Upregulated TRP and enhanced capacitative Ca\(^{2+}\) entry in human pulmonary artery myocytes during proliferation

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have recently been cloned in mammals and humans \((hTRP)\) (4, 39, 44, 45). Expression of TRP genes has been demonstrated to form the \(Ca^{2+}\)-permeable cation channels that are activated by \(Ca^{2+}\) store depletion. This suggests that the TRP-encoded proteins may be the putative channels responsible for CCE (4, 5, 37, 40, 44, 45).

In vascular smooth muscle cells, sustained \(Ca^{2+}\) influx and maintaining sufficient \(Ca^{2+}\) in the SR are both required for cell growth (15, 33). Indeed, removal of extracellular \(Ca^{2+}\) and depletion of the SR \(Ca^{2+}\) significantly inhibited cell growth (33). This study was designed to test the hypothesis that CCE, potentially through TRP-encoded \(Ca^{2+}\)-permeable channels, is an important mechanism to maintain the elevated [\(Ca^{2+}\)]\(_{cyt}\) and [\(Ca^{2+}\)]\(_{SR}\) that are necessary for human pulmonary arterial smooth muscle cell (PASMC) proliferation.

**MATERIALS AND METHODS**

**Cell preparation and culture.** Patients undergoing lobectomy for bronchogenic carcinoma and lung/heart transplantation for cardiopulmonary diseases, who had no evidence of pulmonary hypertension, were the source of control lung tissues. In some cases, patients undergoing lung transplantation with PPH or secondary pulmonary hypertension (SPH; e.g., patients with congenital heart disease and pulmonary thromboembolic disease) were the source of diseased lung tissues. Lung tissues were removed from patients in the operating room and immediately placed in cold (4°C) saline and taken to the laboratory for dissection (41).

Small muscular intrapulmonary arteries were isolated and incubated for 20 min in Hank’s balanced salt solution containing 2 mg/ml collagenase (Worthington). After the incubation, the adventitia was carefully stripped off, and the endothelium was removed. Remaining smooth muscle was digested with 2.0 mg/ml collagenase (Worthington), 0.5 mg/ml elastase, and 1 mg/ml bovine albumin (Sigma) at 37°C. Single PASMC were resuspended, plated onto 25-mm cover slips (for fluorescence microscopy and electrophysiological experiments) were used at passages 4–6–10. Patch pipettes (2–4 MΩ) were fabricated on a Sutter electrode puller using borosilicate glass tubes and fire polished on a microforge (Narishige). For whole cell current recordings, voltage stimuli lasting 300 ms were delivered from a holding potential of 0 mV (to inactive voltage-gated \(Ca^{2+}\) and Na\(^+\) channels) using voltage steps from +80 mV to −80 mV. Traces recorded before the activation of SOCs were used as a template to subtract leak currents. All experiments were performed at room temperature (22–24°C). In both whole cell and single-channel configurations, SOCs were activated by passive depletion of the SR using 10 μM cyclopiazonic acid (CPA; Sigma) dissolved in the \(Ca^{2+}\)-free solution.

**Solution and reagents.** For [\(Ca^{2+}\)]\(_{cyt}\) measurement experiments, the cells were superfused (2–3 ml/min) with physiological salt solution (PSS). The PSS contained (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 10 glucose (pH 7.4 with 5 M NaOH). The bath solution for recording whole cell \(I_{CRAC}\) contained (in mM) 120 sodium methane sulfonate, 20 calcium aspartate, 0.5 3,4-diaminopyridine, 10 glucose, and 10 HEPES (pH 7.4 with methane sulfonic acid). The pipette (intracellular) solution contained (in mM) 138 cesium aspartate, 1.15 EGTA, 1 Ca(OH)\(_2\), 2 Na\(_2\)ATP, and 10 HEPES (pH 7.2). These ionic conditions eliminated the currents through K\(^+\) or Cl\(^−\) channels. In the high-K\(^+\) (40 mM) solution, NaCl was replaced, mole-for-mole, by KCl to maintain the osmolarity of the solution. For cell-attached recording of single-channel \(I_{CRAC}\), the pipette (extracellular) solution contained (in mM) 120 sodium methane sulfonate, 20 calcium aspartate, 0.5 3,4-diaminopyridine, and 10 HEPES (pH 7.4 with methane sulfonic acid). The bath (extracellular) solution contained (in mM) 141 NaCl, 4.7 KCl, 1.2 MgCl\(_2\), 1.8 CaCl\(_2\), 10 glucose, and 10 HEPES (pH 7.4).
CPA and thapsigargin (Sigma) were dissolved into DMSO to make stock solutions of 30 and 10 mM, respectively. Aliquots of the stock solutions were then diluted 1:3,000–10,000 into the bath solution or culture medium to make final concentrations of 10 μM CPA (pH 7.4) and 1 μM thapsigargin. Nifedipine (Sigma) and Ni2+ (Sigma) were directly dissolved in the bath solutions on the day of use. The pH values of all solutions were checked after addition of the drugs and readjusted to 7.4. In [Ca2+]cyt and Icrac measurement experiments, the same amount of DMSO used for dissolving CPA was added to control solutions (0.03%). Vehicle (DMSO) alone had negligible effects on [Ca2+]cyt and Icrac in PASMC.

RT-PCR assay. Total RNA (2–3 μg) prepared from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method, was reverse-transcribed using the First-Strand cDNA synthesis kit (Pharmacia Biotech). The sense/antisense primers were designed from coding regions of hTRP1 [5′-CAAGATTGTGGAAATTTCTCCTTCTGC-3′ (nt. 2,238–2,259)/5′-TGGTGGATAGGGCGATTGGT-3′ (nt. 1,336–1,356); U59019], respectively. The first-strand cDNA reaction mixture (2 μl) was used in a 50-μl PCR reaction consisting of 0.1 μM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 μM of each dNTP, and 2 units of Taq DNA polymerase (Perkin-Elmer). The cDNA samples were amplified in a DNA thermal cycler (Perkin-Elmer) under the following conditions: the mixture was annealed at 50–61°C (for 1 min), extended at 72°C (for 2 min), and denatured at 94°C (for 1 min) for 25 cycles. This was followed by a final extension at 72°C (for 10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1% agarose gel, and amplified cDNA bands were visualized by ethidium bromide staining. The PCR product was sequenced, and the result indicated that the sequence of our PCR product using the sense and antisense primers designed for hTRP1 matched exactly with the hTRP1 sequence (U31110) in GenBank. To quantify the PCR products, channel signals were normalized to the OD values in the control. The OD value for each band on the gel was measured by means of signals; the ratios are expressed as arbitrary units for quantification.

RESULTS

Requirement of extracellular Ca2+ and intracellularly stored Ca2+ for PASMC growth. A rise in [Ca2+]cyt can activate cytoplasmic mitogen-activated protein kinase (6) (which is part of the phosphorylation cascade that leads to activation of DNA synthesis-promoting factor), rapidly increase nuclear [Ca2+] (1), and promote cell proliferation (22). The addition of 2 mM EDTA, a Ca2+ chelator, to the culture medium (which contains 1.6 mM Ca2+) decreases the free [Ca2+] to 59 μM (9) and significantly inhibited human PASMC growth in media containing 5% fetal bovine serum and growth factors (Fig. 1). Depletion of intracellular Ca2+ stores (mainly the SR) with 1 μM thapsigargin further inhibited proliferation of human PASMC cultured in media containing 2 mM EDTA (Fig. 1). These results demonstrate that a sustained Ca2+ influx through sarcoplasmic Ca2+ channels and sufficient [Ca2+] maintained within the SR (33) are both required for PASMC growth.

Increased resting [Ca2+]cyt and CCE in proliferating PASMC. Withdrawal of serum and growth factors from culture media abolished cell growth (Fig. 1). This indicates that the cells cultured in smooth muscle basal medium (without serum and growth factors) are growth-arrested cells, whereas the cells cultured in smooth muscle growth medium (smooth muscle basal medium supplemented with 5% fetal bovine serum and growth factors) are proliferating cells. In the absence of extracellular Ca2+, CPA, by blocking Ca2+ sequestration into the SR, induced a transient [Ca2+]cyt rise due to leakage of Ca2+ from the SR to the cytosol. The CPA-induced [Ca2+]cyt transient declined back to the original baseline level after 5–7 min as the SR Ca2+ was depleted. Under these conditions, restoration of extracellular Ca2+ induced a rise in [Ca2+]cyt, which was obviously due to CCE (Fig. 2A).

The peak increase in [Ca2+]cyt due to CCE was significantly enhanced in proliferating human PASMC (1,712 ± 63 nM, n = 98) compared with growth-arrested cells (369 ± 22 nM, n = 92, P < 0.001) (Fig. 2). Furthermore, the resting [Ca2+]cyt (171 ± 6 vs. 88 ± 4 nM, P < 0.01) and the CPA-mediated Ca2+ transients (due to Ca2+ release from the SR) (614 ± 31 vs. 301 ± 29 nM, P < 0.01) were also significantly higher in proliferating PASMC (n = 98) than in growth-arrested cells (n = 92).

Inhibitory effects of Ni2+ on CCE and PASMC growth. Blockade of L-type VDCC with nifedipine (1 μM) abolished the 40 mM K+-induced increase in...
CCE, TRP, AND CELL GROWTH

Fig. 2. Enhancement of the capacitative Ca\(^{2+}\) entry (CCE) induced by cyclopiazonic acid (CPA)-mediated store depletion in proliferating human PASMC. A: representative records showing the time course of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) changes in growth-arrested [(-)serum-GF] and proliferating [(+)serum-GF] PASMC. CPA (10 \(\mu\)M) was applied to the cells in Ca\(^{2+}\)-free (0Ca) and Ca\(^{2+}\)-containing solutions. B: fura 2 fluorescence (\(F_{360}\)) images showing PASMC from which [Ca\(^{2+}\)]\(_{\text{cyt}}\) were measured and pseudocolor Ca\(^{2+}\) images showing [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC cultured in media with [(+)serum-GF, right] and without [(-)serum-GF, left] serum and growth factors. The pseudocolor images were acquired respectively at the time corresponding to a, b, c, and d in A. Bar = 15 \(\mu\)m.

Fig. 3. Inhibitory effects of Ni\(^{2+}\) on the CCE-induced increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) and human PASMC growth. A, top: representative record of [Ca\(^{2+}\)]\(_{\text{cyt}}\) in response to 40 mM K\(^{+}\) (40K), CPA (10 \(\mu\)M), nifedipine (1 \(\mu\)M), Ni\(^{2+}\) (0.5 mM), and SKF-96365 (10 \(\mu\)M) in the presence or absence (0Ca) of extracellular Ca\(^{2+}\). A, bottom: summarized data showing the CCE-mediated increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in human PASMC (n = 41) before (control), during (Ni), and after (recovery) application of 0.5 mM Ni\(^{2+}\). Data are means \pm SE. ***P < 0.001 vs. control. B: cell numbers were determined before (basal) and after 2-day incubation of human PASMC in SMGM [(+)serum-GF] with (+) or without (-) 0.5 mM Ni\(^{2+}\). Data are means \pm SE (n = 6). **P < 0.01 vs. (-) Ni.

Increased SOC activity in proliferating PASMC. The flux of Ca\(^{2+}\) ions through SOCs has been defined electrophysiologically as \(I_{\text{CRAC}}\) (14, 17, 18, 20, 27). Whole cell \(I_{\text{CRAC}}\) were recorded in cells superfused with solutions containing 120 mM Na\(^{+}\) and 20 mM Ca\(^{2+}\) and dialyzed with solutions containing 138 mM Cs\(^{+}\) and \(~100\) mM free Ca\(^{2+}\). Holding potential was set at 0 mV to inactivate voltage-gated Na\(^{+}\) and Ca\(^{2+}\) channels. Whole cell \(I_{\text{CRAC}}\) were elicited by 300-ms voltage steps from \(-80\) to \(+80\) mV before and after application of 10
carried by $\Ca^2+$ (or $\Na^+$) through SOCs (Fig. 5A). The slope conductance of this current, calculated from the current-voltage relationship curve, is 5.34 pS (Fig. 5B).

Enhanced hTRP1 expression in proliferating PASMC. It has been demonstrated that TRP-encoded proteins may be responsible for CCE (4, 5, 37, 44, 45). Human PASMC from nonpulmonary hypertension patients expressed hTRP1 but not hTRP3 (Fig. 6A). The mRNA level of hTRP1 was significantly higher in proliferating cells than in growth-arrested cells (Fig. 6). The enhanced mRNA expression was selective to hTRP1 because the mRNA level of the hVDCC $\beta_2^-$ subunit was unaltered in proliferating PASMC (Fig. 6B). These results are consistent with the electrophysiological and fluorescence microscopy experiments showing that $I_{\text{CRAC}}$ and CCE were enhanced in human PASMC during proliferation.

**Temporal relationship between hTRP1 expression and PASMC proliferation.** Whether the mRNA expression of hTRP1 was temporally related to the cell proliferation was examined by comparing the time courses of the change of hTRP1 mRNA levels and the cell growth rate. The cells were first growth arrested by incubation in smooth muscle basal medium (the basal medium without serum and growth factors) for 24–36 h and then cultured in smooth muscle growth medium (smooth muscle basal medium supplemented with 5%}

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\text{hTRP1 expression was temporally related to cell proliferation.}
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Fig. 4. Whole cell $\Ca^{2+}$ release-activated $\Ca^{2+}$ currents ($I_{\text{CRAC}}$) were enhanced in proliferating PASMC. A: currents elicited by 300-ms voltage steps from $-80$ to $+80$ mV in 20-mV increments before (control) and after 10-min application of CPA (10 $\mu$M) in growth arrested [a, (-serum-GF) and proliferating [b, (+)-serum-GF] PASMC. The cells were held at 0 mV to minimize voltage-gated $\Ca^{2+}$ and Na$^+$ currents. Right: subtraction ($I_{\text{CRAC}}$) between the currents recorded before and after application of CPA. B: Left: current-voltage relationships (I-V curves) for $I_{\text{CRAC}}$ recorded in PASMC cultured in media with (+) or without (-) serum and growth factors (GF). Right: summarized data showing amplitudes of $I_{\text{CRAC}}$ elicited by test potentials of $-80$ and $+80$ mV, in growth arrested [(-)-serum-GF] and proliferating [(+-)-serum-GF] human PASMC. Data are means ± SE. *$P < 0.05$ vs. [(-)-serum-GF].
fetal bovine serum and growth factors) for up to 4 days. As shown in Fig. 7, the cell number in culture dishes (A) and the mRNA level of hTRP1 (B) were both increased after incubating the cells in media containing serum (5% fetal bovine serum) and growth factors (smooth muscle growth medium). However, the time courses are quite different (Fig. 7C): the mRNA expression of hTRP1 was significantly increased after 6 h of incubation (by 10.1 ± 3.4%, P < 0.05) in the smooth muscle growth medium (5% fetal bovine serum and growth factors), whereas the cell growth was not significantly increased until 24 h (by 15.4 ± 2.4%, P < 0.001) (Fig 7C). The calculated times for the 50% increase in the hTRP1 mRNA expression and the cell growth are 18.36 and 46.56 h, respectively (Fig. 7C). These results suggest that enhancement of hTRP1 mRNA expression precedes the onset of cell proliferation in the presence of fetal bovine serum and growth factors. The precise temporal relationship between hTRP1 expression and actual proliferating process (e.g., different cell cycle phases) needs further study.

Elevated resting [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{SR}$ in growth-arrested PASMC from patients with PPH. Patients with SPH (n = 18) and PPH (n = 7), from whom PASMC were isolated, had similar mean pulmonary
arterial pressure (48 ± 4 vs. 53 ± 4 mmHg, P = 0.30) and total vascular resistance (13.6 ± 3 vs. 11.2 ± 1.7 mmHg·l⁻¹·min⁻¹, P = 0.48) (41). However, the resting [Ca²⁺]ᵣₑᵗ in quiescent (growth arrested) PASMC (n = 16) from PPH patients was significantly higher than in cells (n = 19) from SPH patients (Fig. 8, A and C).

In the presence of extracellular Ca²⁺, the CPA-induced peak increase in [Ca²⁺]ᵣₑᵗ was significantly greater in PPH PASMC (600 ± 70 nM) than in SPH PASMC (350 ± 25 nM, P < 0.01) (Fig. 8, B, top, and C, right). The CPA-induced transient [Ca²⁺]ᵣₑᵗ rise in the absence of extracellular Ca²⁺ (by Ca²⁺ mobilization from the SR) and the CCE-mediated [Ca²⁺]ᵣₑᵗ increase while extracellular Ca²⁺ was restored (193 ± 23 nM, n = 16, vs. 96 ± 8 nM, n = 19, P < 0.01) were both greater in PPH PASMC than in SPH PASMC. These results indicate that resting [Ca²⁺]ᵣₑᵗ, [Ca²⁺]ᵣₛᵣ, and CCE are all higher in PASMC from PPH patients than in cells from SPH patients.

**DISCUSSION**

In human PASMC, chelation of extracellular Ca²⁺ and depletion of intracellularly stored Ca²⁺ markedly inhibited cell growth in media containing serum and growth factors, whereas resting [Ca²⁺]ᵣₑᵗ was much higher in proliferating cells than in growth-arrested cells. These results imply that elevated [Ca²⁺]ᵣₑᵗ and [Ca²⁺]ᵣₛᵣ, due to a constant Ca²⁺ influx, are necessary for human PASMC proliferation. CCE is a critical mechanism involved in maintaining sustained Ca²⁺ influx and refilling Ca²⁺ into the SR (4, 27, 36). Indeed, the CCE-mediated increase in [Ca²⁺]ᵣₑᵗ, whole cell Iᵣₑᵗ, and hTRP1 mRNA expression were all significantly enhanced in PASMC during proliferation. Inhibition of SOCs with Ni²⁺ reduced CCE and significantly attenuated human PASMC growth. These results suggest that CCE, potentially through the up-regulated hTRP₁ expression and the resultant increase in Iᵣₑᵗ (4, 27, 36). Indeed, the CCE-mediated increase in [Ca²⁺]ᵣₑᵗ, whole cell Iᵣₑᵗ, and hTRP1 mRNA expression were all significantly enhanced in PASMC during proliferation. Inhibition of SOCs with Ni²⁺ reduced CCE and significantly attenuated human PASMC growth. These results suggest that CCE, potentially through the up-regulated hTRP₁ expression and the resultant increase in Iᵣₑᵗ is a critical mechanism required to maintain the elevated [Ca²⁺]ᵣₑᵗ and [Ca²⁺]ᵣₛᵣ in PASMC during proliferation.

**Regulation of cytosolic free [Ca²⁺].** In vascular smooth muscle cells, including PASMC, [Ca²⁺]ᵣₑᵗ is regulated by Ca²⁺ influx through Ca²⁺-permeable channels in the plasma membrane and Ca²⁺ release from intracellular Ca²⁺ stores, such as the sarco(endo)plasmic reticulum (34, 36). Many vasoactive agonists stimulate smooth muscle proliferation by increasing [Ca²⁺]ᵣₑᵗ (6, 16, 23, 28, 32). The mitogen-induced changes in [Ca²⁺]ᵣₑᵗ usually consist of an initial release of Ca²⁺ from the SR followed by a sustained Ca²⁺ influx through sarcolemmal Ca²⁺ channels. In human PASMC, resting membrane potential is approximately −45 mV, and resting [Ca²⁺]ᵣₑᵗ is 50–150 nM. Thus the observations that extracellular Ca²⁺ chelation inhibited human PASMC growth imply that continuous Ca²⁺ influx is essential for human PASMC.

There are at least three classes of Ca²⁺-permeable channels in the plasma membrane: 1) VDCC (24, 2) receptor-operated channels (36); and 3) SOCs (4, 27, 36). Membrane potential regulates [Ca²⁺]ᵣₑᵗ by governing Ca²⁺ influx via VDCC (24, 42). The agonist-induced Ca²⁺ influx is mainly caused by the receptor-mediated activation of receptor-operated channels and store depletion-mediated opening of SOCs (4, 27, 36). It is likely that depolarization-activated VDCC, agonist-activated receptor-operated channels, and store depletion-activated CCE all contribute to the sustained Ca²⁺ influx in PASMC during proliferation. Inhibition of CCE by the SOC blocker Ni²⁺ significantly attenuated human PASMC growth, suggesting that CCE through SOCs is a critical mechanism involved in maintaining sustained Ca²⁺ influx and refilling Ca²⁺ into the SR, which are both necessary for PASMC growth.

**Molecular identification of CCE.** Electrophysiological studies on the store depletion-activated Iᵣₑᵗ suggest that SOCs are complex and heterogeneous in molecular composition and in their cellular regulation (4). There are at least seven members (TRP₁–TRP₇) in the mouse TRP gene family, of which at least four (TRP₁, TRP₃, TRP₄, and TRP₆) have been identified in human tissues (4, 26, 44, 45). Expression of TRP genes results in the formation of the Ca²⁺-permeable cation channels that are activated by Ca²⁺ store depletion, suggesting that SOCs may be composed of subunits encoded in TRP genes (3–5, 25, 37, 44, 45). SOCs are hypothesized to be heterotetramers and/or homotetramers made of different TRPs (3, 4, 27). In mammalian tissues, it has been recently demonstrated that TRP₁, TRP₂, TRP₄, and TRP₅ may encode the endogenous SOCs that are activated by store depletion, whereas TRP₃, TRP₆, and TRP₇ may encode the channels that are activated by inositol 1,4,5-trisphosphate (3, 4, 21, 26) and store depletion.

Patch-clamp studies on the store depletion-activated Iᵣₑᵗ indicate that there are multiple SOCs based on the single-channel conductance (0.2–110 pS) (14, 17, 18, 20, 27). In human PASMC, only the hTRP₁ transcript (but not hTRP₃ transcript) was identified using RT-PCR, suggesting that hTRP₁ is a critical TRP gene that encodes SOCs in human PASMC. In proliferating PASMC, we observed a 5.34-pS channel that was activated by CPA-induced depletion of the SR Ca²⁺. These observations suggest that the 5.34-pS channel may be an important SOC that participates in regulating human PASMC growth.

**Role of Ca²⁺ in PASMC Proliferation.** Cytosolic Ca²⁺ stimulates cell proliferation (22, 23, 28, 32), whereas [Ca²⁺]ᵣₛᵣ may regulate cell proliferation by modulating the amplitude and frequency of Ca²⁺ signals in the cytosol and nucleus (6, 8, 19, 33). Maintaining a sufficient level of Ca²⁺ in the SR is also critical for cell growth; indeed, depletion of the SR Ca²⁺ stores induces growth arrest (15, 33). In the cell cycle, there are four Ca²⁺-sensitive steps: transitions 1) from the G₀ (resting state) to the G₁ phase (the beginning of DNA synthesis); 2) from the G₁ to the S phase (an interphase
during which replication of the nuclear DNA occurs); 3) from the G2 to the M phase (mitosis); and 4) through the M phase (22). Thus a rise in \([Ca^{2+}]_{cyt}\) plays a critical role in stimulating PASMC proliferation.

A critical signal transduction pathway upon activation of membrane receptors by mitogenic agonists is

![Diagram](image)

**Fig. 9.** Schematic diagram depicting the proposed mechanisms involved in the CCE- and \(Ca^{2+}\)-mediated pulmonary vasoconstriction and PASMC growth. When the SR is depleted during activation of mitogenic receptors, CCE, potentially through TRP-encoded channels, is activated to maintain the sustained increases in \([Ca^{2+}]_{cyt}\) and nuclear \([Ca^{2+}]_{n}\), and to refill \(Ca^{2+}\) into the SR. The expression of TRPs, induced by genetic and endogenous factors, and the function of TRP channels contribute to the regulation of \([Ca^{2+}]_{cyt}\) by governing \(I_{CRAC}\) and CCE. The resultant increases in cytosolic, nuclear, and intracellularly stored \([Ca^{2+}]_{cyt}\) may promote cell growth by 1) activating cytosolic signal transduction proteins (e.g., CREB and mitogen-activated protein kinase) and transcription factors (e.g., c-Fos and c-Jun), 2) moving quiescent cells into the cell cycle and propelling the proliferating cells through mitosis, and 3) enhancing protein and lipid processing in the SR. The intracellular \(Ca^{2+}\) serves as a shared signal transduction element that leads to pulmonary vasoconstriction and vascular medial hypertrophy, two major contributors for the elevated pulmonary vascular resistance (PVR) and arterial pressure (PAP) in patients with PPH.

**Fig. 8.** Resting \([Ca^{2+}]_{cyt}\) and CPA-induced \([Ca^{2+}]_{cyt}\) increases in growth-arrested PASMC from secondary (SPH) and primary pulmonary hypertension (PPH) patients. **A:** fura 2 fluorescence (F_360) images (top) showing PASMC from which \([Ca^{2+}]_{cyt}\) were measured and pseudocolor \(Ca^{2+}\) images (bottom) showing \([Ca^{2+}]_{cyt}\) in the cells. Bar = 15 μm. **B:** time courses of the CPA-induced \([Ca^{2+}]_{cyt}\) changes in the presence (top) or absence (0 Ca; bottom) of extracellular \(Ca^{2+}\) in PASMC from SPH (left) and PPH (right) patients. **C:** summarized data (means ± SE) showing the resting \([Ca^{2+}]_{cyt}\) (left) and amplitudes of the CPA-induced transient increases in \([Ca^{2+}]_{cyt}\) (right) in the presence of extracellular \(Ca^{2+}\). **P** < 0.01 vs. SPH.
may play an important role in regulating PASMC growth via modulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ (10).

**Mechanisms involved in elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC from PPH patients.** Pulmonary vasoconstriction and vascular smooth muscle proliferation greatly contribute to the increased pulmonary vascular resistance in patients with PPH (31, 35, 38). Elevated pulmonary arterial pressure in patients with SPH is often caused by primary diseases, such as left to right shunt in congenital heart diseases and vascular embolism in pulmonary thromboembolic disease. The results from this study demonstrate that resting $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ are higher, and CCE is greater, in PASMC from PPH patients than in the cells from SPH patients. These data suggest that increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ due to enhanced CCE may also play a role in the development of pulmonary vascular smooth muscle proliferation in patients with PPH (Fig. 9).

We previously reported that the activity of voltage-gated $K^+$ channels is significantly decreased in PASMC from PPH patients compared with PASMC from SPH patients (41, 43). The resultant membrane depolarization increases $[\text{Ca}^{2+}]_{\text{cyt}}$ by opening VDCC and may play an important role in initiating pulmonary vasoconstriction in PPH patients (41, 43). In PASMC isolated from SPH and PPH patients, 60 mM $K^+$-induced rises in $[\text{Ca}^{2+}]_{\text{cyt}}$ are similar to the two cell types (41). High-$K^+$ -induced rises in $[\text{Ca}^{2+}]_{\text{cyt}}$ mainly result from opening of VDCC via membrane depolarization (24, 42). These results suggest that VDCC function normally in PPH PASMC or at least similarly in SPH and PPH PASMC. The observations from the current study demonstrate that the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to CCE is much greater in PPH PASMC than SPH PASMC. Therefore, the elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ in PPH PASMC could be provoked by 1) indirect activation of VDCC via membrane depolarization and 2) direct augmentation of CCE through the $\text{hTRP1}$-encoded SOCs.

In summary, resting $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{SR}}$ as well as store depletion-mediated $I_{\text{CRAC}}$ and CCE are significantly enhanced in normal human PASMC during proliferation (compared with growth-arrested cells). Blockade of SOCs diminishes CCE and significantly inhibits human PASMC growth in media containing serum and growth factors. These results suggest that CCE, a novel mechanism essential for agonist-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and for refilling $\text{Ca}^{2+}$ into the SR, may play an important role in mediating PASMC growth and stimulating pulmonary vascular medial hypertrophy in patients with PPH (Fig. 9).

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**REFERENCES**


