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Plasma membrane associated membranes (PAM) from Jurkat cells contain STIM1 protein
Is PAM involved in the capacitative calcium entry?

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ABSTRACT

A proper cooperation between the plasma membrane, the endoplasmic reticulum and the mitochondria seems to be essential for numerous cellular processes involved in Ca^{2+} signalling and maintenance of Ca^{2+} homeostasis. A presence of microsomal and mitochondrial proteins together with those characteristic for the plasma membrane in the fraction of the plasma membrane associated membranes (PAM) indicates a formation of stabile interactions between these three structures. We isolated the plasma membrane associated membranes from Jurkat cells and found its significant enrichment in the plasma membrane markers including plasma membrane Ca^{2+}-ATPase, Na^+, K^+-ATPase and CD3 as well as sarco/endoplasmic reticulum Ca^{2+} ATPase as a marker of the endoplasmic reticulum membranes. In addition, two proteins involved in the store-operated Ca^{2+} entry, Orai1 located in the plasma membrane and an endoplasmic reticulum protein STIM1 were found in this fraction. Furthermore, we observed a rearrangement of STIM1-containing protein complexes isolated from Jurkat cells undergoing stimulation by thapsigargin. We suggest that the inter-membrane compartment composed of the plasma membrane and the endoplasmic reticulum, and isolated as a stabile plasma membrane associated membranes fraction, might be involved in the store-operated Ca^{2+} entry, and their formation and rebuilding have an important regulatory role in cellular Ca^{2+} homeostasis.

1. Introduction

Many cellular processes require a proper cooperation between the plasma membrane (PM) and subcellular vesicular networks such as the mitochondria and the endoplasmic reticulum (ER). It has been shown that along with their functional relations, relatively stabile contacts exist between the mitochondria and the ER as well as between both of these organelles and the PM. These structures, composed of the pieces of plasma membrane connected with fragments of intracellular membranes (ER and/or the outer mitochondrial membrane, and also recently reported mitochondrial contact sites between outer and inner mitochondrial membranes (Wieckowski et al., 2006)) are known as the plasma membrane-associated membranes (PAM). They can be isolated and purified as a subcellular fraction reflecting structural and presumably functional associations between the ER, mitochondria and PM (Lebiedzinska et al., 2009).

Isolation of PAM using cell fractionation method was originally described for yeast by Pichler et al. (2001). The same authors characterized protein composition of PAM. Moreover a possible function of these structures related to phospholipids (particularly phosphatidylserine and phosphatidylinositol) synthesis and transportation within a cell was also considered. More recently it was evidenced that store-operated Ca^{2+} entry (SOCE) into non-electrically excitable cells needs functional and structural interactions between the ER and the plasma membrane. It

Abbreviations: PAM, plasma membrane associated membranes; MAM, mitochondria associated membranes; ER, endoplasmic reticulum; SOCE, store-operated calcium entry; CCE, capacitative calcium entry.

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seems that multimembranous structures (resembling PAM fraction described for yeast) are relevant to Ca^{2+} homeostasis, especially to SOCE. This hypothesis was supported by Wu and co-workers, who showed that the distance between the ER and the PM varies between 10 and 25 nm. The distance smaller than 30 nm between the organelles could be regarded as not accidental association (Wu et al., 2006). So far, several models have been proposed to characterize the molecular basis of the interactions between the ER and the PM during SOCE (Parekh and Putney, 2005). Recently postulated model of conformational coupling is based on an assumption that structural associations of these membranes are maintained by direct interaction of specific protein located in the ER, known as stromal-interacting molecule 1 (STIM1) and the PM protein called ORAi (Wang et al., 2008). This model was supported by fluorescence microscopy observation, that upon depletion of the ER of Ca^{2+}, the discrete fraction of the ER moves to the cell periphery where it interacts with the PM. Ca^{2+} release from ER increases the number of contact sites between the ER and PM by 62% (1/3 of junctions are newly formed whereas two thirds of them pre-existed in unstimulated cells) (Wu et al., 2006).

Previously it was found that certain regions of the ER interact with mitochondria, forming structures which may be isolated as so called mitochondria-associated membranes (MAM) fraction. Formation of such structures was previously characterized by biochemical means by Gaigg et al. (1995). Data shown by Achleitner and colleagues indicated that interactions between ER and mitochondria occur more often than between other organelles (Achleitner et al., 1999). Formation of functional intermembrane structures between the ER and the mitochondria was demonstrated in yeast (Gaigg et al., 1995) and in mammalian cells (Vance, 1990; Shiao et al., 1995). The distance between interacting membranes, calculated on a basis of electron micrographs (Achleitner et al., 1999), is small enough to allow stabile contacts formation by proteins located on the surface of both organelles. The contacts between ER and mitochondria were also observed using high resolution electron tomography (Marsh et al., 2001) and using GFP targeted probes and high resolution digital imaging microscopy (Rizzuto et al., 1998a,b). Interestingly, close contacts between smooth subdomains of ER and mitochondria have been shown to be required for both maintaining mitochondrial structure and lipid transfer between the two organelles (Vance, 1990; Prinz et al., 2000).

Co-isolation of ER, mitochondria and PM suggests that dynamic associations between these membranes could be fairly tight. The aim of the study presented in this paper was to isolate PAM fraction from Jurkat lymphoidal cells, and using various biochemical approaches, investigate its role in SOCE. It was confirmed that stimulation of Jurkat cells by thapsigargin results in the increased amount of STIM1 protein found next to the plasma mebrane. Moreover, STIM1 was identified in the PAM fraction and upon addition of thapsigargin it was detected in protein complexes of much higher molecular mass than those in which it is present in unstimulated cells. It may be concluded that PAM are important intermembrane structures, of which the formation and rearrangement, following the ER depletion of Ca^{2+}, accompanies activation of SOCE. The participation of STIM1 in such rebuilding was confirmed.

2. Materials and methods

2.1. Cell culture

Human lymphoblastoid T cells (Jurkat) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL), 2 mM glutamine (Gibco BRL), penicillin (100 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2-95% air at 37 °C.

2.2. Induction of store-operated Ca^{2+} entry

Cells washed twice with PBS, were suspended in nominally Ca^{2+}-free buffer consisting of 132 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 1 mM pyruvate, 5 mM glucose, 0.05 mM EGTA; the pH was adjusted to a desired value with NaOH. After incubation of cells with 100 nM thapsigargin for 8 min at 37 °C 3 mM the mixture was supplemented with 2 mM $CaCl_2$ to induce CCE. After next 3 min the cells were used for subfractionation.

2.3. Subfractionation of Jurkat cells

Isolation of cellular fractions from Jurkat cells was made according to the method previously described for yeasts by Pichler et al. (2001) and adapted for Jurkat cells in our laboratory. Briefly, the control and SOCE induced cells were washed in PBS, resuspended in cold (4 °C) homogenization buffer containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, protein protease inhibitor cocktail, 5 mM Tris–HCl pH 7.4 and homogenized in the ice-cold water bath using glass Potter–Elvehjem homogenizer with a motorized teflon pestle. The efficiency of homogenization was monitored under the microscope and homogenization was stopped when disintegration of 90% of cells was reached. The homogenate was centrifuged at 600 × *g* for 3 min twice. The final supernatant was centrifuged at 20,000 × *g* for 20 min. Supernatant containing microsomes and cytosolic proteins was centrifuged at 100,000 × *g* for 1 h to sediment microsomes. Crude membrane fraction containing plasma membrane, mitochondria and PAM fraction was suspended in 5 mM Bis–Tris, 0.2 mM EDTA, pH 6.0 and subjected to separation on a discontinuous 38%, 43% and 53% sucrose gradient made on the base of the solution: 5 mM Bis–Tris, 0.2 mM EDTA, pH 6.0. A low-density band localized on the top of 38% sucrose (denoted as the PAM fraction), middle-density band characterized as a crude mitochondrial fraction localized at the 38/43% sucrose interface and a high-density band, 43/53% sucrose interface, (denoted as plasma membrane) were collected and diluted threefold with 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 5 mM Tris–HCl pH 7.4. PAM fraction was sedimented at 100,000 × *g* for 45 min. Purified plasma membrane was centrifuged at 10,000 × *g* for 10 min to remove contaminating mitochondria and then sedimented at 48 000 × *g* for 20 min. Crude mitochondrial fraction was obtained by centrifugation at 13 000 × *g* for 10 min. All isolated subcellular fractions were suspended in 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 5 mM Tris–HCl pH 7.4 using a loose-fitting Dounce homogenizer. Protein concentration in the fractions was determined according to Bradford's method using Bio-Rad protein estimation kit.

2.4. Isolation of the plasma membrane – PAM enriched fraction and pure plasma membrane by biotinylation of cell-surface proteins from Jurkat cells

Crude plasma membrane (PM-PAM) enriched fraction was isolated as described previously (Pichler et al., 2001). For biotinylation of the cell surface proteins EZ-Link Sulfo-NHS-LC Biotin (Pierce, Rockford, US) was used. 1×10^8 of Jurkat cells suspended in 5 ml PBS (pH 8.0) supplemented with1 mM pyruvate and 5 mM glucose was incubated with 2.5 mg of biotinylating reagent. After 30 min at RT the cells were washed three times with ice cold PBS. Than cells were suspended and incubated in hypotonic lysis buffer (10 mM Tris, 10 mM NaCl, pH 7.0) in the presence of protease inhibitors on ice for 3–5 min. The cell debris was sedimented for 10 min at 1000 × *g*, 4 °C. Cell lysates were incubated with immobilized NeutrAvidin beads (Pierce). Beads with bound proteins were washed six times with hypotonic buffer (10 mM Tris, 10 mM NaCl, 1 mM $MgCl_2$ pH 7.0) and incubated with Laemmli sample buffer supple-

mented with 100 mM DTT at 50 °C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blot.

To purify PM form PAM fraction, NeutrAvidin beads with bound PM/PAM proteins were incubated with 7 M urea/2 M tiourea for 15 min in RT. Beads with PM proteins were prepared for SDS-PAGE as described before.

2.5. Estimation of the protein profile of subcellular fractions by SDS-PAGE

40 μg of the protein of each fraction was separated on 4–12% SDS-PAGE with the size of 1 mm/16 cm/20 cm. Proteins were visualized by the colloidal Coomassie staining according to the Anderson procedure (Anderson and Anderson, 1991).

2.6. Blue native electrophoresis and second dimension (SDS-PAGE) of the BN-PAGE

The cells (30×10^6) washed twice with PBS or of PAM fraction (75 μg of protein) were resuspended in the aminocaproic acid buffer (1.5 M 6-aminocaproic acid, 50 mM Bis–Tris pH 7.0) and supplemented with *n*-dodecyl-β-D-maltoside (1% final concentration). Samples were incubated on ice for 20–30 min and then centrifuged at 100,000 × *g* for 15 min. to remove unsolubilized material. Protein concentration in the supernatant containing solubilized protein complexes was determined according to the Bradford's method using Bio-Rad protein estimation kit. The supernatant was mixed with Serva Blue G (final concentration 0.5%) and subjected to electrophoresis on the acrylamide gel gradient of 5–12% and the size of 1 mm/16 cm/20 cm. At the beginning the separation was conducted slowly at 50 V for 30 min to 1 h until the samples entered the 4% stacking gel. Then the voltage was increased to 300 V. When the front of separated samples reached 1/3 of the gel length, the Coomassie blue containing cathode buffer was replaced by the another, colorless one (without Coomassie). The electrophoresis was continued at 300 V.

The lanes of the first-dimension gel were separated and incubated in a dissociating solution (1% SDS and 1% 2-mercaptoethanol), for 0.5 h at room temperature. Then were washed in electrophoresis buffer, stacked over a 10% SDS-PA gel and separated at a constant current of 35 mA until a front of the blue dye reached the end of the gel. Proteins were visualized with the colloidal Coomassie staining or transfered on to the PVDF membrane using standard procedure.

2.7. Western blot analysis

For SDS-PAGE analysis subcellular fractions were separated on 10% SDS-polyacrylamide gels and identified with specific antibodies as follows: αIP3R, non isotype specific monoclonal antibody, 1:200, Calbiochem; recognizing all three isoforms; Orai 1, 1:500 rabbit polyclonal, Abcam; ACSL-4, goat polyclonal, 1:1000, Santa Cruz; SOD2, goat polyclonal, 1:1000, Santa Cruz; SERCA2 (IID8), mouse monoclonal, 1:2000, Santa Cruz; PMCA, mouse monoclonal, 1:2000, Santa Cruz; PMCA4b (JA3), mouse monoclonal, 1:2500, Santa Cruz; Na/K ATPase mouse monoclonal, 1:2000, Abcam; COX II, mouse monoclonal, 1:2000, MitoSciences; ANT goat polyclonal, 1:1000, Santa Cruz; αVDAC1, 1:5000; Calbiochem, monoclonal anti-porin 31HLHuman; CD3, 1:2500, rabbit polyclonal, DAKO Cytomation; GOK1/STIM, 1:500, BD Transduction Laboratories. After hybridization with peroxidase (HRP) conjugated secondary antibody, the signal was revealed using ECL Plus Western blot detection reagent (Amersham Pharmacia Biotech). Samples containing 70 μg total cellular protein per well were run on a Laemmli-type 8% sodium dodecyl sulfate-polyacrylamide gel and proteins were electroblotted onto nitrocellulose membranes. Blocking of nonspecific protein binding to nitrocellulose was performed by incubating the membranes for 30 min with Tris-buffered saline (TBS)-0.1% Tween-2% milk.

2.8. Measurement of the cystosolic Ca^{2+} concentration

Cytosolic free Ca^{2+} was measured with Fura-2 (Grynkiewicz et al., 1985). The cells were loaded with 1 μM Fura-2/AM in the culture medium at 37 °C for 15 min. After centrifugation the cells were resuspended in the solution consisting of 132 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 1 mM pyruvate, 5 mM glucose, 0.1 mM $CaCl_2$, and centrifuged again. Next, the cells were suspended in the same but nominally calcium free solution ($CaCl_2$ replaced with 0.05 mM EGTA) and used for experiments. Fluorescence of Fura-2 was measured at 30 °C with Shimadzu RF 5301PC spectrofluorimeter set in the ratio mode using 340 nm and 380 nm as excitation wavelengths and 510 nm as the emission wavelength. The time resolution of the measurements was 1 s.

2.9. Immunocytochemical detection of STIM1 protein

After activation of SOCE as described above, the cells (as well as the control ones not stimulated by thapsigargin) were fixed with 4% p-formaldehyde (15 min, at room temperature), washed in PBS and permealized by 0,1% Triton X-100 containing 0.1 M glycine (15 min). All steps of the immunostaining were performed in suspension. After washing in PBS, the cells incubated in 5% horse serum for 30 min. The primary antibody against Stim1 (monoclonal, BD Transduction Laboratories) was applied at dilution 1:100, 4 °C, overnight. Cells were then washed in PBS and incubated in secondary antibody (Alexa Fluor 488, Donkey Anti-Rabbit IgG) diluted 1:200 for 2 h, at room temperature. After immunostaining, the cell pellets were dehydratated respectively in 50%, 70%, 96% and 100% ethanol (in each one for 15 min). Cells were then incubated twice in propylene oxide for 15 min, at room temperature and infiltrated with propylene oxide/epon at a ratio as follows: 2:1, 1:1, 1:2 (for 1 h at each one). Next, infiltration was performed overnight in 100% epon. After addition of a new portion of pure epon, the samples were left for polymerization at 60 °C for 48 h. Cell immobilized in polymerized epon were cut with use of ultramicrotome (Leica) into 500 nm-thick plastic sections. Images were acquired using confocal microscope (Leica TCS SP2). Images were subjected to background-subtraction and deconvolution, using Huygens 3.1 software (maximum-likelihood estimation algorithm), followed by brightness adjustment (in Corel Photo Paint), to cover full dynamic range of pixel-intesities.

2.10. Analysis of mitochondrial and endoplasmic reticulum structure

Cells were plated on glass coverslips coated with Matrigel™ (BD Biosciences), diluted 3× with RPMI medium, and centrifuged by 700 g 4 min. A group of cells was washed with KRB containing 1 mM $CaCl_2$ (controls), whereas another group was washed once with KRB/CaCl2, then incubated sequentially in KRB/EGTA (100 μM, 3 min), KRB/EGTA/Tg (1 μM, 5 min), and KRB/CaCl2 (2 min, Tg treated cells). After treatment cells were immediately fixed with 4% p-formaldehyde (15 min, 37 °C). Immunofluorescence staining was performed according to standard protocols. Briefly, cells were washed in PBS, permeabilized by 0.1% Triton X-100 (10 min), blocked in 1% BSA (20 min), before primary antibodies were applied for 4 h at RT in PBS/BSA 1%. The primary antibodies used were grp-75 (goat polyclonal, Santa Cruz, 1:100) and STIM1 (monoclonal, BD Transduction Laboratories 1:200). Cells were then washed in PBS, and secondary antibodies (Alexa Fluor 488, Goat Anti–Mouse IgG for STIM and Alexa Fluor 594 Rabbit Anti-Goat IgG for grp-75, 1:800) were applied for 30 min at RT. Image acquisition was

made using an inverted microscope (Axiovert 200 M; Carl Zeiss MicroImaging, Inc.), using a 63 × /1.4 Plan-Apochromat objective, a Cool-SNAP HQ interline charge-coupled device camera (Roper Scientifi c) and the MetaMorph 5.0 software (Universal Imaging Corp.). 3D reconstruction and co-localization analysis were performed on a wide-field digital imaging system as previously described (Szabadkai et al., 2004, 2006). 3D image deconvolution was performed using a custom made software developed by the Biomedical Imaging Group of the University of Massachusetts (Worcester, MA, USA) based on measured point spread function (Rizzuto et al., 1998a,b). This setup thus allowed the resolution of 250 nm in the xy, and 500 nm in the z direction. For 3D reconstruction and co-localization analysis of fluorescence signals the *data analysis and visualization environment* (DAVE) software, developed by the same group (Carrington et al., 1995; Rapizzi et al., 2002).

3. Results

3.1. Activation of SOCE induces changes in STIM1 protein localization

Translocation of STIM1 from ER to PM accompanying of SOCE was previously shown in various cell types (Serrano, 1988). In our experiments, a complete depletion of the ER of Ca^{2+}, which is a prerequisite for SOCE activation, was achieved due to inhibition of SERCA by 100 nM thapsigargin. It resulted in a transient increase in the cytosolic Ca^{2+} concentration (Fig. 1A). Such treatment of cells resulted also in accumulation of large STIM1-immunoreactive granules at the cell periphery, sometimes forming linear aggregates (Fig. 1B). They may reflect an accumulation of vesicles separated from the plasma membrane by the distance smaller than the optical microscopy resolution (~250 nm). Notably, the specimen was prepared by distortion-free embedding of cells in the resin, followed by cutting of semi-thin sections. Such a procedure preserves the native 3D arrangement of cellular compartments. This observation, confirming data coming from other laboratories, led us to further studies focused on a transient formation of specialized structures consisting of the ER and PM, putatively engaged in the store-operated Ca^{2+} entry.

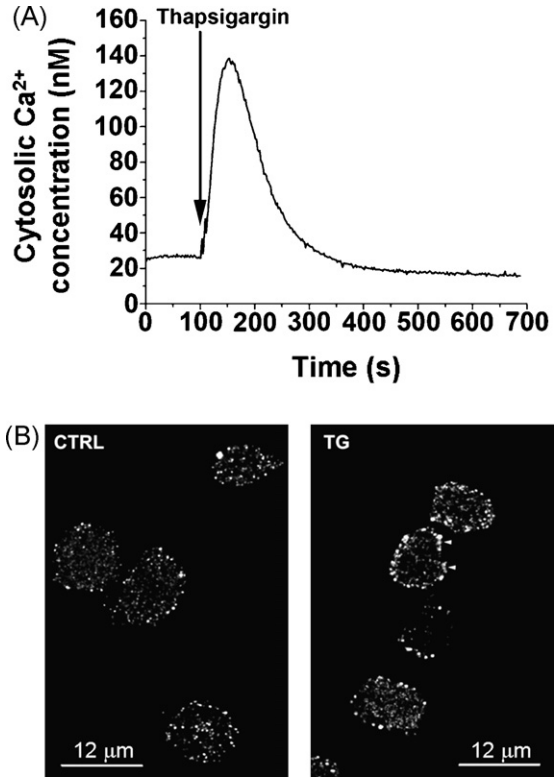

Fig. 1. Intracellular localization of STIM1 in control and SOCE-activated cells. (A) Representative trace showing a thapsigargin-evoked transient increase of the cytosolic Ca^{2+} in Jurkat cells suspended in the Ca^{2+}-free solution. (B) Translocation of STIM1 to the plasma membrane in Jurkat cells in upon addition of thapsigargin.

3.2. Isolation and characterization of PAM fraction from Jurkat cells

To isolate the PAM fraction, we took an advantage of a method developed by Gaigg et al. (1995) using sucrose density gradient centrifugation that allowed to fractionate the crude PM fraction to PAMs and the purified PM (Gaigg et al., 1995; Serrano, 1988). To improve the quality and efficiency of the purification procedure we slightly modified the original protocol. Four subcellular fractions: mitochondria, ER, PM and PAM fraction were isolated from human lymphoblastoid T cells (Jurkat cells). For details of our protocol see Section 2 and Supplementary Figure 1.

According to the data available in the literature, divalent cations modify PAM composition and increase stability of interactions between the PM and the ER, and/or the mitochondria (Pichler et al., 2001). Moreover, a slightly acidic medium (ca. pH 6.0) also seems to be critical for these interactions. Therefore, in all experiments described in this paper, PAM fraction was isolated in the presence of divalent cations chelators in buffers, and pH of sucrose gradient was adjusted to 6.0. Protein composition of the PAM fraction was compared with the protein profiles of the mitochondria, ER and PM isolated from Jurkat cells, using 1D SDS-PAGE technique. As expected, protein profile of the PAM fraction (Supplementary Figure 2) differs from others, but some of protein bands of the PAM have counterparts in the ER and mitochondria.

To identify interactions between the organelles, and find protein "fingerprint" characteristic for the PAM fraction obtained from Jurkat cells, we used various antibodies against proteins which are specific markers of particular subcellular fractions. As it is shown in Fig. 2, the PAM fraction contains both ER and PM proteins as well as some mitochondrial markers such as porin (VDAC), present in the outer mitochondrial membrane (OMM) and ADP/ATP carrier (ANT) located in the inner mitochondrial membrane (IMM). The relatively small amount of cytochrome c oxidase (COX), another integral IMM protein, in the PAM fraction, suggests that discrete mitochondrial contact sites (containing, among other proteins, VDAC and ANT but not COX) could be involved in PAM formation. Superoxide dismutase 2 (SOD2) located in the mitochondrial matrix is not detected in the PAM. Such specific protein composition of the PAM fraction indicates non-accidental presence of selected IMM proteins. Interestingly plasma membrane markers (including plasma membrane Ca^{2+}-ATPase (PMCA), sodium-potassium pump (Na^+/K^+-ATPase,))are more abundant in the PAM fraction than in the PM fragments not engaged in PAM formation. These data correspond well to results published by Lillemeier and co-workers, and could be explained by the presence of plasma membrane proteins forming clusters attached to cytoskeleton. The PAM fraction isolated from Jurkat cells can be formed by specific plasma membrane sub-regions which could be involved in interactions with intracellular organelles (Lillemeier et al., 2006). The presence of ER and PM markers in the crude mitochondrial fraction may reflect formation of the MAM fraction usually accompanying mitochondrial preparations (Lebiedzinska et al., 2009; Vance, 1990; Vance et al., 1997), whereas high amount of proteins derived from the outer and inner mitochondrial membranes and the mitochondrial matrix found in the PM fraction could be unexpected until it was found by Chen and co-workers (Chen et al., 2007). Quantification of Western blots presented in Fig. 2 is shown in Supplementary Figure 3.

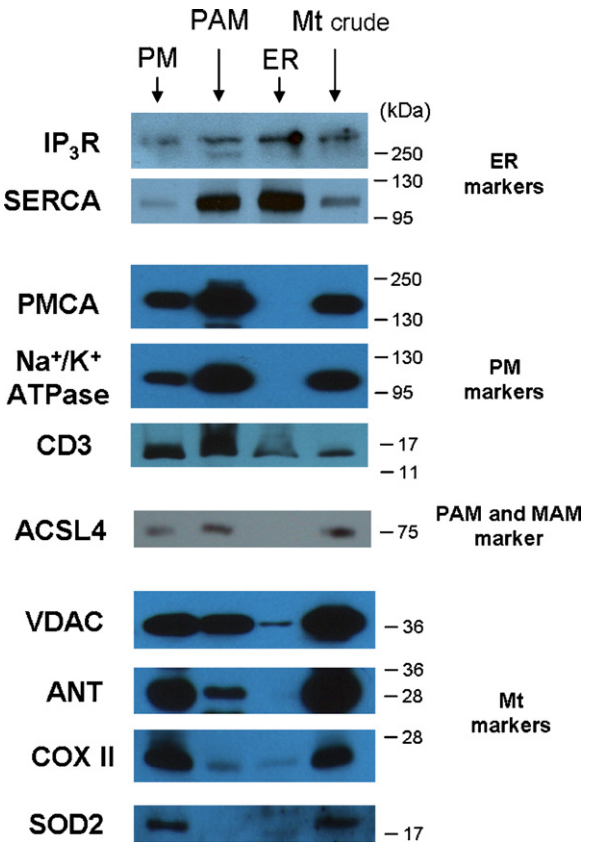

Fig. 2. Immunoblot analysis of protein components of subcellular fractions prepared from Jurkat cells. Subcellular fractions from Jurkat cells were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membrane and incubated with corresponding antibodies. Representative blots showing the relative amounts of characteristic protein markers in isolated subcellular fractions are shown. ER markers: IP_3R, SERCA. PM markers: Na^+/K^+-ATPase, PMCA, CD3R. Mitochondrial markers: VDAC, ANT, COXI, SOD2. Quantification of Western blots is presented in Supplementary Fig. 3.

Furthermore, an introduction of additional steps to the PM isolation/purification procedure, consisting of hypertonic treatment of the PM fraction followed by centrifugation at 48,000 × *g* for 20 min, did not improve its purity (not shown). This might indicate that the plasma membrane and mitochondria form stabile structures distinct from the PAM formed predominantly by the endoplasmic reticulum and plasma membrane. The presence of ACSL4 protein in the PM fraction supports this hypothesis but it needs further confirmation.

Co-localization of selected proteins typical for different intracellular structures in the PAM fraction supports the idea that PAM is a structure which reflects stable bridges between PM and ER. Such a close positioning of ER to the PM enables fast and discrete cross-talk between these two structures e.g. to help or maintain SOCE (Lebiedzinska et al., 2009).

3.3. STIM1and Orai1 proteins are present in the PAM fraction

STIM1 has been shown to be a Ca^{2+} sensor within the ER stores and its role in the store-operated Ca^{2+} signaling pathway was firmly established (Zhang et al., 2005; Roos et al., 2005; Spassova et al., 2006). This protein interacts with Orai1 protein located in the PM, which is another crucial component of the CCE machinery. To test whether both CCE participants, STIM1 and Orai1 are components of the PAM fraction, an intracellular localization of these proteins was investigated. We estimated the relative amount of STIM1 and Orai1 in selected cellular fractions. As shown in Fig. 3A a high amount of STIM1 is detected in both ER and PAM fractions, whereas a relatively low amount of this protein was detected in the PM fraction. Orai1 protein was predominantly detected in the PAM and PM fraction. Using this isolation procedure two bands of STIM1 have been always observed. The lower band corresponds to the STIM1 which is deglycosylated and the upper to the glycosylated form. Occurrence of this two forms of STIM1 is in agreement with the data recently published by our group (Czyż et al., 2009). Interestingly, in PAM fraction only deglycosylated STIM1 has been observed what additionally supports our hypothesis of the involvement of PAM fraction in the CCE.

As STIM1 (but not Orai1) is thought to undergo intracellular translocation between the ER and the PM upon depletion of the intracellular Ca^{2+} stores, the further experiments were focused on this protein. To verify the previously reported observation that STIM1 is present not only in the ER but also can be an integral PM protein (Zhang et al., 2005) an alternative method based on cell surface protein biotinylation was used for isolation of the crude PM fraction. It consist of both "bright" ("pure") PM free of other membrane fragments and PAM fraction. Incubation of the collected material with 7 M urea and 2 M thio-urea resulted in an dissociation of the high purity PM. The PM fraction prepared by this method is free of ER marker (Fig. 3B) whereas the mitochondrial markers (ANT, COX and SOD2) were not detected either in the PM + PAM fraction prior to or in the PM fraction after the urea + thiourea treatment (not shown). Western blot analysis of such highly purified PM clearly indicates that STIM1 is also present in the PM (Fig. 3B). The comparison of both methods used for purification of the PM and PAM fractions confirms that the biotinylation technique allows isolating of PM of much higher purity than that based on the centrifugation in sucrose gradient (Peirce et al., 2009).

3.4. Protein profile of Jurkat cells is unchanged during CCE but STIM1 is present in different protein complexes

An activation of SOCE is an indispensable element of signaling cascade that follows lymphocyte stimulation. Depletion of the ER Ca^{2+} stores induced by an extracellular stimulus results in opening of SOCs located in the PM. Recently it has been shown that this latter step needs close apposition of the ER and PM proteins, STIM1 and Orai1, respectively. For a qualitative description of such relationships between PM and ER under resting condition and during SOCE activation, the rearrangement of STIM1-containing protein complexes were analyzed.

Protein profiles visualized by Coomassie staining after 1D SDS-PAGE of the PAM fraction isolated from resting and thapsigargin treated Jurkat cells were identical (not shown). Hence, it was assumed that possible changes could occur on a more discrete level and deal with differences in protein interaction rather than in a total protein composition. To test this hypothesis the Blue Native polyacrylamide gel electrophoresis (BN-PAGE), originally described by Schägger and von Jagov, was employed. This technique allows studying of protein complexes formation and estimating their size and composition (Schägger and von Jagow, 1991). The typical pattern of the total cellular proteins obtained by sequential use of BN-PAGE and SDS-PAGE, in resting cells and after SOCE induction is shown in Fig. 4A. Proteins were visualized by Colloidal Coomassie staining. As molecular mass markers the mitochondria respiratory chain complexes were used. The results obtained indicate that the short-term activation of SOCE was not followed by global remodeling of protein complexes. However, a typical 2D BN/SDS PAGE shows significant changes in STIM1 localization upon SOCE activation (Fig. 4B). Particularly, stimulation of cells by thapsigargin resulted in the increase of proportion of STIM1 localized in the complexes of relatively higher molecular mass than in control cells and the decreased amount of STIM1 "dispersed" among numer-

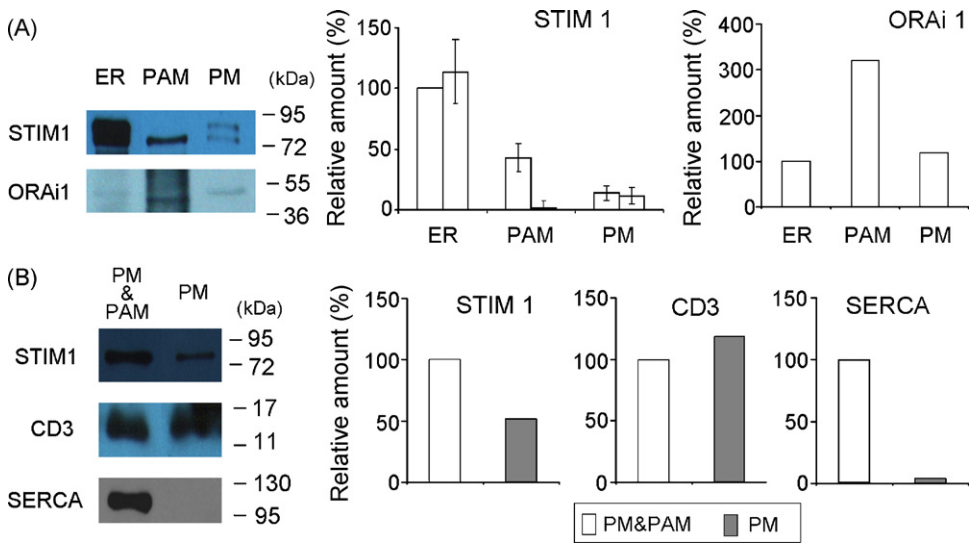

Fig. 3. Subcellular localization of STIM1 protein. (A) Subcellular fractions were prepared as is described in Material and methods. 30 μg proteins were loaded on 10% SDS-polyacrilamide gels. A typical result of immunoblots are shown. Bar chart showing quantified levels of STIM1 and ORAi1 in subcellular fractions isolated from Jurkat cells. Each bar chart represents means ± S.E.M. $n = 3$. For ORAi1 presented values are means from two independent experiments. Western blot scans were analyzed using NIH ImageJ software; (B) Crude plasma membrane (enriched in PAM fraction, PM/PAM) and pure plasma membrane (PM) were isolated by biotinylation of the cell-surface proteins from Jurkat cells and separated on 10% SDS-polyacrilamide gel. A typical results of immunoblot are shown. Bar chart showing quantified levels of STIM1 and CD3 and SERCA. Presented values are means from two independent experiments. Western blot scans were analyzed using NIH ImageJ software.

ous complexes of relatively high, medium and low molecular mass. These changes indicate that STIM1-containing protein complexes are modified during SOCE. Such a remodeling might be important for the initiation and/or execution of Ca^{2+} entry. To distinguish between global and local changes in protein profiles, a participation of STIM1 in different protein complexes located in the PAM fraction was investigated. Results presented in Fig. 4C show remodeling of STIM1-containing complexes during CCE in PAM fraction.

3.5. 3D morphological imaging reveals physical association of STIM1 with plasma membrane and mitochondria

It has been previously shown that following SOCE induction by thapsigargin, mitochondria shifted towards the plasmamembrane in Jurkat cells (Quintana et al., 2006). However, no data concerning the relationship between mitochondria and STIM1 during activation of the SOCE pathway have been published so far. In order to determine the interaction of STIM1 with the mitochondrial network in the control and thapsigargin treated cells we applied a 3D digital imaging approach previously described to assess mitochondrial-ER interactions (Rizzuto et al., 1998a,b). To acquire Z-series of images of the mitochondrial marker grp75 (present in the mitochondrial matrix and the OMM surface) (Szabadkai et al., 2006) and STIM1, cells were immobilized on glass coverslips, fixed either without treatment or immediately after induction of capacitative Ca^{2+} influx (see Section 2), and the proteins were visualized by immunofluorescence. As shown on Fig. 5A, mitochondria form a partially continuous network in a narrow space between the nucleus and PM in both control and Tg treated cells, without any gross change in their structure. Co-localization analysis of the STIM1 and grp75 signals (Fig. 5B) revealed a low level of interaction in control cells (0.27%), presumably reflecting the previously demonstrated ER-mitochondrial contact sites (Rizzuto et al., 1998a,b). Interestingly, in Tg stimulated cells we observed a significant increase in the formation of such STIM1 containing contact sites between mitochondria and the ER and presumably the PM (0.57%). These results thus further confirm the findings that STIM1 co-purifies with mitochondrial, ER and PM markers, coalescing into dynamic protein complexes comprising all three organelles.

In conclusion, our data confirm a close physical coupling between ER and PM which leads to formation of PAM structures, plays a crucial role in activation and/or maintenance of SOCE.

4. Discussion

Previously it was shown that the depletion of Ca^{2+} stores of Ca^{2+} results in enhanced interaction of the ER with mitochondria (Rizzuto et al., 1998a,b). Recently, we have identified protein components of the MAM fraction, and pointed out a possible participation of IP3R, VDAC and MOT2 proteins in the Ca^{2+} transport between these organelles (Szabadkai et al., 2006). Moreover, we defined the mitochondrial proteins in contact sites between the mitochondria and ER in the MAM fraction, and highlighted their role in modulating Ca^{2+} cross talk between the mitochondria and ER (Wieckowski et al., 2006). Furthermore, we also found that the MAM fraction isolated during mitochondria purification from HeLa cells or rat liver contains not only mitochondrial and ER markers but also plasma membrane Ca^{2+} ATPase (PMCA).

More recently, similar interaction between the PM and ER were described in control and stimulated cells, suggesting that formation of direct contacts between selected regions of different cellular membranes is necessary for activation of Ca^{2+} channels (Zhang et al., 2005; Luik et al., 2008). Altogether, these observations indicate that stimulation of Ca^{2+} signal in electrically non-excitable cells needs very complex interactions between membranes of three cellular structures: plasma membrane, endoplasmic reticulum and mitochondria (Lebiedzinska et al., 2009).

In the present study we analyzed interactions between the ER and PM from "the plasma membrane point of view". In other words these studies were focused on contact sites between the ER and PM previously described in yeast as PAM. We observed changes in the distribution of STIM1 protein upon cell stimulation by thapsigargin and hypothesized that PAM structures are the loci of specific proteins participating in the formation of dynamic interactions between PM and ER which are thought to be involved in SOCE activation in animal cells. Two different experimental approaches were applied for separation of selected subcellular fractions, including PM and PAM. The first relied on the differential centrifugation of

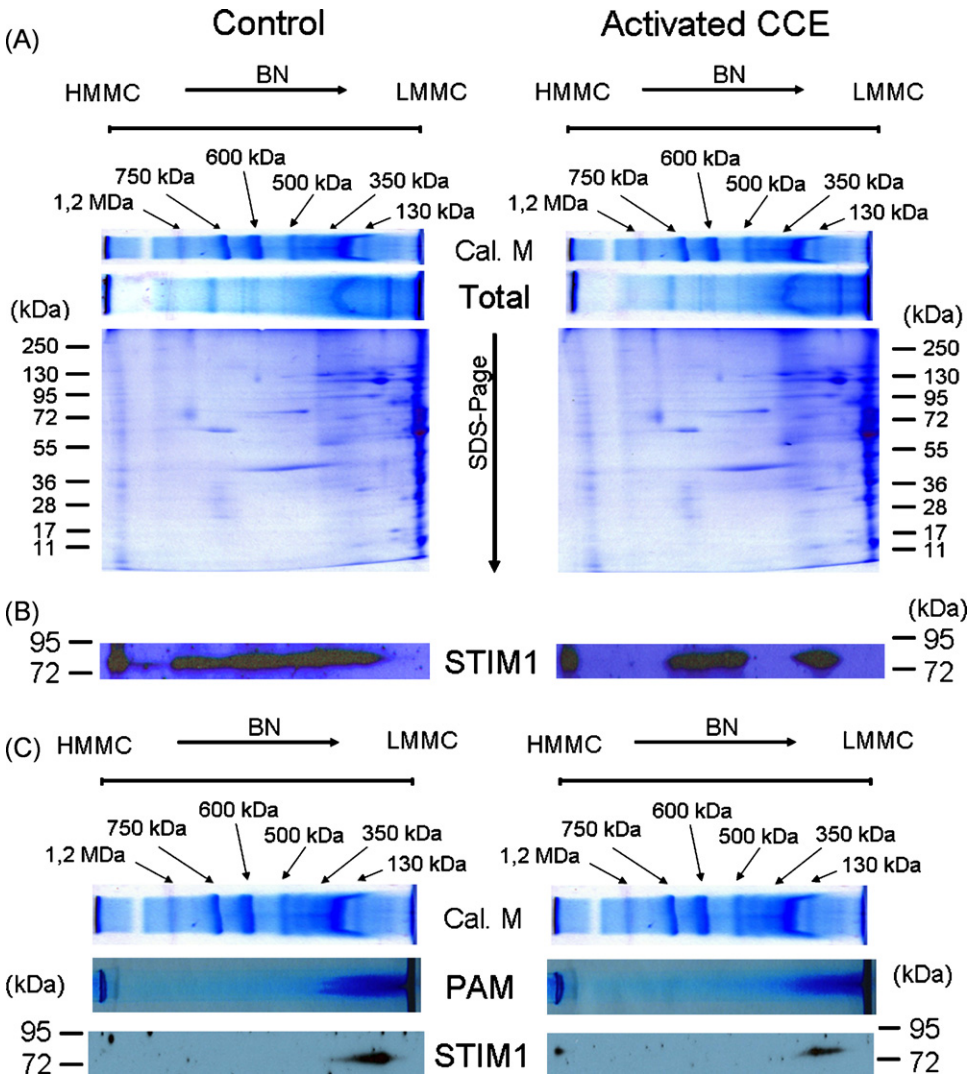

Fig. 4. Blue Native Electrophoresis and Western blot analysis of the total cellular lysate and PAM fraction from Jurkat cells in resting state and after CCA activation. Jurkat cells or native PAM fractions were separated on a 5–12% acrylamide gel in the first dimension. Then, the native gels were incubated with a dissociating solution, stacked over a 10% SDS-PA gel and electrophoreticaly separated as described in Material and methods section. (A) BN-PAGE/SDS-PAGE of control and "stimulated" "total proteom" from Jurkat cells. Calibration marker–BN-PAGE of rat heart mitochondria used as a molecular marker to calibrate separation in the gel. TOTAL–BN-PAGE of "total proteom" stained by Coomassie Brillant Blue G250. (B) Western blot of BN-PAGE/SDS-PAGE separated "total proteom" of Jurkat cells developed against STIM1 protein. C) BN-PAGE/SDS-PAGE of PAM fraction isolated from control and "stimulated" Jurkat cells. Calibration marker–BN-PAGE of rat heart mitochondria used as a molecular marker to calibrate separation in the gel. PAM–BN-PAGE of PAM fraction stained by Coomassie Brillant Blue G250. STIM1-Western blot of BN-PAGE/SDS-PAGE separated PAM fraction developed against STIM1 protein. (B and C) – a typical results of an immunoblot from two separate experiments shown.

cellular homogenate in non-linear sucrose gradient. The second one based on a biotinylation of the intact cell surface proteins and isolation of biotinylated PM subfractions from cellular homogenate. Difference in the proportion of the selected mitochondrial and ER markers in the purified PM and PAM in comparison to that found in the purified ER or mitochondria excludes unspecific contamination of the both PM-containing fractions. It allows regarding "bright" PM and PAMs as a specific subfraction of the total plasma membrane. As we shown in Fig. 2, PAM fraction contains relatively high amount of the outer mitochondrial membrane marker VDAC and the inner mitochondrial membrane marker ANT what is in a good agreement with findings of Pichler et al. (2001). Previously, we have shown the lack of COX II, a subunit of cytochrome c oxidase (a marker of the inner mitochondrial membrane) in the MAM fraction (Szabadkai et al., 2006). Similarly in this case, the absence COX I subunit in the PAM fraction indicates a selective participation of ANT in PAM. ANT present in the PAM fraction comes from the mitochondrial contact sites where VDAC as a link can interact with proteins either from PM or ER. It suggests that only specific regions of the outer and inner mitochondrial membranes are involved in the PAM formation. The detection of the only selected mitochondrial and ER proteins in the PAM fraction suggests the specificity of these complex interorganelle interactions.

It is probable that the presence of mitochondrial proteins in the PAM reflects a role of mitochondria in the transmission of Ca^{2+} signals from the endoplasmic reticulum to the plasma membrane. It was suggested that, mitochondria located in the vicinity of Ca^{2+} channels can readily take up Ca^{2+} coming from both ER and the extracellular space upon cell stimulation (Malli et al., 2005). Moreover mitochondrial buffering Ca^{2+} next to the SOCs channels decreases a local Ca^{2+} concentration and thereby prevents feedback inactivation Ca^{2+} influx. Hence, energized mitochondria support store-operated Ca^{2+} entry (Duszynski et al., 2006). It was proposed that sustained activity of SOC requires translocation of mitochondria to the plasma membrane (Quintana et al., 2006). The presence of selected mitochondrial proteins in the PAM fraction or some PM proteins in crude mitochondria (containing MAM fraction) is in agreement with this hypothesis (Lebiedzinska et al., 2009).

3D reconstruction of the mitochondrial structure in control and SOCE activated Jurkat cells shows no significant differences in mitochondrial shape (see Fig. 5A). This observation is in agreement with the data presented by Quintana and co-workers, who pointed out that translocation of mitochondria to the PM regions was observed in the late phase of Ca^{2+} influx (after 8–10 min. when Ca^{2+} concentration in the cytosol reached plateau) (Quintana et al., 2006). In our experimental model cells were fixed 2 min after initiation of the Ca^{2+} influx. Thus, it probably was to short period for mitochondrial translocation to be observed. In control cells and during early phase of SOCE mitochondria form a partially continuous network in the narrow space between the nucleus and the PM. STIM1 is localized as irregular patches in the ER membranes, often surrounding the mitochondrial particles. We found an increased degree of co-localization of STIM1 and mitochondrial network in stimulated cells (see Fig. 5B). Furthermore, immunocytochemical analysis of the excited cells has revealed that STIM1 shifted towards the edge of the cells what corresponds with the data obtained by Wu et al. indicating that STIM1 accumulates in clusters formed in these areas of ER which are positioned closely to PM (Wu et al., 2006). Increased co-localization of the ER and mitochondria in the excited cells suggests that the abundance of the mitochondria-ER contact sites is maintained at a higher level not only during Ca^{2+} release phase but also upon early phase of the capacitative Ca^{2+} entry.

Analysis of the subcellular fractions isolated from Jurkat cells showed that the PAM fraction is enriched in the PM markers in comparison to the distribution of these proteins in the PM not involved in PAM. It was previously reported that the plasma membrane proteins in Jurkat cells are clustered into separated “islands”. This may indicate that the PAM fraction isolated from these cells preferentially contains these sub-regions of the plasma membrane which enriched in proteins which are responsible for interaction with intracellular organelles (Lillemeier et al., 2006). Low contamination level of PM by ER markers together with the lack of the PM markers in the ER fraction suggests that the composition of PAM fraction is unique and not accidental. It has previously been reported by Pichler and co-workers that PAM fraction isolated from yeast contains markers of both PM and ER. They found that the plasma membrane ATPase activity in PAM fraction is 100-fold lower in comparison to the pure PM. The authors have left open the question whether the protein composition of the PAM fraction is the same as or different then the subfraction of the ER in the proximity to the plasma membrane (Pichler et al., 2001). In contrary to these data our results obtained for Jurkat cells indicate that PAM fraction exhibits high similarity to the PM.

The hypothesis suggesting that PAM can be involved in the SOCE was additionally supported by our finding indicating that the PAM obtained from Jurkat cells by sucrose gradient centrifugation, contained significant amount of STIM1 protein. STIM1 is the ER protein though its localization in other subcellular compartments has also been suggested (Zhang et al., 2005). Furthermore, data obtained from the experiments based on fractionation of cells with the use of the biotinylation technique, confirmed that STIM1 could also be found in the PM. This result is in good agreement with observation described by Spassova et al. (2006). It was also reported that depletion of the ER of Ca^{2+} caused translocation of STIM1 to the PM. (Peirce et al., 2009). Our data may support a hypothesis that STIM1 present in the PAM fraction is involved in the interaction between ER and PM and formation/activation of SOC channels (see Fig. 6). On the other hand, they may became controversial in a view of other reports evidencing that translocation of STIM1 to PM during SOCE does not occur (Wu et al., 2006; Luik et al., 2006; Patterson et al., 1999). The interactional model of SOCE activation assumes that the opening of Ca^{2+} channels needs a specific interaction of STIM1

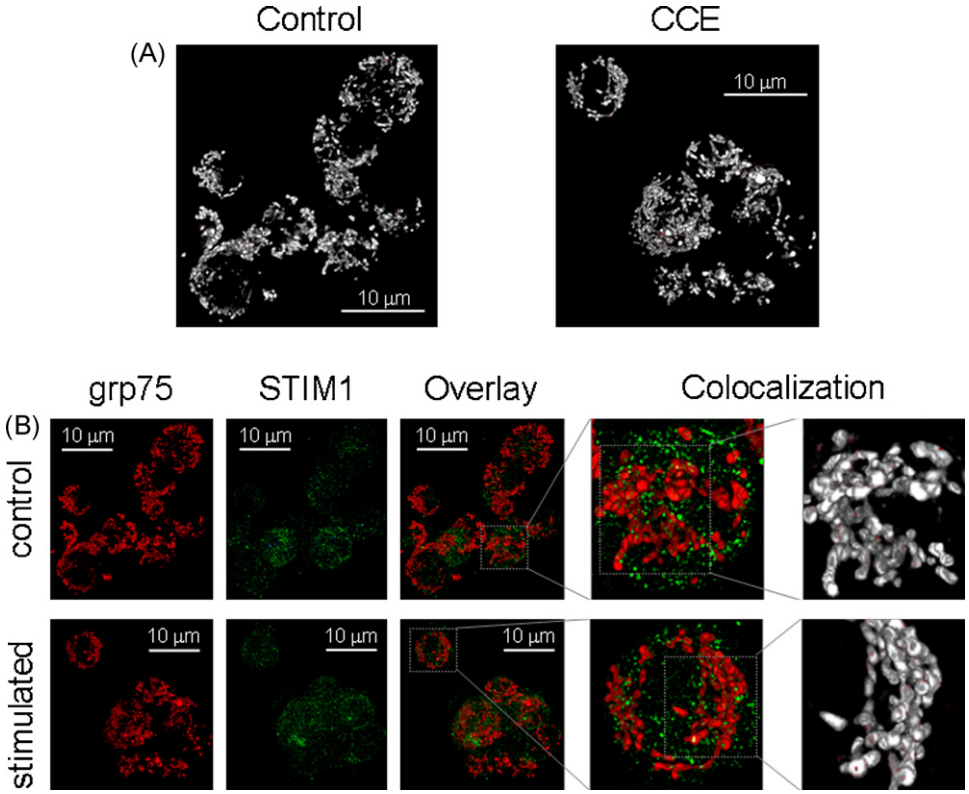

Fig. 5. (A) 3D structure of mitochondria in resting state and upon capacitative calcium entry in Jurkat cells. Cells were seeded on glass coverslips, fixed; cells were labeled and imaged as described in the Materials and Methods section. (B) Co-localization analysis of immunolabelled STIM1 and the mitochondrial network on the same preparation as in (A). Mitochondria are shown in red, STIM1 in green. Magnified inset shows the 3D structure of mitochondria (grey) superimposed with the voxels colocalizing with the STIM1 signal (red spots) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

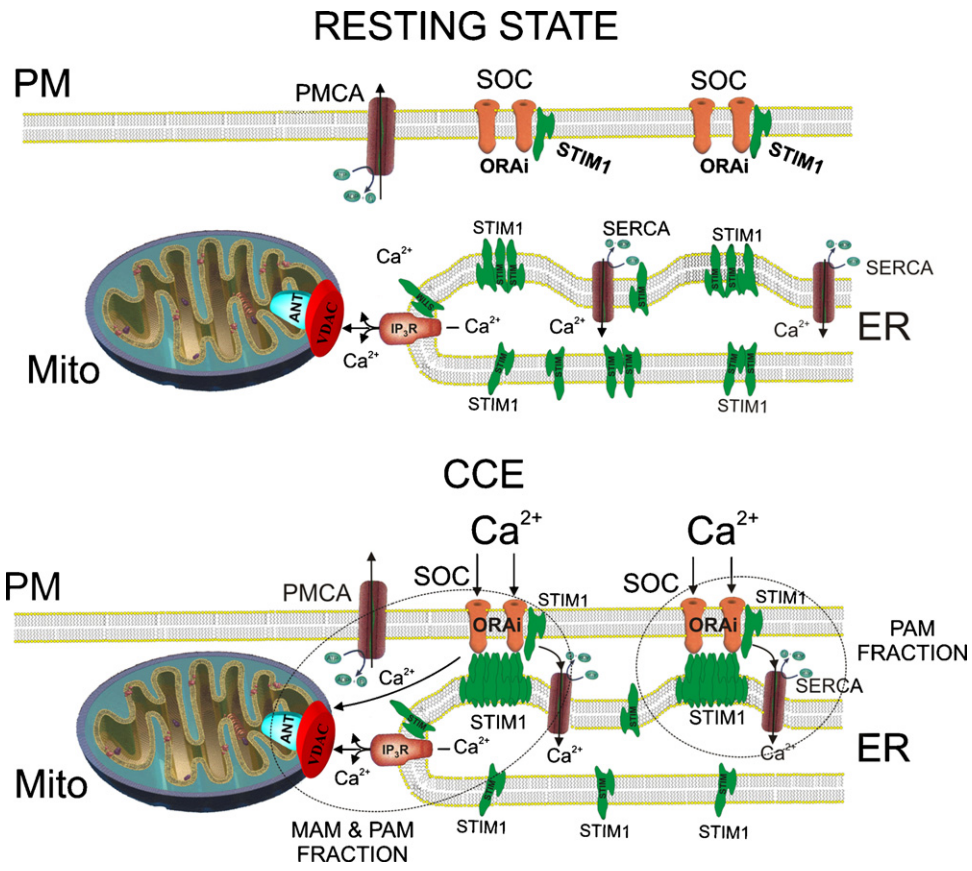

Fig. 6. Model of capacitative calcium entry initiated by aggregation o STIM1 present in PAM fraction in to high molecular weight complexes. Interaction of STIM1 aggregates with ORAi protein results in activation of SOC channels. Close positioning of mitochondria to the SOC reduces Ca^{2+}-dependent channel inactivation. SERCA presence in the vicinity of Ca^{2+} transmission areas enables fast and efficient ER refilling.

and proteins of the PM (Venkatachalam et al., 2002; Dziadek and Johnstone, 2007). Using TIRF microscopy, Wu et al. (2006) observed that STIM1 aggregated in the ER may be positioned at the distance of 10–20 nm from the PM. This is close enough for direct interaction between STIM1and PM proteins (Wu et al., 2006). It is suggested that STIM1 interacts with ORAi1 protein or, alternatively, with STIM1 located in the PM. Such explanation in the line with our observation that, activation of SOCE accompanies the remodeling of STIM1-containing complexes of a molecular masses ranging between 800 kDa–1.5MDa and 300–500 kDa. (Fig. 4B). Unfortunately, in the total native lysate we could not observe an appearance of the very high molecular weight STIM1-containing complexes, which formation was postulated by Dziadek and Johnstone in their model of conformational changes in STIM1 protein (Dziadek and Johnstone, 2007). On the other hand, our results obtained with the use of PAM fraction isolated from Jurkat cells correspond well to the conformational model of SOCE. Detection of STIM1 in the higher molecular mass complexes concomitantly with its decreased participation in the low molecular mass complexes indicates serious changes in proteins interrelationships upon stimulation of cells by thapsigargin. The presence of some amount of the low molecular mass STIM1-containing complexes in PAM fraction after induction of SOCE indicates that not all STIM1 present in the ER or PAM is involved in the activation and/or maintenance of the Ca^{2+} entry (see Fig. 4C low/right panel). It seems that STIM1 accumulation near the PM does not need a massive movement of ER toward PM and is in an agreement with the data presented by Wu et al. that only 1/3 of contacts is newly formed and the rest comes from already preexisting ones. This could emphasize a potential role of PAM fraction in SOCE (Wu et al., 2006). Recently it has been reported that close apposition of ER membrane to the PM can also be important for refilling of ER stores with minimal changes in cytosolic Ca^{2+} level (Jousset et al., 2007). The authors tested the hypothesis that Ca^{2+} influx during SOCE occurs in clusters containing SOC channels, STIM1 and SERCA proteins.

Taking together, we propose that PAM are subcellular structures involved in the store-operated Ca^{2+} entry. It must be emphasized that they not only reflect the functional interactions between the PM, ER and mitochondria, but also are stabile enough to allow purifying them as a defined subfraction of the total plasma membrane. However, the mode of interaction between mitochondria, ER and PM is still far from being fully understood and identification of other components of contact sites between these organelles needs additional studies. More information about the role of PAM and MAM fraction you can find in our recent review, see ref. Lebiedzinska et al. (2009).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.07.003.

References

Achleitner G, Gaigg B, Krasser A, Kainersdorfer E, Kohlwein SD, Perktold A, et al. Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. Eur J Biochem 1999;264:545–53.

Anderson NL, Anderson NG. A two-dimensional gel database of human plasma proteins. Electrophoresis 1991;12:883–906.

Carrington WA, Lynch RM, Moore ED, Isenberg G, Fogarty KE, Fay FS. Superresolution three-dimensional images of fluorescence in cells with minimal light exposure. Science 1995;268:1483–7.

Chen P, Zhang L, Li X, Wang X, Cao R, Liu Z, et al. Evaluation of strategy for analyzing mouse liver plasma membrane proteome. Sci China series: C Life Sci 2007;50:731–8.

Czyż A, Brutkowski W, Fronk J, Duszynski J, Zablocki K. Tunicamycin desensitizes store-operated Ca2+ entry to ATP and mitochondrial potential. Biochem Biophys Res Commun 2009;381:176–80.

Duszynski J, Koziel R, Brutkowski W, Szczepanowska J, Zablocki K. The regulatory role of mitochondria in capacitative calcium entry. Biochim Biophys Acta 2006;1757:380–7.

Dziadek MA, Johnstone LS. Biochemical properties and cellular localization of STIM proteins. Cell Calcium 2007;42:123–32.

Gaigg B, Simbeni R, Hrastnik C, Paltauf F, Daum G. Characterization of a microsomal subfraction associated with mitochondria of the yeast. Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. Biochim Biophys Acta 1995;1234:214–20.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. J Biol Chem 1985;260:3440–50.

Jousset H, Frieden M, Demaurex N. STIM1 knockdown reveals that store-operated Ca^{2+} channels located close to sarco/endoplasmic Ca^{2+} ATPases (SERCA) pumps silently refill the endoplasmic reticulum. J Biol Chem 2007;282:11456–64.

Lebiedzinska M, Szabadkai G, Jones AWE, Duszynski J, Wieckowski MR. Interactions between the endoplasmic reticulum, mitochondria, plasma membrane and other subcellular organelles. Internat J Biochem Cell Biol 2009;41:1805–6.

Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. Proc Natl Acad Sci USA 2006;103:18992–7.

Luik RM, Wang B, Prakriya M, Wu MM, Lewis RS. Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. Nature 2008;454:538–42.

Luik RM, Wu MM, Buchanan J, Lewis RS. The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. J Cell Biol 2006;174:815–25.

Malli R, Frieden M, Trenker M, Graier WF. The role of mitochondria for Ca^{2+} refilling of the endoplasmic reticulum. J Biol Chem 2005;280:12114–22.

Marsh BJ, Mastronarde DN, Buttle KF, Howell KE, McIntosh JR. Organellar relationships in the Golgi region of the pancreatic beta cell line, HIT-T15, visualized by high resolution electron tomography. PNAS 2001;98:2399–406.

Parekh AB, Putney Jr JW. Store-operated calcium channels. Physiol Rev 2005;85:757–810.

Patterson RL, van Rossum DB, Gill DL. Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. Cell 1999;98:487–99.

Peirce MJ, Cope AP, Wait R. Proteomic analysis of the lymphocyte plasma membrane using cell surface biotinylation and solution-phase isoelectric focusing. Methods Mol Biol 2009;528:135–40.

Pichler H, Gaigg B, Hrastnik C, Achleitner G, Kohlwein SD, Zellnig G, et al. A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. Eur J Biochem 2001;268:2351–61.

Prinz WA, Grzyb L, Veenhuis M, Kahana JA, Silver PA, Rapoport TA. Mutants affecting the structure of the cortical endoplasmic reticulum in *Saccharomyces cerevisiae*. J Cell Biol 2000;150:461–74.

Quintana A, Schwarz EC, Schwindling C, Lipp P, Kaestner L, Hoth M. Sustained activity of calcium release-activated calcium channels requires translocation of mitochondria to the plasma membrane. J Biol Chem 2006;28:40302–9.

Rapizzi E, Pinton P, Szabadkai G, Wieckowski MR, Vandecasteele G, Baird G, et al. Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca^{2+} microdomains to mitochondria. J Cell Biol 2002;159:613–24.

Rizzuto R, Carrington W, Tuft RA. Digital imaging microscopy of living cells. Trends Cell Biol 1998a;8:288–92.

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science 1998b;280:1763–6.

Roos J, Di Gregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, et al. STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 2005;169:435–45.

Schägger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991;199:223–31.

Serrano R. H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol 1988;157:533–44.

Shiao YJ, Lupo G, Vance JE. Evidence that phosphatidylserine is imported into mitochondria *via* a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. J Biol Chem 1995;270:11190–8.

Spassova MA, Hewavitharana T, Xu W, Soboloff J, Gill DL. A common mechanism underlies stretch activation and receptor activation of TRPC6 channels. Proc Natl Acad Sci USA 2006;103:16586–91.

Szabadkai G, Bianchi K, Varnai P, De Stefani D, Wieckowski MR, Cavagna D, et al. Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca^{2+} channels. J Cell Biol 2006;175:901–11.

Szabadkai G, Simoni AM, Chami M, Wieckowski MR, Youle RJ, Rizzuto R. Drp-1 dependent division of the mitochondrial network blocks intraorganellar Ca^{2+} waves and protects against Ca^{2+} mediated apoptosis. Mol Cell 2004;16:59–68.

Vance JE, Stone SJ, Faust JR. Abnormalities in mitochondria-associated membranes and phospholipid biosynthetic enzymes in the mnd/mnd mouse model of neuronal ceroid lipofuscinosis. Biochim Biophys Acta 1997;1344:286–99.

Vance JE. Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem 1990;265:7248–56.

Venkatachalam K, van Rossum DB, Patterson RL, Ma HT, Gill DL. The cellular and molecular basis of store-operated calcium entry. Nat Cell Biol 2002;4:E263–272.

Wang Y, Deng X, Hewavitharana T, Soboloff J, Gill DL, Stim ORAI. TRPC channels in the control of calcium entry signals in smooth muscle. Clin Exp Pharmacol Physiol 2008;35:1127–33.

Wieckowski MR, Szabadkai G, Wasilewski M, Pinton P, Duszynski J, Rizzuto R. Overexpression of adenine nucleotide translocase reduces Ca^{2+} signal transmission between the ER and mitochondria. Biochem Biophys Res Commun 2006;348:393–9.

Wu MM, Buchanan J, Luik RM, Lewis RS. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J Cell Biol 2006;174:803–13.

Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, et al. STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 2005;437:902–5.