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Canonical Transient Receptor Potential 5 Channel in Conjunction with Orai1 and STIM1 Allows Sr$^{2+}$ Entry, Optimal Influx of Ca$^{2+}$, and Degranulation in a Rat Mast Cell Line$^1$

Hong-Tao Ma,$^{2*}$ Ze Peng,$^{3*}$ Takaaki Hiragun,$^{4*}$ Shoko Iwaki,$^{7}$ Alasdair M. Gilfillan,$^7$ and Michael A. Beaven$^{2*}$

Degranulation of mast cells in response to Ag or the calcium mobilizing agent, thapsigargin, is dependent on emptying of intracellular stores of Ca$^{2+}$ and the ensuing influx of external Ca$^{2+}$, also referred to as store-operated calcium entry. However, it is unlikely that the calcium release-activated calcium channel is the sole mechanism for the entry of Ca$^{2+}$ because Sr$^{2+}$ and other divalent cations also permeate and support degranulation in stimulated mast cells. In this study we show that influx of Ca$^{2+}$ and Sr$^{2+}$ as well as degranulation are dependent on the presence of the canonical transient receptor potential (TRPC) channel protein TRPC5, in addition to STIM1 and Orai1, as demonstrated by knock down of each of these proteins by inhibitory RNAs in a rat mast cell (RBL-2H3) line. Overexpression of STIM1 and Orai1, which are known to be essential components of calcium release-activated calcium channel, allows entry of Ca$^{2+}$ but not Sr$^{2+}$, whereas overexpression of STIM1 and TRPC5 allows entry of both Ca$^{2+}$ and Sr$^{2+}$. These and other observations suggest that the Sr$^{2+}$-permeable TRPC5 associates with STIM1 and Orai1 in a stoichiometric manner to enhance entry of Ca$^{2+}$ to generate a signal for degranulation. The Journal of Immunology, 2008, 180: 2233–2239.

An increase in cytosolic Ca$^{2+}$ levels in mast cells provides an essential signal for exocytotic release of granules whether cells are stimulated via the IgE receptor (FceRI) or thapsigargin (1, 2). The increase in cytosolic Ca$^{2+}$ is associated with release from intracellular stores and influx of external Ca$^{2+}$ (3, 4) by a mechanism, originally referred to as capacitative calcium entry but is now often referred to as store-operated calcium entry (SOCE),$^5$ in which depletion of intracellular stores of Ca$^{2+}$ leads to entry of Ca$^{2+}$ (5, 6). Ca$^{2+}$ entry was initially thought to occur through a calcium release-activated calcium (CRAC) current ($I_{\text{CRAC}}$) that was first identified in mast cells by patch-clamp techniques (7, 8). $I_{\text{CRAC}}$ is a Ca$^{2+}$-selective current of low conductance that requires external Ca$^{2+}$ to achieve maximal conductance. Replacement of Ca$^{2+}$ with Sr$^{2+}$ or Ba$^{2+}$ results in a decline in $I_{\text{CRAC}}$ activity in T lymphocytes (9, 10). Nevertheless, it appears unlikely that $I_{\text{CRAC}}$ is the sole mechanism for conductance of Ca$^{2+}$ as early studies of calcium-dependent exocytosis clearly demonstrated that stimulated mast cells become highly permeable to a variety of divalent cations including Sr$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$ and that such ions can support degranulation in the absence of Ca$^{2+}$ (11–13).

Members of the subfamily of transient receptor potential (TRP) channels, especially the canonical TRP (TRPC) channels, were initially considered as candidates for mediating SOCE (14, 15). The TRPC channels are activated as a consequence of inositol 1,4,5-trisphosphate generated through phospholipase C (PLC). Some TRPC channel proteins, such as TRPC1 and TRPC4, are activated by store depletion via inositol 1,4,5-trisphosphate or thapsigargin as demonstrated in cells deficient in TRPC1 or TRPC4 (16) or by overexpression (14, 15, 17–19) or knock down (18, 20–25) of these proteins. TRPC5 may be regulated by a variety of signals, including Ca$^{2+}$ store depletion (26), although there are contrary reports (27, 28). On the basis of structure and function, TRPC1, TRPC4, and TRPC5 appear to form one category of TRPC channel proteins and TRPC3, TRPC6, and TRPC7 form another (29). The latter category appears to be activated primarily by diacylglycerides (i.e., receptor-operated) rather than store depletion (30, 31). However, TRPC channels can conduct divalent cations such Sr$^{2+}$ and Ba$^{2+}$ in addition to Ca$^{2+}$ and none of the TRPC channel proteins appear to have the exact electrophysiological features of CRAC (32).

The molecular identity of the $I_{\text{CRAC}}$ putative channel CRAC has been clarified recently with the identification of two proteins, STIM1, a Ca$^{2+}$ sensor, and Orai1, a channel protein, which are essential for CRAC channel activity. STIM1 was first identified as a component of CRAC channel in Drosophila S2 cells and Jurkat

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$^1$Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute and $^2$Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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$^1$Address correspondence and reprint requests to Dr. Michael A. Beaven or Dr. Hong-Tao Ma, Laboratory of Molecular Immunology, Room 8N109, Building 10, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda MD 20892-1760. E-mail addresses: beavenn@nhlbi.nih.gov or mah@nhlbi.nih.gov

$^2$Current address: Division of Hematology, Center for Biologies Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892. 

$^3$Current address: Department of Dermatology, Hiroshima University Faculty of Medicine, 1-2-3, Kasumi, Minami-ku 734-8551, Hiroshima, Japan.

$^4$Abbreviations used in this paper: SOCE, store-operated calcium entry; TRP, transient receptor potential; TRPC, canonical TRP, CRAC, calcium release-activated calcium; $I_{\text{CRAC}}$, CRAC current; eYFP, enhanced yellow fluorescent protein; PLC, phospholipase C; siRNA, small inhibitory RNA; shRNA, short hairpin RNA.

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T cells by use of inhibitory RNAs (33). Of the two known mammalian homologs (STIM1 and STIM2), STIM1 appears to be the primary sensor of Ca\(^{2+}\) in intracellular stores in mammalian cells (33–37). STIM1 is strategically located in the endoplasmic reticulum and knock down of STIM1, but not of STIM2, by small inhibitory RNA (siRNA) abolishes SOCE and CRAC following Ca\(^{2+}\)-store depletion. The second component of the CRAC channel was identified as a mammalian homolog of Drosophila Orai (38) also referred to as CRACM (39). A key discovery was the presence of an inactivating mutation of Orai in a patient with severe immunodeficiency (SCID) that was associated with low CRAC channel activity in T cells (38, 40). Subsequent studies showed that coexpression of Orai1 and STIM1 dramatically enhanced CRAC and SOCE (41–43). A paradoxical and unexplained finding was that overexpression of Orai1 by itself suppresses Ca\(^{2+}\) entry. In addition, the electrophysiological features of coexpressed Orai1 and STIM1 did not match those originally described for CRAC, which left the possibility that additional molecules might be involved (32). Recent work now indicates that TRPC channels may associate with STIM1 and Orai1 and thus alter channel properties as well as enhance Ca\(^{2+}\) entry (44–46). For example, transfection of cells with exogenously tagged proteins indicated that STIM1 can interact with TRPC1, TRPC4, and TRPC5 to regulate their activities (46). STIM1 also colocalizes with both TRPC1 and Orai1 and thus regulates SOCE (44). Interestingly, TRPC3 and TRPC6, which normally operate as receptor-activated and store-independent channels, appear to operate in a store-dependent manner when coexpressed with Orai1 (45).

We investigated the potential role of endogenous TRPC channels in supporting entry of Ca\(^{2+}\) and Sr\(^{2+}\) as well as degranulation in a rat mast cell line RBL-2H3 by overexpression or knock down of TRPC channel proteins, Orai1, and STIM1. As reported in this study, we found that, among the various TRPC channel proteins expressed in these cells, TRPC5 permitted influx of Sr\(^{2+}\), optimal influx of Ca\(^{2+}\), and degranulation. However, TRPC5 function was dependent on Orai1 and STIM to suggest that TRPC5 acted in conjunction with these two proteins.

### Materials and Methods

#### Materials

Reagents were purchased from the following: medium, culture reagents, and Platinum Blue PCR SuperMix from Invitrogen Life Technologies; mouse monoclonal anti-DNP IgE, dimethylphenylamylamine human serum albumin, and p-nitrophenyl-N-acetyl-\(\beta\)-D-glucosaminide from Sigma-Aldrich; thapsigargin, 1-oxethyl-2-acetyl-sul-glycerol, U73122, and RH80267 from Calbiochem; Abs against TRPC1, TRPC3, and TRPC5 from Alomone Laboratories and against TRPC2 from Chemicon International; TRPC7 Ab from Bethyl Laboratories; Abs against Orai1 from ProSci; Abs against STIM1 from BD Biosciences; and fura 2-AM ester from Molecular Probes. Anti-TRPC3 siRNA from OriGene Technologies. Although all available shRNA constructs from OriGene Technologies were tested, representative data are shown with the following constructs (product number): T1548839, T1100018, T1502171, T1304029, and T1336191 to knock down TRPC1, TRPC5, TRPC7, Orai1, and STIM1, respectively. All other chemicals were molecular biology grade from several sources.

#### Anti-TRPC3 siRNA

The siRNA targeted against rat TRPC3 was generated with the Silencer Express kit, pSCE-neo (Ambion), and the oligonucleotide (the target sequence was 5′–561 of rat TRPC3, AB022351) sense 5′-GTGCTACACAAACACACCTACTACGAAAGCCTGGTTGCCTCCCTTCAAGG-3′ and anti-sense 5′-CGCGCAAGAGCTCTTCTTTCACAAAAGCATTCTGACAGTGCTCACACAAACAC-3′, according to the manufacturer’s instruction. A negative control siRNA was generated by using the oligonucleotides that were provided in the Silencer Express kit.

### Detection of mRNA for TRPCs by RT-PCR

RBL-2H3 cells (5 × 10\(^6\) cells) were used for extraction of RNA as described elsewhere (47). The Advantage RT-for-PCR kit (Clontech Laboratories) was used to transcribe 1 μg of RNA to cDNA, of which 1 μg was used for PCR with gene-specific primers for rat TRPC family members (48) (from Lofstrand Labs) and β-actin. PCR amplification was performed with Platinum Blue PCR SuperMix (Invitrogen Life Technologies) under the following conditions for 40 cycles: 30 s at 94°C (denature), 30 s at 56°C (anneal), 1 min at 72°C (extend), and final extension at 10 min at 72°C. Rat brain mRNA (Ambion) was processed with the same protocol and was used as a positive control for TRPC expression.

#### Transient transfection, stimulation of cells, and immunoblotting

RBL-2H3 and the muscarinic m1 receptor-bearing RBL-2H3(m1) cells (49, 50) were cultured in complete growth medium as previously described (51). Cells were transiently transfected by electroporation (Cell Line Nucleofector kit L, Amaxa transfection system) with the plasmids or inhibitory RNAs (1 μg/10\(^6\) cells) along with the expression vector that encodes enhanced yellow fluorescent protein (eYFP) (pd2EYFP-N1; Clontech Laboratories) in the ratio of 5:1. Cells were examined 24 or 48 h after transfection with the inhibitory RNAs or plasmids, respectively. Transfection efficiency ranged from 50% to 80%. Where necessary, cells were incubated overnight with IgE (50 ng/ml) to achieve 100% occupancy of FceRI. Cells were stimulated with 100 ng/ml Ag (dinitrophenylated human serum albumin) or 1 μM thapsigargin to achieve maximal responses. Immunoblotting of whole cell lysates were performed as described elsewhere (51).

#### Measurement of degranulation

Cells in 24-well plates (2 × 10\(^4\) cells/0.4 ml/well) were washed and the medium replaced with a PIPES-buffered medium (25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM MgCl\(_2\), 1 mM CaCl\(_2\), 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum) before stimulation. Where indicated, Ca\(^{2+}\) was omitted from the medium or 3 mM Sr\(^{2+}\) was substituted for Ca\(^{2+}\). Degranulation was determined by measurement of the release of the granule marker, β-hexosaminidase, by use of a colorimetric assay in which release of p-nitrophenol from p-nitrophenyl N-acetyl-\(\beta\)-D-glucosaminide was measured (1). Values were expressed as the percentage of intracellular β-hexosaminidase that was released into the medium.

#### Imaging of intracellular calcium in single cells

After transfection, cells were grown on coverslips in complete growth medium for 48 h. Medium was then replaced with PIPES-buffered medium (see previous section) and loaded with fura 2-AM (2 μM) for 25 min at 25°C. Cells were washed, and the dye was allowed to de-esterify for a minimum of 15 min at 25°C. The coverslips were placed in Ca\(^{2+}\)-free PIPES-buffered medium and 1 mM CaCl\(_2\) or 3 mM SrCl\(_2\) was added as shown in the experiments. Cytosolic Ca\(^{2+}\) was measured in individual transfected and nontransfected cells in the InCyt dual wavelength fluorescence imaging system (Intracellular Imaging). eYFP served as the transfection marker in cells and was detected at an excitation wavelength of 485 nm. Nontransfected cells (not expressing eYFP) were identified from the same field and served as control cells. After cell identification, fluorescence emission at 505 nm was monitored with alternating excitation at wavelengths 340 and 380 nm. Relative intracellular content of divalent cation (\(\text{Ca}^{2+}\) or \(\text{Sr}^{2+}\)) is depicted as the ratio of fluorescence (340/380 nm) for 10–20 single transfected and nontransfected cells as previously described (52). It should be noted that Sr\(^{2+}\) has much lower affinity for fura 2 than Ca\(^{2+}\) (estimated 
\(K_a\) = 0.23 μM for Ca\(^{2+}\) and 2.6 μM or greater for Sr\(^{2+}\)) (53–55), although the fluorescence ratio is similar for both ions at half maximal saturation of fura 2 (55). Therefore, the relative increases in the fluorescence ratios for Sr\(^{2+}\) and Ca\(^{2+}\) understate the actual concentration of cytosolic Sr\(^{2+}\) compared with that of Ca\(^{2+}\) in the data shown in this study. All measurements are representative of three or more independent experiments.

### Results

\(\text{Sr}^{2+}\) entry is dependent on store depletion

Previous findings that Sr\(^{2+}\) can substitute for Ca\(^{2+}\) to support degranulation in stimulated RBL-2H3 cells (13) raise the possibility that Ca\(^{2+}\)-conducting channels also conduct Sr\(^{2+}\). To examine whether entry of Sr\(^{2+}\), like Ca\(^{2+}\), is dependent on depletion of...
Identification of TRPC5 as a channel for store-dependent Sr\(^{2+}\) entry

Intracellular stores of Ca\(^{2+}\), we compared the effects of thapsigargin to those of Ag and the muscarinic agonist, carbachol, in RBL-2H3 cells, a stably transfected cell line made to express the G protein-coupled muscarinic m1 receptor (49, 50). In contrast to thapsigargin, which minimally stimulates PLC in RBL-2H3 cells (56), Ag and carbachol activate PLC\(\gamma\) (57) and PLC\(\beta\) (58), respectively. Both Ag and carbachol induced a transient Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores in the absence of external Ca\(^{2+}\) followed by Sr\(^{2+}\) or Ca\(^{2+}\) entry when either of these ions were added externally (Fig. 1, A and B). Depletion of the intracellular Ca\(^{2+}\) stores by thapsigargin, which does so by blocking uptake of cytosolic Ca\(^{2+}\) into these stores (6), also induced Sr\(^{2+}\) and Ca\(^{2+}\) entry (Fig. 1C). These results suggested that entry of Sr\(^{2+}\) and Ca\(^{2+}\) was dependent on depletion of Ca\(^{2+}\) stores regardless of the mechanism of depletion.

Another indication that entry of Sr\(^{2+}\), as well as Ca\(^{2+}\), can occur independently of PLC was that the PLC inhibitor, U73122, inhibited release of intracellular Ca\(^{2+}\) and reduced entry of Sr\(^{2+}\) and Ca\(^{2+}\) when cells were stimulated by either Ag or carbachol (Fig. 1, D and E) but it failed to suppress entry of these ions when Ca\(^{2+}\) stores were depleted with thapsigargin (Fig. 1F). However, the entry of both Sr\(^{2+}\) and Ca\(^{2+}\) in response to all three stimuli was blocked by La\(^{3+}\) (Fig. 1, G-I) at a concentration (3 \(\mu\)M) that is known to block CRAC activity in mast cells (7).

Identification of TRPC5 as a channel for store-dependent Sr\(^{2+}\) and Ca\(^{2+}\) entry

Messenger RNAs for several TRPC channel proteins were identified in RBL-2H3 cells by RT-PCR. These included TRPC1, TRPC2, TRPC3, TRPC5, and TRPC7 (Fig. 2A). Of these, TRPC1, TRPC3, and TRPC5 could be detected at the protein level (Fig. 2B), whereas TRPC2, a pseudo-gene in some but not all species (59, 60), was not detectable in RBL-2H3 cells. Abs against TRPC7 lacked sufficient specificity for this purpose.

Knock down of TRPC1, TRPC3, TRPC5 (Fig. 2B), and TRPC7 with shRNAs indicated that only knock down of TRPC5 substantially impaired entry of Sr\(^{2+}\) and Ca\(^{2+}\) in thapsigargin-stimulated cells (Fig. 3, A–E). Entry of these cations was also impaired by prior transfection of cells with empty vector (EV) or shRNA against the indicated TRPC protein to assess the effect of the shRNA on TRPC expression. Data are the percentage of decrease in expression as determined by densitometry.

Although entry of Sr\(^{2+}\) and Ca\(^{2+}\) is dependent on store depletion in RBL-2H3 cells (Fig. 1) and TRPC5 is reported to be activated by store-depletion (26), overexpressed TRPC5 can be activated in a PLC/diacylglycerol-dependent manner (61). However, we found that the diacylglycerol cell-permeant analog, 1-oleoyl-2-acetyl-sn-glycerol, or the use of the lipase inhibitor RHC80267 to increase diacylglycerol levels (30), failed to stimulate activate entry of Ca\(^{2+}\) or Sr\(^{2+}\) (data not shown). Together, these results suggested that of the TRPC family members, TRPC5 is a likely component of a SOCE channel in RBL-2H3 cells, although a minor role for TRPC1 cannot be excluded.
Endogenous STIM1 and Orai1 also regulate store-operated Sr\(^{2+}\) and Ca\(^{2+}\) entry

STIM1, the sensor of free Ca\(^{2+}\) in calcium stores in the endoplasmic reticulum, was originally believed to regulate SOCE through its interaction with Orai1 to activate CRAC (32). Recent studies now suggest that STIM1 can also interact with TRPC proteins individually or in combination with Orai1.

We found that endogenous STIM1 as well as Orai1 were essential for TRPC5 function because knock down or either STIM1 or Orai1 by use of shRNA significantly suppressed thapsigargin-induced Sr\(^{2+}\) as well as Ca\(^{2+}\) entry (Fig. 4). Therefore, both TRPC5 and Orai1 are required for Sr\(^{2+}\) and Ca\(^{2+}\) entry. Presumably, STIM1 serves as an essential sensor for the state of depletion of calcium stores.

Effects of overexpressed Orai1 or TRPC5 with STIM1 on store-operated Sr\(^{2+}\) and Ca\(^{2+}\) entry

To verify that Orai1, TRPC5, and STIM1 acted in combination to facilitate entry of either Ca\(^{2+}\) or Sr\(^{2+}\), we examined the effects of

FIGURE 3. Knock down of TRPC channel proteins by shRNA shows that entry of Sr\(^{2+}\) and Ca\(^{2+}\) is dependent on TRPC5. Cells were cotransfected with empty vector (EV) (A) or the indicated inhibitory RNA (B–E) and eYFP 48 h before measurement of Ca\(^{2+}\) and Sr\(^{2+}\) in fura 2-loaded cells. Measurements were made in eYFP-positive cells (green traces) and -negative cells (red traces) to compare responses in transfected and nontransfected cells, respectively. The traces show changes in ion levels 10 min after stimulation with 1 \(\mu\)M thapsigargin (Tg) in Ca\(^{2+}\)-free medium when intracellular stores were depleted of Ca\(^{2+}\). Cells were then exposed to medium containing Sr\(^{2+}\) (3 mM) or Ca\(^{2+}\) (1 mM) where labeled. Data show traces for individual cells that are representative of a field of at least 10 cells for both eYFP-positive and -negative cells. Similar results were obtained in two additional experiments.

FIGURE 4. Knock down of STIM1 and Orai1 also suppress entry of Sr\(^{2+}\) and Ca\(^{2+}\) in thapsigargin-stimulated cells. A, Cells were cotransfected with the indicated shRNA and eYFP 48 h before measurement of Ca\(^{2+}\) and Sr\(^{2+}\) in fura 2-loaded cells. Measurements were made in eYFP-positive cells (green traces) and -negative cells (red traces) to compare responses for STIM1 (A) and Orai1 (B) in transfected and nontransfected cells, respectively. The traces show changes in ion levels 10 min after stimulation with 1 \(\mu\)M thapsigargin (Tg) in Ca\(^{2+}\)-free medium when intracellular stores were depleted of Ca\(^{2+}\). Cells were then exposed to medium containing Sr\(^{2+}\) (3 mM) or Ca\(^{2+}\) (1 mM) where labeled. Data show traces for individual cells that are representative of a field of at least 10 cells for both eYFP-positive and -negative cells. Similar results were obtained in two additional experiments.

FIGURE 5. Overexpression of STIM1 and Orai1 reveal different features for the entry of Sr\(^{2+}\) and Ca\(^{2+}\). Cells were cotransfected with eYFP and STIM1, Orai1, or STIM1 plus Orai1 in combination as indicated 48 h before measurement of Ca\(^{2+}\) and Sr\(^{2+}\) in fura 2-loaded cells. Measurements were made in eYFP-positive cells (green traces) and -negative cells (red traces) to compare responses in transfected and nontransfected cells, respectively. The traces show changes in intracellular ion levels before and after addition of Sr\(^{2+}\) (3 mM) or Ca\(^{2+}\) (1 mM) as labeled. Two sets of experiments (A, D, and G and B, E, and H) show traces before addition of thapsigargin. C, F, and I, Traces 10 min after depletion of intracellular Ca\(^{2+}\) stores with 1 \(\mu\)M thapsigargin (Tg). Data show traces for individual cells that are representative of a field of at least 10 cells for both eYFP-positive and -negative cells. Similar results were obtained in two additional experiments.
overexpression of these molecules in RBL-2H3 cells. Of note, overexpression of STIM1 along with Orai1 or TRPC5 permitted constitutive entry of these cations in nonstimulated cells. This finding is illustrated in Figs. 5 and 6, which show typical traces for fura 2-loaded cultures before and after depletion of intracellular Ca\textsuperscript{2+} stores with thapsigargin.

Overexpression of STIM1 by itself resulted in little or no constitutive entry of Sr\textsuperscript{2+} but did result in modest influx of Ca\textsuperscript{2+} in the absence of stimulation (Fig. 5, A and B). Otherwise, the overexpression of STIM1 did not affect the stimulated entry of either Sr\textsuperscript{2+} or Ca\textsuperscript{2+} after depletion of Ca\textsuperscript{2+} stores with thapsigargin (Fig. 5C). In contrast to STIM1, overexpression of Orai1 did not permit constitutive entry of Sr\textsuperscript{2+} or Ca\textsuperscript{2+} and suppressed entry of these ions in thapsigargin-stimulated cells (Fig. 5, D–F). However, coexpression of STIM1 with Orai1 permitted constitutive entry of Ca\textsuperscript{2+}, but not Sr\textsuperscript{2+} (Fig. 5, G and H), and fully restored entry of Ca\textsuperscript{2+} after depletion of Ca\textsuperscript{2+} stores with thapsigargin, whereas Sr\textsuperscript{2+} entry remained blocked (Fig. 5I). The suppression of thapsigargin-induced Ca\textsuperscript{2+} entry following overexpression of Orai1 and restoration of Ca\textsuperscript{2+} entry on coexpression with STIM1 has been noted by other investigators in different cell lines (41–43). Our results show, in addition, that the coexpression of STIM1 and Orai1 allows substantial constitutive entry of Ca\textsuperscript{2+} (Fig. 5H), but excludes entry of Sr\textsuperscript{2+} under all conditions (Fig. 5I).

In contrast to Orai1, overexpression of TRPC5 resulted in some constitutive entry of Sr\textsuperscript{2+} and Ca\textsuperscript{2+} as well as degranulation in Ag-stimulated cells (Fig. 6A) and suppressed entry of both ions in thapsigargin-treated cells but to a lesser extent than Orai1 (Fig. 6B). In combination with STIM1, the constitutive entry of Sr\textsuperscript{2+} and Ca\textsuperscript{2+} was substantially enhanced (Fig. 6C) and entry of both ions was fully restored in thapsigargin-treated cells (Fig. 6D).

These data demonstrate that transient coexpression of Orai1 and STIM1 allows influx of only Ca\textsuperscript{2+} (Figs. 5 and 6), whereas coexpression of TRPC5 and STIM1 allows influx of Sr\textsuperscript{2+} as well as Ca\textsuperscript{2+}. The studies with shRNAs (Figs. 3 and 4) had indicated that endogenous TRPC5 as well as STIM1 and Orai1 are required for optimal entry Sr\textsuperscript{2+} and Ca\textsuperscript{2+} following depletion of Ca\textsuperscript{2+} stores with thapsigargin to suggest that all three proteins interact with each other.

**RESULTS**

**TRPC5, STIM1, and Orai1 are essential for entry of Sr\textsuperscript{2+} and Ca\textsuperscript{2+} as well as degranulation in Ag-stimulated cells.** As with thapsigargin-stimulated cells, influx of Sr\textsuperscript{2+} and Ca\textsuperscript{2+} was significantly suppressed when cells were stimulated with Ag following knock down of TRPC5, STIM1, or Orai1 (Fig. 7, B–D) as compared with empty vector transfected cells (Fig. 7A). Ag-stimulated release of intracellular Ca\textsuperscript{2+} was unaffected. Knockdown of each of these proteins also significantly impaired degranulation in

**FIGURE 6.** Overexpression of STIM1 and TRPC5 reveal that TRPC5 conducts both Sr\textsuperscript{2+} and Ca\textsuperscript{2+}. Cells were cotransfected with eYFP and TRPC5 alone or in combination with STIM1 as indicated 48 h before measurement of Ca\textsuperscript{2+} and Sr\textsuperscript{2+} in fura 2-loaded cells. Measurements were made in eYFP-positive cells (green traces) and -negative cells (red traces) to compare responses in transfected and nontransfected cells, respectively. The traces show changes in intracellular levels of Ca\textsuperscript{2+}-free medium without stimulation. Cells were exposed to medium containing Sr\textsuperscript{2+} (1 mM) or Ca\textsuperscript{2+} (1 mM) as labeled. A and C. Traces before stimulation. B and D. Traces 10 min after addition of 1 μM thapsigargin (Tg) when intracellular stores were depleted of Ca\textsuperscript{2+} in the absence of extracellular Ca\textsuperscript{2+}. Cells were then exposed to medium containing Sr\textsuperscript{2+} (3 mM) or Ca\textsuperscript{2+} (1 mM) where labeled. Data show traces for individual cells that are representative of a field of at least 10 cells for both eYFP-positive and -negative cells. Similar results were obtained in two additional experiments.

**FIGURE 7.** TRPC5, STIM1, and Orai1 are essential for entry of Sr\textsuperscript{2+} and Ca\textsuperscript{2+} as well as degranulation in Ag-stimulated cells. A–D. As in previous experiments, changes in intracellular Ca\textsuperscript{2+} and Sr\textsuperscript{2+} were determined in fura 2-loaded cells (in Ca\textsuperscript{2+}-free medium) following addition of Ag, 1 mM Ca\textsuperscript{2+}, or 3 mM Sr\textsuperscript{2+} where labeled. Cells were previously transfected with empty vector (EV) or shRNA against TRPC5, STIM1, or Orai1 along with eYFP to allow selection of transfected (green trace) and nontransfected (red trace) cells for imaging. E. Degranulation was determined in the presence of 1 mM Ca\textsuperscript{2+} by measurement of the release of the granule marker, β-hexosaminidase, into the medium in nonstimulated (NS) and Ag-stimulated cultures 15 min after addition of Ag. Cells had been transfected without vector or the indicated shRNA. *p < 0.01, indicating significant decrease in degranulation. F. The extent of degranulation in normal RBL-2H3 cells in the absence or presence of 1 mM Ca\textsuperscript{2+}, 1 mM Sr\textsuperscript{2+}, or 3 mM Sr\textsuperscript{2+} is shown. The transfection efficiency was 70–80% for the experiments shown in E and F. Data are the mean ± SEM of three cultures. Similar results were obtained in two additional experiments.
Ag-stimulated cells (Fig. 7E). As in previous studies (13), Sr^{2+} could substitute for Ca^{2+} in promoting degranulation, partially so at 1 mM and fully so at 3 mM Sr^{2+} (Fig. 7F). If TRPC5 is the carrier of Sr^{2+} as the preceding data suggests, TRPC5 would appear to be essential for degranulation as well as SOCE.

Discussion

The original studies of Ca^{2+}-dependent exocytosis (11, 12) and our early studies (2, 13) showed that Sr^{2+} and other divalent cations are taken up by stimulated mast cells and can replace Ca^{2+} in promoting degranulation. These observations preclude CRAC as the sole mechanism for conveyance of Ca^{2+} promoting degranulation. These interactions have been demonstrated by coimmunoprecipitation of STIM1 with various TRPC channel proteins (46). These interactions have been demonstrated by coinmunoprecipitation of expressed tagged proteins (44–46) or, in one case, endogenous TRPC1 with STIM1 (44). Our attempts to demonstrate similar associations for endogenous proteins in RBL-2H3 cells were unsuccessful because of the limitations of the available Abs. As noted earlier, overexpression of Orai1 suppresses Ca^{2+} entry (41–43). This and our finding that suppression of Sr^{2+} or Ca^{2+} influx by overexpression of Orai1 (Fig. 5D) or TRPC5 (Fig. 6B) could indicate that channel competency requires stoichiometric interaction of Orai1 with TRPC5 or STIM1. One possibility is that overexpressed Orai1 may preferentially oligomerize with itself (64, 65) rather than with TRPC5, and vice versa, with loss of conductance of Sr^{2+} and Ca^{2+} through a TRPC5-Orai1 complex. A caveat is that overexpression may lead to protein interactions that do not normally occur. The constitutive entry of cations noted in this study with overexpressed STIM1 in conjunction with Orai1 or TRPC5 might indicate such interactions.

A possible indication that Orai1 and TRPC5 are both necessary for cation influx in RBL-2H3 cells is that overexpression of either protein with STIM1 did not further enhance thapsigargin-induced Ca^{2+} influx (Figs. 5 and 6). This finding is in contrast to studies in human embryonic kidney HEK293 cells (41–43) where such influx was enhanced by overexpression of Orai1 and STIM1. However, thapsigargin fails to stimulate influx of Sr^{2+} in HEK293 cells (66) and it is unlikely that TRPC proteins interact with Orai1 for Ca^{2+} entry in these cells. If, however, both Orai1 and TRPC5 are components of CRAC, as appears to be the case in RBL-2H3 cells, then overexpression of one of these two CRAC proteins would not further enhance entry of Ca^{2+} or Sr^{2+} because ion conductance would still be dependent on endogenous levels of the other CRAC protein.

Although TRPC5 as well as Orai1 and STIM1 are essential for influx of Ca^{2+} and degranulation in RBL-2H3 cells (Fig. 7), we cannot exclude a minor contribution of TRPC1, although this protein is thought not to be an endogenous component of CRAC in RBL-2H3 cells (44). Also, given the phenotypic diversity of mast cells (67, 68) and the possible variations in the combinations of Orai and TRPC family members, it is possible that such combinations may vary among different subtypes of mast cells.

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Disclosures

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