PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression

YING YU, MICHELE SWEENEY, SHEN ZHANG, OLEK SANDR PLATOSHYN, JUDD LANDSBERG, ABRAHAM ROTHMAN, AND JASON X.-J. YUAN

Division of Pulmonary and Critical Care Medicine, Department of Medicine, and Division of Pediatric Cardiology, Department of Pediatrics, University of California, San Diego, California 92103

Submitted 18 March 2002; accepted in final form 26 September 2002

Yu, Ying, Michele Sweeney, Shen Zhang, Oleksandr Platoshyn, Judd Landsberg, Abraham Rothman, and Jason X.-J. Yuan. PDGF stimulates pulmonary vascular smooth muscle cell proliferation by upregulating TRPC6 expression. Am J Physiol Cell Physiol 284: C316–C330, 2003; 10.1152/ajpcell.00125.2002.—Capacitative Ca2⁺ augmented CCE, elicited by passive depletion of Ca2⁺ by PDGF treatment also upregulated TRPC6 expression and protein levels of c-Jun, and stimulated cell proliferation. activator of transcription (STAT3), increased mRNA and ng/ml for 0.5–48 h phosphorylated signal transducer and receptor potential cation channel. In rat PASMC, PDGF (10 ng/ml for 0.5–48 h) phosphorylated signal transducer and activator of transcription (STAT3), increased mRNA and protein levels of c-Jun, and stimulated cell proliferation. PDGF treatment also upregulated TRPC6 expression and augmented CCE, elicited by passive depletion of Ca2⁺ from the SR using cyclopiazonic acid. Furthermore, overexpression of c-Jun stimulated TRPC6 expression and CCE amplitude in PASMC. Downregulation of TRPC6 using an antisense oligonucleotide specifically for human TRPC6 decreased CCE and inhibited PDGF-mediated PASMC proliferation. These results suggest that PDGF-mediated PASMC proliferation is associated with c-Jun/STAT3-induced upregulation of TRPC6 expression. The resultant increase in CCE raises [Ca2⁺]cyt, facilitates return of Ca2⁺ to the SR, and enhances PASMC growth.

store-operated cation channels; pulmonary hypertension; vascular remodeling; platelet-derived growth factor

PLATELET-DERIVED GROWTH FACTOR (PDGF) is an important autocrine and paracrine mitogen for vascular smooth muscle cells, mediating hyperplasia, hypertrophy, endoreduplication, and migration, and for pulmonary vascular remodeling (3, 4, 58, 60, 71). As a tyrosine kinase-coupled receptor agonist, PDGF is not only itself sufficient to initiate DNA synthesis and mitosis, but it is also a stimulus for its own expression (56) and synthesis of other mitogens such as endothelin-1 (ET-1) and heparin-binding epidermal growth factor in vascular smooth muscle cells (4). High levels of PDGF have been implicated in the blood and lung tissues of patients with primary and secondary pulmonary hypertension, suggesting a critical role of PDGF in the elevated pulmonary vascular resistance and pulmonary arterial pressure in these patients. Indeed, the mitogenic effect of PDGF on pulmonary artery smooth muscle cells (PASMC) has been demonstrated to contribute to the progression of pulmonary vascular wall remodeling in patients with pulmonary hypertension (3, 26, 58, 60, 64).

Ionized Ca2⁺ in the cytoplasm, intracellular organelles, and nucleus is a critical signal transduction element in many cell types (5, 57, 61, 62). An increase in cytoplasmic free Ca2⁺ concentration ([Ca2⁺]cyt) is a major trigger for smooth muscle contraction (57, 62) and an important stimulus for smooth muscle cell growth (6–8, 43). Removal (or chelation) of intracellular Ca2⁺ or depletion of intracellularly stored Ca2⁺ in the sarcoplasmic/endoplasmic reticulum ([Ca2⁺]SR) significantly inhibits vascular smooth muscle cell (including PASMC) proliferation in the presence of serum and growth factors (16, 18, 55). These results indicate that a constant influx of extracellular Ca2⁺, which raises [Ca2⁺]cyt and nuclear Ca2⁺ concentration ([Ca2⁺]n) (1), as well as a sufficient [Ca2⁺]SR, is essential for PASMC proliferation. Therefore, the Ca2⁺-permeable channels in the plasma membrane would potentially be an important downstream effector for PDGF to mediate PASMC growth and proliferation.

In PASMC, there are at least three classes of Ca2⁺-permeable channels: 1) voltage-dependent Ca2⁺ channels (VDCC), 2) receptor-operated or ligand-gated Ca2⁺ channels (ROC), and 3) store-operated Ca2⁺ channels (SOC) (11, 34, 46, 61, 72). Opening of VDCC by membrane depolarization and opening of ROC by ligand-receptor interaction greatly contribute to the increase in [Ca2⁺]cyt in PASMC stimulated by membrane depolarizing factors (e.g., high K⁺), vasoconstrictors (e.g., serotonin, ET-1, and phenylephrine), and

Address for reprint requests and other correspondence: J. X.-J. Yuan, Div. of Pulmonary and Critical Care Medicine, UCSD Medical Center, MC 8382, 200 W. Arbor Dr., San Diego, CA 92150-8382 (E-mail: xyuan@ucsd.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
growth factors (e.g., PDGF) (2, 14, 25, 34, 44, 49, 52–54, 68). Opening of SOC by depletion of Ca\(^{2+}\) from the sarcoplasmatic reticulum (SR) leads to capacitative Ca\(^{2+}\) entry (CCE), a mechanism involved in maintaining a sustained elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and returning Ca\(^{2+}\) to the depleted SR (7, 14, 18, 49, 51, 62). The mitogen-mediated rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) usually consists of two distinguishable components: an initial transient increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) mobilization from the SR followed by a sustained increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) influx through SOC (and other Ca\(^{2+}\) channels, e.g., ROC and VDCC). Indeed, the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) is much higher in proliferating cells cultured in medium containing serum and growth factors than in growth-arrested cells cultured in medium without serum and growth factors (18). Therefore, stimulating SOC function and upregulating expression of the genes that encode SOC would be very likely involved in the mitogen-mediated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) and subsequent cell proliferation.

The SOC responsible for CCE in vascular smooth muscle cells are believed to be encoded by transient receptor potential (TRP) channel genes (7, 18, 27, 45, 66, 70). Overexpression of TRP channel gene(s) in heterologous expression systems (e.g., HEK-293, COS, and Chinese hamster ovary cells and Xenopus oocytes) results in the formation of Ca\(^{2+}\)-permeable channels that are activated by depletion of the SR Ca\(^{2+}\) and activation of membrane receptors (7, 12, 55, 40, 63, 76, 77). Inhibition of TRP channel expression using the antisense (AS) oligonucleotides specifically targeting at TRP genes downregulates the mRNA and protein expression of TRP channels, reduces inward Ca\(^{2+}\) currents through SOC, decreases Ca\(^{2+}\) entry, and inhibits many Ca\(^{2+}\)-mediated functions (10, 27, 45, 59, 70).

TRPC6, a member of the short TRP channel gene subfamily (11), is abundantly expressed in lung tissues and pulmonary arteries (8, 45, 66). c-Jun, a member of the activating protein-1 (AP-1) gene family (29), is a transcription factor that associates with cell growth and proliferation in PASMC (38, 39). It has been well demonstrated that PDGF increases [Ca\(^{2+}\)]\(_{\text{cyt}}\) by modulating VDCC and ROC (2, 25, 68) in vascular smooth muscle cells; however, it is unclear whether SOC is involved in PDGF-induced PASMC growth. This study was thus designed to test the hypothesis that PDGF-mediated PASMC proliferation is in part caused by upregulation of TRPC6 gene expression. The subsequent augmentation of Ca\(^{2+}\) entry due to CCE and receptor-mediated Ca\(^{2+}\) influx increases [Ca\(^{2+}\)]\(_{\text{cyt}}\) and maintains [Ca\(^{2+}\)]\(_{\text{SR}}\) by returning Ca\(^{2+}\) to the SR and, ultimately, contributes to stimulation of PASMC proliferation.

**MATERIALS AND METHODS**

**Cell preparation and culture.** PASMC from pulmonary arteries were prepared from male Sprague-Dawley rats (125–250 g) (72, 73). The isolated pulmonary arterial branches (3rd–4th division) were incubated in Hanks’ balanced salt solution (Biofluids) containing collagenase (1.5 mg/ml; Worthington) for 20 min. After incubation, a thin layer of adventitia was carefully stripped off with a fine forceps, and the endothelium was removed by gentle scratching of the intimal surface with a surgical blade. The remaining smooth muscle was then digested with collagenase (2.0 mg/ml) and elastase (0.5 mg/ml; Sigma) for 35–45 min at 37°C. Cells were plated onto 25-mm coverslips (for patch-clamp and fluorescence microscopy experiments) or 10-cm petri dishes (for molecular biological experiments) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS; GIBCO), penicillin (100 U/ml), and streptomycin (100 mg/ml) and cultured in a humidified incubator at 37°C. The cells were passaged by trypsinization with 0.05% trypsin-EDTA (GIBCO) and used for experiments at passages 3–6. Growth of all cells was arrested before experimentation by culture in serum-free DMEM for 24 h.

The purity of PASMC in cultures was confirmed by the specific monoclonal antibody raised against smooth muscle α-actin (Boehringer Mannheim). Cultured cells were first stained with the membrane-permeable nucleic acid stain 4’,6’-diamidino-2-phenylindole (5 μM; Molecular Probes) to estimate total cell numbers in the cultures. All the 4’,6’-stained nuclei of cells were counted. Additionally, cells were costained with the smooth muscle cell α-actin antibody, indicating that the cultures were smooth muscle cells (50). Cell number was determined using a hemocytometer. Cell count in each of the four 1-mm\(^2\) corner squares in the hemocytometer was averaged to calculate total cell number per milliliter in cell suspension. Cell number, normalized by the size of the petri dishes (cells/cm\(^2\)), was used to compare cell growth rate. Cell viability was determined using 0.45% trypan blue (Sigma).

**DNA synthesis.** \[^3^H\]Thymidine incorporation was determined to evaluate DNA synthesis. Briefly, rat PASMC were seeded in 24-well microplates at \(\times 2 \times 10^4\) cells/well and cultured in 10% FBS-DMEM for 24 h, and growth was arrested in DMEM for 24 h. Cells were then incubated in 0.2% FBS-DMEM with or without PDGF (10 ng/ml) for 48 h, with 1 μCi of \[^3^H\]thymidine added to the cells for the last 16 h. Incorporation of radioactivity into trichloroacetic acid-insoluble material was measured by a liquid scintillation counter. For EGTA experiments, the growth-arrested cells were treated with PDGF in the absence or presence of 0–1.5 mM EGTA for 24 h before \[^3^H\]thymidine incorporation was measured by the liquid scintillation counter.

**Bromodeoxyuridine incorporation.** The immunofluorescent staining (intensity) of incorporated bromodeoxyuridine (BrDU) was detected by the BrDU flow kit according to a modification of the manufacturer’s instructions (BD Pharmingen). Briefly, growth-arrested rat PASMC cells were incubated at 37°C in 0.2% FBS-DMEM with and without PDGF (10 ng/ml) for 24 h, with 10 μM BrDU added to the cells for 2 h. Cells were then harvested, fixed, and permeabilized. To expose incorporated BrDU, cells were treated with DNase at 30 μg/100 μl for 1 h at 37°C. Samples were washed twice with Perm/Wash buffer (BD Pharmingen) and then incubated for 20 min with 50 μl of anti-BrDU-FITC solution. After being washed twice with Perm/Wash buffer, the cells were cultured with 20 μl of 7-amino-actinomycin (BD Pharmingen) solution, resuspended in 200 μl of PBS containing 3% FBS and 0.09% sodium azide, and analyzed by FACScalibur flow cytometry using Cell Quest software (Becton Dickinson, Mountain View, CA). Results are displayed as bivariate distribution of BrDU content vs. DNA content.

**Generation of recombinant adenoviral vector and c-jun infection protocol.** An E1 region-deleted recombinant adenoviral vector carrying sense (+c-jun) c-jun cDNA was constructed. A 2.6-kb pair fragment of full-length c-jun cDNA was then subcloned in sense orientation into the pACCMV-
pLPa shuttle vector to yield the sense construct pSR-sense-c-jun. The pSR-sense-c-jun was then cotransfected with pJM17 into HEK-293 cells by calcium phosphate-DNA coprecipitation. For viral plaque assays, the cotransfected HEK-293 cells were overlaid with 0.65% agarose (prepared with 1× DMEM) every 3–4 days. The growth of the E1-deleted adenovirus is limited to the HEK-293 cells. The PCR assay was used for identification of the recombinant adenoviral vectors.

The adenoviral vector expressing sense c-jun was used to infect PASM; detailed methods for c-jun infection have been described elsewhere (38, 39). Briefly, the cells were infected with appropriate virus at 50 plaque-forming units/cell in DMEM containing 0.2% FBS and incubated with gentle swirling every 20–30 min for 3 h. After 7 h of infection, the medium was replaced with the growth medium, 10% FBS-DMEM. Cells were used 24–48 h after adenoviral infection for experimentation. The expression of c-Jun was verified by Western blot analysis.

Western blot analysis. The cells were gently washed twice in cold PBS, scraped into 0.5 ml of lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, and 30 μl/ml aprotinin), and incubated for 30 min on ice. The cell lysates were then sonicated and centrifuged at 12,000 rpm for 10 min, and the insoluble fraction was discarded. In some experiments, cell lysates were treated with the peptide N-glycosidase F (20 U; New England Biolabs) overnight at 4°C (74). The protein concentration in the supernatant was determined by the bicinchoninic acid protein assay using bovine serum albumin as a standard. Ten to 25 μg of proteins were mixed and boiled in SDS-PAGE sample buffer for 5 min. The protein samples separated on 10% SDS-PAGE were then transferred to nitrocellulose membranes by electroblotting in a MINI Trans-Blot Cell transfer apparatus according to the manufacturer's instructions (Bio-Rad). After incubation overnight at 4°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% nonfat dry milk powder, the membranes were incubated with the anti-c-Jun polyclonal antibody (Santa Cruz Biotechnology), anti-TRPC6 polyclonal antibody (Alomone Labs), and antiphosphorylated signal transducer and activator of transcription (STAT3) antibody (Cell Signaling Technology). The membranes were then washed and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG for 90 min at room temperature. The bound antibody was detected using an enhanced chemiluminescence detection system (Amersham).

RT-PCR. Total RNA (3 μg) was prepared from PASM by the acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol reagent (GIBCO) and reverse transcribed using the Superscript Preamplification System (GIBCO). The sense and AS primers were specifically designed from the coding regions of c-Jun, TRPC1, TRPC4, TRPC6, and rat α-actin (Table 1). The fidelity and specificity of the sense and AS oligonucleotides were examined using the BLAST program.

PCR was performed by a GeneAmp PCR System (Perkin-Elmer, Norwalk, CT) using platinum PCR supermix (GIBCO). The first-strand cDNA reaction mixture (3 μl) was used in a 50-μl PCR consisting of 0.2 nmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 200 μM each dNTP, and 2 U of Taq DNA polymerase. The cDNA samples were amplified in a DNA thermal cycler under the following conditions: the mixture was annealed at 55°C (1 min), extended at 72°C (1 min), and denatured at 94°C (1 min) for 25–30 cycles. This was followed by a final extension at 72°C (10 min) 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 semiquantify the PCR products, an invariant mRNA of β-actin was used as an internal control. The net intensity values of cDNA bands measured by a Kodak electrophoresis documentation system were normalized to the net intensity values of the β-actin signals, and the ratios are expressed as arbitrary units for quantitative comparison.

Electrophysiological measurements. Whole cell currents through SOC (I_{SOC}) were recorded with an Axopatch-1D amplifier using patch-clamp techniques (18, 20). 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 −80 to +20 mV. Current traces recorded before the activation of SOCs were used as a template to subtract leak currents. SOCs were activated by passive depletion of the Ca2+ in the SR using 10 μM cyclopiazonic acid (CPA). The bath solution for recording optimal I_{SOC} contained (mM) 120 sodium methane sulfonate, 20 calcium aspartate, 0.5 3,4-diaminopimidine, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 with methane sulfonic acid. The pipette solution contained (mM) 138 cesium aspartate, 1.15 EGTA, 1 Ca(OH)2, 2 Na2-ATP, and 10 HEPES (pH 7.2). These ionic conditions eliminated the currents activated by passive depletion of the Ca2+ in the SR using 10 μM cyclopiazonic acid (CPA). The bath solution was replaced by equimolar sodium aspartate to maintain osmolality. 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). Ni2+ (Sigma) was directly dissolved in the bath solution on the day of use. SK&F-96365 (Sigma) was first dissolved in distilled water to make a stock solution of 50

<table>
<thead>
<tr>
<th>Standard Name</th>
<th>Accession No.</th>
<th>Predicted Size, bp</th>
<th>Sense/Antisense</th>
<th>Location, nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC1</td>
<td>AF061266</td>
<td>425</td>
<td>5'-CTGTGATGAGGTGGTGGAAAG-3'/3'-GTCAGTGGTCTTCGCTGC-5'</td>
<td>1297–1318</td>
</tr>
<tr>
<td>TRPC4</td>
<td>NM_053434</td>
<td>415</td>
<td>5'-CCTCGGATGCTTATGTGCAAAGAGTGTC-3'/3'-AAAGCTTTGTTCGAGCAAATTTCCATTC-5'</td>
<td>1561–1586</td>
</tr>
<tr>
<td>TRPC6</td>
<td>NM_053559</td>
<td>327</td>
<td>5'-AGCAGGTACAGTGGAACAC-3'/3'-AAAGGAAATTCGAGAACCACTGC-5'</td>
<td>1891–1914</td>
</tr>
<tr>
<td>c-Jun</td>
<td>X17163</td>
<td>262</td>
<td>5'-GGATATCC-3'/3'-CCACCAACAGTGATGACAG-5'</td>
<td>128–147</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_031144</td>
<td>244</td>
<td>5'-AGTGTGATGAGGTGGTGGAAAG-3'/3'-GTCAGTGGTCTTCGCTGC-5'</td>
<td>370–383</td>
</tr>
</tbody>
</table>

Accession no., GenBank accession number for sequences used in designing the primers.
mM; aliquots of the stock solution were then diluted into the bath solution or culture medium to make final concentrations of 5, 10, and 50 μM SK&F-96365. The pH values of all solutions were checked after addition of the drugs and readjusted to 7.4.

**Measurement of \([Ca^{2+}]_{\text{cyt}}\).** In single PASMC, \([Ca^{2+}]_{\text{cyt}}\) was measured using the Ca²⁺-sensitive fluorescent indicator fura 2 (19). Cells were loaded with 3 μM fura 2-AM for 30 min in the dark at room temperature (22–24°C) under an atmosphere of 5% CO₂–95% air. The fura 2-loaded cells on coverslips were then transferred to a recording cell chamber on the microscope stage and superfused with modified Krebs solution for 30 min to remove the extracellular fura 2 and to allow cytosolic esterases to cleave fura 2-AM into active fura 2. Fura 2 fluorescence (510-nm light emission excited by 340- and 380-nm illuminations) from the cell, as well as background fluorescence, was collected at 32°C using Nikon UV-Fluor objectives. The fluorescence signals emitted from the cells were monitored continuously using an intracellular imaging fluorescence microscopy system and recorded on an IBM-compatible computer for later analysis. \([Ca^{2+}]_{\text{cyt}}\) was calculated from fura 2 fluorescence emission excited at 340 and 380 nm (F_{340}/F_{380}) using the ratio method based on the following equation: 

\[
\frac{[Ca^{2+}]_{\text{cyt}}}{K_d} = \left( \frac{S_2/S_b}{S_b} \right) \times \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \]

where \(K_d\) (225 nM) is the dissociation constant for Ca²⁺, \(S_2\) and \(S_b\) are emission fluorescence values at 380-nm excitation in the presence of EGTA and Triton X-100, respectively, \(R\) is the measured fluorescence ratio, and \(R_{\text{min}}\) and \(R_{\text{max}}\) are minimal and maximal ratios, respectively (19).

**AS oligonucleotides.** Second-generation AS oligonucleotides that contain nine phosphorothioate DNA linkages to activate RNase H were purchased from Sequitur (Natick, MA). The AS oligonucleotides were designed to cleave mRNA of the rat TRPC6 gene (GenBank accession no. AB051212) 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 nontargeted genes. The negatively charged AS oligonucleotide S13730 (TGGGC-CCTTTGAAACTTCCACTGCA) 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 oligomer and 2.5 g/ml of Lipid 2012-G (for 12 h) in PASMC (55–60% confluence). RT-PCR and Western blot analyses were used to evaluate oligomer activity. An oligonucleotide with the same base composition as S13730, but with scrambled sequence, was used as a control for nonspecific or toxic effects of the oligomers. The sequence of the AS oligonucleotide for rat TRPC6 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.

The oligonucleotides were prepared by one investigator who provided stocks that were coded so that the investigators performing electrophysiological and fluorescence microscopy experiments were unaware of treatment allocation until experiments and data analyses were completed. For each treatment, the cells were first rinsed with Opti-MEM (GIBCO-BRL), and then oligonucleotides in 0.2% FBS-DMEM were added to the cells. After 8–10 h of incubation with the oligomers, the medium was aspirated and replaced with 0.2% FBS-DMEM with or without PDGF in the absence of oligomers for 12–24 h before the experiments were performed. The final concentration of the oligonucleotides was 40 nM.

**RESULTS**

**PDGF stimulates PASMC proliferation.** In rat PASMC cultured in medium containing a low concentration of serum (0.2% FBS), treatment with PDGF (10 ng/ml for 48 h) induced a 2.3-fold increase in cell number, a 1.6-fold increase in [³H]thymidine uptake, and a 2.9-fold increase in BrdU uptake (Fig. 1, A–C). Cell cycle analysis indicated that 46% of the cells were in the S or G₂/M phase after 24 h of treatment with PDGF. These

---

**Fig. 1.** Platelet-derived growth factor (PDGF) stimulates rat pulmonary artery smooth muscle cell (PASMC) proliferation. A and B: cell number and [³H]thymidine uptake in PASMC cultured in 0.2% FBS-DMEM with (PDGF) or without (control) PDGF (10 ng/ml) for 48 h. Values are means ± SE. ***P < 0.001 vs. control. C: flow cytometry analysis of bromodeoxyuridine (BrdU) uptake in PASMC cultured in 0.2% FBS-DMEM with or without PDGF for 24 h. Gates correspond to BrdU-positive cells. Results are representative of 3 independent experiments. 7-AAD, 7-amino-actinomysin. D: [³H]thymidine uptake in PASMC before (basal) and after incubation in 0.2% FBS-DMEM containing PDGF (10 ng/ml) without EGTA (0) or with 0.5, 0.75, 1.0, and 1.5 mM EGTA. Values are means ± SE. **P < 0.01; ***P < 0.001 vs. 0 EGTA.
results suggest that PDGF significantly increases PASMC proliferation.

Chelation of extracellular Ca\(^{2+}\) with 1.5 mM EGTA, which reduces free Ca\(^{2+}\) concentration in the culture media from 1.6 to 0.1 mM (15), markedly inhibited the PDGF-mediated PASMC growth (Fig. 1D). These results suggest that influx of extracellular Ca\(^{2+}\) to the cytosol is required for the PDGF-mediated PASMC proliferation. The next set of experiments was designed to investigate whether PDGF upregulates expression of TRPC6, a TRP channel gene that may participate in forming native Ca\(^{2+}\) channels in vascular smooth muscle cells (8, 27, 28, 45, 66, 67, 70, 74).

**PDGF upregulates mRNA expression of c-Jun and TRPC6.** Among various TRP channel genes, TRPC6 is predominantly expressed in lung tissues (8) and may be an essential subunit that forms native cation channels activated by agonist-receptor interaction and intracellular store depletion in vascular smooth muscle cells (27, 45). TRPC6 is highly expressed in pulmonary artery segments (66), indicating that TRPC6 is a dominant member of the TRP channel family expressed in PASMC.

Treatment of PASMC with PDGF (10 ng/ml) significantly increased mRNA levels of c-Jun (an early responsive gene that associates with PASMC growth) (38, 39) and TRPC6 (Fig. 2). However, the time courses of PDGF-induced mRNA expression of c-Jun and TRPC6 are quite different. The increase in c-Jun mRNA peaked ~1 h after treatment with PDGF, whereas TRPC6 mRNA expression increased 2–4 h after treatment (Fig. 2, B and C). These results indicate that the PDGF-mediated increase in c-Jun expression precedes the increase in TRPC6 mRNA expression.

The tyrosine-phosphorylated STAT3 is a downstream signal transduction protein activated (or phosphorylated) on activation of PDGF receptors (9). Treatment of PASMC with PDGF induced a transient increase in protein levels of phosphorylated STAT3, which reached maximal level ~0.5 h after treatment (Fig. 3, A and Ba). Consistent with the effect on mRNA expression, PDGF also upregulated protein expression of c-Jun and TRPC6 (Fig. 3, A and Bb and Bc). The PDGF-induced increase in c-Jun protein level reached maximal level ~4 h after treatment and was maintained at this level for up to 48 h (Fig. 3Bb). The PDGF-mediated increase in protein expression of TRPC6 started at ~8 h after treatment and gradually reached maximal level after 20 h of treatment (Fig. 3Bc). These results suggest that PDGF-mediated activation (or phosphorylation) of STAT3 may be an important mechanism in upregulating c-Jun and/or TRPC6 expression.

It has been demonstrated that native TRPC6 is highly glycosylated in various cell types (74, 76). In PASMC, two bands were observed using a polyclonal antibody against TRPC6 in the Western blot experiments (Fig. 3A). Deglycosylation of the cell lysates isolated from PASMC shifted the upper band at ~130 kDa close to the lower band at ~110 kDa (Fig. 3B).

These results suggest that the TRPC6 in native rat PASMC is also highly glycosylated and that both bands (at ~130 and ~110 kDa) shown in the Western blot experiments are TRPC6 (74).

Overexpression of c-Jun upregulates mRNA and protein expression of TRPC6. c-Jun is a transcription factor that is itself sufficient to stimulate gene expression (4, 29). c-Jun can also dimerize with STAT3 and concurrently regulate gene expression (75). The mRNA expression of TRPC6 was significantly increased in
PASMC infected with an adenoviral vector carrying sense \( c\text{-}jun \) compared with control cells infected with an empty virus (Fig. 4A). Furthermore, overexpression of \( c\text{-}Jun \) also upregulated the protein expression of TRPC6 (Fig. 4B). These results suggest that homomorphic or heterogenous \( c\text{-}Jun \) dimers (e.g., \( c\text{-}Jun/c\text{-}Fos, c\text{-}Jun/STAT-3 \)) may serve as downstream signal transduction elements in PDGF-mediated TRPC6 upregulation.

**PDGF enhances CCE.** Expression of TRP channel genes in mammalian cells and Xenopus oocytes has been demonstrated to increase CCE activated by intracellular store depletion and to increase \( Ca^{2+} \) influx activated by ligand-receptor interaction, suggesting that the channels responsible for CCE and receptor-operated \( Ca^{2+} \) entry are formed, at least in part, by TRP gene products (7, 11, 12, 23, 27, 35, 40, 45, 70, 76–78). To examine whether PDGF-mediated upregulation of TRPC6 is associated with an increase in CCE, using fluorescence microscopy, we measured and compared the changes in \([Ca^{2+}]_\text{cyt}\) before and after store depletion between control cells and cells treated with PDGF.

In the absence of extracellular \( Ca^{2+} \), application of 10 \( \mu \text{M} \) CPA, which inhibits the \( Ca^{2+}\text{-Mg}^{2+}\text{-ATPase} \) (SERCA) in the sarcoplasmic/endoplasmic reticulum (SR), induced a transient increase in \([Ca^{2+}]_\text{cyt}\) because of leakage of \( Ca^{2+} \) from the SR to the cytosol (Fig. 5A). When the SR was depleted 5–7 min after treatment...
Functional inhibition of CCE attenuates PASMC growth. Whole cell cation current through SOC (I_{SOC}) was elicited in PASMC held at 0 mV (which inactivates voltage-dependent Na\(^+\) and Ca\(^{2+}\) channels) by a series of test potentials ranging from −80 to +20 mV. The inward currents at negative test potentials were mainly generated by Ca\(^{2+}\) influx, because the ratios of Ca\(^{2+}\) to Na\(^+\) permeability (P_{Ca}/P_{Na}) to go through SOC are usually on the order of 10:1 when extracellular Ca\(^{2+}\) concentration is in the millimolar range (48, 76). The outward currents at positive potential were putatively generated by Ca\(^{2+}\) efflux, because the permeability of SOC to Ca\(^{2+}\) is equal to that of other monovalent cations (e.g., Na\(^+\) and K\(^+\)) (31). Extracellular application of 0.5 mM Ni\(^{2+}\) reversibly decreased the Ca\(^{2+}\) currents, potentially through SOC (I_{SOC}; Fig. 6A), and attenuated the increase in [Ca\(^{2+}\)]\(_{cyt}\) due to CCE (data not shown) (42), suggesting that Ni\(^{2+}\) is a potent blocker of native SOC that is responsible for CCE in PASMC (18, 42, 78).

In addition to inhibiting SOC, Ni\(^{2+}\) has been demonstrated to block Ca\(^{2+}\) influx through nonselective cation channels and VDCC (36, 47). To delineate the role of PDGF-induced activation of SOC in PASMC proliferation, we also tested the effect of SK&F-96365 [1-β-(3-(4-methoxyphenyl)propoxy)-4-methoxy-phenethyl]-1H-imidazole hydrochloride), a selective blocker of SOC (14, 17), on whole cell I_{SOC} and cell growth (see below). As shown in Fig. 6B, extracellular application of 50 μM SK&F-96365 significantly reduced whole cell I_{SOC} elicited by test potentials ranging from −100 to +100 mV. The SK&F-96365-mediated inhibitory effect appeared to be greater on inward I_{SOC} at negative potentials (70–75%) than on outward I_{SOC} (~55–60%; Fig. 6B, Bb, and Bc).

Functional blockade of the channels responsible for CCE with 0.5 mM Ni\(^{2+}\) or 50 μM SK&F-96365 significantly attenuated CCE (data not shown) (18, 42) and inhibited PDGF-mediated \(^{3}H\)thymidine incorporation (Fig. 7A) or cell proliferation (Fig. 7B) in PASMC. These results demonstrate that PDGF stimulates PASMC proliferation by upregulating TRPC6 expression. The resultant increase in availability of functional TRPC6-encoded SOC augments CCE, increases intracellular Ca\(^{2+}\) concentration, and stimulates PASMC growth.

Inhibition of endogenous TRPC6 mRNA and protein expression in PASMC using AS oligonucleotides. It has been demonstrated that native SOC is composed of subunits encoded by TRP channel genes (7, 8, 12, 23, 27, 35, 40, 45, 70, 76–78). Therefore, transcription and expression of TRP channel genes should be involved in the regulation of amplitudes of I_{SOC} and CCE by increasing or decreasing the number of channels available for generating Ca\(^{2+}\) currents. The native SOC or TRP channels are believed to be formed by heteromeric tetramers from different TRPC subunits (7, 11). To
investigate whether TRPC6 is involved in forming functional native SOC responsible for CCE, we tested the effect of specific inhibition of TRPC6 gene expression by AS oligonucleotides on \( I_{\text{SOC}} \) and CCE in PASMC.

The optimal transfection condition for AS oligonucleotides was first determined using a fluorescent control oligonucleotide (provided by Sequitur). As shown in Fig. 8A, the oligonucleotide uptake, determined by fluorescence intensity, in PASMC reached a maximal level \( \sim 8-10 \) h after initial transfection and remained

![Fig. 7](image_url) Inhibitory effects of \( \text{Ni}^{2+} \) and SK&F-96365 on PDGF-induced \( [\text{H}] \)thymidine uptake (A) and proliferation (B) in rat PASMC. A: growth-arrested PASMC were incubated in 0.2% FBS-DMEM. \( [\text{H}] \)thymidine incorporation was detected before (basal) and 72 h after treatment of cells with PDGF (10 ng/ml) in the absence (\( \text{Ni}^{2+} \)) or presence (\( \text{Ni}^{2+} \)) of 0.5 mM \( \text{Ni}^{2+} \) in 0.2% FBS-DMEM. Values are means \( \pm \) SE (\( n = 5 \)). *** \( P < 0.001 \) vs. \( \text{Ni}^{2+} \). B: cell numbers before (Cont) and 48 h after (PDGF) treatment with PDGF (10 ng/ml) without (0) or with 5, 10, and 50 \( \mu \text{M} \) SK&F-96365. Values are means \( \pm \) SE. ** \( P < 0.01 \); *** \( P < 0.001 \) vs. 0 SK&F.

**Fig. 6.** Inhibitory effects of \( \text{Ni}^{2+} \) and SK&F-96365 (SK&F) on store-operated \( \text{Ca}^{2+} \) channel current (\( I_{\text{SOC}} \)) induced by CPA-mediated passive store depletion in rat PASMC. Aa: representative currents (\( I \)) elicited by 300-ms voltage steps from \( -80 \) to \( +20 \) mV in 20-mV increments (holding potential 0 mV) before (basal) and after 10-min application of 10 \( \mu \text{M} \) CPA in the absence (control) and presence (\( \text{Ni}^{2+} \)) of 1 mM \( \text{Ni}^{2+} \). Currents recorded after washout of \( \text{Ni}^{2+} \) in the presence of CPA (recovery) are also shown. Ab: summarized data (means \( \pm \) SE) show amplitude of currents at \( -80 \) mV (\( n = 15 \)) before (basal) and after application of CPA in the absence (CPA) and presence (\( \text{Ni}^{2+} + \text{CPA} \)) of \( \text{Ni}^{2+} \). *** \( P < 0.001 \) vs. solid bar. Ba: representative currents elicited by 300-ms voltage steps from \( -100 \) to \( +100 \) mV in 20-mV increments (holding potential 0 mV) before (basal) and after 10-min application of 10 \( \mu \text{M} \) CPA in the absence (CPA) and presence (CPA + SK&F) of 50 \( \mu \text{M} \) SK&F-96365. Bb: summarized data (means \( \pm \) SE) show current-voltage (\( I-V \)) curves of CPA-induced currents before (CPA) and after application of SK&F-96365. \( I-V \) curves are significantly different on the basis of ANOVA. SK&F-96365-mediated inhibition of inward and outward \( I_{\text{SOC}} \) is plotted against different test potentials. Bc: relative differences (%control) of current density measured in SK&F-96365-treated cells vs. that measured in control cells are much greater for inward than for outward \( I_{\text{SOC}} \).
at the maximal level for up to 24 h. Accordingly, the molecular biological, electrophysiological, and fluorescence microscopy experiments were performed in PASMC ~12–24 h after transfection with nonsense (NS) or AS oligonucleotides.

Using RT-PCR and Western blot analyses, we screened four different AS oligonucleotides designed and synthesized by Sequitur. As shown in Fig. 8B, the no. 2 AS oligonucleotide targeting TRPC6 appeared to be much more potent in inhibiting mRNA and protein expression of TRPC6 than other AS oligonucleotides. Furthermore, the no. 2 TRPC6 AS oligonucleotide had a negligible effect on mRNA expression of other TRPC isoforms (Fig. 8C), indicating that the AS oligonucleotide specifically induces cleavage of TRPC6 mRNA. Accordingly, only the no. 2 AS oligonucleotide (S13730, TTGGCCCTTGGCAAACTTCCACTCCA) was used in the following experiments.

**Inhibition of endogenous TRPC6 using the AS oligonucleotide targeting TRPC6 attenuates ISOC in PASMC.** To investigate the inhibitory effects of the TRPC6 AS oligonucleotide on expression of endogenous TRPC6 and activity of endogenous SOC in PASMC, we determined the protein level of TRPC6 and measured the amplitude and current density of whole cell $I_{\text{SOC}}$. In PASMC cultured in media with PDGF, AS treatment not only downregulated basal protein expression of TRPC6 but also attenuated the PDGF-mediated up-regulation of TRPC6 (Fig. 9A). Furthermore, the AS oligonucleotide markedly decreased the amplitude and current density of whole cell $I_{\text{SOC}}$ in PASMC cultured in medium containing PDGF (Fig. 9B). The inhibitory effect of the TRPC6 AS oligonucleotide was much greater on inward $I_{\text{SOC}}$ at negative potentials than on outward $I_{\text{SOC}}$ at positive potentials. These results suggest that TRPC6 participate in forming native SOC that are mainly responsible for inward $I_{\text{SOC}}$ in rat PASMC.

**Inhibition of endogenous TRPC6 using the AS oligonucleotide targeting TRPC6 attenuates CCE in rat PASMC.** It has been demonstrated that overexpression of TRPC6 in heterologous transfection systems, such as COS-7 (74) and HEK-293 (8, 27) cells, did not enhance CCE induced by thapsigargin-mediated passive store depletion but significantly enhanced receptor-mediated Ca$^{2+}$ influx. On the basis of these results, several groups of investigators concluded that homomeric TRPC6 channels are not SOC but, rather, ROC that are activated by ligand-receptor interaction and activation of G protein (8, 23, 27, 28, 74).

The results from this study (Figs. 2–5) show a close association of PDGF-mediated upregulation of TRPC6 and enhancement of CCE in rat PASMC. These observations direct us to speculate that although homomeric TRPC6 may not form store depletion-activated SOC in heterologous transfection systems, TRPC6 may tetramerize heterogeneously with other TRPC channel subunits and form heterogenous SOC that are activated by store depletion in rat PASMC. The next set of experiments was designed to test whether inhibition of endogenous TRPC6 using the TRPC6 AS oligonucleotide decreases the amplitude of CCE in rat PASMC.

Rat PASMC were first transfected with the NS oligonucleotide and the TRPC6 AS oligonucleotide for 12 h and then cultured in regular media for 12–24 h before experimentation. As shown in Fig. 10, treatment of rat PASMC with the TRPC6 AS oligonucleotide for 15–24 h significantly reduced the amplitude of CCE triggered by passive store depletion using 5 μM CPA (Fig. 10A). In PASMC treated with the NS oligonucleotide (which contains the same composition of nucleotides as the AS oligonucleotide), the amplitude of CPA-mediated CCE was ~1.4-fold greater than in cells treated with the TRPC6 AS oligonucleotide (Fig. 10, A and B). CCE amplitude...
In PASMC treated with the AS oligonucleotide was shifted to the left (by $100 \text{ nM}$) compared with cells treated with the NS oligonucleotide (Fig. 10C). Taken together with the effects on $I_{\text{SOC}}$ (Fig. 9), these results suggest that $I_{\text{SOC}}$ and CCE in rat PASMC are, at least partially, generated by Ca$^{2+}$ influx through the SOC that are formed heterogeneously by TRPC6.

Inhibition of endogenous c-Jun using the AS oligonucleotide targeting c-Jun attenuates PDGF-mediated upregulation of TRPC6. To investigate whether overexpression of c-Jun was involved in PDGF-mediated upregulation of TRPC6 in rat PASMC, we examined the effect of the AS oligonucleotide that specifically targets the rat c-Jun gene (Biomol Research Laboratories) in cells treated with PDGF. Inhibition of endogenous c-Jun significantly attenuated the PDGF-mediated upregulation of TRPC6 (Fig. 11). These results, which are in good agreement with the finding that overexpression of c-Jun enhanced TRPC6 expression (Fig. 4), suggest that c-Jun is a transcription factor involved in PDGF-mediated upregulation of TRPC6 in rat PASMC.

Inhibition of endogenous TRPC6 using the AS oligonucleotide targeting TRPC6 attenuates PDGF-mediated PASMC proliferation. Consistent with its inhibitory effects on TRPC6 expression and CCE, the TRPC6 AS oligonucleotide significantly inhibited PDGF-mediated PASMC proliferation. The $[^{3}H]$thymidine uptake
in PASMC transfected with the TRPC6 AS oligonucleotide was reduced by 60% compared with cells transfected with the NS oligonucleotide (Fig. 12). These results provide compelling evidence that endogenous TRPC6 is involved in PDGF-mediated proliferation in rat PASMC.

**DISCUSSION**

Elevated PDGF level in the blood plasma and upregulated PDGF expression in the lung tissues have been implicated in patients and animals with pulmonary arterial hypertension (3, 26, 30, 60). The PDGF-mediated PASMC proliferation as well as PDGF-mediated upregulation of other growth factors (e.g., vascular endothelial growth factor and ET-1) may be involved in pulmonary vascular remodeling, a major contributor to the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with pulmonary hypertension (58, 64). In experiments in vitro shown in this study, PDGF induced PASMC proliferation in media containing low concentration of serum (0.2% FBS); the cell number and [3H]thymidine labeling in media containing low concentration of serum (0.2% FBS) were increased in cells treated with serum (0.2% FBS); the cell number and [3H]thymidine incorporation were increased in cells treated with serum (0.2% FBS). Similar experiments were repeated independently 3 times.

The TRP channel gene-encoded Ca2+-permeable channels have been implicated in forming the channels responsible for CCE and receptor-mediated Ca2+ entry in vascular smooth muscle cells including PASMC (18, 27, 28, 45, 66, 67, 70). CCE appears to be an important pathway for elevating [Ca2+]cyt and returning Ca2+ to the SR, which are required for PASMC proliferation and pulmonary vasoconstriction (18, 42, 45, 70). Treatment of PASMC with PDGF upregulated mRNA and protein expression of TRPC6, a member of the short TRP channel subfamily, which is preferentially expressed in lung tissues and pulmonary arteries (18, 45, 66). The PDGF-mediated upregulation of TRPC6 was associated with an increase in CCE, induced by passive depletion of intracellular Ca2+ stores with CPA. Pharmacological blockade of the SOC channels inhibited the increase in [Ca2+]cym due to CCE and attenuated the PDGF-induced PASMC proliferation. These results suggest that PDGF-induced PASMC proliferation is mediated, at least partially, by upregulating TRPC6 gene expression. The subsequent increase in the number of TRPC6-encoded Ca2+ channels enhances CCE (and mitogen-mediated Ca2+ influx) and raises [Ca2+]cyr and [Ca2+]Sr, which are required for the progression of the cell cycle when PASMC proliferate (6, 42, 49).

The tyrosine-phosphorylated STAT3 is a downstream signal transduction protein activated by PDGF through its receptor (9). PDGF-mediated activation of STAT3 has been demonstrated to upregulate gene expression of c-Jun, an AP-1 transcription activator involved in PASMC growth (38, 39). Moreover, overexpression of c-Jun upregulated TRPC6 expression and enhanced CCE, whereas downregulation of c-Jun inhibited PDGF-mediated upregulation of TRPC6 in rat PASMC. These results suggest that the PDGF-mediated upregulation of TRPC6 is related to activated STAT3 and upregulated c-Jun. STAT3, by forming homodimers as well as heterodimers (e.g., STAT3/STAT1) with other STAT family members, is a DNA binding protein that mediates transcriptional activation of genes containing STAT binding sites. The PDGF-mediated activation of STAT3 may be an important mechanism in upregulating c-Jun expression. STAT3 and c-Jun may bind with the TRPC6 gene promoter independently to upregulate TRPC6 expression. Interaction of c-Jun with STAT3 has been demonstrated to maximize their enhancer function in genes that contain independent but closely spaced DNA binding sites for STAT3 and c-Jun (75). Therefore, STAT3 and c-Jun may form the heteromorphic dimer c-Jun/STAT3 (75), which may in turn regulate TRPC6 gene expression. The precise mechanism involved in the STAT3/c-Jun-mediated transcriptional regulation of TRPC6 remains unclear.

In addition to STATs, activated or dimerized PDGF receptors also provide docking sites for other signaling molecules and proteins with SH2- and phosphotyrosine-binding sites, such as Src, p85, Src homology phosphatase 2, phospholipase C-γ, GTPase-activating protein expression of TRPC6 protein expression in rat PASMC. Western blot analysis of c-Jun and TRPC6 (without deglycosylation treatment) in cells transfected with the NS oligonucleotide and the c-Jun AS oligonucleotide before (Cont) and after (PDGF) treatment with PDGF (10 ng/ml). Similar experiments were repeated independently 3 times.

![Fig. 11. Downregulation of c-Jun inhibits PDGF-mediated upregulation of TRPC6 protein expression in rat PASMC. Western blot analysis of c-Jun and TRPC6 (without deglycosylation treatment) in cells transfected with the NS oligonucleotide and the c-Jun AS oligonucleotide before (Cont) and after (PDGF) treatment with PDGF (10 ng/ml). Similar experiments were repeated independently 3 times.](http://ajpcell.physiology.org/)

![Fig. 12. Inhibitory effect of the TRPC6 AS oligonucleotide on rat PASMC proliferation. [3H]Thymidine incorporation was detected before (basal) and 72 h after treatment of cells with PDGF (10 ng/ml) and NS or AS oligonucleotide. Values are means ± SE (n = 9). ***P < 0.01; ***P < 0.001 vs. basal.](http://ajpcell.physiology.org/)
protein, growth factor receptor-bound protein 2, Src homology 2 domain containing α2-collagen-related protein, and a novel cytoplasmic protein (13). All these proteins can bind to the tyrosine phosphates on the intracellular domain of activated PDGF receptors and activate different downstream signaling pathways. The final outcome, upregulation or downregulation of the targeting genes, depends on the intensity (e.g., number of activated receptors and dose of ligands), duration, and location of the signaling pathways and proteins involved and varies because of different signaling pathways and their cross talk (13). Therefore, there are multiple downstream pathways after activation of PDGF receptor that may participate in the transcriptional and translational regulation of TRPC6 genes in PASMC.

A rise in [Ca$^{2+}$]$_{cyt}$ is a major trigger for smooth muscle contraction and an important stimulus for PASMC proliferation (2, 4–7, 18, 25, 43, 52–54, 62, 67, 68). The mitogen-mediated increase in [Ca$^{2+}$]$_{cyt}$ usually consists of an initial Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores, such as the SR, followed by a sustained Ca$^{2+}$ influx through plasmalemmal Ca$^{2+}$-channels (6–8, 34, 52–54, 61). The store depletion-mediated CCE serves as an important mechanism in maintaining elevated [Ca$^{2+}$]$_{cyt}$ and returning Ca$^{2+}$ to the emptied SR (7, 46, 76). The ratio of cytosolic free to stored Ca$^{2+}$ in the SR is between 1:10,000 and 1:50,000 (7), whereas the nuclear membrane is highly permeable to Ca$^{2+}$ (1). Therefore, a rise in [Ca$^{2+}$]$_{cyt}$ would rapidly increase [Ca$^{2+}$]$_{in}$ and [Ca$^{2+}$]$_{ISR}$, activate cytoplasmic mitogen-activated protein kinase (which is part of the phosphorylation cascade that leads to activation of DNA synthesis-promoting factor), activate nuclear Ca$^{2+}$-sensitive transcriptional factors, and promote cell proliferation (4–7, 17, 21, 22, 43). Removal (or chelation) of extracellular Ca$^{2+}$ or depletion of [Ca$^{2+}$]$_{IS}$ significantly inhibited vascular smooth muscle cell growth in the presence of serum and growth factors (e.g., PDGF) (18, 55, 59). These findings suggest that a constant Ca$^{2+}$ influx, partially maintained by CCE through a TRPC6-encoded SOC, and a sufficient [Ca$^{2+}$]$_{ISR}$, which requires Ca$^{2+}$ for protein and lipid synthesis and sorting, are required for PASMC growth.

Among various TRP channel genes expressed in mammalian and human tissues (11), TRPC6 is a unique member that is abundantly expressed in lung tissues (8, 27, 45) and pulmonary arteries (66) on the basis of Northern blot analysis. The single-channel conductance of TRPC6 has been reported to be 35 pS, and the ion selectivity of the channel is five times greater for Ca$^{2+}$ than for Na$^+$ ($P_{Ca}/P_{Na} = 5$) (65). Overexpression of TRPC6 in mammalian cells enhances agonist-induced Ca$^{2+}$ influx (27, 74), whereas inhibition of TRPC6 using AS oligomers inhibits vascular tone (67). In rat PASMC, our study demonstrates that PDGF, a growth factor that is implicated in the development of pulmonary hypertension (3, 26, 58, 60, 64), mediates cell proliferation partially by upregulating TRPC6 expression and increasing the store depletion-activated CCE. The resultant increases in [Ca$^{2+}$]$_{cyt}$, [Ca$^{2+}$]$_{in}$, and [Ca$^{2+}$]$_{SR}$ would promote cell proliferation by moving quiescent cells into the cell cycle and by propelling proliferating cells through mitosis (Fig. 13). The PDGF-induced transient increase in [Ca$^{2+}$]$_{cyt}$ by Ca$^{2+}$ release from intracellular stores and by Ca$^{2+}$ influx through VDCC and ROC, well documented by other investigators (2, 25, 68), may also contribute to the upregulation of c-Jun, a Ca$^{2+}$-dependent AP-1 transcription factor. The upregulation of TRPC6 and increase of CCE through SOC induced by chronic exposure to PDGF further enhance the PDGF-mediated increase in [Ca$^{2+}$]$_{cyt}$ and PASMC proliferation.

Fig. 13. Schematic diagram depicting proposed mechanisms involved in PDGF-mediated upregulation of TRPC6 and the subsequent effect on PASMC proliferation. On activation of the PDGF receptor and the receptor tyrosine kinase by PDGF ligand, STAT3 protein becomes tyrosine phosphorylated or activated. Two tyrosine-phosphorylated STAT proteins form dimers that translocate to the nucleus, bind to the STAT-binding sites in the promoter of the c-Jun gene, and promote gene transcription. Upregulated c-Jun not only directly binds to its responsive genes (e.g., TRPC6) but also interacts with phosphorylated STAT3 to synergize the transcriptional effect of c-Jun and STAT3. Owing to the existence of activating protein (AP)-1 and STAT-binding sequences in TRPC6 gene, c-Jun and activated STAT3 may directly upregulate TRPC6 transcription, increase TRPC6 protein expression, and increase the number of TRPC6-encoded Ca$^{2+}$ channels (TRPC). When the SR is depleted, CCE, potentially through TRPC6-encoded channels, is activated to maintain the sustained increases in [Ca$^{2+}$]$_{cyt}$ and nuclear [Ca$^{2+}$] ([Ca$^{2+}$]$_{n}$) and to return Ca$^{2+}$ to the SR via the Ca$^{2+}$-Mg$^{2+}$-ATPase in the SR (SERCA). PDGF-mediated upregulation of TRPC6 channel expression contributes to the regulation of [Ca$^{2+}$]$_{cyt}$ by enhancing CCE. Resultant increases in cytosolic, nuclear, and intracellularly stored [Ca$^{2+}$] may promote cell growth by moving quiescent cells into the cell cycle and propelling the proliferating cells through mitosis and by enhancing protein and lipid processing in the SR. Intracellular Ca$^{2+}$ serves as a shared signal transduction element that leads to pulmonary vasoconstriction and vascular medial hypertrophy, two major contributors to elevated pulmonary vascular resistance (PVR) and arterial pressure (PAP) in patients with pulmonary hypertension. $\oplus$, Ca$^{2+}$-calmodulin-sensitive transitions in the cell cycle.
Several studies have determined the properties of homomeric channels formed by recombinant TRPC6 expressed in heterologous systems (e.g., COS-7 and HEK-293 cells). Overexpression of TRPC6 enhanced the agonist-mediated increase in \([Ca^{2+}]_{cyt}\), but failed to enhance the thapsigargin-mediated increase in \([Ca^{2+}]_{cyt}\) (8, 23–25, 27, 74), suggesting that TRPC6 is a nonselective cation channel that is directly activated by receptor-coupled G protein and diacylglycerol, but not by intracellular \(Ca^{2+}\) store depletion.

In the present study, PDGF-mediated PASMC proliferation was associated with significant increases in the mRNA and protein expression of TRPC6 and in the amplitude of CCE activated by passive depletion of intracellular \(Ca^{2+}\) stores using CPA. It has not been reported that CPA activates membrane receptors and signal transduction proteins (e.g., diacylglycerol and G proteins). Therefore, the enhanced CPA-induced \(Ca^{2+}\) influx in PASMC treated with PDGF was mainly due to CCE. Furthermore, inhibition of TRPC6 expression using the specific AS oligonucleotide that enhances TRPC6 mRNA degradation decreased amplitudes of \(I_{SOC}\) and CCE activated by CPA-induced store depletion. In this study, we have demonstrated that TRPC6 is involved in the mechanisms underlying PDGF-mediated PASMC proliferation by enhancing CCE. This conclusion is mainly based on 1) the correlation of PDGF-mediated upregulation of TRPC6 with the enhancement of store depletion-activated \(I_{SOC}\) and CCE and 2) the ability of TRPC6 AS oligomer to inhibit TRPC6 expression, reduce store depletion-activated \(I_{SOC}\) and CCE, and attenuate PDGF-mediated cell proliferation.

In HEK-293 cells stably transfected with TRPC6, transient transfection of a dominant-negative TRPC6 mutant almost abolished TRPC6-dependent currents activated by G protein activation. The dominant-negative TRPC6 mutant also efficiently inhibited TRPC3-dependent currents in HEK-293 cells stably transfected with TRPC3 (24). These results suggest that TRPC6 is able to form heterotetrameric channels with TRPC3 (and TRPC7) and that the expression level of TRPC6 affects expression of TRPC3 and TRPC7 (24).

TRPC1 can also form heterogeneous TRP channels with TRPC3 that are subject to regulation by phospholipase C and \(Ca^{2+}\) (37). These results suggest that PDGF-mediated upregulation of TRPC6 may increase the expression and promote the formation of oligomeric TRP channels that are activated by store depletion. In addition to upregulating TRPC6 expression, PDGF may also increase the TRPC6 channel function (28).

Differential distribution of various TRP channel genes in different types of cells indicates heteromorphism of native SOC in various cell types (11). In rat PASMC, it is possible that TRPC6 is involved in store depletion-mediated CCE by forming heterotetrameric SOC with other TRPC channels (e.g., TRPC1, TRPC3, and TRPC7), which are demonstrated to be store-operated TRPC channels (32, 33, 41, 63, 69, 76). Using RT-PCR, we found that TRPC1, TRPC3, and TRPC7 were expressed in rat PASMC; the mRNA levels of TRPC3 and TRPC7 were much less than the mRNA level of TRPC6 (data not shown). Whether TRPC3/6 or TRPC3/6/7 and TRPC1/3/6 or TRPC1/6/7 heteromeric channels are store operated and functionally involved in the regulation of cytoplasmic and intracellularly stored \(Ca^{2+}\) in PASMC requires further study. Whether TRPC6 forms SOC may also be cell specific. In HEK-293, COS, and rat aortic smooth muscle cells, homotetrameric TRPC6 may not be store operative, whereas in native PASMC, TRPC6 may form heterotetrameric channels that are activated by store depletion and receptor activation.

In summary, the results from the present study indicate that upregulation of TRPC6 is involved in PDGF-mediated PASMC proliferation. The mitogenic effect of PDGF has been demonstrated to associate with pulmonary vascular remodeling in patients with pulmonary hypertension. Developing pharmacological interventions specifically aiming at downregulating gene expression of TRPC6 or at inhibiting function of TRPC6-encoded \(Ca^{2+}\) channels may greatly help in development of new therapeutic approaches for patients with pulmonary vascular diseases.

We thank B. R. Lapp and Y. Zhao for technical assistance. This work was supported in part by National Heart, Lung, and Blood Institute Grants HL-66012, HL-54043, and HL-64945 (to J. X.-J. Yuan).

REFERENCES

C329

PDGF UPRREGULATES TRPC6 IN SMOOTH MUSCLE CELLS


55. Shimoda LA, Sham JS, Shimoda TH, and Sylvester JT.


