Expression of store-operated Ca\(^{2+}\) entry and transient receptor potential canonical and vanilloid-related proteins in rat distal pulmonary venous smooth muscle

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Peng G, Lu W, Li X, Chen Y, Zhong N, Ran P, Wang J. Expression of store-operated Ca\(^{2+}\) entry and transient receptor potential canonical and vanilloid-related proteins in rat distal pulmonary venous smooth muscle. Am J Physiol Lung Cell Mol Physiol 299: L621–L630, 2010. First published August 6, 2010; doi:10.1152/ajplung.00176.2009.—Chronic hypoxia causes remodeling and alters contractile responses in both pulmonary arteries and pulmonary veins. Although pulmonary arteries have been studied extensively in these disorders, the mechanisms by which pulmonary veins respond to hypoxia and whether these responses contribute to chronic hypoxic pulmonary hypertension remain poorly understood. In pulmonary arterial smooth muscle, we have previously demonstrated that influx of Ca\(^{2+}\) through store-operated calcium channels (SOCC) thought to be composed of transient receptor potential (TRP) proteins is likely to play an important role in development of chronic hypoxic pulmonary hypertension. To determine whether this mechanism could also be operative in pulmonary venous smooth muscle, we measured intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) by fura-2 fluorescence microscopy in primary cultures of pulmonary venous smooth muscle cells (PVSMC) isolated from rat distal pulmonary veins. In cells perfused with Ca\(^{2+}\)-free media containing cyclopiazonic acid (10 μM) and nifedipine (5 μM) to deplete sarcoplasmic reticulum Ca\(^{2+}\) stores and block voltage-dependent Ca\(^{2+}\) channels, restoration of extracellular Ca\(^{2+}\) (2.5 mM) caused marked increases in [Ca\(^{2+}\)]\(i\), whereas MnCl\(_2\) (200 μM) quenched fura-2 fluorescence, indicating store-operated Ca\(^{2+}\) entry (SOCE). SKF-96365 and NiCl\(_2\), antagonists of SOCC, blocked SOCE at concentrations that did not alter Ca\(^{2+}\) responses to 60 mM KCl. Of the seven known canonical TRP (TRPC1–7) and six vanilloid-related TRP channels (TRPV1–6), real-time PCR revealed mRNA expression of TRPC1 > TRPC6 > TRPC4 > TRPC2 > TRPC5 > TRPC3, TRPV2 > TRPV4 > TRPV1 in distal PVSMC, and TRPC1 > TRPC6 > TRPC3 > TRPC4 ≈ TRPC5, TRPV2 ≈ TRPV4 > TRPV1 in rat distal pulmonary vein (PV) smooth muscle. Western blotting confirmed protein expression of TRPC1, TRPC6, TRPV2, and TRPV4 in both PVSMC and PV. Our results suggest that SOCE through Ca\(^{2+}\) channels composed of TRP proteins may contribute to Ca\(^{2+}\) signaling in rat distal PV smooth muscle.

**THE VASOMOTOR ACTIVITY OF THE PULMONARY VENOUS SYSTEM plays important roles in regulating both distention and recruitment of blood flow from alveolar wall capillaries, and thus facilitate the ventilation-perfusion matching in the lung. Studies in a variety of species indicate pulmonary veins (PV) exhibit constriction in response to a number of vasoconstrictor stimuli, such as endothelin (2, 3, 5, 26, 34), platelet-activating factor (5, 26), thromboxane (10, 25), and hypoxia (8, 24, 28, 35, 49). During prolonged hypoxic exposure, vasoconstriction and structural alterations occur not only in pulmonary arteries (PA) but also in PV, each contributing a significant portion to total pulmonary vascular resistance (5, 24, 25, 26, 49). Despite the functional importance of both PA and PV in regulating pulmonary circulation being recognized, less attention has been paid to the PV side in searching for the molecular mechanism of pulmonary hypertension.**

In vascular smooth muscle, increases in [Ca\(^{2+}\)]\(i\), is an essential signal for vasoconstriction, as well as for myocyte proliferation (27). A rise in [Ca\(^{2+}\)]\(i\), can be caused by either release of Ca\(^{2+}\) from internal storage sites, such as sarcoplasmic reticulum (SR), and/or Ca\(^{2+}\) influx from an extracellular source. L-type voltage-dependent Ca\(^{2+}\) channels (VDCC), receptor-operated Ca\(^{2+}\) channels (ROCC), and store-operated Ca\(^{2+}\) channels (SOCC) constitute the major Ca\(^{2+}\) influx pathways (11, 30). VDCC act as the main Ca\(^{2+}\) channel in the vascular smooth muscle cell membrane, activated by membrane depolarization, and can be blocked by Ca\(^{2+}\) channel blockers such as nifedipine (11, 21, 22). ROCC are present in many types of smooth muscle cells. They are typically activated by inositol lipid signaling, one of the most widespread signal transduction cascades, which involves G protein-activated phospholipase C and two second messenger diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)). SOCC mediates store-operated Ca\(^{2+}\) entry (SOCE) and thus plays a critical role to refill Ca\(^{2+}\) in SR and to maintain Ca\(^{2+}\) homeostasis. Activation of this pathway can be independent of IP\(_3\) production, since various procedures that deplete internal stores, i.e., using thapsigargin or cyclopiazonic acid (CPA), are able to stimulate Ca\(^{2+}\) entry across the plasma membrane without affecting the intracellular IP\(_3\) level (6, 9).

The molecular components of SOCC have not been completely understood. A growing body of evidence suggests that it is composed of the mammalian homologs of canonical transient receptor potential (TRPC) proteins (19, 20). So far, seven members of TRPC, termed TRPC1–7, have been identified in mammals (20). Presence of various patterns of TRPC isoforms among studies has been reported in PA (16). We recently demonstrated the predominant expression of TRPC1, TRPC4, and TRPC6 in rat PA smooth muscle and PASM (16, 38, 39). Vanilloid-related transient receptor potential (TRPV) proteins, which are composed of six members (TRPV1–6), constitute another subfamily of TRP channels. Recent studies

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have revealed that TRPV channels, i.e., TRPV1, TRPV6, may be involved in mediating SOCE under certain conditions (21, 40). Expression of some of the TRPV proteins has been demonstrated in systemic and pulmonary arterial smooth muscle and smooth muscle cells (40, 46). The expression pattern and role of TRPC and TRPV in PV are unknown.

We recently found that in rat distal pulmonary arterial smooth muscle, SOCE plays roles in hypoxic pulmonary vascular constriction (42), as well as in the development of chronic hypoxic pulmonary hypertension (39). The general goal of this study is to determine whether these mechanisms could also be operative in PV. As an initial step, the present study was designed to define if SOCE occurs in pulmonary venous smooth muscle cells (PVSMC).

METHODS

PVSMC isolation and culture. Animal experiment protocols were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. The isolation and culture of PVSMC was adapted from a procedure as we previously described for PASMC (38). Briefly, proximal (2nd generation) and distal (≥4th generations) PV relative to atrium were dissected from lungs of male Wistar rats (ages 8–10 wk). The thin layer of adventitia was carefully stripped off with fine forceps, and the endothelium was wiped off using a cotton swab. The isolated PV smooth muscle layer was sequentially incubated for 20 min in ice-cold physiological salt solution (PSS), 20 min in reduced-Ca2+ PSS (20 μM CaCl2) at room temperature, and 20–23 min in reduced-Ca2+ PSS containing collagenase, papain, BSA, and dithiothreitol at 37°C to disperse PVSMC into a single-cell suspension. The cells were washed twice with PSS, resuspended in smooth muscle growth medium-2 (Clonetics, Walkersville, MD) containing 5% serum, plated onto 25-mm coverslips, and cultured for 4–6 days in a humidified atmosphere of 5% CO2-95% air at 37°C. Cells were incubated with mouse monoclonal antibody for α-smooth muscle actin (Sigma, St. Louis, MO). Bound antibodies were probed with Cy3-linked goat anti-mouse antibody (Jackson Labs, West Grove, PA). Nuclei of cells were stained with YO-PRO-1 dimeric cyanine dye (Molecular Probes, Eugene, OR). Primary antibody was omitted in control-staining cells. The stained cell coverslips were mounted on slides with FluoroGuard Antifade (Bio-Rad, Hercules, CA). Cells were examined under a Zeiss LSM-510 inverted laser-scanning confocal microscope with a Zeiss Plan-Neofluor ×40 oil-immersion objective (Atlanta, GA).

RNA extraction and real-time quantitative PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) for distal PV tissue and using RNeasy kit (Qiagen, Valencia, CA) for PVSMC as previously described (16). DNA contamination in RNA preparations was removed by on-column DNase I digestion (Qiagen). mRNA was reverse transcribed by standard reaction using iScript cDNA synthesis kit (BioRad). cDNA was quantified with real-time quantitative PCR (qPCR) in an iCyclerIQ machine (BioRad) using Quantitect SYBR Green PCR Master Mix (Qiagen). The qPCR reaction mixture of 25 μl contained 400 nM forward and reverse primers and cDNA template from 6.25 ng of RNA. Primers specifically designed for rat TRPC1–7, TRPV1–6, and β-actin are listed in Table 1. The program of real-time qPCR consisted of three steps including a hot start at 95°C for 15 min, 40 cycles with each containing 94°C for 15 s, 57.5°C for 20 s, and 72°C for 20 s, and melting curves performed at 95°C for 1 min, 55°C for 1 min, and 80 repeats of increment of 0.5°C. Detection threshold cycle (Ct) values were generated by iCyclerIQ software. Relative concentration of each transcript was calculated using the Pfaffl method (16, 23). Data were expressed as a ratio of TRPC or TRPV to β-actin in the same sample. Specificity of

Table 1. Real-time PCR primers for rat TRPCs, TRPVs, and β-actin

| Gene   | Accession No. | Primer Sequence, Left/Right Product Size, bp Location in Sequence Positive Control |
|--------|---------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| TRPC1  | NM_053558     | 5′-AGGCTCTTGAGAAACGAGGA-3′ 5′-AGCCTGACGACGATTCCCAACG-3′ 146 797–942 Brain |
| TRPC2  | NM_022638     | 5′-TTGCTGGAGAGATGTCCTG-3′ 5′-CTCTGATCCAGACGCTCCCTTA-3′ 139 1235–1393 Brain |
| TRPC3  | NM_021771     | 5′-GAGATGTTGAGATGTCCTG-3′ 5′-AAAAGGTCGTTGTTGGGACTT-3′ 127 608–754 Brain |
| TRPC4  | NM_053434     | 5′-GACCAAGACCTTCGGAGAGAC-3′ 5′-GGTGAGCTGAGAACAA-3′ 142 771–912 Brain |
| TRPC5  | NM_080898     | 5′-CCGAGGAGGTTGTAAGG-3′ 5′-TGTGATGCTGCTGGAACAC-3′ 121 643–784 Brain |
| TRPC6  | NM_053559     | 5′-TATCTGCCTGTGCCTTCTGGC-3′ 5′-GAGCCTGGTGGTGTCAAAATG-3′ 141 1267–1407 Brain |
| TRPC7  | XM_225159     | 5′-AACGAGCTGATCCGAAAGAC-3′ 5′-AGCGTTGTCCTGGGCTCAATG-3′ 114 1270–1383 Brain |
| TRPV1  | NM_031982     | 5′-TGTTGATTTGCTGCTGCTG-3′ 5′-GTGTTGATTTGCTGCTGCTG-3′ 79 517–595 Brain |
| TRPV2  | NM_017207     | 5′-GCTGTCCTGCTGCTGCTGCTG-3′ 5′-AGGATGCTGTCCTGCTGCTGCTG-3′ 138 1865–2002 Brain |
| TRPV3  | NM_001025757  | 5′-CTACAGGAAAGAGGAGGA-3′ 5′-AGTTGTCCTGCTGCTGCTGCTG-3′ 122 322–443 Small intestine |
| TRPV4  | NM_023970     | 5′-GAGAACTGAGATGCTGCTGCTG-3′ 5′-TGTGAACTGAGATGCTGCTGCTG-3′ 143 1171–1313 Brain |
| TRPV5  | NM_053787     | 5′-GCTGTCCTGCTGCTGCTGCTG-3′ 5′-GTGTTGATGCTGCTGCTGCTGCTG-3′ 75 1048–1088 Kidney |
| TRPV6  | NM_053686     | 5′-GTGTTGATGCTGCTGCTGCTG-3′ 5′-AGCAGGAGGAGGAAGTCCGATG-3′ 93 605–697 Brain |
| β-actin| NM_031144     | 5′-AGGCTGAGTCCATCATTAC-3′ 5′-TGCCAGAGGATCTAATGTAAC-3′ 90 812–901 Brain |
the PCR products was confirmed by the melting curve, agarose gel electrophoresis, and DNA sequencing.

Western blotting. Samples of PV tissue or PVSVMC were homogenized 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. Protein concentration in the homogenates was quantified using BCA protein assay (Pierce, Rockford, IL). Tissue or cell homogenates were added with dithiothreitol to 150 mM, heated at 95°C for 3 min, and resolved by 10% SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride membranes (pore size 0.45 μM, BioRad) and blotted with rabbit polyclonal antibodies against TRPC1 (Sigma), TRPC6, TRPV2, TRPV4, or mouse monoclonal antibody specific for α-actin (Sigma). Bound antibodies were probed with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (KPL, Gaithersburg, MD) and detected using an enhanced chemiluminescence system (ECL; GE Healthcare, Piscataway, NJ).

Measurement of intracellular Ca²⁺ concentration. As previously described (38), coverslips with PVSVMC were incubated with 5 μM fura-2 AM (Invitrogen) for 60 min at 37°C with 5% CO₂-95% air, desorbed fluorescent antibodies, and then coverslips with PVSVMC were incubated with 5 μM fura-2 AM (Invitrogen) for 60 min at 37°C with 5% CO₂-95% air, desorbed fluorescent antibodies, and then mounted in a closed polycarbonate chamber, and clamped in a heated aluminum platform (PH-2; Warner Instrument, Hamden, CT) on the stage of a Nikon TSE 100 Ellipse inverted microscope (Nikon, Melville, NY). The chamber was perfused at 0.5–1 ml/min with Krebs ringer bicarbonate (KRB) solution equilibrated with 16% O₂-5% CO₂ at 38°C in heated reservoirs. The perfusate was led via stainless steel tubing and a manifold to an inline heat exchanger (SF-28, Warner Instrument) to rewarm the perfusate just before it entered the cell chamber. The temperature of the heat exchanger and chamber platform was controlled by a dual-channel heater controller (TC-344B, Warner Instrument). Cells were perfused for 10–15 min to remove the extracellular dye, and intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was determined at 12– to 30-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 (F₃₄₀/F₃₈₀) 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 IncCyte software (Intracellular Imaging, Cincinnati, OH). [Ca²⁺]ᵢ was estimated from the calibration curve of F₃₄₀/F₃₈₀ measured in standard solutions with [Ca²⁺]² of 0–1,350 nM. For depolarizing cells to assess VDCC, perfusate was supplemented with 60 mM KCl, and NaCl was decreased to 62.7 mM.

Assessment of SOCE. We assessed SOCE in two ways, Ca²⁺ restoration and Mn²⁺ quenching, as previously described (38). Briefly, PVSVMC were perfused for 10–15 min with Ca²⁺-free KRB solution containing 5 μM nifedipine, 10 μM CPA, and 1 mM EGTA. We first measured [Ca²⁺]ᵢ at 0.5-min intervals before and after restoration of extracellular [Ca²⁺]² to 2.5 mM. SOCE were evaluated from the increase in [Ca²⁺]ᵢ caused by restoration of extracellular [Ca²⁺]². Second, we monitored fura-2 fluorescence excited at 360 nm at 0.5-min intervals before and after addition of MnCl₂ (200 μM) to the cell perfusate. Mn²⁺ competes with Ca²⁺ to enter the cell; however, it reduces fura-2 fluorescence on binding to the dye. Fluorescence excited at 360 nm is the same for Ca²⁺-bound and Ca²⁺-free fura-2; therefore, changes in fluorescence can be assumed to be caused by Mn²⁺ alone. SOCE was evaluated from the rate at which fura-2 fluorescence was quenched by Mn²⁺.

Drugs and materials. Unless otherwise specified, all reagents were obtained from Sigma. TRPC6, TRPV2, and TRPV4 antibodies were purchased from Alomone Laboratories (Jerusalem, Israel). Fura-2 AM (Invitrogen) was prepared on the day of the experiment as a 2.5 mM stock solution in pluronic dimethyl sulfoxide (20% DMSO). Stock solutions of SKF-96365, NiCl₂, and mibebradil were made in water at 10, 500, and 30 mM, respectively. Stock solutions of CPA and nifedipine were both made in DMSO at 30 mM.

Statistical analysis. Data are expressed as means ± SE; n is the number of experiments, which equals the number of animals providing veins or PVSVMC. When fura-2 fluorescence was measured, the number of cells in each experiment was 20–30, as indicated in RESULTS and the legends for Figs. 1–9. Statistical analyses were performed using Student’s t-test. Differences were considered significant when P < 0.05.

RESULTS

Characteristics of rat distal PVSVMC in primary culture. In phase-contrast imaging, cells cultured for 2–6 days from distal PV appeared spindle-shaped with elongated fibers extended from a larger central area (Fig. 1A and B). Immunostaining for α-actin showed specific red staining of actin fiber bundles in >98% of cells with green staining for nuclei (Fig. 1C). All cells in the observed fields exhibited increased [Ca²⁺]ᵢ in response to 60 mM KCl, indicating the presence of VDCC (Fig. 1D). These characteristics confirmed that the cells in the primary culture were smooth muscle cells.

SOCE in rat distal PVSVMC. We assessed SOCE by measuring both [Ca²⁺]ᵢ response to extracellular Ca²⁺ restoration and Mn²⁺ quenching of fura-2 fluorescence after store depletion with CPA. As seen in Fig. 2A, in the presence of nifedipine, a specific L-type VDCC blocker, perfusion of PVSVMC with Ca²⁺-free KRB solution containing CPA caused an initial small transient increase in [Ca²⁺]ᵢ, which returned to baseline after 5–10 min, indicating calcium release from SR; subsequent restoration of extracellular Ca²⁺ to 2.5 mM caused a second large increase of [Ca²⁺]ᵢ that went up quickly to a peak
of \([\text{Ca}^{2+}]\) = 365 ± 70 nM (Fig. 2, A and B, n = 8, P < 0.0001 vs. the value in the absence of CPA), followed by a lower plateau with final \([\text{Ca}^{2+}]\) = 187 ± 62 nM (n = 8) before it returned to baseline by perfusion of cells with KRB solution. When CPA was omitted in the perfusate, these changes in \([\text{Ca}^{2+}]\) did not happen (Fig. 2, A and B). In the Mn\(^{2+}\) quenching study, perfusion of PVSMC for 10 min with Ca\(^{2+}\)-free KRB solution containing Mn\(^{2+}\) resulted in 45 ± 4.5% (n = 7) decrease in fura-2 fluorescence in the presence of nifedipine and CPA, whereas only 20 ± 2.6% decrease in the absence of CPA (n = 5; P < 0.0001 vs. the value in the absence of CPA) (Fig. 3, A and B). These data indicate the occurrence of SOCE after store depletion with CPA in PVSMC. Mibefradil, a selective T-type Ca\(^{2+}\) channel antagonist, did not affect SOCE assessed either by Ca\(^{2+}\) restoration or Mn\(^{2+}\) quenching (data not shown), suggesting Ca\(^{2+}\) entry under these assessments was not mediated by T-type Ca\(^{2+}\) channels.

SOCC antagonists, i.e., SKF-96365 and Ni\(^{2+}\), have been shown to block SOCE in various cell types including smooth muscle cells such as PASMC (15, 17, 21, 38, 39); therefore, we evaluated their effects on SOCE assessment to confirm the presence of SOCE in PVSMC. As shown in Fig. 4, although SKF-96365 assessed at 1, 10, and 50 \(\mu\)M or Ni\(^{2+}\) at 50, 200, and 500 \(\mu\)M caused marked attenuation to Ca\(^{2+}\) release by CPA (P < 0.01 compared with control at 189 ± 14.0 nM), the attenuated levels (125 ± 10.3 nM, 106 ± 5.0 nM, and 92 ± 6.5 nM for SKF-96365 at 1, 10, and 50 \(\mu\)M; 128 ± 7.7 nM, 104 ± 8.7 nM, and 101 ± 5.4 nM for Ni\(^{2+}\) at 50, 200, and 500 \(\mu\)M) were not different among different doses (Fig. 4, A–C); however, both SKF-96365 and Ni\(^{2+}\) decreased Ca\(^{2+}\) entry in response to extracellular Ca\(^{2+}\) restoration in a dose-dependent manner, with maximum decrease of the peak of \([\text{Ca}^{2+}]\), response from 338 ± 22.7 nM (n = 8) in untreated control cells to an average of 70 ± 16.5 nM in cells perfused with 50 \(\mu\)M SKF-96365 (n = 5; P < 0.0001 vs. control), and 82 ± 13.8 nM in cells with 500 \(\mu\)M Ni\(^{2+}\) (n = 6; P < 0.0001 vs. control) (Fig. 4, A, B, and D), indicating that SKF-96365 and Ni\(^{2+}\) acted, at least in part, by directly blocking Ca\(^{2+}\) entry. Similarly, in SOCE measured by Mn\(^{2+}\) quenching of fura-2 fluorescence, administration of Mn\(^{2+}\) for 10 min in the perfusate with 50 \(\mu\)M SKF-96365 or 500 \(\mu\)M Ni\(^{2+}\) decreased fura-2 fluorescence by 21 ± 3.3% (n = 5; P < 0.0001 vs. control) and 20 ± 4.1% (n = 5, P < 0.0001 vs. control), respectively; these values were significantly less than the decrease observed in control cells with vehicle (46 ± 5.0%, n = 5) (Fig. 5), confirming that SKF-96365 and Ni\(^{2+}\) blocked Ca\(^{2+}\) entry.

The specificity of 50 \(\mu\)M SKF-96365 or 500 \(\mu\)M Ni\(^{2+}\) on SOCE was verified by testing their alteration on \([\text{Ca}^{2+}]\), response to depolarization with 60 mM KCl. As shown in Fig. 6, A and B, none of them caused significant change in this response (n = 6 each). In contrast, the \([\text{Ca}^{2+}]\), response...
to KCl was abolished by nifedipine (n = 5; P < 0.0001 vs. control) (Fig. 6C).

TRPC and TRPV expression in distal PVSMC and PV. To determine the molecular identity of SOCC responsible for SOCE in distal PVSMC and PV, we screened the expression of TRPC and TRPV transcripts using real-time PCR. Of the seven known isoforms of TRPC, we were able to detect mRNA expression of TRPC1, TRPC2, TRPC3, TRPC4, TRPC5, and TRPC6 with the abundance rank order of TRPC1 > TRPC6 > TRPC4 > TRPC2 = TRPC5 > TRPC3 in distal PVSMC;
relative to TRPC1, the expression abundance of TRPC2, TRPC3, TRPC4, TRPC5, and TRPC6 were 9.5 × 10^{-4}, 3.0 × 10^{-4}, 1.1 × 10^{-2}, 8.2 × 10^{-4}, and 2.4 × 10^{-1}, respectively (Fig. 7). Similarly, in PV tissue, we detected TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 mRNA; the rank order of them was TRPC1 > TRPC6 > TRPC3 > TRPC4 ≈ TRPC5 (Fig. 7). TRPC2 was minimally detected in PVSMC, but not in PV. TRPC7 was not found in either distal PVSMC or PV.

Of the six so far identified subtypes of TRPV (TRPV1–6), we detected the mRNA expression of TRPV1, TRPV2, and TRPV4 in distal PVSMC and PV (Fig. 8). In PVSMC, TRPV2 is 16-fold more abundant than TRPV4, and 110-fold more than TRPV1. In PV tissue, the abundance of TRPV2 and TRPV4 was similar; TRPV1 was three- to fivefold less.

With rat brain as positive control, we demonstrated the protein expression of TRPC1, TRPC6, TRPV2, and TRPV4 by Western blotting, which was found most abundantly expressed among the TRPC and TRPV in both distal PVSMC and PV (Fig. 9).

Reduced expression of TRPC6 in distal PVSMC and PV compared with proximal. As shown in Fig. 7, the mRNA expression profiles and the amount of TRPC and TRPV in proximal and distal PVSMC and PV were similar, with the exception for TRPC5, which was minimally detected in distal PVSMC, but not in proximal cells at all, and for TRPC6, which exhibited reduced expression in distal PV and PVSMC compared with the respective proximal tissue and cells. Western blotting also indicated that the expression of TRPC6 in distal PV and PVSMC is less than proximal; in terms of the expression of TRPC1, TRPV2, and TRPV4, there is no difference between distal and proximal tissue and cells (Fig. 8).

DISCUSSION

The present study demonstrated the occurrence of SOCE and expression of TRPC and TRPV in rat distal PV smooth muscle. Cells isolated and cultured from distal PV were identified to be smooth muscle cells at high purity. To reduce the chance of cardiomyocyte contamination, we chose distal PV to work with, as the main PV that approaches the left atrium was thought to contain cardiac myocytes (18). The cells in the primary culture exhibit the common characteristics of other smooth muscle cells such as PASMC, in terms of the spindle-
shaped morphology, the expression of α-actin, and presence of VDCC (47). In this highly pure PVSMC culture, we assessed the presence of SOCE as the increase in [Ca\(^{2+}\)]\(_i\) following restoration of extracellular [Ca\(^{2+}\)] and as the decreased rate of Mn\(^{2+}\) quenching, with both blocked by SOCC antagonists Ni\(^{2+}\) and SKF-96365. By screening the expression of various isoforms of TRPC and TRPV, we found TRPC1, TRPC6, TRPV2, and TRPV4 were the predominant ones expressed in PVSMC and PV.

The occurrence of SOCE in PVSMC was demonstrated via the following evidence through three approaches. First, immediately after exposure of PVSMC to Ca\(^{2+}\)-free perfusate containing nifedipine and CPA, a small transient rise in [Ca\(^{2+}\)]\(_i\) was observed, indicating Ca\(^{2+}\) release from SR; subsequently, restoring extracellular Ca\(^{2+}\) caused a bigger and sustained increase in [Ca\(^{2+}\)]\(_i\), reflecting Ca\(^{2+}\) influx. These responses were dependent on store depletion, as they did not occur in PVSMC exposed to Ca\(^{2+}\)-free perfusate containing nifedipine but not CPA. Second, to further confirm that depletion of SR Ca\(^{2+}\) stores caused Ca\(^{2+}\) entry, we also determined the rate of Mn\(^{2+}\) quenching on fura-2 fluorescence in PVSMC. Mn\(^{2+}\) is a Ca\(^{2+}\) surrogate; it reduces fura-2 fluorescence upon binding to the dye. The intensity of fluorescence excited at 360 nm is the same (isosbestic) for Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free fura-2; therefore, changes in fluorescence induced by Mn\(^{2+}\) can be assumed to be due to Mn\(^{2+}\) alone. Before exposure of PVSMC to Mn\(^{2+}\), there was a slow decline in fura-2 fluorescence, a phenomenon that was similar as observed in PASMC, thought to be due to photo bleaching of the dye (38); however, Mn\(^{2+}\) caused a marked increase of this decline in cells exposed to CPA, suggesting enhanced entry through SOCC. In cells not exposed to CPA, Mn\(^{2+}\) only increased quenching of fura-2 fluorescence slightly, suggesting the presence of Ca\(^{2+}\) entry pathways other

![Fig. 7. Expression of TRPC mRNA normalized to β-actin in rat proximal and distal PVSMC (top: n = 5) and PV (bottom: n = 5) as determined by real-time quantitative PCR. *P < 0.05 vs. respective proximal cells or tissue.](http://ajplung.physiology.org/)

![Fig. 8. Expression of TRPV mRNA normalized to β-actin in rat proximal and distal PVSMC (top: n = 5) and PV (bottom: n = 5) as determined by real-time quantitative PCR.](http://ajplung.physiology.org/)
than SOCC or L-type VDCC. In these assessments, nifedipine was included in the perfusate to block the activity of VDCC, and CPA, a sarcoplasmic-endoplasmic Ca\textsuperscript{2+}/H\textsuperscript{+}-ATPase (SERCA) pump inhibitor, was used to deplete intracellular stores; the single dose of nifedipine at 5 \textmu M and CPA at 10 \textmu M was chosen because they have been proven to be effective in blocking VDCC or depleting SR Ca\textsuperscript{2+} in various cell types including smooth muscle cells (38). In addition, considering the previous observation that Ca\textsuperscript{2+} influx through SOCC proteins (i.e., TRPC6) could contribute to membrane depolarization, which may subsequently activate low voltage-activated Ca\textsuperscript{2+} channels (i.e., T-type Ca\textsuperscript{2+} channels), mibefradil was included in the perfusate, and we found that SOCE in PVSMC assessed either by Ca\textsuperscript{2+} restoration or Mn\textsuperscript{2+} quenching were not influenced by T-type Ca\textsuperscript{2+} channels. Last, SKF-96365 and Ni\textsuperscript{2+} are commonly used SOCC antagonists and have been used as a pharmacological tool to identify SOCE in various smooth muscle cell preparations or vascular tissues from different species (7, 14, 21, 38, 41). Therefore, here we determined their effects in PVSMC to confirm the presence of SOCE. Indeed, exposure to SKF-96365 (50 \textmu M) or Ni\textsuperscript{2+} (500 \textmu M) blocked Ca\textsuperscript{2+} release initiated by CPA. Hence, the reduced Ca\textsuperscript{2+} influx caused by SKF-96365 or Ni\textsuperscript{2+} could be due to direct inhibition on Ca\textsuperscript{2+} entry through SOCC and incomplete Ca\textsuperscript{2+} store depletion. In fact, divergent effects of SKF-96365 on Ca\textsuperscript{2+} entry and release have been observed in various cell types (14). The mechanisms of how SKF-96365 or Ni\textsuperscript{2+} inhibited Ca\textsuperscript{2+} release in PVSMC need to be further identified. Together, these results provide strong evidence indicating the presence of SOCE in rat distal PVSMC. Our results are consistent with a previous report that SOCE exists in PVSMC using thapsigargin (TG) to inhibit SERCA pump activity. Shimizu et al. (29) observed a transient increase in [Ca\textsuperscript{2+}]\text{\textit{i}} caused by TG and a rapid peak increase in [Ca\textsuperscript{2+}], followed by a sustained increase in [Ca\textsuperscript{2+}], after restoring extracellular Ca\textsuperscript{2+} in canine PVSMC.

Besides the functional demonstration of the presence of SOCE in distal PVSMC, we furthermore found that, of the known isoforms of TRPC, TRPC1 and TRPC6 were the ones predominantly expressed in both distal PVSMC and distal PV smooth muscle. Although we were able to detect the mRNA of TRPC3, TRPC4, TRPC5, and/or TRPC2 in PVSMC and PV, their expression was far less (100-fold in PVSMC and 35-fold in PV) compared with TRPC1. Regardless of their expression level, all of these TRPC channel proteins have been shown to contribute to SOCE under certain conditions (22). For example, overexpression of TRPC1 mediated by adenovirus vectors enhanced SOCE in rat PA rings (13). In contrast, RNA interference for TRPC1 resulted in attenuated SOCE in mouse osteoblasts (1). Tiruppathi et al. (33) reported that mouse microvascular endothelial cells lacking TRPC4 pro-

![Fig. 9. Expression of TRPC and TRPV proteins in rat proximal and distal PVSMC and PV as determined by Western blotting using rat brain as positive control. Data show representative blots for TRPC1, TRPC4, TRPC6, TRPV2, TRPV4, and \beta-actin in rat distal PVSMC (top: \textit{n} = 5) and PV (bottom: \textit{n} = 3). *\textit{P} < 0.05 vs. respective proximal cells or tissue.](http://ajplung.physiology.org/)
duced a similar initial increase of intracellular Ca\textsuperscript{2+} secondary to store depletion, but drastically reduced Ca\textsuperscript{2+} influx induced by agonists such as thrombin compared with cells from TRPC4 wild-type mice. Conversely, increased expression of TRPC4 was stimulated by agonist, i.e., ATP was associated with enhanced SOCE in human PASMC (48). As for TRPC6, although it has been identified as a putative receptor-operated channel mediating store-independent Ca\textsuperscript{2+} currents (9), increased expression of TRPC6 was later on also found to be associated with enhanced SOCE in rat PASMC (47). Thus, it has been assumed that SOCC are likely composed of multiple TRPC isoforms, which associate into complex to form heterogeneous channels (12). The dominant expression of TRPC1 and TRPC6 suggests they likely play a major role in eliciting SOCE in PV smooth muscle.

The understanding of TRPV proteins as components of SOCC is far more elusive than TRPC. Among the six members of TRPV, mRNA of TRPV1, TRPV2, and TRPV4 were found expressed in PVSMC and PV, whereas TRPV3, TRPV5, and TRPV6 were not. A few studies have suggested that TRPV1 may be involved in SOCE through function as Ca\textsuperscript{2+} release channels in SR (36, 37, 44). Chronic hyperpnea increases TRPV1 expression in association with increased SOCE in human PASMC (46). TRPV2 or TRPV4 are found to be activated by very diverse stimuli, including heat, cell swelling, and mechanical stress in different cell types (23). So far, no evidence indicates if TRPV2 or TRPV4 plays a role in SOCE. Among the various TRPV members, the predominant expression of TRPV2 and TRPV4 in PVSMS suggests they may be involved in thermosensing, osmosensing, mechanosensing, and/or basal Ca\textsuperscript{2+} homeostasis in these cells.

In the PV vasculature, intralobar distal PV constitutes the major site of vasomotor responses; therefore, in this study, we focused on determination of the presence of SOCE in cells from the distal part, with an attempt to investigate the role of SOCE in regulating the vasomotor tone of PV under normal and disease conditions for future study. Interestingly, we also found that, while the expression profiles of TRPC and TRPV were similar in proximal and distal PV and PVSNC, the mRNA and protein expression of TRPC6 in distal PV and PVSNC were drastically less than proximal. The functional significance of this reduction is unknown at this time; however, we think it may (or partially) reflect the molecular basis of the differential contractile and relaxant properties of proximal and distal PV, a fact that has been found in a previous report (4). In addition, it is also noteworthy that our results could be age related given that aging was thought to affect the contractile response of PV in various species including rat according to several lines of previous observations (31, 32, 45).

In summary, we demonstrated the presence of SOCE and defined the expression profiles of TRPC and TRPV in rat distal PV smooth muscle. Our study may serve as a basis for future identification of the pathophysiologic function of SOCE and various cation channel proteins in pulmonary venous vascularization.

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**DISCLOSURES**

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

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