Orai1 and TRPC1 have been proposed as core components of store-operated calcium release-activated calcium (CRAC) and store-operated calcium (SOC) channels, respectively. STIM1, a Ca$^{2+}$ sensor protein in the endoplasmic reticulum, interacts with and mediates store-dependent regulation of both channels. We have previously reported that dynamic association of Orai1, TRPC1, and STIM1 is involved in activation of store-operated Ca$^{2+}$ entry (SOCE) in salivary gland cells. In this study, we have assessed the molecular basis of TRPC1-SOC channels in HEK293 cells. We report that TRPC1 + STIM1-dependent SOCE requires functional Orai1. Thapsigargin stimulation of cells expressing Orai1 + STIM1 increased Ca$^{2+}$ entry and activated typical I$_{\text{CRAC}}$ current. STIM1 alone did not affect SOCE, whereas expression of Orai1 induced a decrease. Expression of TRPC1 induced a small increase in SOCE, which was greatly enhanced by co-expression of STIM1. Thapsigargin stimulation of cells expressing TRPC1 + STIM1 activated a non-selective cation current, I$_{\text{ISC}}$, that was blocked by 1 mM Gd$^{3+}$ and 2-APB. Knockdown of Orai1 decreased endogenous SOCE as well as SOCE with TRPC1 alone. siOrai1 also significantly reduced SOCE and I$_{\text{ISC}}$ in cells expressing TRPC1 + STIM1. Expression of R91W,Orai1 or E106Q,Orai1 induced similar attenuation of TRPC1 + STIM1-dependent SOCE and I$_{\text{ISC}}$, whereas expression of Orai1 with TRPC1 + STIM1 resulted in SOCE that was larger than that with Orai1 + STIM1 or TRPC1 + STIM1 but not additive. Additionally, Orai1, E106Q,Orai1, and R91W,Orai1 co-immunoprecipitated with similar levels of TRPC1 and STIM1 from HEK293 cells, and endogenous TRPC1, STIM1, and Orai1 were co-immunoprecipitated from salivary glands. Together, these data demonstrate a functional requirement for Orai1 in TRPC1 + STIM1-dependent SOCE.

Store-operated Ca$^{2+}$ entry (SOCE) is mediated via activation of specific plasma membrane channels in response to depletion of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores (1). Neither the mechanism by which the status of Ca$^{2+}$ in the endoplasmic reticulum is transmitted to the plasma membrane nor the molecular components of the channels have yet been conclusively identified in all cell types. Several reports suggest a diversity in store-operated Ca$^{2+}$ channels in different cell types (2–4). For example, calcium release-activated calcium (CRAC) channel, which is found in T-lymphocytes, RBL, and other hematopoietic cells, is a highly Ca$^{2+}$-selective channel with unique properties (4, 5). Channels in other cell types, including salivary gland, endothelial, and smooth muscle cells, referred to as SOC channels, range from non-selective to relatively Ca$^{2+}$-selective (2–4, 6, 7). It is believed that the difference in channel property is due to differences in the channel components. TRPC1 is reported to form SOC channels that range from being relatively selective for Ca$^{2+}$ to those that are non-selective in various cell types (2, 7–16). With the exception of a few studies (17, 18), TRPCs do not appear to generate I$_{\text{CRAC}}$. Consistent with this, we have shown that TRPC1 does not contribute to the I$_{\text{CRAC}}$ in RBL-2H3 cells (19). Two proteins, STIM and Orai, have emerged as candidate components of the CRAC channel (5, 20). Knockdown of STIM1 expression using siRNA significantly reduced SOCE in a number of cell types (20–23), whereas overexpression only modestly enhanced SOCE. The second protein Orai1 has four transmembrane domains (5, 20, 24). Mutations in Orai1 have been genetically linked to severe combined immunodeficiency (SCID), and T-lymphocytes isolated from SCID patients display decreased I$_{\text{CRAC}}$ activity (20). Although knockdown of Orai1 decreases SOCE, overexpression of the protein attenuates endogenous SOCE. However, co-expression of Orai1 with STIM1 increases SOCE and generates CRAC channel activity in HEK293 cells (25, 26). Further, mutations in the conserved negatively charged residues of Orai1 alter the Ca$^{2+}$ selectivity of CRAC channel (27, 28). Thus, it has been suggested that Orai1 and STIM1 are sufficient for the formation of CRAC channel and that Orai1 is the pore-forming unit. The contribution of Orai1 proteins to SOCE in all cell types is not yet clear. Not all cells that express Orai proteins demonstrate CRAC currents (24, 29, 30). A recent report demonstrated that Orai1 does not form the CRAC channel in mouse T-lymphocytes (31). Thus, it has been suggested that Orai and STIM proteins might serve multiple functions and display very different biophysical properties in different cell types depending on the molecular composition of the channel complexes. Orai channel complexes might consist of not only different Orais and STIMs but also other channel subunits (24). The possibility of Orai1 being associated with a larger protein complex was also suggested (32).
Assembly of TRPC1-dependent SOC Channels

We have previously reported that dynamic assembly of a TRPC1-STIM1-Orai1 complex is involved in SOCE in salivary gland cells (19). We have shown that endogenous Orai1, STIM1, and TRPC1 are partially regulated in these cells. Although heterologous expression of TRPC1 alone does not increase SOCE, co-expression with STIM1 was reported to increase SOCE and SOC channel function in HEK293 cells (33, 34). Further, a suggestion has been made that Orai1 might function as a regulator of TRPC3 and TRPC6, conferring store-dependent activation of these channels (35). In this study, we have examined the molecular basis of TRPC1-dependent SOC channels. We report that in addition to STIM1, there is a functional requirement for Orai1 in the generation of TRPC1-SOC channels.

EXPERIMENTAL PROCEDURES

HEK293 Cell Culture and Transfection—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium and 10% heat-inactivated fetal bovine serum supplemented with 100 units/ml penicillin G and 100 µg/ml streptomycin. Cells were allowed to grow to ~70% confluence and transfected with required DNA at concentrations of 1 µg/ml using Lipofectamine 2000 and protocols supplied by the manufacturer (Invitrogen). Knockdown experiments were carried out by transfection of Orai1 siRNA (sequence number ucaccguaggcaauaga) or control siRNA (Dharmacon, Chicago, IL), transfected at a concentration of 0.8 nmol/ml, using DharmaFECT Duo reagent and protocols supplied by the manufacturer.

Electrophysiology—Coverslips with HEK cells were transferred to the recording chamber and perfused with standard external solution with the following composition (in mM): NaCl, 145; KCl, 5; MgCl2, 1; CaCl2, 1; Hepes, 10; glucose, 10; pH 7.4 (NaOH). The patch pipette had resistances between 3 and 5 milliohm after filling with the standard intracellular solution that contained the following (in mM): cesium methane sulfonate, 145; NaCl, 8; MgCl2, 10; Hepes, 10; EGTA, 10; pH 7.2 (CsOHa). Osmolarity for all the solutions was adjusted with mannose to 300 ± 5 mosm using a vapor pressure Osmometer (Wescor, Logan, UT). Whole cell patch clamp experiments were performed in the standard whole cell configuration at room temperature (22–25 °C) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Generation of the current was assessed by the amplitude at ~80 mV, taken from the currents recorded during voltage ramps ranging from ~90 to 90 mV over a period of 1 s imposed every 4 s (holding potential was 0 mV) and digitized at a rate of 1 kHz. Liquid-junction potentials were less than 8 mV and were not corrected. Capacitative currents and series resistance were determined and minimized. For analysis, current recorded during the first ramp was used for leak subtraction of the subsequent current records.

[Ca2+]i Measurements—Fura2 fluorescence was measured in single HEK cells cultured for 24–48 h in glass bottom MatTek tissue culture dishes (MatTek Corp. Ashland, MA) as well as by 20 µM 2-APB (supplemental Fig. 1), showing that TRPC1 + STIM1 primarily results in SOCE. Importantly, knockdown of endogenous Orai1 attenuated TRPC1- or TRPC1+STIM1-induced SOCE by >50% (i.e. the increase in 340/380 ratio in TRPC1 + STIM1 is about 0.7, whereas that in siOrai1 + TRPC1 + STIM1-expressing cells is 0.35. Note that these values represent the increase in 340/380 ratio above that in control cells following Ca2+ entry in these cells). 340/380 ratio above that in control cells following Ca2+ entry, i.e. due to Ca2+ entry). Thus, knockdown of endogenous Orai1 decreased STIM1-dependent increase in SOCE in TRPC1-expressing cells (SOCE in TRPC1 + STIM1 cells was 1.2 when compared with 0.7 in cells expressing TRPC1 alone, i.e. the increase due to STIM1 is 0.5. With expression of siOrai1 in both sets of cells, this increase was attenuated to 0.2, a 60%
decrease, Fig. 1, panel vii). Thus, endogenous Orai1 is required for exogenously expressed TRPC1 and STIM1 to increase SOCE. The data also show that the increase in TRPC1 function conferred by STIM1 is dependent on endogenous Orai1.

The contributions of TRPC1, STIM1, and Orai1 in SOCE were further examined by measuring store-operated currents. In contrast to the current seen with Orai1+STIM1 (Fig. 1, panels iii and iv), cells expressing TRPC1+STIM1 displayed linear currents following Tg stimulation, which could be blocked by 20 μM 2-APB (Fig. 1, panel ix). Similar inhibition was seen with 1 μM Gd^{3+} (data not shown). Note that about 40% of the cells displayed spontaneous currents, which in some cells could be further increased with Tg. In both cases, the current was fully blocked by 1 μM Gd^{3+} (supplemental Fig. 1). Together, these data demonstrate that TRPC1+STIM1 generate SOC channels that are distinct from CRAC channels in the property of their currents. The characteristics of TRPC1+STIM1 channels in these cells are similar to those described by Yuan et al. (34). Importantly, TRPC1+STIM1-induced $I_{SOCE}$ was significantly reduced by knockdown of endogenous Orai1 (Fig. 1, panels xi and xii, and see Fig. 2, panel x, for average data). Thus, TRPC1+STIM1-dependent SOC channel function also requires endogenous Orai1.

Together, the data shown in Fig. 1 demonstrate that TRPC1 requires overexpression of STIM1 to generate SOC channels in HEK293 cells. This most likely explains why in previous studies there was no increase in function associated with TRPC1 overexpression in these cells (4). A novel finding of the present study is that the ability of TRPC1 and STIM1 to increase SOCE and generate SOC channels is dependent on endogenous Orai1. We detected a small contribution of TRPC1 to endogenous SOCE (data not shown). However, shTRPC1 did not affect Orai1+STIM1-dependent increase in SOCE and $I_{CRAC}$ (data not shown). Consistent with
these findings, we have previously shown that endogenous TRPC1 does not contribute to I_{CRAC} in RBL-293 cells (19). Several recent studies have also suggested lack of a role for TRPC1 in Orai1/H11001 STIM1-generated CRAC channels in HEK293 cells (25, 26). Thus, the molecular requirement for generation of Orai1/H11001 STIM1-CRAC channels appears to be different from those of TRPC1/H11001 STIM1-SOC channels in this cell type. We have previously demonstrated an association between TRPC1- STIM1-Orai1 in human submandibular gland (HSG) cells. The present data would suggest that exogenously expressed STIM1 and TRPC1 associate with endogenous Orai1 to form a functional SOC channel in HEK293 cells. Unfortunately, the latter observation could not be determined biochemically due to the lack of an appropriate Orai1 antibody.

**Functional Orai1 Is Required for TRPC1+STIM1-dependent SOCE**—Severe loss of I_{CRAC} in T-lymphocytes isolated from SCID patients has been linked to a mutation (R91W) near the first TM domain of Orai1. The mutant channel has been shown to be inactive (20). Although exactly why this mutant is not functional is not yet understood, it is interesting that a conserved sequence in the N terminus of Orai1, amino acids 74–90 immediately upstream of the mutation site, has been suggested to be involved in activation of the channel (36). Expression of R91W Orai1 in HEK293 cells induced a decrease in endoge-
nous SOCE (Fig. 2, panel ii, trace not shown). Expression of TRPC1+STIM1 in cells expressing R91WOral1 resulted in SOCE similar to that seen in cells expressing TRPC1+STIM1+siOral1 (Fig. 2, panel i and ii, compare with Fig. 1, vii and viii). Similar inhibition of endogenous SOCE (trace not shown, Fig. 2, panel ii) as well as SOCE in TRPC1+STIM1-expressing cells was seen by expression of E106QOral1 (Fig. 2, panels i and ii). This mutant of Oral1 has decreased ion permeability and has been shown to exert dominant suppression of endogenous SOCE as well as Oral1+STIM1-dependent SOCE in HEK293 cells (27, 28). In contrast, expression of Oral1 together with TRPC1+STIM1 induced SOCE that was larger than that seen with TRPC1+STIM1 or Oral1+STIM1, but not additive (increase was calculated relative to SOCE in control cells, Fig. 2i, average data shown in panel ii). Expression of TRPC1+Oral1 without STIM1 did not change SOCE (data not shown).

Fig. 2, panel iii, shows that R91WOral1, E106QOral1, and Oral1 (all FLAG-tagged) were expressed at similar levels and co-immunoprecipitated with comparable levels of STIM1 as well as TRPC1 (note that none of the proteins were immunoprecipitated in control IPs using anti-FLAG antibody and lysates of non-transfected HEK cells, supplemental Fig. 2). Thus, the functional differences seen in cells expressing the wild type and mutant proteins are not due to differences in their expression levels or their ability to associate with STIM1 or TRPC1. Further, IP of TRPC1 pulls down similar level of STIM1 in cells where Oral1 was not overexpressed (data not shown), indicating that there is no disruption of STIM1-TRPC1 association by expression of Oral1 (also see Ref. 19). The association between the three proteins was also observed in submandibular gland cells. IP of endogenous TRPC1 co-immunoprecipitated endogenous Oral1 and STIM1 (Fig. 2, panel iii, right blot). These data provide strong evidence that there is close association between TRPC1, Oral1, and STIM1.

The requirement of functional Oral1 was assessed by using functionally deficient Oral1 mutants. Co-expression of R91WOral1 (the mutant in SCID patients) or E106QOral1 (the pore-deficient mutant) with TRPC1+STIM1 induced >70% inhibition of ISOC (Fig. 2, panels vi to ix, respectively, also see Fig. 2, panel x, for average data). Since siOral1 also induces similar attenuation of TRPC1+STIM1-dependent SOCE channel activity, it is unlikely that competition for STIM1 accounts for the observed attenuating effect of the mutant Oral1 proteins on TRPC1+STIM1-SOC function. In contrast, co-expression of Oral1 with TRPC1+STIM1 (Fig. 2, panel iv and v) induced non-selective linear current in 8/11 cells, which was 30% larger in amplitude than that seen with TRPC1+STIM1 or Oral1+STIM1 (Fig. 2, panels iv, v, and x). In 3/11 cells, the currents were weakly inwardly rectifying but relatively non-selective, i.e. more like ISOC. Note that when Oral1 cDNA was used during transfection was increased (5 μg instead of 1 μg), 3/7 cells displayed linear non-selective currents, 2/7 displayed ISOC-like currents with right shift in the Frev, and 2/7 displayed ICrAC-like currents (data not shown).

The data reported above demonstrate that co-expression of STIM1 with TRPC1 is required for generation of SOC channels in HEK293 cells. This is similar to the STIM1 requirement reported for the generation of CRAC channels in HEK293 cells by exogenous expression of Oral1 (5). Further, we show that TRPC1+STIM1 generate channels that display characteristics that are distinct from Oral1+STIM1-generated CRAC channels. These data are consistent with previous studies describing TRPC+STIM1-dependent SOC channel activity in HEK293 cells (33, 34). Together, these data also account for the previously reported lack of effect of heterologously expressed TRPC1 on SOCE.

An important and novel finding of the present study is that endogenous Oral1 is required for TRPC1+STIM1-generated SOC channel function. Further, non-functional Oral1 mutant, R91WOral1, or the permeability-defective mutant, E106QOral1, attenuate the function of TRPC1+STIM1-SOC channels, whereas Oral1 increases TRPC1+STIM1-dependent SOCE. Although we do not yet understand how exactly Oral1 contributes to TRPC1+STIM1 channel function, possible mechanisms that can be proposed are: (i) TRPC1 and Oral1 contribute to the same channel, (ii) Oral1 and TRPC1 form distinct channels, whereby the function of Oral1 somehow regulates TRPC1, and (iii) TRPC1 and Oral1 form distinct and independent channels. Although the present data do not exclude the first two possibilities, several of our observations, described here and previously (19), suggest that when co-expressed, TRPC1 and Oral1 do not form distinct and independent channels. First, increase in the current as well as Ca2+ entry obtained in cells expressing Oral1+STIM1+TRPC1 (increase in 340/380 ratio above control = 0.9) is less than that expected if Oral1+STIM1 and TRPC1+STIM1 generated separate channels (together the increase in 340/380 ratio should be 1.4). We do not believe that this is due to a competition between TRPC1 and Oral1 for STIM1 since transfection with different amounts of STIM1 cDNA gave similar results (data not shown). Also, co-IP of exogenously expressed TRPC1 and STIM1 was not affected by co-expression of Oral1 (19). Finally, siOral1 did not increase TRPC1+STIM1-mediated SOCE as would be expected if Oral1 were competing for a limited pool of STIM1. Although more studies are required to determine the precise functional association between TRPC1 and Oral1, based on our data, we propose that the proteins converge on the same SOC channel. Oral1 has been shown to contribute to the ion permeability of CRAC channel, which has been reported to be independent of TRPC1 and other TRPC proteins. However, a recent study showed that several neuronal cells express Oral1 proteins but do not display ICrAC (24), and it was suggested that Oral1 proteins might interact with each other or with other proteins to form diverse SOC channels. An interesting role for Oral1 proposed by Liao et al. (35) was that it serves as a regulatory subunit for TRPC3 and TRPC6 channels and confers coupling to the store by mediating the regulation by STIM1. Our previous study suggested a possible association between Oral1 and TRPC1 in cells expressing SOC channels (19). Here we have shown that functional Oral1 is required for generation of TRPC1-SOC channel. These data do not exclude the possibility that Oral1 and TRPC1 form the same SOC channel, although how this is achieved will need to be addressed in future studies. Alternatively, Oral1-CRAC channels might somehow regulate TRPC1-SOC channels. Although the exact contribution of
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Orai1 in TRPC1-dependent SOC channel function has yet to be determined, we have shown above that the three proteins are closely associated endogenously in salivary glands where TRPC1 has a major role in SOCE (19).

In conclusion, store-operated Ca\(^{2+}\) entry appears to be mediated via distinct channels in different cell types. Although some channels are Orai1+STIM-based, others depend on TRPC+STIM. We recently reported that TRPC1
\(^{-/}\) salivary gland cells had greatly reduced SOCE and I\(_{SOCE}\), which was associated with a significant and stable loss of fluid secretion, although all three Orai transcripts were detected in TRPC1
\(^{-/}\) cells (37). Thus, the Orai proteins were unable to compensate for the loss of TRPC1. Here we show that TRPC1 generates SOC channels in a STIM1-dependent manner and that these channels are distinct from CRAC channels. Importantly, our data demonstrate a novel functional requirement for Orai1 in TRPC1-generated SOC channel. Thus, STIM1 and Orai1 appear to be required for the generation of CRAC as well as SOC channels. Although Orai1+STIM1 appear to be sufficient for CRAC channels, TRPC1, Orai1, and STIM1 concertededly generate SOC channels. Further studies are required to determine the exact molecular interactions between these proteins that determine SOCE.

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