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Original article

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

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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 (hCASMCs), the sarco/endoplasmic reticulum calcium ATPase type 2a (SERCA2a) expressed in the contractile form of the hCASMCs, 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 hCASMCs 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 s in SERCA2a-expressing cells ($n = 39$) vs $1.37 \pm 0.20$/100 s in control cells ($n = 45$), $p < 0.01$); 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.



## 1. 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 is 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)[2] [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 $[Ca^{2+}]$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.

$IP_3/Ca^{2+}$ 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

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

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

## 2. Materials and methods

### 2.1. 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.

### 2.2. 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 B (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 $\alpha_{1C}$ 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).

### 2.3. Adenovirus

The following adenovirus were used: Ad-S2a, encoding human SERCA2a and green fluorescence protein (GFP) under cytomegalovirus (CMV) promoter [24]; Ad-βGal, 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, JMAd-98). Cells were infected with adenovirus at 1 to10 pfu/cell. The efficacy of infection was controlled by GFP fluorescence.

### 2.4. 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 63× 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.

### 2.5. Culture of hCASMCs

Human coronary artery smooth muscle cells (hCASMCs) 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 $CO_2$. Cells were used between passages 2 and 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 hCASMCs 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 ($10^5$/300 μl). The Transwell chambers were then incubated in a humidified incubator with 5% $CO_2$ 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 (Amaxa). 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).

### 2.6. 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 (5 μg/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.

### 2.7. Measurement of intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]i$)

Cells were loaded with 2 μM 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 $MgCl_2$, 5 $NaHCO_3$, 1 $NaH_2PO_4$, 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 s. To record $Ca^{2+}$ oscillations, single images of