RESEARCH ARTICLE

Hypoxia selectively upregulates cation channels and increases cytosolic \([\text{Ca}^{2+}]\) in pulmonary, but not coronary, arterial smooth muscle cells

Xi He,1,2 Shanshan Song,2 Ramon J. Ayon,2 Angela Balisterieri,2 Stephen M. Black,2,3 Ayako Makino,2,3 W. Gil Wier,2 Wei-Jin Zang,1 and Jason X.-J. Yuan2,3

1Department of Pharmacology, Xi'an Jiaotong University Health Science Center, Xi'an, Shannxi Province, China; 2Division of Translational and Regenerative Medicine, Department of Medicine, The University of Arizona College of Medicine, Tucson, Arizona; and 3Department of Physiology, The University of Arizona College of Medicine, Tucson, Arizona

Submitted 6 November 2017; accepted in final form 2 January 2018

ABSTRACT

Hypoxia selectively upregulates cation channels and increases cytosolic \([\text{Ca}^{2+}]\) in pulmonary, but not coronary, arterial smooth muscle cells. The resting cytosolic \([\text{Ca}^{2+}]\) ([Ca\text{cyt}]\) in pulmonary, but not coronary, arterial smooth muscle cells. The resting cytosolic \([\text{Ca}^{2+}]\) ([Ca\text{cyt}]\) and the stored \([\text{Ca}^{2+}]\) in the sarcoplasmic reticulum were not different in CASMC and PASMC. Seahorse measurement showed a similar level of mitochondrial bioenergetics (basal respiration and ATP production) between CASMC and PASMC. Glycolysis was significantly higher in PASMC than in CASMC. The amplitudes of cyclopiazonic acid-induced SOCE and OAG-induced ROCE in CASMC are slightly, but significantly, greater than in PASMC. The frequency and the area under the curve of \([\text{Ca}^{2+}]\) oscillations induced by ATP and histamine were also larger in CASMC than in PASMC. \(\text{Na}^+\)/\([\text{Ca}^{2+}]\) exchanger-mediated increases in \([\text{Ca}^{2+}]\)\text{cyt} did not differ significantly between PASMC and CASMC. The baseline protein expression levels of STIM1/STIM2, Orai1/Orai2, and TRPC6 and increased the \([\text{Ca}^{2+}]\)\text{cyt} due to \([\text{Ca}^{2+}]\)\text{cyt} in vascular smooth muscle cells. An increase in \([\text{Ca}^{2+}]\)\text{cyt} in smooth muscle cells is a key factor in the initiation and maintenance of vascular contraction, and a decrease in \([\text{Ca}^{2+}]\)\text{cyt} due to \([\text{Ca}^{2+}]\)\text{cyt} is vascular smooth muscle cell proliferation and migration (47, 59, 65). In PASMC, \([\text{Ca}^{2+}]\)\text{cyt} is elevated by 1) \([\text{Ca}^{2+}]\) influx through \([\text{Ca}^{2+}]\)\text{cyt}-permeable channels in the plasma membrane, 2) \([\text{Ca}^{2+}]\) release from the intracellular stores (e.g., sarcoplasmic reticulum), and 3) inward \([\text{Ca}^{2+}]\) transport by plasmalemmal \(\text{Na}^+\)/\([\text{Ca}^{2+}]\) exchangers when intracellular \([\text{Na}^+]\) concentration is raised (28, 33, 65, 67, 68). Based on the proposed models of activation, the \([\text{Ca}^{2+}]\)\text{cyt}-permeable channels responsible for \([\text{Ca}^{2+}]\)\text{cyt} influx are classified into three categories: 1) voltage-dependent \([\text{Ca}^{2+}]\)\text{cyt}...
channels (VDCC), which are primarily opened by membrane depolarization; 2) store-operated Ca\(^{2+}\) channels (SOCC), which are activated by active and passive depletion of Ca\(^{2+}\) from the intracellular stores (e.g., the sarcoplasmic or endoplasmic reticulum); and 3) receptor-operated Ca\(^{2+}\) channels (ROCC), which are activated by ligand binding to membrane receptors (23, 43, 47).

The canonical transient receptor potential (TRPC) channels, such as TRPC6, are the major components of ROCC responsible for receptor-operated Ca\(^{2+}\) entry (ROCE) (29). Recent evidence suggests that stromal interacting molecule proteins (e.g., STIM1/2) and Orai1/2 channels are the major components of SOCC leading to store-operated Ca\(^{2+}\) entry (SOCE) (9–11). Some reports also indicate that TRPC channels contribute to forming SOCC and regulating SOCE (5, 35). That Ca\(^{2+}\) signaling plays a critical role in a wide range of cell functions underscores the importance of understanding the mechanisms involved in regulating Ca\(^{2+}\) homeostasis in pulmonary (PASMC) and systemic or coronary arterial smooth muscle cells (CASMC) under both physiologic and pathophysiologic conditions.

Accumulating evidence suggests that increased \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC is the major determinant contributing to pulmonary vasoconstriction induced by acute hypoxia and pulmonary vascular wall thickening (due to PASMC proliferation) induced by chronic hypoxia (28). Studies showed that the increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC exposed to acute hypoxia was due to many mechanisms including 1) membrane depolarization-induced opening of VDCC, 2) active store depletion-induced SOCE through SOCC or STIM/Orai channels, and 3) agonist-mediated ROCE through ROCC or TRPC channels (51, 54). The elevated resting \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC and the pulmonary arterial tone of chronically hypoxic rats can be reduced to the control level by antagonists of nonselective cation channels and blockers of VDCC. Chronic hypoxia not only downregulates voltage-gated K\(^+\) channels, leading to membrane depolarization and activation of VDCC (40), but also upregulates Cav1.2 and Cav3.2 channels to enhance Ca\(^{2+}\) influx through L- and T-type VDCC (53). Furthermore, Ca\(^{2+}\) entries via store- and receptor-operated channels are also crucial for the altered Ca\(^{2+}\) homeostasis in acute hypoxic pulmonary vasoconstriction and chronic hypoxic pulmonary hypertension (29). Enhanced SOCE and ROCE are believed to be responsible for the chronic hypoxia-mediated increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC, which subsequently results in sustained pulmonary vasoconstriction and concentric pulmonary arterial wall thickening (due to enhanced PASMC proliferation and migration).

Although it induces pulmonary vasoconstriction, acute hypoxia causes systemic (e.g., coronary, cerebral and renal) vasoconstriction (32). Chronic hypoxia, or prolonged exposure to hypoxia, also causes different responses between pulmonary and systemic (coronary, cerebral, and mesenteric) arterial smooth muscle cells (4, 7, 66). The distinct changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by hypoxia are closely correlated with the diversity of hypoxic contractile responses in pulmonary and systemic arterial smooth muscle cells. Hypoxia triggered increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC, whereas it resulted in decreases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in renal (systemic) artery smooth muscle cells (57). Likewise, hypoxia can increase \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC, but not in mesenteric artery smooth muscle cells (42). Thus, hypoxia may differentially regulate selected proteins that participate in forming SOCC and ROCC, leading to diverse Ca\(^{2+}\) and contractile responses in pulmonary and systemic artery smooth muscle cells. In this study, we investigated and compared the basic expression and function of various cation channels and transporters, or the machinery of Ca\(^{2+}\) signaling cascade in human CASMC and PASMC, and their response to hypoxia. These data provide important information for our understanding of the molecular mechanisms responsible for the hypoxia-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in PASMC and the diversity of the hypoxic response between pulmonary and coronary artery smooth muscle cells. These data may also help to provide insight into developing novel therapeutic targets for pulmonary hypertension.

**METHODS AND MATERIALS**

**Cell culture and hypoxia treatment.** Human pulmonary artery smooth muscle cells (PASMC) and coronary artery smooth muscle cells (CASMC) were purchased from Lonza (Walkersville, MD). The cells were initially isolated from normal subjects who were of the same sex and race. PASMC and CASMC were cultured at 37°C under a humidified 5% CO\(_2\) atmosphere in the smooth muscle cell growth medium (SmGM) including the smooth muscle cell basal medium (SmBM, CC-3181, Lonza) supplemented with SmGM-2 SingleQuots (CC-4149, Lonza). Cells grown at passage 5–8 were used in the experiments. For in vitro hypoxic experiments, PASMC and CASMC were cultured in an incubator equilibrated with 3% O\(_2\) (in N\(_2\)), while control cells were cultured in an incubator equilibrated with room air (21% O\(_2\)).

\([\text{Ca}^{2+}]_{\text{cyt}}\) measurements. \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements were performed as described previously (46). Briefly, human PASMC and CASMC were grown to confluence on 25-mm round glass coverslips. The cells were loaded with 4 μM fura-2 acetoxyethyl ester (fura-2/AM; Invitrogen/Molecular Probes, Eugene, OR) in the dark for 60 min at room temperature (22–24°C) in normal physiological salt solution (PSS). The PSS contained 140 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 10.0 mM glucose, and 10.0 mM HEPES. A coverslip containing fura-2/AM-loaded cells was placed in a recording chamber mounted on the stage of the Nikon inverted fluorescence microscope (Eclipse Ti-E; Nikon, Tokyo, Japan). The excitation wavelengths were 340 nm and 380 nm, and the emission signal at 520 nm was detected using an EM-CCD camera (Evolve; Photometrics, Tucson, AZ), a Nikon S-Plan Fluor ELWD ×20/0.45 objective lens and NIS Elements 3.2 software (Nikon). \([\text{Ca}^{2+}]_{\text{cyt}}\) within the region of interest (5 × 5 μm), which was positioned at the peripheral region of each cell, was measured as the ratio of fluorescence intensities \(R_{380}/R_{340}\). The following equation was used to calculate the lifetime ratio: \([\text{Ca}^{2+}]_{\text{cyt}} = K_d \times (S_{380}/S_{340}) \times (R - R_{\text{min}})/(R_{\text{max}} - R)\), where \(K_d\) (225 nM) is the dissociation constant of fura-2 for Ca\(^{2+}\) and \(S_{380}\) and \(S_{340}\) are the fluorescent intensities at 380-nm excitation in Ca\(^{2+}\)-free (with EGTA) and Ca\(^{2+}\)-saturated (using ionomycin) solutions, respectively. \(R\) is the measured fluorescence ratio, while \(R_{\text{min}}\) and \(R_{\text{max}}\) are the minimal and maximal ratios that were determined in cells superfused with the Ca\(^{2+}\)-free solution (plus 5 mM EGTA) with 2 μM ionomycin and the bath solution containing 11 mM CaCl\(_2\), respectively. In some ex-

_AJP-Cell Physiol • doi:10.1152/ajpcell.00272.2017 • www.ajpcell.org_
perperiments, Ca²⁺ signals (R/R₀) were determined as the ratio of fluorescence intensities (R) divided by the average baseline ratio (R₀) (10, 47). In Ca²⁺-free solution, CaCl₂ was replaced by equimolar MgCl₂, and 0.1 mM EGTA was added to chelate residual Ca²⁺. All experiments for measurement of [Ca²⁺]ₘ回味 were performed at room temperature (22–24°C).

Protein preparation and Western blotting. Human PASMC and CAMSC were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysate was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatants were used as sample protein. An equal amount of proteins (30 μg) from each cell type was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Munich, Germany). After blocking with 5% skim milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated at 4°C overnight with primary antibody against STIM1 (diluted 1:1,000; catalog no. 4119, Prosci, Poway, CA) (62), STIM2 (diluted 1:1,000; catalog no. S8572, Sigma, St. Louis, MO) (48), Orai1 (diluted 1:10,000; catalog no. ACC-060, Alomone, Jerusalem, Israel) (8, 69), Orai2 (diluted 1:1,000; catalog no. ACC-061, Alomone) (18), and TRPC6 (diluted 1:1,000; catalog no. ACC-120, Alomone) (27). The membranes were then incubated with the appropriate horseshadish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) at 1:5,000 dilution for 30 min at room temperature. The protein level was normalized to β-actin (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). The gel bands were visualized with Amersham ECL prime Western blotting detection reagent (GE Healthcare, Little Chalfont, UK), and then the band density was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence. Human PASMC and CAMSC were seeded onto 14-mm coverslips at a density of 2 × 10⁴ cells per coverslip. After the hypoxia treatment, the cells were washed twice with PBS and fixed with 4% formaldehyde solution for 20 min at room temperature. Cells were then washed twice with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Unspecific binding was reduced by blocking with 5% bovine serum albumin (BSA; Sigma) in PBS for 60 min. Cells were incubated overnight at 4°C with a mouse monoclonal anti-Orai1 antibody (1:100, catalog no. ALM-025, Alomone) (34) and rabbit polyclonal anti-STIM2 antibody (1:200; catalog no. ab59342, Abcam, Cambridge, MA) (3). After incubation, cells were extensively washed with 1% BSA in PBS for 10 min 3 times. The cells were then incubated with secondary antibodies with Hoechst (1:1,000, Thermo Scientific) in 1% BSA in PBS for 60 min at room temperature. A goat anti-mouse IgG highly cross-adsorbed secondary antibody conjugated with Alexa Fluor 594 (1:1,000, Thermo Scientific) was used to display Orai1 fluorescence image, and donkey anti-rabbit IgG highly cross-adsorbed secondary antibody conjugated with Alexa Fluor 488 (1:1,000, Thermo Scientific) was used to display STIM2 fluorescence image.

Statistical analysis. Data are expressed as means ± SE. Statistical significance between two or among multiple groups was performed using Student’s t-test or one-way analysis of variance followed by Tukey’s multiple comparison post hoc test, respectively. Differences were considered to be significant when P < 0.05.

RESULTS

Resting [Ca²⁺]ₘ回味 is comparable in CAMSC and PASMC. We first compared the resting [Ca²⁺]ₘ回味 level between human CAMSC and PASMC. As shown in Fig. 1, A and B, the resting [Ca²⁺]ₘ回味 was not significantly different between CAMSC (85.07 ± 0.71 nM) and PASMC (89.80 ± 0.94 nM) cultured under the same conditions. The histogram of the resting [Ca²⁺]ₘ回味 in CAMSC and PASMC showed a similar distribution with almost overlapped fitting curves in both cell types (Fig. 1A).
Basal metabolism between CASMC and PASMC. In CASMC and PASMC, OCR was measured and compared with estimate mitochondrial bioenergetics. Our data identified a similar level of basal respiration between CASMC and PASMC (Fig. 1C). In addition, there was no significant difference in the decrease in OCR between CASMC and PASMC exposed to oligomycin (Fig. 1C). Extracellular acidification rates (ECAR) were monitored to reflect glycolysis. There was no difference in the distribution of the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} in CASMC (blue bars) and PASMC (red bars). The peak values of the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} based on the fitting curves are 85.07 nM and 89.8 nM in coronary (CASMC) and pulmonary (PASMC) artery smooth muscle cells, respectively. A: histogram showing the distribution of the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} in CASMC (blue bars) and PASMC (red bars). The peak values of the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} based on the fitting curves are 85.07 nM and 89.8 nM in coronary (CASMC) and pulmonary (PASMC) artery smooth muscle cells, respectively. B: summarized data (means ± SE) showing the basal mitochondrial respiration and the O\textsubscript{2} consumed for ATP production in CASMC and PASMC (30,000/0.275 cm\textsuperscript{2}). Summarized data (means ± SE) showing the basal glycolytic rate (ECAR, mpmh/min) in CASMC and PASMC (30,000/0.275 cm\textsuperscript{2}). D-glucose (Gluc, 2 mg/ml), oligomycin (Oligo, 1 μM), and 2-deoxy-D-glucose (2-DG, 100 nM) were added at the time indicated by arrows. E: summarized data (means ± SE) showing the basal glycolytic rate (left) determined as the difference between basal ECAR and that obtained in the presence of D-glucose and the maximal glycolytic capacity (right) determined as the difference between the obtained in the presence of oligomycin and that after the addition of 2-DG (n = 20 measurements). *P < 0.05 vs. CASMC.

The changes in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to CPA-induced SOCE and OAG-induced ROCE in CASMC and PASMC. We first measured and compared the amplitude of increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to SOCE in CASMC and PASMC. In the absence of extracellular Ca\textsuperscript{2+} (0Ca), blockade of the sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) Ca\textsuperscript{2+}-pump (SERCA) with cyclopiazonic acid (CPA) (10 μM) induced a transient increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to Ca\textsuperscript{2+} leakage from the SR in CASMC and PASMC. The amplitude of CPA-induced transient increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} reflects the level of [Ca\textsuperscript{2+}]\textsuperscript{-} stored in the SR, especially the IP\textsubscript{3}-sensitive SR stores. Stored [Ca\textsuperscript{2+}]\textsuperscript{-} in the SR, or the amplitude of CPA-induced transient increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in the absence of extracellular Ca\textsuperscript{2+}, seemed to be comparable in CASMC and PASMC (Fig. 2, A and B). Then we measured and compared the amplitude of SOCE by determining the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} upon reintroduction of 1.8 mM external Ca\textsuperscript{2+} in the presence of CPA. We observed that the second increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to SOCE (upon extracellular Ca\textsuperscript{2+} restoration) in CASMC was slightly, but statistically significantly, greater than in PASMC (Fig. 2, A and B). We also determined the maximum change of [Ca\textsuperscript{2+}]\textsubscript{cyt} in Ri/Ro, which displayed a similar trend (Fig. 2Bb).

Extracellular application of the membrane-permeable diacetylglycerol (DAG) analogue, 1-oleoyl-2-acyetyl-sn-glycerol (OAG, 100 μM), can directly activate ROCC and induce ROCE. The amplitude of OAG-induced ROCE in CASMC was significantly higher compared with PASMC, with Ri/Ro also exhibiting a similar trend (Fig. 2, Bb and Dd). Furthermore, the area under the curve (AUC) of the OAG-mediated increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}, which represents the total amount of Ca\textsuperscript{2+} that enters the cells, was significantly greater in CASMC than in PASMC (Fig. 2, C and D). The proportion of responsive cells in CASMC was also higher than in PASMC (Fig. 2D). These results indicate that the amplitudes of CPA-induced SOCE and the OAG-induced ROCE in CASMC were greater than in PASMC.

ATP-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} is greater in CASMC than in PASMC. ROCE was also evaluated by changes in [Ca\textsuperscript{2+}]\textsubscript{cyt}, upon exposure to the receptor agonist ATP. ATP binds to purinergic receptors, stimulating phospholipase C to produce IP\textsubscript{3} and DAG. DAG is a known second messenger that directly opens ROCC to induce ROCE, while IP\textsubscript{3} activates the IP\textsubscript{3} receptors in the SR, thus producing Ca\textsuperscript{2+} release and store depletion causing SOCE. Of the responding cells, we observed three patterns of ATP-mediated increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} in CASMC and PASMC: 1) a transient increase (pattern 1), 2) a transient increase followed by a plateau or sustained increase (pattern 2), and 3) an oscillatory increase (pattern 3) (Fig. 3A). The amplitude of ATP-induced increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} showed no significant difference between CASMC and PASMC, while the AUC and frequency of Ca\textsuperscript{2+} oscillations induced by ATP were more significant in CASMC than in PASMC (Fig. 3B). Patterns 1, 2, and 3 changes in [Ca\textsuperscript{2+}]\textsubscript{cyt} were analyzed together for calculation of AUC. Most of the CASMC (60%)
and PASMC (67%) exhibited pattern 2 increases in $[Ca^{2+}]_{cyt}$ in response to extracellular application of ATP (Fig. 3C); the proportion of responsive cells in CASMC was also higher than in PASMC (Fig. 3D). The dose-response curves of ATP-induced increases in $[Ca^{2+}]_{cyt}$ showed significant differences between CASMC and PASMC (Fig. 3E). In CASMC, ATP increased $[Ca^{2+}]_{cyt}$ at the dose range of 10^{-8} to 10^{-7} M and the estimated EC_{50} was ~0.2 μM (Fig. 3E). In PASMC, ATP increased $[Ca^{2+}]_{cyt}$ at the concentration ranging from 10^{-6} to 10^{-5} M with an estimated EC_{50} of 15 μM (Fig. 3E). These data indicate that CASMC are more sensitive to ATP than PASMC.

**Histamine-induced increase in $[Ca^{2+}]_{cyt}$ in CASMC and PASMC.** ROCE was also evaluated by changes in $[Ca^{2+}]_{cyt}$ upon exposure to the receptor agonist histamine. Similar to the ATP-mediated changes, there are three patterns of the increases in $[Ca^{2+}]_{cyt}$ in response to histamine in CASMC and PASMC: 1) a transient increase (pattern 1), 2) a transient increase followed by a plateau or sustained increase (pattern 2), and 3) an oscillatory increase (pattern 3) (Fig. 3F). The histamine-mediated increase in $[Ca^{2+}]_{cyt}$ (in terms of both amplitude and AUC) in CASMC is greater than in PASMC (Fig. 3G). More CASMC (72%) exhibited pattern 2 increases in $[Ca^{2+}]_{cyt}$ in response to extracellular application of histamine than PASMC (48%) (Fig. 3H); the percentage of responsive cells in CASMC (97%) was also higher than in PASMC (80%) (Fig. 3I). These results imply that CASMC are more sensitive to histamine than PASMC.

The increase in $[Ca^{2+}]_{cyt}$ due to Na^+/Ca^{2+} exchange in CASMC and PASMC. The activity of Na^+/Ca^{2+} exchangers (NCX) in the plasma membrane depends predominantly on the transmembrane Na^+ gradient. Removal of extracellular Na^+ reverses the transmembrane Na^+ gradient to favor Na^+ extrusion and Ca^{2+} entry via the reverse mode of Na^+/Ca^{2+} exchange. As shown in Fig. 4A, removal of extracellular Na^+ using an equimolar concentration NMDG^+ resulted in two patterns of increases in $[Ca^{2+}]_{cyt}$: 1) a transient increase (pattern 1) and 2) an oscillatory increase (pattern 3). The amplitude of the transient increase in $[Ca^{2+}]_{cyt}$ (pattern 1) and the frequency of the oscillatory increase in $[Ca^{2+}]_{cyt}$ (pattern 3) due to the inward transportation of Ca^{2+} through NCX were not different between CASMC and PASMC; however, the AUC, an indicator of the total amount of Ca^{2+} entering the cell, of the NCX-associated inward transportation of Ca^{2+} in CASMC was significantly larger than in PASMC (Fig. 4B). More CASMC (80%) exhibited Ca^{2+} oscillations (pattern 3) when extracellular Na^+ was removed than PASMC (60%) (Fig. 4C).

The local increase in $[Ca^{2+}]_{cyt}$ due to inward Ca^{2+} transporation via the reverse mode of NCX may trigger the Ca^{2+}-induced Ca^{2+} release (CICR) by activating ryanodine receptors (RYR) on the SR membrane. Therefore, the increase in $[Ca^{2+}]_{cyt}$ by removal of extracellular Na^+ is composed of both inward Ca^{2+} transportation due to NCX and CICR. To specifically determine and compare the increases in $[Ca^{2+}]_{cyt}$ via inward Ca^{2+} transportation through NCX, we repeated the experiments described above in the presence of 50 μM ryanodine (which inhibits RYR at this dose). As shown in Fig. 4, E–H, the amplitude and AUC of the NCX-associated transient increases in $[Ca^{2+}]_{cyt}$ and the frequency of the NCX-associated oscillatory increases in $[Ca^{2+}]_{cyt}$ were comparable in CASMC and PASMC (Fig. 4, E and F). The percentage of cells exhibiting pattern 1 (transient increase) and pattern 3 (oscillatory increase) changes of $[Ca^{2+}]_{cyt}$ is also similar in CASMC and PASMC (Fig. 4G); the percentage of responsive cells, however, was higher in CASMC than in PASMC (Fig. 4H).
The area under the curve (AUC) of RyR-associated increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) was determined by subtracting the AUC of the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by removal of extracellular Na\(^+\) from the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) induced by ryanodine. The increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) by inward \text{Ca}^{2+}\) transpor- tation via NCX was comparable in CASMC and PASMC, while the AUC in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \text{Ca}^{2+}-induced \text{Ca}^{2+}\) release (CICR) via RyR was greater in CASMC than in PASMC. These data suggest that, by inhibition of CICR through RyR, CASMC and PASMC exhibit similar inward \text{Ca}^{2+}\) transportation via NCX. It is possible that RyR is more active or the expression level of RyR is greater in CASMC than in PASMC.

**Hypoxia upregulates protein expression of SOCC and ROCC in PASMC, but not in CASMC.** We examined and compared the protein expression level of STIM1/STIM2 and Orai1/Orai2, which are believed to form SOCC responsible for SOCE, as well as the level of TRPC6, which contributes to forming ROCC, in normoxic control CASMC/PASMC with hypoxic CASMC/PASMC. Consistent with the functional experiments (Fig. 2), our Western blot experiments showed that protein expression levels of STIM1/STIM2, Orai1/Orai2 and TRPC6 were significantly higher in CASMC than in PASMC (Fig. 5, A and B). These results indicate that the basal expression of the proteins participating in the formation of SOCC (e.g., STIM1/2 and Orai1/2) and ROCC (e.g., TRPC6) is higher in CASMC than in PASMC. The Western blot experimental data are consistent with the functional data showing that the amplitudes of CPA-induced SOCE and OAG-induced ROCE are greater in CASMC than in PASMC.

Prolonged hypoxia (for 72 h) markedly upregulated the protein expression of STIM1/STIM2, Orai1/Orai2, and TRPC6.
in PASMC, but not in CASMC (Fig. 5, C and D). These data show that exposure to hypoxia selectively increases expression of proteins involved in SOCE and ROCe in PASMC, but not in CASMC. The hypoxia-induced upregulation of STIM1/2, Orai1/2, and TRPC6 may be one of the unique characteristics of PASMC, in comparison to CASMC.

**Effect of prolonged hypoxia on STIM2 and Orai1 immunofluorescence in CASMC and PASMC.** We also determined and compared the protein expression levels of STIM2 and Orai1 using immunofluorescence assay. The fluorescence intensities of STIM2 and Orai1 are shown in Fig. 6, A and B, which was consistent with the Western blot results shown in Fig. 5, C and D. Chronic or prolonged hypoxia significantly increased the protein expression level of STIM2 and Orai1 in PASMC, but there was no significant difference in the fluorescence intensity of STIM2 and Orai1 between normoxic and hypoxic CASMC. As shown in Fig. 6C, line scans also showed a marked increase in the fluorescence intensities of STIM2 and Orai1 in PASMC exposed to hypoxia (in comparison to normoxic control PASMC), but not in CASMC exposed to hypoxia (in comparison to normoxic control CASMC). The line scan region is indicated in Fig. 6A. STIM2 is a Ca\(^{2+}\) sensor expressed in the SR/ER membrane that is mainly involved in the regulation of the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) and ER Ca\(^{2+}\) levels (8). Previous studies have suggested that compared with STIM1, STIM2 is more sensitive to small changes in the ER [Ca\(^{2+}\)] due to its lower affinity for Ca\(^{2+}\) (6, 38). Ong et al. (38) have demonstrated that STIM2 promotes STIM1 clustering and recruits STIM1 to ER-PM junctions at low stimulus intensities when ER Ca\(^{2+}\) depletion is minimal, thus enhancing interaction with Orai1 and activating SOCE, suggesting that STIM2 is a critical protein that determines STIM1 clustering. Moreover, Orai1 is the main and essential pore-forming subunit of SOCC (41). The next set of experiments was designed to test whether the hypoxia-induced upregulation of STIM2 and Orai1 in PASMC would increase the basal [Ca\(^{2+}\)]\(_{\text{cyt}}\).

**Hypoxia results in significant increase in the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC, but not in CASMC.** We measured and compared the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) in normoxic CASMC/PASMC and hypoxic CASMC/PASMC. Exposure of CASMC to hypoxia had no significant effect on the basal [Ca\(^{2+}\)]\(_{\text{cyt}}\) level (normoxia: 88.53 ± 1.63 nM; hypoxia: 90.95 ± 1.17 nM) (Fig. 7, A and B). There was no shift in the histogram showing the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) distribution in hypoxic CASMC compared with normoxic CASMC (Fig. 7A). In contrast, the basal [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC was significantly increased by exposure to hypoxia (Fig. 7, C and D). The histogram showing the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) distribution in hypoxic PASMC was shifted to the right in
The results from this study demonstrate that: 1) The basal $[\text{Ca}^{2+}]_{\text{cyt}}$ was comparable in CASMC and PASMC under resting conditions; 2) the mitochondrial bioenergetics (e.g., basal respiration and ATP production) were also similar between CASMC and PASMC; 3) glycolysis was significantly higher in PASMC than in CASMC; 4) SOCE/ROCE is slightly, but significantly, greater in CASMC than in PASMC under normoxic conditions, which was due apparently to a higher expression of STIM1/2, Orai1/2, and TRPC6 in CASMC than in PASMC; 5) the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ by inward $\text{Ca}^{2+}$ transpor- tation via $\text{Na}^+/$$\text{Ca}^{2+}$ exchangers was comparable in CASMC and PASMC, while the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release via RyR was greater in CASMC than in PASMC; and 6) most interestingly, hypoxia had a negligible effect on protein expression of SOCC/ROCC (STIM1/STIM2, Orai1/Orai2, and TRPC6) and the resting $[\text{Ca}^{2+}]_{\text{cyt}}$ in CASMC, but significantly upregulated SOCC/ROCC and increased the resting $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC. Collectively, these data imply that it is a unique intrinsic characteristic or feature for PASMC to respond to hypoxia by upregulating cation channels responsible for SOCE/ROCE. These data also provide one of the important explanations why hypoxia causes pulmonary vasoconstriction and induces pulmonary vascular remodeling, but causes coronary vasodilation.

The pulmonary circulation is a high-flow, low-resistance, and low-pressure system; the pulmonary arterial pressure is typically about one-fifth to one-sixth of the systemic arterial pressure. The primary function of the pulmonary artery is to deliver the deoxygenated venous blood to the lung for gas exchange or reoxygenation of the venous blood. The primary function of the coronary artery is to deliver the oxygenated arterial blood to cardiomyocytes (17, 50, 71). Thus, tissue hypoxia causes coronary vasodilation to increase blood flow to provide more oxygen and nutrients to heart muscle for maintaining heart function, whereas alveolar hypoxia causes pulmonary vasoconstriction to maintain an optimal ventilation/perfusion match for maximal oxygenation of the venous blood (15, 56, 57). Prolonged alveolar hypoxia, in patients with chronic obstructive lung disease or inhabitants living at high altitude, can cause sustained pulmonary vasoconstriction and pulmonary vascular remodeling, and ultimately pulmonary hypertension. Thus, the mechanisms by which hypoxia induces pulmonary vasoconstriction and coronary vasodilation are therefore an active area of research. There are, however, still no adequate explanations for the mechanisms responsible for hypoxia-induced pulmonary vasoconstriction and coronary vasodilation.

An increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC due to $\text{Ca}^{2+}$ entry through $\text{Ca}^{2+}$ channels and/or transporters in the plasma membrane is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC migration and proliferation that contribute to pulmonary vascular remodeling. A sustained increase in the resting $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC is one of the indispensable prerequisites for enhancing PASMC proliferation in patients with idiopathic pulmonary arterial hypertension and animals with hypoxia-induced pulmonary hypertension. Recent evidence suggests that STIM1-mediated recruitment of
Orai1/2 forms SOCC in the plasma membrane and induces SOCE. Active depletion of intracellular Ca\(^{2+}\) stores, such as the IP\(_3\)-sensitive SR, by agonists or growth factors that stimulate IP\(_3\) synthesis, or passive depletion of the SR by inhibiting the SR Ca\(^{2+}\)/H\(^{1001}\) pump with CPA, promotes STIM1 to polymerize and then translocate to the puncta area, where STIM1 interacts with Orai1 and facilitates to form tetrameric Orai1 channels to induce SOCE (9, 11, 14, 36). STIM2 shares similar structure with STIM1, but the sensitivity of STIM2 to changes of intracellularly stored [Ca\(^{2+}\)/H\(^{1001}\)] in the SR is much greater than that of STIM1 (8, 70). Therefore, a very small decrease in [Ca\(^{2+}\)/H\(^{1001}\)] in the SR is sufficient to activate STIM2 and ultimately stimulate Ca\(^{2+}\)/H\(^{1001}\) entry through Orai1/2 channels. It has therefore been proposed that STIM1 is mainly involved in the regulation of store depletion-mediated Ca\(^{2+}\)/H\(^{1001}\) entry, while STIM2 is predominantly involved in regulating the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) (8).

TRPC6 forms homotetrameric or heterotetrameric cation channels that are activated by DAG upon ligand-mediated activation of G protein-coupled receptors (GPCR) and tyrosine kinase receptors (21, 24). TRPC6-formed ROCCs can also be opened by the membrane-permeable DAG analogue, OAG, or by other signaling messengers involved in the G protein signal pathway (29). Knockdown of TRPC6 with siRNA decreased DAG-induced cation influx in rat PASMC (29). ATP and histamine can bind to the related GPCR to activate phospholipase C to produce DAG and IP\(_3\), which act synergistically to promote Ca\(^{2+}\)/H\(^{1001}\) entry through ROCC and SOCC. In the present study, we determined the basic expression and function of various cation channels and transporters, or the machinery of Ca\(^{2+}\) signaling cascade, in human CASMC and PASMC. The data showed that the amplitudes of CPA-induced SOCE and OAG-induced ROCE in CASMC are slightly, but significantly, greater than in PASMC. The frequency and the area under the curve (AUC) of Ca\(^{2+}\)/H\(^{1001}\) oscillations induced by ATP were larger in CASMC than in PASMC. Histamine-induced increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (both amplitude and AUC) was also greater in CASMC than in PASMC. The greater increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) to Ca\(^{2+}\)/H\(^{1001}\) entry through ROCC and SOCC are likely due to the slightly higher expression of TRPC6 (ROCC) and STIM1/Orai1/2 (SOCC) in CASMC than in PASMC. The results from

---

**Fig. 6.** Hypoxia increases protein level (determined by immunofluorescence intensity) of STIM2 and Orai1 only in PASMC, but not in CASMC. A and B: representative images (A) and summarized data (B, means ± SE) showing STIM2 (green) and Orai1 (red) level in CASMC (top) and PASMC (bottom) under normoxic (Nor) and hypoxic (Hyp) conditions. The cells were dual-labeled with the anti-STIM2 antibody (green) and Orai1 antibody (red). Nuclei were stained with Hoechst (blue). Experiments were performed in 4 separate immunostaining procedure. **P < 0.01 vs. Nor. C: line scans showing the distribution of green (STIM2) and red (Orai1) fluorescence intensity among selected CASMC (left) and PASMC (right) under normoxic (Nor, blue lines) and hypoxic (Hyp, red lines). The line scan region in the field is shown as a broken white line in each image panel.
between two groups was evaluated using Student's t-test. ***P < 0.001 vs. Nor.

A: histogram showing the distribution of the resting [Ca\textsuperscript{2+}]\text{cyt} in normoxic (dark green bars) and hypoxic (dark red bars) CASMC. The peak values of the resting [Ca\textsuperscript{2+}]\text{cyt} based on the fitting curves are 88.53 nM and 90.95 nM in normoxic (Nor, n = 233 cells from 6 coverslips) and hypoxic (Hyp, n = 371 cells from 9 coverslips) CASMC, respectively. B: summarized data (means ± SE) showing the averaged resting [Ca\textsuperscript{2+}]\text{cyt} in normoxic (n = 233 cells from 6 coverslips) and hypoxic (n = 371 cells from 9 coverslips) CASMC. C: histogram showing the distribution of the resting [Ca\textsuperscript{2+}]\text{cyt} in Nor (dark green bars) and Hyp (dark red bars). The peak values of the resting [Ca\textsuperscript{2+}]\text{cyt} based on the fitting curves are 90.43 nM and 146.78 nM in normoxic (n = 281 cells from 8 coverslips) and hypoxic (n = 281 cells from 8 coverslips) PASMC, respectively. D: summarized data (means ± SE) showing the averaged resting [Ca\textsuperscript{2+}]\text{cyt} in normoxic (n = 320 cells from 8 coverslips) and hypoxic (n = 281 cells from 8 coverslips) PASMC. Statistical significance between two groups was evaluated using Student’s t-test. ***P < 0.001 vs. Nor.

this study also demonstrated that human CASMC and PASMC functionally express Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers. The increase in [Ca\textsuperscript{2+}]\text{cyt} due to the inward transportation of Ca\textsuperscript{2+} through Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers is not significantly different between CASMC and PASMC under the resting conditions.

Intriguingly, one of the major differences between CASMC and PASMC is their response to hypoxia: prolonged hypoxia had a negligible effect on protein expression of SOCC (STIM1/Orai1/2) and ROCC (TRPC6) or the resting [Ca\textsuperscript{2+}]\text{cyt} in CASMC, but it significantly upregulated SOCC and ROCC and increased the resting [Ca\textsuperscript{2+}]\text{cyt} in PASMC. A wealth of data has been accumulated suggesting that STIM1 and Orai1/2 contribute to form SOCC responsible for SOCE and are upregulated in PASMC from patients with idiopathic pulmonary arterial hypertension and animals with experimental pulmonary hypertension, in comparison to controls (9, 11, 46). Our in vitro data showing that hypoxia increases the resting [Ca\textsuperscript{2+}]\text{cyt} in PASMC, but not in CASMC, are consistent with the previous reports demonstrating the increased basal [Ca\textsuperscript{2+}]\text{cyt} in hypoxic rat PASMC (29).

SOCE is one of the Ca\textsuperscript{2+} entry pathways responsible for the elevated basal [Ca\textsuperscript{2+}]\text{cyt} in chronically hypoxic PASMC (29). There is growing evidence supporting the pivotal role of TRPC6 in acute hypoxia-induced pulmonary vasoconstriction (13, 58) and chronic hypoxia-induced pulmonary hypertension (29). Upregulation of TRPC6 and enhanced Ca\textsuperscript{2+} entry through ROCC have also been found in PASMC exposed to chronic hypoxia; knockdown of TRPC6 with siRNA attenuates the hypoxia-induced enhancement of ROCE (29, 39). TRPC6 mediates agonist-induced ROCE in vascular smooth muscle cells (20, 25). The TRPC6-formed ROCC appears to be expressed in systemic (e.g., coronary) vascular smooth muscle cells (19, 56); however, the protein expression cannot be upregulated by hypoxia. This is likely one of the reasons why hypoxia fails to increase [Ca\textsuperscript{2+}]\text{cyt} and result in contractile responses in smooth muscle from the systemic circulation system (e.g., coronary arterial smooth muscle). These data also explain why hypoxia causes pulmonary vasoconstriction and coronary (or systemic) vasodilation and, furthermore, why prolonged hypoxia only causes pulmonary hypertension. Our previous data indicated that that Ca\textsuperscript{2+}-sensing receptor (CaSR) might play a significant pathogenic role in the development and progression of sustained pulmonary vasoconstriction and concentric pulmonary arterial wall thickening in patients with idiopathic pulmonary arterial hypertension (IPAH) and animals with experimental pulmonary hypertension (63, 64). The mRNA and protein expression level of CaSR was upregulated, while the extracellular Ca\textsuperscript{2+}-induced increase in [Ca\textsuperscript{2+}]\text{cyt} was enhanced, in PASMC from patients with IPAH in comparison to normal controls (63). Importantly, CaSR functionally interacted with TRPC6 to mediate extracellular Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} influx and increase in [Ca\textsuperscript{2+}]\text{cyt} in IPAH-PASMC (52). Enhanced Ca\textsuperscript{2+} entry due to upregulated expression and enhanced function of CaSR contributes to enhancing Ca\textsuperscript{2+} signaling and activating Ca\textsuperscript{2+}/CaM-sensitive transcription factors associated with PASMC proliferation and migration. It is therefore important to study the potential role of CaSR (and its functional interaction with STIM1/2, Orai1/2, and TRPC6) in the differential effects of hypoxia on Ca\textsuperscript{2+} signaling in PASMC and CASMC.

The mechanisms underlying differential responses to hypoxia in CASMC and PASMC, regarding protein expression of SOCC (STIM1/Orai1/2) and ROCC (TRPC6), are unknown. Activation of Notch signaling can potentially mediate transcriptional upregulation of genes encoding SOCC (e.g., TRPC6, and Orai1/2) in human PASMC, thus enhancing SOCE (65). Our recent study showed that hypoxia activates Notch signaling and upregulates TRPC6 channels; genetic depletion of the TRPC6 gene, trpc6 (trpc6\textsuperscript{-/-}) significantly attenuates acute hypoxia-induced pulmonary vasoconstriction and chronic hypoxia-induced pulmonary hypertension. These data imply that Notch signaling may transcriptionally regulate TRPC6 and may hence exert a critical role in pulmonary hypertension (44). It is, however, unknown whether hypoxia or prolonged hypoxia activates Notch signaling in systemic arterial smooth muscle cells like CASMC. Furthermore, hypoxia increases hypoxia-inducible factors 1α (HIF-1α) and 2α (HIF-2α) in both systemic and pulmonary arterial smooth muscle cells (2, 26). The downstream targets of HIF-1α and HIF-2α may vary among different types of cells. In PASMC, hypoxia-induced upregulation of TRPC (e.g., TRPC1 and TRPC6) channels can be mediated by HIF-1α and/or HIF-2α, hypoxia-sensitive transcription factors that are known to be upregulated by increased superoxide as well (55, 60). Further studies are needed to define the different mechanisms responsible for hypoxia-me-
C514

DIFFERENT RESPONSE OF PASMC AND CASMC TO HYPOXIA

...ated transcriptional, posttranscriptional, and/or translational regulation of ROCC (e.g., STIM1 and Orai1/2) and SOCC (e.g., TRPC6/TRPV1) channel genes in CASMC and PASMC.

...Although effects of hypoxia on Ca2+ signaling and contractile response in the two types of vascular smooth muscle cells (CASMC and PASMC) have been described, the precise mechanisms involved in hypoxia (or oxygen) sensing and its downstream effectors (dependent or independent of HIF-1α/HIF-2α) are not well known. Substantial evidence has accumulated demonstrating that reactive oxygen species (ROS) can influence vascular smooth muscle [Ca2+]cyt (43, 56) and hypoxia can differentially regulate the production of cytoplasmic and mitochondrial ROS including superoxide in systemic and pulmonary vascular cells (1, 57). A recent report indicates that chronic hypoxia increases H2O2 generation in PASMC, and H2O2 is the pivotal regulator that ultimately acts to promote the expression and interaction of SOCC and function as the functional regulator in response to hypoxia (10). In contrast, hypoxia inhibits the effect of oxidants (including H2O2) and increases cellular reducing capacity, thereby reducing Ca2+ influx in systemic arteries (15). Wu et al. (60) have shown that chronic hypoxia differently regulates the generation of ROS in human pulmonary and coronary artery smooth muscle cells, with PASMC exhibiting an increase in ROS, whereas CASMC is showing a decrease. As one of the downstream effectors of ROS, ion channels including ROCC and SOCC are highly sensitive to the cellular redox status (16, 37); hence, chronic hypoxia may divergently regulate SOCC/ROCC by affecting intracellular ROS generation, which leads to diverse Ca2+ and contractile responses in pulmonary and coronary artery smooth muscles. Mitochondrial-derived ROS may also contribute to the different responses in these two types of vascular smooth muscle cells (CASMC vs. PASMC). Mitochondria in PASMC exhibit lower respiratory rates and higher rates of ROS and H2O2 production compared with the mitochondria in smooth muscle cells of systemic arteries (31). Accordingly, a recent study shows that a specific subunit 4 isoform of the mitochondrial complex IV, the cytochrome-c oxidase subunit 4 isoform 2 (Cox4i2), which is preferentially expressed in lungs, plays an important role in hypoxia-induced superoxide release and acute hypoxic pulmonary vasoconstriction (45). Mitochondria in PASMC also possess unique functional and metabolic properties that are different from systemic vascular smooth muscle cells (72).

In this study, we also determined and compared oxygen consumption profile for oxidative phosphorylation in CASMC and PASMC. No significant difference between CASMC and PASMC in terms of the mitochondrial respiration was observed; both basal respiration and the oxygen consumed for ATP production were comparable between CASMC and PASMC under resting conditions. The glycolysis, however, was higher in PASMC than in CASMC, suggesting that differences in metabolic profiles exist between PASMC and CASMC. Accumulating evidence suggests that mitochondria in PASMC appear structurally and functionally distinct from those in systemic arteries (12, 31). Michelakis et al. (31) showed that, at baseline, lung mitochondria are less active and have lower respiratory rates and higher rates of ROS and H2O2 production than kidney mitochondria. Recently, Zhu et al. (72) provided compelling evidence that transplantation of mitochondria derived from femoral artery smooth muscle cells inhibits acute hypoxia-induced pulmonary vasoconstriction, attenuates chronic hypoxia-mediated pulmonary vascular remodeling, and thus prevents the development of pulmonary hypertension and reverses the established pulmonary hypertension in rats exposed to chronic hypoxia. In the present study, however, we examined CASMC and PASMC under resting conditions. It is possible that, when the cells are exposed to hypoxia or stimulated by other cellular stressors, the mitochondrial function and/or oxygen consumption profile maybe different.

On the basis of data from our current study and others, it is plausible to speculate that glycolytic capacity might at least in part explain the distinct responses of PASMC and CASMC to hypoxia. It should be noted that our data do not prove a direct causal relationship between the mitochondrial diversity and differentially regulating protein expression (and function) of SOCC and ROCC. However, the results from this study are promising and provide clear clues for future in-depth study. Previous studies also implicate that hypoxia markedly increases mitochondrial ROS production in pulmonary, but not in systemic, vascular system. The increased mitochondrial ROS may result in Ca2+ entry through TRPC channels and increase [Ca2+]cyt in PASMC. In contrast, hypoxia has a negligible effect on mitochondrial ROS production to affect the expression and/or function of SOCC/ROCC in systemic vascular smooth muscle cells (56). These data direct us to hypothesize that mitochondrial ROS and glycolytic capacity may be responsible for differentially regulating protein expression (and function) of SOCC and ROCC in CASMC and PASMC, which contribute, at least in part, to the distinction of coronary and pulmonary arterial smooth muscle cells regarding their response to hypoxia.

One potential limitation of the present study is the use of cultured PASMC and CASMC, which may exhibit alterations compared with freshly isolated cells. Thus, caution should be used in drawing conclusions based on experiments using cultured cells as the study subject, as they may have different molecular features compared with freshly isolated cells. More studies using freshly isolated human tissue (when possible) are required to further prove and clarify the conclusions from the present study. Additionally, the previous study showed that modifying culture conditions could preserve the differentiated state of smooth muscle cells (22), which would be a better way to investigate cultured smooth muscle cells when freshly isolated smooth muscle cells are not suitable.

Furthermore, differential regulation of the IP3 and IP3 receptors (IP3R) through allosteric modifiers, for example, or Ca2+ feedback mechanisms, may cause the cells (e.g., CASMC and PASMC) to respond differently to the same agonist at the same concentration and duration. In CASMC and PASMC, we categorized the agonist-induced changes of [Ca2+]cyt into three patterns: pattern 1, a transient increase in [Ca2+]cyt; pattern 2, a transient increase in [Ca2+]cyt followed by a plateau or sustained increase in [Ca2+]cyt; and pattern 3, an oscillatory increase in [Ca2+]cyt. The kinetics and/or pattern of agonist-induced changes of [Ca2+]cyt depend largely on IP3 synthesis and IP3R activity, and IP3 interaction with IP3R. Therefore, differential regulation of IP3 and IP3R through endogenous intracellular regulators would be a central contributor to the three patterns of [Ca2+]cyt changes identified in CASMC and PASMC. More experiments on IP3 synthesis, IP3/IP3R inter-
action, and IP3R regulation in CASMC and PASMC are needed to define whether IP3, and its downstream signaling are the central arbiter of signaling properties involving the three patterns and of the differences between CASMC and PASMC.

An increase in [Ca2+]c, due to Ca2+ entry through upregulated SOCC and ROCC may play an important pathogenic role in the development and progression of pulmonary vascular remodeling and vasoconstriction in pulmonary hypertension. The hypoxia-mediated upregulation of STIM1/2, Orai1/2, and TRPC6 in PASMC may reflect an intrinsic pathophysiological response unique to the pulmonary vasculature, which contributes to sustained pulmonary vasoconstriction and concentric pulmonary arterial wall thickening in patients and animals with hypoxia-induced pulmonary hypertension. Hypoxia-mediated upregulation of STIM1, Orai1/2, and TRPC6 is, however, not observed in CASMC so that hypoxia may result in coronary vasodilation via different mechanisms. Of note, a majority of previously reported studies are performed in animals or using animal cells; this study provides compelling evidence that hypoxia differentially regulates protein expression of ROCC and SOCC in coronary and pulmonary arterial smooth muscle cells, which may help improve our understanding of the molecular mechanisms involved in the functional distinction of the pulmonary and coronary vasculature.

REFERENCES

1. Adesina SE, Kang BY, Bijli KM, Ma J, Cheng J, Murphy TC, Michael Hart C, Suttif RL. Targeting mitochondrial reactive oxygen species to modulate hypoxia-induced pulmonary hypertension. Free Radic Biol Med 87: 36–47, 2015. doi:10.1016/j.freeradbiomed.2015.05.042.

2. Ahmad A, Ahmad S, Malcolm KC, Miller SM, Hendry-Hofer T, Schaub JK, White CW. Differential regulation of pulmonary vascular cell growth by hypoxia-inducible transcription factor-1α and hypoxia-inducible transcription factor-2α. Am J Respir Cell Mol Biol 49: 78–85, 2013. doi:10.1165/rcmb.2012-0107OC.

3. Aytes A, Mollevi DG, Martínez-Juárez M, Nadal M, Vidal A, Morales A, Salazar R, Capella G, Villanueva A. Stromal interaction molecule 2 (STIM2) is frequently overexpressed in colorectal tumors and confers a tumor cell growth suppressor phenotype. Mol Carcinog 51: 746–753, 2012. doi:10.1002/mc.20843.

4. Ball MK, Waypa GB, Mungai PT, Nielsen JM, Czech L, Dudley VJ, Beutissik L, Dettman RW, Berkshamer SK, Steinhor RH, Shah SJ, Schumacker PT. Regulation of hypoxia-induced pulmonary hypertension by vascular smooth muscle hypoxia-inducible factor-1α. Am J Respir Crit Care Med 189: 314–324, 2014. doi:10.1164/rcrm.201302-0302OC.

5. Beech DJ, Xu SZ, McHugh D, Flemming R. TRPC1 store-operated cationic channel subunit. Cell Calcium 33: 433–440, 2003. doi:10.1016/S0969-5252(03)00054-X.

6. Bernal-Erro A, Jardin I, Salido GM, Rosado JA. Role of STIM2 in cell function and physiopathology. J Physiol 595: 3111–3128, 2017. doi:10.1113/JP273889.

7. Boyer L, Chaar V, Pelle G, Maitre B, Chouaid C, Covali-Noroc A, Zerah F, Bucherer C, Lacombe C, Housset B, Dubois-Randé JL, Bozkowski J, Adnot S. Effects of polycythemia on systemic endothelial function in chronic hypoxic lung disease. J Appl Physiol (1985) 110: 1196–1203, 2011. doi:10.1152/japplphysiol.01204.2010.

8. Brandman O, Lion J, Park WS, Meyer T. STIM2 is a feedback regulator that stabilizes basal cytotoxic and endoplasmic reticulum Ca2+ levels. Cell 131: 1327–1339, 2007. doi:10.1016/j.cell.2007.11.059.

9. Chen J, Syoso JR, Singla S, Zhao S, Yamamura A, Valdez-Jasso D, Abbasi T, Shioura KM, Sahni S, Reddy V, Sridhar A, Gao H, Torres J, Camp SM, Tang H, Ye SQ, Comhair S, Dweik R, Hassoun P, Yuan JX, Garcia JGN, Machado RF. Nicotinamide phosphoribosyltransferase promotes pulmonary vascular remodeling and is a therapeutic target in pulmonary arterial hypertension. Circulation 135: 1532–1546, 2017. doi:10.1161/CIRCULATIONAHA.116.024557.

10. Chen TX, Xu XY, Zhao Z, Zhao FY, Gao YM, Yan XH, Wan Y. Hydrogen peroxide is a critical regulator of the hypoxia-induced alterations of store-operated Ca2+ entry into rat pulmonary arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 312: L477–L487, 2017. doi:10.1152/ajplung.00138.2016.

11. Fernandez RA, Wan J, Song S, Smith KA, Gu Y, Tauseef M, Tang H, Makino A, Mehta D, Yuan JX. Upregulated expression of STIM2, TRPC6, and Orai2 contributes to the transition of pulmonary arterial smooth muscle cells from a contractile to proliferative phenotype. Am J Physiol Cell Physiol 308: C581–C593, 2015. doi:10.1152/ajpcell.00202.2014.

12. Firth AL, Gordienko DV, Yuill KH, Smirnov SV. Cellular localization of mitochrondia contributes to Kv channel-mediated regulation of cellular excitability in pulmonary but not mesenteric circulation. Am J Physiol Lung Cell Mol Physiol 296: L347–L360, 2009. doi:10.1152/ajplung.00138.2008.

13. Fuchs B, Rupp M, Ghofrani HA, Schermuly RT, Seeger W, Griminger F, Gudermann T, Dietrich A, Weissmann N. Diacylglyerol regulates acute hypoxic pulmonary vasoconstriction via TRPC6. Respir Res 12: 20, 2011. doi:10.1186/1465-9921-12-20.

14. Guo RW, Yang LX, Li MQ, Pan XH, Liu B, Deng YL. Stim1- and Orai1-mediated store-operated calcium entry is critical for angiotensin II-induced vascular smooth muscle cell proliferation. Cardiovase Res 93: 360–370, 2012. doi:10.1093/cvr/cvs057.

15. Gupte SA, Wolin MS. Oxidant and redox signaling in vascular oxygen sensing: implications for systemic and pulmonary hypertension. Antioxid Redox Signal 10: 1137–1152, 2008. doi:10.1089/ars.2007.1995.

16. Henke N, Albrecht P, Bouchachia I, Ryazantseva M, Koll M, Lenz J, Kaznacheyeva E, Maher P, Metheren A. The plasma membrane channel ORAI1 mediates detrimental calcium influx caused by endogenous oxidative stress. Cell Death Dis 4: e470, 2013. doi:10.1038/cddis.2012.216.

17. Hussain A, Suleiman MS, George SJ, Loubani M, Morice A. Hypoxic pulmonary vasculature in humans: tale or myth. Open Cardiovasc Med J 11: 1–13, 2017. doi:10.2174/1874192401711010001.

18. Inayama M, Suzuki Y, Yamada S, Kurita T, Yamamura H, Ohya S, Giles WR, Imaizumi Y. Orai1-Oral2 complex is involved in store-operated calcium entry in chondrocyte cell lines. Cell Calcium 57: 337–347, 2015. doi:10.1016/j.ceca.2015.02.005.

19. Inoue R, Jensen LJ, Shi J, Morita H, Nishida M, Honda A, Ito Y. Transient receptor potential channels in cardiovascular function and disease. Circ Res 99: 119–131, 2006. doi:10.1161/01.RES.0000233356.10630.8a.

20. Inoue R, Okada T, Onoue H, Harayama K, Shimizu S, Naitoh S, Ito Y, Morii Y. The transient receptor potential protein homologue TRP6 is the essential component of vascular α1-adrenoceptor-activated Ca2+-permeable cation channel. Circ Res 88: 325–332, 2001. doi:10.1161/01.RES.88.3.325.

21. Doi T, Ohashi I, Kato Y, Yamashita N, Terajima Y, Suzuki A, Inoue T, Nishino S, Tsutsumi S, Hasegawa K, Ohkado Y, Fujisawa K. Transient receptor potential channels in the lungs. Am J Resp Physiol Lung Cell Mol Physiol 84: C354–C360, 2002.

22. Isukki T, Imai Y, Hase H, Okamura Y, Inoue R, Morii MX. PLC-mediated P(4,5)P2 hydrolysis regulates activation and inactivation of TRPC6/7 channels. J Gen Physiol 143: 183–201, 2014. doi:10.1085/jgp.201311033.
22. Jarrousse C, Lodis N, Michel F, Bali JP, Magoun R. Cultured gastrointestinal smooth muscle cells: cell response to contractile agonists depends on their phenotypic state. *Cell Tissue Res* 316: 221–232, 2004. doi:10.1007/s00441-004-0859-9.

23. Jiao HX, Mu YP, Gui LX, Yan FR, Lin DC, Sham JS, Lin MJ. Increase in caveolin and caveolin-1 expression modulates agonist-induced contraction and store- and receptor-operated Ca\(^{2+}\) entry in pulmonary arteries of pulmonary hypertensive rats. *Vasc Pharmacol* 48: 55–66, 2010. doi:10.1016/j.v磷酸.2009.06.004.

24. Ju M, Shi J, Saleh SN, Albert AP, Large WA. Intracellular Ca\(^{2+}\) modulation in pulmonary arteries of pulmonary hypertensive rats. *Am J Physiol Lung Cell Mol Physiol* 280: L801–L812, 2001. doi:10.1152/ajplung.2001.280.4.L801.

25. Jung S, Strotmann R, Schultz G, Plant TD. TRPC6 is a candidate channel involved in receptor-stimulated calcium currents in A757 smooth muscle cells. *Am J Physiol Cell Physiol* 282: C347–C359, 2002. doi:10.1152/ajpcell.00283.2001.

26. Jürgenssen JS, Rosenberger C, Wiesener MS, Warnecke C, Hörstrup JH, Gräfe M, Philipp S, Griethe W, Maxwell PH, Frei U, Bachmann S, Willenbrock R, Eckardt KU. Persistent induction of HIF-1α and -2α in cardiomyocytes and stromal cells of ischemic myocardium. *FASEB J* 18: 1135–1145, 2004. doi:10.1096/fj.03-1130com.

27. Kim EY, Alvarez-Baron CP, Drye SE. Canonical transient receptor potential channel (TRPC) regulatory TRPC6 associate with large-conductance Ca\(^{2+}\)-activated K\(^+\) (BKCa) channels: role in BKCa trafficking to the surface of cultured podocytes. *Mol Pharmacol* 75: 466–477, 2009. doi:10.1124/mol.108.051912.

28. Kuhr FK, Smith KA, Song MY, Levitan I, Yuan JX. New mechanisms of pulmonary arterial hypertension: role of Ca\(^{2+}\) signaling. *Am J Physiol Heart Circ Physiol* 307: H1154–H1162, 2012. doi:10.1152/ajpcell.00750.2011.

29. Lin MJ, Leung GP, Zhang WM, Yang XR, Yip KP, Tse CM, Sham JS. Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca\(^{2+}\) channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension. *Circ Res* 95: 496–505, 2004. doi:10.1161/01.RES.0000139522.16382.ad.

30. Makino A, Firth AL, Yuan JX. Endothelial and smooth muscle cell ion channels in pulmonary vasomotor dysfunction and vascular remodeling. *Comb Pharm* 1: 1555–1602, 2011. doi:10.1002/cphy.c100023.

31. Michelakis ED, Hampl V, Nsaar A, Wu X, Harry G, Haromy A, Gurtu R, Archer SL. Diversity in mitochondrial function explains differences in vascular oxygen sensing. *Circ Res* 90: 1307–1315, 2002. doi:10.1161/01.RES.0000093868.61265.6c.

32. Moradkhan R, Spitnale B, McQuillan P, Hogeman C, Gray KS, Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, Kuhr FK, Smith KA, Voiriot G, Tang H, Fraidenburg DR, Song S, Yamamura H, Guo Q, Wan J, Polli NM, Pohl NM, Bodmer R, Oechslin E, Thistlethwaite PA, Pohl FM, Powell FL, Makino A, Mehta D, Yuan JX. Notch activation of Ca\(^{2+}\) signaling in the development of pulmonary vasohypertension and pulmonary hypertension. *Am J Respir Cell Mol Biol* 53: 355–367, 2015. doi:10.1165/rcell.2014-0235OC.

33. Sommer N, Hüttemann M, Pak O, Scheibe S, Knoepp F, Sinkler C, Maleczky M, Gierhardt M, Esfandiary A, Kraut S, Jonas F, Veith C, Arredouani M, Nysedyk A, Akebrahimshokordi N, Giehl K, Hecker M, Brandes RP, Seeger W, Grimfinger F, Ghoftoli HA, Schermuly RT, Grossmann LI, Weissemn N. Mitochondrial complex IV subunit 4 isofrom 2 is essential for acute pulmonary oxygen sensing. *Circ Res* 121: 424–438, 2017. doi:10.1161/CIRCRESAHA.116.310482.

34. Song MY, Makino A, Yuan JX. STIM2 contributes to enhanced store- and receptor-operated Ca\(^{2+}\) entry in pulmonary arterial smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. *Pulm Circ* 1: 84–94, 2011. doi:10.1080/20458932.78106.

35. Song S, Ayon RJ, Yamamura A, Yamamura H, Dash S, Babicheva A, Tang J, Sun X, Corderoy AG, Khalpey Z, Black SM, Desai AA, Rischarf F, McDermott KM, Garcia J, Makino A, Yuan JX. Capsaicin-induced Ca\(^{2+}\) signaling is enhanced via upregulated TRPV1 channel in coronary arteries of pulmonary hypertensive rats. *Pulm Circ* 7: 443–453, 2017. doi:10.1177/20458932.170222.

36. Sompolinska I, Pak O, Strelkov I, Hutmacher D, Weissmann N. Recent advances in oxygen sensing and signal transduction in hypoxic pulmonary vasomotor function. *Am J Physiol Lung Cell Mol Physiol* 312: L309–L325, 2017. doi:10.1152/ajplung.00357.2016.

37. Stanisz H, Saul S, Müller CS, Kappl R, Niemeyer BA, Vogt T, Hoth M, Roessch A, Bogske I. Inverse regulation of melanoma growth and migration by Oral1/STIM2-dependent calcium entry. *Pigment Cell Melanoma Res* 27: 442–453, 2014. doi:10.1111/pcrm.12222.

38. Taniguchi H, Naka S, Shimoda LA, Weissmann N. Role of STIM in oxygen sensing and signal transduction in hypoxic pulmonary vasomotor function. *Am J Physiol Lung Cell Mol Physiol* 310: L846–L859, 2016. doi:10.1152/ajplung.00050.2016.

39. Tan W, Madhavan K, Hunter KS, Park D, Stennark KR. Vascular stiffening in pulmonary hypertension: cause or consequence? (2013 Grover Conference series). *Pulm Circ* 4: 560–580, 2014. doi:10.1177/20458932.135018.

40. Tang C, To WK, Meng F, Wang Y, Gu Y. A role for receptor-operated Ca\(^{2+}\) entry in human pulmonary artery smooth muscle cells in response to hypoxia. *Pulm Circ* 5: 909–918, 2010.

41. Tang H, Yamamura A, Yamamura H, Song S, Fraidenburg DR, Chen J, Gu Y, Polli NM, Zhou T, Jiménez-Pérez L, Ayon RJ, Desai AA, Goltzman D, Rischarf F, Kralpely Z, Black SM, Garcia J, Makino A, Yuan JX. Pathogenic role of calcium-sensing receptors in the development and progression of pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 305: L154–L164, 2013. doi:10.1152/ajplung.00313.2012.

42. Wang J, Shimoda LA, Weigand L, Wang Y, Sun D, Sylvester JT. Acute hypoxia increases intracellular [Ca\(^{2+}\)] in pulmonary arterial smooth muscle cells.
muscle by enhancing capacitative Ca\(^{2+}\) entry. *Am J Physiol Lung Cell Mol Physiol* 288: L1059–L1069, 2005. doi:10.1152/ajplung.00448.2004.

55. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA. Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca\(^{2+}\) in pulmonary arterial smooth muscle cells. *Circ Res* 98: 1528–1537, 2006. doi:10.1161/01.RES.0000227551.68124.98.

56. Wang YX, Zheng YM. Role of ROS signaling in differential hypoxic Ca\(^{2+}\) and contractile responses in pulmonary and systemic vascular smooth muscle cells. *Respir Physiol Neurobiol* 174: 192–200, 2010. doi:10.1016/j.resp.2010.08.008.

57. Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D, Schumacker PT. Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ Res* 106: 526–535, 2010. doi:10.1161/CIRCRESAHA.109.206334.

58. Weisemann N, Dietrich A, Fuchs B, Kalwa H, Ay M, Dumitrascu R, Olschewski A, Storch U, Mederos y Schnitzler M, Ghofrani HA, Schermuly RT, Pinkenburg O, Seeger W, Grimminger F, Guzy R, Mungai PT, Schriewer J, Dokic D, Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D, Schumacker PT. Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ Res* 106: 526–535, 2010. doi:10.1161/CIRCRESAHA.109.206334.

59. Wu K, Zhang Q, Wu X, Lu W, Tang H, Liang Z, Gu Y, Song S, Aoyon RJ, Wang Z, McDermott KM, Balistrieri A, Wang C, Black SM, Garcia GJN, Makino A, Yuan JX, Wang J. Chloroquine is a potent pulmonary vasodilator that attenuates hypoxia-induced pulmonary hypertension. *Br J Pharmacol* 174: 4155–4172, 2017. doi:10.1111/bph.13990.

60. Wu W, Platoshyn O, Firth AL, Yuan JX. Hypoxia divergently regulates production of reactive oxygen species in human pulmonary and coronary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 293: L952–L959, 2007. doi:10.1152/ajplung.00203.2007.

61. Xia Y, Yang XR, Fu Z, Paudel O, Abramowitz J, Birnbaumer L, Shami JS. Classical transient receptor potential 1 and 6 contribute to hypoxic pulmonary hypertension through differential regulation of pulmonary vascular functions. *Hypertension* 63: 173–180, 2014. doi:10.1161/HYPERTENSIONAHA.113.01902.

62. Xu Y, Zhang S, Niu H, Ye Y, Hu F, Chen S, Li X, Luo X, Jiang S, Liu Y, Chen Y, Li J, Xiang R, Li N. STIM1 accelerates cell senescence in a remodeled microenvironment but enhances the epithelial-to-mesenchymal transition in prostate cancer. *Sci Rep* 5: 11754, 2015. doi:10.1038/srep11754.

63. Yamamura A, Guo Q, Yamamura H, Zimnicka AM, Pohl NM, Smith KA, Fernandez RA, Zeifman A, Makino A, Dong H, Yuan JX. Enhanced Ca\(^{2+}\)-sensing receptor function in idiopathic pulmonary arterial hypertension. *Circ Res* 111: 469–481, 2012. doi:10.1161/CIRCRESAHA.112.266361.

64. Yamamura A, Yamamura H, Guo Q, Zimnicka AM, Wan J, Ko EA, Smith KA, Pohl NM, Song S, Zeifman A, Makino A, Yuan JX. Dihydropyridine Ca\(^{2+}\) channel blockers increase cytosolic Ca\(^{2+}\) by activating Ca\(^{2+}\)-sensing receptors in pulmonary arterial smooth muscle cells. *Circ Res* 112: 640–650, 2013. doi:10.1161/CIRCRESAHA.113.308997.

65. Yamamura H, Yamamura A, Ko EA, Pohl NM, Smith KA, Zeifman A, Powell FL, Thistlethwaite PA, Yuan JX. Activation of Notch signaling by short-term treatment with Jagged-1 enhances store-operated Ca\(^{2+}\) entry in human pulmonary smooth muscle cells. *Am J Physiol Cell Physiol* 306: C871–C878, 2014. doi:10.1152/ajpcell.00221.2013.

66. Yu L, Hales CA. Effect of chemokine receptor CXCR4 on hypoxia-induced pulmonary hypertension and vascular remodeling in rats. *Respir Res* 12: 21, 2011. doi:10.1186/1465-9921-12-21.

67. Zhang S, Dong H, Rubin LJ, Yuan JX. Upregulation of Na\(^{+}\)/Ca\(^{2+}\) exchanger contributes to the enhanced Ca\(^{2+}\) entry in pulmonary artery smooth muscle cells from patients with idiopathic pulmonary arterial hypertension. *Am J Physiol Cell Physiol* 292: C2297–C2305, 2007. doi:10.1152/ajpcell.00383.2006.

68. Zhang S, Yuan JX, Barrett KE, Dong H. Role of Na\(^{+}\)/Ca\(^{2+}\) exchange in regulating cytosolic Ca\(^{2+}\) in cultured human pulmonary artery smooth muscle cells. *Am J Physiol Cell Physiol* 288: C245–C252, 2005. doi:10.1152/ajpcell.00411.2004.

69. Zhang X, Zhang W, González-Cobos JC, Jardin I, Romanin C, Matrougui K, Trebak M. Complex role of STIM1 in the activation of store-independent Orai1/Ca\(^{2+}\) channels. *J Gen Physiol* 143: 345–359, 2014. doi:10.1085/jgp.201311084.

70. Zheng L, Stathopulos PB, Li GY, Ikura M. Biophysical characterization of the EF-hand and SAM domain containing Ca\(^{2+}\) sensory region of STIM1 and STIM2. *Biophys Chem* 139: 240–246, 2008. doi:10.1016/j.bcp.2007.12.129.

71. Zheng YM, Park SW, Stokes L, Tang Q, Xiao JH, Wang YX. Distinct activity of BK channel \(\beta\)-subunit in cerebral and pulmonary artery smooth muscle cells. *Am J Physiol Cell Physiol* 304: C780–C789, 2013. doi:10.1152/ajpcell.00006.2012.

72. Zhu L, Zhang J, Zhou J, Lu Y, Huang S, Xiao R, Yu X, Zeng X, Liu B, Liu F, Sun M, Dai M, Hao Q, Li J, Wang T, Li T, Hu Q. Mitochondrial transplantation attenuates hypoxic pulmonary hypertension. *Oncotarget* 7: 48925–48940, 2016. doi:10.18632/oncotarget.10596.