Published in final edited form as:

J Mol Cell Cardiol. 2011 April ; 50(4): 621–633. doi:10.1016/j.yjmcc.2010.12.016.

SERCA2a controls the mode of agonist-induced intracellular Ca^{2+} signal, transcription factor NFAT and proliferation in human vascular smooth muscle cells

Regis Bobe^a, Lahouaria Hadri^{b,*}, Jose J. Lopez^{a,*}, Yassine Sassi^c, Fabrice Atassi^c, Ioannis Karakikes^b, Lifan Liang^b, Isabelle Limon^d, Anne-Marie Lompré^c, Stephane N. Hatem^c, Roger J. Hajjar^b, and Larissa Lipskaia^{b,c}

^a INSERM U770, CHU Bicêtre, Le Kremlin-Bicêtre, 94276, France

^b Mount Sinai School of Medicine, Department of Cardiology, New York, NY 10029-6574, USA

^c INSERM UMRS 956, UPMC-Paris 6, Paris, 75013 France

^d Univ Paris 06, UR4, Paris, France

Abstract

In blood vessels, tone is maintained by agonist-induced cytosolic Ca^{2+} oscillations of quiescent/contractile vascular smooth muscle cells (VSMCs). However, in synthetic/proliferative VSMCs, Gq/phosphoinositide receptor-coupled agonists trigger a steady-state increase in cytosolic Ca^{2+} followed by a Store Operated Calcium Entry (SOCE) which translates into activation of the proliferation-associated transcription factor NFAT. Here, we report that in human coronary artery smooth muscle cells (hCASCs), the sarco/endoplasmic reticulum calcium ATPase type 2a (SERCA2a) expressed in the contractile form of the hCASCs, controls the nature of the agonist-induced Ca^{2+} transient and the resulting down-stream signaling pathway. Indeed, restoring SERCA2a expression by gene transfer in synthetic hCASCs 1) increased Ca^{2+} storage capacity; 2) modified agonist-induced IP_3R Ca^{2+} release from steady-state to oscillatory mode (the frequency of agonist-induced IP_3R Ca^{2+} signal was $11.66 \pm 1.40/100$ sec in SERCA2a-expressing cells ($n=39$) vs $1.37 \pm 0.20/100$ sec in control cell ($n=45$), $p<0.01$); 3) suppressed SOCE by preventing interactions between SR calcium sensor STIM1 and pore forming unit ORAI1; 4) inhibited calcium regulated transcription factor NFAT and its down-stream physiological function such as proliferation and migration.

This study provides evidence for the first time that oscillatory and steady-state patterns of Ca^{2+} transients have different effects on calcium-dependent physiological functions in smooth muscle cells.

Correspondence to L. Lipskaia: Mount Sinai School of Medicine, Atran Laboratory Building, One Gustave L. Levy Place, Box 1030, New York, NY 10029-6574, USA. Tel: 212-241-5737; Fax: 212-241-4080; larissa.lipskaia@mssm.edu.

*LH & JLL contributed equally to this work.

SERCA2b differs from SERCA2a by an extension of 46 amino acids that forms an additional transmembrane domain setting the C terminus of SERCA2b in the SR lumen [41].

Disclosures

None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

SERCA; NFAT; proliferation; signal transduction; calcium oscillations; store-operated calcium entry

Introduction

The primary function of vascular smooth muscle cells (VSMCs) in mature vessels is to control the vascular tone [1]. In differentiated VSMCs, contraction is triggered by entry of the Ca^{2+} through voltage-dependent L-type Ca^{2+} channels (LTCC) [2]. However, VSMCs maintain also considerable plasticity throughout life and can exhibit a diverse range of phenotypes in response to changes in local environment [3]. During vascular pathologies including atherosclerosis and post-angioplasty restenosis, VSMCs transit towards a synthetic/proliferating status characterized by the down-regulation of contractile proteins [3] as well as proteins regulating the excitation-contraction coupling process. These include the L-type Ca^{2+} channels [4,5], the sarco(endo)plasmic reticulum (SR/ER) Ca^{2+} channel, the ryanodine receptor 2 (RyR2) and the sarco(endo)plasmic reticulum calcium ATPase type 2a (SERCA2a) [6–9]. Interestingly, the synthetic/proliferating status of VSMCs are also associated with an up-regulation of certain molecular entities, particularly those interfering directly or indirectly with the plasma membrane-localized Ca^{2+} release-activated Ca^{2+} channel (CRAC)¹ [10,11]; we refer to the inositol-1,4,5-triphosphate (IP_3) receptor (IP_3R), proteins form the CRAC complex and in turn regulate the I_{CRAC} (such as the pore forming units ORAI1-3 and the SR/ER sensor of $[\text{Ca}^{2+}]_i$ - stromal interaction molecule 1 (STIM1) [12,13]). Similar observations have been made in the transient receptor potential protein C (TRPC) 1/3/4/5/6, involved in the formation of multi-protein complexes responsible for store-operated Ca^{2+} entry (SOCE) [12–14]. These data suggested a change in calcium handling in synthetic/proliferating VSMCs.

$\text{IP}_3/\text{Ca}^{2+}$ signaling pathway leading to VSMC proliferation translates into the transcription factor NFAT (standing for nuclear factor of activated T-lymphocytes) translocation to the nucleus [6,7] and its subsequent activation. This occurs through its dephosphorylation mediated by the Ca^{2+} /calmodulin-activated phosphatase PP2B (calcineurin) induced by a low steady-state increase in cytosolic Ca^{2+} [15] and allows the NFAT control of cell-cycle-related proteins such as Cyclin D1, Cyclin D2, c-myc and pRb, required for the passage of G1/S checkpoint [6,16]). Consistent with NFAT involvement in VSMC proliferation, *in vitro* disruption of NFAT signaling pathway either by silencing STIM1, ORAI1 or TRPC1 or by expressing the NFAT competing peptide VIVIT, inhibits this cell response [6,17–19]. Because SERCA2a gene transfer inhibited VSMC proliferation *in vitro* and prevented restenosis in animal models in a very similar way to what has been observed using VIVIT or shSTIM gene transfer [6,16,20,21], a role for SERCA2a in control of NFAT has been suggested. We previously reported that, in rat VSMCs, SERCA2a inhibits NFAT transcriptional activity preventing the formation of active PP2B/calmodulin complex required for NFAT dephosphorylation [6]. However, how SERCA2a modifies Ca^{2+} homeostasis to specifically inhibit proliferation-associated PP2B/NFAT signaling pathway remained largely unknown.

Here, we provide evidence that SERCA2a increases the rate of Ca^{2+} store refilling, maintaining a high SR Ca^{2+} concentration. Furthermore, we demonstrated that SERCA2a inhibits the activation of STIM1/ORAI1 dependent SOCE and the downstream PP2B/NFAT

¹The CRAC is known to be responsible for the 2h cytosolic Ca^{2+} increase required to induce proliferation [10].

signaling pathway by modifying the agonist-induced intracellular Ca^{2+} transient from steady-state to an oscillatory mode.

Materials and Methods

Human samples

Fragments of left anterior descending coronary artery were dissected from human explanted hearts. The artery segments were immediately immersed in physiological saline solution, placed at 4°C and used within a few hours.

Materials

The following primary antibodies were used: IID8 (sc-53010, Santa Cruz Biotechnology), anti-SERCA2a and anti-SERCA2b [22], anti-RyR2 [23], anti-non-muscular myosin heavy chain β (NM-B) (Ab 684, Abcam), anti-smooth muscle myosin heavy chain (MHC) (M3558, Dako Cytomation), anti-Cyclin D1 (556470, BD Biosciences), anti-PP2B (calcineurin, 556350, BD Biosciences), anti-STIM1 (ACC-63, Alomone labs), anti-Orai2 (ACC-061, Alomone labs), anti-ORAI1 (ACC-60, Alomone lab), anti-ORAI1 (sc-68895), anti-Cav1.2 calcium channel (L-type Ca^{2+} channel α_{1C} subunit) (75053, NeuroMab); anti-h-calponin (C2687, Sigma-Aldrich), anti-caldesmon (C4562, Sigma-Aldrich); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-47424, Santa Cruz Biotechnology).

Adenovirus

The following adenovirus were used: Ad-S2a, encoding human SERCA2a and green fluorescence protein (GFP) under cytomegalovirus (CMV) promoter [24]; Ad- β Gal, encoding β -galactosidase and GFP under CMV promoter [24]; Ad-VIVIT, encoding NFAT competing peptide VIVIT and GFP under CMV promoter [25,26]; AdNFAT-GFP, encoding human cDNA for NFATc1 fused to GFP under CMV promoter (Seven Hill Bioreagent, JMAAd-98). Cells were infected with adenovirus at 1 to 10 pfu/cell. The efficacy of infection was controlled by GFP fluorescence.

Confocal microscopy

Immunocytochemistry was performed on acetone-fixed sections according to a standard protocol. Slides were examined with a Leica TCS4D confocal scanning laser microscope equipped with a 25 mW argon laser and a 1 mW helium-neon laser, using a Plan Apochromat 63X objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505–550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.4 μm along the z-axis. All settings were kept constant to allow comparison. For double immunofluorescence, dual excitation using the multitrack mode (images taken sequentially) was achieved using the argon and He/Ne lasers.

Culture of hCASCs

Human Coronary Artery Smooth Muscle Cells (hCASCs) were isolated from the medial layer of coronary by enzymatic digestion. After dissection, the fragments of media were incubated in SMCBM2 medium (Promocell) with collagenase (CLS2, 50 U/mL, Worthington) and pancreatic elastase (0.25 mg/mL, Sigma) for 4–6 h at 37°C. After periods of 30 min, the suspension was centrifuged at 1000 rpm for 3 min, and the cells were collected and placed in SMCBM2 + 20% Supplement Mix (SM). The cells obtained in the first 30 min period were discarded. Those obtained in the other cycles were pooled and

cultured in SMCBM2 containing SM (5%) and antibiotics at 37°C and 5% of CO₂. Cells were used between passages 2 to 8. *Proliferation* was measured by BrdU incorporation during 24 or 48 h using Cell Proliferation ELISA, BrdU (colorimetric) assay kit (Roche) or by using the CellTiter96® Cell Proliferation Assay kit (Promega), according to manufacture instructions. *Migration* of hCASCs was assessed using a micro Boyden Chamber QCM™ 24-Well Colorimetric Cell Migration Assay (ECDM 508, Chemicon International). Briefly, different concentrations of serum medium were added to the lower chamber of the apparatus. Cells were infected for 3 days with virus and then serum-starved and spread to the upper chamber (10⁵/300 µl). The Transwell chambers were then incubated in a humidified incubator with 5% CO₂ for 18 h. After incubation, the inserts were incubated with cell stain solution for 20 min and rinsed with water and swabbed with a cotton swab to remove non-migrated cells. Subsequently migrated cells were extracted and detected on a microplate reader at 560 nm by colorimetric assay. All experiments were performed in triplicate and expressed as the percentages of βGal infected cells. For *NFAT-reporter gene assay*, cells were transfected with NFAT-promoter-luciferase construct by electroporation using Basic Nucleofector® Kit Prim. Smooth Muscle Cells (Amara). The luciferase activity was measured by using “the luciferase assay kit” (Promega) and normalized to total protein. It was expressed as percent of control in relative luciferase units (RLU).

Co-immunoprecipitation and Western blot

Total cell lysates were prepared according to a standard protocol (Upstate) and were separated by SDS-PAGE to perform Western blot analysis. Proteins were visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). For co-immunoprecipitation total lysates were incubated with prewashed protein A-agarose beads (50 µl, Sigma Aldrich Corp. St. Louis, MO) for 1 h, prior to incubation with primary antibody anti-STIM1 antibody (5µg/ml, Alomone labs) overnight at 4°C with gentle shaking. Prewashed agarose beads were further incubated with lysate/antibody mixture for 1–2 h and subsequently washed three times in ice-cold washing buffer. Proteins were resolved by 7.5 % SDS-PAGE and subsequent Western blot analysis.

Measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]_i)

Cells were loaded with 2µM Fura-2-AM for 45 min at 37°C and kept in serum free medium for 30 min before the experiment. HEPES buffer (in mmol/L: 116 NaCl, 5.6 KCl, 1.2 MgCl₂, 5 NaHCO₃, 1 NaH₂PO₄, 20 HEPES pH 7.4) was used for the experiments. Single images of fluorescent emission at 510 nm under excitation at 340 and 380 nm were taken every 5 sec. To record Ca²⁺ oscillations, single images of fluorescent emission at 510 nm under single excitation at 380 nm were recorded at the rate of 7 images per second. Changes in [Ca²⁺]_i in response to the indicated agonist were calculated using the Fura-2 340/380 fluorescence ratio according to the equation of Grynkiewicz or using the ratio 380em/380em (basal).

Dynamic measurements of NFAT-GFP subcellular distribution

Cells were infected with Ad-NFATc1-GFP adenovirus for 6 h in the presence of serum. Then cells were cultured 12 h in virus-free and serum-free medium (synchronized in G₁). To visualize NFAT-GFP, cells were excited at 488 nm and cellular emission was recorded between 500nm and 520 nm. Subcellular distribution of NFAT-GFP was quantified as the ratio NFAT_{NUC}/NFAT_{CYT} using a region of interest (ROI) that covered the area of the nucleus (NFAT_{NUC}) and a cytoplasmic ROI (NFAT_{CYT}) as described [27]. The average fluorescence of a particular ROI was analysed using Metamorph. The individual cellular ratio NFAT_{NUC}/NFAT_{CYT} was measured every 10 sec.

Quantitative real-time PCR

Total RNA was prepared with RNeasy Mini kits (Invitrogen), and 1 µg was reverse transcribed using a standard protocol. Gene specific primers were used to amplify mRNA by quantitative PCR on an Mx3005 apparatus (Stratagen) using Qiagen SYBR Green Master Mix using the following conditions: 95 °C for 15 min and 40 cycles, each at 94°C for 30 sec, 60°C for 30 sec, and 72° for 30 sec. The following human forward and reverse primer sequences were used for real-time PCR analysis: SERCA2a: 5'-GTTTCGGTTGCGTGCATGTGCG-3' and 5'-ATGTGGCGACTTGGCTGACGG-3'; SERCA2b: 5'-TCATCTTCCAGATCACACCGC-3' and 5'-TCAAGACCAGAACATATCGC-3'; TRPC1: 5'-GATGCATTCCATCCTACACT-3' and 5'-TACACAGTCCTTCTGCTCCT-3'; TRPC4: 5'-GGACTTCAGGACTACATCCA-3' and 5'-ACGCAGAGAACTGAAGATGT-3'; TRPC5: 5'-CCGCAAGGAGGTGGTAGG-3' and 5'-TGTGATGTCTGGTGTGAACTC-3'; ORAI1: 5'-GACTGGATCGGCCAGAGTTA-3' and 5'-CACTGAAGGCGATGAGCAG-3'; ORAI2: 5'-GTCACCTCTAACCACCACTCG-3' and 5'-CGGGTACTGGTACTGCGTCT-3'; ORAI3: 5'-GCACGTCTGCCTTGCTCT-3' and 5'-GAGGTTGTGGATGTTGCTCA-3'; STIM1: 5'-GTGGAAGAAAGTGATGAGTTCCT-3' and 5'-TGTGATCAGCCACTGTACCA-3'; and RPL32 5'-GCCCCAAGATCGTCAAAAAGA-3' and 5'-GTCAATGCCTCTGGGTTT-3'.

Statistical analysis

All quantitative data are presented as mean of at least 3 independent experiments ± SEM. One-way ANOVA tests were performed for multiple comparisons of values. Statistical comparisons of 2 groups were done by an unpaired Student's t-test. Differences were considered significant when $P < 0.05$.

Results

1. SR Ca^{2+} handling proteins expression in SMCs from healthy coronary arteries

Two distinct populations of SMCs have been described in human normal and pathological coronary arteries (CA): the “contractile” (differentiated) and “synthetic” (proliferative) [28]. These populations of VSMCs are respectively present in the media and the subendothelial intima². To identify whether in human coronary arteries SERCA2a expression is associated with specific SMC phenotype, healthy segments of CA obtained from 5 patients with dilated cardiomyopathy were studied. Adventitia (a), media (m) and subendothelial intima (si) were identified on cross sections by hematoxylin/eosin staining (Fig. 1A) and elastin autofluorescence (Fig. 1B). The non-muscular myosin heavy chain B, NM-B, was visualized in the media and the subendothelial layers; the smooth muscle mynosin heavy chain (MHC), a marker of terminal differentiation [3], was only detected in the media. Thus, SMCs from the media presented a “contractile” phenotype whereas those of the subendothelial space displayed a “synthetic/proliferative” phenotype. Consistent with this, the intima exhibited a considerable amount of Cyclin D1-positive cells (Fig. 1B). Of note: Cyclin D1 expression in subendothelial intima was heterogeneous along the arteries, with zones of high expression and zones of low expression, in accordance with previous observations [32]. RyR2 and SERCA2a were both expressed in medial contractile SMCs but no positive labeling could be detected in the subendothelial intima; conversely, the ubiquitous SERCA2b isoform was present in both types of SMCs. As attested by the expression of contractile SMC markers [3], such as MHC, h-calponin and caldesmon, freshly isolated medial hCAsMCs displayed a differentiated phenotype (Fig. S1A&B); they also expressed SERCA2a, SERCA2b and

²Of note: the subendothelial SMCs present even in normal human coronary arteries are believed the most likely source of intimal growth in atherosclerosis, restenosis, and bypass graft intima hyperplasia [29, 30]; normal human coronary arteries contain a subendothelial intima, composed of 5 to 10 layers of SMCs and extracellular matrix [31].

RyR2 (Fig. S1A&B). Noteworthy, no difference between SERCA2a and SERCA2b subcellular localization was detected by confocal microscopy (Fig. S1A). When cultured in the presence of serum, hCASCs both proliferated - as attested by BrdU incorporation (not shown) and the emerging expression of Cyclin D1 - and dedifferentiated as illustrated by the loss of MHC, h-calponin and caldesmon (Fig. S1A&B). Consistent with previous observations made for rat VSMCs [4,5], the expression of L-type Ca^{2+} channel α_{1C} subunit dramatically decreased (Fig. S1B). Moreover, the expression of PP2B increased in keeping with the activation of PP2B/NFAT signaling [7]. Real-time-PCR analysis revealed a similar pattern of expression of SERCA2a and SERCA2b when compared to that visualized on coronary artery segments. Indeed, SERCA2a mRNA expression significantly diminishes in proliferating hCASCs comparing to coronary artery hCASCs, whereas that of SERCA2b remains unchanged (Fig. 1C). Similar observations could be made when examining SERCA protein expression (Fig. 1D, 2A, S1). Of note: total SERCA2 protein (IID8) was not significantly modified.

2. SERCA2a controls hCASC proliferation and migration *via* the inhibition of PP2B/NFAT signaling pathway

To assess the role of SERCA2a in controlling proliferation and migration, we studied the consequences of restoring its expression in synthetic hCASCs on PP2B/NFAT signaling pathway and cyclin D1 expression. Cells were transduced by means of an adenovirus encoding SERCA2a (Ad-S2a); importantly the infection was pursued until obtaining a detectable level of terminally differentiated human SMCs in coronary arteries (Fig. 2A). Adenovirus encoding β -galactosidase, (Ad- β Gal) (used as control) altered neither the expression of SERCA2a nor that of SERCA2b (Fig. 2A). Moreover, Ad- β Gal did not alter the proliferation of these cells (BrdU incorporation: 100.00 ± 4.48 , in control *vs* 84.83 ± 4.38 , in Ad- β Gal infected cells, ns). Hence, Ad- β Gal infected cells were used as a baseline in these experiments (Fig. 2B–E).

As shown figure 2B, the expression of cyclin D1 was decreased in SERCA2a infected cells while that of PP2B remains unaltered. Adenovirus delivering the NFAT competing peptide VIVIT³ provided similar results (Fig. 2B). NFAT-reporter assay showed that NFAT transcriptional activity was blocked in both SERCA2a infected and VIVIT infected cells compared to β -Gal infected cells (Fig. 2C). As expected, NFAT-dependent physiological functions, proliferation and migration induced by serum, were significantly inhibited in SERCA2a and VIVIT-infected cells when compared to β -Gal infected cells (Fig. 2D, E). These data suggested that in hCASCs SERCA2a controls NFAT-dependent proliferation and migration *via* inhibition of PP2B and are consistent with previously reported results obtained in rats aortic SMC [6].

3. PP2B/NFAT signaling pathway is controlled by both SR Ca^{2+} release and influx of extracellular SOCE Ca^{2+}

To identify the source of calcium regulating PP2B/NFAT signaling pathway, we analyzed the effects of various Ca^{2+} channel inhibitors on hCASC proliferation and NFAT-luciferase activity induced by serum. As shown Fig. 3A&B, Diltiazem (Dil), a specific L-type Ca^{2+} channel inhibitor did not have any effect either on cell proliferation or on NFAT activity. This was consistent with the down-regulation of LTCC in synthetic hCASCs. In contrast, carboxyamidotriazole (CAI) and 2- aminoethoxydiphenyl borate (2-APB), two inhibitors of SOCE, inhibited both NFAT-luciferase activity and cell proliferation similarly to the PP2B inhibitor cyclosporine A (CsA), used here as a control (Fig. 3A&B). Of note: SOCE inhibitors were effective only at 50 μM , a concentration which is supposed to inhibit

³Synthetic peptide VIVIT interacts with PP2B in competition with NFAT [25,26].

IP₃R Ca²⁺ release and SOCE [33, 34]. Serum-induced proliferation translated into a long lasting increase in cytosolic Ca²⁺ followed by an increase of cytosolic Ca²⁺ basal levels as recorded by the use of a fluorescent calcium probe FURA-2 (Fig. 3C). In the absence of extracellular Ca²⁺, the [Ca²⁺]_i increase was systematically lower (Fig. 3D); addition of extracellular Ca²⁺ results in a rise of cytosolic [Ca²⁺]_i related to extracellular Ca²⁺ influx. Same results were obtained using the Gq/phosphoinositide receptor-coupled agonist thrombin (THR) (Fig. 3E&F) except that cytological [Ca²⁺]_i seems to return to the basal level after stimulation. Of note: thrombin has been previously shown to induce NFAT activation and proliferation of VSMCs [35]. These data indicated that, in synthetic hCASCs, the serum-induced steady-state calcium signal activating NFAT-luciferase activity and cell proliferation consisted of SR Ca²⁺ release and extracellular Ca²⁺ influx. Next, the identification of the calcium signal required for PP2B/NFAT activation was evaluated by discriminating calcium origin buffering extracellular calcium with EGTA (100 μM) or by adding Ca²⁺ in the cell media. Here, PP2B/NFAT activation was measured by calculating NFAT_{NUC}/NFAT_{CYT} ratio as an index of NFAT nuclear translocation. This was performed in cells stimulated either by serum or by thrombin, using a NFAT-GFP fusion protein in the presence of extracellular calcium. The NFAT_{NUC}/NFAT_{CYT} ratio was arbitrary set to 1.00 for each cell at the beginning of the recording. As displayed in (Fig. 4A.) the NFAT_{NUC}/NFAT_{CYT} ratio reached 1.99 ± 0.20 (n=26), within 4 min after the addition of serum; a slight increase could also be noticed until the end of recording (15 min). The increase of NFAT-luciferase activity was observed rapidly 30 min after stimulation (data not shown). Although the rate of translocation was lower (NFAT_{NUC}/NFAT_{CYT} ratio reached 1.58 ± 0.09 (n=19)), similar data were obtained when the cells were incubated with thrombin: (Fig. 4C). In the absence of extracellular Ca²⁺ (EGTA 100 μM), stimulation with serum (Fig. 4B) or thrombin (Fig. 4D) caused IP₃R mediated Ca²⁺ release from SR and rapid (within 3 min) NFAT1c-GFP nuclear translocation. Indeed, NFAT_{NUC}/NFAT_{CYT} ratios were increased up to 1.33 ± 0.04 (n=36), and 1.54 ± 0.05 (n=101), respectively. When influx of extracellular Ca²⁺ was activated (by adding Ca²⁺ in the extracellular medium), a second spurt of NFAT nuclear accumulation was recorded in both cases (NFAT_{NUC}/NFAT_{CYT}: with serum, 2.02 ± 0.16 , n=36; with thrombin, 1.94 ± 0.08 , n=101, these data were calculated 3 min after extracellular calcium addition (Fig. 4B&D). Fig 4E shows images of the NFAT intracellular localization at different time points of figure 4D. Of note: in non-stimulated cells, NFATc1-GFP was preferentially localized in the cytoplasm (Fig. 4E). Thrombin was added in the absence of extracellular Ca²⁺ at 200 sec time point; at the 250 sec time point, NFAT-GFP nuclear accumulation was already clearly observed (Fig. 4E). These data demonstrated that, in synthetic hCASCs, NFAT is activated by a steady-state increase in cytosolic Ca²⁺ arising from SR Ca²⁺ release and SOC-mediated extracellular Ca²⁺ influx. However, SR Ca²⁺ release was sufficient to initiate NFAT nuclear translocation and SOCE-induced Ca²⁺ rise enhanced the activation of PP2B/NFAT signaling pathway.

4. Effect of SERCA2a expression on calcium handling and Store Operated Calcium Entry in hCASCs

In order to study the effects of SERCA2a on the serum-induced calcium homeostasis, we examined Ca²⁺ transient in SERCA2a-infected synthetic cells and compared it to βGal infected cells. In synthetic βGal infected cells, the serum induced a steady-state intracellular Ca²⁺ increase followed by a sustained increase of basal Ca²⁺ level in presence of extracellular Ca²⁺ (Fig. 5A&B). This was similar to that observed in synthetic non-infected cells (Fig. 3A). In SERCA2a expressing cells, the serum triggered persistent and rapid oscillations of cytosolic Ca²⁺ without any increase in basal Ca²⁺ level (Fig 5C&D). When removing extracellular calcium (by EGTA) in βGal-infected cells, the serum induced a steady-state increase of intracellular calcium corresponding to the IP₃R-induced Ca²⁺

release (Fig. 5E&F); conversely, in SERCA2a expressing cells, the serum-induced IP₃R Ca²⁺ release occurred as rapid oscillations of cytosolic Ca²⁺ (Fig. 5G&H). When extracellular calcium was applied, a rise of calcium illustrating SOCE was observed in βGal- but not in SERCA2a-expressing cells (Fig. 5E–H). The absence of SOCE could not be due to impaired SOC function; indeed, when SERCA pumps were inhibited by thapsigargin⁴ full SOCE was observed whether in βGal- or in SERCA2a- expressing cells (Fig 5I&J). Thapsigargin-induced Ca²⁺ mobilization from SR was higher in SERCA2a expressing-cells as compared to βGal expressing cells ([Ca²⁺]_i, nM: 443.40 ± 52.24, n=72 vs 282.80 ± 27.28, n=62, P=0.01). Besides guaranteeing the functionality of the calcium pump, this illustrated an increase of Ca²⁺ storage capacity in SERCA2a- expressing cells.

When experiments were performed with thrombin, similar results were obtained (Fig. 6A&B, video 1S online supplement). The rapid recording revealed that SERCA2a expressing cells effectively mobilized intracellular Ca²⁺ in absence of extracellular calcium. The pattern of thrombin-induced Ca²⁺ transients was different in control cells (a) than in Ad-S2a infected cells (b) (Fig. 6B); indeed, thrombin induced rapid oscillations of intracellular Ca²⁺ only in SERCA2a expressing cells (Fig. 6B&D). The frequency of calcium peak was 11.66 ± 1.40/100 sec in SERCA2a-expressing cells (n=39) vs 1.37 ± 0.20/100 sec in control cells (n=45), p<0.01. Addition of extracellular Ca²⁺ failed to induce SOCE in more than 90% of the cell population (Fig. 6C&D). Interestingly, when thapsigargin was added 3 min after thrombin stimulation, the quantity of Ca²⁺ that was re-mobilized from intracellular store, was significantly higher in SERCA2a expressing hCASCs than in control cells (Fig. 6E). This observation suggested that, in SERCA2a expressing cells, the calcium released from SR during the thrombin stimulation was rapidly recaptured without any loss in the extracellular medium and further ruled out that extracellular calcium is necessary to refill calcium store in SERCA2a- expressing cells.

Altogether, these data demonstrated first that SERCA2a modifies the mode of agonist induced IP₃R calcium release and prevents SOCE. Furthermore it reveals that the absence of SOC response in these cells is clearly due to the activity of the SERCA2a proteins since SOCE was observed in SERCA2a expressing hCASCs when the Ca²⁺ pumps were inhibited with thapsigargin. The difference observed between responses to Tg and thrombin indicated that the use of SERCA inhibitors can only provide information concerning the possible existence of SOCE but cannot be relevant to what really happens in cells in which all SERCA activity is maintained.

5. SERCA2a prevents the formation of STIM1/ORAI complex in cultured hCASCs

Finally, we investigated the effect of SERCA2a on the different SOC sub-unit expression and association. As shown in Fig. 7A, the increase of SERCA2a expression (evidenced by real-time PCR as ~ 100-fold, data not shown) did not modify the mRNA levels regardless SOC sub-units (ORAI1/2/3, TRPC1/4/5 and STIM1). Same data were obtained for STIM1, ORAI1 and ORAI2 when examining their protein levels (Fig. 7B). By performing co-immunoprecipitation studies with an anti-STIM1 antibody, we demonstrated an interaction between STIM1 and ORAI1 or ORAI2, in proliferating non-infected or βGal-infected hCASCs. Both interactions were strongly inhibited in SERCA2a- infected cells (Fig. 7C). Since STIM1/ORAI1 complex was identified as an essential component of the *I_{CRAC}*, required for proliferation and migration of VSMCs [13], these results demonstrated that SERCA2a expression prevented SOCE activation in hCASCs *via* inhibition of STIM1/ORAI association.

⁴Thapsigargin (Tg) is a common SERCA (calcium pump) inhibitor

Figure 8 combines the data obtained and summarized them. More specifically, it evidences that the loss of SERCA2a in proliferating SMCs result in lesser Ca^{2+} uptake which translate into a peripheral STIM1 relocalization leading to functional association of STIM1-ORAI1 complex and activation of SOCE. In presence of SERCA2a, Ca^{2+} depletion of SR is not sufficient or not long enough to induce STIM1 delocalization. In support of that, the spontaneous interactions between STIM1 and/ ORAI1/2 are reduced in SERCA2a-expressing hCAsMCs.

Discussion

In this study we demonstrated that increasing the Ca^{2+} luminal loading of the SR by restoring of SERCA2a expression in synthetic SMCs was sufficient to modify the nature of agonist-induced Ca^{2+} transient. Indeed, SERCA2a forced expression transforms the steady-state SR Ca^{2+} release into an oscillatory signal, characteristic of contractile vascular SMCs [36].

SERCA2a expression in synthetic SMCs modifying the mode of agonist-induced Ca^{2+} transient matches results obtained in human endothelial cells showing that, increasing store loading by SERCA2a gene transfer increased the frequency of histamine-induced oscillations [37]. Moreover, it is consistent with Berridge's model (referred as "store loading model of calcium oscillations") in which the speed with which the SR internal store is loaded plays a critical role in Ca^{2+} oscillations frequency; by setting the sensitivity of the IP_3R , determining timing of the next Ca^{2+} spike [10,38–40]. The fact that the addition of thapsigargin after thrombin stimulation produced a larger response in SERCA2a expressing cells, indicating that in these cells the concentration of luminal Ca^{2+} was higher than in cells lacking SERCA2a, also reinforced the luminal loading mechanism. Whether SERCA2b⁵ could also participate in the Ca^{2+} oscillations could be considered as a possibility. It is, however, unlikely since SERCA2a has a higher catalytic turnover due to a higher rate of dephosphorylation and a lower affinity to Ca^{2+} [42,43]. In addition, SERCA2b, in contrast to SERCA2a isoform, is ubiquitous [44].

Our study also shows that serum or thrombin-induced persistent oscillations could occur in SERCA2a expressing synthetic SMCs without any extracellular calcium, in absence of SOCE and STIM1/ORAI1 complex. These data highlight that Ca^{2+} oscillations can persist without extracellular Ca^{2+} influx in a closed system based solely on lumen/cytosol Ca^{2+} turnover, challenging the idea as to whether extracellular Ca^{2+} influx is absolutely required for refilling the stores between each oscillatory cycle [10].

Several studies have reported that the extracellular Ca^{2+} influx is required for transcriptional activation of NFAT [45]. Based on our measurements of NFAT-GFP subcellular localization clearly demonstrating that the initial IP_3 -induced Ca^{2+} release from SR intracellular store is sufficient to induce NFAT nuclear translocation, we now suggest that Ca^{2+} influx through plasma membrane channels acts as an enhancer -rather as an inductor- of NFAT mobilization. This mechanism is further reinforced by the fact that modifying the mode of IP_3R -induced Ca^{2+} transient expressing SERCA2a prevented NFAT activation.

In our study we found that diltiazem had no effect on NFAT signaling pathway in hCAsMCs. Our results are different than those of Nieves-Cintrón and coworkers who showed that diltiazem blocked PP2B/ NFAT signaling through the inhibition of persistent calcium sparklets in mouse and rat arterial myocytes. In contractile arterial SMC, persistent Ca^{2+} sparklets refer to sustained Ca^{2+} influx and are mediated by clusters of L-type Ca^{2+} channels operating in a high open probability mode. In fact persistent Ca^{2+} sparklet activity is required for activation of PP2B/NFAT signaling in contractile SMCs

[46–49]. Considering that hCAsMCs, as opposed to the rodent arterial VSMCs, display low level of L-type Ca^{2+} channels and that, the major route of extracellular calcium influx is the SOCs, this may explain the differing results result sin our study.

In addition to modifying the intracellular agonist-induced Ca^{2+} transient, the rescue of SERCA2a expression in synthetic hCAsMCs disrupted functional association of STIM1/ORAI1 and that of STIM1/ORAI2. SERCA2a control of ORAI isoform activity is consistent with SERCA2a regulating STIM function. Though it is generally admitted that STIM1/ORAI complex is responsible for SOCE in VSMCs [13], the functional properties of STIM1/ORAI2 protein complex remains controversial. Potier *et al.*, (2009) reported that silencing of either STIM1 or ORAI1 in synthetic VSMCs greatly reduced SOCE, whereas that of ORAI2, ORAI3, had no effect [13]; however, Mercer *et al.*, (2006) showed that the co-expression of ORAI1 or ORAI2 in combination with STIM1 resulted in substantial increase in I_{CRAC} , ORAI3 failing to produce any detectable Ca^{2+} selective currents [50]. Additional experiments would be needed to clarify the role of STIM1/ORAI2 association in synthetic hCAsMCs

In conclusion, we have demonstrated that SERCA2a is involved in the frequency dependence of intracellular Ca^{2+} signaling which leads to the control of SOCE and NFAT pathways and eventually in the proliferation of VSMCs. This study is, to our knowledge, the first evidence for oscillatory and steady-state increases in cytosolic calcium having different effects on calcium dependent signaling processes in muscle cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Michael J. Berridge (The Babraham Institute, Cambridge, UK) for helpful discussion, Bruno Constantine (University of Poitiers, France) for critical reading of this manuscript, Susan Kraner and Christopher M. Norris (Sanders-Brown Center on Aging, Lexington, KY-USA) for providing AdVIVIT, Frank Wuytack (University of Leuven, Belgium) for the anti-SERCA2a and anti-SERCA2b antibodies.

Sources of Funding

This work is supported by AHA SDG 0930116N (LL);, by NIH R01 HL080498, & HL083156 (RJH), by Leducq Foundation through the Caerus network (05 CVD 03, AML and RJH); the Association Française Contre les Myopathies, AFM (RB); by MEC-FEDER BFU2010-C02-01 (RB and JJJ), by K01 HL1031176-01 (LH); JJJ was supported by a postdoctoral fellowship from the Junta de Extremadura (POS0922).

References

1. Wamhoff BR, Bowles DK, Owens GK. Excitation-transcription coupling in arterial smooth muscle. *Circulation research*. 2006 Apr 14; 98(7):868–78. [PubMed: 16614312]
2. House SJ, Potier M, Bissaillon J, Singer HA, Trebak M. The non-excitabile smooth muscle: calcium signaling and phenotypic switching during vascular disease. *Pflugers Arch*. 2008 Aug; 456(5):769–85. [PubMed: 18365243]
3. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev*. 2004 Jul; 84(3):767–801. [PubMed: 15269336]
4. Gollasch M, Haase H, Ried C, Lindschau C, Morano I, Luft FC, et al. L-type calcium channel expression depends on the differentiated state of vascular smooth muscle cells. *Faseb J*. 1998 May; 12(7):593–601. [PubMed: 9576486]

5. Quignard JF, Harricane MC, Menard C, Lory P, Nargeot J, Capron L, et al. Transient down-regulation of L-type Ca^{2+} channel and dystrophin expression after balloon injury in rat aortic cells. *Cardiovasc Res*. 2001 Jan; 49(1):177–88. [PubMed: 11121810]
6. Lipskaia L, del Monte F, Capiod T, Yacoubi S, Hadri L, Hours M, et al. Sarco/endoplasmic reticulum Ca^{2+} -ATPase gene transfer reduces vascular smooth muscle cell proliferation and neointima formation in the rat. *Circulation research*. 2005 Sep 2; 97(5):488–95. [PubMed: 16081870]
7. Lipskaia L, Pourci ML, Delomenie C, Combettes L, Goudouneche D, Paul JL, et al. Phosphatidylinositol 3-kinase and calcium-activated transcription pathways are required for VLDL-induced smooth muscle cell proliferation. *Circulation research*. 2003 May 30; 92(10):1115–22. [PubMed: 12730091]
8. Massaeli H, Austria JA, Pierce GN. Lesions in ryanodine channels in smooth muscle cells exposed to oxidized low density lipoprotein. *Arterioscler Thromb Vasc Biol*. 2000 Feb; 20(2):328–34. [PubMed: 10669627]
9. Lipskaia L, Pinet C, Fromes Y, Hatem S, Cantaloube I, Coulombe A, et al. Mutation of delta-sarcoglycan is associated with Ca^{2+} -dependent vascular remodeling in the Syrian hamster. *Am J Pathol*. 2007 Jul; 171(1):162–71. [PubMed: 17591963]
10. Berridge MJ. Inositol trisphosphate and calcium signalling mechanisms. *Biochimica et biophysica acta*. 2009 Jun; 1793(6):933–40. [PubMed: 19010359]
11. Lewis RS. The molecular choreography of a store-operated calcium channel. *Nature*. 2007 Mar 15; 446(7133):284–7. [PubMed: 17361175]
12. Berra-Romani R, Mazzocco-Spezia A, Pulina MV, Golovina VA. Ca^{2+} handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. *American journal of physiology*. 2008 Sep; 295(3):C779–90. [PubMed: 18596214]
13. Potier M, Gonzalez JC, Motiani RK, Abdullaev IF, Bisailon JM, Singer HA, et al. Evidence for STIM1- and Orai1-dependent store-operated calcium influx through ICRCAC in vascular smooth muscle cells: role in proliferation and migration. *Faseb J*. 2009 Aug; 23(8):2425–37. [PubMed: 19364762]
14. Albert AP, Saleh SN, Peppiatt-Wildman CM, Large WA. Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells. *The Journal of physiology*. 2007 Aug 15; 583(Pt 1):25–36. [PubMed: 17615095]
15. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature*. 1997 Apr 24; 386(6627):855–8. [PubMed: 9126747]
16. Liu Z, Zhang C, Dronadula N, Li Q, Rao GN. Blockade of nuclear factor of activated T cells activation signaling suppresses balloon injury-induced neointima formation in a rat carotid artery model. *The Journal of biological chemistry*. 2005 Apr 15; 280(15):14700–8. [PubMed: 15681847]
17. Peel SE, Liu B, Hall IP. ORAI and store-operated calcium influx in human airway smooth muscle cells. *Am J Respir Cell Mol Biol*. 2008 Jun; 38(6):744–9. [PubMed: 18239188]
18. Takahashi Y, Watanabe H, Murakami M, Ono K, Munehisa Y, Koyama T, et al. Functional role of stromal interaction molecule 1 (STIM1) in vascular smooth muscle cells. *Biochem Biophys Res Commun*. 2007 Oct 5; 361(4):934–40. [PubMed: 17689489]
19. Sweeney M, Yu Y, Platoshyn O, Zhang S, McDaniel SS, Yuan JX. Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. *Am J Physiol Lung Cell Mol Physiol*. 2002 Jul; 283(1):L144–55. [PubMed: 12060571]
20. Yu H, Sliedregt-Bol K, Overkleeft H, van der Marel GA, van Berkel TJ, Biessen EA. Therapeutic potential of a synthetic peptide inhibitor of nuclear factor of activated T cells as antirestenotic agent. *Arterioscler Thromb Vasc Biol*. 2006 Jul; 26(7):1531–7. [PubMed: 16675727]
21. Aubart FC, Sassi Y, Coulombe A, Mougenot N, Vrignaud C, Leprince P, et al. RNA interference targeting STIM1 suppresses vascular smooth muscle cell proliferation and neointima formation in the rat. *Mol Ther*. 2009 Mar; 17(3):455–62. [PubMed: 19107116]

22. Eggermont JA, Wuytack F, Verbist J, Casteels R. Expression of endoplasmic-reticulum Ca²⁺(+)-pump isoforms and of phospholamban in pig smooth-muscle tissues. *Biochem J.* 1990 Nov 1; 271(3):649–53. [PubMed: 2244871]
23. Marty I, Robert M, Villaz M, De Jongh K, Lai Y, Catterall WA, et al. Biochemical evidence for a complex involving dihydropyridine receptor and ryanodine receptor in triad junctions of skeletal muscle. *Proc Natl Acad Sci U S A.* 1994 Mar 15; 91(6):2270–4. [PubMed: 8134386]
24. del Monte F, Harding SE, Schmidt U, Matsui T, Kang ZB, Dec GW, et al. Restoration of contractile function in isolated cardiomyocytes from failing human hearts by gene transfer of SERCA2a. *Circulation.* 1999 Dec 7; 100(23):2308–11. [PubMed: 10587333]
25. Aramburu J, Garcia-Cozar F, Raghavan A, Okamura H, Rao A, Hogan PG. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. *Mol Cell.* 1998 Apr; 1(5):627–37. [PubMed: 9660947]
26. Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science (New York, NY).* 1999 Sep 24; 285(5436):2129–33.
27. Rinne A, Banach K, Blatter LA. Regulation of nuclear factor of activated T cells (NFAT) in vascular endothelial cells. *Journal of molecular and cellular cardiology.* 2009 Sep; 47(3):400–10. [PubMed: 19540841]
28. Hao H, Gabbiani G, Bochaton-Piallat ML. Arterial smooth muscle cell heterogeneity: implications for atherosclerosis and restenosis development. *Arterioscler Thromb Vasc Biol.* 2003 Sep 1; 23(9):1510–20. [PubMed: 12907463]
29. Schwartz SM, deBlois D, O'Brien ER. The intima. Soil for atherosclerosis and restenosis. *Circulation research.* 1995 Sep; 77(3):445–65. [PubMed: 7641318]
30. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W Jr, Richardson M, et al. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb.* 1992 Jan; 12(1):120–34. [PubMed: 1731855]
31. Reikhter MD, Simari RD, Work CW, Nabel GJ, Nabel EG, Gordon D. Gene transfer into normal and atherosclerotic human blood vessels. *Circulation research.* 1998 Jun 29; 82(12):1243–52. [PubMed: 9648720]
32. Gueguen M, Keuylian Z, Mateo V, Mougnot N, Lompre AM, Michel JB, et al. Implication of adenylyl cyclase 8 in pathological smooth muscle cell migration occurring in rat and human vascular remodelling. *The Journal of pathology.* Jul; 221(3):331–42. [PubMed: 20527026]
33. Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM. 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca²⁺ entry but an inconsistent inhibitor of InsP₃-induced Ca²⁺ release. *Faseb J.* 2002 Aug; 16(10):1145–50. [PubMed: 12153982]
34. Faehling M, Kroll J, Fohr KJ, Fellbrich G, Mayr U, Trischler G, et al. Essential role of calcium in vascular endothelial growth factor A-induced signaling: mechanism of the antiangiogenic effect of carboxyamidotriazole. *Faseb J.* 2002 Nov; 16(13):1805–7. [PubMed: 12354692]
35. Yellaturu CR, Ghosh SK, Rao RK, Jennings LK, Hassid A, Rao GN. A potential role for nuclear factor of activated T-cells in receptor tyrosine kinase and G-protein-coupled receptor agonist-induced cell proliferation. *Biochem J.* 2002 Nov 15; 368(Pt 1):183–90. [PubMed: 12188924]
36. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *The Journal of physiology.* 2008 Nov 1; 586(Pt 21):5047–61. [PubMed: 18787034]
37. Hadri L, Bobe R, Kawase Y, Ladage D, Ishikawa K, Atassi F, et al. SERCA2a gene transfer enhances eNOS expression and activity in endothelial cells. *Mol Ther.* Jul; 18(7):284–92.
38. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature.* 1993 Jan 28; 361(6410):315–25. [PubMed: 8381210]
39. Berridge, MJ. Inositol trisphosphate and calcium oscillations. *Biochemical Society symposium;* 2007. p. 1-7.
40. Berridge MJ, Dupont G. Spatial and temporal signalling by calcium. *Curr Opin Cell Biol.* 1994 Apr; 6(2):267–74. [PubMed: 7517689]

41. Campbell AM, Kessler PD, Fambrough DM. The alternative carboxyl termini of avian cardiac and brain sarcoplasmic reticulum/endoplasmic reticulum Ca^{2+} -ATPases are on opposite sides of the membrane. *The Journal of biological chemistry*. 1992 May 5; 267(13):9321–5. [PubMed: 1533629]
42. Dode L, Andersen JP, Leslie N, Dhitavat J, Vilsen B, Hovnanian A. Dissection of the functional differences between sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) 1 and 2 isoforms and characterization of Darier disease (SERCA2) mutants by steady-state and transient kinetic analyses. *The Journal of biological chemistry*. 2003 Nov 28; 278(48):47877–89. [PubMed: 12975374]
43. Dally S, Bredoux R, Corvazier E, Andersen JP, Clausen JD, Dode L, et al. Ca^{2+} -ATPases in non-failing and failing heart: evidence for a novel cardiac sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 isoform (SERCA2c). *Biochem J*. 2006 Apr 15; 395(2):249–58. [PubMed: 16402920]
44. Bobe R, Bredoux R, Corvazier E, Lacabartz-Porret C, Martin V, Kovacs T, et al. How many Ca^{2+} -ATPase isoforms are expressed in a cell type? A growing family of membrane proteins illustrated by studies in platelets. *Platelets*. 2005 May-Jun; 16(3–4):33–50.
45. Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A. Signalling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. *Cell calcium*. 2007 Aug; 42(2):145–56. [PubMed: 17572487]
46. Navedo MF, Amberg GC, Nieves M, Molkentin JD, Santana LF. Mechanisms underlying heterogeneous Ca^{2+} sparklet activity in arterial smooth muscle. *J Gen Physiol*. 2006 Jun; 127(6): 611–22. [PubMed: 16702354]
47. Nieves-Cintrón M, Amberg GC, Navedo MF, Molkentin JD, Santana LF. The control of Ca^{2+} influx and NFATc3 signaling in arterial smooth muscle during hypertension. *Proc Natl Acad Sci U S A*. 2008 Oct 7; 105(40):15623–8. [PubMed: 18832165]
48. Amberg GC, Bonev AD, Rossow CF, Nelson MT, Santana LF. Modulation of the molecular composition of large conductance, Ca^{2+} activated K^{+} channels in vascular smooth muscle during hypertension. *The Journal of clinical investigation*. 2003 Sep; 112(5):717–24. [PubMed: 12952920]
49. Wellman GC, Santana LF, Bonev AD, Nelson MT. Role of phospholamban in the modulation of arterial Ca^{2+} sparks and Ca^{2+} -activated K^{+} channels by cAMP. *American journal of physiology*. 2001 Sep; 281(3):C1029–37. [PubMed: 11502581]
50. Mercer JC, Dehaven WI, Smyth JT, Wedel B, Boyles RR, Bird GS, et al. Large store-operated calcium selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *The Journal of biological chemistry*. 2006 Aug 25; 281(34):24979–90. [PubMed: 16807233]

Figure 1. Characterization of SMCs of healthy human coronary arteries (CA)

A. Representative haematoxylin/eosin staining of human CA cross- sections. a - adventitia, m - media, si - subendothelial intima, ec - endothelial cells, iel - internal elastic lamina.

B. Confocal immunofluorescence (red) of human CA cross sections. Antibodies used are given in experimental procedure. Abbreviations antibodies MHC – anti-smooth muscle myosin heavy chain 1 and 2; NM-B, anti- non-muscular myosin heavy chain B; RyR2, anti-Ryanodine Receptor isoform 2; SERCA2a or SERCA2b, anti-sarco/endoplasmic reticulum calcium ATPase 2a or 2b; IID8, pan anti-SERCA2 (a and b); GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase.

Green - elastin autofluorescence. Abbreviations for the different compartments of the vessel wall are the same as that of mentioned in A.

C. Quantitative real-time PCR of SERCA2a and 2b mRNA expression in human CA and cultured hCASCs. Values represent the mean of values obtained from 3 donors. Levels of mRNA are normalized to the value obtained in coronary arteries.

D. Western blot of SERCA2 isoform expression in freshly dissociated and cultured hCASCs. Total protein extracts (50µg) were loaded. Upper panel shows a representative immunoblot; lower panel: histograms showing the relative ratio of SERCA normalized to GAPDH in three independent experiments.

Figure 2. SERCA2a prevents SMC proliferation and migration *via* the inhibition of the Ca^{2+} -regulated transcription factor NFAT

A. Left panel: representative immunoblot of SERCA2 isoform expression in freshly dissociated and cultured hCASCs infected or not during 4 days with Ad-βGal or Ad-S2a. Total protein extracts (50μg) were loaded. **Right panel:** histograms showing the relative ratio of SERCA normalized to GAPDH. Values represent the mean of three independent experiments.

B. Left panel: representative immunoblot of PP2B and Cyclin D1 expression in cultured hCASCs infected during 4 days with Ad-βGal, Ad-S2a or AdVIVIT. Total protein extracts (50μg) were loaded. **Right panel:** histograms showing the relative ratio of PP2B and Cyclin D1 normalized to GAPDH. Values represent the mean of three independent experiments.

C. Promoter-reporter assay of NFAT transcriptional activity. 24 h after NFAT-Luc plasmid transfection, cells were infected with adenovirus during 48h and NFAT activity was induced by adding 5% of serum in the cell culture medium. Data are expressed in relative luciferase units (RLU) as a percentage of value in Ad-βGal infected cells.

D. Effect on hCASC proliferation. Cells were infected with the above mentioned virus for 48h and then cultured for 48h in virus-free BrdU containing medium supplemented with serum (5%). Bars represent the mean of BrdU incorporation of 8 independent experiments performed on SMCs from 4 donors in triplicate. Data are expressed as a percentage of value in Ad-βGal infected cells.

E. Effect on hCASMCM migration. Cells were infected for 3 days with the above mentioned virus and spread on the upper chamber of transwell apparatus (Chemicon International). Migration was induced by addition of serum (5%) in the medium of the lower chamber for 18h. Values are plotted as the percentage of change with respect to control (Ad- β Gal, 0% S). Bars represent mean $\pm$ SEM of at least 3 experiments in triplicate.

Figure 3. Analysis of Ca^{2+} signal required for induction of proliferation in synthetic hCASCs
 Effect of various Ca^{2+} channels blockers on serum-induced proliferation (**A**) and NFAT-Luc activity (**B**) of hCASCs. Abbreviations used are: Dil - diltiazem, CAI - carboxyamidotriazole, 2APB - 2-aminoethoxydiphenyl borate, CsA - cyclosporine A. Concentrations used are indicated the figure. For proliferation assay, cells were cultured in presence of BrdU and different drugs during 48h. For NFAT activity assay, cells were transfected with NFAT-Luc plasmid, cultured 24h in serum-free medium and were stimulated by adding 5% serum and different drugs during 4h. Data (A&B) are expressed as a percentage of value issued of control wells (0% serum). Bars represent mean $\pm$ SEM of at least 3 experiments in triplicate. *** $P < 0.001$; * $P < 0.05$ vs 5% S.

C–F. Intracellular calcium imaging in FURA-2 loaded cells. Typical traces representative of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) recorded in single cell. Cells were synchronized in G_1 phase of cell cycle by removing of serum from culture medium for 24 h before experiments. Cells were treated with serum (**C**) or thrombin (THR, 1U/ml) (**E**) in the presence of extracellular Ca^{2+} (300 μM). To record SOCE activation, cells were treated with 5% serum (**D**) (the Ca^{2+} present in the serum was buffered with 2mM EGTA) extracellular Ca^{2+} or THR (1U/ml) (**F**) in the absence of calcium (EGTA 100 μM) and then (Ca^{2+} 300 μM) was then added (**D&F**).

Figure 4. Involvement of SR Ca^{2+} release and SOCE in the synthetic hCASM C NFAT activation
A–D. NFATc1-GFP infected cells were cultured during 12h in serum-free medium before monitoring of NFATc1-GFP nuclear translocation induced by 5% of serum (**A&B**) or 1U/ml of thrombin (**C&D**) in the presence (**A&C**) or absence (**B&D**) of extracellular Ca^{2+} (2 mM), to discriminate between SR Ca^{2+} release and SOCE, as described in Fig. 3.

E. The images corresponding to D curve at the indicated time are presented in pseudocolor reflecting the fluorescence intensity increase (blue < green < yellow < red < white). Thrombin was added at 200 sec in the presence of EGTA (100 μ M); extracellular Ca^{2+} was added at 1200 sec.

Figure 5. SERCA2a alters intracellular Ca^{2+} handling in hCASCs

$[\text{Ca}^{2+}]_i$ imaging was recorded in FURA-2 loaded cells representative of 3 experiments obtained with 3 independent infections. HCASCs were infected with Ad- β Gal (**A, B, E, F**) or Ad-S2a (**C, D, G, H**) for 2 days and then cultured 24h in virus-free and serum-free medium before recording. Fluorescence intensity was only recorded in response to one excitation wavelength (380 nm) in order to increase the acquisition rate up to 7 images per second. **Left panels:** traces of the mean of several cell recording; **right panels:** traces of individual cell recording. **A–D:** record of global Ca^{2+} signal in cells treated with 5% of serum (S) and extracellular Ca^{2+} (300 μM , CaCl_2). **E–H:** record of SR Ca^{2+} release and SOCE activation in cells treated with 5% serum buffered with 2mM EGTA in absence of extracellular calcium (EGTA 100 μM); extracellular Ca^{2+} (CaCl_2 , 300 μM) was then added at the indicated time.

I. HCASCs were infected with Ad- β Gal or Ad-S2a for 2 days and then cultured 24h in virus-free and serum-free medium before recording. Cells were treated in the absence of extracellular Ca^{2+} (EGTA 100 μM) with thapsigargin (Tg, 1 μM); then extracellular Ca^{2+} (CaCl_2 , 300 μM) was added.

J. Histograms showing $[\text{Ca}^{2+}]_i$ SOCE peak (means $\pm$ SEM) observed after the addition of extracellular Ca^{2+} (CaCl_2 , 300 μM) to cells either treated with thapsigargin (1 μM) alone or with thapsigargin (1 μM) and ionomycin (Iono, 50nM).

Figure 6. SERCA2a modifies the nature of thrombin-induced Ca^{2+} transient and inhibits SOCE

A. Ad-S2a-infected cells were identified by GFP fluorescence (**upper**); FURA-2 fluorescence (**lower**) was similar in both infected and non-infected cells. Two areas were monitored for FURA-2 fluorescence recording: (a) - for non-infected cells and (b) - for SERCA2a-infected cells.

B. Typical traces (representative of the $[Ca^{2+}]_i$) recorded in single non-infected (a) or infected cell (b). Cells were treated, in the absence of calcium (EGTA, 100 μ M), with 1U/ml of thrombin and Ca^{2+} ($CaCl_2$, 300 μ M). In order to detect $[Ca^{2+}]_i$ oscillations, fluorescence intensity was only recorded in response to one excitation wavelength (380 nm) as in Fig. 5. The full recording is presented as Online supplement Video.

C. Bar graphs comparing the $[Ca^{2+}]_i$ peak corresponding to SOCE (means $\pm$ SEM) recorded when extracellular Ca^{2+} (300 μ M) was added after stimulation with thrombin (1U/mL) of hCASMCs infected or not with ad-S2a or ad- β Gal. The data are mean $\pm$ SEM of 3 experiments (**p < 0.01 vs control).

D. Bar graphs comparing the percentage of SERCA2a-expressing to that of and control cells that displayed an oscillatory response to thrombin and a SOCE upon extracellular Ca^{2+} (300 μ M) addition. This has been recorded during 6 experiments.

E. Ca^{2+} store content after thrombin-induced Ca^{2+} response. Cells were treated, in the absence of extracellular Ca^{2+} (EGTA, 100 μ M), with 1U/ml thrombin for 3 min. Then, thapsigargin (Tg, 1 μ M) was added and the following Ca^{2+} mobilization was quantified as $\Sigma[Ca^{2+}]_i \cdot \text{time}$ (in sec)/number of measurements ($\delta[Ca^{2+}]_{Tg} \cdot s$). The data are mean $\pm$ SEM for 3 experiments (**p < 0.01 vs control).

Figure 7. SERCA2a prevents the formation of STIM-1/ORAI1 complex in cultured hCASCs
A. Effect of SERCA2a gene transfer on the expression of SOC sub-units. mRNA level quantified by real-time PCR was normalized to the value obtained in Ad-βGal-infected cells. Histograms show the means $\pm$ SEM of three experiments.
B. Cells were infected for 4 days with Ad-βGal or Ad-S2a. Total protein extracts (50μg) were loaded. **Left panel:** western blot showing the expression of ORAI1, ORAI2 and STIM1 in whole-cell lysates. **Right panel:** histograms showing the relative ratio of ORAI1, ORAI2 and STIM1 normalized to GAPDH in three experiments.
C. Whole-cell lysates were immunoprecipitated (IP) with an anti-STIM1 antibody, resolved on SDS/PAGE and immunoblotted for ORAI1 or ORAI2. Membranes were reprobed for STIM1 for protein loading control. Histograms showing the mean (n=4) relative ratio of ORAI1 (left panel) and ORAI2 (right panel) normalized to STIM1 and arbitrary considered as 100% for Ad-βGal infected cells.

Figure 8. Schematic representation of the involvement of SERCA2a in the physiological control of SOCE

GPCR - G-protein coupled receptor; PLC - phospholipase C; NFAT - nuclear factor of activated T lymphocytes; P - phosphate; IP₃ - inositol-1,4,5-trisphosphate, IP₃R - IP₃ receptor; SR/ER sarco/endoplasmic reticulum; SERCA - SE/ER Ca²⁺ATPase; STIM1 - Stromal Interaction Molecule 1, ORAI1 - the pore forming unit.