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 vasconstriction (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\(^{2+}\) in distal PASMC, such as the change in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) induced by hypoxia (5, 46, 57, 59), K\(^{+}\) channel function (3, 4, 26, 48, 51) and expression (4, 9), CI\(^{–}\) channel function (48), Ca\(^{2+}\) channel density and O\(_{2}\) 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\(^{2+}\)]\(i\), 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, 43, 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 differs between proximal and distal PASMC is unknown.

Stromal interacting molecule 1 (STIM1), a 90-kDa transmembrane 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\(^{2+}\)]\(i\)) 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

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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 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).

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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 RNaseasy 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 Quantitec SYBR Green PCR Master Mix (Qiagen) in an iCyclerIQ real-time PCR detection system (Bio-Rad) using 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 were 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 80 increments of 0.5°C at 10-s intervals. Real-time qPCR detection threshold cycle values were generated by iCyclerIQ 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...
antibody was obtained from BD Biosciences (Franklin Lakes, NJ). Fura 2-AM (Invitrogen) was prepared on the day of the experiment as a 2.5 mM stock solution in pluronic dimethyl sulfoxide (DMSO). Stock solutions (30 mM) of CPA and nifedipine were made in DMSO.

Statistical analysis. Data are expressed as means ± SE; n is the number of experiments, which equals the number of animals providing arteries or cells. When fura 2 fluorescence was measured, the number of experiments, which equals the number of animals providing arteries or cells. When fura 2 fluorescence was measured, the number of experiments, which equals the number of animals providing arteries or cells. When fura 2 fluorescence was measured, the number of experiments, which equals the number of animals providing arteries or cells. When fura 2 fluorescence was measured, the number of experiments, which equals the number of animals providing arteries or cells.

F-ratios were obtained with the former, pairwise comparison of means when significant differences were considered significant when P < 0.05.

RESULTS

[Ca2+]i responses to hypoxia and KCl in PASMC. Acute hypoxia (4% O2) caused a rapid sustained increase in [Ca2+]i in both proximal and distal PASMC (Fig. 1A); however, the increase in distal PASMC was ~2.5-fold greater than that in proximal PASMC (peak Δ[Ca2+]i = 123 ± 18 vs. 32 ± 5 mM; P < 0.001). In contrast, responses to KCl (Fig. 1B) were the same in proximal and distal cells (peak Δ[Ca2+]i = 307 ± 84 vs. 313 ± 63 mM).

SOCE in PASMC. SOCE in proximal and distal PASMC was assessed in two ways. First, we measured the peak increase in [Ca2+]i caused by restoration of extracellular Ca2+ to 2.5 mM in PASMC perfused with Ca2+-free KRBS containing CPA and nifedipine. CPA caused an initial transient increase in [Ca2+]i during Ca2+-free perfusion (Fig. 2, A and B), which tended to be greater in distal than proximal PASMC (220 ± 45 vs. 140 ± 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 [Ca2+]i caused by restoration of extracellular Ca2+ was greater in distal than proximal PASMC (562 ± 65 vs. 410 ± 83 nM during normoxia and 798 ± 65 vs. 471 ± 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>
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TRPC, canonical transient receptor potential; STIM1, stromal interacting molecule 1.

Table 1. Primers for rat TRPC, STIM1, and β-actin used in real-time quantitative PCR
Because the increase in $[Ca^{2+}]_i$ caused by restoration of extracellular $Ca^{2+}$ 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 $Ca^{2+}$ 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).

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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.
Fig. 4. Expression of canonical transient receptor potential (TRPC) mRNA relative to β-actin in proximal and distal PASMC (top; n = 6 in each group) and pulmonary arteries (PA; bottom; n = 11 for TRPC1, TRPC4, and TRPC6 in each group, and n = 6 for TRPC5 and TRPC3 in each group) as determined by real-time quantitative PCR. TRPC2 and TRPC7 were not detected. Note expanded scales for TRPC5 and TRPC3. Brackets indicate ± SE.

DISCUSSION

Acute hypoxia increased \([\text{Ca}^{2+}]_{i}\) 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 \([\text{Ca}^{2+}]_{i}\) signaling in PASMC. In contrast, \([\text{Ca}^{2+}]_{i}\) 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 \([\text{Ca}^{2+}]_{i}\) responses to hypoxia.

Recent evidence indicates that the \([\text{Ca}^{2+}]_{i}\) 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 \([\text{Ca}^{2+}]_{i}\) (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 \([\text{Ca}^{2+}]_{i}\) stores and activate SOCC and nifedipine to block \([\text{Ca}^{2+}]_{i}\) 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.
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) \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\)-free KRBS was not different during normoxia and hypoxia (Fig. 2), suggesting similar SR \([\text{Ca}^{2+}]_i\) release and therefore similar store depletion. One possible explanation for this finding is that CPA caused complete depletion of SR \([\text{Ca}^{2+}]_i\) 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). In contrast to hypoxia, the effect of vessel locus on CPA-induced \([\text{Ca}^{2+}]_i\) release approached significance, i.e., \(\Delta[\text{Ca}^{2+}]_i\), caused by CPA during perfusion with \([\text{Ca}^{2+}]_i\)-free KRBS tended to be greater in distal than proximal PASMC (\(P = 0.06\); Fig. 2C), suggesting greater release and greater store depletion in distal cells. Potential reasons for such a difference include larger stores and/or more leakage of SR \([\text{Ca}^{2+}]_i\) in distal PASMC. Of course, an augmented CPA response could also be the result of lower \([\text{Ca}^{2+}]_i\) efflux via sarcolemmal \([\text{Ca}^{2+}]_i\)-ATPase or \(\text{Na}^+/\text{Ca}^{2+}\) exchange, lower \([\text{Ca}^{2+}]_i\) uptake in mitochondria, or less \([\text{Ca}^{2+}]_i\) buffering in distal PASMC. Further investigation is needed to confirm the different effects of CPA in proximal and distal PASMC, and to clarify underlying mechanisms.

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 \([\text{Ca}^{2+}]_i\) 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 in distal PASMC and PA (Figs. 4 and 5). We also detected TRPC3 and TRPC5, which were not identified in our previous studies (61, 63). This inconsistency is probably because of the use of real-time qPCR in the present study, which facilitated detection of less abundant isoforms. TRPC1 was by far the most abundant, followed by TRPC6 and TRPC4, and then much more distantly by TRPC3 and TRPC5 (Fig. 4). This rank order was the same in PASMC and PA, indicating that relative abundance was not...
TRPC5 mRNA was lower in distal than proximal PA tissue. Perhaps inconsistent with this possibility, expression of TRPC6 increased with mitogens decreased or inhibited ATP-induced proliferation in human PASMC (75). If TRPC6 knockout abolished early HPV by eliminating DAG-dependent influx of Ca$^{2+}$ through receptor-operated channels composed of TRPC6. We do not know whether receptor-operated mechanisms contributed to augmentation of the [Ca$^{2+}$]$_i$ response to hypoxia in distal PASMC (Fig. 1A), which exhibited enhanced TRPC6 expression (Figs. 4–5) but were not primed with vasoactive agents.

Several hypotheses have been proposed to explain how depletion of SR Ca$^{2+}$ activates SOCC, including 1) SR release of “Ca$^{2+}$ influx factor,” which liberates sarcoplasmic lysosphospholipids through activation of phospholipase A$_2$ (41, 49, 50, 56); 2) decreased [Ca$^{2+}$]$_i$, Mn$^{2+}$ quenching of fura 2 fluorescence, or cation currents through pathways other than K$^+$ channels or L-type VOCC, whereas all of these variables were increased by hypoxia in wild-type PASMC (65). In addition, the transient early (0–30 min) phase of HPV was abolished in lungs from TRPC6$^{-/-}$ mice. Because R-59949, a DAG kinase inhibitor, increased [Ca$^{2+}$]$_i$, in PASMC from wild-type but not TRPC6$^{-/-}$ mice and caused sarcolemmal accumulation of DAG in both wild-type and TRPC6$^{-/-}$ cells, the authors concluded that TRPC6 knockout abolished early HPV by eliminating DAG-dependent influx of Ca$^{2+}$ through receptor-operated channels composed of TRPC6.

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 region (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 sarcolemma (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 cation 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.

REFERENCES


