

Previously published data had suggested that SOCE-mediating channels may be heteromeric formed of both TRPC and Orai proteins (3–5). The plausibility of this composition of SOCE channels was underscored by the findings that ROCE channels are heteromeric being formed of up to three TRPCs (7) and of more than one type of channel as in heteromeric TRPV4 and TRPC1 channels in human vascular endothelial cells (22). It became important to test SOCE in cells in which the Ca^{2+} entry channel subunit composition is known. 35-cycle RT-PCR analysis for presence of Orai and STIM mRNAs in SV40 immortalized wt MEF cells showed that this type of cells expresses mRNA coding for the three mouse Orai proteins (Orai1, -2, and -3) as well as mRNA coding for the two mouse STMs (STIM1 and -2) (Fig. 1 *B* and *C*). In agreement with the disruption of the Orai1 and STIM1 genes, Orai1 KO MEF cells lack Orai1 mRNA (Fig. 1*B*2) and STIM1 KO MEF cells lack STIM1 mRNA (Fig. 1 *C* and *D*). In contrast to Orai and STIM, wt MEF cells express only three of the seven TRPC genes (C1, C2, and C6), of which Orai1 KO MEF cells express only two (TRPC1 and TRPC2).

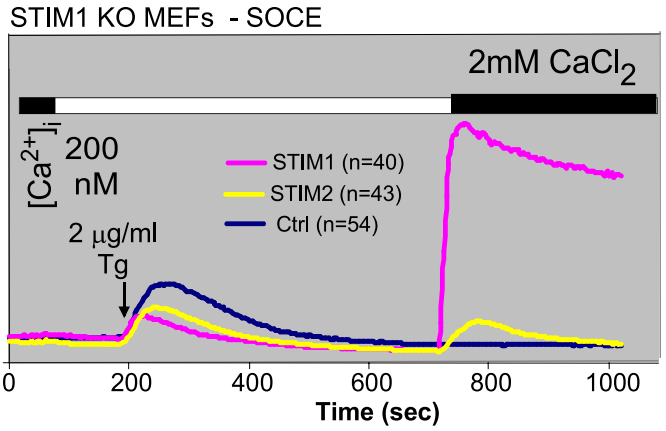

Fig. 8. Failure of store depletion to trigger Ca^{2+} entry in STIM1 KO MEFs and reconstitution of SOCE in STIM1 KO MEFs by expression of exogenous STIM1 or STIM2. Tg-activated SOCE in STIM1 KO MEF cells transfected with STIM1 or STIM2 cDNAs.

We tested for a requirement of TRPC proteins in SOCE and ROCE by creating a TRPC-null mouse done by breeding. Fig. 2*A* shows the results of genotyping such a mouse, done in this case, by monitoring electrophoretic mobility to differentiate the amplicons from each other, Mouse #4537-7, a female, was the first mouse to have all of its seven TRPC genes disrupted. She was mated to a multi-KO mouse which had only one allele of Trpc4 remaining (Trp4+/–), Mouse #4537-7 had a litter of 8 pups, of which 4 were homozygous KO for all but Trpc4 which, as their father, were Trpc4+/–, The other 4 pups were TRPC heptaKO and served to establish a strain of TRPC heptaKO mice. Mouse #34537-7 with her 8-pup litter is shown in Fig. 2*B*.

Dominant Nature of Orai1. To investigate the importance of Orai in SOCE and ROCE we prepared SV40-immortalized embryonic fibroblasts from Orai1$^{-/-}$ mouse embryos (Orai1 KO MEFs) and tested these cells for presence of SOCE and ROCE using standard

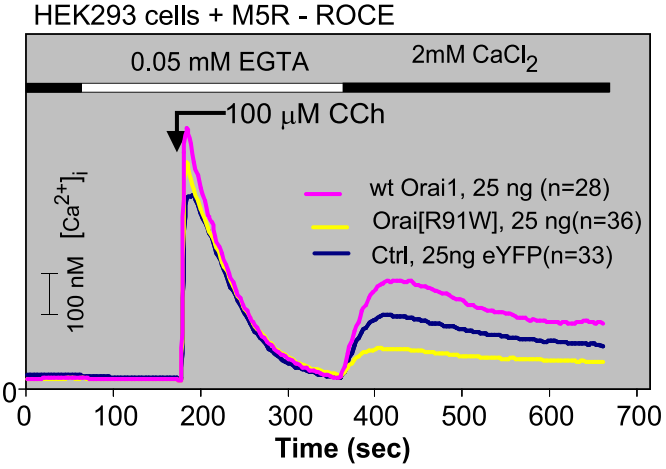

Fig. 9. Inhibition of M5R triggered ROCE by expression of the SCID-causing Orai1 mutant [R91W] Orai1 and enhancement of ROCE by wt Orai1. HEK293 cells were transfected with 25 ng pcDNA3 driving the expression of the indicated Orai1 molecules, the M5R, and TRPC6 N-terminally tagged with EYFP.

two-step SOCE and ROCE protocols. To test SOCE, intracellular stores are depleted of Ca^{2+} by addition of the SERCA pump inhibitor thapsigargin, in the absence of extracellular Ca^{2+}, followed 8 to 10 min later by addition of extracellular Ca^{2+} and monitoring Ca^{+2} influx due to thapsigargin addition, i.e., store operated Ca^{2+} entry (SOCE). To test ROCE, stores are depleted by activation of a Gq-coupled receptor (muscarinic M5R or vasopressin V1aR) with the respective ligand in the absence of external Ca^{2+}, allowing $[Ca^{2+}]$ i to rise and return to basal levels, after which cells are exposed to extracellular Ca^{2+} in the continuous presence of receptor ligand, and receptor-operated Ca^{2+} entry, ROCE, is monitored.

When SOCE and ROCE were tested in Orai1 KO cells we found that in the absence of Orai1, MEF cells are essentially unable to mount SOCE and ROCE responses to store depletion (Fig. 3). Reconstitution of SOCE in Orai1 KO MEFs by expression of the individual Orai proteins revealed that Orai2 and Orai3 are able to form SOCE channels, and that Orai1-mediated SOCE is dominant when compared to SOCE mediated by Orai2 or Orai3. Indeed, its loss cannot be compensated by Orai2 or Orai3, as Orai1 KO MEFs express Orai2 and Orai3 (Fig. 1*B*).

STIM1 and STIM2 differ in their ability to promote the assembly of Orai-based SOCE channels and to potentiate Ca^{2+} entry. STIM1 is better than STIM2 (Fig. 4).

The ability of the each of the three Orai proteins to assemble into store-depletion-activated Ca^{2+} permeable cation channels was also examined in patch-clamp experiments in which Orai1 KO MEFs were transfected with mCherry STIM1 and the desired Orai (1, -2, or -3) tagged N-terminally with a fluorescent protein (CFP or YFP), loaded with 8 mM BAPTA and monitored for development of inward currents (CRAC currents) recorded at a holding potential of -100 mV in an extracellular Ringer solution containing 20 mM Ca^{2+}. As shown in Fig. 5 *A–C*, the three Orai proteins assemble into CRAC channels that, when switched to a divalent cation-free (DVF) solution showed conduction of Na^{+}. Voltage ramps of 100 ms from −100 mV to +100 mV showed an inwardly rectifying current consistent with I_{CRAC}. As previously published CRAC currents are blocked by 100 mM La^{3+} and undergo Ca^{2+}-dependent inactivation (CDI) (Fig. 5*D*) which varies for each of the Orai proteins, being most marked for Orai3, less for Orai2, and even less for Orai1. 2-APB is generally considered as a SOCE and Icrac inhibitor. However, tests of the effect in Orai1 KO MEFs expressing transfected recombinant Orai proteins revealed that 2-APB affects Ca^{2+} entry differently depending on the presence of the Orai isoform (Fig. 5 *A–C*): for Orai1 it evoked an initial short-lived stimulation followed by strong inhibition, for Orai2 it is weakly inhibitory and for Orai3 it is stimulatory. Fura2 does not allow for detection of the biphasic response in Orai1 expressing cells, but readily reveals the stimulation of Orai3-mediated Ca^{2+} entry. The rate at which 2-APB stimulates Orai3-based store operated Ca^{2+} entry is also affected by the nature of STIM molecules driving the assembly of the Ca^{2+} entry channels (Fig. 6). Furthermore, unactivated STIM proteins are not totally silent as they influence the rate and extent to which 2-APB activates Ca^{2+} entry in Orai1 KO MEF cells without depletion of endogenous Ca^{2+} stores (Fig. 6). The reason for this difference requires further experimentation.

Expression of high levels of Orai1 inhibits SOCE (23). This inhibition can be relieved by exogenous STIM1 (Fig. 7). a property exploited in the above study of CRAC currents reconstituted in Ora1 KO MEF cells. STIM proteins differ in their ability to relieve inhibition by excess Orai1. As expected, SOCE and ROCE not only depend on Orai1 but also on STIM1, the ER Ca^{2+} sensor. Thus, STIM1 KO MEFs are unable to mount store depletion induced Ca^{2+} entry. SOCE is reconstituted by expression of the missing STIM1. Whereas transfected STIM1 is highly effective in reconstituting SOCE in STIM1 KO MEFs, transfected STIM2 is barely able to replace STIM1 (Fig. 8) and natural levels of STIM2 present in STIM1 KO MEF cells cannot replace the missing STIM1.

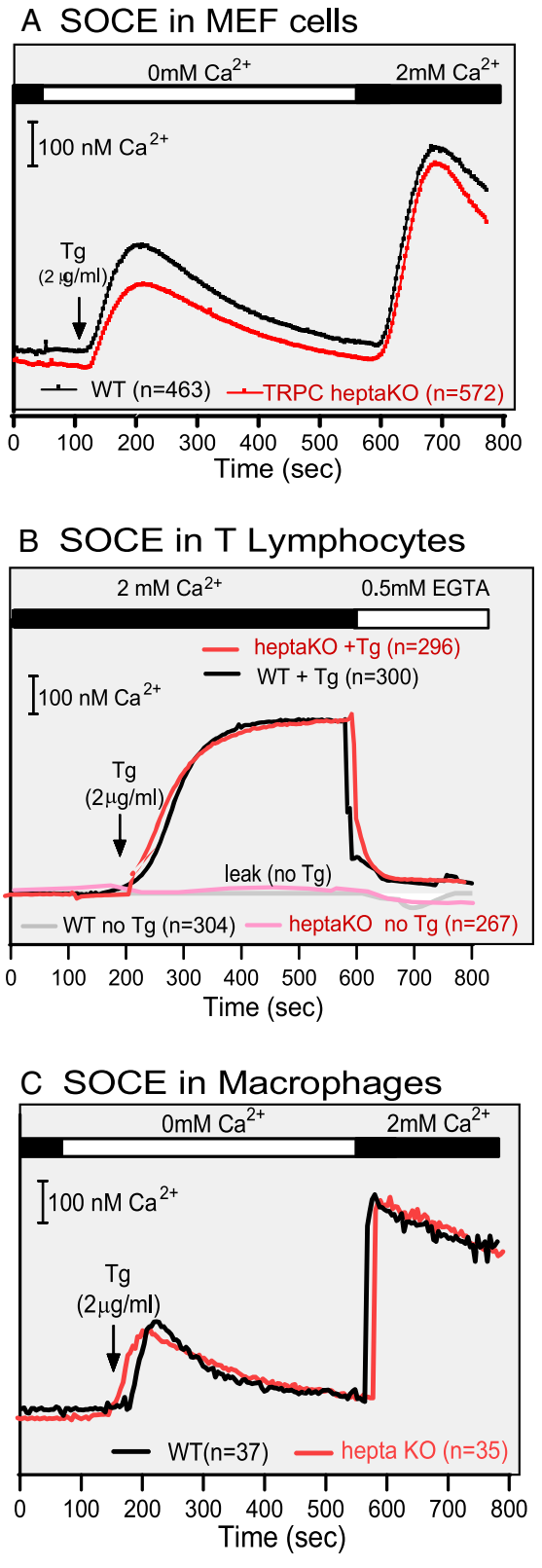

Fig. 10. SOCE is unaffected by the absence of all TRPC proteins. (*A*) Intact SOCE in TRPC-HeptaKO MEFs. (*B*) Intact SOCE in naïve T cells of TRPC heptaKO mice. (*C*) Intact SOCE in bone marrow–derived macrophages (BMDM) of TRPC heptaKO mice.

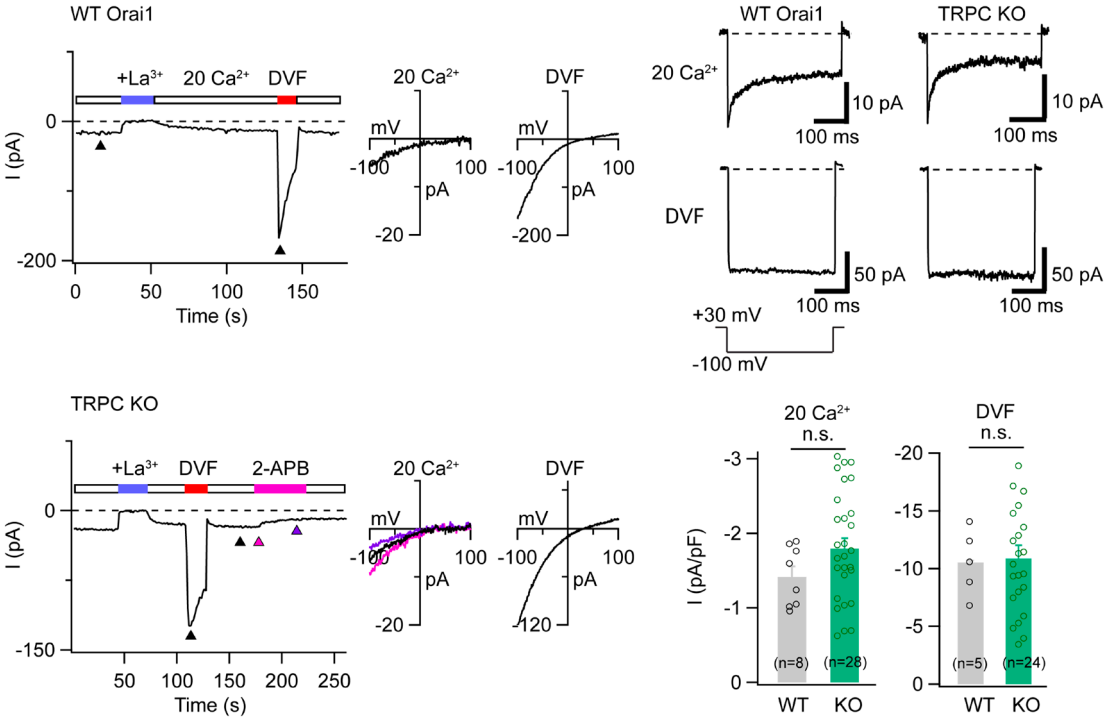

Fig. 11. CRAC channel currents are unaffected in TRPC heptaKO T cells. (*A* and *B*) Leak-corrected CRAC channel currents in WT and TRPC heptaKO MEF cells. The graph on the *Left* shows the peak CRAC current amplitude measured during hyperpolarizing steps to −100 mV plotted against time. The I–V plots of Ca^{2+} and monovalent currents are shown on the *Right*. Currents were induced by pretreating cells with thapsigargin prior to the recording (5 min). The extracellular Ringer solution (20 mM Ca^{2+}) was periodically switched with a Na^+ -based DVF (divalent-free) solution, revealing permeation of Na^+ ions in the absence of extracellular divalent ions. I_{CRAC} is completely inhibited by 100 mM $LaCl_3$. I–V relationships were measured at times indicated by the arrowheads. 2APB (50 μM) inhibits I_{CRAC} in the TRPC heptaKO cells, in line with the known effects of 2APB on CRAC currents in cells expressing Orai1. I_{CRAC} is slightly enhanced immediately following 2-APB administration and this is followed by inhibition of current. (*C*) Both WT and TRPC heptaKO cells show characteristic CDI (Ca^2-dependent inhibition) of CRAC currents. The traces show CRAC currents during hyperpolarizing steps (300 ms) to −100 mV in the presence of either 20 mM $Ca^{2+}{}_o$ or of DVF solution. CDI is absent in DVF solution indicating that the process is Ca^{2+}-dependent. (*D*) Summary graphs showing the current densities of I_{CRAC} in 20 mM $Ca^{2+}{}_o$ and in DVF solutions in WT and TRPC heptaKO cells. The current densities of WT and TRPC heptaKO cells in 20 mM $Ca^{2+}{}_o$ are −1.41 ± 0.14 pA/pF (n = 8) and −1.8 ± 0.14 pA/pF (n = 28), respectively ($P > 0.05$); in the presence of DVF, the current densities are −10.6 ± 1.27 pA/pF (WT, n = 5) and −10.5 ± 0.97 pA/pF (TRPC heptaKO, n = 24), respectively ($P > 0.05$). Values are mean ± SEM. Mouse CD4 T cells were isolated from the spleen and activated with anti-CD3 and anti-CD28 antibodies for 3 d as described above in *Materials and Methods*. Analysis of current amplitudes was performed by measuring the peak currents during the 100-ms pulse to −100 mV. For the bar graphs shown, current amplitudes were determined by averaging the maximal current densities in each cell. Averaged results are presented as the mean value ± SEM. Statistical comparisons were performed using unpaired *t* tests.

No Role for TRPC Proteins in SOCE. Previous studies had suggested a role for TRPCs in SOCE (3–5, 24). Here, we show a role for Orai1 in both SOCE and ROCE, as both are absent in Orai1KO MEFs (Fig. 3) and restored upon exogenous expression of low levels of the missing Orai1 (Fig. 7). As expected, the reverse is also true: STIM1 KO MEF cells are unable to trigger SOCE. The question as to whether TRPCs operate as TRPC–Orai complexes remained unanswered. We sought to gain an insight into the possible participation of Orai as a component of activated TRPCs by testing whether Orai1[R91W], responsible for a Severe Combined Immunodeficiency (SCID) affects ROCE, i.e., under conditions where Orai1 will be inhibited. As shown in Fig. 9, ROCE is inhibited by the dominant negative form of Orai1: 25 ng of Orai1[R91W] plasmid per well of a 6-well plate were used in the transfection mixture; wt Orai1 is not inhibitory at this concentration; in fact, it stimulates ROCE (Fig. 9).

Taken together, our previous studies and the results presented here indicate that TRPC channels depend for their function on Orai proteins, primarily Orai1. As mentioned in the Introduction, it was not known whether Orai channels depend on TRPC protein(s) for their assembly, as tests probing for Orai functions had never been done in the absence of TRPC proteins. This required cells without functioning TRPC proteins, i.e., a cell in which all seven TRPC genes were inactivated. We thus combined by breeding the individual KO alleles, see *Materials and Methods*, and obtained a mouse strain lacking functional TRPC proteins. We refer to mice of this TRPC-null strain as TRPC heptaKO mice. Genotyping PCR analysis of the first TRPC null mouse, tag # 4537-7, is shown in Fig. 2*A*. Fig. 1*B* shows the genotyping result of embryonic fibroblasts derived from the established TRPC heptaKO strain, confirming the TRPC null genotype of the new strain.

As reported elsewhere, tests of angiotensin II triggered ROCE in cardiac fibroblasts from TRPC heptaKO mice exhibit small but significant responses to angiotensin II, statistically indistinguishable from responses triggered by angiotensin II in control WT cardiac fibroblasts (25).

Tests for SOCE in MEF cells, in naïve T cells and in bone marrow–derived macrophages (BMDM) obtained from TRPC HeptaKO mice (Fig. 10) showed that SOCE is also unaffected by loss of all TRPCs. As expected from this result, the absence of TRPC proteins also did not affect the Orai1-based electrophysiological correlate of SOCE, the CRAC currents both amplitude and ion selectivity (Fig. 11). Further, in agreement with the observed loss of ROCE and SOCE in Orai1 KO MEF cells (Fig. 3), pharmacologic suppression of Orai1 by GSK7975A (26) inhibited not only SOCE but also the development of ROCE in response to activation of the Gq/11 signaling pathway by the carbachol-stimulated M5 muscarinic acetylcholine receptor. This

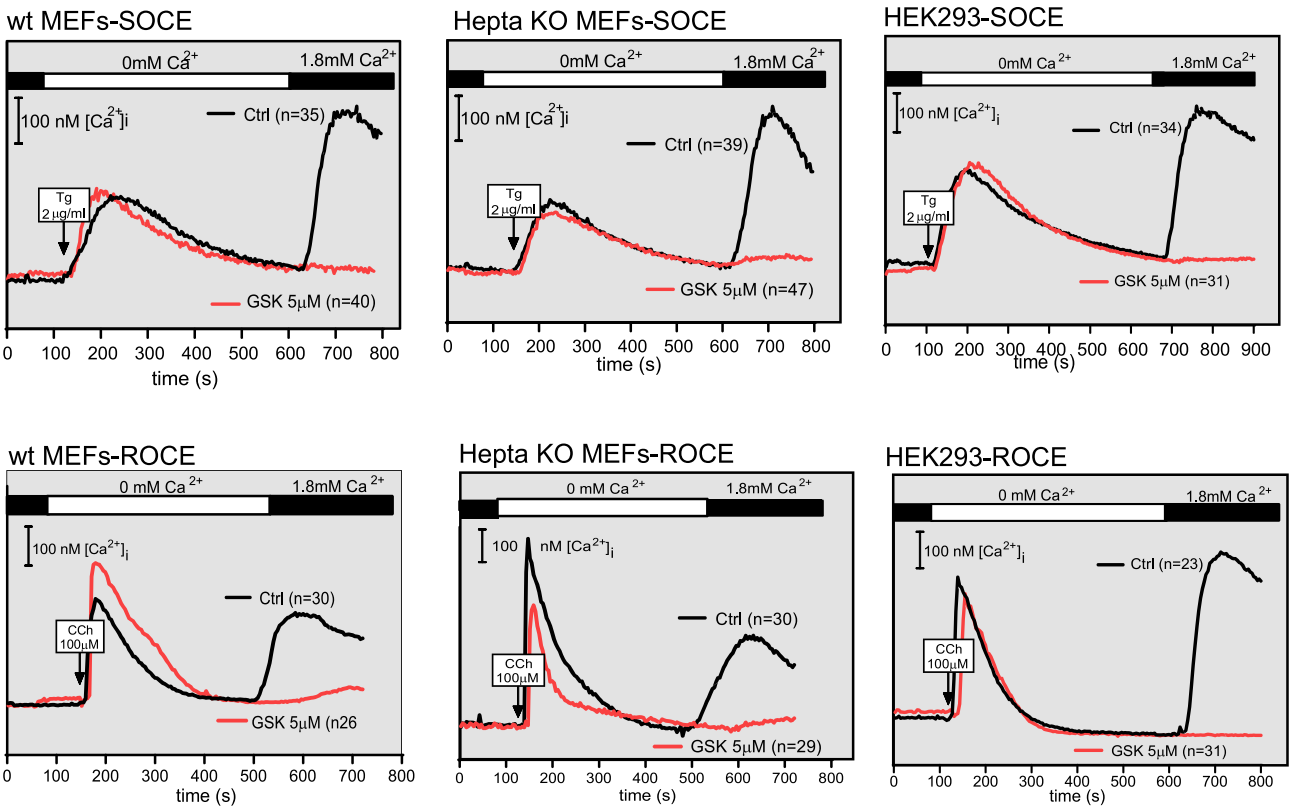

Fig. 12. Inhibition of ROCE and SOCE by CRAC-inhibitor GSK7975A. The figure shows the inhibition of ROCE and SOCE in wt and HeptaKO MEF cells and in human HEK293 cells, all transfected with the rat muscarinic M5 acetyl choline Receptor.

is shown in Fig. 12 for two cell systems: murine embryonic fibroblasts (MEFs) and human HEK293 cells.

The results shown above indicate that TRPCs do not participate in events related to Ca^{2+} store depletion. Given that a wide spectrum of phenotypes appears in TRPC KO mice (2, 27), often involving changes in Ca^{2+}, it follows that an activation mechanism must exist by which TRPC proteins are activated. Activation by diacylglycerols generated by β and γ PLCs fit this role. First discovered for TRPC3 and TRPC6 (28), direct gating of TRPC channels by diacylglycerols (DAGs) has been shown for all: TRPC1 (29), TRPC2 (30), TRPC4 and TRPC5 (31), and TRPC7 (32). Activation of TRPCs by DAGs has not received the attention it requires possibly because it is regulated in diverse and complex ways. TRPC1 is strongly inhibited by extracellular Ca^{2+}, this inhibition is relieved by association with TRPC3 likely by forming heterotetrameric Ca^{2+} conducting channels (29). Activation of TRPC5 and TRPC4 became apparent only when the interactions of two C-terminally located PDZ-binding motifs with the PDZ domains of two scaffolding proteins NHERF1 and -2, (Na^+/H^+ exchanger regulatory factor 1 and -2) were studied (31). Depletion of membrane phosphatidylinositol 4,5-bis phosphate and Inhibition of constitutive NHERF phosphorylation by PKC led to NHERF dissociation and uncovered activation of the TRPC4 and -C5 channels by the DAG analog OAG (oleyl-acetyl-glycerol).

In summary, the main conclusions emerging from the present work are 1) that TRPC proteins are not involved in assembly and ion channel properties of the Ca^{2+} release-activated Ca^{2+} entry as seen by electrophysiologic means using patch clamp techniques, or by monitoring changes in intracellular levels of Ca^{2+} using the calcium reporter dye Fura2; 2) that the molecular makeup of the channels allowing Ca^{2+} entry triggered by emptying ER delimited Ca^{2+} stores includes under normal conditions Orai1 and does not include a TRPC molecule; and 3) that activation of TRPCs by diacylglycerols must play a dominant role in the wide spectrum of physiologic and pathologic consequences seen in live TRPC KO mice.

Data, Materials, and Software Availability. All study data are included in the main text.

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