THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 15, pp. 11456–11464, April 13, 2007

STIM1 Knockdown Reveals That Store-operated Ca^{2+} Channels Located Close to Sarco/Endoplasmic Ca^{2+} ATPases (SERCA) Pumps Silently Refill the Endoplasmic Reticulum*[S]

Received for publication, October 10, 2006, and in revised form, January 16, 2007 Published, JBC Papers in Press, February 5, 2007, DOI 10.1074/jbc.M609551200

Hélène Jousset, Maud Frieden, and Nicolas Demaurex[1]

From the Department of Cell Physiology and Metabolism, University of Geneva Medical School, 1 Michel-Servet, CH-1211 Geneva 4, Switzerland

Stromal interaction molecule (STIM) proteins are putative ER Ca^{2+} sensors that recruit and activate store-operated Ca^{2+} (SOC) channels at the plasma membrane, a process triggered by the Ca^{2+} depletion of the endoplasmic reticulum (ER). To test whether STIM1 is required for ER refilling, we used RNA interference and measured Ca^{2+} signals in the cytosol, the ER, and the mitochondria of HeLa cells. Knockdown of STIM1 (mRNA levels, 73%) reduced SOC entry by 73% when sarco/endoplasmic Ca^{2+} ATPases (SERCA) were inhibited by thapsigargin but did not prevent Ca^{2+} stores refilling when cells were stimulated by physiological agonists. Stores could be fully refilled by increasing the external Ca^{2+} concentration above physiological values, but no cytosolic Ca^{2+} signals were detected during store refilling even at very high Ca^{2+} concentrations. $[Ca^{2+}]_{ER}$ measurements revealed that the basal activity of SERCA was not affected in STIM1 knockdown cells and that $[Ca^{2+}]_{ER}$ levels were restored within 2 min in physiological saline following store depletion. Mitochondrial inhibitors reduced ER refilling in wild-type but not in STIM1 knockdown cells, indicating that ER refilling does not require functional mitochondria at low STIM1 levels. Our data show that ER refilling is largely preserved at reduced STIM1 levels, despite a drastic reduction of store-operated Ca^{2+} entry, because Ca^{2+} ions are directly transferred from SOC channels to SERCA. These findings are consistent with the formation of microdomains containing not only SOC channels on the plasma membrane and STIM proteins on the ER but also SERCA pumps and mitochondria to refill the ER without perturbing the cytosol.

Ca^{2+} signals generated by the release of Ca^{2+} ions from the endoplasmic reticulum (ER)[2] regulate essential cellular functions such as secretion, contraction, and gene transcription. The depletion of ER Ca^{2+} stores, in turn, activates Ca^{2+}-permeable channels at the plasma membrane to ensure long term signaling. This mechanism of store-operated Ca^{2+} entry was described 20 years ago (1), and the prototypic store-operated channel CRAC (for Ca^{2+} release-activated Ca^{2+} channel) was extensively characterized (2). However, the mechanism that activates SOC and CRAC channels upon ER depletion has long remained elusive (3).

Two protein families, STIM and Orai, were identified recently as essential for SOC activity (4, 5). STIM1 is a highly conserved type I ER membrane protein containing a luminal EF-hand domain and several cytosolic protein-protein interaction domains. Several evidences indicate that STIM1 is the ER Ca^{2+} sensor that regulates the activity of SOC channels: STIM1 redistributes into ER puncta located 10–25 nm from the plasma membrane upon Ca^{2+} store depletion, and puncta formation precedes the activation of CRAC channels by several seconds, consistent with a causal role of STIM1 in SOC activation (6). STIM1 EF-hand mutations constitutively activate SOC and induce puncta formation (4, 7). Finally, STIM1 associates with the CRAC channel pore subunit Orai1, an interaction increased by store depletion (8), and also with the hTRPC1 channel (9).

The other protein, Orai1 (also known as CRACM1), was identified by genetic linkage in a subset of patients with severe combined immunodeficiency, who lack functional CRAC channels (10). As observed for STIM1, knockdown of Orai1 drastically reduced both store-operated Ca^{2+} entry and CRAC current (11, 12). Co-expression of STIM1 and Orai1, but not expression of either protein alone, reconstituted store-operated Ca^{2+} entry and generated massive CRAC currents (12–15). All three mammalian Orai homologues synergized with STIM1 to augment store-operated Ca^{2+} entry in the potency order Orai1 → Orai2 → Orai3 (15). Mutagenesis studies then conclusively showed Orai1 to be the CRAC channel pore as point mutations in Orai1 transformed the Ca^{2+}-selective, inwardly rectifying channel into an outwardly rectifying channel permeable to monovalent cations (8, 16, 17).

During store depletion, STIM1 and Orai1 move in a coordinated fashion to form closely apposed ER-plasma membrane clusters, and the clusters are associated with highly localized

* This work was supported by operating Grant 31-068317.02 from the Swiss National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures.

[1] To whom correspondence should be addressed. Tel.: 41-22-379-5399; Fax: 41-22-379-5338; E-mail: Nicolas.Demaurex@medecine.unige.ch.

[2] The abbreviations used are: ER, endoplasmic reticulum; $[Ca^{2+}]_{ER}$, endoplasmic reticulum free Ca^{2+} concentration; $[Ca^{2+}]_{mit}$, mitochondrial matrix free Ca^{2+} concentration; SERCA, sarco/ER Ca^{2+}-ATPase; STIM1, stromal interaction molecule 1; YC, yellow cameleon; PM, plasma membrane; dsRNA, double-stranded ribonucleic acid; SOC, store-operated Ca^{2+}; CRAC, calcium release-activated calcium; siRNA, small interfering RNA; RT, reverse transcription; TAMRA, tetramethylrhodamine; FAM, 6-carboxyfluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

increases in subplasmalemmal [Ca^{2+}] (18). The STIM-Orai interaction thus restricts Ca^{2+} influx to specific regions of the plasma membrane located 10 nm away from the ER. This cellular structure resembles the synaptic cleft and creates a diffusion barrier that prevents the escape of Ca^{2+} ions from the cleft. In these conditions, Ca^{2+} influx should be barely detectable with cytosolic Ca^{2+} dyes. Yet cytosolic Ca^{2+} dyes are used routinely to measure store-operated Ca^{2+} entry. One possible explanation is that SERCA inhibitors are often used to activate SOC. With SERCA inhibited, Ca^{2+} ions entering across SOC channels cannot be transferred to the ER and are thus more readily detected in the cytosol. Alternatively, STIM1 might bring together the ER and plasma membrane only in cells that express CRAC channels, such as Jurkat cells.

Here, we tested the hypothesis that SOC influx occurs in membrane clusters closely apposed to the ER by measuring the impact of STIM1 levels on cytosolic, ER, and mitochondrial Ca^{2+} handling. STIM1 knock-down with siRNA markedly decreased SOC activity in HeLa cells but had surprisingly little impact on ER Ca^{2+} homeostasis because all incoming Ca^{2+} ions were directly taken up by SERCA pumps. These findings are consistent with the formation of clusters containing SOC channels on the plasma membrane and both STIM and SERCA proteins on the closely apposed ER membrane to enable efficient ER refilling with minimal changes in cytosolic Ca^{2+}.

EXPERIMENTAL PROCEDURES

Reagents—Minimum essential medium, fetal calf serum, penicillin, streptomycin, and Lipofectamine 2000 transfection reagent were obtained from Invitrogen. Histamine, thapsigargin, oligomycin, and rotenone were obtained from Sigma, CGP-37157 was from Calbiochem, and UTP was from GE Healthcare. $YC3.6_{cyto}$ and $D1_{ER}$ were kindly provided by Drs. Amy Palmer and Roger Tsien, and $YC2.1_{2mit}$ was provided by Dr. Tullio Pozzan.

RNA Interference—Small double-stranded RNAs (dsRNA) were purchased from Ambion. All experiments were performed with the previously described hSTIM1–1140 siRNA (sense, 5′GGCUCUGGAUACAGUGCUCtt3′, antisense, 5′-GAGCACUGUAUCCAGAGCCtt3′) (4, 5). The scrambled dsRNA (sense, 5′GUGCGACUGCUGGACUACUtt3′, antisense, 5′AGUAGUCCAGCAGUCGCACtt3′) was used as a negative control.

Cell Culture and Transfection—HeLa cells were grown in minimum essential medium containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin at 37 °C and 5% CO_2. For all experiments, cells were plated on 25-mm-diameter glass coverslips and co-transfected with plasmids (2 μg) coding for YC probes and dsRNA (80 nM) using Lipofectamine 2000. Cytosolic, ER, and mitochondrial recordings were performed using $YC3.6_{cyto}$, $D1_{ER}$, and $YC2.1_{2mit}$, respectively. All experiments were performed 2 days after transfection.

Cytosolic Ca^{2+} Measurements—Experiments were performed in HEPES buffer solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 20 Hepes, 10 glucose, pH 7.4, with NaOH. Ca^{2+}-free solution contained 1 mM EGTA instead of $CaCl_2$. Glass coverslips were inserted in a thermostatic chamber (Havard Apparatus, Holliston, MA) equipped with gravity inlets and vacuum outlets for solution changes. Cells were imaged on an Axiovert s100 TV using a ×40, 1.3 NA oil-immersion objective (Carl Zeis AG, Feldbach, Switzerland) and a cooled, 16-bit CCD back-illuminated frame transfer MicroMax camera (Roper Scientific, Trenton, NJ). For dual emission imaging of $YC3.6_{cyto}$, $D1_{ER}$, and $YC2.1_{2mit}$, cells were excited at 430 nm with a monochromator (DeltaRam, PTI, Monmouth Junction, NJ) through a 455-nm dichroic mirror and imaged sequentially at 475 and 535 nm using a filter wheel (455DRLP, 475DF15, and 535DF25, Omega Optical, Brattleboro, VT). Fura-2 fluorescence was imaged using alternate excitation at 340 and 380 nm and a 510WB40 emission filter. Image acquisition and analysis were performed with the Metafluor 6.2 software (Universal Imaging, West Chester, PA).

Quantitative RT-PCR—Two day after co-transfection, cells were harvested by trypzination, washed twice with phosphate-buffered saline, resuspended in phosphate-buffered saline, and subjected to cytofluorometric analysis. Green fluorescent protein-positive cells were sorted using a FACStar+ (BD Biosciences). Total RNA was isolated from the sorted cells using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), and 0.5 μg of Dnase-treated RNA was used to synthesize cDNA using QuantiTec reverse transcription kit (Qiagen, Hombrechtikon, Switzerland). RT-PCR assays were carried out in an iCycler (Bio-Rad Laboratories) using the TaqMan system in a final volume of 25 μl. The reaction mix included 12.5 μl of ABsolute QPCR mixes (ABgene), 0.5 μM primers, and 0.1 μM specific Taqman probe. The sequences of the primer used were as followed: *GAPDH*, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′; *STIM1*, 5′-TGACAGGGACTGTGCTGAAG-3′ and 5′-AAGAGAGGAGGCCCAAAGAG-3′; *GAPDH* fluorogenic probe, 5′-FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3′; *STIM1* fluorogenic probe, 5′-FAM-ACAGACCGGAGTCATCGGCAAGAAG-BHQ1–3′. Taqman fluorogenic probe are labeled with 6-carboxyfluorescein (FAM) at the 5′-end and with the fluorescent black hole quencher 1 (BHQ1) or with tetramethylrhodamine (TAMRA) used as fluorescent quencher at the 3′-end. For quantification, relative standard curves were created for each gene product, and a housekeeping gene, *GAPDH*, was used for normalization of the concentration. Relative expression was calculated using the $2^{-\Delta\Delta C}{}_T$ method.

Statistics—The significance of differences between means was established using the Student's *t* test for unpaired samples. The level of significance was defined as $p < 0.05$.

RESULTS

Effect of STIM1 Knockdown on Calcium Release and Influx—To decrease cellular STIM1 levels, double-stranded RNAs designed against STIM1 were transiently transfected in HeLa cells, together with a "cameleon" Ca^{2+}-sensitive fluorescent protein (YC3.6) that was used as a marker of transfection. Fluorescent cells were sorted by flow cytometry to select cells that presumably received the dsRNA, and quantitative RT-PCR was performed. As shown in Fig. 1*A*, STIM1 mRNA levels were decreased by 73% in fluorescent cells exposed to STIM1 dsRNA when compared with cells exposed to control, scramble

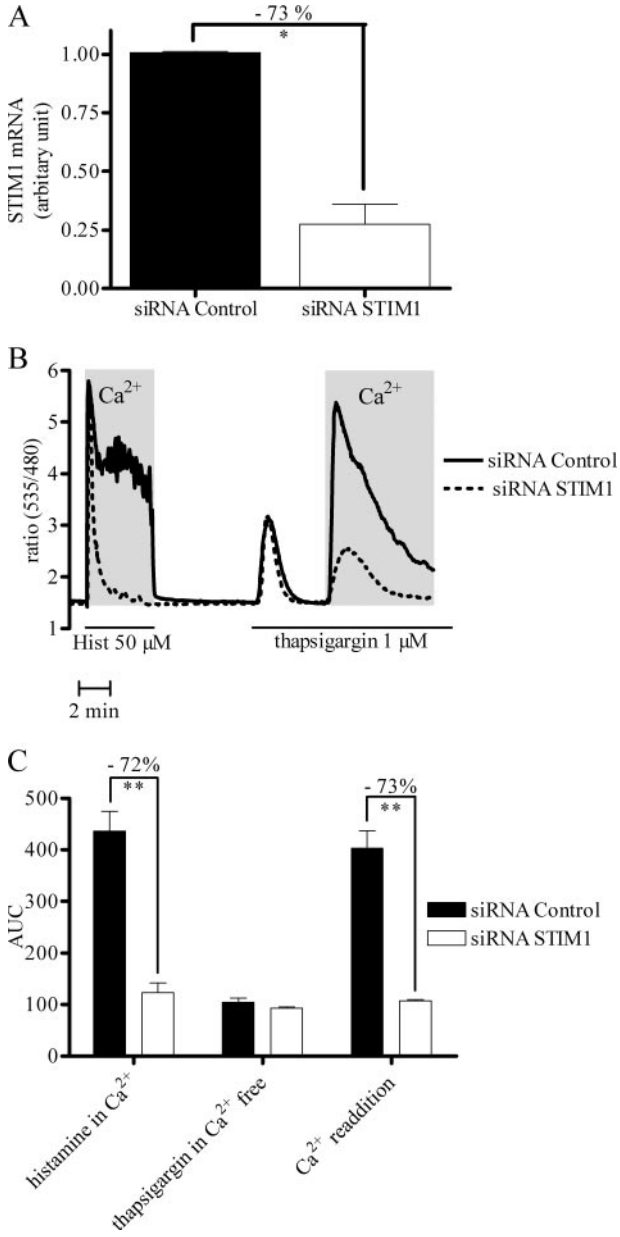

FIGURE 1. **Effect of STIM1 knockdown on store-operated Ca^{2+} influx.** HeLa cells were co-transfected with YC3.6 and either STIM1 or control (scramble) siRNA. *A*, quantification of STIM1 knockdown by real-time RT-PCR. Cells were sorted by fluorescence-activated cell sorter for YC3.6 fluorescence, mRNA was isolated, and real-time RT-PCR was performed. *Bars* are mean ± S.E. ($n = 3$) of the relative amount of STIM1 mRNA (relative to GAPDH). *B*, Ca^{2+} responses elicited by histamine (*Hist*) and thapsigargin in control (*thick line*) and STIM1 knockdown cells (*hatched line*). Cells were stimulated first with histamine in Ca^{2+} medium and then with thapsigargin in Ca^{2+}-free medium to deplete Ca^{2+} stores, and Ca^{2+} was then readded to monitor Ca^{2+} influx. *C*, statistical evaluation of the integrated Ca^{2+} responses (area under the curve (*AUC*)) shown in *panel B*. *Bars* are mean ± S.E. of 28 control and 30 STIM1 knockdown cells; *, $p = 0.001$ and **, $p < 0.0001$ *versus* control.

dsRNA. To test whether STIM1 knockdown altered Ca^{2+} handling, we measured the cytosolic Ca^{2+} responses elicited by agonists and by SERCA inhibitors using ratio imaging of the YC3.6 fluorescence. As shown in Fig. 1*B*, the initial peak of the histamine response was identical in cells that received control or STIM1 dsRNA, whereas the subsequent plateau phase, which reflects Ca^{2+} influx, was nearly abrogated in STIM1 knockdown cells. As a result, the integrated Ca^{2+} response to histamine was inhibited by 72% in STIM1 knockdown cells (Fig. 1*C*). Consistent with a decreased Ca^{2+} influx but preserved Ca^{2+} store content, the addition of thapsigargin in Ca^{2+}-free medium to passively deplete Ca^{2+} stores elicited similar responses in control and STIM1 knockdown cells, whereas the response elicited by the further readdition of Ca^{2+} was severely inhibited in STIM1 knockdown cells, the area under the curve being reduced by 73% (Fig. 1, *B* and *C*). These observations confirm previous results showing that Ca^{2+} influx, but not Ca^{2+} release from stores, is affected by STIM1 silencing.

Effect of STIM1 Knockdown on the Refilling of Ca^{2+} Stores—The strongly reduced SOC influx but preserved Ca^{2+} store content suggested that STIM1 knockdown cells were still able to refill their internal Ca^{2+} stores. This observation is surprising because the primary role of SOC influx is to sustain ER refilling during physiological stimulations. To study the impact of STIM1 knockdown on the refilling of Ca^{2+} stores, we extensively depleted Ca^{2+} stores without altering the activity of SERCA. For this purpose, cells were repeatedly stimulated with histamine in the absence of external Ca^{2+} (Fig. 2*A*, *phase i*). As shown in Fig. 2, the amplitude and kinetics of the agonist-induced Ca^{2+} release were equivalent in control and STIM1 knockdown cells, confirming that STIM1 knockdown cells retained a normal Ca^{2+} store content (Fig. 2, *B* and *C*, *phase i*). After this extensive store depletion, Ca^{2+} was transiently readded to allow store refilling (Fig. 2*A*, *phase ii*), and cells were stimulated again with histamine to assess the content of Ca^{2+} stores (Fig. 2*A*, *phase iii*). Remarkably, no Ca^{2+} changes were observed in STIM1 knockdown cells during Ca^{2+} readmission, whereas the expected increase was observed in control cells (Fig. 2*B*, *phase ii*). As a result, the integrated "Ca^{2+} influx" response was nearly abrogated in STIM1 knockdown cells (−99%, Fig. 2*C*, *phase ii*). Despite the lack of visible Ca^{2+} influx, however, the final histamine stimulation elicited a large cytosolic Ca^{2+} increase in STIM1 knockdown cells (Fig. 2*B*, *phase iii*), the integrated Ca^{2+} response averaging 58% of control cells (Fig. 2*C*, *phase iii*). To verify that histamine efficiently depleted Ca^{2+} stores, we repeated this experiment using ATP and UTP as Ca^{2+}-mobilizing agonists. The Ca^{2+} responses elicited by UTP and ATP were smaller than the responses induced by histamine, and Ca^{2+} influx remained undetectable in STIM1 knockdown cells (data not shown). When added to cells previously stimulated with histamine, UTP elicited a very small Ca^{2+} response, indicating that the agonist mobilizable store was efficiently depleted by histamine (supplemental Fig. S1). Importantly, Ca^{2+} influx remained abrogated in STIM1 knockdown cells, even when UTP was added in combination with histamine (supplemental Fig. S1). Thus, although no Ca^{2+} changes were observed in the cytosol during Ca^{2+} readmission, Ca^{2+} stores were able to refill efficiently in STIM1 knockdown cells.

Effect of External Ca^{2+} Concentration on the Refilling of Ca^{2+} Stores—The ability of STIM1 knockdown cells to remobilize Ca^{2+} from internal stores after the depletion/readmission protocol indicated that a "silent" supply of Ca^{2+} ions sustained the activity of SERCA pumps. To reveal this silent influx pathway, we varied the external Ca^{2+} concentration applied during the 5-min readmission phase, from 0 to 50 mM. As expected, when no Ca^{2+} was present during the

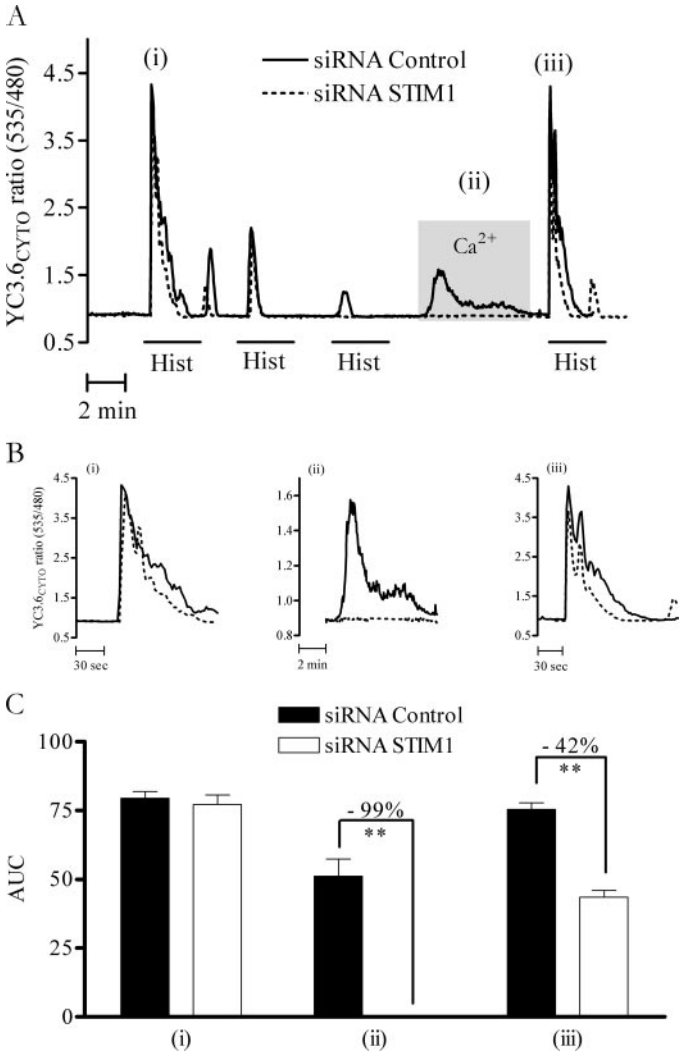

FIGURE 2. **Effect of STIM1 knockdown on the refilling of Ca^{2+} stores.** HeLa cells were co-transfected with YC3.6 and either control or STIM1 siRNA, and Ca^{2+} responses were measured by single cell imaging. Cells were stimulated three times with histamine (*Hist*) in Ca^{2+}-free medium to completely deplete Ca^{2+} stores. Ca^{2+} was then added transiently, and the cells were stimulated again with histamine to assess store refilling. *A*, representative recordings of the Ca^{2+} responses elicited by this protocol. *B*, enlargement of three phases of *panel A*: *phase i*, 1st histamine stimulation; *phase ii*, Ca^{2+} readdition; *phase iii*, last histamine stimulation. *C*, statistical evaluation of the integrated Ca^{2+} responses shown in *panel B*. *Bars* are mean ± S.E. of 89–103 control and 62 STIM1 knockdown cells; **, $p < 0.0001$ *versus* control. *AUC*, area under the curve.

refilling period, histamine could not remobilize Ca^{2+} from stores (Fig. 3*D*). Interestingly, at 0.2 mM $[Ca^{2+}]$, the responses were identical in control and STIM1-invalidated cells. In both conditions, Ca^{2+} influx was undetectable, and histamine could remobilize an identical amount of Ca^{2+} from internal stores (Fig. 3, *C* and *D*). The responses diverged markedly at higher Ca^{2+} concentrations, however. In control cells, Ca^{2+} responses increased in a dose-dependent manner with the external $[Ca^{2+}]$, both during Ca^{2+} readmission and during Ca^{2+} remobilization from stores (Fig. 3, *C* and *D*). In STIM1 knockdown cells, only minimal changes were observed during the readmission phase even at the highest Ca^{2+} concentration, but the amount of Ca^{2+} that could be remobilized by histamine increased with the amount of external Ca^{2+} supplied (Fig. 3, *C* and *D*). The response obtained in STIM1 knockdown cells with 50 mM Ca^{2+} approached the response obtained in control cells with 2 mM Ca^{2+} (Fig. 3*D*), indicating that Ca^{2+} stores could be completely replenished despite the lack of visible influx. Thus, increasing the external $[Ca^{2+}]$ enabled complete refilling of Ca^{2+} stores in STIM1 knockdown cells, but the entering Ca^{2+} ions remained undetectable in the cytosol.

Effect of STIM1 Knockdown on ER Ca^{2+} Homeostasis—The major intracellular Ca^{2+} store is the ER, where the STIM1 protein resides (6). The experiments shown in Figs. 2 and 3 indicate that external Ca^{2+} ions can reach the ER without altering cytosolic Ca^{2+} levels in STIM1 knockdown cells. To verify this observation, we directly measured the changes in the free ER Ca^{2+} concentration, $[Ca^{2+}]_{ER}$, using a cameleon probe targeted to the ER, $D1_{ER}$ (kindly provided by Dr. Amy Palmer, Boulder, CO). As illustrated in Fig. 4, *A* and *B*, the $D1_{ER}$ recordings showed that $[Ca^{2+}]_{ER}$ levels decreased upon histamine stimulation and returned to resting levels upon Ca^{2+} readmission, both in wild-type and in STIM1 knockdown cells. The $[Ca^{2+}]_{ER}$ changes correlated temporally with the changes in cytosolic Ca^{2+} measured simultaneously with Fura-2 during our depletion/readmission protocol (supplemental Fig. S2). The amplitude of the $[Ca^{2+}]_{ER}$ changes during Ca^{2+} release and uptake was similar in all conditions (Fig. 4*C*), but detailed analysis of the $D1_{ER}$ responses revealed that the kinetics of ER Ca^{2+} refilling were slower in cells transfected with STIM1 siRNA (τ = 31 s) than in cells transfected with scramble siRNA (τ = 11 s, Fig. 4, *D* and *F*). In contrast, the kinetics of histamine-induced Ca^{2+} release were identical for both conditions (supplemental Fig. S3). Further stimulation with histamine elicited a second decrease in $[Ca^{2+}]_{ER}$, whereas a final stimulation with thapsigargin to extensively deplete Ca^{2+} stores elicited a slower and monotonic decrase in $[Ca^{2+}]_{ER}$. Importantly, $[Ca^{2+}]_{ER}$ decreased with identical kinetics in wild-type and STIM1 knockdown cells exposed to thapsigargin (Fig. 4*E*). This indicates that the Ca^{2+} permeability of the ER was not affected, and by inference, that the activity of SERCA that counterbalances the ER Ca^{2+} leak was also unaffected. Thus, although Ca^{2+} ions reach the ER more slowly in STIM1 knockdown cells, SERCA activity is normal at low STIM1 levels, and resting $[Ca^{2+}]_{ER}$ levels are restored in 2 min upon Ca^{2+} readmission.

Effect of STIM1 on Mitochondrial Ca^{2+} Handling—The silent ER refilling revealed by STIM1 knockdown suggested that an organelle could relay Ca^{2+} ions from membrane channels to SERCA pumps on the ER. Mitochondria rapidly accumulate and release Ca^{2+} and thereby can provide such a Ca^{2+} relay mechanism. In addition, Ca^{2+} buffering by mitochondria has been shown to favor SOC influx by preventing the Ca^{2+}-dependent inhibition of SOC channels (19). To investigate whether mitochondria participate in Ca^{2+} influx and ER refilling in STIM1 knockdown cells, we used a combination of rotenone (an inhibitor of complex I from the respiratory chain) and oligomycin (an inhibitor of the H^+ ATPase) to dissipate the mitochondrial membrane potential and prevent mitochondrial Ca^{2+} uptake. In wild-type cells, the histamine-induced Ca^{2+} influx was severely inhibited by the

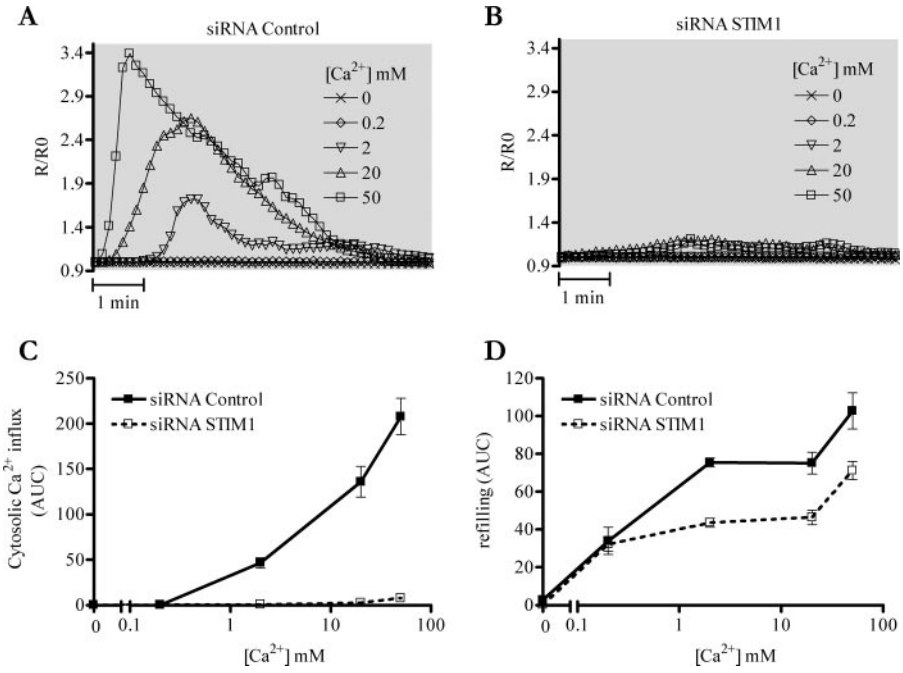

FIGURE 3. **Effect of increasing external Ca^{2+} concentrations during Ca^{2+} stores refilling.** Cells were stimulated as in Fig. 2, and different external Ca^{2+} concentrations were applied during the Ca^{2+} readmission phase. *A* and *B*, representative responses elicited by Ca^{2+} readmission to cells transfected with control and STIM1 siRNA. *C*, the integrated Ca^{2+} readmission signal (Fig. 2, *phase ii*) is plotted as a function of the Ca^{2+} concentration. Data are mean ± S.E. of 18–118 control cells and 16–74 STIM1 knockdown cells. *AUC*, area under the curve. *D*, the integrated Ca^{2+} response elicited by the last histamine stimulation (Fig. 2, *phase iii*) is plotted as a function of the Ca^{2+} concentration applied during Ca^{2+} readmission. Data are mean ± S.E. of 18–104 control cells and 16–72 STIM1 knockdown cells.

mitochondrial inhibitors, which were applied shortly before Ca^{2+} readmission (Fig. 5*A*). The response during Ca^{2+} readmission was reduced by 69%, and the refilling efficiency, assessed as in Fig. 2, was reduced by 37% (Fig. 5, *B* and *C*). In contrast, in STIM1 knockdown cells, the refilling efficiency was essentially insensitive to mitochondrial inhibitors (Fig, 5*C*). As previously reported (20), mitochondrial inhibition significantly decreased thapsigargin-induced Ca^{2+} influx in control cells (Fig. 6, *A* and *B*). In this case, however, SOC influx was nearly abolished in STIM1 knockdown cells exposed to mitochondrial inhibitors, the integrated Ca^{2+} response averaging 11% of the response of control, untreated cells (Fig. 6, *A* and *B*). Thus, the residual channels of STIM1 knockdown cells do not appear to require functional mitochondria when SERCA are active but are sensitive to mitochondrial inhibitors when SERCA are inhibited by thapsigargin. To test whether the effect of oligomycin and rotenone reflected reduced Ca^{2+} uptake by mitochondria, we used CGP-37157, an inhibitor of mitochondrial Na^+/Ca^{2+} exchange. This compound greatly reduces mitochondrial Ca^{2+} buffering without altering the ability of mitochondria to produce ATP (21). As shown in Fig. 6, *C* and *D*, CGP-37157 prevented thapsigargin-induced Ca^{2+} influx as efficiently as the combination of oligomycin and rotenone in wild-type cells but had no effect on SOC entry in STIM1 knockdown cells.

To check whether mitochondria were taking up the Ca^{2+} ions entering across SOC channels, we directly measured Ca^{2+} signals within the mitochondrial matrix, $[Ca^{2+}]_{mit}$, using a cameleon bearing a mitochondrial targeting sequence ($YC2.1_{2mit}$, kindly provided by Dr. T. Pozzan). As shown in Fig. 6, *E* and *F*, large changes in $[Ca^{2+}]_{mit}$ were observed upon Ca^{2+} readmission in control cells treated with thapsigargin. In STIM1 knockdown cells, the $[Ca^{2+}]_{mit}$ response was much smaller, consistent with the cytosolic responses shown in Fig. 1, the integrated $[Ca^{2+}]_{mit}$ and $[Ca^{2+}]_{cyt}$ responses being reduced by 89 and 73%, respectively. Nonetheless, the $[Ca^{2+}]_{mit}$ response was clearly visible in STIM1 knockdown cells during thapsigargin-induced influx, confirming that mitochondria were taking up Ca^{2+} at low STIM1 levels. These experiments indicate that mitochondria are located close to SOC channels and that SOC influx depends not only on STIM1 levels but also on the activity of SERCA and of neighboring mitochondria.

DISCUSSION

In this study, we decreased the levels of the protein STIM1, a key activator of store-operated Ca^{2+} influx, to study how Ca^{2+} ions entering across membrane channels are transferred to the ER. We confirmed that the STIM1 protein is an important regulator of SOC influx; reducing STIM1 mRNA levels by 73% with siRNA caused a 73% decrease in the integrated Ca^{2+} response to thapsigargin, a robust and very sensitive assay for SOC activity. The excellent correlation between STIM1 mRNA levels and the magnitude of the thapsigargin response confirms previous studies showing that the flux of Ca^{2+} ions entering across SOC channels is limited by the amount of STIM1 proteins (4, 5). Despite the drastic reduction in SOC influx, however, Ca^{2+} handling was essentially normal in STIM1 knockdown cells, which maintained normal resting cytosolic Ca^{2+} levels, released a normal amount of Ca^{2+} from internal stores, and were able to replenish their Ca^{2+} stores efficiently. The major difference observed was that at low STIM1 levels, store refilling occurred without detectable changes in cytosolic $[Ca^{2+}]$. Store reloading at resting $[Ca^{2+}]_{cyt}$ was reported previously when the amplitude of Ca^{2+} influx was reduced pharmacologically with SOC blockers (22) or with receptor antagonists (23) or when Ca^{2+} influx was spatially restricted to one side of the cell with a patch pipette (24). Our data confirm these early studies and show that a decrease in cellular STIM1 levels "silences" Ca^{2+} entry but has minimal impact on store refilling.

The efficiency of Ca^{2+} stores refilling was quantified by two distinct approaches: 1) by remobilizing Ca^{2+} from stores after a brief refilling period and 2) by measuring Ca^{2+} changes within the lumen of the endoplasmic reticulum. The first approach revealed that store refilling was reduced by 42% in STIM1 knockdown cells kept for 5 min in physiological saline. Stores

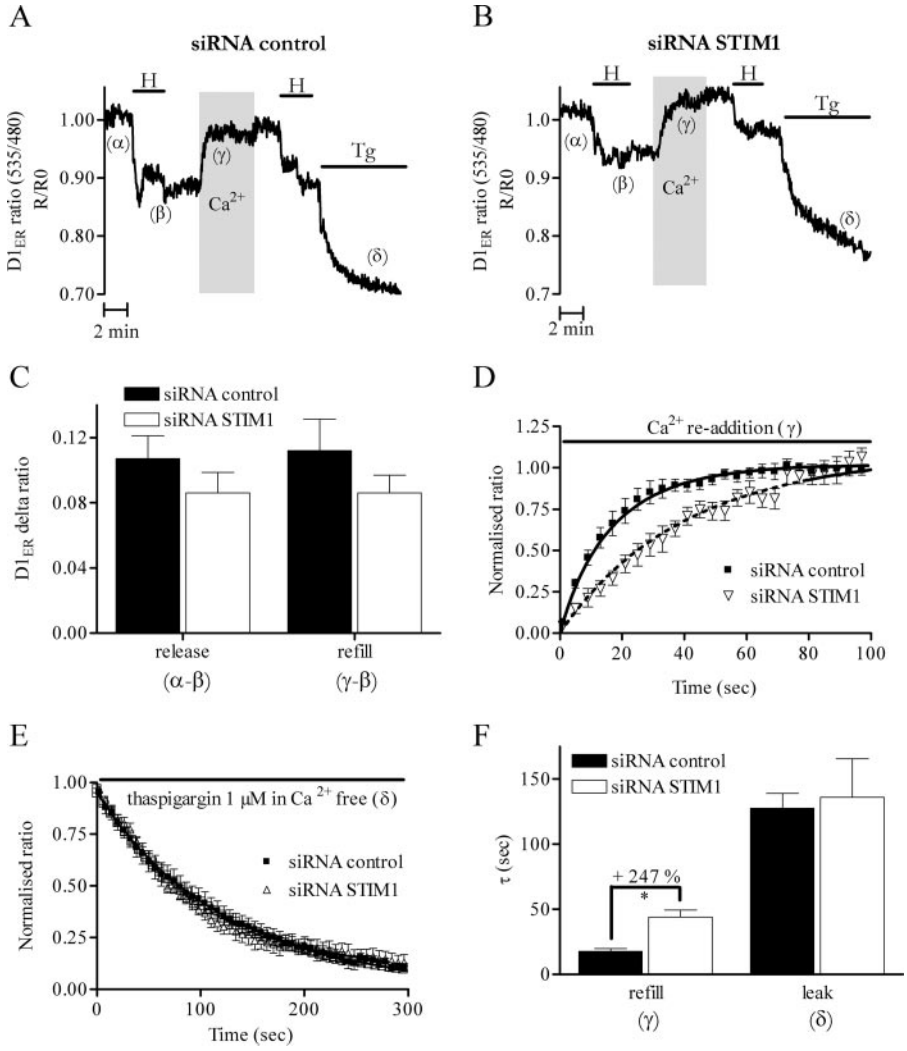

FIGURE 4. **Effect of STIM1 knockdown on ER Ca^{2+} release and uptake.** Cells were transfected with the ER-targeted cameleon probe $D1_{ER}$ to measure ER Ca^{2+} changes. Cells were kept for 2 min in the absence of external Ca^{2+} to monitor resting ER Ca^{2+} levels (α), stimulated with 50 μM histamine (*H*) to mobilize Ca^{2+} from stores (β), and Ca^{2+} added back transiently to refill ER Ca^{2+} stores (γ). Cells were then stimulated sequentially with histamine and thapsigargin (*Tg*) to extensively deplete Ca^{2+} stores (δ). *A* and *B*, representative recordings of ER Ca^{2+} responses in control (*A*) and STIM1 knockdown cells (*B*). *C*, mean amplitude of the $[Ca^{2+}]_{ER}$ changes during Ca^{2+} release and ER Ca^{2+} refilling. The changes in $D1_{ER}$ ratio elicited by histamine (α-β) and by Ca^{2+} readmission (γ-β) are shown. *Bars* are mean ± S.E. of 30–32 cells. *D* and *E*, time course of the $[Ca^{2+}]_{ER}$ responses during ER refilling (*D*) and during passive store depletion with thapsigargin (*E*). The $D1_{ER}$ responses were fitted with an exponential function to extract the time constants (τ). *F*, averaged time constants (τ) of the ER Ca^{2+} refilling and ER Ca^{2+} leak processes, derived from the fits shown in *panels D* and *E*. Data are mean ± S.E. of 6–7 experiments comprising 23–32 cells; **, $p < 0.0001$ *versus* control.

could be fully refilled by increasing the Ca^{2+} concentration from 2 to 50 mM during the Ca^{2+} readmission period, but even at this very high Ca^{2+} concentration, Ca^{2+} stores refilled without any visible cytosolic signal. When measured from within the ER, store refilling was very rapid, and $[Ca^{2+}]_{ER}$ returned to resting levels within 2 min upon Ca^{2+} readmission. ER refilling proceeded more slowly in STIM1 knockdown cells than in wild-type cells, but in both cases, $[Ca^{2+}]_{ER}$ returned within 2 min to prestimulatory levels in physiological saline. The kinetics of histamine- and thapsigargin-induced Ca^{2+} release were not altered by STIM1 knockdown, indicating that the permeability and InsP3 sensitivity of Ca^{2+} stores were normal in these cells. These observations indicate that cells can maintain normal levels of Ca^{2+} within the ER even with drastically reduced SOC activity. When Ca^{2+} influx is limiting, the few Ca^{2+} ions entering across the plasma membrane are rapidly carried to the ER without impacting on cytosolic Ca^{2+} homeostasis.

In cells containing a normal amount of STIM1, however, the large flux of Ca^{2+} ions through SOC channel clusters exceeds the capacity of subplasmalemmal SERCA. In this case, a significant fraction of the entering Ca^{2+} is taken up by nearby mitochondria and redirected to SERCA located farther from the plasma membrane (25, 26). This trans-mitochondrial Ca^{2+} flux ensures that only a minimal fraction of the Ca^{2+} entering via SOC channels diffuses in the cytosol (Fig. 7*A*). The combined capacity of mitochondria and SERCA is fairly large, and in wild-type cells, cytosolic flooding is not observed when the external Ca^{2+} concentration is reduced to 0.2 mM. This mechanism ensures that during stimulation with physiological agonists, nearly all the entering Ca^{2+} ions are used by SERCA to refill the ER, either directly or indirectly via mitochondria. The situation is very different when influx through SOC channels is limiting because in STIM1 knockdown cells, the silent ER refilling was insensitive to mitochondrial inhibitors. This indicates that all the entering Ca^{2+} ions were directly captured by subplasmalemmal SERCA and not relayed by mitochondria. Mitochondria, however, were still located close to the active channels because a mitochondrial Ca^{2+} signal was detected when SERCA were inhibited by thapsigargin (Fig. 6). This indicates that, at low STIM1 levels, the Ca^{2+} uptake capacity of SERCA exceeds the flux of Ca^{2+} ions across the remaining functional SOC channels (Fig. 7*B*).

Our data thus highlight a major role of SERCA in buffering subplasmalemmal Ca^{2+} ions, a role that is intrinsic to their Ca^{2+} pumping activity but was not fully appreciated before. SOC channels inactivate rapidly at high cytosolic Ca^{2+} concentrations, and their sustained activity requires efficient buffering systems to prevent the buildup of Ca^{2+} near the mouth of the channel. Mitochondria were shown to prevent the Ca^{2+}-dependent inactivation of SOC channels by buffering subplasmalemmal Ca^{2+} (19). SERCA can perform the same function, as shown in patch clamp studies (24, 27) and in cells expressing

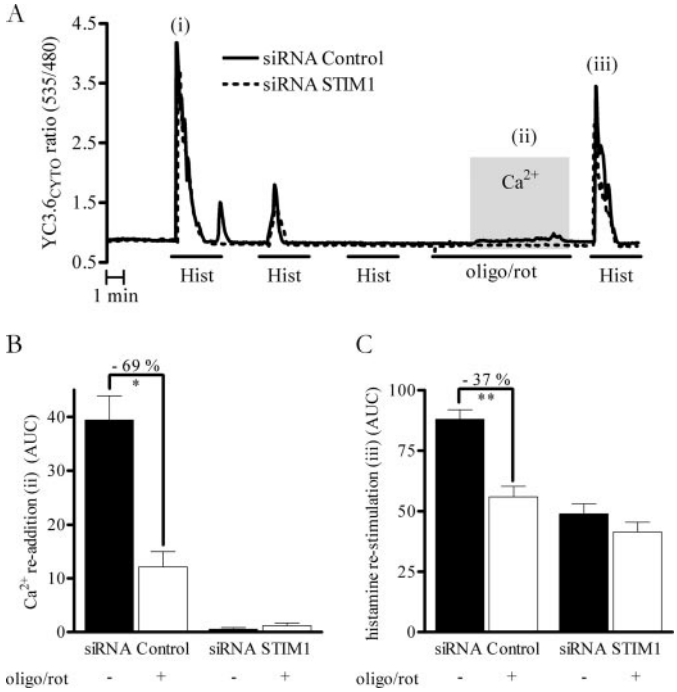

FIGURE 5. **Effect of mitochondrial inhibitors on ER Ca^{2+} refilling.** *A*, the protocol of Fig. 2 was used to deplete and refill Ca^{2+} stores, and mitochondrial inhibitors (*oligo/rot*, 5 μg/ml oligomycin and 25 μM rotenone) were added 2 min before Ca^{2+} readmission. *B* and *C*, integrated Ca^{2+} responses measured during Ca^{2+} readmission (*B*, *phase ii*) and during Ca^{2+} remobilization with histamine (*C*, *phase iii*). *Bars* are mean ± S.E. of 58–156 control cells and 59–70 STIM1 knockdown cells; *, $p < 0.001$, and **, $p < 0.0001$ *versus* control. *AUC*, area under the curve.

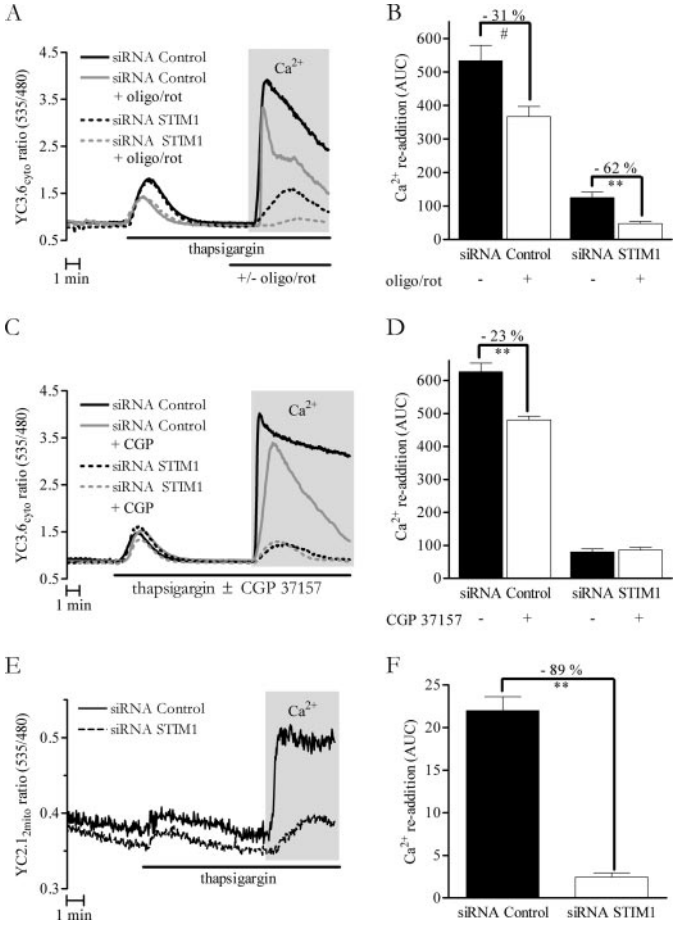

FIGURE 6. **Effect of mitochondrial ATP production and Ca^{2+} buffering on Ca^{2+} influx.** *A*, effect of inhibitors of mitochondrial ATP production (*oligo/rot*, 5 μg/ml oligomycin and 25 μM rotenone) on thapsigargin-induced Ca^{2+} responses. *B*, integrated Ca^{2+} responses during Ca^{2+} readmission. *Bars* are mean ± S.E. of 35–38 control cells and 35–45 STIM1 knockdown cells. *AUC*, area under the curve. *C*, effect of mitochondrial Na/Ca^{2+} exchange inhibition (15 μM CGP-37157 (*CGP*)) on thapsigargin-induced Ca^{2+} influx. *D*, integrated Ca^{2+} responses during Ca^{2+} readmission. *Bars* are mean ± S.E. of 43–79 control cells and 32–61 STIM1 knockdown cells. *E* and *F*, effect of STIM1 knockdown on mitochondrial Ca^{2+} uptake. Cells were transfected with a mitochondrial-targeted cameleon ($YC2.1_{2mit}$) together with either control or STIM1 siRNA. *E*, representative recordings of the mitochondrial Ca^{2+} responses elicited by the thapsigargin/readmission protocol. *F*, integrated mitochondrial Ca^{2+} responses during Ca^{2+} readmission. *Bars* are mean ± S.E. of 52 control and 32 STIM1 knockdown cells; #, $p < 0.01$ and **, $p < 0.0001$ *versus* control.

transient receptor potential channels (28). Our data show that, at low STIM1 levels, SERCA take up all the Ca^{2+} ions entering across SOC channels, thereby preventing channel inactivation and enabling efficient ER refilling. A similar conclusion was reached by Malli *et al.* (29) using high K^+ to reduce the electrochemical driving force for Ca^{2+} and thus the magnitude of Ca^{2+} influx. When Ca^{2+} influx exceeds the capacity of subplasmalemmal SERCA, mitochondria take up the excess Ca^{2+} and relay it to deeper ER regions. Finally, when both SERCA and mitochondria are saturated, the Ca^{2+}-dependent inactivation of SOC channels shuts down the supply of Ca^{2+} ions. Thus, as depicted in the diagram of Fig. 7, three mechanisms contribute to minimize the diffusion of Ca^{2+} ions in the cytosol in the vicinity of SOC channels: 1) the presence of active SERCA on the juxtaposed ER membrane, which under physiological conditions take up most of the entering Ca^{2+} ions; 2) the presence of neighboring mitochondria, which scavenge the remaining Ca^{2+} ions; and 3) the Ca^{2+}-dependent inactivation of SOC channels, which terminates Ca^{2+} entry when both Ca^{2+} scavenging systems are saturated. At low STIM1 levels, SOC influx did not require mitochondrial Ca^{2+} buffering but nevertheless required functional mitochondria (Fig. 6), indicating that local ATP production is also required for SOC channel activity. Because SERCA were inhibited by thapsigargin in this experiment, the mitochondrial ATP was not required as energy supply but most likely as a mobile Ca^{2+} buffer, as recently shown in lymphocytes (30). The mitochondria located near the ER-PM junction are thus particularly important because they supply ATP used to buffer the entering Ca^{2+} ions and to energize SERCA and scavenge the excess of Ca^{2+} ions when SOC influx exceeds the capacity of SERCA. Our STIM1 knockdown study thus highlights the important role of the mitochondria located near the narrow and extended ER-PM junctions.

Our $[Ca^{2+}]_{ER}$ measurements further show that removing STIM1 proteins is well tolerated by the ER Ca^{2+} handling machinery. This is somewhat unexpected given the dominant role of STIM1 in the regulation of SOC entry. STIM1 contains a lumenal EF-hand domain that acts as a $[Ca^{2+}]_{ER}$ sensor and a cytosolic domain that is both necessary and sufficient for the activation of SOC channels (31). STIM1 interacts with Orai1 and TRP Ca^{2+} channels at the plasma membrane via its cytosolic ERM domain, but it is not known whether the only partners of STIM1 are plasma membrane Ca^{2+} channels or whether STIM1 also interacts with Ca^{2+} transporters on the ER membrane and modulate their activity. Our data indicate that

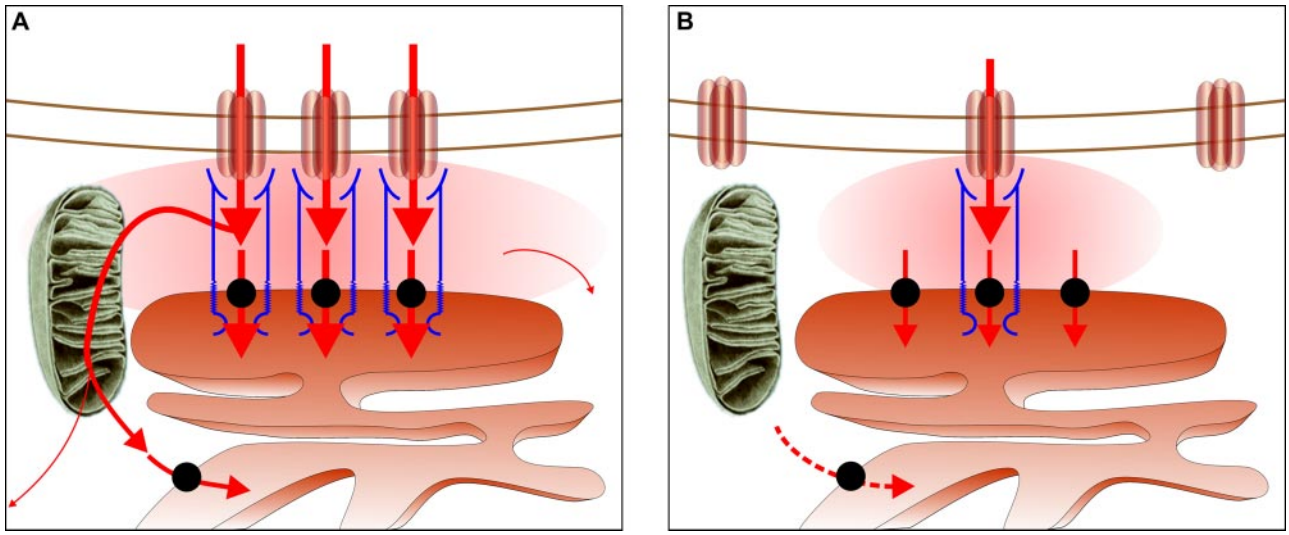

FIGURE 7. **Model of the STIM1 microdomains linking SOC channels to SERCA.** *A*, in wild-type cells, the SOC channels (*red cylinders*) clustered by STIM1 (*blue anchors*) sustain a massive influx of Ca^{2+} ions that exceeds the capacity of SERCA pumps (*black circles*) located on nearby ER cisternae. As a result, a significant fraction of the entering Ca^{2+} ions are captured by subplasmalemmal mitochondria and relayed either to the cytosol or to SERCA located farther from the plasma membrane. *B*, at low STIM1 levels, the limited influx of Ca^{2+} ions entering across the residual SOC channels are entirely taken up by neighboring SERCA, and Ca^{2+} ions do not spill over to mitochondria or to the cytosol. The close proximity of SOC channels to SERCA allows an extremely rapid and efficient ER Ca^{2+} refilling with only a few SOC channels, without perturbing the cytosol.

STIM1 has minimal impact on the basal activity of ER Ca^{2+} transporters because decreasing STIM1 levels did not significantly alter resting $[Ca^{2+}]_{ER}$ levels or the passive Ca^{2+} permeability of the ER. Since the $[Ca^{2+}]_{ER}$ homeostasis is maintained by a "pump and leak" mechanism, this implies that the basal activity of SERCA Ca^{2+} pumps and of the Ca^{2+} leak pathway (which reflects the basal Ca^{2+} permeability of InsP3 receptors and other pathways, see Ref. 32) is not affected by the decrease in STIM1 levels. Thus, our data indicate that removing STIM1 proteins has minimal impact on ER Ca^{2+} homeostasis, confirming a recent study in *Caenorhabditis elegans* (33). In this model system, STIM1 knockdown inhibited store-operated Ca^{2+} entry but had no effects on Ca^{2+} oscillations and waves and did not induce ER depletion. Our data confirm these findings and show that STIM1 regulates the activity of SOC channels at the plasma membrane without modifying the activity of Ca^{2+} transporters located on the ER. The structure formed by the juxtaposition of the ER and the plasma membrane ensures the optimal delivery of Ca^{2+} ions to the reticulum and minimizes the Ca^{2+} contamination of the cytosol, creating a privileged pathway for ER refilling.

Acknowledgments—We acknowledge the support of all members of the Demaurex laboratory and specifically of Cyril Castelbou for excellent technical assistance. We thank N. Dupont for brilliant graphic work. We thank Drs. R. Y. Tsien, A. Palmer, and T. Pozzan for providing the cameleon constructs, Drs. M. Rossier and S. Lenglet for helping in the quantitative RT-PCR experiments, and Dr. W. Graier for critical reading of the manuscript.

REFERENCES

1. Putney, J. W., Jr. (1986) *Cell Calcium* **7,** 1–12
2. Hoth, M., and Penner, R. (1992) *Nature* **355,** 353–356
3. Parekh, A. B., and Putney, J. W., Jr. (2005) *Physiol. Rev.* **85,** 757–810
4. Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr., and Meyer, T. (2005) *Curr. Biol.* **15,** 1235–1241
5. 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., Velicelebi, G., and Stauderman, K. A. (2005) *J. Cell Biol.* **169,** 435–445
6. Wu, M. M., Buchanan, J., Luik, R. M., and Lewis, R. S. (2006) *J. Cell Biol.* **174,** 803–813
7. Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. J., Ellisman, M. H., Stauderman, K. A., and Cahalan, M. D. (2005) *Nature* **437,** 902–905
8. Yeromin, A. V., Zhang, S. L., Jiang, W., Yu, Y., Safrina, O., and Cahalan, M. D. (2006) *Nature* **443,** 226–229
9. Lopez, J. J., Salido, G. M., Pariente, J. A., and Rosado, J. A. (2006) *J. Biol. Chem.* **281,** 28254–28264
10. Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S. H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006) *Nature* **441,** 179–185
11. Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) *Science* **312,** 1220–1223
12. Zhang, S. L., Yeromin, A. V., Zhang, X. H., Yu, Y., Safrina, O., Penna, A., Roos, J., Stauderman, K. A., and Cahalan, M. D. (2006) *Proc. Natl. Acad. Sci. U. S. A.*
13. Peinelt, C., Vig, M., Koomoa, D. L., Beck, A., Nadler, M. J., Koblan-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J. P. (2006) *Nat. Cell Biol.* **8,** 771–773
14. Soboloff, J., Spassova, M. A., Tang, X. D., Hewavitharana, T., Xu, W., and Gill, D. L. (2006) *J. Biol. Chem.* **281,** 20661–20665
15. Mercer, J. C., Dehaven, W. I., Smyth, J. T., Wedel, B., Boyles, R. R., Bird, G. S., and Putney, J. W., Jr. (2006) *J. Biol. Chem.* **281,** 24979–24990
16. Prakriya, M., Feske, S., Gwack, Y., Srikanth, S., Rao, A., and Hogan, P. G. (2006) *Nature* **443,** 230–233
17. Vig, M., Beck, A., Billingsley, J. M., Lis, A., Parvez, S., Peinelt, C., Koomoa, D. L., Soboloff, J., Gill, D. L., Fleig, A., Kinet, J. P., and Penner, R. (2006) *Curr. Biol.* **16,** 2073–2079
18. Luik, R. M., Wu, M. M., Buchanan, J., and Lewis, R. S. (2006) *J. Cell Biol.* **174,** 815–825
19. Hoth, M., Fanger, C. M., and Lewis, R. S. (1997) *J. Cell Biol.* **137,** 633–648
20. Frieden, M., James, D., Castelbou, C., Danckaert, A., Martinou, J. C., and Demaurex, N. (2004) *J. Biol. Chem.* **279,** 22704–22714
21. Arnaudeau, S., Kelley, W. L., Walsh, J. V., Jr., and Demaurex, N. (2001) *J. Biol. Chem.* **276,** 29430–29439

22. Byron, K. L., Babnigg, G., and Villereal, M. L. (1992) *J. Biol. Chem.* **267,** 108–118
23. Takemura, H., and Putney, J. W., Jr. (1989) *Biochem. J.* **258,** 409–412
24. Mogami, H., Nakano, K., Tepikin, A. V., and Petersen, O. H. (1997) *Cell* **88,** 49–55
25. Malli, R., Frieden, M., Osibow, K., Zoratti, C., Mayer, M., Demaurex, N., and Graier, W. F. (2003) *J. Biol. Chem.* **278,** 44769–44779
26. Frieden, M., Arnaudeau, S., Castelbou, C., and Demaurex, N. (2005) *J. Biol. Chem.* **280,** 43198–43208
27. Liu, X., and Ambudkar, I. S. (2001) *J. Biol. Chem.* **276,** 29891–29898
28. Lemonnier, L., Trebak, M., Lievremont, J. P., Bird, G. S., and Putney, J. W., Jr. (2006) *FASEB J* **20,** 503–505
29. Malli, R., Frieden, M., Hunkova, M., Trenker, M., and Graier, W. F. (2007) *Cell Calcium* **41,** 63–76
30. Montalvo, G. B., Artalejo, A. R., and Gilabert, J. A. (2006) *J. Biol. Chem.* **281,** 35616–35623
31. Huang, G. N., Zeng, W., Kim, J. Y., Yuan, J. P., Han, L., Muallem, S., and Worley, P. F. (2006) *Nat. Cell Biol.* **8,** 1003–1010
32. Lomax, R. B., Camello, C., Van Coppenolle, F., Petersen, O. H., and Tepikin, A. V. (2002) *J. Biol. Chem.* **277,** 26479–26485
33. Yan, X., Xing, J., Lorin-Nebel, C., Estevez, A. Y., Nehrke, K., Lamitina, T., and Strange, K. (2006) *J. Gen. Physiol.* **128,** 443–459