TRPC1 and Orai1 interact with STIM1 and mediate capacitative Ca$^{2+}$ entry caused by acute hypoxia in mouse pulmonary arterial smooth muscle cells

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The present study aimed to determine if transient receptor potential canonical 1 (TRPC1) and Orai1 interact with STIM1 and mediate CCE caused by acute hypoxia in mouse PASMCs. In primary cultured PASMCs loaded with fura-2, acute hypoxia caused a transient followed by a sustained rise in intracellular Ca$^{2+}$ concentration ($[Ca^{2+}]_{i}$). The transient but not sustained rise in $[Ca^{2+}]_{i}$ was partially inhibited by nifedipine. Acute hypoxia also increased the rate of Mn$^{2+}$ quench of fura-2 fluorescence that was inhibited by SKF 96365, Ni$^{2+}$, La$^{3+}$, and Gd$^{3+}$, and exhibiting pharmacological properties characteristic of CCE. The nifedipine-insensitive rise in $[Ca^{2+}]_{i}$, and the increase in Mn$^{2+}$ quench rate were both inhibited in cells treated with TRPC1 antibody or TRPC1 small interfering (si)RNA, in STIM1 siRNA-transfected cells and in Orai1 siRNA-transfected cells. Moreover, overexpression of STIM1 resulted in a marked increase in $[Ca^{2+}]_{i}$ and Mn$^{2+}$ quench rate caused by acute hypoxia, and they were reduced in cells treated with TRPC1 antibody and in cells transfected with Orai1 siRNA. Furthermore, TRPC1 and Orai1 coimmunoprecipitated with STIM1 and the precipitation levels of TRPC1 and Orai1 were increased in cells exposed to acute hypoxia. Immunostaining showed colocalization of TRPC1-STIM1 and Orai1-STIM1, and the colocalizations of these proteins were more apparent in acute hypoxia. These data provide direct evidence that TRPC1 and Orai1 channels mediate CCE through activation of STIM1 in acute hypoxic mouse PASMCs.

TRPC1; Orai1; STIM1; capacitative Ca$^{2+}$ entry; acute hypoxia

Hypoxic pulmonary vasoconstriction (HPV) diverts blood perfusion from poorly ventilated areas of the lung to well-ventilated regions to optimize gas exchange. This process is essential during local alveolar hypoxia because it optimizes arterial oxygenation by matching local ventilation with perfusion (47). HPV is activated by an increase in intracellular Ca$^{2+}$ concentration ($[Ca^{2+}]_{i}$) in pulmonary arterial smooth muscle cells (PASMCs) during hypoxia. This increase in $[Ca^{2+}]_{i}$ can be due to the inhibition of voltage-gated potassium channels (Kv) caused by hypoxia, leading to the membrane depolarization and activation of Ca$^{2+}$ entry through voltage-operated Ca$^{2+}$ channels (VOCCs; Refs. 34, 37, 56). Hypoxia has also been found to cause Ca$^{2+}$ release from the intracellular sarcoplasmic reticulum (SR) Ca$^{2+}$ stores leading to the rise in $[Ca^{2+}]_{i}$ (8, 14, 28, 42). More recently, store-depletion due to release of Ca$^{2+}$ from the SR caused by acute hypoxia has been shown to activate store-operated calcium entry or capacitative calcium entry (CCE) in PASMCs (30, 31, 50), intact pulmonary arteries (40) and also in isolated lungs (52). These findings suggest a significant role of store-operated channels (SOCs) in contributing to the rise in $[Ca^{2+}]_{i}$ underlying HPV. However, the molecular candidates forming SOCs and the molecular signal(s) that activate these channels during hypoxia remain unclear.

In PASMCs, several studies have confirmed the existence of CCE and confirmed the expression of several homologs of transient receptor potential canonical (TRPC) channels, putative candidates for SOCs (29, 48, 49). Among all TRPC members, TRPC1 has been extensively studied and is suggested to be an important molecular candidate for SOCs in PASMCs (11, 18, 44). Interestingly, expression of TRPC1 and TRPC6 were significantly elevated in chronic hypoxia and TRPC1 were found to mediate CCE in rat PASMCs (22), suggesting a potential role of TRPC1 channel in chronic hypoxia. However, the functional role of TRPC channels in mediating CCE caused by acute hypoxia remains unclear.

Recent advancement in the study of SOCs has led to the discovery of two transmembrane proteins, Orai1 and stromal interacting molecule 1 (STIM1). Orai1 was found to be an essential pore subunit of Ca$^{2+}$ release-activated Ca$^{2+}$ channel, a putative store-operated channel in nonexcitable cells (10, 39). On the other hand, STIM1 was found in the SR to act as a sensor of the Ca$^{2+}$ stores and it may also activate Ca$^{2+}$ release-activated Ca$^{2+}$ channel in the plasma membrane (41, 57). When the stores are depleted of Ca$^{2+}$, STIM1 aggregates and form clusters or so-called “puncta” in junctional endoplasmic reticulum located 10–25 nm from the plasma membrane so that STIM1 may interact directly with the SOCs in the plasma membrane (23, 26, 27). So far, STIM1 and Orai1 are only found in certain vascular smooth muscle cell preparations and have been shown to mediate CCE. Small interfering (si)RNA knockdown of STIM1 protein resulted in a reduction of Ca$^{2+}$ entry and whole cell current activated by store-depletion in cultured saphenous vein cells (19), cultured coronary artery smooth muscle cells (45), mouse aorta smooth muscle cells (7), and cultured rat aorta smooth muscle cells (38), whereas siRNA knockdown of Orai1 inhibited Ca$^{2+}$ entry and whole cell current activated by store-depletion in cultured aorta smooth muscle cells (3, 38).

More recently, STIM1 was found to mediate CCE in cultured rat PASMCs (24) and our recent study in cultured mouse...
PASMCs reveals that STIM1 functionally interacts with TRPC1 and Orai1 to mediate CCE (32, 33). Interestingly, STIM1 was shown to mediate CCE caused by acute hypoxia in cultured rat PASMCs (25). However, there is no direct evidence that TRPC1 and Orai1 interact with STIM1 and play a role in acute hypoxia. Therefore, the aims of the present study were to determine if acute hypoxia causes activation of CCE in mouse PASMCs and determine whether TRPC1 and Orai1 interact with STIM1 and mediate CCE in acute hypoxia.

**MATERIALS AND METHODS**

**PASMCs isolation and cell culture.** Male C57BL/6 mice were killed by inhalation of 5% isoflurane in oxygen followed by cervical dislocation, as approved by the university of Nevada Reno Institutional Care and Use Committee. The heart and lungs were removed, and the second and third branches of the intrapulmonary artery were dissected in a low-Ca2+ physiological salt solution (PSS) composed of the following (mM): 125 NaCl, 5.36 KCl, 0.34 Na2HPO4, 0.44 K2HPO4, 1.2 MgCl2, 11 HEPES, 10 glucose, and 0.05 CaCl2 (pH 7.4 adjusted with Tris). To disperse cells, pulmonary arterial tissue was incubated with the low-Ca2+ PSS containing (in mg/ml): 1 collageenate type XI, 2 trypsin inhibitor, 0.45 protease, 1.3 taurine, 2 BSA (fat free) for 30 min at 5°C followed by 8 min at 33°C. The tissue was then transferred to an enzyme-free, low-Ca2+ PSS and triturated with a fire-polished Pasteur pipette. The resulting dispersed PASMCs were subjected to cell culture as previously described (32). Freshly dispersed PASMCs were plated onto a 60-mm cell-cultured dish and incubated with DMEM cell culture medium containing 10% newborn calf serum (NCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were cultured in a humidified atmosphere of 5% CO2 in air at 37°C and grown to 90–95% confluence. These primary cultured cells were then trypsinized and passaged onto a coverslip and incubated at room temperature for 20 min to allow formation of a cell monolayer. Then, 10 µl of OPTIMEM I (Invitrogen, Carlsbad, CA) was added to the cell monolayer, followed by reexposure of cells to 2 mM CaCl2-PSS for another 10–15 min. Ca2+-free PSS was identical to 2 mM Ca2+-PSS but with CaCl2 omitted and 1 mM EGTA added. An elevation in [Ca2+]i above basal fluorescence was recorded during excitation at 360 nm in nominally Ca2+-free PSS containing 10 µM nifedipine. Nominally Ca2+-free solutions were similar to Ca2+-free PSS but with EGTA omitted.

Transfection of PASMCs with siRNAs. PASMCs were transiently transfected with TRPC1 siRNA (ID: s75482, Silencer Select Predesigned siRNA; Ambion, Austin, TX), STIM1 siRNA (ID: s74488, Silencer Select Predesigned siRNA; Ambion), or Orai1 siRNA (ID: s97224, Silencer Select Predesigned siRNA; Ambion) using siPORT Amine transfection reagent (Ambion) as previously described (32, 33). For every 35-mm culture dish of cells, 10 µl of siPORT Amine were diluted in 90 µl of OPTIMEM I (Invitrogen, Carlsbad, CA). Then, 10 µl of siPORT Amine were diluted in 90 µl of OPTIMEM I and mixed with the diluted siRNA. The mixture (200 µl) was incubated at room temperature for 20 min to allow formation of transfection complexes. Primary cultured PASMCs were then trypsinized and incubated in DMEM cell culture medium containing 10% NCS and 0.4% antibiotics, and the cells were subsequently passaged onto three 35-mm cell-cultured dishes. To each culture dish, 100 µl of siPORT Amine was added to the cell monolayer, followed by reexposure of cells to 2 mM CaCl2-PSS but with CaCl2 omitted and 1 mM EGTA added. An elevation in [Ca2+]i above basal fluorescence was recorded during excitation at 360 nm in nominally Ca2+-free PSS containing 10 µM nifedipine. Nominally Ca2+-free solutions were similar to Ca2+-free PSS but with EGTA omitted.

In experiments where the effect of acute hypoxia was investigated, hypoxia was induced by switching normoxic gas mixture to hypoxic PSS, which continuously superfused the cells in the recording chamber as previously described (30, 31). Hypoxic PSS was prepared by continuous gassing with certified gas mixture containing 21% O2-5% CO2-74% N2 (Sierra Welding, Sparks, NV). In experiments where the effect of acute hypoxia was investigated, hypoxia was induced by switching normoxic gas mixture to hypoxic PSS, which continuously superfused the cells in the recording chamber as previously described (30, 31). Hypoxic PSS was prepared by continuous gassing with certified gas mixture containing 95% N2-5% CO2 (Sierra Welding, Sparks, NV). The uncertified gas mixture contained minimal amount of oxygen, which equilibrated with PSS to avoid exposure of cells to anoxic condition. All solutions were placed in a water bath at 37°C and saturated with either normoxic or hypoxic gas mixtures for at least 30 min before the start of perfusion and maintained at pH 7.4. The PO2 was measured in preliminary experiments with an O2 sensitive electrode (MI-730; Microelectrodes, Bedford, NH) to be 145 ± 1 mmHg during normoxic PSS perfusion and fell to 15 ± 1 mmHg within 79 ± 2 s of hypoxic exposure. The PO2 of hypoxic solutions was measured at the end of each experiment and was found to be 15–18 mmHg, ensuring that the PO2 did not approach anoxia during recording of each experiment. The effect of acute hypoxia was determined in cells incubated in Ca2+-free PSS for 10 min following reexposure of cells to 2 mM Ca2+-PSS for another 10–15 min. Ca2+-free PSS was identical to 2 mM Ca2+-PSS but with CaCl2 omitted and 1 mM EGTA added. An elevation in [Ca2+]i above basal levels during 2 mM Ca2+ readdition was used as a marker of hypoxia-induced extracellular Ca2+ entry. In experiments where the Ca2+ influx pathway was studied, the rate of Mn2+-induced quenching of fura-2 fluorescence was recorded during excitation at 360 nm in nominally Ca2+-free PSS containing 10 µM nifedipine. Nominally Ca2+-free solutions were similar to Ca2+-free PSS but with EGTA omitted.
another 24 h before experimental use. For negative control, the cells were transacted with a scrambled siRNA (Silencer Negative Control #1 siRNA; Ambion) using the same transfection method.

**Generation of recombinant STIM1 adenovirus.** STIM1 adenoviruses were generated as previously described (32). STIM1 cDNA was isolated from mouse brain and cloned into pcDNA3.1 and the STIM1 construct was confirmed using terminator cycle sequencing. Recombinant adenoviruses for STIM1 were then produced in a PAdTrack-CMV/pAdEasy recombinant containing green fluorescent protein (Ad-GFP-STIM1), purified, and amplified by using the AdEasy adenoviral vector system (Stratagene, La Jolla, CA). To produce adenoviruses, the STIM1 adenovirus recombinants were transfected into a viral packaging cell line using the MBS mammalian transfection kit (Stratagene). Adenoviruses were then harvested, plaque purified, and titred by an agarose overlay plaque assay. The same procedure was used to generate a control adenovirus containing GFP (Ad-GFP) with no insertion of STIM1 gene. The titer for Ad-GFP and Ad-GFP-STIM1 were $1.4 \times 10^{10}$ plaque-forming units/ml and $1.8 \times 10^{10}$ plaque-forming units/ml, respectively. For infection, cultured PASMCs were incubated with adenovirus in DMEM containing 0.1% NCS for 24 h. The cells were then washed with fresh 0.1% NCS medium for another 24 h. Infected cells were monitored by observing the number of green cells under fluorescence microscope and were subsequently used for Western blot analysis or calcium imaging study.

In experiments where Ad-GFP and Ad-GFP-STIM1 cells were used for calcium imaging, simultaneous excitation of GFP and fura-2 during experimental recording was avoided to ensure that GFP did not affect fura-2 signals. The GFP-positive cells were preselected by exciting the cells at 488 nm and emission at 510 nm before experimental recording. After the GFP-positive cells were selected and imaged using the imaging software, the measurement of fura-2 fluorescence in each designated cell was started by exciting the cells at 340 and 380 nm and emission at 510 nm.

**Western blot analysis, coimmunoprecipitation, and colocalization studies.** Total protein was obtained from cultured mouse PASMCs by using RIPA extraction buffer containing protease and phosphatase inhibitors as previously described (32, 33). For Western blot analysis, equal amounts of total protein (40 μg) were resolved by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes for 90 min at 24 V (Gene blower; Iden) and blocked in 5% non-fat dry milk, 0.1% NCS medium for 2 h. The membranes were then blocked for 1 h with LI-COR blocking solution (LI-COR, Lincoln, NE) and probed with a rabbit polyclonal TRPC1 antibody (1:100; Alomone), rabbit polyclonal Orai1 antibody (1:100; ProSci, Poway, CA), or mouse monoclonal STIM1 antibody (1:100; BD Biosciences, Bedford, MA). The membranes were simultaneously probed with mouse monoclonal GAPDH antibody (1:20,000; Ambion) as an internal control. The primary antibodies were incubated overnight at 4°C, and after washout, membranes were incubated with two secondary antibodies in LI-COR solution for 45 min at room temperature: one coupled to an infrared fluorescence marker with emission wavelength of 800 nm (1:10,000, IR 800; Rockland Immunomethods, Gilbertsville, PA), and the other coupled to an infrared fluorescence marker with emission wavelength of 680 nm (1:100,000; Alexa Fluor 680; Molecular Probes, Eugene, OR). Immunoblots were then scanned to obtain double-color fluorescent images with an Odyssey scanner (LI-COR).

For coimmunoprecipitation of STIM1 and TRPC1, or STIM1 and Orai1, 0.40 mg of total protein was first diluted with an equal volume of PBS (with protease inhibitors) and mixed with 10 μg of STIM1 antibody (EXBIO, Vestec, Czech Republic) and incubated with agitation at 4°C for 2 h. Then, 100 μl of slurry of agarose beads conjugated to goat-anti-mouse antibodies (Sigma-Aldrich, St. Louis, MO) were washed with 1 ml PSS and incubated overnight with the protein/antibody complex at 4°C on an end-over-end mixer. The beads-protein-antibody complex was then washed three times with 1 ml of PSS. The protein was released from the beads by addition of 35 μl of 4× SDS loading buffer and incubated for 20 min at room temperature before loading on a 10% SDS gel. After gel electrophoresis, the separated protein was transferred onto nitrocellulose membrane. To demonstrate immunoprecipitation of STIM1, the blot was probed with STIM1 antibody (1:100; BD Biosciences). To demonstrate coimmunoprecipitation of STIM1 and TRPC1, or STIM1 and Orai1, the blot was subsequently probed with TRPC1 antibody (1:100; Alomone) or Orai1 antibody (1:100; ProSci). For control, 10 μg of mouse IgG1 (eBioscience, San Diego, CA) were used for immunoprecipitation and the blot was subsequently probed with STIM1, TRPC1, or Orai1 antibody using the same protocol.

For colocalization study, dual labeling of endogenous STIM1 and TRPC1, or STIM1 and Orai1 proteins were performed using immunostaining method as previously described (33). Cultured mouse PASMCs were fixed in 4% paraformaldehyde and stained with a rabbit polyclonal TRPC1 (1:100, Alomone) or rabbit polyclonal Orai1 antibody (1:100; ProSci) and mouse monoclonal STIM1 antibody (1:100; EXBIO) using Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instruction. A secondary antibody conjugated with Alexa Fluor 488 (1:200; Molecular Probes) was used to display TRPC1 or Orai1 fluorescence image (excited at 495 nm and emitted at 519 nm), and secondary antibody conjugated with Alexa Fluor 546 (1:200; Molecular Probes) was used to display STIM1 fluorescence image (excited at 556 nm and emitted at 573 nm). For control experiments, the cells were treated similarly in the absence of primary antibodies. The cells were then mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) to stain cells nuclei. The cells were examined under a Bio-Rad Radiance 2100 inverted laser-scanning confocal microscope with a Nikon Plan-Fluor ×60 oil immersion objective. In experiments where the morphology of cultured mouse PASMCs was studied, a similar immunostaining procedure was performed by using smooth muscle α-actin antibody (1:200; Sigma-Aldrich). A secondary antibody conjugated with Alexa Fluor 488 (1:200; Molecular Probes) was used to display smooth muscle α-actin image, and DAPI was used to stain cells nuclei.

**Drug solutions and data analysis.** Nifedipine, MnCl₂, NiCl₂, LaCl₃, GdCl₃, and smooth muscle α-actin antibody were obtained from Sigma-Aldrich. Ionomycin and SKF 96365 were obtained from Calbiochem. TRPC1 antibody was obtained from Alomone. Orai1 antibody was obtained from ProSci. STIM1 antibodies were obtained from BD Biosciences and EXBIO. Mouse IgG₁ was obtained from eBioscience (San Diego, CA, USA). GAPDH antibody, TRPC1 siRNA, Orai1 siRNA, STIM1 siRNA, and negative control siRNA were obtained from Ambion. Nifedipine and ionomycin were dissolved in dimethylsulphoxide. Other drugs were dissolved in deionized water. Data are expressed as means ± SE of n cells from at least five cell culture dishes passed from three primary cultured dishes of separate seedings. Statistical comparisons employed Student’s paired t-tests, Student’s unpaired t-tests or one-way ANOVA as appropriate. A value of P < 0.05 was considered significant.

**RESULTS**

**Mouse PASMCs retain the characteristics of smooth muscle cell with culture.** To determine if mouse PASMCs retain the smooth muscle cell characteristics with culture, we first investigated the expression of smooth muscle α-actin in cultured mouse PASMCs using immunostaining study. Figure 1A shows expression of smooth muscle α-actin in cultured mouse PASMC. For negative control, no smooth muscle α-actin staining was observed in mouse PASMC when the α-actin antibody was omitted (Fig. 1B). In addition, we studied the effects of acute hypoxia on these cells by exposing the cells to hypoxic PSS in the presence of 2 mM external Ca²⁺ for ~10 min. We found that acute hypoxia caused a transient increase in [Ca²⁺], (244 ± 42 nM; ΔR = 0.66 ± 0.14) followed by a...
sustained increase in $[\text{Ca}^{2+}]_i$ (50 ± 10 nM; $\Delta R = 0.13 ± 0.02$) above basal levels (Fig. 1, C and D; $n = 22$). These data confirm that acute hypoxia causes an increase in $[\text{Ca}^{2+}]_i$ in cultured mouse PASMCs as is characteristic of PASMCs.

Acute hypoxia causes activation of VOCCs and CCE in mouse PASMCs. When applied in Ca$^{2+}$-free solution, acute hypoxia caused a transient increase in $[\text{Ca}^{2+}]_i$ in cultured mouse PASMCs indicative Ca$^{2+}$ release from the intracellular stores (Fig. 2A). The transient rise in $[\text{Ca}^{2+}]_i$ decayed slowly to a mean level below baseline. Subsequent addition of 2 mM Ca$^{2+}$ in acute hypoxia elicited a significant transient rise in $[\text{Ca}^{2+}]_i$ (278 ± 8 nM; $\Delta R = 1.06 ± 0.05$) followed by a sustained rise in $[\text{Ca}^{2+}]_i$ (94 ± 8 nM; $\Delta R = 0.35 ± 0.03$) above basal levels (Fig. 2, A and B; $n = 165$; $P < 0.01$). Part
of the transient rise in \([\text{[Ca}^{2+}])\), caused by hypoxia was mediated by \([\text{Ca}^{2+}]) influx through VOCCs because nifedipine significantly reduced the transient rise in \([\text{[Ca}^{2+}])\), to 243 ± 8 nM (Fig. 2, A and B; \(\Delta R = 0.78 ± 0.03; n = 363; P < 0.01\)). However, nifedipine did not affect the sustained rise in \([\text{[Ca}^{2+}])\), (Fig. 2, A and B). In control experiments, cells were exposed to normoxic solutions throughout the protocol (data not shown). Removal of extracellular \([\text{Ca}^{2+}]) caused a decrease in \([\text{[Ca}^{2+}])\), and subsequent addition of 2 mM \([\text{Ca}^{2+}]) elicited a very small transient rise in \([\text{[Ca}^{2+}])\), 97 ± 8 nM (\(\Delta R = 0.42 ± 0.03\)) above basal levels (\(n = 67\); \(P < 0.01\)), which decayed slowly to the baseline.

To determine if acute hypoxia increases \([\text{Ca}^{2+}])\), by recruiting a \([\text{Ca}^{2+}]) influx pathway similar to CCE, the effect of hypoxia on Mn\(^{2+}\) quench of fura-2 fluorescence was tested in the presence of 10 \(\mu\text{M}\) nifedipine. Figure 2C shows that acute hypoxia caused a marked 144 ± 22\% (\(n = 505; P < 0.01\)) increase in Mn\(^{2+}\) quench rate in the presence of nifedipine compared with the rate before hypoxic exposure, indicating hypoxia activation of a nifedipine-insensitive \([\text{Ca}^{2+}])\) entry pathway. Figure 2D shows that SOC blockers SKF96365, Ni\(^{2+}\), La\(^{3+}\), and Gd\(^{3+}\) abolished the hypoxia-activated Mn\(^{2+}\) quench of fura-2 fluorescence to 25 ± 4\% (\(n = 161\)), 10 ± 4\% (\(n = 111\)), −16 ± 2\% (\(n = 196\)), and 13 ± 4\% (\(n = 131\)), respectively (\(P < 0.01\)).

**TRPC1, STIM1, and Orai1 mediate CCE in acute hypoxic mouse PASMCs.** To determine if TRPC1 channels are responsible for CCE caused by acute hypoxia in mouse PASMCs, the effects of TRPC1 antibody were investigated in cells subjected to acute hypoxia in the presence of 10 \(\mu\text{M}\) nifedipine. The anti-TRPC1 antibody from Alomone is raised against the extracellular amino acid sequence 557 to 571, which is predicted to lie in the pore-forming region of the protein (1). This antibody is widely used to study CCE in many various cell types, including pulmonary artery cells (18, 32), endothelial cells (1, 15), and glomerular mesangial cells (9). In control experiments, TRPC1 antibody was preadsorbed with TRPC1 antigen peptide and incubated with the cells at 37°C for 24 h before recording. Figure 3A confirms the endogenous expression of TRPC1 in cultured mouse PASMCs. In control cells, acute hypoxia caused a nifedipine-insensitive transient and sustained increase in \([\text{[Ca}^{2+}])\), amounted to 203 ± 22 nM (\(\Delta R = 0.70 ± 0.03\); \(n = 117\)) and 73 ± 14 nM (\(\Delta R = 0.17 ± 0.02\); \(n = 117\)), respectively (Fig. 3, B and C). Both the nifedipine-insensitive transient and sustained increases in \([\text{[Ca}^{2+}])\), were reduced in TRPC1 antibody-treated cells to 152 ±

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**Fig. 3.** Transient receptor potential canonical 1 (TRPC1) mediates CCE in acute hypoxic mouse PASMCs. **A:** TRPC1 protein and GAPDH were detected in cultured mouse PASMCs using Western blot analysis. A negative control was performed by preincubating TRPC1 antibody with the antigen peptide. Experiments were performed in 3 separate Western blot analyses. **B:** TRPC1 antibody (1:100) inhibited the hypoxia-induced transient and sustained increase in fura-2 fluorescence ratio in the presence of 10 \(\mu\text{M}\) nifedipine. **C:** bar graph showing mean changes in transient and sustained increase in \([\text{[Ca}^{2+}])\), caused by acute hypoxia after readdition of 2 mM \([\text{Ca}^{2+}])\) in the presence of 10 \(\mu\text{M}\) nifedipine, in control cells (filled bars, TRPC1 Ab + peptide; \(n = 117\)), and in cells treated with TRPC1 antibody (open bars; \(n = 207\)). *\(P < 0.05\), **\(P < 0.01\) (unpaired t-test). **TRPC1 antibody (1:100) inhibited the increase in Mn\(^{2+}\) quench of fura-2 fluorescence caused by acute hypoxia in the presence of 10 \(\mu\text{M}\) nifedipine, in control cells (filled bar, TRPC1 Ab + peptide; \(n = 116\)), and in cells treated with TRPC1 antibody (open bar; \(n = 127\)). **\(P < 0.01\) (unpaired t-test).
10 nM (ΔR = 0.44 ± 0.03; n = 207) and 23 ± 9 nM (ΔR = 0.09 ± 0.02; n = 207), respectively (Fig. 3, B and C; P < 0.01). To confirm that TRPC1 mediates CCE in acute hypoxic mouse PASMCs, we compared the effects of acute hypoxia on Mn$^{2+}$ quench of fura-2 fluorescence in control cells to cells treated with TRPC1 antibody. Figure 3D shows that acute hypoxia caused a marked 106 ± 9% (n = 116) increase in Mn$^{2+}$ quench rate in the presence of 10 μM nifedipine in control cells. This increase in Mn$^{2+}$ quench rate was significantly reduced to 29 ± 6% (Fig. 3, D and E; n = 127; P < 0.01) in cells treated with TRPC1 antibody.

We also used the approach of siRNA knockdown of TRPC1 to further confirm the role of TRPC1 in mediating CCE in acute hypoxic cultured mouse PASMCs. First, we verified if siRNA knockdown of TRPC1 mRNA reduced the expression level of TRPC1 protein in cultured mouse PASMCs. Figure 4, A and B, shows that endogenous TRPC1 protein was detected at similar levels in nontransfected cells and in cells transfected with 200 nM scrambled siRNA (negative control). Expression of TRPC1 but not GAPDH reduced significantly in cells transfected with 200 nM TRPC1 siRNA compared with nontransfected cells (negative control, Fig. 4C). The protein level was significantly reduced by ~60–70% in cells transfected with 200 nM TRPC1 siRNA compared with nontransfected cells and cells transfected with scrambled siRNA (P < 0.01). To confirm that endogenous TRPC1 mediates CCE in acute hypoxic mouse PASMCs, we compared the effects of acute hypoxia on Mn$^{2+}$ quench of fura-2 fluorescence between control cells transfected with scrambled siRNA and cells transfected with 200 nM TRPC1 siRNA. Figure 4D shows that acute hypoxia caused a 159 ± 20% (n = 33) increase in Mn$^{2+}$ quench rate in the presence of 10 μM nifedipine in negative control cells. This increase in Mn$^{2+}$ quench rate was significantly reduced to 9 ± 15% (Fig. 4, C and D; n = 20; P < 0.01) in cells transfected with 200 nM TRPC1 siRNA.

To determine if STIM1 protein mediates CCE in mouse PASMCs during acute hypoxia, we first verified if siRNA knockdown of STIM1 mRNA reduced the expression level of STIM1 protein in cultured mouse PASMCs. Figure 5, A and B, shows that endogenous STIM1 protein was detected at similar levels in nontransfected cells and in cells transfected with 200 nM scrambled siRNA (negative control). Expression of STIM1 siRNA further confirms the role of TRPC1 in mediating CCE in acute hypoxic cultured mouse PASMCs. Figure 4E shows that acute hypoxia caused a 100 ± 11% (n = 66) increase in Mn$^{2+}$ quench rate in the presence of 10 μM nifedipine in negative control cells. This increase in Mn$^{2+}$ quench rate was significantly reduced to 32 ± 7% (Fig. 5, E and F; n = 118; P < 0.01) in cells transfected with 200 nM STIM1 siRNA.

To determine if Orai1 channels play a role in mediating CCE caused by acute hypoxia in mouse PASMCs, the effects of Orai1 knockdown were investigated in cells exposed to acute hypoxia. We first showed that endogenous Orai1 protein was detected at similar levels in nontransfected cells and in cells transfected with 200 nM scrambled siRNA (negative control, Fig. 6, A and B). The protein level was significantly reduced by...
Fig. 5. siRNA knockdown of stromal interacting molecule 1 (STIM1) reduces CCE in acute hypoxic mouse PASMCs. A and B: STIM1 protein and GAPDH were detected in nontransfected mouse PASMCs and in PASMCs transfected with 200 nM scrambled siRNA (negative control). Expression of STIM1 but not GAPDH reduced significantly in cells transfected with 200 nM STIM1 siRNA. Experiments were performed in 3 separate Western blot analyses (**P < 0.01, ANOVA). C: siRNA knockdown of STIM1 reduced the hypoxia-induced transient and sustained increase in fura-2 fluorescence between control cells transfected with 200 nM scrambled siRNA (negative control). Both the transient and sustained increases in \([\text{Ca}^{2+}]_i\) were significantly reduced in cells transfected with 200 nM scrambled siRNA (negative control). The transient and sustained increase in \([\text{Ca}^{2+}]_i\) caused by acute hypoxia after readDITION of 2 mM Ca\(^{2+}\) in the presence of 10 \(\mu\)M nifedipine, in negative control cells (filled bars; \(n = 77\)), and in STIM1 siRNA-transfected cells (open bars; \(n = 69\)). **P < 0.01 (unpaired t-test). E: siRNA knockdown of STIM1 reduced the increase in Mn\(^{2+}\) quench of fura-2 fluorescence caused by acute hypoxia in the presence of 10 \(\mu\)M nifedipine. F: bar graph showing percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 \(\mu\)M nifedipine, in negative control cells (filled bar; \(n = 66\)), and in STIM1 siRNA-transfected cells (open bar; \(n = 118\)). **P < 0.01 (unpaired t-test).

~50% in cells transfected with 200 nM Orai1 siRNA compared with nontransfected cells and cells transfected with scrambled siRNA. We then examined the effect of Orai1 siRNA on hypoxia-induced rise in \([\text{Ca}^{2+}]_i\) in the presence of 10 \(\mu\)M nifedipine. Figure 6C shows that acute hypoxia caused an increase in nifedipine-insensitive transient and sustained increase in \([\text{Ca}^{2+}]_i\) in cells transfected with 200 nM scrambled siRNA (negative control). Both the transient and sustained increases in \([\text{Ca}^{2+}]_i\) were significantly reduced in Orai1 siRNA-transfected cells from 235 ± 17 nM (ΔR = 0.79 ± 0.06; \(n = 159\)) and 65 ± 12 nM (ΔR = 0.27 ± 0.03; \(n = 159\); \(P < 0.01\)) to 148 ± 10 nM (ΔR = 0.55 ± 0.03; \(n = 171\)) and 33 ± 5 nM (ΔR = 0.17 ± 0.02; \(n = 171\); \(P < 0.01\)), respectively (Fig. 6, C and D). To confirm that endogenous Orai1 mediates CCE in acute hypoxic mouse PASMCs, we compared the effects of acute hypoxia on Mn\(^{2+}\) quench of fura-2 fluorescence between control cells transfected with scrambled siRNA and cells transfected with Orai1 siRNA. Figure 6E shows that acute hypoxia caused a 110 ± 27% (\(n = 88\)) increase in Mn\(^{2+}\) quench rate in the presence of 10 \(\mu\)M nifedipine in negative control cells. This increase in Mn\(^{2+}\) quench rate was significantly reduced to 46 ± 8% (Fig. 6, E and F; \(n = 113\); \(P < 0.01\)) in cells transfected with 200 nM Orai1 siRNA.

**STIM1 functionally associates with TRPC1 and Orai1 to mediate CCE in acute hypoxic mouse PASMCs.** To examine if STIM1 protein functionally interacts with TRPC1 and Orai1 to mediate CCE in acute hypoxia, we first determined if overexpression of STIM1 protein in cultured mouse PASMCs increases CCE activated by acute hypoxia. Then, we determined if this increase in CCE is affected by TRPC1 inhibition or Orai1 knockdown. Figure 7A shows that endogenous STIM1 and TRPC1 proteins were detected in cells infected with adenovirus containing GFP (Ad-GFP). The STIM1 protein level was significantly increased in cells infected with STIM1-GFP adenovirus (Ad-GFP-STIM1), but expression of TRPC1 protein was not affected by Ad-GFP-STIM1. Preincubation of TRPC1 antibody with TRPC1 antigen peptide was performed as a control to show specificity of TRPC1 antibody. To determine if STIM1 is functionally associated with TRPC1 in mediating CCE, the effects of TRPC1 antibody were investigated in STIM1-overexpressed cells subjected to acute hypoxia in the presence of 10 \(\mu\)M nifedipine. In control experiments, TRPC1 antibody was preadsorbed with TRPC1 antigen.
peptide and incubated with Ad-GFP cells for 24 h before recording. Figure 7B shows that acute hypoxia caused an increase in nifedipine-insensitive transient and sustained rise in [Ca\(^{2+}\)]\(_i\) in the Ad-GFP cells under control condition. The transient and sustained increase in [Ca\(^{2+}\)]\(_i\) was augmented in STIM1-overexpressed cells from 184 ± 15 nM (ΔR = 0.48 ± 0.06; n = 59) to 431 ± 49 nM (ΔR = 1.04 ± 0.09; n = 60; P < 0.01) and 26 ± 7 nM (ΔR = 0.07 ± 0.03; n = 59) to 55 ± 5 nM (ΔR = 0.18 ± 0.02; n = 60; P < 0.01), respectively (Fig. 7, B and C). These increases in transient and sustained rise in [Ca\(^{2+}\)]\(_i\) were significantly reduced in STIM1-overexpressed cells treated with TRPC1 antibody to 241 ± 19 nM (ΔR = 0.75 ± 0.06; n = 59) and 20 ± 7 nM (ΔR = 0.08 ± 0.03; n = 59; P < 0.01), respectively. To further confirm that STIM1 is functionally associated with TRPC1 in acute hypoxic mouse PASMCs, we compared the effects of acute hypoxia on Mn\(^{2+}\) quench of fura-2 fluorescence among Ad-GFP cells treated with TRPC1 antibody-antigen peptide, Ad-GFP-STIM1 cells treated with TRPC1 antibody-antigen peptide, and Ad-GFP-STIM1 cells treated with TRPC1 antibody (Fig. 7, D and E). Figure 7D shows that acute hypoxia caused a 108 ± 11% (n = 104) increase in Mn\(^{2+}\) quench rate in the presence of 10 μM nifedipine in Ad-GFP cells under control condition. This increase in Mn\(^{2+}\) quench rate was significantly enhanced to 382 ± 77% (n = 87; P < 0.01) in cells overexpressed with STIM1. This increase in Mn\(^{2+}\) quench rate was reduced to 75 ± 15% (n = 73; P < 0.01) in STIM1-overexpressed cells treated with TRPC1 antibody (Fig. 7, D and E).

To examine the functional interaction between STIM1 and Orai1 in the contribution of CCE caused by acute hypoxia, we studied the effects of acute hypoxia in the presence of 10 μM nifedipine in STIM1 overexpressing cells transfected with Orai1 siRNA. Figure 8, A and B, shows that endogenous STIM1 and Orai1 protein was detected in scrambled siRNA-transfected cells (negative siRNA) (open bars; n = 171), **P < 0.01 (unpaired t-test). E: siRNA knockdown of Orai1 reduced the increase in Mn\(^{2+}\) quench of fura-2 fluorescence caused by acute hypoxia in the presence of 10 μM nifedipine. F: bar graph showing percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine, in negative control cells (filled bars; n = 88), and in Orai1 siRNA-transfected cells (open bar; n = 113). **P < 0.01 (unpaired t-test).
Comparison with filled and open bars (ANOVA).

crease in Mn2± treated with TRPC1 antibody (open bar; cells transfected with 200 nM scrambled siRNA. Both the tide (shaded bar; cells transfected with 200 nM Orai1 siRNA. To further confirm that STIM1 is

n2± were performed in 3 separate Western blot analyses. B: overexpression of STIM1 in cells transfected with TRPC1 antibody-antigen peptide (shaded bar; cells transfected with 200 nM scrambled siRNA, acute hypoxia caused a 78

0.01, compared with filled and open bars (ANOVA). C: bar graph showing mean changes in transient and sustained rise in fura-2 fluorescence ratio in the presence of 10 μM nifedipine. The increase in fluorescence ratio was reduced in TRPC1 antibody-treated cells overexpressed with STIM1. D: graph showing percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine in GFP-infected cells preincubated with TRPC1 antibody-antigen peptide (filled bars; n = 60) and in STIM1 overexpressing cells treated with TRPC1 antibody (open bars; n = 59). **P < 0.01, compared with filled and open bars (ANOVA). D: overexpression of STIM1 in cells preincubated with TRPC1 antibody-antigen peptide caused an increase in hypoxia-induced increase in Mn2± quench of fura-2 fluorescence in the presence of 10 μM nifedipine. The increase in Mn2± quench of fura-2 fluorescence was reduced in TRPC1 antibody-treated cells overexpressed with STIM1. E: bar graph showing percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine in GFP-infected cells preincubated with TRPC1 antibody-antigen peptide (filled bar; n = 104), in STIM1 overexpressing cells preincubated with TRPC1 antibody-antigen peptide (shaded bar; n = 87), and in STIM1 overexpressing cells treated with TRPC1 antibody (open bar; n = 73). **P < 0.01, compared with filled and open bars (ANOVA).

Fig. 7. STIM1 associates with TRPC1 to mediate CCE in acute hypoxic mouse PASMCs. A: TRPC1, STIM1, and GAPDH proteins were detected in cells infected with adenovirus containing GFP (Ad-GFP). The expression of STIM1 but not TRPC1 or GAPDH increased markedly in cells infected with STIM1-GFP-adenovirus (Ad-GFP-STIM1). A negative control was performed by preincubating TRPC1 antibody with the antigen peptide in STIM1 overexpressing cells. Experiments were performed in 3 separate Western blot analyses. B: overexpression of STIM1 in cells transfected with TRPC1 antibody-antigen peptide (shaded bar; cells transfected with 200 nM scrambled siRNA, acute hypoxia caused a 78

was significantly increased to 205 ± 33% (n = 99; P < 0.01) in Ad-GFP-STIM1 cells transfected with scrambled siRNA (Fig. 8, E and F). Knockdown of Orai1 protein in Ad-GFP-STIM1 cells significantly reduced the Mn2± quench rate to 63 ± 10% (Fig. 8, E and F; n = 91; P < 0.01).

To ensure that incubation of cells with TRPC1 antibody-antigen peptide or transfection of cells with scrambled siRNA did not affect the function of cells infected with Ad-GFP or Ad-GFP-STIM1, we performed control experiments by studying the effects of acute hypoxia on cells overexpressed with GFP alone and STIM1 alone. Then, we compared the effects of acute hypoxia on the overexpressed cells, overexpressed cells treated with TRPC1 antibody-antigen peptide, and overexpressed cells transfected with 200 nM scrambled siRNA. Figure 9A shows that acute hypoxia caused an increase in nifedipine-insensitive transient and sustained rise in Ca2±, in Ad-GFP cells. Both the transient and sustained increases in Ca2±, were significantly enhanced in STIM1-overexpressed cells from 165 ± 16 nM (ΔR = 0.41 ± 0.03; n = 138) to 351 ± 41 nM (ΔR = 0.84 ± 0.04; n = 155; P < 0.01) and 37 ± 5 nM (ΔR = 0.01) increase in Mn2±, were significantly increased to 368 ± 25 nM (ΔR = 0.89 ± 0.05; n = 99; P < 0.01) and 71 ± 9 nM (ΔR = 0.22 ± 0.02; n = 99; P < 0.01), respectively, in Ad-GFP-STIM1 cells transfected with 200 nM scrambled siRNA. These transient and sustained increases in Ca2± were respectively reduced to 278 ± 13 nM (ΔR = 0.65 ± 0.03; n = 93; P < 0.01) and 21 ± 6 nM (ΔR = 0.07 ± 0.02; n = 93; P < 0.01) in Ad-GFP-STIM1 cells transfected with 200 nM Orai1 siRNA. To further confirm that STIM1 is functionally associated with Orai1 in acute hypoxic mouse PASMCs, we compared the effects of acute hypoxia on Mn2± quench of fura-2 fluorescence among Ad-GFP cells transfected with scrambled siRNA, Ad-GFP-STIM1 cells transfected to scrambled siRNA, and Ad-GFP-STIM1 cells transfected with Orai1 siRNA. In Ad-GFP cells transfected with 200 nM scrambled siRNA, acute hypoxia caused a 78 ± 13% (n = 101) increase in Mn2± quench rate in the presence of 10 μM nifedipine (Fig. 8, E and F). This increase in Mn2± quench rate...
The effects of acute hypoxia on Mn2⁺ quench rate caused by acute hypoxia in Ad-GFP cells were not significant different from Ad-GFP cells treated with TRPC1 antibody-antigen peptide or Ad-GFP cells transfected with 200 nM scrambled siRNA. Expression of Orai1 but not STIM1 or GAPDH was reduced significantly in STIM1 overexpressing cells transfected with 200 nM Orai1 siRNA. Experiments were performed in 3 separate Western blot analyses (**P < 0.01, ANOVA). C: overexpression of STIM1 in scrambled siRNA-transfected cells caused an increase in hypoxia-induced transient and sustained rise in fura-2 fluorescence ratio in the presence of 10 μM nifedipine. The increase in fluorescence ratio was reduced in Orai1 siRNA transfected cells overexpressed with STIM1. D: bar graph showing percent change in fura-2 fluorescence ratio in the presence of 10 μM nifedipine. The increase in fluorescence ratio caused by acute hypoxia after readdition of 2 mM Ca²⁺ in the presence of 10 μM nifedipine in GFP-infected cells transfected with scrambled siRNA (filled bars; n = 99) and in STIM1 overexpressing cells transfected with scrambled siRNA (shaded bars; n = 99) and in STIM1 overexpressing cells transfected with Orai1 siRNA (open bars; n = 93). **P < 0.01, compared with filled and open bars (ANOVA). E: overexpression of STIM1 in scrambled siRNA-transfected cells caused a hypoxia-induced increase in Mn²⁺ quench of fura-2 fluorescence in the presence of 10 μM nifedipine. The increase in Mn²⁺ quench of fura-2 fluorescence was reduced in Orai1 siRNA-transfected cells overexpressed with STIM1. F: bar graph showing percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine in GFP-infected cells transfected with scrambled siRNA (shaded bar; n = 101), in STIM1 overexpressing cells transfected with scrambled siRNA (shaded bar; n = 99), and in STIM1 overexpressing cells transfected with Orai1 siRNA (open bar, n = 91). **P < 0.01, compared with filled and open bars (ANOVA).

0.12 ± 0.02; n = 138) to 117 ± 40 nM (ΔR = 0.18 ± 0.02; n = 155; P < 0.05), respectively (Fig. 9, A and B). We also studied the effects of acute hypoxia on Mn²⁺ quench of fura-2 fluorescence in Ad-GFP cells and Ad-GFP-STIM1 cells. Figure 9C shows that acute hypoxia caused a 130 ± 34% (n = 21) increase in Mn²⁺ quench of fura-2 in the presence of 10 μM nifedipine in Ad-GFP cells. This increase in Mn²⁺ quench rate was significantly increased to 300 ± 46% (Fig. 9, C and D; n = 37; P < 0.01) in Ad-GFP-STIM1 cells.

Figure 10, A and B, shows comparison of the effects of acute hypoxia on Ad-GFP cells, Ad-GFP cells treated with TRPC1 antibody-antigen peptide, and Ad-GFP cells transfected with 200 nM scrambled siRNA. We found that the transient and sustained increases in [Ca²⁺], as well as the increase in Mn²⁺ quench rate caused by acute hypoxia in Ad-GFP cells were not significant different from Ad-GFP cells treated with TRPC1 antibody-antigen peptide or Ad-GFP cells transfected with 200 nM scrambled siRNA (Fig. 10, A and B; P > 0.05). In addition, we compared the effects of acute hypoxia on Ad-GFP-STIM1 cells, Ad-GFP-STIM1 cells treated with TRPC1 antibody-antigen peptide, and Ad-GFP-STIM1 cells transfected with 200 nM scrambled siRNA (Fig. 10, C and D). We found that the transient and sustained increases in [Ca²⁺], as well as the increase in Mn²⁺ quench rate caused by acute hypoxia in Ad-GFP-STIM1 cells were not significant different from Ad-GFP-STIM1 cells treated with TRPC1 antibody-antigen peptide or Ad-GFP-STIM1 cells transfected with 200 nM scrambled siRNA (Fig. 10, C and D; P > 0.05). These data confirm no significant difference in the response to acute hypoxia, whether or not the Ad-GFP cells or Ad-GFP-STIM1 cells were treated with TRPC1 antibody-antigen peptide or scrambled siRNA.

Acute hypoxia enhances the physical interaction of STIM1 with TRPC1, and STIM1 with Orai1 in mouse PASMCs. To investigate if acute hypoxia affects the expression levels of TRPC1, STIM1, and Orai1, we compared the expression levels of TRPC1, STIM1 and Orai1 between normoxic cells and cells exposed to acute hypoxia. In normoxic cells, the cells were
incubated with Ca\(^{2+}\)-free PSS saturated with normoxic gas for 10 min followed by readmission of 2 mM Ca\(^{2+}\)-PSS saturated with normoxic gas for 15 min. In cells subjected to acute hypoxia, the cells were incubated with Ca\(^{2+}\)-free PSS saturated with hypoxic gas for 10 min followed by readmission of 2 mM Ca\(^{2+}\)-PSS saturated with hypoxic gas for 15 min. We found that acute hypoxia did not affect the expression levels of TRPC1 (Fig. 11, A and B), STIM1 (Fig. 11, C and D), or Orai1 (Fig. 11, E and F) as compared with normoxic cells. To determine if STIM1 is physically associated with TRPC1 channels in mouse PASMCs, a communoprecipitation study was performed. Figure 11G shows that STIM1 coimmunoprecipitates with TRPC1, indicating a molecular complex formed between STIM1 proteins and TRPC1 channels in mouse PASMCs. Interestingly, more TRPC1 was coimmunoprecipitated with STIM1 in cells subjected to acute hypoxia as compared with normoxic cells (Fig. 11, G and H, \(P < 0.05\)).

Communoprecipitation study was also performed to determine if STIM1 is physically associated with Orai1 channels in mouse PASMCs. Figure 11I shows that STIM1 coimmunoprecipitates with Orai1, indicating a molecular complex formed between STIM1 proteins and Orai1 channels in mouse PASMCs. Similarly, more Orai1 was coimmunoprecipitated with STIM1 in cells exposed to acute hypoxia as compared with normoxic cells (Fig. 11, I and J, \(P < 0.01\)). In addition, IgG control experiments were performed to show nonspecific binding and we found that no STIM1, TRPC1, or Orai1 was detected in these experiments (Fig. 11, G and J). Taken together, these data suggest that acute hypoxia did not affect the expression levels of TRPC1, STIM1, or Orai1, but it enhanced the association of STIM1 with TRPC1, and STIM1 with Orai1 in mouse PASMCs.

To confirm the physical interaction of TRPC1 with STIM1, and Orai1 with STIM1 in the contribution of CCE in acute hypoxia, we examined colocalization of these proteins in cultured mouse PASMCs. Figure 12A shows no staining of TRPC1 or STIM1 in the absence of primary antibodies under normoxic condition. Figure 12, B–D, shows three single permeabilized PASMCs labeled with TRPC1 (green) and STIM1 antibody (red). Partial colocalization of TRPC1 and STIM1 (yellow/orange) was found in mouse PASMCs, but independent expression of TRPC1 and STIM1 was also apparent under normoxic condition. In cells exposed to acute hypoxia, no staining of TRPC1 or STIM1 was found in the absence of primary antibodies (Fig. 12E). Acute hypoxia appeared to cause redistribution of TRPC1 and STIM1 from a diffuse pattern to a more visible aggregated or “punctate” appearance at the cell periphery (Fig. 12, F–H). Colocalization of TRPC1 and STIM1 was more apparent (yellow/orange) in cells exposed to acute hypoxia (Fig. 12F, inset). Similarly, no staining of Orai1 or STIM1 was found in the absence of primary antibodies under normoxic condition (Fig. 13A). Figure 13, B–D, shows three single permeabilized PASMCs labeled with Orai1 (green) and STIM1 antibody (red). Partial colocalization of Orai1 and STIM1 (yellow/orange) was found in mouse PASMCs, but independent expression of Orai1 and STIM1 was also apparent under normoxic condition. In cells exposed to acute hypoxia, no staining of Orai1 or STIM1 was found in the absence of primary antibodies (Fig. 13E). Acute hypoxia appeared to cause redistribution of Orai1 and STIM1 to form aggregates or “punctae” at the cell periphery (Fig. 13, F–H). Colocalization of Orai1 and STIM1 was more apparent (yellow/orange) in cells exposed to acute hypoxia (Fig. 13F, inset).

**DISCUSSION**

Over the past 10 years, CCE has gained considerable amount of attention in the study of the mechanisms underlying HPV in...
pulmonary hypertension (16, 25, 30, 40, 50). Although CCE has recently been shown to be mediated by TRPC1, STIM1, and Orai1 in PASMCs (24, 32, 33) and TRPC1 channels are found to be upregulated and mediate CCE in chronic hypoxia (22, 51), there is little evidence for these proteins to play a role in acute hypoxia and the molecular interaction of these proteins in linking store-depletion to the activation of CCE in acute hypoxia remains unknown. Our present study provides the first direct evidence that acute hypoxia causes activation of CCE, which is mediated by the interaction of STIM1 with TRPC1 and Orai1 in mouse PASMCs. We found that acute hypoxia caused intracellular Ca2+ release as indicated by a transient rise in [Ca2+]i in Ca2+-free solution (Fig. 2). Subsequent addition of 2 nM Ca2+ elicited a nifedipine-sensitive and nifedipine-insensitive rise in [Ca2+]i (Fig. 2). In addition, acute hypoxia caused an increase in Mn2+-quench of fura-2 fluorescence and the increase in fura-2 quench rate was inhibited by putative store-operated channel blockers SKF 96365, Ni2+, La3+, and Gd3+ (Fig. 2). These data in mouse PASMCs is consistent with our previous findings in canine PASMCs (31) and another study in rat PASMCs (50) that acute hypoxia causes Ca2+ release from the intracellular Ca2+ stores leading to the activation of CCE. More interestingly, TRPC1 antibody, siRNA knockdown of TRPC1, STIM1, or Orai1, significantly reduced the nifedipine-insensitive rise in [Ca2+]i and the increase in Mn2+-quench of fura-2 fluorescence caused by acute hypoxia (Figs. 3–6). These data confirm that TRPC1, STIM1 and Orai1 play an important role in mediating CCE caused by acute hypoxia in mouse PASMCs. A recent study in rat PASMCs has also shown that knockdown of STIM1 markedly inhibited CCE and abolished the sustained [Ca2+]i response to acute hypoxia (25), supporting our present study that STIM1 mediates CCE in acute hypoxic PASMCs. Nonetheless, we provide the first direct evidence that TRPC1 and Orai1 are also important candidates in mediating CCE in acute hypoxia.

Perhaps the most important finding in the present study is that STIM1 functionally and physically associates with TRPC1 and Orai1 and mediates CCE in mouse PASMCs during acute hypoxia. This is evident from our present finding that overexpression of STIM1 enhanced the increase in [Ca2+]i, and Mn2+-quench rate caused by acute hypoxia, and these responses were reduced in cells treated with TRPC1 antibody (Fig. 7) or Orai1 siRNA (Fig. 8). Moreover, we found that STIM1 coimmunoprecipitated with TRPC1 or Orai1 and the precipitation level of TRPC1 or Orai1 increased after exposure of cells to acute hypoxia (Fig. 11). Furthermore, STIM1 colocalized with}

![Fig. 10. Comparison of the effects of acute hypoxia on overexpressed cells, overexpressed cells treated with TRPC1 antibody-antigen peptide and overexpressed cells transfected with scrambled siRNA. A: bar graph showing no significant difference in acute hypoxia-induced transient and sustained rise in [Ca2+]i, in the presence of 10 μM nifedipine among Ad-GFP cells (filled bars; n = 138), Ad-GFP cells treated with TRPC1 antibody-antigen peptide (shaded bars; n = 59), and Ad-GFP cells transfected with 200 nM scrambled siRNA (open bars; n = 94), P > 0.05 (ANOVA). B: bar graph showing no significant difference in percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine among Ad-GFP cells (filled bar; n = 21), Ad-GFP cells treated with TRPC1 antibody-antigen peptide (shaded bar; n = 104), and Ad-GFP cells transfected with 200 nM scrambled siRNA (open bar; n = 101). P > 0.05 (ANOVA). C: bar graph showing no significant difference in acute hypoxia-induced transient and sustained rise in [Ca2+]i, in the presence of 10 μM nifedipine among Ad-GFP-STIM1 cells (filled bars; n = 155), Ad-GFP-STIM1 cells treated with TRPC1 antibody-antigen peptide (shaded bars; n = 60), and Ad-GFP-STIM1 cells transfected with 200 nM scrambled siRNA (open bars; n = 99). P > 0.05 (ANOVA). D: bar graph showing no significant difference in percent change in fura-2 quench rate after exposure of cells to acute hypoxia in the presence of 10 μM nifedipine among Ad-GFP-STIM1 cells (filled bar; n = 37), Ad-GFP-STIM1 cells treated with TRPC1 antibody-antigen peptide (shaded bar; n = 87), and Ad-GFP-STIM1 cells transfected with 200 nM scrambled siRNA (open bar; n = 99). P > 0.05 (ANOVA).]
TRPC1 and Orai1 in mouse PASMCs under normoxic condition but the colocalizations of STIM1-TRPC1 and STIM1-Orai1 were more apparent after exposure of cells to acute hypoxia (Figs. 12 and 13). These findings are consistent with our previous studies that store-depletion causes formation of STIM1-TRPC1 (32) and STIM1-Orai1 (33) complexes, which mediate CCE in mouse PASMCs. Interestingly, STIM1-TRPC1 and STIM1-Orai1 complexes appear to be redistributed and changed to puncta-like structures at the cell periphery after exposure of cells to acute hypoxia (Fig. 12 and 13, F-H). Thus it is likely that acute hypoxia causes store-depletion, which in turns causes clustering of STIM1 in the SR and translocates into a localized area of the SR close to the plasma membrane. The puncta-like localization of TRPC1 and Orai1 could be due to the clustering of TRPC1 or Orai1 by STIM1 binding at the SR-plasma membrane junction, where the interaction between STIM1-TRPC1 and STIM1-Orai1 complexes causes the activation of CCE (19, 36, 54, 55). These molecular complexes have not been described in acute hypoxia in any pulmonary artery preparations. Here, we provide the first functional and physical evidence for an interaction between STIM1 and TRPC1, and STIM1 and Orai1 in mediating CCE activated by acute hypoxia in mouse PASMCs.

Although the present study reveals the formation of STIM1-TRPC1 and STIM1-Orai1 complexes in mediating CCE activated by acute hypoxia, the question remains on how TRPC1 and Orai1, the two potentially distinct pore-forming proteins, contribute to CCE. Several possible mechanisms have previously been discussed (33): 1) TRPC1-STIM1 and Orai1-STIM1 could function as two distinct channels and contribute independently to CCE (43); 2) Orai1 and TRPC1 form distinct channels, whereby Orai1 regulates the function of TRPC1 (5); and 3) TRPC1, STIM1, and Orai1 form a ternary complex to mediate CCE (5, 17, 20, 35). We would like to propose two possible models for CCE based on our previous findings in mouse PASMCs subjected to store-depletion and our present.
findings in PASMCs exposed to acute hypoxia. In cells subjected to store-depletion, TRPC1 antibody did not affect the transient but inhibited the sustained component of CCE (32), while knockdown of Orai1 protein significantly reduced the transient but not the sustained component of CCE (33). Both transient and sustained components of CCE are reduced by STIM1 knockdown (32, 33). These findings suggest that TRPC1 and Orai1 may be two distinct channels, which interact with STIM1 and contribute independently to CCE. Interestingly, the present study shows that TRPC1 antibody (Fig. 3), TRPC1 siRNA (Fig. 4), STIM1 siRNA (Fig. 5), and Orai1 siRNA (Fig. 6) significantly inhibited both transient and sustained components of CCE, suggesting a tenary complex formed by these proteins to mediate CCE in acute hypoxia. We currently have no direct evidence to support these models, but the discrepancies found in store-depletion and acute hypoxia suggest an involvement of a different signaling mechanism in the activation of SOCs caused by acute hypoxia. One possible mechanism could be that acute hypoxia causes production of reactive oxygen species (ROS) and STIM1 can be activated through cysteine modification induced by ROS (13). Thus it is likely that STIM1 can be modified by ROS during acute hypoxia, which leads to the modification of its interaction with TRPC1 and Orai1 from outside to inside of the lipid raft to form a tenary complex (20, 21, 46). Future studies are warranted to determine if TRPC1 functionally and physically interacts with Orai1 in acute hypoxia. Thus SOCs may exist as different molecular complexes assembled by a combination of TRPC channels, STIM1, and/or Orai proteins (see Ref. 46 for review). Such formation of various molecular complexes of SOCs in the same cell type is not impossible because they may be responsible for different physiological functions depending on how CCE is activated (e.g., store-depletion vs. hypoxia).

Fig. 12. STIM1 colocalizes with TRPC1 in acute hypoxic mouse PASMCs. A–D: staining of mouse cultured PASMCs after exposure of live cells with normoxic solution. A: omission of TRPC1 and STIM1 antibody resulted in no TRPC1 or STIM1 staining. B–D: 3 representative cells dual-labeled with anti-TRPC1 antibody (green) and STIM1 antibody (red). TRPC1 and STIM1 colocalization (yellow/orange) is shown in the merged images. E–H: staining of mouse cultured PASMCs after exposure of live cells with hypoxic solution. E: omission of TRPC1 and STIM1 antibody resulted in no TRPC1 or STIM1 staining. F–H: 3 representative cells dual-labeled with anti-TRPC1 antibody (green) and STIM1 antibody (red). Colocalization of TRPC1 and STIM1 is more apparent (yellow/orange) after exposures the cells to acute hypoxia as shown in the merged images. Nuclei were stained with DAPI (blue). Experiments were performed in 3 separate immunostaining procedures, each with duplicate coverslips. Scale bars = 20 μm.
Despite the importance of CCE in the contribution of hypoxia-induced rise in [Ca\(^{2+}\)], Ca\(^{2+}\) entry through VOCCs was also found to mediate the [Ca\(^{2+}\)] response to acute hypoxia because nifedipine partially inhibited the transient rise in [Ca\(^{2+}\)], caused by acute hypoxia in mouse PASMCs (Fig. 2, A and B). A similar observation has also been reported in canine PASMCs (30, 31). This is not surprising because hypoxia causes membrane depolarization leading to Ca\(^{2+}\) entry through VOCCs has long been considered as a component of HPV in many pulmonary artery preparations (2, 14, 40, 53). It is not our present interest to study the mechanisms underlying these events but our present study that acute hypoxia causes Ca\(^{2+}\) release from the SR and the involvement of TRPC1 in mediating CCE may suggest the following possibilities: 1) hypoxic release of Ca\(^{2+}\) from the SR may inhibit Kv channels, leading to membrane depolarization and subsequent activation of VOCCs (37); 2) Ca\(^{2+}\) release from the SR caused by hypoxia may activate Ca\(^{2+}\)-dependent Cl\(^{-}\) channels, leading to the membrane depolarization and activation of VOCCs (6, 29); and 3) TRPC1 is a nonselective cation channel that also permeable to Na\(^{+}\) besides Ca\(^{2+}\) (4). Na\(^{+}\) entering the cells through TRPC1 may cause depolarization of the membrane potential and result in the activation of VOCCs.

In conclusion, acute hypoxia causes depletion of intracellular Ca\(^{2+}\) stores leading to the activation of CCE and VOCCs. The activation of CCE caused by acute hypoxia requires the functional and physical interaction of STIM1 with TRPC1 and Orai1.

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TRPC1, ORAI1, and STIM1 MEDIATE CCE IN ACUTE HYPOXIC PASMCs

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DISCLOSURES
No conflicts of interest, financial or otherwise, are disclosed by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


