Published in final edited form as:

Biochim Biophys Acta. 2017 December ; 1862(12): 1481–1490. doi:10.1016/j.bbalip.2017.09.005.

Cholesterol modulates the cellular localization of Orai1 channels and its disposition among membrane domains

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Abstract

Store Operated Calcium Entry (SOCE) is one of the most important mechanisms for calcium mobilization in to the cell. Two main proteins sustain SOCE: STIM1 that acts as the calcium sensor in the endoplasmic reticulum (ER) and Orai1 responsible for calcium influx upon depletion of ER. There are many studies indicating that SOCE is modulated by the cholesterol content of the plasma membrane (PM). However, a myriad of questions remain unanswered concerning the precise molecular mechanism by which cholesterol modulates SOCE.

In the present study we found that reducing PM cholesterol results in the internalization of Orai1 channels, which can be prevented by overexpressing caveolin 1 (Cav1). Furthermore, Cav1 and Orai1 associate upon SOCE activation as revealed by FRET and coimmunoprecipitation assays. The effects of reducing cholesterol were not limited to an increased rate of Orai1 internalization, but also, affects the lateral movement of Orai1, inducing movement in a linear pattern (unobstructed diffusion) opposite to basal cholesterol conditions where most of Orai1 channels moves in a confined space, as assessed by Fluorescence Correlation Spectroscopy, Cav1 overexpression inhibited these alterations maintaining Orai1 into a confined and partially confined movement.

These results not only highlight the complex effect of cholesterol regulation on SOCE, but also indicate a direct regulatory effect on Orai1 localization and compartmentalization by this lipid.

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Author contributions

Arlette Bohórquez, Enrico Gratton, Jonathan Pacheco, Alexander Asanov and Luis Vaca performed experiments and data analysis. Arlette Bohórquez and Jonathan Pacheco prepared figures. All authors contributed to the writing of the manuscript.

Conflict of interests

Alexander Asanov is the President of TIRFLabs, the Ig-TIRFM system is property of TIRFLabs.

Data and materials availability

All non-commercial plasmids described in this study are available through an MTA.

Keywords

Cholesterol; Orai1; SOCE; Caveolae; Fluorescence Correlation Spectroscopy (FCS)

1. Introduction

One of the most important mechanisms of calcium entry in non-excitable cells is the Store Operated Calcium Entry (SOCE). SOCE is regulated by the calcium content at the lumen of the Endoplasmic Reticulum (ER). The release of calcium from the ER activates calcium influx from the extracellular space through store-operated channels (SOC) like Orai1 channels [1–8], and it involves the communication between two spatially independent membranes in the cell: the ER and plasma membrane (PM). The calcium depletion of the ER is sensed by the Stromal Interaction Molecule 1 (STIM1) [9,10], a multi-domain protein responsible of communicating the depleted state of the calcium repository (ER) to Orai1 channels [11,12], which are located at the PM. STIM1 undergoes a series of conformational modifications, that result in the association to Orai1 channels, activating a calcium entry from the extracellular space [13–16]. SOCE has been implicated in a wide variety of physiological and pathological processes related to malfunction and deregulation of calcium entry and calcium homeostasis such as immune responses, respiratory diseases, and cancer [17–20].

It is known that numerous ionic channels and receptors are regulated by plasma membrane cholesterol, including those involved in SOCE [21–28]. The cholesterol content of the PM plays an important role in the regulation, maintenance and activation of SOCE [29–32]. Even more, the plasma membrane cholesterol content affects SOCE in a differential manner.

The effects of cholesterol on SOCE are complex, on one hand it has been reported that reducing PM cholesterol increases SOCE due to an increase in the interaction of STIM1 with the amino terminus of Orai1 [33,34]. Other reports indicate that the presence of low cholesterol content at the PM before SOCE activation (prior to the assembly of the STIM1-Orai complex), results in reduced calcium entry [30,31,35–37]. Our group, has recently identified a cholesterol recognition amino acid consensus (CRAC) domain in STIM1, specifically in the SOAR domain, which is the minimum sequence required for Orai1 activation [37,38] and that cholesterol binding at the SOAR domain within STIM1 reduces STIM1-Orai1 interaction [37].

The functions of several SOCE components such as STIM1, Orai1 channels and TRPC1 channels are regulated by its interaction with PM domains enriched in cholesterol (the so-called lipid rafts) [19,29,39–42].

Caveolae are a special subset of lipid rafts (LR) with a distinctive omega shape ranging from 50 to 100 nm in diameter [43–46]. The principal component of caveolae is Caveolin 1 (Cav1) [45,47]. Cav1 is not only the main component of caveolae, but it is necessary for the formation of these structures. Cav1 possesses different structural domains that interact with a myriad of proteins, grouping and regulating them [14,19,44,47,48].

Caveolae have been identified as PM domains that recruit calcium regulatory components [44,46], even more it has been reported that in airway smooth muscle (ASM), the PM fraction enriched in caveolae contains several calcium regulatory proteins and that silencing Cav1 reduces SOCE [19,49]. Also, the interplay between Cav1 and SOCE during *Xenopus* egg meiosis has been reported [14,47]. Nevertheless, the role of caveolae in the regulation of SOCE, particularly of Orai1 channels and the way both proteins interact remain poorly understood. So far, we know that PM cholesterol regulates SOCE in a differential manner depending on the SOCE activation state, our group demonstrated that diminishing PM cholesterol after SOCE activation (after the assembly of the STIM1-Orai complex) lead to an increment in calcium entry and enhanced STIM1-Orai1 associations [37]. Other groups, including ours, found that reducing PM cholesterol before SOCE activation (prior to the assembly of the STIM1-Orai complex) leads to a decrease in SOCE [30,31,37,41,50]. Also, it has been reported that the overexpression of SOCE components masks the effects of cholesterol reduction [31].

In agreement with other studies here we show that reducing PM cholesterol reduces SOCE, when the reduction is performed previous to the formation of the Orai1-STIM1 complex, using cells with endogenous SOCE components only. PM cholesterol depletion triggers the internalization of Orai1 channels (under resting conditions, when SOCE is not active), which results in the reduction of SOCE. Overexpression of Cav1 prevents the internalization of Orai1 induced by the reduction on PM cholesterol. Furthermore, SOCE activation increases the Cav1-Orai1 interaction. Reducing cholesterol not only induces Orai1 internalization, but also alters the diffusion of Orai1 at the PM. Our results provide a molecular mechanism as to why PM cholesterol reduction results in diminished SOCE.

2. Material and methods

2.1. Reagents

All reagents were analytical grade purchased from Sigma (Saint Louis, MO) and Invitrogen (Waltham, Massachusetts), unless otherwise stated.

2.2. Cell culture and transfection

Human Embryonic Kidney 293 cells (HEK293) were cultured using DMEM supplemented with 10% (V/V) fetal bovine serum, 50 µg/ml penicillin/streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO₂.

Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer instruction using cells seeded to 80% confluence. Plasmid mCherry/GFP-Orai1 was a generous gift from S. Muallem, c-Myc-Orai1, Cav1-GFP and Kaede were purchased from Addgene (Cambridge, Massachusetts). The expression of all genes was driven by the strong viral cytomegalovirus early promoter (CMV).

2.3. Cholesterol manipulation and quantification

The cells were rinsed twice using calcium free Krebs-Ringer Buffer (KRB) containing 119 mM NaCl, 2.5 mM KCl, 1 mM NaH₂PO₄, 1.3 mM MgCl₂, 20 mM HEPES, 11 mM glucose

and adjusted to 7.4 pH. For all the experiments the cholesterol removal was performed incubating the cells 40 min in a solution of 10 mM of β -Methyl Cyclodextrin, M β CD (Sigma) in calcium free KRB at room temperature (RT), the controls were incubated in calcium free KRB for 40 min at RT.

The cholesterol replenishment assays were performed incubating the cells 40 min in a solution of 10 mM of β -Methyl Cyclodextrin, M β CD (Sigma) in calcium free KRB at room temperature (RT), then the cells were incubated with a solution containing water soluble cholesterol (Sigma) in calcium free KRB 40 min at 37 °C. We used three concentrations of water soluble cholesterol, 5, 10 and 15 mM.

The cholesterol content was measured using Amplex® Red cholesterol assay kit (Thermo Fisher Scientific, Waltham Massachusetts) according to manufacturer instructions and normalized against protein concentration. The measurements were performed using a synergy Mx microplate reader (BioTek Instruments, Inc.).

2.4. Cytotoxicity quantification

The cytotoxicity levels of M β CD (10 mM, 40 min, RT) treatment and acceptor photobleaching protocols were measured, using the LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific), according to manufacturer instructions. The cells were cultured in 96 well plates, treated with Ethanol as a negative control (all cells dead, red), PBS as positive control (cells live, green) or M β CD 10 mM and then analyzed using a Synergy™ Mx Microplate Reader (BioTek Instruments, Inc.).

2.5. Calcium measurements

Cells were seeded in 25 mm round glass coverslips prior transfection. After the protocol for cholesterol manipulation or the incubation in calcium free KRB for the control group, the cells were rinsed once with calcium free KRB and incubated 30 min at 27 °C in calcium free KRB containing FLUO4-AM 2 μ M (Molecular Probes). The cells were rinsed with calcium free KRB and incubated 15 min at RT. Afterwards they were maintained in calcium free KRB with EGTA (500 μ M). Once the experiment started, calcium stores were depleted at minute 1:30, using thapsigargin (TG) to a final concentration of 5 μ M. Then at minute 7 a solution of calcium was added to a final concentration of 1.8 mM. Calcium dynamics were measured in individual cells (at least 20 per coverslip) using a wide-field inverted IX81 Olympus® microscope with a 40 $\times$ 1.30 NA oil immersion objective, MT-20 illumination system, 484/25 excitation filter, 520 nm/40 bandpass emission filter with an EMCCD camera iXon-897 (Andor Technology South Windsor, CT, USA). The acquired images were analyzed using the microscope software, Olympus Cell^R.

2.6. Fluorescence microscopy

2.6.1. Confocal microscopy—The internalization measurements were performed using an Olympus® FLUOVIEW FV10i microscope, using the 60 $\times$ NA 1.35 oil immersion objective. The images were analyzed using the microscope software, FV10ASW.

Cells were seeded in 25 mm round glass coverslips and transfected with mCherry-Orai1; the measurements were performed 24 h after transfection. After placing the sample on the microscope, we added Wheat Germ Agglutinin (WGA), Alexa Fluor® 488 conjugate (Thermo Fisher Scientific), at a final concentration of 5 µg/ml, to label the plasma membrane, WGA selectively binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues. This marker does not internalize when cholesterol is depleted or at basal cholesterol conditions after 60 min of incubation.

First, the entire cell was acquired in z from top to bottom, this measurement represents the basal cholesterol conditions (Basal), and then a solution of MβCD (10 mM final concentration) was added and incubated for 40 min at RT, after this incubation, a second acquisition of the same cell was performed, this will be treated as low plasma membrane cholesterol group (MβCD). We analyzed three z slices, away from the top and bottom of the cell and the average fluorescence, of the three slices, at each condition was calculated. The cytoplasm was delimited using a ROI (Region of Interest), avoiding the region marked by the WGA.

2.6.2. TIRFM—Total Internal Reflection Fluorescence Microscopy (TIRFM) was used to acquire images for the acceptor photobleaching FRET and FCS analysis. In TIRFM, the evanescent wave of totally internally reflected light selectively excites fluorescent molecules in cell regions that are located in close proximity to the glass of the coverslip or culture dish. In TIRFM only the molecules within a few nanometers from the glass are excited, since the evanescent wave decays exponentially with the distance [51,52]. This microscopy method not only provides surface selectivity but also brings very high signal-to-noise ratios eliminating the fluorescence from the bulk solution.

2.6.3. Single plane illumination microscopy—All the measurements were performed using an Olympus® IX71 microscope with inclined selective plane illumination and Photometrics Evolve camera (LFD, UCI), with an acquisition speed of 100 frames/s and pixel size of 142 nm. Using this configuration, the light sheet is around 1.5 µm with approximately 1 mm of depth and sectioning of 1 µm.

Briefly, the cells were seeded in a glass strip prior to transfection with GFP-Orai1 to allow the observation; cells were treated with MβCD (10 mM, 40 min at RT) or observed at basal cholesterol conditions incubated 40 min at room temperature (RT) before observations. We acquired a stack of frames at the same z plane, at the cell equator, for 100 s. The acquired images were analyzed using Image J, measuring the fluorescence on a ROI of the cytoplasm at three different time points; an average of fluorescence of these measurements is presented.

2.6.4. Kaede photoconversion—For the measurements of Orai1 internalization we used Kaede fused to the Orai1 amino terminus (Kaede-Orai1), Kaede is green fluorescent after synthesis but upon UV light irradiation it converts to red. The plasmid for Kaede was acquired from Addgene (Plasmid #54726), the Kaede-Orai1 construction was fully sequence prior to conducting all the experiments and transfected into HEK293 cells. Images were acquired using an FV1000 Olympus® confocal microscope, equipped with a solid-state 405 nm laser and a 1.45 NA TIRFM 100X objective (Olympus, Japan).

First, the photoconversion of Kaede was performed by exciting a ROI in the plasma membrane using 405 nm wavelength at 60% laser power for 2 min or until photoconversion was obtained. After the photoconversion, the M β CD was added to a final concentration of 10 mM and incubated for 20 min. Then, we acquired 3 frames per minute for 20 min of the plasma membrane and cytoplasm of the cell, the redistribution of the red Kaede (photoconverted) was analyzed and compared to the green Kaede (not photoconverted) localization.

2.7. Cell surface protein biotinylation

We used Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) to label and isolate cell surface proteins in accordance to the manufacturer's instructions. First, cells are labeled with a thiol-cleavable amine-reactive biotinylation agent then cells are lysed with mild detergent and the labeled proteins.

The Orai1 concentration in total cell lysate was analyzed using WB to ensure equal amounts of Orai1 were loaded in the column. The amount of biotinylated Orai1 was analyzed using an Orai1 specific antibody (ab59330).

2.8. Western blot

Biotinylated proteins were separated using SDS-Page at standard conditions, 12% acrylamide gels were used, and the transference of proteins into the membrane was performed at 120 V 80 min in a wet chamber. Suitable antibodies were used at previously identified concentrations, Orai1 (ab59330) and Cav1 (ab2910) both purchased from Abcam and c-myc (MA1-21316), purchased from Thermo Fisher Scientific. Primary antibody was incubated ON with agitation at 4 °C. The secondary antibody was incubated for 1 h with agitation at RT. The signal was analyzed with a C-Digit Blot scanner (LI-COR) and the signal was quantified using the scanner software, Image Studio. Additionally, signal was acquired using an X-ray films and then the image was digitalized and analyzed using ImageJ software.

2.9. FRET measurements

The cells were seeded in 25 mm coverslips and cotransfected with Cav1-GFP and mCherry-Orai1. We performed Förster Resonance Energy Transfer (FRET) measurements using both the acceptor photobleaching and sensitized emission methodology, with Cav1-GFP as donor and mCherry-Orai1 as acceptor.

For acceptor photobleaching the images were acquired with an inverted IX81 Olympus® microscope equipped with the CrestOptics Integrated FRAP (fluorescence recovery after photobleaching) module (Crest Optics, Via Mattia Battistini, 184/D - 00167 Roma). The photobleaching of the acceptor was performed exposing the selected ROI to laser, at 50% of its maximum intensity, at a wavelength of 580 nm. This wavelength does not affect the donor as the excitation of GFP beyond 550 nm is negligible. The photobleaching protocol consisted of continuous excitation at 580 nm for 4 min, during this time, the fluorescence emission of GFP and mCherry was monitored using TIRFM. Photobleaching excitation was alternated with image acquisition of GFP (510 nm) and mCherry (640 nm). We used a 1.45

NA TIRFM 100X objective (Olympus, Japan) for image acquisition. For acceptor photobleaching we used both fixed and live cells, with indistinguishable results [53]. For fixed cells we used a standard fixing procedure consisting in incubating the transfected cells for 20 min at room temperature in 3.7% paraformaldehyde diluted in $1 \times$ PBS [54]. For living cells, after the photobleaching procedure, cells viability was determined as described in (2.4 Cytotoxicity quantification). Cell viability remained unaffected by the photobleaching protocol (data not shown).

FRET efficiency, which indicates the percentage of excitation photons that contribute to FRET, was calculated measuring the fluorescence intensity in a ROI, before and after photobleaching the acceptor (mCherry-Orai1), we also measured the fluorescence of acceptor and donor in a different ROI (without photobleaching protocol applied) to correct for photobleaching that might occur during acquisition unrelated to the photobleaching protocol.

For sensitized emission the images were acquired in a wide-field inverted IX81 Olympus® microscope equipped with 60×1.42 NA objective with a MT-20 illumination system and an EMCCD camera iXon-897 (Andor Technology South Windsor, CT, USA).

FRET experiments consisted in raw images (the images obtained directly from the instrument) with 3-channels; Channel D (GFP), with excitation at 470 nm and emission collected at 520 nm/40 bandpass, channel A (mCherry) with excitation at 520 nm and emission collected at 605 nm/70 bandpass and channel F (FRET) consisted in excitation at 470 nm and emission 605 nm/70 bandpass respectively. Apparent FRET efficiency (E_{app}) was calculated using raw images. First, spectral bleed-through (SBT) was calculated, for donor and acceptor, using images of cells expressing donor or acceptor, using the three channels, D, A and F. SBT was calculated from the ratio of signal from F and D channels (F_d/D_d) for cells transfected only with Cav1-GFP, for cells transfected with mCherry-Orai1 SBT was calculated using F and A channels (F_a/A_a).

A raw FRET (a FRET signal prior normalization for protein expression levels) index was calculated by subtracting SBT of donor and acceptor from FRET channel (F_{da}) of co-transfected cells. Then, these FRET values were normalized (NFRET) against signal of donor channel (D_{da}) to eliminate variations due to different expression levels. Finally, E_{app} was computed using the ratio of NFRET divided by NFRET plus the factor G. We used a constant G value of 1.0. All images were analyzed pixel-to pixel using custom-made ImageJ plugin. To reduce artifacts of overexpression of a specific protein, we only used images that accomplish a 1.0 ± 0.3 ratio of donor and acceptor signal ($D_{da}/A_{da} = 1.0 \pm 0.3$). Pixel areas that did not comply with this restriction were blacked out and not included in the analysis to avoid overestimating FRET.

2.10. Orai1/Cav1 Coimmunoprecipitation

HEK 293 cells overexpressing Cav1-GFP and c-Myc-Orai1 were washed twice with PBS, and then the lysis of cells was done with TNI lysis buffer during 30 min with agitation at 4°C and sonicated using a bath sonicator (55 kHz, 5 min). The samples were centrifuged 40 min (4°C , $18,000 \times g$) and the supernatant was incubated overnight (ON) with sepharose

resin beads coupled to c-Myc antibody. Proteins were recovered by centrifuging the beads (4 °C, 500 × g). Bound proteins were eluted, incubating twice with elution buffer during 30 min at 37 °C and neutralized with Tris [55]. The proteins were analyzed using western blot, the molecular weight of endogenous Orai1 is 50 kDa and of Cav1 is 20 kDa.

2.11. Electrophysiology

HEK293 cells expressing the different constructs described in the legend of Fig. 4 were placed on coverslips coated with poly-lysine (Sigma). Cells were measured between 24 and 48 h post-transfection. Coverslips were mounted on an open perfusion chamber (TIRF Labs). Where indicated, cells were incubated for 40 min with 10 mM Methyl- β -cyclodextrin (M β CD) prior to initiating whole-cell studies.

The patch clamp amplifier used for whole-cell recordings was the EPC-10 (HEKA Elektronik, Germany). The patch clamp pipettes were prepared from Corning 7052 glass and had a resistance of 1–5 M Ω when filled with the pipette solution (see below). An Ag/AgCl electrode was utilized to attain electrical continuity and was connected to the bath solution via a KCl agar bridge. TG was applied using a multibarrel perfusion system driven by gravity (TIRF Labs).

To study Orai1 whole-cell currents, the pipette solution contained: Cesium aspartate 120 mM, EGTA 5 mM, HEPES 10 mM, MgCl₂ 2 mM and NaCl 8 mM. pH to 7.2 adjusted with CsOH. The bath solution contained: NaCl 120 mM, Tetraethylammonium chloride (TEA-Cl) 10 mM, CaCl₂ 10 mM, MgCl₂ 2 mM, Glucose 30 mM and HEPES 10 mM. pH to 7.2 adjusted with NaOH. Osmolarity of both solutions was adjusted to 320 mosM with mannitol (Sigma). Current density was obtained by dividing the current from each cell by the cell capacitance (measured directly from the amplifier readout). The amplifier provided current density in real time calculated via the Patchmaster software and EPC10 electronics (HEKA Elektronik, Germany). Leak values were obtained after gigaseal formation using the automatic mode from the EPC10. These values were used to perform leak subtraction cell by cell. For time courses studying Orai1 whole-cell current activation, TG was applied while the cell membrane potential was held at –100 mV. All whole-cell currents illustrated in Fig. 4 represent the mean $\pm$ standard deviation (SD) from at least 25 independent cells obtained from 3 different days and transfections.

Orai1 whole-cell currents were imported into Igor Pro v. 7 (WaveMetrics, Oregon) for further analysis and plotting.

2.12. Fluorescence correlation spectroscopy

Cells were seeded in 35 mm glass bottom dishes (MatTek Corporation Massachusetts, USA.) and transfected with mCherry-Orai1.

Fluorescence Correlation Spectroscopy (FCS) images acquisition was performed using an Olympus IX81 microscope with TIRFM illuminator and objectives (Laboratory of Fluorescence Dynamics, UCI). The acquisition was at 50 frames/s on TIRF mode and 1500 frames were collected. The size of pixel was: 1 $\times$ 0.18, 2 $\times$ 0.36 μ m, depending on the zoom.

The analysis was made using SimFCS software (developed by Enrico Gratton). The acquired images from each cell were analyzed selecting a ROI (128 pixels).

iMSD analysis was performed using SimFCS, which gives the protein diffusion and particle size measurement when the iMSD is plotted versus time. The image mean-square displacement technique (iMSD) is based on the calculation of the spatial temporal (nm/s) image correlation function determining the population behavior of all the molecules in a given region [56,57], an example of the diffusion models and the distribution in the analyzed cells can be found in Supplementary Fig. 5.

First, fast imaging on the membrane was performed, then for each time delay an ROI of 32×32 pixel was used for the calculation of the correlation function, which was and fitted to a Gaussian. In each ROI the diffusion law of the molecule was obtained [56,58], identifying the corresponding diffusion model (linear, confined, transiently confined, or directed diffusion) [57,59]. The linear model involves pure isotropic movement, confined diffusion is delimited by boundaries that cannot be crossed, in transiently confined diffusion the molecule is able to escape the confinement (in and out) and the directed diffusion is regulated by a transporter [56,57]. The ROI was moved to another region so as to cover the entire image.

For the identification of the diffusion model, the analysis of the different models proceeds by first calculating the iMSD only the quadratic term (velocity) from the inverse amplitude term. In the case that the iMSD, has an upward curvature (positive V) from the inverse amplitude term, then the linear model (diffusion only) is also calculated. If the correlation coefficient of the quadratic fit is larger (by at least 0.001) of that of the linear fit, then the directed motion model is accepted, and no further calculation is done. Note that the velocity values are always calculated from the shift of the autocorrelation function (ACF), not from this upward curvature, so this procedure is only used for the rankings of the model. In case the directed motion is not accepted, then the 3 other models are tested. The iMSD data were fitted to three models of diffusion:

- 0) The directed motion is detected by the positive curvature of the iMSD plot
- 1) Diffusion only (linear, isotropic), the linear term is the only term in the equation
- 2) Confined only, the exponential saturation term is the only term in the equation
- 3) Confined and diffusion (transiently confined), both terms are included in the fit.

Examples of curves obtained for each type of diffusion are presented in Supplementary Fig. 5.

Note that model 3 will always be better than model 1 and 2 because it includes these two models. So, the issue is to rank them and estimate how “better” one model is with respect to the others. For this purpose, we use the correlation coefficient of the fit. The ranking is examined starting with the diffusion model, which is assumed by default. If the confined model gives a better correlation value (higher value) by at least 0.001, then the confined model is accepted. Then the full model is tested. It is accepted if the correlation parameter

for the full model is better by at least 0.001 of the confined model [60]. As a result of this analysis we obtained:

- a. Diffusion model
- b. Confinement size
- c. Particle (aggregate) size

The particle (aggregate) size corresponds to Orai1 subunits associated. As showed by di Rienzo [56], the size of the particle (aggregate) can be obtained from the iMSD plot against time when the size of particle/domain is not negligible (e.g., large protein clusters, vesicles, large domains), the correlation function includes.

2.13. Data analysis

The acquired data were analyzed using two-tailed, unpaired Student's t-test (GraphPad Prism, GraphPad Software Inc.). Unless otherwise indicated data are presented as mean $\pm$ s.e.m. Electrophysiology data was analyzed using Igor Pro v 7 (WaveMetrics, Oregon, USA). Data is presented as mean $\pm$ standard deviation. Significance was set at *** $p < 0.001$, ** $p < 0.01$ or * $p < 0.05$.

3. Results

3.1. Cholesterol depletion induces Orai1 internalization

To understand the molecular mechanisms behind the reduction of SOCE after cholesterol depletion, we used HEK293 cells expressing only endogenous SOCE components, treated with M β CD (10 mM, 40 min, RT), then loaded with FLUO4-AM and treated with the inhibitor of ER calcium ATPase thapsigargin (TG) in the absence of extracellular calcium (Fig. 1A, B). As reported in previous studies, we observed a marked reduction in SOCE in cells with low PM cholesterol (Fig. 1A, B). These studies confirmed previous observations indicating that SOCE is reduced in cells with low PM cholesterol (when cholesterol is reduced prior to the formation of the STIM1-Orai1 complex). We found that the treatment with M β CD reduced approximately 50% of the total cholesterol content in the cells (Supplementary Fig. 2D) without greatly affecting cell viability (Supplementary Fig. 1), the treatment with M β CD, reduces only around 10% the number of live cells compared to the number of live cells in positive control (PBS). To ensure that the effect of M β CD treatment was specific to cholesterol removal, we performed cholesterol replenishment assays (Supplementary Fig. 1) and found that the cholesterol replenishment using water-soluble cholesterol (M β CD loaded with cholesterol) restored the calcium entrance (Fig. 1A, B and Supplementary Fig. 2A–C). We found differences in calcium depletion from the ER induced by TG treatment in cholesterol-replenished cells (Supplementary Fig. 2B). These differences might be related to the long protocol of cholesterol removal and replenishment, which lasted around 135 min. During this time there might be passive depletion of the ER induced by the sustained low extracellular calcium protocol. We did not explore further this observation.

One possible mechanism by which SOCE is reduced in cells with low cholesterol content might involve the internalization of store operated channels (SOC), as reported for the

acetylcholine receptor, which has been shown to be internalized upon depletion of PM cholesterol [22]. We explored this possibility using fluorescence microscopy.

For these experiments we used HEK293 cells, overexpressing Orai1 fused to a fluorescent protein (dsRed, EGFP, Kaede). Using confocal microscopy the cells treated with M β CD (10 mM, 40 min) showed an increase in cytosolic fluorescence signal corresponding to mCherry-Orai1 as measured using confocal microscopy (Supplementary Fig. 3), these results showed an increase in fluorescence signal at the cytosol (Supplementary Fig. 3B) and a decrease in the colocalization index between a PM marker and mCherry-Orai1 (Supplementary Fig. 3C). To access to intracellular space with high spatial resolution, we used single plane illumination microscopy (SPIM) to excite only a thin optical section of the cell (Fig. 1C, Supplementary video 1). Using SPIM we observed an increase in fluorescent signal at the cytosol when cells were treated with M β CD. To further confirm if the increment of cytosolic Orai1 upon cholesterol depletion was the result of internalization from the PM we performed photoconversion studies with Kaede-Orai1 a fusion protein formed by the photoconvertible protein Kaede and Orai1 (Kaede-Orai1). Newly synthesized Kaede-Orai1 emits light in the green spectra (peak at 525 nm). Upon UV irradiation Kaede switches its emission to the red spectra (peak at 590 nm). For this purpose, we selected a region of interest (ROI) at the PM and excited exclusively this ROI with UV light (405 nm), until the emission of Kaede-Orai1 switched from green to red (Fig. 1D). After this photoconversion maneuver the cell was exposed to M β CD treatment (10 mM, 40 min, RT). The presence of red Kaede-Orai1 (photoconverted Kaede) at the cytosol indicated the translocation of Kaede-Orai1 from the PM where it was photoconverted to red into the cytosol upon M β CD treatment (Fig. 1D).

The internalization of Orai1 was further confirmed with biotinylation assays designed to label membrane proteins. For these assays we used HEK293 cells with endogenous SOCE components only. In agreement with the microscopy studies, the biotinylation assays show a decrease in endogenous Orai1 present at the plasma membrane of cells treated with M β CD (Fig. 1E).

With all these results we can conclude that the decrease of SOCE induced by reducing PM cholesterol is the result of Orai1 translocation from the PM to the cytosol.

3.2. Cav1 overexpression rescue SOCE and prevents Orai1 internalization in cells with low plasma membrane cholesterol

Several SOCE components are associated with cholesterol enriched plasma membrane domains [14,29,40,44], these domains include a special subset, named caveolae, mainly formed by caveolin 1 protein (Cav1).

We performed calcium measurement experiments using cells with endogenous SOCE components and overexpressing Cav1, the cells were treated with M β CD prior calcium measurements (Fig. 2A). Cav1 overexpression prevented the effects of M β CD on SOCE (Fig. 2A–B), also, we compared the cells overexpressing Cav1 with basal cholesterol levels against cells with endogenous Cav1 only (Fig. 2A, B) and found no difference in calcium replenishment.

Furthermore, using HEK 293 cells overexpressing mCherry-Orai1 and Cav1-GFP we found that the overexpression of Cav1 prevented the internalization of mCherry-Orai1, as measured by confocal microscopy (Fig. 2C–D). These results were confirmed by biotinylation assays designed to label plasma membrane proteins. These results show that the overexpression of Cav1 reduced the amount of Orai1 channels internalized when PM cholesterol is decreased (Fig. 2E).

From these results we can conclude that the overexpression of Cav1 rescue SOCE in cells with low PM cholesterol by inhibiting the internalization of Orai1 channels.

3.3. SOCE activation induces the association of Orai1 and Cav1

Since Cav1 overexpression prevents the effects of cholesterol reduction on SOCE and Orai1 internalization, we decided to explore if Cav1 and Orai1 channels interact.

First, we evaluated Cav1-Orai1 interaction using Förster Resonance Energy Transfer (FRET) to explore protein-protein interactions in living cells, we used two FRET methodologies: sensitized emission and acceptor photobleaching. For these experiments we used cells overexpressing mCherry-Orai1 and Cav1-GFP.

In our hands the apparent FRET (E_{app} , a global FRET efficiency) measured by sensitized emission (Supplementary Fig. 4) showed low values, however the results were very consistent. Low FRET efficiency may be the result of a non-optimal alignment of the dipolar moments from both fluorescent proteins. Sensitized emission FRET showed an increased interaction between Orai1 and Cav1 when SOCE is activated (Supplementary Fig. 4), this interaction was not affected when cells were treated with M β CD. To confirm the interaction between both proteins we used an alternative methodology to compute FRET efficiency, the acceptor photobleaching method (Fig. 3) in which only the donor intensity is used to measure FRET efficiency, in this way the spectra crosstalk is avoided. We found a small but significant FRET between Cav1 and Orai1 in control cells (basal, cells not exposed to TG or M β CD). The FRET efficiency increased 2-fold when the SOCE was activated with TG (Fig. 3B) in cells not exposed to M β CD.

The activation of SOCE (with TG) induced an increased FRET signal between Cav1 and Orai1 of 2-fold (Fig. 3B) compared to cells where SOCE is not activated. The interaction between Caveolin1 and Orai1 channel was confirmed by co-immunoprecipitation assay using cells overexpressing Orai1 and Cav1 in basal and depleted cholesterol conditions (Fig. 3C). In cells at basal cholesterol conditions, activation of SOCE with TG resulted in a 4-fold increment in co-immunoprecipitated Cav1 (using Orai1 as a bait), the concentration of co-immunoprecipitated Cav1 had a small decrease in cells with low cholesterol treated with TG. In cells with SOCE not activated the concentration of co-immunoprecipitated Cav1 remains the same compared to cells with basal cholesterol and M β CD treated cells.

Taken together these results indicate an interaction between Cav1 and Orai1, this interaction is strongly enhanced by SOCE activation. PM cholesterol content has a negligible effect on Cav1-Orai1 interaction in basal conditions (SOCE not activated), but Cav1-Orai1 interaction is reduced under low PM cholesterol when SOCE is activated (Fig. 3C).

3.4. The effect of cholesterol depletion on TG-induced whole-cell currents is overcome by Cav1 overexpression

Since we already had the Fluo4-AM fluorescence measurements, which provide information about cytosolic calcium content fluctuations. We wanted to explore in greater detail the effects of cholesterol depletion on Orai1 activity. To do so we conducted whole-cell electrophysiological measurements of Orai1 currents.

The PM cholesterol reduction reduced drastically TG-induced endogenous (WT) whole-cell currents (Fig. 4A, left panel). This reduction was rescued by overexpressing Cav1 (Fig. 4A, left panel). Similar results were obtained in cells overexpressing Orai1 and STIM1 (Fig. 4A, right panel), the removal of PM cholesterol also induced a reduction in TG-induced whole-cell currents, this reduction was avoided by Cav1 overexpression. Thus, Cav1 overexpression prevented whole-cell current reduction in control cells (WT currents) and in cells overexpressing Orai1 and STIM1 (Fig. 4B). Overexpression of Cav1 alone (in the absence of M β CD) had no effect on TG-induced whole-cell WT or overexpressing Orai1 and STIM1 currents (Fig. 4B).

The current-voltage relation indicates that whole-cell currents were reduced at all voltages explored (-100 to $+100$ mV), for wild type (WT) currents or currents measured in cells overexpressing Orai1 and STIM1 (Fig. 4C).

These results indicate that Cav1 overexpression rescued the whole cell currents in cells with endogenous SOCE components when PM cholesterol is reduced, the same effect is observed in cells overexpressing STIM1 and Orai1. The SOCE current is not affected by Cav1 overexpression with basal PM cholesterol levels.

3.5. Cholesterol reduction modifies Orai1 diffusion patterns, and the size of aggregates of Orai1 channels

PM cholesterol has shown to be an important lipid that mediate lateral diffusion of membrane components [61,62].

TIRFM images that were analyzed with fluorescence correlation spectroscopy (FCS), which is an analysis methodology of the acquired images that allow us to get an insight in protein dynamics (Supplementary Fig. 5), using this methodology we were able to find how the lateral movement of Orai1 and Cav1 was.

FCS analysis showed that Orai1 moves inside a confined compartment (Fig. 5A, left) in 90% of analyzed cells with basal cholesterol, while 10% presented random motion (linear iMSD), examples of the obtained graphs for motion models are show in Supplementary Fig. 5A.

The treatment with M β CD (Fig. 5A, right) reduced the number of Orai1 moving inside a confined compartment to 42%, while increasing the proportion of cells presenting the linear model motion (random motion) from 7% to 45% (Fig. 5A, right panel).

The overexpression of Cav1 modified Orai1 dynamics, increasing the proportion of Orai1 moving inside a confinement, thus eliminating the cells showing Orai1 random motion (linear iMSD) (Fig. 5B). Even more, in 70% of analyzed cells Orai1 channels move inside a

confined compartment and the number of cells with partially confined movement also increased ten times compared to cells with endogenous Cav1 (Fig. 5B).

Cholesterol depletion does not influence confinement size as depicted in Fig. 5D while the overexpression of Cav1 increased cluster size (Orai1 molecules) (Fig. 5C) and confinement size by 4-fold (Fig. 5D). An example of the obtained images for Orai1 and Cav1 is presented in Supplementary Fig. 5B.

These results show that lowering PM cholesterol not only induces Orai1 channels internalization, but also modifies the lateral movement of Orai1, from trapped to linear. Again, this effect is rescued by Cav1 overexpression.

4. Discussion

The regulation of SOCE by plasma membrane cholesterol content has been shown in several studies [29–31,37,40], nevertheless the molecular mechanisms underlying these modulations remain poorly understood and there are contradictory results. A large number of reports show that SOCE is strongly sensitive to PM cholesterol levels, and that upon PM cholesterol reduction SOCE decreases [30,31,35–37]. The inhibition of punctae formation was proposed as responsible for this phenomenon [32,63,50].

The effect of cholesterol on SOCE depends on whether cholesterol reduction takes place before or after the STIM1-Orai1 complex is formed (Before or after SOCE activation). In general, most studies agree that cholesterol reduction before SOCE activation results in a decreased calcium entry whereas cholesterol reduction after SOCE activation enhances calcium entry.

We have recently identified a cholesterol-binding domain inside STIM1 (within SOAR). This cholesterol-binding domain is responsible for the enhanced SOCE observed when cholesterol is depleted after the STIM1-Orai1 complex is formed [37].

Another important regulatory component beside the activation state of SOCE at the moment of cholesterol depletion is the expression levels of SOCE components, which play an important role in SOCE reduction upon cholesterol depletion, the overexpression of STIM1 and Orai1 might mask the depletion effect as showed by Gwozdz *et al.* [31], these effects might explain the contradictory results of different groups.

In the present study we focused on the molecular mechanisms responsible for SOCE reduction when cholesterol is depleted before the STIM1-Orai1 complex is formed in cells with only endogenous STIM1 and Orai1.

Using a wide variety of methods, we show evidence indicating that Orai1 is internalized upon cholesterol reduction. This result is in agreement with the reduction of SOCE under these conditions. If there are less Orai1 channels at the PM, one would expect reduced calcium entry, which is exactly what we and others have found when only endogenous SOCE components are present. Most interestingly, Cav1 overexpression prevents Orai1 internalization and the reduction of SOCE upon cholesterol depletion.

The present study provides (to the best of our knowledge) the first explanation of the mechanism responsible for SOCE reduction upon plasma membrane cholesterol depletion through modulation of Orai1 channels and also by modifying the spatial organization of Orai1 molecules.

The cholesterol content of PM affects ionic channels in different forms by altering open probability, structural stability or by inducing internalization [22,25–27,48,64]. It has been shown that PM cholesterol content controls the internalization rate of acetylcholine receptors in a dose dependent manner [22]. We found that low PM cholesterol content induces the internalization of a large portion of Orai1 channels, from the plasma membrane into intracellular vesicles. This internalization results in a reduction of calcium influx and whole-cell currents by preventing the STIM1-Orai1 interaction. The internalization phenomena of Orai1 channels is described here for the first time as a response to low PM cholesterol content, the internalization of Orai1 channels was extensively confirmed with microscopy and biotinylation studies (Fig. 1).

An increasing number of reports indicate that several SOCE components are associated to cholesterol enriched domains [29,30,40], particularly caveolae, which have been implicated in the regulation of calcium signaling [19,42,44,47,65,66]. In spite the numerous studies; there were no evidences of a direct interaction between Orai1 and caveolae until now. Here, we present evidence linking caveolae to Orai1 in a differential manner dependent on the SOCE activation state (Fig. 3), this interaction circumvents the effects of cholesterol depletion on SOCE (Fig. 2) and prevents Orai1 internalization (Fig. 2C–E).

The interaction between Orai1 and Cav1 (Fig. 3) is enhanced by the activation of SOCE. The increased interaction between Orai1 and Cav1 when SOCE is activated may reflect Orai1 being recruited into caveolae. The recruitment of Orai1 into caveolae may explain also the effects of Cav1 overexpression on Orai1 diffusion patterns (see below).

The effects of reducing cholesterol were not limited to increasing the internalization of Orai1 channels; additionally, it altered the diffusion patterns of Orai1 at the PM, changing the movement from a confined space, at basal PM cholesterol content, to a movement in a linear pattern (unobstructed diffusion) at low PM cholesterol levels. Moreover, Cav1 overexpression returned Orai1 into a confined and partially confined space (Fig. 5). The confined movement of Orai1 may reflect the recruitment of this channel into caveolae.

The confined movement of Orai1 channels (when Cav1 is overexpressed) is consistent with the increased association of Orai1 to Cav1, as demonstrated by our FRET and CoIP studies. These results strongly suggest that the confined space in which Orai1 is moving are caveolae. Furthermore, our results show that Orai1 cannot be internalized when sequestered into caveolae (when associated to Cav1). Our results suggest also that Orai1 might be present in the PM in at least two different subpopulations at basal PM cholesterol:

1. A population that moves inside cholesterol enriched domains, different to caveolae (nonresistant to M β CD treatment).
2. A population that moves inside caveolae (resistant to M β CD treatment).

The first subpopulation changes its diffusion pattern from confined to linear and partially confined diffusion when PM cholesterol is depleted, while the second subpopulation retains a confined diffusion (remains inside caveolae). The first population of Orai1 is eventually internalized at low PM cholesterol conditions. These results provide a more detailed understanding of the Orai1 subpopulations proposed by Baird *et al.* [32], defining them also by the nature of the domain to which they associate.

The nature of the vesicles where Orai1 is internalized also deserves special attention and is subject of ongoing investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge the technical support from Dr. Alicia Samperi.

Funding

This work was supported by grants from CONACYT (127822), Secretaria de Ciencia, Tecnología e Innovación de la Ciudad de México (SECITI47/2015) y la Dirección General de Asuntos del Personal Académico (DGAPA47/2015) to LV. Arlette Bohórquez Hernández is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 230945 from CONACYT.

Several experiments reported in this paper were performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of California, Irvine (UCI). The LFD is supported jointly by the National Institutes of Health grants (2P41GM103540 and NIH P50-GM076516) and UCI.

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Figure 1. SOCE reduction is induced by Orai1 internalization

(A) Calcium response measurements of cells with basal, low concentration of cholesterol and cholesterol replenished at the plasma membrane. (B) Area under the curve (AUC) of calcium entry after depletion of the ER with TG. (C) SPIM measurements of increment of fluorescent signal in the cytoplasm due to the redistribution of mCherry-Orai1. The black ROI define cytoplasm (D) Kaede-Orai1 is photoconverted (from green to red emission) in PM and change localization to the cytoplasm of cells exposed to MβCD. (E) Amount of Orai1 in PM of cells with basal concentration and low concentration of cholesterol at the plasma membrane. Black: Basal cholesterol, Red: Low cholesterol, Blue: cholesterol replenished. $n = 20$ cells, $n = 10$ independent biotinylation experiments, error bars: S.E.M. *** $p < 0.001$ or ** $p < 0.01$.

Figure 2. Cav1 overexpression prevents the effects of cholesterol depletion on SOCE
 (A) Calcium response measurements of cells overexpressing Cav1-GFP, with basal concentration (black line) low concentration of cholesterol at the plasma membrane (red line) and cells with only endogenous Cav1-GFP (green line). (B) Area under the curve (AUC) of calcium entry in response to ER depletion induced by TG. (C) Localization of mCherry-Orai1 in control cells (Basal) and low concentration of cholesterol at the plasma membrane (MβCD). (D) Change of fluorescent signal (mCherry-Orai1) localization measurements in cytoplasm measured with confocal microscopy. (E) Amount of biotinylated Orai1 in control cells and low concentration of cholesterol at the PM. All the cells overexpressed Cav1-GFP. Black: Basal cholesterol, Red: Low cholesterol. $n = 20$ cells, $n = 10$ independent biotinylation experiments, error bars: S.E.M.

Figure 3. Orai1 and Cav1 interaction is influenced by SOCE activation

(A) Representative images of FRET efficiency measurements using acceptor photobleaching methodology between mCherry-Orai1 and Cav1-GFP obtained by TIRF. Top panels show the pre photobleaching, bottom post photobleaching. Yellow circle: control with no photobleaching protocol, yellow square: area with acceptor photobleaching protocol (B) FRET efficiency plot at different conditions, from left to right; Basal, TG, MβCD, and MβCD + TG. (C) Top panel shows representative western blot membranes, upper and bottom membrane, show respectively co-immunoprecipitated Cav1-GFP (47 kDa) and Orai1 (50 kDa) at different conditions, from left to right; Basal, MβCD, TG and MβCD + TG. Lower panel shows the signal of co-immunoprecipitated Cav1, normalized with Orai1

concentration at each condition. Basal (black), M β CD (red), TG (dark blue), and M β CD + TG (light blue). FRET $n = 70$ cells, Pseudocolor scale maps showing the FRET efficiency (%), obtained in the control (yellow circle, eff = 0) area and the acceptor photobleached (yellow square) area. CoIP $n = 10$ independent assays. Error bars: S.E.M. *** $p < 0.001$, ** $p < 0.01$ or * $p < 0.05$.

Figure 4. Cholesterol depletion reduces TG-induced whole-cell currents

(A) Example of whole cell patch clamp recordings, left panel, endogenous Orail and STIM1, right panel overexpressing Orail and STIM1, at basal (blue line, $n = 54$), Cav1 overexpressed (pink line, $n = 56$ cells) and cholesterol depleted conditions (red line, $n = 61$ cells), cholesterol depleted conditions overexpressing Cav1 (black line, $n = 58$ cells). (B) Bar graph summarizing current densities measured at -100 mV, same color code as A. (C) Current-voltage relationships (I/V) for TG induced currents. Number of cells explored is indicated at each plot. Error bars: S.E.M. *** $p < 0.001$.

Figure 5. Cholesterol depletion and Cav1 overexpression influence Orai1 diffusion and cluster size

(A) Change of mCherry-Orai1 diffusion models at basal cholesterol conditions (left panel) compared with low cholesterol conditions (right panel). (B) mCherry-Orai1 diffusion models in basal cholesterol conditions when Cav1 is overexpressed. The diffusion models are presented as percentage of total analyzed cells, partially confined (orange), confined (red), linear (green). For the models used please refer to Material and Methods. (C) Change in mCherry-Orai1 aggregate size (D) Changes in mCherry-Orai1 confinement size at different conditions. Basal (black), MβCD (red), Cav1 overexpressed (green). $n = 50$ cells, in 6 independent experiments. Error bars: S.E.M. *** $p < 0.001$, ** $p < 0.01$ or * $p < 0.05$.