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**Canonical Transient Receptor Potential 5 Channel in Conjunction with Orai1 and STIM1 Allows Sr²⁺ Entry, Optimal Influx of Ca²⁺, and Degranulation in a Rat Mast Cell Line**

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

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Degranulation of mast cells in response to Ag or the calcium mobilizing agent, thapsigargin, is dependent on emptying of intracellular stores of Ca\textsuperscript{2+} and the ensuing influx of external Ca\textsuperscript{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\textsuperscript{2+} because Sr\textsuperscript{2+} and other divalent cations also permeate and support degranulation in stimulated mast cells. In this study we show that influx of Ca\textsuperscript{2+} and Sr\textsuperscript{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\textsuperscript{2+} but not Sr\textsuperscript{2+}, whereas overexpression of STIM1 and TRPC5 allows entry of both Ca\textsuperscript{2+} and Sr\textsuperscript{2+}. These and other observations suggest that the Sr\textsuperscript{2+}-permeable TRPC5 associates with STIM1 and Orai1 in a stoichiometric manner to enhance entry of Ca\textsuperscript{2+} to generate a signal for degranulation. The Journal of Immunology, 2008, 180: 2233–2239.

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

Replacement of Ca\textsuperscript{2+} with Sr\textsuperscript{2+} or Ba\textsuperscript{2+} results in a decline in I\textsubscript{CRAC} activity in T lymphocytes (9, 10). Nevertheless, it appears unlikely that I\textsubscript{CRAC} is the sole mechanism for conductance of Ca\textsuperscript{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\textsuperscript{2+}, Ba\textsuperscript{2+}, and Mn\textsuperscript{2+} and that such ions can support degranulation in the absence of Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} and Ba\textsuperscript{2+} in addition to Ca\textsuperscript{2+} and none of the TRPC channel proteins appear to have the exact electrophysiological features of CRAC (32).

The molecular identity of the I\textsubscript{CRAC} putative channel CRAC has been clarified recently with the identification of two proteins, STIM1, a Ca\textsuperscript{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
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 Orai1 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 STIM1 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, dinitrophenylated human serum albumin, and p-nitrophenyl N-acetyl-β-D-glucosaminide from Sigma-Aldrich; thapsigargin, 1-octyl-2-acetyl-sn-glycerol, U73122, and RH80267 from Calbiochem; Abs against TRPC1, TRPC3, and TRPC5 from Alomone Laboratories and against TRPC2 from Chemicon International; TRPC7 from Bethyl Laboratories; Abs against Orai1 from ProSci; Abs against STIM1 from BD Biosciences; and fura 2-AM ester from Molecular Probes. Although all available Abs were obtained from OriGene Technologies. All other chemicals were purchased from the following: medium, culture reagents, and degranulation. However, TRPC5 function was dependent on Orai1 and STIM1 to suggest that TRPC5 acted in conjunction with these two proteins.

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’-GCTACACAAAACCACACCTGACTGAAAGCTGTGGTTGCCCTTCCACAAG-3’ and anti-sense 5’-CGGCCGAAGCTTTTCTCCCAAAGAAGCTGTCGTAAGCTGCTACACAAAACCAC-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 murine mast cells were transfected with pcDNA3 (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 FcεRI. 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-β-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 washed 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 (Ca\(^{2+}\) or Sr\(^{2+}\) ) is depicted as the average ratio of fluorescence (340/380 nm) in 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\(_{d}\) ~ 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

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...
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 expression 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 unimpaired by prior transfection of cells with empty vector (Fig. 3A) or with shRNA against TRPC2 (data not shown). A small decrease in entry of cations was observed with knockdown of TRPC1 although the expression of this protein was decreased to a similar extent as was TRPC5 (Fig. 2B). Other control experiments showed that shRNA against TRPC5 had no effect on expression of TRPC1 (data not shown).

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.

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

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We found that endogenous STIM1 as well as Orai1 were essential for TRPC5 function because knockdown of 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 µM 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 µM 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 µM 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.

Effects of overexpressed Orai1 or TRPC5 with STIM1 on store-operated Sr$^{2+}$ and Ca$^{2+}$ entry
The traces show changes in intracellular ion levels in Ca\(^{2+}\) to compare responses in transfected and nontransfected cells, respectively. 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 in Ca\(^{2+}\)-free medium without stimulation. Cells were exposed to medium containing Sr\(^{2+}\) (3 mM) or Ca\(^{2+}\) (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\(^{2+}\) in the absence of extracellular 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.

Our results show, in addition, that the coexpression of STIM1 and Orai1 allows substantial constitutive entry of Ca\(^{2+}\) (Fig. 5H), but excludes entry of Sr\(^{2+}\) under all conditions (Fig. 5I).

In contrast to Orai1, overexpression of TRPC5 resulted in some constitutive entry of Sr\(^{2+}\) and Ca\(^{2+}\) 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\(^{2+}\) and Ca\(^{2+}\) 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\(^{2+}\) (Figs. 5 and 6), whereas coexpression of TRPC5 and STIM1 allows influx of Sr\(^{2+}\) as well as Ca\(^{2+}\). 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\(^{2+}\) and Ca\(^{2+}\) following depletion of Ca\(^{2+}\) stores with thapsigargin to suggest that all three proteins interact with each other.

TRPC5, STIM1, and Orai1 are required for cation influx and degranulation in Ag-stimulated cells

As with thapsigargin-stimulated cells, influx of Sr\(^{2+}\) and Ca\(^{2+}\) 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\(^{2+}\) was unaffected. Knock down of each of these proteins also significantly impaired degranulation in

In contrast to Orai1, overexpression of TRPC5 resulted in some constitutive entry of Sr\(^{2+}\) and Ca\(^{2+}\) as well as degranulation in Ag-stimulated cells. A–D. As in previous experiments, changes in intracellular Ca\(^{2+}\) and Sr\(^{2+}\) were determined in fura 2-loaded cells (in Ca\(^{2+}\)-free medium) after addition of Ag, 1 mM Ca\(^{2+}\), or 3 mM Sr\(^{2+}\) where labeled (Fig. 6A). 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\(^{2+}\) 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 with empty 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\(^{2+}\), 1 mM Sr\(^{2+}\), or 3 mM Sr\(^{2+}\) 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\(^{2+}\) into the cell because of the high Ca\(^{2+}\) selectivity of CRAC (62, 63) and the diminution of CRAC activity on replacement of Ca\(^{2+}\) with other divalent cations (9, 10).

It has been suggested that the channel properties of Orai1 may be modified by other proteins because the properties of CRAC, especially its low conductivity and specificity for Ca\(^{2+}\), are not entirely recapitulated by overexpressed Orai1 and STIM1 (32). We suggest that TRPC5 may be one such protein that allows entry of Sr\(^{2+}\) and Ca\(^{2+}\) into RBL-2H3 cells. Although influx of these two ions is readily distinguishable (Figs. 5 and 6), entry of both are dependent on TRPC5 (Fig. 3D), STIM1 (Fig. 4A), and Orai1 (Fig. 4B). A plausible scenario is that entry is dependent on formation of a ternary complex of TRPC5/Orai1/STIM1 in which STIM1 acts (44). A possible indication that Orai1 and TRPC5 are both necessary store-operated Ca\(^{2+}\) entry in these cells. If, however, both Orai1 and TRPC5 are present, the channel competence 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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