Orai1, a critical component of store-operated Ca\textsuperscript{2+} entry, is functionally associated with Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and plasma membrane Ca\textsuperscript{2+} pump in proliferating human arterial myocytes

Sergey G. Baryshnikov,* Maria V. Pulina,* Alessandra Zulian, Cristina I. Linde, and Vera A. Golovina

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

Submitted 30 June 2009; accepted in final form 7 August 2009

Baryshnikov SG, Pulina MV, Zulian A, Linde CI, Golovina VA. Orai1, a critical component of store-operated Ca\textsuperscript{2+} entry, is functionally associated with Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and plasma membrane Ca\textsuperscript{2+} pump in proliferating human arterial myocytes. Am J Physiol Cell Physiol 297: C1103–C1112, 2009. First published August 12, 2009; doi:10.1152/ajpcell.00283.2009.—Ca\textsuperscript{2+} entry through store-operated channels (SOCs) in the plasma membrane plays an important role in regulation of vascular smooth muscle contraction, tone, and cell proliferation. The C-type transient receptor potential (TRPC) channels have been proposed as major candidates for SOCs in vascular smooth muscle. Recently, two families of transmembrane proteins, Orai [also known as Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel modulator (CRACM)] and stromal interacting molecule 1 (STIM1), were shown to be essential for the activation of SOCs mainly in nonexcitable cells. Here, using small interfering RNA, we show that Orai1 plays an essential role in activating store-operated Ca\textsuperscript{2+} entry (SOCE) in primary cultured proliferating human aortic smooth muscle cells (hASMCs), whereas Orai2 and Orai3 do not contribute to SOCE. Knockdown of Orai1 protein expression significantly attenuated SOCE. Moreover, inhibition of Orai1 downregulated expression of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger type 1 (NCX1) and plasma membrane Ca\textsuperscript{2+} pump isoform 1 (PMCA1). The rate of cytosolic free Ca\textsuperscript{2+} concentration decay after Ca\textsuperscript{2+} entry into plasma membrane Ca\textsuperscript{2+}-free medium was also greatly decreased under these conditions. This reduction of Ca\textsuperscript{2+} extrusion, presumably via NCX1 and PMCA1, may be a compensation for the reduced SOCE. Immunocytochemical observations indicate that Orai1 and NCX1 are clustered in plasma membrane microdomains. Cell proliferation was attenuated in hASMCs with disrupted Orai1 expression and reduced SOCE. Thus Orai1 appears to be a critical component of SOCE in proliferating vascular smooth muscle cells, and may therefore be a key player during vascular growth and remodeling.

C-type transient receptor potential proteins; sarcoplasmic reticulum Ca\textsuperscript{2+} stores; proliferation

STORE-OPERATED CA\textsuperscript{2+} ENTRY (SOCE) plays an important role in shaping cytoplasmic Ca\textsuperscript{2+} signals in a variety of cell types including vascular smooth muscle cells (SMCs) (2, 6, 9, 17, 21, 58). There is accumulating evidence that Ca\textsuperscript{2+} entry through store-operated channels (SOCs) in the plasma membrane (PM) may be involved in regulating vascular smooth muscle contraction, tone, and cell proliferation (2, 9, 17, 19, 34, 40, 57). Despite extensive research, the molecular identity of SOCs remains controversial (3, 7). Numerous reports indicate that C-type transient receptor potential (TRPC) proteins, mammalian homologs of the Drosophila transient receptor potential (trp) channel, are important components of SOCs in vascular SMCs (3, 7, 12, 33, 50). In particular, TRPC1, TRPC4, and TRPC5 may form, or be part of, the SOCs activated by sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} store depletion (7, 42, 62, 63). In contrast to these SOCs, there is a related class of receptor-operated Ca\textsuperscript{2+} channels (ROCs), composed of other TRPC proteins, TRPC3/6/7 (23, 35, 45). These channels are activated by diacylglycerols in a store depletion-independent manner (25, 35). Nevertheless, both SOCs (TRPC1/4/5) and ROCs (TRPC3/6/7) have important functions in vascular smooth muscle; they participate in hyperplasia, remodeling, and the regulation of arterial blood pressure (5, 8, 13, 16, 32, 60, 65).

One of the first store depletion-activated channels identified was the Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channel in mast cells (27). Recently, two families of transmembrane proteins, Orai [also known as CRAC channel modulator (CRACM)] and stromal interacting molecule 1 (STIM1), were shown to be essential for the activation of SOCs mainly in nonexcitable cells (15, 28, 51, 59, 64). The role of Orai1 in SOCE was also confirmed in human airway SMCs (44) and in rat “synthetic” aortic myocytes (47). Orai1 may form the Ca\textsuperscript{2+} selectivity filter of the CRAC channel (64), which may be another type of SOC (42). A point mutation in the gene encoding Orai1 results in defects in T lymphocyte function and severe immunodeficiency in humans (15). There are two other potential homologs of Orai1 in the mammalian genome, Orai2 and Orai3 (15). Orai2 may also constitute or contribute to SOCs (39) but not in all tested cells (22, 24). The role of Orai3 in SOCE is less clear (11, 24). Orai3, however, can rescue SOCE when Orai1 is knocked down in HEK-293 cells (39).

Recently, we demonstrated (9) that expression of each of the three members of the Orai family in homogenates of human aorta is negligible. All Orai proteins are, however, readily detected in cultured, proliferating human aortic smooth muscle cells (hASMCs) (9). STIM1, the putative Ca\textsuperscript{2+} sensor in the SR, regulates SOCs and CRAC channels (28, 43, 56). STIM1 and Orai1 may interact with TRPC proteins (28, 41); the dynamic assembly of a TRPC1-STIM1-Orai1 ternary complex is involved in SOC activation in human salivary glands (41). Although the role of Orai proteins has been extensively investigated in T lymphocytes, mast cells, and various heterologous expression systems, there is no evidence to date that Orai proteins play a role in SOCE in human vascular smooth muscle.

Here, using fura-2 imaging, RNA interference, and Western blot analysis, we demonstrate that Orai1 is an essential component of SOCs in human primary cultured proliferating aortic smooth muscle cells (hASMCs). In contrast, Orai2 and Orai3 do not contribute to SOCE. Moreover, Orai1 is functionally

* S. G. Baryshnikov and M. V. Pulina contributed equally to this work.

Address for reprint requests and other correspondence: V. A. Golovina, Dept. of Physiology, Univ. of Maryland School of Medicine, 685 W. Baltimore St., HSF1, Rm. 565, Baltimore, MD 21201 (e-mail: v golovin@umaryland.edu).
associated with Na\textsuperscript+/Ca\textsuperscript{2+} exchanger type 1 (NCX1) and PMCA\textsubscript{1}.

**MATERIALS AND METHODS**

**Primary cultured ASMCs.** Primary human aortic myocytes were purchased from Lonza Walkersville (Walkersville, MD). Cells were cultured in smooth muscle basal medium (SmBM) containing 5% fetal bovine serum (FBS) at 37\(^\circ\)C in a humidified atmosphere of 5% CO\textsubscript{2}. hASMCs from passages 2–4 were used for the experiments. Cells were plated on 25-mm glass coverslips for use in fluorescent microscopy experiments, on coverslips with a lettered grid for counting, or on 100-mm cell dishes for biochemical experiments. The medium was changed on days 3 and 6. Experiments were performed on subconfluent cultures on days 6–7 in vitro.

**Calcium imaging.** Cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) was measured with fura-2 by digital imaging. Details of fluorescence imaging and analysis techniques are published elsewhere (9). Primary cultured ASMCs were loaded with fura-2 by incubation for 35 min in culture medium containing 3.3 \mu M fura-2 AM (20–22\(^\circ\)C, 5% CO\textsubscript{2}, 95% O\textsubscript{2}). After dye loading, the coverslips were transferred to a tissue chamber mounted on a microscope stage, where cells were superfused for 15–20 min (35–36\(^\circ\)C) with physiological salt solution (PSS) to wash away extracellular dye. The PSS contained (in mM) 140 NaCl, 5.0 KCl, 1.2 Na\textsubscript{2}HPO\textsubscript{4}, 5 NaHCO\textsubscript{3}, 1.4 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 11.5 glucose, and 10 HEPES (pH 7.4). In Ca\textsuperscript{2+}-free PSS, CaCl\textsubscript{2} was replaced by equimolar MgCl\textsubscript{2} and 50 \mu M EGTA was added to chelate residual Ca\textsuperscript{2+}. In low Na\textsuperscript{+} PSS, NaCl was replaced by equimolar N-methyl-D-glucamine (NMDG\textsuperscript{+}). Cells were studied for 40–60 min during continuous superfusion with PSS (35\(^\circ\)C).

The imaging system included a Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY). The dye-loaded cells were illuminated with a diffraction grating-based system (Polychrome V, TILL Photonics). Fluorescent images were recorded with a CoolSnap HQ2 charge-coupled device (CCD) camera (Photometrics, Tucson, AZ). Image acquisition and analysis were performed with a MetaFluor/MetaMorph Imaging System (Molecular Devices, Downingtown, PA). [Ca\textsuperscript{2+}]\textsubscript{cyt} was calculated by determining the ratio of fura-2 fluorescence excited at 380 and 360 nm as described previously (9, 19).

**Immunoblot analysis.** Membrane proteins were solubilized in sodium dodecyl sulfate (SDS) buffer containing 5% 2-mercaptoethanol and were separated by polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (9). The following antibodies were used: rabbit polyclonal anti-Orai1 and anti-Orai2 (Allomone Laboratories, Jerusalem, Israel), rabbit polyclonal anti-Orai3 (Sorbie, Powy, CA), monoclonal anti-NCX1 (clone R5F1; Swant, Bellinzona, Switzerland), polyclonal anti-PMCA1 (Affinity Bioreagents, Rockford, IL), rabbit monoclonal anti-TRPC1 (Epitomics, Burlingame, CA), rabbit polyclonal anti-TRPC4 (Allomone Laboratories), and mouse monoclonal anti-TRPC5 (Abnova, Taipei, Taiwan). Gel loading was controlled with monoclonal or polyclonal anti-\beta-actin antibodies ( Sigma-Aldrich, St. Louis, MO) or monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Abcam, Cambridge, MA). After being washed, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 h at room temperature. The immune complexes on the membranes were detected by Enhanced Chemiluminescence-Plus (Amersham Biosciences) and exposure to X-ray film (Eastman Kodak, Rochester, NY). Quantitative analysis of immunoblots was performed with a Kodak DC120 digital camera and 1D Image Analysis Software (Eastman Kodak).

**Small interfering RNA knockdown.** Primary cultured hASMCs were transfected with the small interfering (si)RNA ON-Target plus Smart pool (20 \mu M) designed against Orai1, Orai2, Orai3 or siCONTROL (Dharmacon, Lafayette, CO). The sequences of the Orai1/siRNA duplexes were as follows: 5'AGACGCUAAAGAAGACGGCUU-3', 5'-CAUGACGCCGAAACAGGGCUU-3', 5'-GUUGAACGGCGCAAAUCUCU-3', and 5'-UGCAUGGUGGUCGCUU-3'. The sequences of the Orai2/siRNA duplexes were 5'-UUAGAGGUGGCAAGUUCUU-3', 5'-CAGAUGGAGCGGUACGCU-3', 5'-GUCCCCGGUAACU-3', and 5'-AGAUGGAGGCAAGGGCUU-3'. The sequences of the Orai3/siRNA duplexes were 5'-UCAUGUCCUGCU-UGUAACGU-3', 5'-CAUGAUGGCAAGAGUGCUU-3', 5'-AACCAACAGGAGCAUCCU-3', and 5'-GACUAAGGAGGAGGACU- CACU-3'. Twenty-four hours before treatment, ASMCs were placed in the culture medium (SmBM) without antibiotics and further transfected with siRNA and Lipofectamine 2000 reagent in Opti-MEM (Invitrogen). After 24-h incubation, the medium was aspirated and replaced with SmBM without siRNA for 7 h before Ca\textsuperscript{2+} measurements or Western blot analysis was performed.

**Immunocytochemistry.** hASMCs were immunolabeled as described previously (20). Briefly, cells were fixed in cyclohexylamine-formaldeyde fixative consisting of 0.45% (wt/vol) formaldehyde and (in mM) 75 cyclohexylamine, 75 NaCl, 10 EGTA, 10 MgCl\textsubscript{2}, and 10 PIPES. After fixation, the cells were permeabilized in fixative containing 0.5% polyoxyethylene 20 cetyl ether (Brij 58) and were then incubated (4–17 h) in antibody buffer containing antibodies against Orai1 (Allomone Laboratories) and NCX1 (clone R5F1, Swant). FITC-labeled donkey anti-mouse IgG or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was used to visualize the primary antibodies. The fluorescence from the secondary antibody in the absence of primary antibody (positive control) did not exceed 2–3% of the fluorescence in the presence of antiserum.

**Materials.** SmBM was purchased from Lonza (Walkersville, MD). Jurkat whole cell lysate was obtained from Abcam. Fura-2 AM was obtained from Molecular Probes (Invitrogen Detection Technologies, Eugene, OR). Cyclopiiazonic acid (CPA), dimethyl sulfoxide, and nifedipine were purchased from Sigma. All other reagents were analytic grade or the highest purity available.

**Statistical analysis.** Numerical data presented are means \pm SD from \( n \) single cells (1 value per cell). Western blot experiments were repeated at least four to six times for each protein. Data from five to seven transfections were obtained for most siRNA protocols as were consistent for all two to four passages. Statistical significance was determined by Student's t-test and ANOVA. Differences were considered to be significant when \( p < 0.05 \).

**RESULTS**

Orai1 is an essential contributor to SOCE in human primary cultured proliferating aortic cells. Proliferating hASMCs express all three Orai family members at the protein level. Western blot analysis of Orai1, Orai2, and Orai3 (Fig. 1A, 5A, and 6A) revealed prominent bands close to their predicted molecular masses of 33, 28, and 32.5 kDa, respectively (24). To determine whether Orai proteins are involved in SOCE in hASMCs, we used siRNA-mediated silencing of Orai(s). Transfection with Orai1/siRNA resulted in 82 ± 4% knockdown of Orai1 protein (Fig. 1A and B).

To examine SOCE, Ca\textsuperscript{2+} stores were depleted in the absence of extracellular Ca\textsuperscript{2+} with CPA, a specific inhibitor of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA). Then, after store depletion, Ca\textsuperscript{2+} was added back and the rise in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to SOCE was measured (Fig. 1C). In hASMCs, a large SOCE is activated when SR Ca\textsuperscript{2+} stores are depleted with 10 \mu M CPA (1,142 ± 71 nM, \( n = 52 \); not shown) (9). Cell transfection with nontargeting siRNA (siControl) (Fig. 1, C and D) or cell treatment only with the transfection reagent, Lipofectamine 2000 (not shown), did not affect SOCE. Selective inhibition of Orai1 protein expression, however, significantly attenuated the SOC-mediated rise of [Ca\textsuperscript{2+}]\textsubscript{cyt} (232 ± 15 vs. 1,084 ± 77 nM in cells treated with siControl RNA; \( n = 6 \).
Fig. 1. Knockdown of the Orai1 gene markedly reduces store-operated Ca\(^{2+}\) entry (SOCE) and human aortic smooth muscle cell (hASMC) proliferation. 

A: Western blot showing knockdown of endogenous Orai1 protein in hASMCs treated with Orai1 small interfering (si)RNA. Contr, cells treated with nontargeting siRNA. Membrane proteins (50\(\mu\)g/lane) were loaded and probed with specific anti-Orai1 antibodies. Blots were later incubated with anti-\(\beta\)-actin antibodies to verify uniform protein loading.

B: data are normalized to the amount of \(\beta\)-actin and expressed as means ± SE from 7 Western blots. *\(P < 0.001\) vs. Orai1 protein expression in control cells.

C: representative records showing the time course of cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) changes in control hASMC (siControl) and cell treated with Orai1/siRNA. Cyclopiazonic acid (CPA; 10\(\mu\)M) was applied to the cells in the absence and presence of extracellular Ca\(^{2+}\), as indicated. Nifedipine (10\(\mu\)M) was applied 10 min before the traces shown and was maintained throughout the experiment.

D: summarized data showing resting [Ca\(^{2+}\)]\(_{cyt}\), the CPA-induced transient Ca\(^{2+}\) peak in the absence of extracellular Ca\(^{2+}\), and the amplitude of SOCE in control hASMCs and cells treated with Orai1/siRNA. Data are means ± SE (\(n = 122\) cells transfected with nontargeting siRNA and \(n = 94\) cells transfected with Orai1/siRNA; 56 coverslips). *\(P < 0.001\) vs. control.

E, G, and I: Western blot analysis of Orai2 (E; 50\(\mu\)g/lane), Orai3 (G; 30\(\mu\)g/lane), and C-type transient receptor potential (TRPC)1 (I; 40\(\mu\)g/lane) protein expression in control hASMCs and cells treated with Orai1/siRNA. F, H, and J: data are normalized to the amount of \(\beta\)-actin and expressed as means ± SE from 4 (F), 6 (H), and 4 (J) Western blots. K: transfection with Orai1/siRNA inhibits hASMC proliferation. Cell numbers were determined before (Basal) and after incubation for 77 h in control growth medium (Control) or medium containing siControl or Orai1/siRNA.

Data are presented as % of control (Basal) cell number (100%) and expressed as means ± SE from 4 experiments/transfections. *\(P < 0.05\) vs. siControl.
Knockdown of Orai1 gene downregulates expression of NCX1 and PMCA1 and alters Ca\(^{2+}\) extrusion. The rate of [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay following the initial Ca\(^{2+}\) transient in Ca\(^{2+}\)-free medium was greatly reduced in cells transfected with Orai1/siRNA. Both control and siRNA-treated cells retained normal morphology, but hASMC proliferation was markedly inhibited in the Orai1/siRNA-treated group (Fig. 1K). The results indicate that Orai1 is essential for activation of arterial SOCE, and that Orai1 and SOCE play an important role in hASMC proliferation.

Taking logarithms:

\[
\ln([\text{Ca}^{2+}]_{\text{cyt}}(t)) = \ln([\text{Ca}^{2+}]_{\text{cyt}}(0)) - (t/\tau_f + t/\tau_s)
\]

where [Ca\(^{2+}\)]\(_{\text{cyt}}(0)\) is [Ca\(^{2+}\)]\(_{\text{cyt}}\) at time 0. Figure 2B shows an example of the analysis of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay in the gray boxed area in Fig. 2A in cells transfected with siControl or Orai1/siRNA. Lines in Fig. 2B are the fitted exponentials representing the fast and slow components of [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay in control hASMCs (blue) and cells transfected with Orai1/siRNA (red). The average values of \(\tau_f\) and \(\tau_s\) were significantly larger for cells transfected with Orai1/siRNA than for control cells (3.37 ± 0.56 and 15.58 ± 2.78 vs. 1.63 ± 0.17 and 10.21 ± 1.43 min, respectively; means ± SD, \(n = 20\) cells).

The slower kinetics of [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay in hASMCs with disrupted Orai1 expression may result from reduced Ca\(^{2+}\) sequestration in CPA-resistant organelles and/or decreased Ca\(^{2+}\) extrusion from the cytosol by NCX and/or PMCA. Indeed, Western blot analysis revealed that Orai1 knockdown greatly reduces the expression of NCX1 (Fig. 1, C and D) and PMCA1 (Fig. 2, E and F). Thus reduced Ca\(^{2+}\) extrusion by both NCX1 and PMCA1 can explain the significant slowing of the decline in [Ca\(^{2+}\)]\(_{\text{cyt}}\) after CPA-induced Ca\(^{2+}\) transients in Ca\(^{2+}\)-free medium in cells transfected with Orai1/siRNA (Fig. 1C, Fig. 2, A and B).

Immunocytochemistry was used to elucidate the relationship between the specific location of Orai1 and NCX1 proteins in the PM (Fig. 3). High-power images of a portion of an hASMC show that the Orai1 labeling pattern (Fig. 3A, inset) is remarkably similar to the pattern observed with antibodies directed against the NCX1 (Fig. 3B, inset). Indeed, when Fig. 3A (red) is overlaid on Fig. 3C (green), extensive overlap of the labels is observed (Fig. 3Cc), as indicated by the large amount of

\[
[A_0]_{\text{siControl}} = A_{\text{siControl}} + B_{\text{siControl}} + C_{\text{siControl}} + D_{\text{siControl}} + E_{\text{siControl}}
\]

\[
[A_0]_{\text{Orai1/siRNA}} = A_{\text{Orai1/siRNA}} + B_{\text{Orai1/siRNA}} + C_{\text{Orai1/siRNA}} + D_{\text{Orai1/siRNA}} + E_{\text{Orai1/siRNA}}
\]

where \(A_0\) represents the fast and slow time constants.

Fig. 2. Knockdown of the Orai1 gene greatly slows down [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay following CPA-induced Ca\(^{2+}\) transients in Ca\(^{2+}\)-free medium and downregulates expression of Na\(^+\)/Ca\(^{2+}\) exchanger I (NCX1) and plasma membrane Ca\(^{2+}\) pump 1 (PMCA1) in hASMCs. A: representative records showing the time course of [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes in control hASMC (siControl) and cell treated with Orai1/siRNA. The [Ca\(^{2+}\)]\(_{\text{cyt}}\) decline in the gray boxed portion was fitted to 2 exponentials (Eqs. 1 and 2), and the result of the fitting is plotted in B. B: plot of the fast (\(\tau_f\)) and slow (\(\tau_s\)) components of [Ca\(^{2+}\)]\(_{\text{cyt}}\) decay for cells treated with siControl and Orai1/siRNA. C and E: Western blot analysis of NCX1 (C; 50 µg/lane) and PMCA1 (E; 30 µg/lane) protein expression in control hASMCs and cells treated with Orai1/siRNA. D and F: data are normalized to the amount of β-actin and expressed as means ± SE from 5 (D) and 5 (F) Western blots. *P < 0.001 vs. control.

\[\text{AJP-Cell Physiol} \cdot \text{VOL 297} \cdot \text{NOVEMBER 2009} \cdot \text{www.ajpcell.org}\]
yellow in the image. Notably, reactivity was not detected in the PM in the absence of the primary anti-Orai1 (Fig. 3D) or anti-NCX1 (not shown) antibodies.

It was previously proposed that the reverse mode of NCX contributes to Ca\(^{2+}\) entry and may be involved in refilling of SR Ca\(^{2+}\) stores (36). Na\(^{+}\) entry via TRPC-related nonselective cation channels and the consequent Na\(^{+}\) accumulation in a restricted space between the PM and adjacent SR may raise the 

\[ \text{Ca}^{2+} \text{cyt} \]

value in the gray boxed area in Fig. 4A similar to the analysis shown in Fig. 2B. Lines in Fig. 4B are the fitted exponentials representing the fast and slow components of 

\[ \text{Ca}^{2+} \text{cyt} \]

decay under control conditions (140 mM [Na\(^{+}\)\(_{o}\)] and at low extracellular Na\(^{+}\) (5 mM). SOCE was not, however, significantly changed at low [Na\(^{+}\)\(_{o}\)] (Fig. 4, A and C). The results indicate that NCX1 is involved in [Na\(^{+}\)\(_{o}\)]-dependent Ca\(^{2+}\) extrusion from the cytosol but apparently does not contribute to the rise in [Ca\(^{2+}\)\(_{cyt}\)] during Ca\(^{2+}\) readmittance in cultured hASMCs.

**Orai2 and Orai3 do not contribute to SOCE in hASMCs.** Transfection with Orai2/siRNA resulted in 64 ± 4% knockdown of Orai2 protein expression (Fig. 5, A and B) without affecting Orai1 (Fig. 5, E and F) and Orai3 (Fig. 5, G and H). Moreover, selective inhibition of Orai2 did not affect the SOC-mediated rise in [Ca\(^{2+}\)\(_{cyt}\)] (1.084 ± 77 vs. 1.056 ± 60 nM in cells treated with nontargeting siRNA) (Fig. 5, C and D). The resting [Ca\(^{2+}\)\(_{cyt}\)] level and the amplitude of CPA-induced Ca\(^{2+}\) transients in Ca\(^{2+}\)-free medium also were not changed significantly under these conditions (Fig. 5D).

Cell transfection with siRNA targeted to the Orai3 gene resulted in 68 ± 7% knockdown of Orai3 protein expression (Fig. 6, A and B) without affecting expression of Orai1 (Fig. 6, E and F) or Orai2 (Fig. 6, G and H). Inhibition of Orai3 expression (Fig. 6, A and B) did not affect SOCE (1.084 ± 77 vs. 1.026 ± 125 nM in cells treated with nontargeting siRNA).

**Fig. 4. NCX does not contribute to SOCE in hASMCs.** A: representative records showing time course of [Ca\(^{2+}\)\(_{cyt}\)] changes in response to CPA (10 μM) in absence and presence of extracellular Ca\(^{2+}\). hASMCs were superfused with solutions containing 140 mM or 5 mM extracellular Na\(^{+}\) concentration ([Na\(^{+}\)\(_{o}\)]). The [Ca\(^{2+}\)\(_{cyt}\)] decline in the gray boxed area was fitted to 2 exponentials (Eqs. 1 and 2), and the result of the fitting is plotted in B. Nifedipine (10 μM) was applied 10 min before the traces shown and was maintained throughout the experiment. B: plot of \(\tau_{f}\) and \(\tau_{s}\) of [Ca\(^{2+}\)\(_{cyt}\)] decay at 140 mM [Na\(^{+}\)\(_{o}\)] and 5 mM [Na\(^{+}\)\(_{o}\)]. Values of \(\tau_{f}\) and \(\tau_{s}\) were significantly larger in cells perfused with solution containing 5 mM Na\(^{+}\) (conditions that block NCX1-mediated Ca\(^{2+}\) extrusion) than in cells bathed in control solution (140 mM Na\(^{+}\)) (2.55 and 23.81 min vs. 1.78 and 17.54 min, respectively). C: summarized data showing the amplitude of SOCE in cells bathed in 140 mM Na\(^{+}\) solution or in 5 mM Na\(^{+}\) solution (n = 44 and 48 cells, respectively; 10 coverslips).
C1108  ORAI1 IS FUNCTIONALLY ASSOCIATED WITH Na⁺/Ca²⁺ EXCHANGER

Fig. 5. Knockdown of the Orai2 gene does not affect SOCE in hASMCs. A: Western blot of Orai2 expression in hASMCs treated with siControl (Contr) and Orai2/siRNA. Membrane proteins (10 μg/lane) were loaded and probed with specific anti-Orai2 antibodies. Blots were later incubated with anti-β-actin antibodies to verify uniform protein loading. B: data are normalized to the amount of β-actin and expressed as means ± SE from 4 Western blots. *P < 0.001 vs. Orai2 protein expression in control cells. CPA (10 μM) was applied to the cells in the absence and presence of extracellular Ca²⁺, as indicated. Nifedipine (10 μM) was applied 10 min before the traces shown and was maintained throughout the experiment. C: representative records showing the time course of [Ca²⁺]cyt changes in control hASMC (siControl) and cell treated with Orai2/siRNA. CPA (10 μM) was applied to the cells in the absence and presence of extracellular Ca²⁺, as indicated. Nifedipine (10 μM) was applied 10 min before the traces shown and was maintained throughout the experiment. D: summarized data showing resting [Ca²⁺]cyt, the CPA-induced transient Ca²⁺ peak in the absence of extracellular Ca²⁺, and the amplitude of SOCE in control hASMCs (n = 88 cells) and cells treated with Orai2/siRNA (81 cells). Data are means ± SE (n = 24 coverslips). E and G: Western blot analysis of Orai1 (E; 20 μg/lane) and Orai3 (G; 50 μg/lane) protein expression in control hASMCs and cells treated with Orai2/siRNA. F and H: data are normalized to the amount of β-actin and expressed as means ± SE from 4 (F) and 4 (H) Western blots.

(Fig. 6, C and D). Resting [Ca²⁺]cyt and stored Ca²⁺ levels, evaluated by measuring peak amplitudes of CPA-induced Ca²⁺ transients in Ca²⁺-free medium, were also not changed in cells treated with Orai3/siRNA (Fig. 6, C and D). In contrast to knockdown of Orai1, inhibition of Orai2 or Orai3 did not affect proliferation of hASMCs significantly (data not shown). The results indicate that Orai2 and Orai3 do not play a role in activation of SOCE in proliferating hASMCs.

DISCUSSION

Molecular identification of SOCs is essential for studying the function of SOCE in various types of cells. Over the 20 years since the concept of “capacitative” or store-operated Ca²⁺ entry was proposed, TRPC channels have been considered the main candidates for SOCs (42). Some reports, however, indicate that the implication of TRPC channels in SOCE can vary substantially in different types of cells or under different experimental conditions (48). The recent discovery that Orai proteins are pore-forming subunits of CRAC channels in T lymphocytes and other hematopoietic cells does not, however, indicate that these proteins are involved in activation of SOCE in human vascular SMCs. Here, we provide such evidence. Using siRNAs, we show that Orai1 plays an essential role in activation of SOCE in primary cultured proliferating hASMCs, whereas Orai2 and Orai3 do not contribute to SOCE. Moreover, Orai1 is functionally associated with NCX1 and PMCA1. This conclusion is also supported by immunocytochemical observations showing that Orai1 and NCX1 proteins are localized in the PM in close proximity.

The role of Orai1 and STIM1, as critical components of SOCE, was initially discovered through genomewide RNA interference screens in Drosophila (15, 51, 59). The mammalian homolog of Orai1 is located in the PM. This homolog appears to have four transmembrane domains that form the pore of CRAC channels in hematopoietic cells and in a variety of heterologous expression systems (15, 28, 41). STIM1 is a Ca²⁺-binding protein located mainly in the endoplasmic reticulum (ER) membrane with a single transmembrane region (48,
The Ca\textsuperscript{2+}/H\textsuperscript{+}-sensing domain is an EF-hand in the NH\textsubscript{2} terminal that resides in the lumen of the ER. Evidence indicates that depletion of intracellular Ca\textsuperscript{2+}/H\textsuperscript{+} stores triggers STIM1 to translocate into defined ER-PM "junctional" areas in which coupling to Orai proteins can occur (54). Coexpression of Orai1 and STIM1 in HEK-293 cells generates Ca\textsuperscript{2+}/H\textsuperscript{+} release-activated Ca\textsuperscript{2+}/H\textsuperscript{+} current (\textit{I}_{\text{CRAC}}) and significantly increases SOCE (24, 64). Expression of Orai1 alone, however, decreases SOCE, whereas expression of STIM1 does not affect SOCE (10). STIM1 associates not only with Orai1 but also with TRPC1 (28, 41), suggesting that SOCs and CRAC channels are regulated by similar molecular components (4). The majority of functional TRPC channels are heterotetrameric complexes of different TRPC subunits (3, 7). TRPC1 associates with TRPC4 and TRPC5 to determine the PM expression and function of TRPC-containing channels (4, 18, 26). When one of the TRPC subunits is suppressed, compensatory upregulation of other subunits can be observed (13). Recent studies revealed that Orai1 mediates the interaction between STIM1 and TRPC1 and regulates activation of TRPC1-forming Ca\textsuperscript{2+} channels in human platelets (29) and in HEK-293 cells (10, 38).

SOCs in vascular SMCs and \textit{I}_{\text{CRAC}} in nonexcitable cells have striking differences in biophysical properties, permeabilities to Ca\textsuperscript{2+}, and activation mechanisms (3, 21, 37, 53). This indicates that the molecular composition of SOCs and \textit{I}_{\text{CRAC}} may be different. It has been proposed that \textit{I}_{\text{CRAC}} consist of Orai proteins, whereas SOCs may rather represent heterotetrameric TRPC structures (3). This view is supported by our observation that native arteries readily express TRPC1 and TRPC5 proteins but do not express Orai proteins (9). Expression of Orai1 and Orai3 mRNA also was not detected in mouse aorta, although very low-level expression of Orai2 mRNA was previously demonstrated (55). Therefore, SOCE in native arterial myocytes is attributable to the activity of TRPC1 and TRPC5 channels. In cultured proliferating human aortic cells, which abundantly express Orai2, Orai3, and much less Orai1 (9), the role of these proteins in SOCE can be more significant. Indeed, the present study demonstrates that Orai1 is involved in SOCE in primary cultured proliferating hASMCs. Cell transfection with Orai1/siRNA reduced by \textapprox 80\% expression of Orai1 protein (Fig. 1, A and B) and SOCE (Fig. 1, C and D). Orai2 and Orai3, however, do not contribute to SOCE in...
hASMCs (Figs. 5 and 6). Expressions of TRPC1, TRPC4, and TRPC5 proteins were not changed in hASMCs treated with Orai1/siRNA. Particularly noteworthy is the fact that inhibition of Orai1 greatly reduces the rate of [Ca^{2+}]_{cyt} decay after CPA-induced Ca^{2+} transients in Ca^{2+}-free medium (Fig. 2, A and B). The double-exponential fits of the [Ca^{2+}]_{cyt} decay data show that τ_1 was increased by approximately twofold and τ_2 by ~50% in cells transfected with Orai1/siRNA. The data are consistent with the observed approximately twofold downregulation of NCX1 and PMCA1 (Fig. 2, C–F). These are the primary mechanisms of Ca^{2+} extrusion from the cytosol after cell activation. Ultimately, [Ca^{2+}]_{cyt} is determined by the balance between Ca^{2+} influx and extrusion. In proliferating vascular SMCs, SOCE is the main pathway through which myocytes gain Ca^{2+} when SR Ca^{2+} stores are depleted (9,19).

The contribution of L-type voltage-gated Ca^{2+} channels, critical in regulating smooth muscle contraction, is markedly decreased in dedifferentiated, proliferating aortic myocytes (49). Therefore, downregulation of NCX1 and PMCA1 and the reduction of Ca^{2+} extrusion in hASMCs with inhibited Orai1 and SOCE may compensate for the reduced SOCE in order to maintain sufficient Ca^{2+} levels in the cytosol and within the SR.

It was previously reported that NCX1 operating in the Ca^{2+}-entry mode may contribute to SOCE in cultured human pulmonary artery and rat aorta myocytes and in T3-9 cells (46, 52, 67). For example, SOCE in T3-9 cells was substantially suppressed by 5 μM KB-R7943, an inhibitor of NCX (52). KB-R7943 is, however, nonselective, e.g., it also blocks currents through TRPC3/5/6 (IC_50 = 0.46–1.38 μM) (31). When experiments were performed at low (5 mM) [Na^+]_o, SOCE was much less diminished (52). Our data indicate that NCX1 is involved in [Na^+]_o-dependent Ca^{2+} extrusion from the cytosol (Fig. 4, A and B) but apparently does not contribute to the SOCE in cultured hASMCs. These differences may be explained by relative differences in the contribution of NCX to SOCE in different cell types.

The functional interaction of NCX1 and Orai1 raises the possibility of their clustering in the PM-junctional SR regions. Notably, that NCX1 (30) and TRPC channels (20) are confined to the PM microdomains that overlie the closely apposed junctional SR where STIM1 accumulates after store depletion (61). Moreover, colocalization experiments provide evidence for association of NCX1 with TRPC3 in protein complexes in HEK-293 cells (52). In the present study immunocytochemistry with anti-Orai1 and anti-NCX1 antibodies revealed the close proximity of Orai1 and NCX1 proteins in the PM (Fig. 3). These findings indicate that PM microdomains that include Orai1-containing channels and NCX1 function as integrated units that help to regulate Ca^{2+} signals in vascular SMCs.

Previously, we demonstrated (19, 21) that proliferation of human pulmonary artery SMCs is associated with enhanced SOCE. The present study shows that SOCE also plays an essential role in proliferation of human aortic SMCs. Inhibition of SOCE in cells with disrupted Orai1 protein expression significantly attenuated hASMC proliferation (Fig. 1F). Knockdown of Orai2 or Orai3, however, did not affect SOCE and hASMC proliferation. The data confirm a previous report demonstrating an essential role of Orai1 but not Orai2 or Orai3 in proliferation and migration of rat aortic SMCs (47). Augmented expression of Orai proteins in proliferating aortic myocytes (9) might also implicate Orai in arterial phenotype modulation.

In conclusion, one of the key results of this study is that downregulation of Orai1 dramatically reduces SOCE in primary cultured proliferating hASMCs. This establishes the role of Orai1 as a critical component of arterial SOCE, which is essential for cell proliferation. The implication is that Orai1 is required for activation of SOCE not only in nonexcitable cells, such as T lymphocytes and other hematopoietic cells, but also in proliferating human vascular SMCs. Moreover, Orai1 and NCX1 proteins are localized in the PM in close proximity. Such specialized distribution of Orai1 and NCX1 facilitates their functional interaction. The data suggest that Orai1 may play a critical role in an altered Ca^{2+} handling associated with cell proliferation, for example, during vascular growth and remodeling.

ACKNOWLEDGMENTS

We thank Dr. M. P. Blaustein for insightful discussion. Present address of M. V. Pulina: Div. of Cardiology, Dept. of Medicine, Weill Cornell Medical College, New York, NY 10021.

GRANTS

This work was supported by National Institutes of Health Grant NS-048263 and Grant HL-PO1-078870 Project 2 (to V. A. Golovina) and by funds from the University of Maryland School of Medicine.

REFERENCES

ORAI1 IS FUNCTIONALLY ASSOCIATED WITH Na+/Ca2+ EXCHANGER

C111


