Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca\(^{2+}\) responses to hypoxia

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Lu W, Wang J, Shimoda LA, Sylvester JT. Differences in STIM1 and TRPC expression in proximal and distal pulmonary arterial smooth muscle are associated with differences in Ca\(^{2+}\) responses to hypoxia. Am J Physiol Lung Cell Mol Physiol 295: L104–L113, 2008. First published April 18, 2008; doi:10.1152/ajplung.00058.2008.—Hypoxic pulmonary vasoconstriction (HPV) requires Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels (SOCC) in pulmonary arterial smooth muscle cells (PASMC) and is greater in distal than proximal pulmonary arteries (PA). SOCC may be composed of canonical transient receptor potential (TRPC) proteins and activated by stromal interacting molecule 1 (STIM1). To assess the possibility that HPV is greater in distal PA because store-operated Ca\(^{2+}\) entry (SOCE) is greater in distal PASMC, we measured intracellular Ca\(^{2+}\) of extracellular Ca\(^{2+}\), such as the change in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) induced by hypoxia (5, 46, 57, 59), K\(^{+}\) channel function (3, 4, 26, 48, 51) and expression (4, 9), Cl\(^{-}\) channel function (48), Ca\(^{2+}\) channel density and O2 sensitivity (13), Ca\(^{2+}\) stores in sarcoplasmic reticulum (SR) (58), and expression and function of endothelin receptors (10, 54). One assumption of these studies is that differences between proximal and distal PASMC could yield clues to the mechanisms of HPV, which remain elusive.

Recent results suggest that [Ca\(^{2+}\)]\textsubscript{i} responses to hypoxia in distal PASMC (31, 62) and HPV in isolated lungs (64) require Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels (SOCC). SOCC are activated by release of Ca\(^{2+}\) from stores in SR and are thought to be heterotetrameric assemblies of so-called canonical transient receptor potential (TRPC) proteins (33, 34). Of the seven TRPC isoforms (TRPC1–7) thus far identified (29), all have been detected in pulmonary arterial smooth muscle (14, 18, 21, 27, 30, 43, 53, 60, 61, 63, 65, 71, 72, 74); however, the pattern of expression has varied considerably among studies. TRPC1, TRPC4, and TRPC6 are the most consistently identified isoforms, whereas TRPC2, TRPC3, TRPC5, and TRPC7 have been detected less frequently. A single quantitative polymerase chain reaction analysis (qPCR) of mouse distal PASMC revealed that TRPC1 and TRPC6 were more abundant than other TRPC isoforms (65). Whether TRPC expression differences between proximal and distal PASMC is unknown.

Stromal interacting molecule 1 (STIM1), a 90-kDa trans-membrane Ca\(^{2+}\)-binding protein found in sarco(endo)plasmic reticulum and plasma membrane, is thought to play a pivotal role in activation of SOCC (15, 20, 28, 39). According to this hypothesis, decreased Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\textsubscript{i}) in the SR lumen caused by Ca\(^{2+}\) release leads to dissociation of Ca\(^{2+}\) from an EF-hand motif in the intraluminal NH2-terminal region of STIM1, aggregation of STIM1 proteins at “puncta” in portions of the SR membrane close to plasma membrane, and physical interaction of STIM1 with SOCC and/or associated regulatory proteins, leading to channel activation (15, 68, 76). Although originally demonstrated in HeLa (22) and Drosophila S2 cells (44), STIM1 is now known to be required for store-operated Ca\(^{2+}\) entry (SOCE) in many cell types, including smooth muscle (12, 36, 55); however, evidence of STIM1 expression in PASMC has not been reported.

In this study, we quantified expression of TRPC and STIM1 in primary cultures of proximal and distal PASMC and freshly formed pulmonary arteries (PA) (24), and pulmonary arterial smooth muscle cells (PASMC) (25) indicates that hypoxic pulmonary vasoconstriction (HPV) is greater in distal (“resistance”) PA than in proximal (“conduit”) PA. This difference in HPV has been associated with other differences between proximal and distal arterial smooth muscle (12, 36, 55); however, evidence of STIM1 expression in PASMC has not been reported.

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isolated deendothelialized proximal and distal PA. To assess whether differences in myocyte expression of TRPC and STIM1 have functional consequences in these vessels, we also measured the effects of hypoxia or KCl on [Ca\(^{2+}\)]; and the effects of hypoxia on SOCE in proximal and distal PASMC.

**METHODS**

**Preparation of PA and PASMC.** Animal protocols were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. As previously described (61–63, 66), proximal (main trunk) and distal (≥4th generation) intrapulmonary arteries were dissected from lungs removed from male Wistar rats (300–500 g body wt) after anesthesia with pentobarbital sodium (65 mg/kg ip). Endothelium was denuded from the isolated PA by opening the vessel longitudinally and rubbing the luminal surface with a cotton swab. PASMC were harvested from these vessels enzymatically, plated on 25-mm cover slips in six-well dishes, and cultured for 3–5 days to 40–60% confluence in smooth muscle growth media (Clonetics, Walkersville, MD) in a humidified atmosphere of 5% CO\(_2\)-95% air at 37°C. Before an experiment (24 h), culture media were changed to smooth muscle basal media (Clonetics) containing 0.3% serum to stop cell growth. Cellular purity was >95%, as assessed by morphological appearance under phase-contrast microscopy and immunofluorescence staining for α-actin (61).

**Measurement of intracellular [Ca\(^{2+}\)].** As previously described (61, 66), cover slips with myocytes were incubated with 5 μM fura 2-AM (Invitrogen, Carlsbad, CA) for 60 min at 37°C under an atmosphere of 5% CO\(_2\)-95% air, then mounted in a closed polycarbonate chamber clamped in a heated aluminum platform (PH-2; Warner Instrument, Hamden, CT) on the stage of a Nikon TSE 100 Eclipse inverted microscope (Nikon, Melville, NY), and perfused at 0.5 ml/min with Krebs Ringer bicarbonate solution (KRBS) that contained (in mM) NaCl, 118; KCl, 4.7; CaCl\(_2\), 2.5; MgSO\(_4\), 0.57; KH\(_2\)PO\(_4\), 1.18; NaHCO\(_3\), 25; and glucose, 10. Perfusion was equilibrated in heated reservoirs with 5% CO\(_2\) and either 16% O\(_2\) (normoxia) or 4% O\(_2\) (hypoxia) and led to the chamber through stainless steel tubing. For depolarization of cells, perfuse KCl was increased to 60 mM, and NaCl was decreased to 62.7 mM. Chamber temperature was maintained at 37°C with an in-line heat exchanger and dual-channel heater controller (models SF-28 and TC-344B; Warner Instrument).

After removal of extracellular dye by 10 min of normoxic perfusion, [Ca\(^{2+}\)], was determined at 30- to 60-s intervals from the ratio of fura 2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F\(_{\text{510/F340}}\)) measured in 20–30 cells using a xenon arc lamp, interference filters, electronic shutter, ×20 fluorescence objective, and a cooled charge-coupled device imaging camera. Data were collected online with InCyte software (Intracell Imaging, Cincinnati, OH). [Ca\(^{2+}\)], was estimated from F\(_{\text{510/F340}}\) measured in vitro in calibration solutions with [Ca\(^{2+}\)] of 0–1,350 nM (Invitrogen). As previously described, hypoxia did not alter behavior of fura 2 in PASMC (62).

**Measurement of SOCE.** We perfused PASMC for at least 10 min with Ca\(^{2+}\)-free physiological salt solution containing 0.5 mM EGTA to chelate residual Ca\(^{2+}\), 5 μM nifedipine (Sigma Chemical, St. Louis, MO) to prevent calcium entry through L-type voltage-operated Ca\(^{2+}\) channels (VOCC), and 10 μM cyclopiazonic acid (CPA; Sigma Chemical) to deplete SR Ca\(^{2+}\) stores. SOCE was assessed in two ways, as described previously (61). First, we measured [Ca\(^{2+}\)], before and after restoration of extracellular [Ca\(^{2+}\)] to 2.5 mM. SOCE was evaluated from the peak increase in [Ca\(^{2+}\)], caused by restoration of extracellular Ca\(^{2+}\) in the continued presence of nifedipine and CPA. Second, we monitored fura 2 fluorescence excited at 360 nm at 30-s intervals before and after addition of MnCl\(_2\) (200 μM) to the cell perfusate; SOCE was evaluated from the rate at which fura 2 fluorescence was quenched by Mn\(^{2+}\), which enters the cell as a Ca\(^{2+}\) surrogate and reduces fura 2 fluorescence upon binding to the dye.

Fluorescence excited at 360 nm is the same for Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free fura 2; therefore, changes in fluorescence are caused by Mn\(^{2+}\) alone.

**RNA isolation and measurement by real-time qPCR.** Total RNA in deendothelialized distal or proximal PA was isolated using the TRIzol method, as previously described (61, 63). Briefly, samples were placed in ice-cold TRIzol reagent (Invitrogen) at 1 ml/20–50 mg tissue and homogenized at 20,000 rpm (model PT3100; Polytron, Kinematica, Switzerland). After incubation at 30°C for 5 min and addition of chloroform (0.2 ml/ml TRizol), samples were centrifuged at 4°C and 8,000 g for 15 min. The upper aqueous phase of centrifuged samples was mixed with 100% isopropanol (0.5 ml/ml TRizol), incubated at 30°C for 10 min, and recentrifuged at 4°C and 12,000 g for 10 min. The clear gel-like RNA precipitate was washed one time with 1 ml of 75% ethanol, dissolved in diethylpyrocarbonate-treated water, and stored at −80°C. Total RNA in PASMC was extracted using the RNeasy kit (Qiagen, Valencia, CA). DNA contamination in RNA preparations was removed by on-column DNAse digestion using an RNeasy column and RNAse-free DNase (Qiagen).

Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The reaction mixture contained 1 μg total RNA in a 20-μl volume. cDNA was quantified by real-time qPCR using Quantitect SYBR Green PCR Master Mix (Qiagen) in the following conditions: 95°C for 15 min and 45 cycles, each at 94°C for 15 s, 57.5°C for 20 s, and 72°C for 20 s. The volume of each real-time qPCR reaction mixture was 25 μl containing 300 nM forward and reverse primers and cDNA template from 50 ng RNA. Primer sequences specific for rat TRPC, STIM1, or β-actin were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 1. Identity of the qPCR products was confirmed by a single sharp peak in the melting curve performed after cDNA amplification; 2) a single band of the expected size resolved by agarose gel electrophoresis; and 3) the correct DNA sequence. Melting curves were performed at 95°C for 1 min and 55°C for 1 min, followed by 50 increments of 0.5°C at 10-s intervals. Real-time qPCR detection threshold cycle values were generated by iCyQgene software. Relative concentrations of each transcript were calculated using the Pfaffl (37) method. Efficiency for each gene was determined from five-point serial dilutions of positive control cDNA samples (PA or PASMC for TRPC1, TRPC4, TRPC6, and STIM1; rat brain for TRPC2, TRPC3, TRPC5, and TRPC7). Mean efficiencies of each gene were used to quantify expression of TRPC and STIM1 relative to β-actin in the same sample.

**Protein isolation and measurement by Western blotting.** Deendothelialized proximal or distal PA or PASMC samples were homogenized by sonication in Laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% protease inhibitor cocktail, 1 mM EDTA, and 200 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride. Total protein concentration in the homogenates was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using BSA as a standard. Homogenates were denatured by adding dithiothreitol to 150 mM and heating at 95°C for 3 min. Homogenate proteins were resolved by 10% SDS-PAGE calibrated with prestained protein molecular weight markers (Precision Plus; Bio-Rad, Carlsbad, CA). Separated proteins were transferred to polyvinylidene difluoride membranes (pore size 0.45 μm; Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20, blotted with affinity-purified polyclonal antibodies specific for TRPC proteins, or monoclonal antibody specific for STIM1 or β-actin. The membranes were then washed for 15 min three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG for 1 h. Bound antibodies were detected using an enhanced chemiluminescence system (ECL; GE Healthcare, Piscataway, NJ).

**Materials and drugs.** Unless otherwise specified, all reagents were obtained from Sigma Chemical. TRPC antibodies other than TRPC1 were obtained from Alomone Laboratories (Jerusalem, Israel). STIM1...
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\[ \text{[Ca}^{2+}\text{]}_i \]

Fig. 1. Changes in intracellular \( \text{[Ca}^{2+}\text{]}_i \) responses to hypoxia and KCl in PASMC. Acute
hypoxia (4% O2) caused a rapid sustained increase in \( \text{[Ca}^{2+}\text{]}_i \) in both proximal and distal PASMC (Fig. 1A); however, the increase in distal PASMC was \( \sim 2.5 \)-fold greater than that in proximal PASMC (peak \( \Delta [\text{Ca}^{2+}]_i \) = 123 \( \pm \) 18 vs. 32 \( \pm \) 5 nM; \( P < 0.001 \)). In contrast, responses to KCl (Fig. 1B) were the same in proximal and distal cells (peak \( \Delta [\text{Ca}^{2+}]_i \) = 307 \( \pm \) 84 vs. 313 \( \pm \) 63 nM).

SOCE in PASMC. SOCE in proximal and distal PASMC was assessed in two ways. First, we measured the peak increase in \( \text{[Ca}^{2+}\text{]}_i \) caused by restoration of extracellular \( \text{[Ca}^{2+}\text{]}_i \) to 2.5 mM in PASMC perfused with \( \text{Ca}^{2+}\text{-free} \) KRBS containing CPA and nifedipine. CPA caused an initial transient increase in \( \text{[Ca}^{2+}\text{]}_i \) during \( \text{Ca}^{2+}\text{-free} \) perfusion (Fig. 2, A and B), which tended to be greater in distal than proximal PASMC (220 \( \pm \) 45 vs. 140 \( \pm \) 19 nM, \( P = 0.06 \); Fig. 2C). This response was not altered by hypoxia, which began at the same time as CPA. The increase in \( \text{[Ca}^{2+}\text{]}_i \) caused by restoration of extracellular \( \text{Ca}^{2+} \) was greater in distal than proximal PASMC (562 \( \pm \) 65 vs. 410 \( \pm \) 83 nM during normoxia and 798 \( \pm \) 65 vs. 471 \( \pm \) 33 nM during hypoxia, \( P < 0.03 \); Fig. 2D). Hypoxia increased the response to restoration in distal PASMC (\( P < 0.02 \); Fig. 2D), but not proximal PASMC.

Table 1. Primers for rat TRPC, STIM1, and \( \beta \)-actin used in real-time quantitative PCR

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<th>Gene</th>
<th>Accession No.</th>
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<th>Product Size, bp</th>
<th>Location in Sequence</th>
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<td>90</td>
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TRPC, canonical transient receptor potential; STIM1, stromal interacting molecule 1.

**RESULTS**

**[Ca}^{2+}\text{]}_i \) responses to hypoxia and KCl in PASMC.** Acute hypoxia (4% O2) caused a rapid sustained increase in \( \text{[Ca}^{2+}\text{]}_i \) in both proximal and distal PASMC (Fig. 1A); however, the increase in distal PASMC was \( \sim 2.5 \)-fold greater than that in proximal PASMC (peak \( \Delta [\text{Ca}^{2+}]_i \) = 123 \( \pm \) 18 vs. 32 \( \pm \) 5 nM; \( P < 0.001 \)). In contrast, responses to KCl (Fig. 1B) were the same in proximal and distal cells (peak \( \Delta [\text{Ca}^{2+}]_i \) = 307 \( \pm \) 84 vs. 313 \( \pm \) 63 nM).

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**Fig. 1.** Changes in intracellular \( \text{Ca}^{2+} \) concentration (\( \text{[Ca}^{2+}\text{]}_i \)) caused by 4% O2 in rat distal pulmonary arterial smooth muscle cells (PASMC: Distal, \( n = 7 \) experiments in 205 cells) and proximal PASMC (Proximal, \( n = 7 \) experiments in 201 cells; A); or 60 mM KCl in rat distal (\( n = 7 \) experiments in 198 cells) and proximal (\( n = 7 \) experiments in 190 cells) PASMC (B). Responses to hypoxia, but not KCl, were different in distal and proximal PASMC (\( P < 0.001 \) and 0.95, respectively). Brackets indicate \( \pm \) SE.

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Because the increase in $[\text{Ca}^{2+}]_{i}$ caused by restoration of extracellular $[\text{Ca}^{2+}]$ can be affected by factors other than SOCE, such as changes in efflux and membrane potential, we also measured the rate at which Mn$^{2+}$ quenched fura 2 fluorescence, which is thought to be a more specific index of $\text{Ca}^{2+}$ influx (Fig. 3). Mn$^{2+}$ quenching, expressed as the percentage decrease in fluorescence at 5 min, was greater in distal than proximal PASMC during both normoxia and hypoxia (20% vs. 11% during normoxia and 44% vs. 19% during hypoxia, $P < 0.01$; Fig. 3B). Acute hypoxia, quenching increased in both distal and proximal PASMC ($P < 0.002$; Fig. 3B), and the increase was significantly greater in distal cells ($P < 0.04$; Fig. 3B).

**TRPC and STIM1 expression in PASMC and PA.** Using qPCR, we detected transcripts of TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 in proximal and distal PASMC and PA.

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**Fig. 2.** Effects of restoration of extracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]$) to 2.5 mM in proximal and distal PASMC perfused with $\text{Ca}^{2+}$-free Krebs Ringer bicarbonate solution (KRBS; 0 $\text{Ca}^{2+}$) containing cyclopiazonic acid (CPA, 10 $\mu$M) and nifedipine (5 $\mu$M) during normoxia ($16\%\text{O}_2$; $n = 9$ experiments in 265 cells for proximal PASMC, 9 experiments in 261 cells for distal PASMC; A) and hypoxia ($4\%\text{O}_2$; $n = 5$ experiments in 138 cells for proximal PASMC, 5 experiments in 141 cells for distal PASMC; B). C: maximum initial increase in $[\text{Ca}^{2+}]$, in response to CPA during $\text{Ca}^{2+}$-free perfusion tended to be greater in distal PASMC ($P = 0.06$). D: maximum increase in $[\text{Ca}^{2+}]$, after restoration of extracellular $[\text{Ca}^{2+}]$ was greater in distal PASMC ($P < 0.03$) and further increased by hypoxia in distal cells ($P < 0.02$). Brackets indicate ± SE.

**Fig. 3.** Quenching of fura 2 fluorescence at 360 nm by 200 $\mu$M Mn$^{2+}$ in PASMC perfused with $\text{Ca}^{2+}$-free KRBS (0 $\text{Ca}^{2+}$) containing nifedipine (5 $\mu$M) and CPA (10 $\mu$M). A: time course of fluorescence normalized to fluorescence at time 0 ($F/F_0$) during normoxia ($16\%\text{O}_2$; $n = 8$ experiments in 211 cells for proximal PASMC and 8 experiments in 218 cells for distal PASMC) and hypoxia ($n = 4$ experiments in 98 cells for proximal PASMC and 4 experiments in 92 cells for distal PASMC). B: Mn$^{2+}$ quenching, expressed as the percentage decrease in fluorescence from time 0, was greater in distal PASMC ($P < 0.01$) and increased by hypoxia ($P < 0.002$). The increase caused by hypoxia was also greater in distal cells ($P < 0.04$). Brackets indicate ± SE.
but not TRPC2 or TRPC7. In all preparations, the rank order of abundance was TRPC1 > TRPC6 > TRPC4 >> TRPC3 ≈ TRPC5. In particular, TRPC1 was 2- to 4-fold more abundant than TRPC6, 10- to 30-fold more abundant than TRPC4, and 1,000- to 10,000-fold more abundant than TRPC3 or TRPC5. Expression of TRPC1, TRPC6, and TRPC4 mRNA was about twofold greater in distal than proximal PASMC and PA (P < 0.01; Fig. 4). Expression of other TRPC mRNA was not different with respect to vessel locus except TRPC5 in PA, which was less abundant in distal arteries. We did not compare TRPC mRNA expression between PASMC and PA because reference gene (β-actin) expression was different in these preparations. In proximal and distal PASMC and PA, Western blotting confirmed protein expression of TRPC1, TRPC4, and TRPC6, the most abundant TRPC isoforms identified by qPCR (Fig. 5). In all cases, distal expression exceeded proximal expression. We were unable to detect TRPC3 and TRPC5 proteins, presumably because of low protein abundance and/or inadequate antibody specificity. Expression of TRPC2 and TRPC7 proteins was not tested because mRNA was not detected. Differences in antibody-antigen interactions in Western blotting precluded determination of the rank order of abundance among TRPC proteins.

STIM1 mRNA was detected by qPCR in proximal and distal PASMC and PA. In both PASMC and PA, distal expression was two- to threefold greater than proximal expression (P < 0.001; Fig. 6). Western blotting confirmed that STIM1 protein expression was greater in distal PASMC and PA (Fig. 7).

DISCUSSION

Acute hypoxia increased [Ca\(^{2+}\)] in both proximal and distal PASMC, but the increase was greater in distal cells (Fig. 1A). This result is consistent with previous observations in PASMC (25) and suggests that differences in the magnitude of HPV between proximal and distal PA (24, 45, 47) could be caused by differences in Ca\(^{2+}\) signaling in PASMC. In contrast, [Ca\(^{2+}\)], responses to KCl were the same in proximal and distal PASMC (Fig. 1B). This result suggests that activation of VOCC by depolarization was similar in proximal and distal cells and that another signaling pathway must have been responsible for the different [Ca\(^{2+}\)], responses to hypoxia.

Recent evidence indicates that the [Ca\(^{2+}\)], response to hypoxia requires SOCE in distal PASMC (31, 62). To determine if SOCE was different in proximal and distal PASMC, we measured the effects of restoration of extracellular Ca\(^{2+}\) (Fig. 2) or addition of extracellular Mn\(^{2+}\) (Fig. 3) on fura 2 fluorescence in PASMC perfused with Ca\(^{2+}\)-free KRBS. The perfusate contained CPA to deplete SR Ca\(^{2+}\) stores and activate SOCC and nifedipine to block Ca\(^{2+}\) entry through L-type VOCC. Responses to both interventions were greater in distal than proximal PASMC, indicating greater SOCE in distal cells. Furthermore, hypoxia increased Mn\(^{2+}\) quenching in proximal and distal PASMC, and this effect was greater in distal cells.

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These results indicate that SOCE and the effects of hypoxia on SOCE were greater in distal than proximal PASMC. This difference could explain why $[\text{Ca}^{2+}]_{i}$ responses to hypoxia were greater in distal cells (Fig. 1A).

SOCE could be increased because of increases in 1) $\text{Ca}^{2+}$ release from SR or 2) the number and activation of SOCC. With respect to the first possibility, the transient increase in $[\text{Ca}^{2+}]_{i}$ caused by CPA during perfusion with $\text{Ca}^{2+}$-free KRBS was not different during normoxia and hypoxia (Fig. 2), suggesting similar SR $\text{Ca}^{2+}$ release and therefore similar store depletion. One possible explanation for this finding is that CPA caused complete depletion of SR $\text{Ca}^{2+}$ stores. In this case, the increase in SOCE caused by hypoxia in the presence of CPA (Fig. 3) could be the result of enhanced activation of SOCC through effects of hypoxia on the channels themselves or transduction mechanisms linking store depletion to channel activation (62).

To evaluate the second possibility, we quantified expression of TRPC, which are thought to compose SOCC (33, 34), and STIM1, which is thought to sense SR $\text{Ca}^{2+}$ and transduce activation of SOCC (12, 15, 20, 28, 36, 39, 55). Consistent with our previous results (61, 63) and reports of other investigators (18, 21, 65, 71, 72), we detected mRNA and protein expression for TRPC1, TRPC4, and TRPC6 (Fig. 4). This rank order was the same in PASMC and PA, indicating that relative abundance was not

<fig5>Fig. 5. Expression of TRPC protein relative to $\alpha$-actin in distal (Dist) and proximal (Prox) PASMC (top) and PA (bottom) as determined by Western blotting. A and C: representative blots for TRPC1, TRPC4, TRPC6, and $\alpha$-actin in proximal (Prox) and distal (Dist) PASMC and PA. B and D: mean protein expression for TRPC1, TRPC4, and TRPC6 relative to $\alpha$-actin ($n = 3$ experiments on samples from 3 animals). Brackets indicate ± SE.

<fig6>Fig. 6. Expression of stromal interacting molecule 1 (STIM1) mRNA relative to $\beta$-actin in proximal and distal PASMC (top; $n = 6$ in each group) and PA (bottom; $n = 6$ in each group) as determined by real-time quantitative PCR. Brackets indicate ± SE.
altered by cell isolation and culture. It was also similar to that measured by qPCR in mouse distal PASMC (65) and standard PCR in human PASMC (71), but slightly different from standard PCR measurements in rat distal PASMC, where TRPC3 but not TRPC4 was detected (21). Consistent with our previous results (61, 63), but not those of others (21, 65, 71), we did not detect TRPC2 or TRPC7 by qPCR in PASMC or PA, even though both isoforms were detected in positive control tissue (rat brain). The reasons for these discrepancies are not clear.

Like SOCE, expression of TRPC1, TRPC6, and TRPC4 was greater in distal than proximal PASMC and PA (Figs. 4 and 5). Accumulating evidence suggests that these TRPC isoforms may function as SOCC in pulmonary arterial smooth muscle (7, 11, 42). For example, treatment with an antisense oligonucleotide targeted to TRPC1 reduced TRPC1 gene expression and SOCE in human PASMC (53), whereas overexpression of human TRPC1 in rat pulmonary arterial rings enhanced contractile responses to CPA, but not KCl (19). Knockdown of TRPC1 with small interfering RNA (siRNA) in rat PASMC attenuated cation influx induced by thapsigargin (21). Decreasing TRPC6 expression with an antisense oligonucleotide or increasing TRPC6 expression with mitogens decreased or increased, respectively, Ca\(^{2+}\) entry induced by CPA in rat distal PASMC (72). siRNA targeted to TRPC4 attenuated ATP-mediated increases in TRPC4 expression and SOCE and inhibited ATP-induced proliferation in human PASMC (75). If TRPC1, TRPC4, and TRPC6 were components of SOCC in our PASMC, the data shown in Figs. 4 and 5 suggest that the number of SOCC could be higher in distal cells and lead to greater SOCE and thus greater [Ca\(^{2+}\)]\(_i\) response to hypoxia. This suggests that TRPC6 may be both the sensor of ER Ca\(^{2+}\) release and the SOCC activator (15, 52, 68, 76). This conformational change is thought to trigger aggregation of STIM1 and TRPC6 in regions of SOCC-containing vesicles with sarclemma, which increases the number of SOCC at the cell surface (1, 70); and 4) decreased binding of Ca\(^{2+}\) to regions of inositol trisphosphate receptors within the depleted SR lumen, which leads to “conformational coupling” of the receptor’s cytoplasmic regions with sarclemmal SOCC (8, 17, 35, 40). None of these hypotheses, however, has achieved general acceptance (34).

Recently, it was proposed that depletion of Ca\(^{2+}\) in endoplasmic reticulum (ER) is sensed by the Ca\(^{2+}\)-binding protein STIM1, which spans the ER membrane and undergoes structural alteration when Ca\(^{2+}\) dissociates from its EF-hand domain within the ER lumen (15, 20, 22, 28, 39, 44). This conformational change is thought to trigger aggregation of STIM1 proteins in regions of ER membrane close to the plasma membrane, where interaction with SOCC or its associated regulatory proteins causes channel activation (15, 52, 68, 76). Thus STIM1 may be both the sensor of ER Ca\(^{2+}\) and transducer of SOCC activation.

Much of the work on STIM1 has been carried out in nonexcitable cells; however, recent evidence indicates that STIM1 is also required for SOCE in excitable cells, including smooth muscle. In human airway, STIM1 was highly expressed in smooth muscle, and siRNA targeted to STIM1 decreased STIM1 mRNA and protein, reduced SOCE, and virtually abolished CPA-induced inward current amplitudes (36).
In human coronary arterial smooth muscle, STIM1 protein was localized predominantly to SR but was also detected in sarcotubular (55). Furthermore, knockdown of STIM1 with STIM1-targeted siRNA drastically decreased SOCE. In mouse aortic smooth muscle cells, RNA interference decreased STIM1 expression and inhibited thapsigargin-induced store-operated calcium influx (12). As far as we know, expression of STIM1 in PASMC has not been documented.

We found STIM1 to be highly expressed in both PA and PASMC (Figs. 5–6). Furthermore, like SOCE (Fig. 2–3) and expression of TRPC1, TRPC6, and TRPC4 (Fig. 4–5), STIM1 expression was greater in distal than proximal smooth muscle. If STIM1 sensed SR Ca\(^{2+}\) and transduced SOCC activation in PASMC, as it is thought in other smooth muscles, this difference could contribute to greater SOCE (Figs. 2–3), greater augmentation of SOCE by hypoxia (Fig. 3), and greater [Ca\(^{2+}\)]\(_i\) responses to hypoxia in distal PASMC.

The coordinate upregulation of STIM1 and TRPC in distal pulmonary arterial smooth muscle is interesting, since interaction of STIM1 with TRPC seems to be essential for the latter to function as SOCC (2, 16, 32, 55, 67, 73). In HEK cells, the structural features necessary for STIM1 to activate SOCC were found to be the same as those required for STIM1 to bind and activate TRPC1 channels (16). In human platelets, Ca\(^{2+}\) store depletion stimulated rapid STIM1 surface expression and association with endogenously expressed TRPC1 (23). In human coronary arterial smooth muscle, an EF-hand mutant of STIM1, STIM1(E87A), markedly increased SOCE, and this increase was abolished by cotransfection with siRNA targeted to TRPC1 (55). These findings indicate that SOCC composed of TRPC1 require STIM1 for activation, and the same may be true for other TRPC (67). Whether similar conclusions apply to PASMC remains to be determined.

In summary, we found that basal SOCE, the increases in SOCE and [Ca\(^{2+}\)]\(_i\), caused by hypoxia, and expression of STIM1 and the most abundant TRPC isoforms (TRPC1, TRPC6, TRPC4) were greater in distal than proximal pulmonary arterial smooth muscle. Because SOCC may be composed of TRPC and activated by STIM1, and SOCE is required for [Ca\(^{2+}\)]\(_i\) responses to hypoxia in PASMC and HPV in isolated lungs, these results suggest that HPV is greater in distal PA because greater numbers and activation of SOCC generate bigger [Ca\(^{2+}\)]\(_i\) responses to hypoxia in distal PASMC. Further investigation of the roles played by SOCE, TRPC, and STIM1 in HPV is warranted.

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