The Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is the third element in capacitative calcium entry*

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STIM1 and Orai1 are the principal players in capacitative calcium entry (CCE). STIM senses the Ca^{2+} content inside the endoplasmic reticulum (ER) and, when it decreases, it activates Orai1, a store-operated calcium channel (SOC) in the plasma membrane that promotes Ca^{2+} entry and increases cytosolic Ca^{2+} . The final destination of the entering Ca^{2+} is not the cytosol but the ER, which refills very efficiently (capacitatively) with it. We propose here that the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is the third element of CCE, to which is tightly coupled. Close proximity between SOC and SERCA favours rapid Ca^{2+} pumping from the high Ca^{2+} microdomains generated at the cytoplasmic mouth of SOCs to the ER. We find that, on depletion of the intracellular Ca^{2+} stores, SERCA colocalizes with STIM1 at punctae. Adequate coupling of CCE and Ca^{2+} pumping into the ER requires correct proportions of STIM1, Orai1 and SERCA. Overexpression of Orai1 produced a modest decrease of CCE and a dramatic fall of Ca^{2+} uptake into ER, measured selectively with aequorins targeted to cytosol and ER, respectively. This inhibition of ER refilling was rescued by STIM1 coexpression. The inhibition could also be reversed by increasing external Ca^{2+} . In permeabilized cells, Ca^{2+} uptake into the ER was the same in the Orai1-expressing as in the control cells. We propose that, in the intact cells, excess Orai1 uncouples SERCA from CCE by disturbing the fine topology of Ca^{2+} pumping within the ER-plasma membrane junctions.

Capacitative Ca^{2+} entry (CCE) [1] is essential for Ca^{2+} homeostasis. It keeps the ER adequately filled with Ca^{2+} , thus making possible sustained signalling by Ca^{2+} mobilization. CCE rests on a feedback mechanism activated by the decrease of the Ca^{2+} inside ER ($[\text{Ca}^{2+}]_{\text{ER}}$) that triggers Ca^{2+} entry through the plasma membrane (reviewed by [2]). This store-operated Ca^{2+} entry (SOCE) takes place through store-operated Ca^{2+} channels (SOC), which may differ somewhat among different cell types. The best characterized Ca^{2+} current is CRAC (Ca^{2+} -release activated current), first described in mast cells [2, 3]. In the original model, the incoming Ca^{2+} was proposed to enter directly into the stores, bypassing the cytosol [1]. It has been proved later that the entering Ca^{2+} do pass through the cytosol [4-6], but there seems to be a preferential connexion between the extracellular medium and the ER that makes refilling very efficient [7]. The Ca^{2+} sensor of the ER, STIM1 [8, 9], and the plasma membrane (PM) channel, Orai1 [10-13], have been identified recently. Mutations of STIM1 or Orai1 cause immunodeficiencies [10, 14] and perturbation of ER filling affects not only Ca^{2+} signalling but also protein handling in the ER and may lead to ER stress, unfolding protein response (UPR) and cell death [15, 16].

The decrease of $[\text{Ca}^{2+}]_{\text{ER}}$ favours dissociation of Ca^{2+} from STIM1, which aggregates and translocates to ER junctions closely apposed to the PM [17]. There, STIM and Orai associate into clusters visible under the optic microscope (punctae), which co-localize with the places of PM Ca^{2+} entry (reviewed by [18]). Close interactions between STIM and Orai have been confirmed by Förster resonance energy transfer (FRET) measurements [19-22], and assembly of the SOC

complex has been followed in great detail by combining light-guided TIRF microscopy with FRET [22]. In the present study, we focus on functional measurements of Ca^{2+} transport by the ER during activation of CCE using targeted aequorins for real-time selective monitoring of Ca^{2+} within this compartment. Our results indicate that SERCA organizes together with STIM1 and Orai1 within the clusters responsible for CCE, thus, optimizing refilling of the ER with the incoming Ca^{2+} .

EXPERIMENTAL PROCEDURES

DNA constructs- Chimeric GFP-aequorins targeted to cytosol (cytGA) or to ER reticulum (erGA) were generated as previously described [23, 24]. STIM1 and Orai1 were purchased from Origene. The following tagged proteins were used: STIM1-YFP and Orai1-YFP (from A. Rao, Harvard University) [19], mCherry-Orai1 (from A. Tepikin, University of Liverpool, U.K.) [25] and Orai-EGFP (from L. Chen & T. Xu, National Key Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Science, Beijing, China) [26]. The PCR-amplified fragments of EGFP or mCherry were fused in frame to the N-terminus of SERCA2b, obtained from P. Camacho, University of Texas Health Science Center, San Antonio) [27].

Cell culture and transfection- HEK293T and HeLa cells were cultured and handled as described before [23, 24] and seeded at about 5×10^5 on 6 well-plates. Transfection was performed using Lipofectamine (Invitrogen) with 0.1-0.4 μg of cDNA. After 12-24 h cells were removed and re-seeded in poly-L-lysine coated 12 mm coverslips (5×10^4 cells/coverslip).

Expression of fluorescent proteins- Transfected cells were treated with standard medium containing (in mM): NaCl, 145; KCl, 5; MgCl_2 , 1; glucose, 10; sodium-HEPES, 10; pH 7.4 and either 1 mM CaCl_2 or 0.5 mM EGTA plus 1 μM thapsigargin for 10 min. at room temperature and fixed with 4% paraformaldehyde for 20 min. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Images were captured in a Zeiss Axioplan Z microscope using a 63x/1.20w Korr objective. GFP/YFP and mCherry were monitored by imaging the green (Ex, 490/20;

Em, 540/50 nm) and the red fluorescence (Ex, 560/40; Em, 615/45 nm). The Zeiss ApoTome^R system was used for optical sectioning. Images were analyzed using ImageJ software (<http://rsb.info.nih.gov/ij/>). Colocalization and pixel by pixel intensity correlation were performed as described [28]. FRET between GFP or YFP and the mCherry-fluorescent protein was evaluated by the sensitized emission protocol using an ImageJ plugin [29].

Calcium measurements- Imaging was performed in cells loaded with fura-4F as described before [30]. For aequorin bioluminescence measurements, GFP-aequorin fusion proteins targeted either to cytosol (cytGA) or to ER (erGA) were used to determine $[\text{Ca}^{2+}]_C$ or $[\text{Ca}^{2+}]_{ER}$, respectively. Aequorin was reconstituted with either native (cytGA) or n-type (erGA) coelenterazine in standard medium containing either 1 mM CaCl_2 (filled stores) or 0.5 mM EGTA and 10 μM of 2,5-ditert-butyl-benzohydroquinone (TBH; depleted stores) for about 1h [23, 31]. In the experiments with permeabilized cells, an intracellular-like solution containing (in mM) KCl, 140; KH_2PO_4 , 1; MgCl_2 , 1; Mg-ATP, 1; Na-succinate, 2; sodium-HEPES, 20; pH 7.0 was used for perfusion. The cells were permeabilized with 60 μM digitonin in a Ca^{2+} -free (2 mM EGTA) medium for 60 s; after washing with Ca^{2+} -free medium, the solution was switched to the intracellular-like solution containing 100 nM Ca^{2+} buffered with EGTA [32] to allow Ca^{2+} refilling of the ER. All the measurements were performed at 22 °C. Other details were as described previously [33].

RESULTS

Co-expression of STIM1 and Orai1 resulted in magnified CCE, with an increase of the Ca^{2+} overshoot in fura-4F-loaded cells whose intracellular Ca^{2+} stores had been depleted of Ca^{2+} by prior treatment with thapsigargin (TG) in Ca^{2+} -free medium (Fig. 1A). This is consistent with previous results [11, 34, 35]. At optimal Orai1:STIM1 ratio (1:1), the $\Delta[\text{Ca}^{2+}]_C$ on re-addition of Ca^{2+} was 8-fold larger than in non-transfected controls (Fig. 1A). Increasing the Orai1:STIM1 ratio decreased CCE (3.2-fold at 4:1 and only 1.3-fold at 10:1; results not shown). Expressing only Orai1 decreased moderately CCE. Fig. 1B compares the effects of the expression of either Orai1 alone or Orai1 together with STIM1 on CCE by TG-treated cells, now measured using cytosolic aequorin (cytGA). A high Ca^{2+}

concentration (5 mM) was used to force Ca^{2+} entry during the overshoot. When both proteins were co-expressed, the overshoot was about 5-fold larger than in the control cells. In contrast, expression of Orai1 alone produced a small (28%) inhibition.

Ca^{2+} uptake into the ER during the depletion/overshoot protocol can be conveniently measured using an ER-targeted aequorin (erGA). In Fig. 1C the ER had been depleted of Ca^{2+} by treatment, in Ca^{2+} -free medium, with the reversible SERCA inhibitor TBH [36]. Then, TBH was washed and 1 mM Ca^{2+} was added to induce Ca^{2+} entry. The Ca^{2+} uptake by ER was the fastest in the cells co-transfected with Orai1 and STIM1 (mean $\pm$ s.e.m, 40 ± 9 $\mu\text{M/s}$ compared to 14 ± 2 $\mu\text{M/s}$ in the control). This outcome is not surprising as the stimulation of Ca^{2+} entry increases $[\text{Ca}^{2+}]_c$ (Fig. 1A & 1B) and this stimulates SERCA pumping into the ER. Interestingly, in the cells transfected with Orai1 alone the Ca^{2+} uptake into the ER was exceedingly slow (Fig. 1C; 1.8 ± 0.3 $\mu\text{M/s}$). Such a drastic effect cannot be explained by the modest decrease of the Ca^{2+} overshoot in the cytosol (Fig. 1B)

The decrease of Ca^{2+} uptake into the ER caused by Orai1 overexpression was then studied in permeabilized cells (Fig. 1D), where $[\text{Ca}^{2+}]_c$ and other cytosolic properties can be precisely controlled by perfusing with intracellular-like solutions containing Ca^{2+} buffers. Permeabilized cells still formed punctae when treated with TG. After PM permeabilization with digitonin, ER uptake was studied in an intracellular-like environment containing ATP and 100 nM Ca^{2+} , a concentration close to the resting $[\text{Ca}^{2+}]_c$. Ca^{2+} was rapidly taken up by control cells to reach steady state $[\text{Ca}^{2+}]_{ER}$ levels close to 10^{-3} M within less than 5 min (Fig. 1D). The behaviour of the ER was quite analogous to the one found in the intact cells (compare to Fig. 1C). Surprisingly, the Orai1-expressing cells behaved, once permeabilized, similarly to the control cells (Fig. 1D). In 7 similar experiments the initial uptake rates were (in $\mu\text{M/s}$; mean $\pm$ s.e.m.) 7.6 ± 0.7 in the control and 5.9 ± 1.1 $\mu\text{M/s}$ in the experimental condition and the steady state levels, measured 5 min after Ca^{2+} addition, were 803 ± 65 μM for the controls and 715 ± 59 μM for the Orai1-

expressing cells. These results, so contrasting with the ones obtained in intact cells (Fig. 1C), indicate that the interaction between Orai1 and SERCA requires cell integrity.

The discrepancy between the results with intact and permeabilized cells was so striking that we designed new independent experiments for further investigation. HEK293T cells express both purinergic P2Y and cholinergic receptors, which are able, via IP_3 , to release Ca^{2+} from the ER with production of the concomitant $[\text{Ca}^{2+}]_c$ peak [37]. The size of the $[\text{Ca}^{2+}]_c$ peak is proportional to the Ca^{2+} contents of the ER [38]. In the experiments of Fig. 1E, the intracellular Ca^{2+} stores of cells expressing cytGA were first emptied by treatment with TBH in Ca^{2+} -free medium. When TBH was washed and Ca^{2+} readmitted, there was an increase of $[\text{Ca}^{2+}]_c$ that reflects activation of CCE. As shown above (Fig. 1B), expression of Orai1 decreased only moderately (24%) the Ca^{2+} overshoot. Incubation with the Ca^{2+} -containing medium was continued for 5 min to allow ER refilling by Ca^{2+} uptake from the cytosol. Then, the cells were challenged with ATP and carbachol to induce maximal release of the stored Ca^{2+} . A large $[\text{Ca}^{2+}]_c$ peak was obtained in the control, but not in the Orai1-expressing cells (Fig. 1E), suggesting that the ER had not refilled. The mean $\Delta[\text{Ca}^{2+}]_c$ value in 8 experiments was ($\pm$ s.e.m.) 0.03 ± 0.03 μM compared to 1.45 ± 0.22 μM in the controls. These results are consistent with the ones of Fig. 1C and demonstrate that the ER of the cells with excess Orai1 refills with great difficulty. In parallel experiments, cells incubated with 1 mM Ca^{2+} for 1-2 hours (without store depletion) responded to the ATP/carbachol challenge (Fig. 1F), demonstrating the IP_3 receptors are functional. The peak was decreased by about 50% in the Orai-1 expressing cells with regard to controls, suggesting that the amount of Ca^{2+} stored into the ER at the steady state may be smaller, although the effect on the initial rate of uptake (Fig. 1E) is much more striking.

Finally, we designed experiments to see whether the inhibition of ER Ca^{2+} uptake induced by Orai1 overexpression could be circumvented by raising the Ca^{2+} entry. Faster influx was achieved by increasing the extracellular Ca^{2+} concentration from 1 to 5 or 15 mM. CCE was activated by emptying the intracellular Ca^{2+} stores with TBH and the overshoot following external Ca^{2+} readdition was measured both in the cytosol (using cytGA; Fig. 1G) and in the ER (using erGA; Fig.

1H). The results were quite different. In 4 similar experiments, the peak $[Ca^{2+}]_C$ values achieved during CCE were (mean $\pm$ s.e.m.) 0.87 ± 0.07 , 1.45 ± 0.09 and 2.09 ± 0.25 μ M at 1, 5 and 15 mM external Ca^{2+} , respectively. The uptake into the ER was more deeply affected (Fig. 1H). The rates of the $[Ca^{2+}]_{ER}$ increases at 1, 5 and 15 mM external Ca^{2+} were (mean $\pm$ s.e.m.) 0.81 ± 0.06 , 2.82 ± 0.64 and 15.97 ± 0.97 μ M/s, respectively. These results demonstrate that the inhibition of ER uptake by Orai1 is functional and can be rescued by increasing Ca^{2+} entry.

Putative physical interactions between STIM1, Orai1 and SERCA2b were investigated by studying FRET among fluorescent fusions of these proteins expressed in HEK293T or HeLa cells treated with TG. Results were similar with both cell types. We were able to document FRET between Orai1-YFP and mCherry-Orai1 (C-Orai1) (Results not shown; consistent with [20]) or between STIM1-YFP and C-Orai1 (consistent with [19-21]). However, we could not demonstrate systematic FRET between m-Cherry-SERCA (C-SERCA) and either Orai1-YFP or STIM1-YFP (Results not shown). As previously reported (reviewed by [18]), STIM1 migrated from its cytoplasmic location towards the plasma membrane upon stimulation with TG (compare Fig. 2A and Fig. 2B). We also observed subtler changes in SERCA distribution in the TG-treated cells. SERCA tended to distribute close to STIM1 in the subplasmalemmal region (Fig. 2C1), and a tight inspection demonstrated an accumulation at that location (Fig. 2C2, arrows). In addition, distribution at punctae regions showed an excellent colocalization of STIM1 and SERCA accumulations (Fig. 2D1, D2 & D3). In the image shown, the pixel by pixel correlation coefficient between the expression of STIM1 and SERCA was 0.81 (Fig. 2D4). The tendency to colocalization at the subplasmalemmal region was also evident in volume reconstructions. Both SERCA and STIM1 distributed uniformly through the cytoplasm in the untreated cells, but upon stimulation with TG, SERCA tended to co-accumulate with STIM1 at the subplasmalemmal region (Fig. 2E).

DISCUSSION

The final destination of Ca^{2+} entering through CCE is not the cytosol but the ER, which must be refilled to become ready for response to new stimuli. Our experiments show two primary results: i) excess of Orai1 slows down very much the filling of ER in cells with activated CCE; and ii) when CCE is activated by TG, SERCA allocates very close to STIM1 and Orai1 in the subplasmalemmal region. Both observations can be explained within a wider hypothesis by postulating that STIM1, Orai1 and SERCA form well defined complexes that secure efficient refilling of the ER using Ca^{2+} entering from the extracellular medium through active SOC. The privileged relation and interactions between STIM1 and Orai1 have been studied in detail in the last three years (reviewed in [18, 39]). We now propose that SERCA is the third element required for efficient filling of the ER via CCE.

Whereas expression of Orai1 along with STIM1 increased Ca^{2+} uptake into the ER, expression of Orai1 alone dramatically inhibited it (Fig. 1C & E). This result could not be explained by the inhibition of Ca^{2+} influx through the PM (Fig. 1B), which was much smaller than the block of the ER uptake. A non-specific side-effect of Orai1 expression on SERCA function is unlikely, as ER refilling in permeabilized cells was the same with or without Orai1 overexpression (Fig. 1D). On the other hand, increasing the extracellular Ca^{2+} concentration could compensate the blockade (Fig. 1H). These results indicate that the inhibition of SERCA pumping by Orai1 is functional and that it requires the intact cell structure. We propose that inhibition of ER uptake by Orai1 is due to the uncoupling between PM Ca^{2+} entry and SERCA. The building up of high Ca^{2+} microdomains near the membrane of cytoplasmic organelles can accelerate very much the uptake of Ca^{2+} [40]. When $[Ca^{2+}]_{ER}$ decreases, STIM1 aggregates in punctae, which become very close to Orai1 in the subplasmalemmal region (reviewed by [18]), so close that there is FRET between both proteins [19-22]. Although it was initially proposed that STIM1 moved from the ER to the PM, it is now widely accepted that it is the whole ER membrane, including STIM within it, what comes in close contact with the PM and forms discrete protein aggregates [41]. It has been suggested that interactions between Orai1 homomultimers and STIM1 oligomers take place at the perimeter of these tight ER-PM junctions [42]. In this context, we propose that a third player, SERCA, would also distribute close to the STIM-Orai aggregates. This would permit optimal Ca^{2+}

uptake into the ER from the high Ca^{2+} microdomains formed near the points of Ca^{2+} entry at the PM. As a matter of fact, we find, in activated cells, a close spatial relation between STIM1 and SERCA at the subplasmalemmal region and colocalization of both proteins at punctae (Fig. 2). Sampieri *et al.* [22] have reported recently similar findings using a shallow fluorescence procedure and co-immunoprecipitation of STIM1 and SERCA2a in TG-treated cells. We were unable to show systematic FRET between either STIM1 or Orai1 and SERCA, although the same fluorescent fusions did produce Orai1-Orai1 and STIM1-Orai1 FRET. Sampieri *et al.* [22] neither could evince FRET between STIM1 and SERCA. These results suggest that SERCA may locate somewhat more distant than the other two proteins, thus surpassing the FRET distance. Based on elegant experiments using spacers of different sizes, Balla's group has proposed that there is a gap of 9-14 nm between ER and PM during activation of CCE [42]. In this model, we can envisage SERCA forming an outer ring around Orai-STIM

aggregates in the close ER-PM junctions. The proximity to the high Ca^{2+} microdomains formed around STIM1-Orai1 complex would allow rapid Ca^{2+} pumping inside ER.

Orai1 overexpression decreased dramatically Ca^{2+} transport by the ER, indicating that the right proportion of every one of the three players is required. Our interpretation is that Orai overexpression disrupts the functional topology of the CCE complex by keeping SERCA away (too far) from the high Ca^{2+} microdomains, formed at the Ca^{2+} entry points. This uncouples ER uptake from PM entry. Consistently with this interpretation, the inhibition was rescued by restoring the right protein stoichiometry by overexpression of STIM1 along with Orai1 (Fig. 1C).

In summary, our results add to STIM and Orai, the key players of CCE, a third element, SERCA, that has to be physically close and functionally coupled to STIM1 and Orai1 in order to allow efficient re-filling of the intracellular calcium stores using the Ca^{2+} entering through the capacitative mechanism.

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³The abbreviations used are: GFP, green fluorescent protein; cytGA, chimeric cytosolic GFP-Aequorin protein; ER, Endoplasmic Reticulum; erGA, chimeric GFP-Aequorin protein targeted to the ER; $[Ca^{2+}]_C$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, Ca^{2+} concentration inside ER; TBH, 2,5-di-*tert*-butylbenzohydroquinone; TG, Thapsigargin; PM, Plasma Membrane; CCE, Capacitative Calcium Entry; SOC/E, Store-Operated calcium Channel/Entry; CRAC, Ca^{2+} -Release Activated Current; SERCA, Sarco/Endoplasmic Reticulum Ca^{2+} ATPase.

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FIGURE LEGENDS

FIGURE 1. Effects of Orai1 expression on capacitative Ca^{2+} entry in HEK293T cells. (A) Cells were cotransfected with STIM1-YFP and Orai1 (ratio 1:1). Transfected cells were identified by yellow fluorescence and compared to the non-transfected in the same coverslip. Cells were loaded with 4 μ M fura-4F/AM and ER was depleted of Ca^{2+} by treatment with 1 μ M thapsigargin (TG) for 5 min in Ca^{2+} -free medium. At the time shown, 1 mM Ca^{2+} was added. The traces are the average of 15 transfected (discontinuous line) and 4 controls, untransfected cells (continuous line). (B) Comparison of the effects of transfection with STIM1 and Orai1 together (1:1), or with Orai1 alone on the uptake of Ca^{2+} measured with cytGA. Cells were pre-treated with 1 μ M TG in a Ca^{2+} -free medium and then 5 mM $CaCl_2$ was added to trigger CCE, as shown by the arrow. The traces are average values of 4-8 experiments. (C) Uptake into ER, measured with erGA. Cells were transfected with STIM1 plus Orai1 or Orai1 alone, as in (B), and depleted of Ca^{2+} by incubation with 10 μ M TBHQ for 60 min in Ca^{2+} -free medium. TBH was then removed and 1 mM Ca^{2+} was added as indicated. Each trace is the mean of 4-12 experiments. (D) ER uptake in permeabilized cells. Cells were treated as in (C) and then they were permeabilized in an intracellular-like Ca^{2+} -free medium with digitonin (60 μ M) as shown (DIG). At the time indicated, the cells were perfused with an intracellular-like medium containing 100 nM Ca^{2+} buffered with EGTA. Each trace is the average of 7 experiments. (E) Confirmation of the lack of refilling of the intracellular Ca^{2+} stores in intact cells expressing Orai1. $[Ca^{2+}]_C$ was measured with cytGA. Store-emptying and Ca^{2+} addition as in (C). After 5 min refilling, the cells were challenged with 100 μ M ATP plus 100 μ M carbachol (marked "ATP" to simplify). Each trace is the mean of 8 experiments. (F) $[Ca^{2+}]_C$ peak produced by release of stored intracellular Ca^{2+} by ATP plus carbachol (ATP) in cells whose stores were allowed to refill during the aequorin reconstitution for 1-2 hours in Ca^{2+} -containing medium. Measurements performed with cytGA. Each trace is the average of 3-4 experiments. (G) and (H) Kinetics of Ca^{2+} uptake into the cytosol (G, measured with cytGA) and into the ER (H, measured with erGA) in cells expressing Orai1 challenged with increasing extracellular Ca^{2+} concentrations. Cells transfected with the Ca^{2+} probe and Orai1 were Ca^{2+} -depleted by treatment with 10 μ M TBH in a Ca^{2+} -free medium for 60 min. Then, Ca^{2+} was added at the time marked by the arrow at 1 (dotted line), 5 (dashed line) or 15 mM (continuous line).

FIGURE 2. Colocalization of STIM1 and SERCA2b during activation of CCE in HeLa cells.

Cells were transfected with mCherry-SERCA (C-SERCA, in red), STIM1-YFP, (STIM-Y, in green) and untagged Orai1, with a ratio 2:1:1. Nuclei were counterstained with DAPI (in blue). (A) Resting, unstimulated cells. (B), (C), (D) Cells activated with 1 μ M thapsigargin in Ca^{2+} -free medium for 15 min. (B) Typical field showing redistribution of STIM-Y in punctae in equatorial plane. (C1) Inhomogeneous distribution of STIM-Y and C-SERCA. (C2) C-SERCA corresponding to the merged image in C1. Arrows show co-localization of SERCA with STIM1 in the subplasmalemmal region. (D) Colocalization of STIM-Y (D1,) and C-SERCA (D2,) in punctae. The image was taken at the plane of the cell-coverslip interphase. The merged image (D3) shows colocalization (in yellow). (D4) Shows correlation of the green and red fluorescent pixels performed using an ImageJ plugin [28]. The

slope was 0.89 and $r=0.81$. **(E)** Volume renderings of 12 $0.7\ \mu\text{m}$ -sections of a control (CONT) and a TG-activated cell, performed using Volume J [\[43\]](#). **(E1)** STIM-Y, **(E2)** C-SERCA and **(E3)** merged images. Both STIM-Y and C-SERCA tend to concentrate at the subplasmalemmal space in the TG-treated cell.

Figure 2