Overexpression of Orai1 and STIM1 Proteins Alters Regulation of Store-operated Ca\(^{2+}\) Entry by Endogenous Mediators*\(^\text{a,b}\)

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Background: Store-operated Ca\(^{2+}\) entry (SOCE) is essential for cell function.
Results: We discovered cross-talk between expression of molecules that determine SOCE and demonstrated that the role of endogenous mediators may be altered in overexpressed system.
Conclusion: iPLA\(_2\)\(\beta\) is an important regulator of endogenous SOCE, but its role can be obscured by Orai1 and STIM1 overexpression.
Significance: Mediators of endogenous SOCE are important for Ca\(^{2+}\) homeostasis in health and disease.

Orai1 and STIM1 have been identified as the main determinants of the store-operated Ca\(^{2+}\) entry (SOCE). Their specific roles in SOCE and their molecular interactions have been studied extensively following heterologous overexpression or molecular knockdown and extrapolated to the endogenous processes in naïve cells. Using molecular and imaging techniques, we found that variation of expression levels of Orai1 or STIM1 can significantly alter expression and role of some endogenous regulators of SOCE. Although functional inhibition of Ca\(^{2+}\)-independent phospholipase A\(_2\)\(\beta\) (iPLA\(_2\)\(\beta\) or PLA2g6A), or depletion of plasma membrane cholesterol caused a dramatic loss of endogenous SOCE in HEK293 cells, these effects were attenuated significantly when either Orai1 or STIM1 were overexpressed. Molecular knockdown of iPLA\(_2\)\(\beta\) impaired SOCE in both control cells and cells overexpressing STIM1. We also discovered important cross-talk between expression of Orai1 and a specific plasma membrane variant of iPLA\(_2\)\(\beta\) but not STIM1. These data confirm the role of iPLA\(_2\)\(\beta\) as an essential mediator of endogenous SOCE and demonstrate that its physiological role can be obscured by Orai1 and STIM1 overexpression.

Endogenous store-operated Ca\(^{2+}\) entry (SOCE)\(^3\) has fundamental importance for the vast majority of eukaryotic cells: it ensures timely refilling of ER stores, which is essential for functional integrity and ultimately cell survival (for review, see Refs. 1 and 2). A major breakthrough in understanding SOCE mechanism was triggered by identification of STIM1 (3, 4) as a Ca\(^{2+}\) sensor in the ER and Orai1 (5, 6) as the plasma membrane channel (historically called CRAC, or Ca\(^{2+}\) release-activated CRAC CA\(^{2+}\) channel (1)) that responds to depletion of Ca\(^{2+}\) stores and mediates endogenous SOCE. Molecular down-regulation of either component leads to effective inhibition of SOCE in a wide variety of cell types, and genetic deficiency in either Orai1 or STIM1 was shown to result in major pathologies in mice and humans (for review, see Ref. 7). However, it is important to mention that the cells lacking either Orai1 or STIM1 still remain viable and do not lose the ability to proliferate and sustain most of their functions.

The mechanism of Orai1 and STIM1 involvement in SOCE has been studied extensively in heterologous overexpression systems (for recent reviews, see Refs. 2 and 8–12). Rigorous studies of overexpressed Orai1 and STIM1 (11–19), together with in vitro experiments with purified proteins, resulted in the conclusion that direct conformational coupling of STIM1 to Orai1 can be required and is sufficient for SOCE activation in heterologous systems. However, these experiments could not tell much about what else is required for signal transduction from STIM1 to Orai1 in naïve cells when both molecules are expressed at the normal endogenous levels, which are significantly lower than that in heterologous systems. Thus, the question remains open on how closely endogenous SOCE mechanism may be represented by SOCE created in heterologous systems, and if there are any features of endogenous SOCE pathway that may be lost when Orai1 and/or STIM1 are overexpressed. This question is particularly important as additional molecules and mechanisms have been shown to participate intimately in endogenous SOCE (20), but their role in SOCE was questioned in view of a direct conformational coupling model established for overexpressed Orai1 and STIM1.

One such essential component is Ca\(^{2+}\)-independent phospholipase A2 (iPLA\(_2\)\(\beta\) or PLA2G6A). Its important role in endogenous SOCE was demonstrated in vascular smooth muscle cells (24–28), platelets (21), Jurkat T lymphocytes (21), RBL-
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2H3 cells (29, 30), neuroblastoma/glioma cells (22), as well as in astrocytes (23), keratinocytes (24), skeletal muscle cells (25), fibroblasts (26), prostate cancer cells (27), and endothelial cells (28). In all of these studies, inhibition of the catalytic activity or molecular knockdown of iPLA₂β caused dramatic impairment of endogenous SOCE, leaving little doubt about its important role in the SOCE process (for review, see Ref. 20). Importantly, results of a genetic screen of Drosophila melanogaster (5), which identified STIM1 and Orai1 as essential components of SOCE, also picked up an ortholog of iPLA₂β encoded by the CG6718 gene, which is highly homologous to the human PLA₂G6 (up to 85% in the main structural domains). Knockdown of the iPLA₂β ortholog (CG6718) showed a significant impact on SOCE activation, which was identical to that of STIM1 (see supplemental data in Ref. 5). This result clearly confirmed the role of iPLA₂β as one of the major molecular determinants of endogenous SOCE. However, the importance of iPLA₂β for SOCE was questioned by the finding that functional inhibition of iPLA₂β with bromoeno lactone (BEL, a suicidal inhibitor of catalytic activity) failed to inhibit CRAC current in HEK293 cells overexpressing Orai1 and STIM1 (29). This result pointed to the possibility that overexpressed Orai1 and STIM1 create SOCE that may not fully reflect the properties of the endogenous process.

The current study presents new evidence that some properties of endogenous SOCE indeed change in response to overexpression of STIM1 and Orai1. We also tested a novel idea that there may be a cross-talk between expression levels of the major molecular determinants of endogenous SOCE and found that expression of endogenous Orai1 determines and depends upon expression levels of a specific plasma membrane variant of iPLA₂β but not STIM1. These findings highlight the complexity of the SOCE mechanism and the role of its endogenous mediators, which may be lost in overexpression systems.

EXPERIMENTAL PROCEDURES

Cells and Transfections—Human embryonic kidney 293 (HEK293) cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium containing 4.5 mg/ml glucose, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 units/ml penicillin and 10 mg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂.

HEK293 cells were transiently transfected with plasmid DNA using jetPEI (PolyPlus) or with siRNA using Lipofectamine 2000 (Invitrogen). In experiments that required down-regulation of one protein along with overexpression of another protein, Lipofectamine 2000 was used for co-transfection of siRNA and plasmid DNA. Transfection factors were used according to the protocols suggested by the manufacturers. The standard transfection rate was ~70–90%, 24 h after transfection.

DNA Constructs—Constructs encoding human Orai1 (GenBank™ accession no. NM032790) and STIM1 (GenBank™ accession no. NM003156) tagged with green fluorescent protein (EGFP) were described previously (30). Briefly, cDNAs of Orai1 and STIM1 were amplified by PCR and cloned in-frame into the pEGFP-N1 and/or pmCherry-N1 vectors (Clontech) using BglII and EcoRI restriction sites.

Molecular Down-regulation and Expression Analysis—To down-regulate STIM1, Orai1, or iPLA₂β, HEK293 cells were transfected with following stealth siRNAs (100 nM): 5’-AGAAGGACCUAGAUCACUauu-3’ (IDT) for STIM1 and 5’-CCCCUCGGCCUCUAUCAUCUGCU-3’ (Invitrogen) for Orai1 and 5’-GGAUCCUAUGCACAUCACUAtt-3’ (Ambion, catalog no. 4390825) for iPLA₂β. The siRNA for iPLA₂β was targeted to the common part of short (iPLA₂β-S) and long (iPLA₂β-L) variants. Scrambled siRNA (Ambion) was used in each experiment as a control. Cells were studied 24–72 h after transfection.

Total RNA was purified from HEK293 cells with a High Pure RNA isolation kit (Roche Applied Science), and reverse transcription to cDNA was performed using a high capacity RNA-to-cDNA kit (Applied Biosystems). The concentration of RNA during isolation was determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Protein extracts were isolated from the cells 96 h after transfection with scrambled RNA or siRNA against iPLA₂β, separated on a 7.5% SDS-polyacrylamide gel, and subsequently analyzed by Western blotting. Briefly, membranes were blocked in 5% milk powder in phosphate-buffered saline with 0.1% Tween for 1 h at room temperature. Custom-made rabbit polyclonal antibody specific to the long variant of human iPLA₂β (1:1000) was used for iPLA₂β detection, and monoclonal anti-actin antibody (Sigma, clone AC-15, 1:2000) was used for actin staining. Secondary antibodies were HRP-linked anti-rabbit (1:2000, Cell Signaling, catalog no. 7074) or IRDye® 800CW conjugated anti-mouse (1:20,000, LI-COR Bioscience, catalog no. 926-32210), respectively.

Expression of iPLA₂β-L, iPLA₂β-S, STIM1, and Orai1 was determined by quantitative real-time PCR with a StepOnePlus real-time PCR system (Applied Biosystems) with the following TaqMan gene expression assays (Applied Biosystems): Hs00895670_m1 (iPLA₂β-S), Hs00899715_m1 (iPLA₂β-L), Hs00963373_m1 (STIM1), Hs00385627_m1 (Orai1), and 4333767F (human glucouronidase β, GUSB). Samples for the analysis were collected before (0 h), 24, 48, and 72 h after transfection. mRNA levels of housekeeping gene (GUSB) were always determined for each target in the same experiment. Data were analyzed with StepOne Software (version 2.0) using the relative standard curve method and normalized to the GUSB and control samples (cells transfected with scrambled siRNA). Data are presented as an average (S.E.) of three to four independent experiments.

Imaging Experiments—A Nikon TE2000 wide field system with a 60× oil immersion objective (1.4 numerical aperture) was used to image individual cells as previously described (30). Prior to the imaging experiments, cells were passed onto glass-bottomed dishes (MatTek) and studied 24–36 h after transfection. During imaging experiments cells were maintained in extracellular buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 20 mM HEPES, 10 mM glucose, and 1.5 mM CaCl₂, pH 7.4) at room temperature. Fluorescence was monitored using following filter sets (Chroma): 1) ET470/40 (excitation), ET525/50 (emission) and T495LP (dichroic) for GFP, and 2) ET560/40 (excitation), ET630/75 (emission), and T585LP (dichroic) for mCherry. Stacks of images were taken at Z intervals of 0.3 μm.
and then deconvolved using the AutoQuant Module Media Cybernetics) in NIS-Elements software (Nikon).

**Ca**$^{2+}$/H11001 Influx Studies—HEK293 cells were grown on glass coverslips and loaded with fura2/AM (Invitrogen), and changes in intracellular Ca$^{2+}$ (measured as $F_{340}/F_{380}$ ratio) were monitored as described previously (30). Briefly, a dual-excitation fluorescence imaging system (Intracellular Imaging) was used for studies of individual cells. The changes in intracellular Ca$^{2+}$ were expressed as Aratio, which was calculated as the difference between the peak values of the ratio after extracellular Ca$^{2+}$ was added and ratio right before Ca$^{2+}$ addition. After transfection, the cells were plated on the coverslips and kept in culture. 72 h after transfection, Ca$^{2+}$ was recorded simultaneously from 20–40 cells from each coverslip, which after transfection represented a mixed population of GFP-positive (transfected) and GFP-negative (non-transfected) cells. To determine the effect of (S)-BEL and MCD on Ca$^{2+}$ influx, the cells on each coverslip were pretreated with 50 μM (S)-BEL or 10 μM MCD for 30 min at 37 °C, and the drugs were washed away prior to the Ca$^{2+}$ experiment. Data were summarized from the large number of individual cells (as indicated on the bar graphs): 10–40 cells were analyzed in each single run (representative traces are shown in Figs. 1 and 3), repeated in three to five independent experiments for each condition using at least three different cell transfections. In each experiment, GFP-positive (transfected) and GFP-negative (non-transfected) cells were analyzed and presented separately.

**RESULTS**

Functional Inhibition of iPLA$_{2β}$ Blocks SOCE in Naïve Cells, but Not in Cells Overexpressing Orai1 or STIM1—To compare the role of iPLA$_{2β}$ in endogenous SOCE in naïve HEK293 cells with its role in SOCE mediated by overexpressed Orai1 or STIM1, we started with testing the effects of inhibition of catalytic activity of iPLA$_{2β}$ on SOCE evoked by either buffering free Ca$^{2+}$ in ER with TPEN (see Fig. 1) or by depleting ER stores as a result of SERCA inhibition with thapsigargin (TG, Fig. 2). As in our earlier studies (24, 25), inhibition of iPLA$_{2β}$ activity was achieved by pretreatment of the cells with (S)-BEL (31).

Similarly to a wide variety of cells (for review, see Ref. 20), pretreatment of HEK293 with (S)-BEL (50 μM for 30 min) resulted in 86.6 ± 1.9% and 83.8 ± 8.2% inhibition of endogenous SOCE induced by TPEN (1 mM for 5 min) and TG (5 μM for 5 min), respectively. Surprisingly, we noticed that when...
either Orai1\textsuperscript{GFP} or STIM1\textsuperscript{GFP} was overexpressed in HEK293 cells, treatment with (S)-BEL produced virtually no effect. To assess this phenomenon in detail and to exclude the possibility that mere transfection of the cells may change their sensitivity to (S)-BEL, we systematically investigated effects of (S)-BEL on SOCE in HEK293 cells transfected with either GFP or Orai1\textsuperscript{GFP} or STIM1\textsuperscript{GFP}. In each cell preparation, we simultaneously recorded \(\text{Ca}^{2+}\) responses in transfected (GFP-positive) and non-transfected (GFP-negative) cells. We found that transfection of the cells with plain GFP did not change SOCE sensitivity to (S)-BEL, and Fig. 1A shows that TPEN-induced SOCE was inhibited by \(\sim 80\%\) in both GFP-positive and GFP-negative cells. In contrast, in cells overexpressing either Orai1\textsuperscript{GFP} (Fig. 1B) or STIM1\textsuperscript{GFP} (Fig. 1C), (S)-BEL failed to inhibit SOCE in GFP-positive (transfected) cells. Importantly SOCE in adjacent GFP-negative cells (that did not express Orai1\textsuperscript{GFP} or STIM1\textsuperscript{GFP}) was inhibited, respectively, by 85.3 \(\pm\) 3.3\% and 82.5 \(\pm\) 1.9\%, which was similar to what was observed in naïve cells (Fig. 1A).

Simultaneous study of control cells and cells overexpressing Orai1\textsuperscript{GFP} ensured that both cell types were treated and investigated under the same conditions, and yet, endogenous SOCE in control cells was inhibited, whereas SOCE in Orai1/STIM1-overexpressing cells remained untouched.

Similar results were obtained when SOCE was triggered by TG, as demonstrated in Fig. 2. In HEK293 cells overexpressing GFP, pretreatment with (S)-BEL inhibited SOCE by 84.4 \(\pm\) 3.7\% (Fig. 2A), whereas in cells overexpressing Orai1\textsuperscript{GFP} or STIM1\textsuperscript{GFP} (Fig. 2B and C, respectively), it had no effect. Again, in GFP-negative cells adjacent to those expressing Orai1\textsuperscript{GFP} or STIM1\textsuperscript{GFP}, pretreatment with (S)-BEL resulted in profound inhibition of TG-induced SOCE: 68.5 \(\pm\) 3.9\% for Orai1\textsuperscript{GFP}-negative and 76.9 \(\pm\) 4.0\% for STIM1\textsuperscript{GFP}-negative cells (data not shown). Moreover, pretreatment with (S)-BEL had no visible effect on the dynamics of overexpressed STIM1\textsuperscript{mCherry} and Orai1\textsuperscript{GFP}, which formed puncta even in the cells in which catalytic activity of iPLA\(_2\)\(\beta\) was inhibited (Fig. 3).

Depletion of Cholesterol with \(\beta\)-Methylcyclodextran Impairs Endogenous SOCE in Naïve Cells, but Has No Effect on SOCE in Cells Overexpressing Orai1 or STIM1—To determine whether overexpression of Orai1/STIM1 impairs only SOCE dependence on functional activity of iPLA\(_2\)\(\beta\) or may change some other features of endogenous SOCE, we have tested SOCE dependence on plasma membrane cholesterol abundance. \(\beta\)-Methylcyclodextran (\(\beta\)-MCD) is used widely to deplete plasma membrane cholesterol and was shown to inhibit SOCE in RBL-2H3 cells (32). Fig. 4 shows that incubation of GFP-transfected HEK293 cells with \(\beta\)-MCD inhibited TG-induced SOCE (Fig. 4A) in both GFP-positive (by 81.5 \(\pm\) 5.3\%) and GFP-negative cells (by 93.5 \(\pm\) 1.4\%). However, the effect of MCD was abolished completely by overexpression of either Orai1\textsuperscript{GFP} (Fig. 4B) or STIM1\textsuperscript{GFP} (Fig. 4C). Importantly, SOCE in GFP-negative adjacent cells was inhibited by 83.9 \(\pm\) 4.9\% (Orai1\textsuperscript{GFP}, Fig. 4B) and 65.9 \(\pm\) 4.4\% (STIM1\textsuperscript{GFP}, Fig. 4C). These results indicate that the overexpression of either Orai1 or STIM1 significantly changes the properties of endogenous SOCE and can make it

![FIGURE 2. Inhibition of iPLA\(_2\)\(\beta\) with (S)-BEL impairs TG-induced SOCE in naïve HEK293 cells but not in cells overexpressing either STIM1 or Orai1. Summary data show the average TG-induced \(\text{Ca}^{2+}\) influx recorded in HEK293 cells overexpressing GFP (+GFP; A), Orai1 (+Orai1\textsuperscript{GFP}; B), or STIM1 (+STIM1\textsuperscript{GFP}; C) pretreated with vehicle (control) or (S)-BEL (+(S)-BEL, 50 \(\mu\)M for 30 min at 37 °C). Cells were treated with TG (5 \(\mu\)M for 5 min) in the absence of extracellular \(\text{Ca}^{2+}\) followed by addition of 2 mM Ca\(^{2+}\). Data are presented for GFP-positive cells, as shown in the middle panels of Fig. 1.](image-url)

![FIGURE 3. Inhibition of iPLA\(_2\)\(\beta\) with (S)-BEL does not affect puncta formation by Orai1 and STIM1 in overexpressing system. Fluorescent images of representative HEK293 cells co-expressing Orai1 (tagged with GFP) and STIM1 (tagged with mCherry) preincubated with (S)-BEL (50 \(\mu\)M for 30 min at 37 °C). Treatment with TG (5 \(\mu\)M for 5 min) induced translocation of Orai1 and STIM1 into characteristic puncta. Panels on the right show indicated regions at 3-fold magnification.](image-url)
resistant to inhibitors of different cellular processes that are essential components of endogenous SOCE.

Down-regulation of iPLA2\textsubscript{H9252} Protein Inhibits Not Only Endogenous SOCE, but also SOCE in STIM1-overexpressing Cells—Our earlier studies showed that in the endogenous SOCE pathway, iPLA2\textsubscript{H9252} is required downstream from STIM1, so the results of the present study raised a question as to whether overexpression of STIM1 may overcome only an acute catalytic deficiency of iPLA2\textsubscript{H9252} (caused by (S)-BEL) or may create a shortcut in SOCE pathway that may function even in the longer term absence of the iPLA2\textsubscript{H9252} protein. To answer that question, we used an siRNA approach to knockdown iPLA2\textsubscript{H9252} protein and compared its effects on endogenous SOCE in control HEK293 (at physiological levels of STIM1 expression) with its effects on SOCE in cells overexpressing STIM1GFP.

Transfection of HEK293 cells with siRNA targeting all splice variants of iPLA2\textsubscript{H9252} caused up to an 88\% decrease in mRNA levels of iPLA2\textsubscript{H9252} at 72 hours after transfection, followed by only 30\% loss of protein (supplemental Fig. 1). Fig. 5\textit{A} shows that even such incomplete down-regulation of iPLA2\textsubscript{H9252} caused a 52\% reduction in TG-stimulated Ca\textsuperscript{2+} entry. Importantly, significant (33\% ± 5\%) inhibition of SOCE was also observed in siRNA-treated cells overexpressing STIM1\textsuperscript{GFP} (Fig. 5\textit{B}). These results suggest that even partial deficiency in the iPLA2\textsubscript{H9252} protein can impair not only endogenous SOCE but also SOCE in the cells in which STIM1 is overexpressed. The reasons for the different sensitivity of SOCE to acute loss of catalytic activity versus prolonged siRNA-induced deficiency in iPLA2\textsubscript{H9252} protein in STIM1-overexpressing cells are yet to be determined.

**Cross-talk between Expression Levels of Plasma Membrane Variant of iPLA2\textsubscript{H9252} and Orai1—**To investigate whether there may be cross-talk between expression of different components of the SOCE mechanism, mRNA levels of endogenous iPLA2\textsubscript{H9252}, Orai1, and/or STIM1 were assessed in resting HEK293 cells after each of these molecules was knocked down using specific siRNA. Fig. 6\textit{A} shows that siRNA-induced knockdown of both splice variants of iPLA2\textsubscript{H9252} (plasma membrane-associated iPLA2\textsubscript{H9252}(L), and cytosolic iPLA2\textsubscript{H9252}(S)) caused progressive increase in mRNA levels of both Orai1 and STIM1: when mRNA of iPLA2\textsubscript{H9252} was down by 88\% ± 3\%, mRNAs for Orai1 and STIM1 increased 2-fold. Strikingly, knockdown of Orai1 (Fig. 6\textit{B}) dramatically boosted expression levels of the long variant of iPLA2\textsubscript{H9252}, with no significant change in mRNA levels of either STIM1, or the short variant of iPLA2\textsubscript{H9252}: 72 h after cell transfection with siRNA specific to Orai1, mRNA levels of iPLA2\textsubscript{H9252}(L) increased >4-fold. Molecular knockdown of STIM1 (Fig. 6\textit{C}) did not cause any significant changes in mRNA levels of Orai1 or either variant of iPLA2\textsubscript{H9252}. These results for the first time demonstrate strong
cross-talk (inverse correlation) between expression levels of Orai1 and the plasma membrane variant of iPLA2.

DISCUSSION

This study presented the first evidence for cross-talk between expression levels of the major molecular determinants of endogenous SOCE and demonstrated that overexpression of Orai1 and STIM1 may change SOCE regulation by endogenous mediators. The important new finding is the existence of an inverse correlation between the expression of endogenous Orai1 and the specific plasma membrane variant of iPLA2. This further supports the idea of a close functional relationship between these two components of the endogenous SOCE mechanism. Here, we demonstrated that molecular down-regulation of Orai1 leads to a dramatic 4-fold increase in expression of the plasma membrane but not cytosolic variant of iPLA2 and not STIM1. It is tempting to hypothesize that up-regulation of one specific variant of iPLA2 could be a defensive response of the cell to the loss of significant amount of Orai1 channels. Indeed, we have earlier shown (33, 34) that mere activation of plasma membrane-associated iPLA2 and/or enrichment of the plasma membrane with its lysophospholipid products can activate Orai1 channels and SOCE bypassing Ca2+ store depletion. Boosting the expression or functional activity of endogenous iPLA2 may help sustain activity of endogenous Orai1 channels and help prevent a potentially lethal loss of Orai1-mediated Ca2+ entry, thus ensuring cell survival when Orai1 expression is down-regulated.

The absence of any detectable cross-talk between expression levels of Orai1 and STIM1 was rather unexpected and warrants further investigation. One may anticipate at least some interdependence of these two major determinants of SOCE that have been shown to directly associate with each other. Because STIM1 is essential for initiation of the signaling cascade leading to SOCE activation, it was surprising to find that knockdown of STIM1 had no effect on either Orai1 or iPLA2β expression. However, it is important to mention that there is a precedent in a neuroblastoma/glioma cell line (NG115) in which STIM1 is absent, and despite the fact that TG fails to produce any significant SOCE, NG115 cells are able to grow and proliferate (22). These data suggest that endogenous activation of Orai1 by iPLA2β or some other yet to be determined regulator(s) may be sufficient for resting cell survival. This does not contradict the fact that molecular knock-down of either Orai1 or STIM1 or
iPLA₂β are all known to dramatically impair SOCE activated by TG or other stimuli. One may speculate that significant depletion of the stores (induced naturally by agonists or experimentally by TG) may require a full activation of the SOCE mechanism to refill empty stores: deficiency in either component of SOCE will significantly affect its maximum capacity and can impair agonist-induced SOCE and related cell function. In contrast, full capacity of all SOCE components may not be needed for sustaining background SOCE in resting cells, and cross-talk of endogenous Orai1 and iPLA₂β on expression levels may become especially important for cell adaptation and function under resting conditions.

Although direct coupling of ER-resident STIM1 to plasma membrane-resident Orai1 may be rightfully considered as the straightforward mechanism for signal transduction, there is a significant body of evidence for the presence of additional mediators and regulators of the endogenous SOCE mechanism (for review, see Ref. 20). One of them is iPLA₂β, which was found to be functionally required downstream from STIM1 and preceding Orai1. It is important to emphasize that existence of additional steps in SOCE mechanism, and molecular intermediate(s) between STIM1 and Orai1 do not contradict the studies that demonstrated their spatial proximity and eventual conformational coupling. Indeed, endogenous mediators may be simply needed to set up a stage that will allow such interactions. In contrast to overexpression systems, in naïve cells under physiological conditions, there are a limited number of Orai1 channels in the plasma membrane and ER Ca²⁺ sensors, and endogenous mediators (similar to iPLA₂β, lysophospholipids, and other factors) may facilitate their targeting to specific areas where the ER comes into close proximity with the plasma membrane. Our results suggest that this process may be also dependent on plasma membrane cholesterol content, as its disturbance, which in our studies was achieved by cholesterol depletion by MCD, significantly impaired endogenous SOCE but not in cells overexpressing Orai1 or STIM1. This result is fully in line with an earlier report (35) of the lack of an effect of MCD on the whole-cell Orai1-mediated current in STIM1-overexpressing cells. The absence of the MCD effect in this case was suggested to be a result of MCD-induced changes in membrane potential. However, the striking differences in MCD effects on SOCE in intact naïve and overexpressing cells (Fig. 4) found in this study do not seem to favor such an explanation. Indeed, naïve and overexpressing cells in our study have been treated with MCD in the same way and studied simultaneously, so, if membrane potential is a factor in the MCD effects, one would expect the MCD effects to be the same in naïve and overexpressing cells. The dramatically different effects of MCD and (S)-BEL in naïve and overexpressing cells may favor the idea that overexpression of Orai1 in the plasma membrane or STIM1 in the ER can saturate the SOCE pathway and eliminate the need for mediating and coordinating their proper targeting toward each other. The results of our studies, together with some earlier reports (36, 37) clearly demonstrate that overexpression of Orai1, and/or STIM1 may significantly alter SOCE sensitivity to pharmacological inhibitors or molecular manipulations that are known to impair endogenous SOCE in naïve cells.

Discovery that molecular down-regulation of iPLA₂β leads to a 2-fold increase in expression of Orai1 and STIM1, strongly suggests that endogenous up-regulation of Orai1 and STIM1 expression may be needed to compensate for the loss of iPLA₂β as an endogenous regulator/mediator that is important for signal transduction from STIM1 to Orai1. This idea is further supported by the striking differences we found in SOCE in naïve and overexpression conditions. Indeed, experimental overexpression of Orai1 and STIM1 may create special conditions for their direct interaction/conformational coupling as described in heterologous systems, in which the need for functional activity of iPLA₂β as a mediator of signal transduction may be reduced significantly or lost completely. Thus, despite the great importance of heterologous studies, overexpression of major SOCE components may obscure or alter some endogenous processes that are important for the native SOCE mechanism.

In summary, results of these studies demonstrated that there is cross-talk between expression levels of endogenous Orai1 and iPLA₂β and that some properties of endogenous SOCE may change in response to experimental overexpression of STIM1 and Orai1. Our findings highlight the complexity of the endogenous SOCE mechanism and the important role of iPLA₂β and plasma membrane cholesterol in endogenous signal transduction, which can be obscured by overexpression of Orai1 or STIM1.

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REFERENCES

1. Parekh, A. B., and Putney, J. W., Jr. (2005) Store-operated calcium channels. Physiol. Rev. 85, 757–810
2. Parekh, A. B. (2010) Store-operated CRAC channels: Function in health and disease. Nat. Rev. Drug Discov. 9, 399–410
3. Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Veličelebi, G., and Stauderman, K. A. (2005) STIM1, an essential and conserved component of store-operated Ca²⁺ channel function. J. Cell Biol. 169, 435–445
4. Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr., and Meyer, T. (2005) STIM is a Ca²⁺ sensor essential for Ca²⁺ store depletion-triggered Ca²⁺ influx. Curr. Biol. 15, 1235–1241
5. Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblau-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca²⁺ entry. Science 312, 1220–1223
6. Peinelt, C., Vig, M., Koomoa, D. L., Beck, A., Nadler, M. J., Koblau-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J. P. (2006) Amplification of CRAC current by STIM1 and CRACM1 (Orai1). Nat. Cell Biol. 8, 771–773
7. Feske, S. (2009) Orai1 and STIM1 deficiency in human and mice: Roles of store-operated Ca²⁺ entry in the immune system and beyond. Immunol. Rev. 231, 189–209
8. Feske, S. (2010) CRAC channelopathies. Pflugers Arch. 460, 417–435
9. Hogan, P. G., Lewis, R. S., and Rao, A. (2010) Molecular basis of calcium signaling in lymphocytes: STIM and Orai. Annu. Rev. Immunol. 28, 491–533
10. Clapham, D. E. (2009) A STIMulus package puts Orai calcium channels to work. Cell 136, 814–816
11. Fahrner, M., Muik, M., Derler, L., Schindl, R., Fritsch, R., Frischauer, I., and
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Romanin, C. (2009) Mechanistic view on domains mediating STIM1-Orai coupling. *Immunol. Rev.* **231**, 99–112.

Frischauf, I., Schindl, R., Derler, I., Bergsmann, J., Fahrner, M., and Romanin, C. (2008) The STIM/Orai coupling machinery. *Channels* **2**, 261–268.

Park, C. Y., Hoover, P. J., Mullins, F. M., Bachhawat, P., Covington, E. D., Raunser, S., Walz, T., García, K. C., Dolmetsch, R. E., and Lewis, R. S. (2009) STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. *Cell* **136**, 876–890.

Luik, R. M., Wang, B., Prakriya, M., Wu, M. M., and Lewis, R. S. (2008) Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454**, 538–542.

Schindl, R., Muik, M., Fahrner, M., Derler, I., Fritsch, R., Bergsmann, J., and Romanin, C. (2009) Recent progress on STIM1 domains controlling Orai activation. *Cell Calcium* **46**, 227–232.

Yuan, J. P., Zeng, W., Dorwart, M. R., Choi, Y. J., Worley, P. F., and Muallem, S. (2009) SOAR and the polybasic STIM1 domains gate and regulate Orai channels. *Nat. Cell Biol.* **11**, 337–343.

Muik, M., Frischauf, I., Derler, I., Fahrner, M., Bergsmann, J., Eder, P., Schindl, R., Hesch, C., Polzinger, B., Fritsch, R., Kahr, H., Madl, J., Gruber, H., Groschner, K., and Romanin, C. (2008) Dynamic coupling of the putative coiled-coil domain of Orai1 with STIM1 mediates Orai1 channel activation. *J. Biol. Chem.* **283**, 8014–8022.

Li, Z., Lu, J., Xu, P., Xie, X., Chen, L., and Xu, T. (2007) Mapping the interacting domains of STIM1 and Orai1 in Ca2+ release-activated Ca2+ channel activation. *J. Biol. Chem.* **282**, 29448–29456.

Vármai, P., Tóth, B., Tóth, D. J., Hunyady, L., and Balla, T. (2007) Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 complex. *J. Biol. Chem.* **282**, 29678–29690.

Bolotina, V. M. (2008) Orai, STIM1, and iPLA2β: A view from a different perspective. *J. Physiol.* **586**, 3035–3042.

Smani, T., Zakharov, S. I., Leno, E., Csutora, P., Trepakova, E. S., and Bolotina, V. M. (2003) Ca2+-independent phospholipase A2 is a novel determinant of store-operated Ca2+ entry. *J. Biol. Chem.* **278**, 11909–11915.

Csutora, P., Peter, K., Kilic, H., Park, K. M., Zakhariv, V., Gwozdz, T., and Bolotina, V. M. (2008) Novel role for STIM1 as a trigger for calcium influx factor production. *J. Biol. Chem.* **283**, 14524–14531.

Singaravelu, K., Lohr, C., and Deitmer, J. W. (2006) Regulation of store-operated calcium entry by calcium-independent phospholipase A2 in rat cerebellar astrocytes. *J. Neurosci.* **26**, 9579–9592.

Ross, K., Whitaker, M., and Reynolds, N. J. (2007) Agonist-induced calcium entry correlates with STIM1 translocation. *J. Cell. Physiol.* **211**, 569–576.

Boittin, F. X., Petermann, O., Hirn, C., Mittaud, P., Dorchies, O. M., Roulet, E., and Ruegg, U. T. (2006) Ca2+-independent phospholipase A2 enhances store-operated Ca2+ entry in dystrophic skeletal muscle fibers. *J. Cell Sci.* **119**, 3733–3742.

Martinez, J., and Moreno, J. I. (2005) Role of Ca2+-independent phospholipase A2 and cytochrome P-450 in store-operated calcium entry in 3T6 fibroblasts. *Biochim. Pharmacol.* **70**, 733–739.

Vanden Abeele, F., Lemonnier, L., Thébault, S., Lepage, G., Parys, J. B., Shibuya, Y., Smyrna, R., and Prevorskaya, N. (2004) Two types of store-operated Ca2+ channels with different activation modes and molecular origin in LNCaP human prostate cancer epithelial cells. *J. Biol. Chem.* **279**, 30326–30337.

Boittin, F. X., Gribi, F., Serir, K., and Bény, J. L. (2008) Ca2+-independent PLA2 controls endothelial store-operated Ca2+ entry and vascular tone in intact aorta. *Am. J. Physiol. Heart Circ. Physiol.* **295**, H2466–H2474.

Derler, I., Fritsch, R., Schindl, R., and Romanin, C. (2008) CRAC inhibitors: identification and potential. *Exp. Opin. Drug Discov.* **3**, 787–800.

Gwozdz, T., Dutko-Gwozdz, J., Zakarivskiy, V., Peter, K., and Bolotina, V. M. (2008) How strict is the correlation between STIM1 and Orai expression, puncta formation, and ICRAC activation? *Am. J. Physiol. Cell Physiol.* **295**, C1133–1140.

Hazan, S. L., Zapan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A2. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A2. *J. Biol. Chem.* **266**, 7227–7232.

Kato, N., Nakanishi, M., and Hirashima, N. (2003) Cholesterol depletion inhibits store-operated calcium currents and exocytotic membrane fusion in RBL-2H3 cells. *Biochemistry* **42**, 11808–11814.

Csutora, P., Zakarivskiy, V., Peter, K., Monje, F., Smani, T., Zakharov, S. I., Litvinov, D., and Bolotina, V. M. (2006) Activation mechanism for CRAC current and store-operated Ca2+ entry: Calcium influx factor and Ca2+-independent phospholipase A2β-mediated pathway. *J. Biol. Chem.* **281**, 34926–34935.

Smani, T., Zakharov, S. I., Csutora, P., Leno, E., Trepakova, E. S., and Bolotina, V. M. (2004) A novel mechanism for the store-operated calcium influx pathway. *Nat. Cell Biol.* **6**, 113–120.

DeHaven, W. L., Jones, B. F., Petranka, J. G., Smyth, J. T., Tomita, T., Bird, G. S., and Putney, J. W. (2009) TRPC channels function independently of STIM1 and Orai. *J. Physiol.* **587**, 2275–2298.

Smyth, J. T., DeHaven, W. L., Bird, G. S., and Putney, J. W., Jr. (2008) Ca2+-store-dependent and -independent reversal of Stim1 localization and function. *J. Cell Sci.* **121**, 762–772.

DeHaven, W. L., Smyth, J. T., Boyles, R. R., Bird, G. S., and Putney, J. W., Jr. (2008) Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry. *J. Biol. Chem.* **283**, 19265–19273.