Inhibition of endogenous TRP1 decreases capacitative Ca\(^{2+}\) entry and attenuates pulmonary artery smooth muscle cell proliferation

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Sweeney, Michele, Ying Yu, Oleksandr Platoshyn, Shen Zhang, Sharon S. McDaniel, and Jason X.-J. Yuan. Inhibition of endogenous TRP1 decreases capacitative Ca\(^{2+}\) entry and attenuates pulmonary artery smooth muscle cell proliferation. Am J Physiol Lung Cell Mol Physiol 283: L144–L155, 2002. First published February 8, 2002; 10.1152/ajplung.00412.2001.—Pulmonary vascular medial hypertrophy due to proliferation of pulmonary artery smooth muscle cells (PASMC) greatly contributes to the increased pulmonary vascular resistance in pulmonary hypertension patients. A rise in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]\) is an important stimulus for cell growth in PASMC. Resting [Ca\(^{2+}\)\(_{\text{cyt}}\)], intracellularly stored [Ca\(^{2+}\)], capacitative Ca\(^{2+}\) entry (CCE), and store-operated Ca\(^{2+}\) currents (I\(_{\text{SOC}}\)) are greater in proliferating human PASMC than in growth-arrested cells. Expression of TRP1, a transient receptor potential gene proposed to encode the channels responsible for CCE and I\(_{\text{SOC}}\), was also upregulated in proliferating PASMC. Our aim was to determine if inhibition of endogenous TRP1 gene expression affects I\(_{\text{SOC}}\) and CCE and regulates cell proliferation in human PASMC. Cells were treated with an antisense oligonucleotide (AS, for 24 h) specifically designed to cleave TRP1 mRNA and then returned to normal growth medium for 40 h before the experiments. Then, mRNA and protein expression of TRP1 was downregulated, and amplitudes of I\(_{\text{SOC}}\) and CCE elicited by passive depletion of Ca\(^{2+}\) from the sarcoplasmic reticulum using cyclopiazonic acid were significantly reduced in the AS-treated PASMC compared with control. Furthermore, the rate of cell growth was decreased by 50% in AS-treated PASMC. These results indicate that TRP1 may encode a store-operated Ca\(^{2+}\) channel that plays a critical role in PASMC proliferation by regulating CCE and intracellular [Ca\(^{2+}\)\(_{\text{cyt}}\)]\.

PULMONARY VASOCONSTRICTION and vascular smooth muscle cell proliferation greatly contribute to the elevated pulmonary vascular resistance and arterial pressure in patients with pulmonary hypertension (45, 52). Vasoconstriction and cellular proliferation may share a common pathway, involving signaling processes that result in parallel intracellular events in pulmonary vascular remodeling and in the development of pulmonary hypertension (58). Intracellular Ca\(^{2+}\) is a critical signal transduction element in regulating muscle contraction (51), cell proliferation (3, 15, 33), and gene expression (19). Cytoplasmic ionized Ca\(^{2+}\) diffuses rapidly between cytosol and nucleus (1); therefore, a rise in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]) not only activates Ca\(^{2+}\)-dependent function occurring in the cytosol (e.g., contraction) but also activates Ca\(^{2+}\)-sensitive events in the nucleus (e.g., expression of the nuclear proteins that are related to the cell cycle) (4, 6, 19, 51).

In pulmonary artery smooth muscle cells (PASMC), [Ca\(^{2+}\)\(_{\text{cyt}}\)] is increased mainly by Ca\(^{2+}\) influx through Ca\(^{2+}\)-permeable channels in the plasma membrane and Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, such as the sarcoplasmic reticulum (SR). Mitogen- or agonist-induced increases in [Ca\(^{2+}\)\(_{\text{cyt}}\)] usually consist of an initial release of Ca\(^{2+}\) from the SR followed by a sustained Ca\(^{2+}\) influx through sarcolemmal Ca\(^{2+}\)-permeable channels (6, 12, 15, 31, 41, 48). Removal or chelation of extracellular Ca\(^{2+}\) abolishes pulmonary vasoconstriction and significantly inhibits PASMC growth (16, 35), suggesting that a constant influx of Ca\(^{2+}\) from extracellular fluid to the cytosol is required for vasoconstriction and PASMC proliferation. There are at least three classes of Ca\(^{2+}\)-permeable channels functionally expressed in the plasma membrane in vascular smooth muscle cells: 1) voltage-dependent Ca\(^{2+}\) channels (VDCC), which are regulated by membrane potential, 2) receptor-operated Ca\(^{2+}\) channels, which are regulated by binding ligand with respective receptors, and 3) store-operated Ca\(^{2+}\) channels (SOC), which are regulated by capacity of Ca\(^{2+}\) in the SR (5, 37, 40, 43, 54). Depletion of Ca\(^{2+}\) from the SR activates SOC and triggers capacitative Ca\(^{2+}\) entry (CCE), a mechanism involved in maintaining sustained Ca\(^{2+}\) influx and refilling Ca\(^{2+}\) into the SR (5, 6, 40, 43). In other words, CCE is an important mechanism that links intracellularly stored [Ca\(^{2+}\)\(_{\text{cyt}}\)] in the SR to membrane Ca\(^{2+}\) permeability. The amplitude of CCE is
mainly dependent on 1) the level of \([\text{Ca}^{2+}]\) in the SR, 2) activity of the sarcolemmal \(\text{Ca}^{2+}\) channels that are activated by store depletion (SOC), 3) total number of functional SOC in the plasma membrane, and 4) signal transduction from emptied SR to the membrane SOC.

The molecular basis of SOC responsible for CCE is still not completely understood. It has been demonstrated that SOC may be composed of subunits encoded in the transient receptor potential (TRP) channel genes. Indeed, expression of TRP genes in mammalian cells and Xenopus oocytes results in the formation of \(\text{Ca}^{2+}\)-permeable cation channels, which are activated by \(\text{Ca}^{2+}\) store depletion, whereas the blockade of TRP channel gene expression attenuates the cation currents through the store-depletion-activated \(\text{Ca}^{2+}\) channels and the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to CCE (5, 7, 38, 56, 57, 61, 64–66). TRP1, a member of the short TRP subfamily (10, 20), is highly expressed in the plasma membrane of vascular smooth muscle cells (16, 59, 61). Overexpression of TRP1 in mammalian cell lines and Xenopus oocytes enhances CCE induced by store depletion. This suggests that the TRP1 gene encodes subunits involved in forming native SOC, which is believed to be composed of four heterogenous or homologous TRP subunits (10, 23, 32, 53, 60, 64–66). In human PASMC, we previously demonstrated that the mRNA and protein expression of TRP1 and the amplitude of SOC currents \(I_{\text{SOC}}\) and CCE were much greater in proliferating cells than growth-arrested cells (16). These results suggest that TRP1 gene product participates in forming SOC that are involved in elevating \([\text{Ca}^{2+}]_{\text{cyt}}\) through CCE when cells proliferate. In this study, using an antisense (AS) oligonucleotide that specifically cleaves mRNA of the human TRP1 gene, we examined whether inhibition of the endogenously expressed TRP1 affected activity of SOC, amplitude of SOC, and PASMC proliferation in the presence of serum and growth factors.

**MATERIALS AND METHODS**

**Cell culture and AS oligonucleotide.** Human PASMC from normal subjects were purchased from Clonetics (Clonetics, BioWhittaker) and used at the 4th–6th passage. The cells were plated onto coverslips (for electrophysiological and fluorescence microscopy experiments) or petri dishes (for molecular biological experiments) and were cultured at 37°C in smooth muscle growth medium (SMGM), which is composed of smooth muscle basal medium (SMBM), 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Cells were subcultured or plated onto 25-mm coverslips using trypsin-EDTA buffer (Clonetics) when 70–90% confluence was achieved. The cells isolated for study were all synchronized or growth arrested by incubating them in SMBM for 48 h before biochemical characterization of TRP1 expression and functional assessment of SOC activity.

Second Generation AS oligonucleotides that contain nine phosphorothioate DNA linkages to activate RNase H were purchased from Sequitur (Natick, MA). The AS oligonucleo-
tides were designed to cleave mRNA of the human TRP1 gene (GenBank accession number U31110) by activating endogenous RNase H and have a unique combination of specificity, efficacy, and reduced toxicity. The AS oligonucleotides were screened against the GenBank database, and no matches were found to other non-targeted genes. The negatively charged oligonucleotide S12174 (TRP1 Ultramer) from Sequitur was transfected into cells with Lipid 2012-G according to the manufacturer’s protocol (Sequitur). The transfection efficiency, determined by measuring uptake of a fluorescent control oligomer in a separate plate, was ~95% using 40 nM of the oligomer and 2.5 g/ml of Lipid 2012-G (for 12 h) in human PASMC (65–70% confluence). An oligonucleotide with the same base composition as S12174, but with scrambled sequence, was used as a control for nonspecific or toxic effects of the oligomers. The sequence of the AS oligonucleotide for human TRP1 was compared with known sequences in GenBank using the National Center for Biotechnology Information web-blasting program to ensure that no homologies to any other human TRP genes were found.

For each treatment, the cells were first rinsed with OptiMEM (GIBCO-BRL), and then oligos in SMGM were added to the cells. After 24 h of incubation with the oligomers, the medium was aspirated and replaced with SMGM without oligomers for 48–72 h before the experiments were performed. The final concentration of the oligos was 40 nM.

**Cell cycle analysis.** The human PASMC cell cycle distribution was analyzed by flow cytometry. Briefly, cells were first cultured in SMBM or SMGM (Clonetics) for 24–42 h. Then, the cells were trypsinized, washed once with PBS, and fixed with 70% ethanol for at least 1 h at 4°C. The fixed cells were washed with PBS and incubated with a solution containing 0.05 mg/ml propidium iodine, 0.1% sodium citrate, and 50 μg/ml RNase A for 30 min at 4°C in the dark. The stained cells were analyzed by FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

**Electrophysiological measurements.** Whole cell \(I_{\text{SOC}}\) was recorded with an Axopatch-1D amplifier using patch-clamp techniques (16, 35). Patch pipettes (2–4 MΩ) were made on a Sutter electrode puller using borosilicate glass tubes and fire-polished on a Narishige microforge. Voltage stimuli were delivered from a holding potential of 0 mV using voltage steps from −100 to 0 or +100 mV. Current traces recorded before the activation of SOC were used as a template to subtract leak currents. SOC was activated by passive depletion of the SR Ca\(^{2+}\) using 10 μM cyclopiazonic acid (CPA). The bath (extracellular) solution for recording optimal \(I_{\text{SOC}}\) contained (mM) 120 Na methane sulfonate, 20 Ca aspartate, 0.5 3,4-diaminopyridine, 10 glucose, and 10 HEPES (pH 7.4) containing 0.05 mg/ml propidium iodine, 0.1% sodium citrate, and 50 μg/ml RNase A for 30 min at 4°C. These ionic conditions eliminated the currents through K\(^+\) and Cl\(^-\) channels. In Ca\(^{2+}\)-free bath solution, Ca aspartate was replaced by equimolar Na aspartate to maintain osmolarity. In Na\(^+\)-free bath solution, Na methane sulfonate was replaced by equimolar N-methyl-D-glucamine (NMDG\(^+\)). CPA was dissolved into DMSO to make a stock solution of 30 mM. Aliquots of the stock solution were then diluted 1:3,000 into the bath solution or culture medium to make a final concentration of 10 μM CPA (pH 7.4). Ni\(^{2+}\) (Sigma) was directly dissolved in the bath solution on the day of use. The pH values of all solutions were checked after addition of the drugs and readjusted to 7.4.

**Measurement of cytosolic [Ca\(^{2+}\)].** \([\text{Ca}^{2+}]_{\text{cyt}}\) in single human PASMC was measured using the Ca\(^{2+}\)-sensitive fluorescent indicator fura 2 (16, 17). Cells on 25-mm coverslips were loaded with the acetoxymethyl ester form of fura 2, fura 2-AM (3 μM for 30 min), in the dark at room temperature (22–24°C) under an atmosphere of 5% CO\(_2\)-95% air. The fura 2-loaded cells were then transferred to a recording cell chamber.
ber on the microscope stage and superfused with physiological salt solution (PSS) for 30 min to remove extracellular dye and allow intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. The PSS contained (mM) 141 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose, buffered to pH 7.4 with 5 M NaOH. In Ca²⁺-free PSS, CaCl₂ was replaced by equimolar MgCl₂, and 0.1 mM EGTA was added to chelate residual Ca²⁺. Fura 2 fluorescence (510 nm light emission excited by 340- and 380-nm illuminations) from the cells, as well as background fluorescence, was collected at 32°C using the Nikon UV-Fluor objectives and a charge-coupled device camera. The fluorescence signals emitted from the cells were monitored and recorded continuously on an Intracellular Imaging fluorescence microscopy system and recorded in an IBM-compatible computer for later analysis. The 340- to 380-nm nm ratio (R) of the fluorescence images were then calculated and subsequently calibrated. On the basis of these R, [Ca²⁺]cyt was calculated by the equation

\[ [Ca^{2+}]_{cyt} = K_d \times (S_0/S_{max}) \times (R - R_{min})/(R_{max} - R) \]

where \( S_0 \) and \( S_{max} \) are the emission fluorescence values at 380-nm excitation in the presence of EGTA and Triton X-100, respectively; \( K_0 \) (225 nM) is the dissociation constant of the Ca²⁺-fura 2 complex; and \( R_{max} \) and \( R_{min} \) were calculated according to Grynkiewicz et al. (17).

**RT-PCR.** Total RNA (3 μg) was first reverse-transcribed using random hexamers [pd(N)₆ primer]. The sense [5'-CAAGATTTTTGAAAAATTTTTG-3'; nucleotide (nt) 2338–2359] and AS [5'-TTGTGCTTCTATGTTTTGCTAT-3'; nt 2689–2709] primers were designed from the coding region of the human TRP1 gene (U31110). The sense and AS primers for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (M33197) are 5'-CTTGTGTTATGTTGAAGCCT-3' (nt 561–582) and 5'-CTTCTCCTTGGTCTTGGCTG-3' (nt 1,089–1,110). The sense and AS primers for the human β-actin gene (M10277) are 5'-GACGGGCTCACCCACACTGCGCCATCTA-3' (nt 2134–2162) and 5'-CGAAGGATTGCGTGAGGATGAG-3' (nt 2971–3000). PCR was performed by a GeneAmp PCR System using AmpliTaq DNA polymerase and accompanying buffers. Three microliters of the first-strand cDNA reaction mixture were used in a 50-μl PCR reaction consisting of 0.2 nmol/l of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 200 μM each of 2-deoxynucleotide 5’-triphosphates, and 2 units of Taq DNA polymerase. The cDNA samples were amplified in a DNA thermal cycler under the following conditions: the mixture was annealed at 52–61°C, extended at 72°C, and denatured at 94°C for 20–30 cycles. This was followed by a final extension at 72°C to ensure complete product extension. The PCR products were electrophoresed through a 2% agarose gel, and amplified cDNA bands were visualized by ethidium bromide staining. To quantify the PCR products of TRP1, we used an invariant mRNA of smooth muscle β-actin as an internal control. Immediately after each experiment, the optical density (OD) value for each band on the gel was measured by a gel documentation system. The OD values in the TRP1 signals were normalized to the OD values in the β-actin or GAPDH signals. The normalized values are expressed as arbitrary units for quantitative comparison.

**Immunoblot analysis.** Cells were washed with PBS, scraped into PBS (2 ml/dish), and centrifuged at 3,500 rpm. The cell pellets were homogenized in 10 mM HEPES-KOH (pH 7.0) containing the protease inhibitor cocktail (Complete tablets) for 10 s at 7,000 rpm. Nuclei and plasma membrane were removed by centrifugation in a microfuge at 4°C for 10 min (10,000 rpm). Protein concentrations were determined by the Coomassie BA protein assay, using bovine serum albumin as a standard. Proteins solubilized in SDS-sample buffer were separated by SDS-PAGE on 10% gels, which were calibrated with prestrained protein molecular weight markers. The separated proteins were then transferred to the Hybond-C extra nitrocellulose membrane. The efficiency of the transfer was verified by Ponceau S staining. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 for 40 min at room temperature. The blots were then incubated with the affinity-purified polyclonal antibodies specific for TRP1 (Alomone Labs, Jerusalem, Israel) for ~1 h at room temperature. The membranes were washed (3× 5 min) and incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 h, and an enhanced chemiluminescence detection system was used for detection of the bound antibody. Finally, the filters were placed in a plastic sheet protector and exposed to Kodak X-Omat film for 10–60 s.

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed using the unpaired Student’s t-test or ANOVA and post hoc tests (Student-Newman-Keuls) as indicated. Differences are considered to be significant when \( P < 0.05 \).

**RESULTS**

Enhancement of TRP1 expression during PASMC proliferation. Expression of TRP genes in mammalian cells enhances CCE, suggesting that TRP-encoded proteins may form the putative SOC responsible for CCE (5, 7, 38, 56, 57, 61, 64–66). TRP1 is a member of the short TRP channel subfamily, which is ubiquitously expressed in a variety of tissue types, including lung, heart, and brain (10). It has been demonstrated that TRP1 encodes subunits involved in forming native SOC, which are activated by store depletion in many cell types, including vascular smooth muscle cells (8, 10, 23, 32, 53, 60, 64–66).

In human PASMC cultured in SMGM, which contains serum and growth factors, 36.3% of the cells were in G₀/G₁ phase (Fig. 1A). This suggests that the cells cultured in SMGM are proliferating cells, whereas the cells cultured in SMBM are mostly growth-arrested cells. To examine the possible association of TRP1 expression and SOC activity with PASMC proliferation, we measured and compared 1) mRNA and protein expression of TRP1 and 2) whole cell cation currents through store depletion-activated channels in proliferating and growth-arrested PASMC.

Using the primers specifically designed for human TRP1 (nt 2,238–2,709), we detected a band of 372 bp using cDNA prepared from human PASMC (Fig. 1B). The sequence analysis of the PCR product indicated a 100% match with the sequence of human TRP1 in GenBank (U31110). The density of this 372-bp band in proliferating PASMC cultured in SMGM was 2.6-fold greater than in growth-arrested cells cultured in basal medium (SMBM) (Fig. 1B).

In Western blotting of brain membranes, the anti-TRP1 polyclonal antibody purchased from Alomone Labs revealed two bands with significantly different density: 1) a faint band at ~117 kDa, which is some-
protein because the proteins (e.g., TRP1) containing multiple transmembrane domains tend to form polymers in SDS. Both bands were abolished by preadsorption of the TRP1 antibody with a control peptide antigen. In human PASMC, we could detect only the ~220-kDa band using the antibody from Alomone; it was probably because of the low expression level of TRP1 in these cells compared with brain tissues. As shown in Fig. 1C, the protein level of TRP1 was significantly higher in proliferating PASMC cultured in SMGM than in growth-arrested cells cultured in SMBM. The increase in TRP1 protein level (Fig. 1C) in proliferating cells was consistent with the increase in TRP1 mRNA level (Fig. 1B), suggesting that the mRNA and protein expression of TRP1 is both increased in human PASMC during proliferation.

Enhancement of SOC activity and CCE amplitude during PASMC proliferation. Activity of SOC, or the whole cell cation current through SOC ($I_{\text{SOC}}$), in PASMC is partially determined by the total number of functional SOC proteins expressed in the plasma membrane. If TRP1 encodes the protein subunits involved in forming native SOC, the upregulated expression of TRP1 would increase whole cell $I_{\text{SOC}}$ in PASMC during proliferation. To determine whether the upregulated TRP1 expression associates with an increase in SOC activity, we measured and compared whole cell $I_{\text{SOC}}$ in proliferating and growth-arrested PASMC.

Whole cell $I_{\text{SOC}}$ was elicited in PASMC held at 0 mV to inactivate voltage-dependent Na$^+$ and Ca$^{2+}$ channels by a series of test potentials ranging from −100 to +100 mV. The inward currents at negative test potentials were mainly generated by Ca$^{2+}$ (and/or Na$^+$) influx, and the outward currents at positive potentials were putatively generated by Cs$^+$ efflux because 1) permeability ratios for Ca$^{2+}$ and Na$^+$ ($P_{\text{Ca}}/P_{\text{Na}}$) are usually on the order of 10:1 under normal physiological conditions with extracellular [Ca$^{2+}$] in the millimolar range (2, 42, 64) and 2) the permeability of SOC to Na$^+$, K$^+$, and Cs$^+$ is equal ($P_{\text{Na}}=P_{\text{K}}=P_{\text{Cs}}=1:1:1$) (26).

The amplitude of whole cell $I_{\text{SOC}}$ activated by passive depletion of the SR Ca$^{2+}$ by 10 μM CPA was much greater in proliferating PASMC than in growth-arrested cells (Fig. 2A, a and b). Interestingly, the proliferation-associated percent increase in inward $I_{\text{SOC}}$ appeared to be significantly greater than in outward $I_{\text{SOC}}$ (Fig. 2A, c); this suggests that 1) the inward and outward $I_{\text{SOC}}$ may be generated by cation flux through SOC encoded by different TRP genes (10, 46, 63) and 3) permeability of TRP1-encoded SOC may change when human PASMC undergo phenotypical changes from a quiescent phenotype to a proliferating phenotype. These results provide strong evidence that upregulation of TRP1 and increased SOC activity are associated with proliferation of PASMC in the presence of serum and growth factors.

CPA, by blocking Ca$^{2+}$ sequestration into the SR, induced a transient increase in [Ca$^{2+}$]$_{\text{cyt}}$ because of leakage of Ca$^{2+}$ from the SR to the cytosol in the

**Fig. 1.** The mRNA and protein expression of transient receptor potential gene (TRP1) is upregulated when human pulmonary artery smooth muscle cells (PASMC) proliferate. A: flow cytometry histograms of cell-cycle analysis for cells cultured in media with [smooth muscle growth medium (SMBM)] or without [smooth muscle basal medium (SMGM)] serum and growth factors by propidium iodide staining. B: PCR products for TRP1 (372 bp) and β-actin (661 bp) in growth-arrested (cultured in SMBM) and proliferating (cultured in SMGM) cells (top panels). The PCR was performed in the absence (−) or presence (+) of RT. M, 100-bp DNA ladder. Bottom: summarized data (means ± SE) showing the mRNA level of TRP1 that was normalized to the amount of β-actin in growth-arrested (SMBM) and proliferating (SMGM) cells (top panels). Molecular mass markers are indicated on left (in kDa). C: Western blot analyses of TRP1 and α-actin proteins in growth-arrested (SMBM) and proliferating (SMGM) cells. PCR (B) and Western blot (C) experiments were repeated 4 times independently in each group, ***$P < 0.001$ vs. SMBM.

what larger than the 87 kDa expected for TRP1 (50) potentially because of extensive glycosylation of native TRP1 channel proteins and 2) an intensive band of ~220 kDa, which seems to be a dimer of the ~117-kDa
absence of extracellular Ca\textsuperscript{2+} (Fig. 2B, a). The CPA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} transients in cells bathed in Ca\textsuperscript{2+}-free solution declined back to the baseline level after 4–5 min because the SR Ca\textsuperscript{2+} was depleted. Under these conditions, restoration of extracellular Ca\textsuperscript{2+} induced a rise in [Ca\textsuperscript{2+}]\textsubscript{cyt}, which was apparently due to CCE (Fig. 2B, a). The store depletion-mediated CCE in proliferating PASMC (808 ± 59 nM, n = 42) was much greater than in growth-arrested cells (131 ± 11 nM, n = 25; P < 0.001) (Fig. 2B, b). These results indicate that the TRP1 gene is upregulated when human PASMC proliferates. The subsequent increase in protein expression of the TRP1 gene product, a putative SOC, augments $I_{\text{SOC}}$ and enhances CCE. The influx of Ca\textsuperscript{2+} through CCE may serve as an important mechanism to maintain a sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{cyt} and sufficient [Ca\textsuperscript{2+}] in the SR during PASMC proliferation (15, 16, 49).

**Inhibition of endogenous TRP1 expression reduced $I_{\text{SOC}}$ and CCE.** It has been demonstrated that SOC is composed of subunits encoded in TRP genes (5, 7, 38, 56, 57, 61, 64–66). Therefore, gene transcription and expression of TRPs should be involved in long-term electrophysiological change of SOC. Similar to molecular topology of voltage-gated K\textsuperscript{+} channels, TRP1 has six transmembrane domains (S1–S6) with both carboxy and amino termini located intracellularly and a pore-forming loop between the S5 and S6 domains. The native TRP channels are believed to be formed heteromerically by different TRP subunits (5, 10). To test whether TRP1 is involved in forming functional native SOC, we measured and compared whole cell $I_{\text{SOC}}$ and CCE in control PASMC and cells in which TRP1 gene expression was inhibited.

To selectively inhibit the gene expression of TRP1, we used the AS oligonucleotide (S12174, TRP1 Ultra-mer) specifically targeted on the human TRP1 gene (U31110). Treatment of human PASMC with the AS oligonucleotide (40 nM, for 24 h) caused a 67% decrease in mRNA level (0.896 ± 0.015 arbitrary units, $n = 8$, $P < 0.001$) and a 40% decrease in protein level (1.097 ± 0.029 vs. 0.667 ± 0.081 arbitrary units, $n = 6$, $P < 0.01$) of TRP1 channels but had little effect on mRNA and protein expression of GAPDH and α-actin (Fig. 3).

Whole cell currents ($I_{\text{soc}}$) generated by Ca\textsuperscript{2+} influx through TRP-encoded SOC are a function of the number of cytosolic free Ca\textsuperscript{2+} (1.8 mM) in growth-arrested (SMBM) and proliferating (SMGM) cells. Summarized data (means ± SE, $b$) showing the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to CCE in growth-arrested (SMBM) and proliferating (SMGM) cells. ***$P < 0.001$ vs. SMBM.

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**Fig. 2.** Increased whole cell store-operated Ca\textsuperscript{2+} currents ($I_{\text{SOC}}$) and enhanced capacitative Ca\textsuperscript{2+} entry (CCE) in proliferating human PASMC. A: representative families of superimposed currents ($a$), elicited by 300-ms voltage steps ranging from −100 to +100 mV in 20-mV increments, before (Control) and after 15-min application of cyclopiazonic acid (CPA, 10 μM) in cells cultured in media with (SMGM) or without (SMBM) serum and growth factors. The cells were held at 0 mV to minimize voltage-gated Ca\textsuperscript{2+} and Na\textsuperscript{+} currents. Right panels: the subtraction ($I_{\text{SOC}}$) between the currents recorded before and after application of CPA. Summarized data (means ± SE, $b$) showing the current-voltage ($I$-$V$) relationships of $I_{\text{SOC}}$, elicited by a series of test potentials ranging from −100 to +100 mV (holding potential, 0 mV), in growth-arrested (SMBM) and proliferating (SMGM) cells. The $I$-$V$ curves are significantly different ($P < 0.001$) between growth-arrested and proliferating cells. The ratio of inward and outward $I_{\text{SOC}}$ at different test potentials in proliferating ($I_{\text{SOC-SMGM}}$) and growth-arrested ($I_{\text{SOC-SMBM}}$) PASMC is shown in $c$. B: representative records ($a$) showing the time course of cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) changes induced by CPA (10 μM) in the absence (0Ca) or presence of extracellular Ca\textsuperscript{2+} (250 μM). The Ca\textsuperscript{2+} -free solution declined back to the baseline level after 4–5 min because the SR Ca\textsuperscript{2+} was depleted. Under these conditions, restoration of extracellular Ca\textsuperscript{2+} induced a rise in [Ca\textsuperscript{2+}]\textsubscript{cyt}, which was apparently due to CCE (Fig. 2B, a). The store depletion-mediated CCE in proliferating PASMC (808 ± 59 nM, n = 42) was much greater than in growth-arrested cells (131 ± 11 nM, n = 25; $P < 0.001$) (Fig. 2B, b). These results indicate that the TRP1 gene is upregulated when human PASMC proliferates. The subsequent increase in protein expression of the TRP1 gene product, a putative SOC, augments $I_{\text{SOC}}$ and enhances CCE. The influx of Ca\textsuperscript{2+} through CCE may serve as an important mechanism to maintain a sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{cyt} and sufficient [Ca\textsuperscript{2+}] in the SR during PASMC proliferation (15, 16, 49).
Fig. 3. Effects of the antisense (AS) oligonucleotide for the human TRP1 gene on mRNA and protein expression of TRP1 in human PASMC cultured in media containing serum and growth factors. RT-PCR products (A, top panels) for TRP1 (372 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 550 bp) as well as Western blot analysis (B, top panels) of TRP1 and α-actin in cells treated with control (Cont) or AS oligos in SMGM. Bottom panels: summarized data (means ± SE) showing the mRNA (A, n = 8) and protein (B, n = 6) levels of TRP1 normalized to the amounts of GAPDH (for PCR analysis) and α-actin (for immunoblot analysis). **P < 0.01 and ***P < 0.001 vs. Cont.

ber (N) of functional TRP channels expressed in the plasma membrane and the single-channel activity based on the equation: \( I = N \times i \times P_{\text{open}} \), where \( i \) is the amplitude of single-channel current and \( P_{\text{open}} \) is the steady-state open probability of the channel. When TRP channel expression is decreased or \( N \) is declined, the whole cell \( I \) through the channels would be reduced. Indeed, the AS-induced inhibition of mRNA and protein expression of TRP1 caused a 64% decrease in current density at \(-80 \text{ mV} \) (\(-29 ± 3 \text{ pA/pF} \)) vs. \(-10 ± 1 \text{ pA/pF} \), \( n = 12, P < 0.001 \) of \( I_{\text{SOC}} \) activated by passive store depletion with 10 \( \mu \text{M} \) CPA (Fig. 4). The AS-induced percent decrease of inward \( I_{\text{SOC}} \) at negative test potentials (\(-100 \text{ to } -20 \text{ mV} \)) was much greater than the decrease of outward \( I_{\text{SOC}} \) at positive potentials (Fig. 4D). To confirm that the AS-mediated inhibition of \( I_{\text{SOC}} \) attenuates \( \text{Ca}^{2+} \) influx in proliferating cells, we measured and compared \( I \) whole cell \( I_{\text{SOC}} \) in PASMC superfused with \( \text{Na}^+ \)-free and \( \text{Ca}^{2+} \)-containing solution and 2) the increase in \( [	ext{Ca}^{2+}]_{\text{cyt}} \) due to CCE.

When extracellular \( \text{Na}^+ \) (140 mM) was replaced by equimolar NMDG\(^+\), whole cell \( I_{\text{SOC}} \), activated by passive store depletion by 10 \( \mu \text{M} \) CPA, was \( 15 ± 1 \text{ pA/pF} \) at \(-80 \text{ mV} \) in PASMC treated by a control oligonucleotide (NS, which has the same base composition as the TRP1 AS oligonucleotide, but with scrambled sequence). Treatment of the cells with the TRP1 AS oligos significantly reduced whole cell \( I_{\text{SOC}} \) (from \(-15.0 ± 1.2 \text{ to } -5.1 ± 0.5 \text{ pA/pF} \) at \(-80 \text{ mV} \), \( P < 0.001 \) in the absence of extracellular \( \text{Na}^+ \) (Fig. 5, A–C). The AS-induced percent decrease of inward \( I_{\text{SOC}} \) at negative test potentials from \(-100 \text{ to } -20 \text{ mV} \), which was generated solely by \( \text{Ca}^{2+} \) influx through activated SOC, was much greater than the decrease of outward \( I_{\text{SOC}} \) at positive potentials (Fig. 5D). These results demonstrate that the TRP1-encoded protein is in-

\[ \text{Bath (mM):} \]
- 120 Na methane sulfonate
- 20 Ca aspartate
- 0.5 dithiociglydine

\[ \text{Pipette (mM):} \]
- 128 Cs aspartate
- 1.15 EGTA
- 1.0 Ca(OH)\(_2\)
- 2.0 Na\(_2\)ATP

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Fig. 5. Effect of the AS oligonucleotide for the human TRP1 gene on whole cell $I_{\text{SOC}}$ in proliferating human PASMC superfused with Na⁺-free solution. A: representative records showing time course of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in cells treated with control (Cont) or AS oligos. CPA (10 μM) was applied to the cells in the absence (0Ca) and presence of extracellular Ca²⁺ (1.8 mM). B: summarised data (means ± SE, n = 21–26 cells) showing the amplitudes of CCE in cells treated with control or AS oligos. **P < 0.01 vs. Cont.

Fig. 6. Effect of the AS oligonucleotide for the human TRP1 gene on CCE in human PASMC cultured in media containing serum and growth factors. A: representative records showing time course of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in cells treated with control (Cont) or AS oligos. CPA (10 μM) was applied to the cells in the absence (0Ca) and presence of extracellular Ca²⁺ (1.8 mM). B: summarised data (means ± SE, n = 21–26 cells) showing the amplitudes of CCE in cells treated with control or AS oligos. **P < 0.01 vs. Cont.

Involved in forming native SOC and inhibition of endogenous TRP1 expression attenuates Ca²⁺ influx through store depletion-activated SOC in human PASMC. The amplitude of CCE is partially dependent on the activity of SOC that are activated by store depletion. The selective inhibition of gene expression of TRP1 (Fig. 3) and $I_{\text{SOC}}$ (Figs. 4 and 5) was also associated with a significant decrease in the amplitude of CCE (Fig. 6). In human PASMC treated with control oligos, the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to CCE was 618 ± 106 nM (n = 21), whereas in cells treated with AS oligos, the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to CCE was 279 ± 54 nM (n = 26; P < 0.01). The AS-mediated decrease (~55%) in the amplitude of CCE was consistent with the decrease in $I_{\text{SOC}}$ (60–64%).

In addition to contributing to the maintenance of a sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, CCE is believed to be required for refilling Ca²⁺ to the emptied SR (12, 40, 43). Therefore, inhibition of CCE should also reduce the level of $[\text{Ca}^{2+}]_{\text{SR}}$. As shown in Fig. 6A, extracellular application of CPA induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of extracellular Ca²⁺, which was mainly due to the leakage of Ca²⁺ from the SR to the cytosol. The amplitude of the CPA-induced transient increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ is thus proportionally related to the level of $[\text{Ca}^{2+}]_{\text{SR}}$. In human PASMC treated with control oligos, the CPA-induced rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to the initial mobilization of Ca²⁺ from the SR was 370 ± 32 nM (n = 21), whereas in cells treated with AS oligos, the rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to the Ca²⁺ mobilization was reduced to 240 ± 34 nM (n = 26, P < 0.01). These results indicate that the AS-mediated attenuation of CCE is also associated with a decrease in the level of $[\text{Ca}^{2+}]_{\text{SR}}$. 
It has been demonstrated that removal (or chelation) of extracellular Ca^{2+} or depletion of the SR Ca^{2+} significantly inhibits vascular smooth muscle cell proliferation in the presence of serum and growth factors (16, 49). Consistent with the inhibitory effect on CCE and the reducing effect on [Ca^{2+}]_{SR}, the AS-mediated inhibition of the endogenously expressed TRP1 also attenuated cell proliferation in the presence of serum and growth factors (Fig. 7A). The time course of PASMC growth in the presence of serum and growth factors indicates that the cells (at a density of 11,000 cells/cm^2) spent ~24–36 h in a lag phase before undergoing log phase growth. The AS-induced growth inhibition appeared to start from the lag phase (Fig. 7B). These results suggest that TRP1 gene product is an endogenous SOC that contributes to CCE in human PASMC, and gene expression of TRP1 regulates PASMC proliferation by modulating I_{SOC} and CCE.

Pharmacological blockade of SOC with Ni^{2+} decreased CCE and inhibited PASMC growth. Ni^{2+} is a potent cationic blocker of SOC (16, 67). In proliferating human PASMC cultured in SMGM, extracellular application of Ni^{2+} decreased I_{SOC} in a dose-dependent manner; the EC_{50} is ~0.38 mM at ~80 mV (Fig. 8A). Moreover, extracellular application of Ni^{2+} reversibly attenuated the increase in [Ca^{2+}]_{cyt} due to CCE (Fig. 8B), suggesting that Ni^{2+} is a potent blocker of native SOC that are responsible for CCE in these cells. Treatment of PASMC with SMGM containing 0.5 mM Ni^{2+} for 12–72 h significantly reduced the resting [Ca^{2+}]_{cyt} (Fig. 8C) and inhibited cell proliferation in the presence of serum and growth factors (Fig. 8D).

Blockade of VDCC with nifedipine (1 μM) abolished the 50 mM K^{+}-induced increase in [Ca^{2+}]_{cyt} but had little effect on the CCE-mediated increase in [Ca^{2+}]_{cyt} (data not shown) (16), whereas blockade of SOC with Ni^{2+} (0.5 mM) significantly inhibited the CCE-mediated increase in [Ca^{2+}]_{cyt} (Fig. 8B). Treatment of the cells with both Ni^{2+} and nifedipine decreased resting [Ca^{2+}]_{cyt} and inhibited cell growth to a greater extent than treatment with Ni^{2+} or nifedipine alone (Fig. 8, C and D). The additive inhibitory effects of nifedipine on the resting [Ca^{2+}]_{cyt} (Fig. 8C) and cell growth (Fig. 8D) in the presence of Ni^{2+} indicate that the Ni^{2+}-sensitive SOC and the nifedipine-sensitive VDCC are both involved in the increase in resting [Ca^{2+}]_{cyt} in human PASMC during proliferation. It has been emphasized that, since Ni^{2+} also blocks Ca^{2+}-permeable channels other than SOC, we cannot exclude the possibility that the inhibitory effect of Ni^{2+} on PASMC proliferation might be partially due to blockade of other Ca^{2+} channels, such as voltage-gated and receptor-operated Ca^{2+} channels, which are highly expressed in human PASMC.

DISCUSSION

An important signal transduction pathway upon activation of receptors by mitogenic agonists is the increase in [Ca^{2+}]_{cyt} due to Ca^{2+} release from the SR and Ca^{2+} influx via Ca^{2+} channels in the plasma membrane (5, 15, 31, 41, 48, 51, 54). Ionized or free Ca^{2+} diffuses quickly between the cytosol and nucleus (1); therefore, a rise in [Ca^{2+}]_{cyt} would rapidly increase nuclear [Ca^{2+}]. Modulation of the amplitude or frequency of Ca^{2+} signals (or spatially distinguishable Ca^{2+} signals) and sustained increases in [Ca^{2+}]_{cyt} are all involved in regulating gene expression and cell growth (11–15, 18, 25, 49). Ca^{2+} is believed to be necessary for transitions from G0 to G1 phase, G1 to S phase, and G2 to M phase, and for going through mitosis in the cell cycle (3, 33). Maintenance of sufficient Ca^{2+} within the SR, an intracellular organelle important in the synthesis of many membrane lipids and proteins, is also required for cell growth; depletion of the SR Ca^{2+} store induces growth arrest (16, 25, 49) and triggers apoptosis (21). When ionized Ca^{2+} is depleted from the SR by inositol 1,4,5-trisphosphate (IP3) during activation of mitogenic receptors, the emptied SR needs to be refilled to further (or ensure) the mitogen-mediated cell proliferation and prevent cell apoptosis.

CCE generated by Ca^{2+} influx through SOC is a critical mechanism involved in maintaining sustained increase in [Ca^{2+}]_{cyt} and in refilling Ca^{2+} into the SR (5, 6, 40, 43). In human PASMC, amplitudes of CCE and I_{SOC} were both increased when cells proliferated, suggesting that an increase in expression and/or function of SOC is involved in cell growth. Because of the diversity and variability of the biophysical and pharmacological properties of whole cell and single-channel SOC (16, 24, 27, 30, 34), SOC are believed to be complex

![Fig. 7. Effect of the AS oligonucleotide for the human TRP1 gene on cell growth in human PASMC cultured in media containing serum and growth factors. A: cell numbers (means ± SE, n = 6 experiments) were determined before (Basal) and after 60-h incubation in SMBM (hatched bar) or SMGM containing control (solid bar) or AS (gray bar) oligos. ***P < 0.001 vs. Cont. B: summarized data (means ± SE) showing cell numbers before (day 0) and after incubating the cells in SMGM with control oligos (NS) or AS oligos (AS) for 1–4 days. The time course curve for the AS-treated cells is significantly different from the curve for control cells (P < 0.001).](http://ajplung.physiology.org/)

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and heterogeneous in molecular composition and in cellular regulation (5, 10).

What the native SOC are molecularly made of has been a question for many investigators. Electrophysiological studies demonstrate that expression of TRP genes in mammalian cells results in the formation of Ca$^{2+}$-permeable cation channels that are activated by store depletion. This suggests that SOC may be composed of subunits encoded in TRP genes (5, 7, 38, 56, 57, 64–66). The TRP gene family can be divided into three subfamilies based on sequence homology: short TRP, long TRP, and osm-9-like TRP (10, 20). TRP1 is a member in short TRP subfamily (termed as TRPC1), which contains an amino terminal ankyrin domain and a proline-rich cytosolic segment in the proximal carboxy terminus. Some of the six transmembrane domains (S1–S6) of TRP channels are homologous to transmembrane segments of voltage-dependent channels; however, the putative S4 domain of TRP channels lacks the positive amino acids involved in the voltage-sensing function (5, 10). SOC are thought to be heterotetramers or homotetramers made up of different TRP subunits (5, 10, 20, 32, 53).

In vascular smooth muscle cells isolated from human mammary arteries and aortas, Xu and Beech (61) provided compelling evidence that TRP1 gene encodes native subunits of SOC, which are responsible for CCE. In pulmonary vascular endothelial cells, Stevens and his associates (8, 36, 39) demonstrated that endogenously expressed TRP1 channel subunits contribute to form a Ca$^{2+}$-selective, store-operated Ca$^{2+}$ entry pathway that plays a central role in regulating endothelial permeability and in response to inflammation. These results also indicate that expression of TRP1 gene and function of the SOC-containing TRP1 subunit play critical roles in regulating [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{SR}$ in many cell types.

TRP1 is highly expressed in PASMC isolated from humans (16) and animals (59). We have demonstrated that gene expression of TRP1 and SOC activity are related to PASMC proliferation (16). The mRNA and protein levels of TRP1 and amplitudes of $I_{SOC}$ were both significantly enhanced in proliferating human PASMC cultured in growth medium containing serum and growth factors, compared with growth-arrested cells cultured in basal medium without serum and growth factors (see Figs. 1 and 2A). Moreover, the rise in [Ca$^{2+}$]$_{cyt}$ due to CCE, elicited by passive depletion of Ca$^{2+}$ from the SR with CPA (Fig. 2B) and thapsigargin (data not shown), was greatly augmented in proliferating PASMC, in which more than one-third of the cells undergo DNA synthesis and mitosis. These results suggest that, when PASMC are activated by mitogens, the native subunits of SOC, which are responsible for CCE.
the gene expression of TRP1 is triggered to produce more TRP1 channels for the need of Ca\(^{2+}\) in cytosol, nucleus, and the SR.

The molecular identity of native SOC in human PASMC is poorly understood, although multiple TRP gene transcripts have been described in lung tissues or PASMC isolated from humans and animals (10, 16, 59, 61). On the basis of the AS oligomer specifically targeted on the human TRP1 gene (Figs. 3–7), the observations from this study provide crucial evidence for the important role of endogenously expressed TRP1 in forming native SOC in human PASMC. Inhibition of endogenous TRP1 gene expression not only reduced activity of SOC but also decreased amplitude of CCE mediated by store depletion. The reduced I\(_{\text{SOC}}\) and CCE also led to a reduced [Ca\(^{2+}\)]\(_{\text{SR}}\). The CPA-induced initial increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in the absence of extracellular Ca\(^{2+}\) is mainly due to leakage of Ca\(^{2+}\) from the SR to the cytosol. Its amplitude is thus positively proportional to the level of [Ca\(^{2+}\)]\(_{\text{SR}}\). In cells treated with the AS oligomer specifically targeted on the human TRP1 gene, the CPA-induced Ca\(^{2+}\) mobilization from the SR was markedly reduced compared with control cells. Because refilling Ca\(^{2+}\) into the SR is an important function of CCE, a reduced number of functional TRP1 channels may likely be involved in the decreased [Ca\(^{2+}\)]\(_{\text{SR}}\). These results suggest that, in human PASMC, 1) TRP1-encoded proteins are subunits of native SOC, 2) upregulated TRP1 expression plays an important role in cell growth, and 3) development of drugs specifically targeted on the gene expression of TRP1 may be a potential therapeutic approach for inhibiting the progression of pulmonary vascular remodeling in patients with primary and secondary pulmonary hypertension.

Removal of extracellular Ca\(^{2+}\) almost abolished PASMC growth in the presence of serum and growth factors (16, 49), whereas pharmacological blockade of SOC and CCE using Ni\(^{2+}\) only partially inhibited cell growth (by 50\%, Fig. 8D). This suggests that Ca\(^{2+}\) influx through CCE or SOC is not the only pathway for raising [Ca\(^{2+}\)]\(_{\text{cyt}}\) (and/or [Ca\(^{2+}\)]\(_{\text{SR}}\)) during cell proliferation. In addition to SOC, PASMC also express voltage-dependent (VDCC) and receptor-operated Ca\(^{2+}\) channels (37, 44). By governing Ca\(^{2+}\) influx via VDCC, membrane potential plays a critical role in regulating [Ca\(^{2+}\)]\(_{\text{cyt}}\) (37). The additive effect of nifedipine, a dihydropyridine blocker of VDCC, on Ni\(^{2+}\)-induced inhibition of human PASMC growth (Fig. 8D) indicates that multiple Ca\(^{2+}\) influx pathways (e.g., via SOC and VDCC) are involved in raising [Ca\(^{2+}\)]\(_{\text{cyt}}\) when cells proliferate (29, 47, 62).

Pulmonary vascular remodeling due to smooth muscle proliferation and hypertrophy greatly contributes to the elevated pulmonary vascular resistance observed in patients with pulmonary hypertension. The high levels of vasoactive substances (e.g., endothelin-1, serotonin) and growth factors in plasma and lung tissues have been implicated in pulmonary hypertension (9, 22, 28, 55). The agonist- and mitogen-mediated cell growth depends on extracellular Ca\(^{2+}\) and on the level of intracellularly stored [Ca\(^{2+}\)]. CCE, potentially and partially through TRP1-encoded subunits contained in heterotetrameric SOC, is an important mechanism involved in maintaining the agonist-induced increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) and in refilling Ca\(^{2+}\) into the SR emptied by IP\(_3\) in human PASMC. Thus expression of the TRP1 gene and function of TRP1-encoded SOC may also play critical roles in regulating the progression and regression of pulmonary vascular remodeling in patients with pulmonary hypertension via modulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{SR}}\) in hypertrophied PASMC.

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