Overexpression of TRPC1 enhances pulmonary vasoconstriction induced by capacitative Ca\textsuperscript{2+} entry

Naomi Kunichika, Ying Yu, Carmelle V. Remillard, Oleksandr Platoshyn, Shen Zhang, and Jason X.-J. Yuan
Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92093

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Kunichika, Naomi, Ying Yu, Carmelle V. Remillard, Oleksandr Platoshyn, Shen Zhang, and Jason X.-J. Yuan. Overexpression of TRPC1 enhances pulmonary vasoconstriction induced by capacitative Ca\textsuperscript{2+} entry. *Am J Physiol Lung Cell Mol Physiol* 287: L962–L969, 2004. First published June 25, 2004; doi:10.1152/ajplung.00452.2003.—Transient receptor potential (TRP) cation channels are a critical pathway for Ca\textsuperscript{2+} entry during pulmonary artery (PA) smooth muscle contraction. However, whether canonical TRP (TRPC) subunits and which TRP channel isoforms are involved in store depletion-induced pulmonary vasoconstriction in vivo remain unclear. This study was designed to test whether overexpression of the human TRPC1 gene (hTRPC1) in rat PA enhances pulmonary vasoconstriction due to store depletion-mediated Ca\textsuperscript{2+} influx. The hTRPC1 was infected into rat PA rings with an adenoviral vector. RT-PCR and Western blot analyses confirmed the mRNA and protein expression of hTRPC1 in the arterial rings. The amplitude of active tension induced by 40 mM K\textsuperscript{+} in PA rings infected with an empty adenoviral vector (647 ± 88 mg/mg) was similar to that in PA rings infected with hTRPC1 (703 ± 123 mg/mg, *P* = 0.3). However, the active tension due to capacitative Ca\textsuperscript{2+} entry (CCE) induced by cyclopiazonic acid was significantly enhanced in PA rings overexpressing hTRPC1 (91 ± 13% of 40K-induced contraction) compared with rings infected with an empty adenoviral vector (61 ± 14%, *P* < 0.001). Endothelial expression of hTRPC1 was not involved since the CCE-induced vasoconstriction was also enhanced in endothelium-denuded PA rings infected with the adenoviral vector carrying hTRPC1. These observations demonstrate that hTRPC1 is an important Ca\textsuperscript{2+}-permeable channel that mediates pulmonary vasoconstriction when PA smooth muscle cell intracellular Ca\textsuperscript{2+} stores are depleted.

PULMONARY VASOCONSTRUCTION is one of the important causes for the elevated pulmonary vascular resistance (PVR) and increased pulmonary arterial pressure (PAP) in patients with primary and secondary pulmonary arterial (PA) hypertension (18, 36, 52). An increase in cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in PA smooth muscle cells (PASMC), due to Ca\textsuperscript{2+} influx through plasmalemmal Ca\textsuperscript{2+} channels and/or Ca\textsuperscript{2+} release from intracellular stores (e.g., the sarcoplasmic reticulum (SR)), is a major trigger for pulmonary vasoconstriction (41) and may play an important role in the development of PA hypertension (18, 36, 52, 55). Sustained pulmonary vasoconstriction is also involved in initiating pulmonary vascular remodeling by stimulating PASMC proliferation and hypertrophy (24).

In vascular smooth muscle cells stimulated by circulating agonists and mitogens via an endocrine mechanism and by intercellular agonists and mitogens via an autocrine or paracrine mechanism, activation of G protein-coupled receptors (e.g., α-adrenergic receptors and endothelin receptors) (39) and/or tyrosine kinase receptors (12) in the plasma membrane stimulates synthesis and production of the second messengers, inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG) (3, 9, 10). IP\textsubscript{3} induces Ca\textsuperscript{2+} release from the SR, causing a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} (4, 40). Depletion of Ca\textsuperscript{2+} from the SR triggers the opening of store-operated Ca\textsuperscript{2+} channels (SOC) in the plasma membrane and causes so-called capacitative Ca\textsuperscript{2+} entry (CCE), a unique Ca\textsuperscript{2+} influx mechanism that not only contributes to maintaining the elevated [Ca\textsuperscript{2+}]\textsubscript{i} but also is required for refilling of Ca\textsuperscript{2+} stores (2, 33, 38, 40). In addition to CCE, IP\textsubscript{3} and DAG are also involved in activating second messenger-operated Ca\textsuperscript{2+} channels (SMOC) and/or receptor-operated Ca\textsuperscript{2+} channels (ROC) in the plasma membrane via direct or indirect mechanisms (3, 25, 30), also causing Ca\textsuperscript{2+} influx and increasing [Ca\textsuperscript{2+}]\textsubscript{i}. Therefore, the activation of SOC and ROC, as well as SMOC, plays a critical role in agonist-mediated increase in [Ca\textsuperscript{2+}]\textsubscript{i} and pulmonary vasoconstriction.

The molecular composition of SOC of ROC (or SMOC) in vascular smooth muscle cells remains unclear; however, the transient receptor potential (TRP) gene products have been demonstrated to form Ca\textsuperscript{2+}-permeable cation channels that are activated by store depletion (1, 30, 35, 45, 47, 48, 56, 57) or by receptor activation (5, 27, 45, 47). TRPC1 is an isoform of the canonical TRP subfamily (TRPC) that can form not only homomeric tetramers but also heteromeric tetramers with many other isoforms of TRPC channels (e.g., TRPC4 and TRPC5) (26). In animal and human PASMC, we previously identified transcripts of TRPC1, TRPC4, TRPC5, and TRPC6 using RT-PCR analysis (32, 42, 54), suggesting that these TRPCs may contribute to form homo- and heterotetrameric channels that are regulated by agonist-mediated receptor activation and/or store depletion in human PASMC. This study was designed to test the hypothesis that TRPC1 in PASMC is an important canonical TRP isoform involved in forming functional native SOC that contribute to the regulation of pulmonary vascular contractility.

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MATERIALS AND METHODS

Isolation of PA rings and tension measurement. Animal use was approved by the University of California, San Diego Animal Care and Use Committee. The right and left branches (2nd division) of the main PA as well as the intrapulmonary arteries (3rd–4th division) were isolated from male Sprague-Dawley rats (100–250 g). The adipose and connective tissues were carefully removed, and the remaining muscular arteries were cut into 2-mm-long rings. In some of the experiments, the endothelium of the PA rings was removed by gently rubbing the inner lumen of the vessels with a rough wooden stick. Functional removal of the endothelium was confirmed by the loss of relaxant response to acetylcholine (ACh, 10 μM). Also, this procedure appeared not to damage the vessels because it did not significantly affect high K⁺-mediated contractions.

Two stainless steel hooks (0.1 mm in diameter) were inserted through the lumen of the PA rings. One hook was mounted in a perfusion chamber, and the other hook was connected to an isometric transducer (Harvard Apparatus). Isometric tension was continuously monitored and recorded using DATALOG data acquisition software (DATASOFT Instruments). Resting passive tension, i.e., that offering maximal tension in rings exposed to 40 mM K⁺ (40K), was 600–625 mg. The rings were equilibrated for 1 h at resting tension and then challenged three times with 40K perfusate to obtain a stable contractile response.

Isolated PA rings were superfused with modified Krebs solution (MKS, at 37°C) consisting of (in mM) 138 NaCl, 1.8 CaCl₂, 4.7 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5 HEPES, and 10 glucose (pH 7.4). For Ca²⁺-free (0Ca MKS), CaCl₂ was replaced by equimolar MgCl₂, and 1 mM EGTA was added to chelate residual Ca²⁺ and then challenged three times with 40K perfusate to obtain a stable 600–625 mg. The rings were equilibrated for 1 h at resting tension and then challenged three times with 40K perfusate to obtain a stable contractile response.

Preparation of recombinant adenosinovirus. We prepared a recombinant adenosinovirus containing the human TRPC1 gene (Adv-hTRPC1). The cDNA of hTRPC1 (U31110) contained in the pcDNA3 vector was kindly provided by Dr. M. Zhu (Ohio State University, Columbus, OH). The coding sequence of the hTRPC1 gene was subcloned into multiple cloning sites of the adenoviral recombination cassette vector (pACCMVP/Lpa plasmid). The expression cassette, TRPC1- pACCMVP/Lpa, and the plasmid pJM17 were cotransfected into isolated PA rings by incubating the endothelium-vessel preparations with adenovirus at a multiplicity of infection of 100. The titer of the virus was determined by plaque forming units/ml at 37°C in serum-free Opti-MEM I medium (Invitrogen) for 5–7 h. Medium containing the adenovirus construct was then washed out to remove excess/untransfected adenosinovirus. Contractile experiments were conducted 24–48 h after the initial infection. Another set of PA rings, isolated from the same branches as those that were infected with Adv-hTRPC1, was incubated with Adv-empty under the same conditions as controls. Transfection of human TRPC1 in isolated PA rings using the Adv-hTRPC1 vector was determined by a fluorescent objective lens coupled to a digital camera in a Nikon microscope.

Histological preparation. After contraction experiments, the PA rings were fixed overnight in 10% neutral buffered formalin. The formalin-fixed rings were serially cut for microscopic examination. The vessel tissues were routinely processed and embedded in paraffin blocks in an automatic tissue processor (Sakura Tissue-Tek VIP; Sakura Finetek). The paraffin-embedded tissues were cut in 5-μm-thick sections for standard hematoxylin-eosin staining. Selected sections were also stained with trichrome and elastin.

Western blot analysis. Cells (rat PASMC and HEK-293 cells) or tissues (isolated PA rings) were gently washed twice in cold PBS and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1 mM EDTA, 0.4% deoxycholate, 1% Nonidet P-40, 0.2% SDS, and 50 mM Tris, pH 8.0) with protease inhibitors on ice. The cell lysates were then sonicated and centrifuged at 12,000 rpm for 10 min, and the insoluble fraction was discarded. The protein concentration in the supernatant was determined by the bicinchoninic acid protein assay using BSA as a standard. Proteins (10–25 μg) 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 (Bio-Rad Laboratories). After incubation overnight at 4°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% milk powder, the membranes were incubated with a polyclonal rabbit anti-TRPC1 antibody (1:200, Alomone Labs) and a monoclonal mouse α-actin antibody (1:1,000, Sigma Chemical). The membranes were then washed and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories) for 90 min at room temperature. The bound antibody was detected using an enhanced chemiluminescence detection system (Amersham Biotech).

RT-PCR. Total RNA was extracted from isolated rat PA rings with or without endothelium as described previously (54). RT was performed using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR was performed by a Gene amp PCR system using AmpliTaq DNA polymerase; cDNA samples were amplified in a DNA thermal cycler (Perkin-Elmer). The PCR products were separated by electrophoresis. To quantify the amounts of mRNA of human TRPC1, a branched DNA probe of smooth muscle cell β-actin was used as an internal control.

Measurement of [Ca²⁺]cyt. Rat PASMC were loaded with the membrane-permeable acetoxyethyl ester form of fura-2 (fura-2 AM, 10%), penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were passaged by trypsinization and used for experiments at passages 3–6. HEK-293 and rat PASMC were grown in high-glucose DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C in a humidified atmosphere. At 70–80% confluence, cells were infected with the purified Adv-hTRPC1 viral plaques. Infected cells were then harvested, subjected to three freeze-thaw cycles, and then purified by CsCl gradient centrifugation. The titer of the virus was determined by a limiting dilution plaque assay. The transfection efficiency was consistently >60% in rat PASMC cells using the Adv-hTRPC1 vector.

Infection of adenosinovirus in isolated PA rings. hTRPC1 was transiently transfected into isolated PA by incubating the endothelium-intact or denuded PA rings with the Adv-hTRPC1 vector (1×10⁸ plaque-forming units/ml) at 37°C in serum-free Opti-MEM I medium (Invitrogen) for 5–7 h. Medium containing the adenovirus construct was then washed out to remove excess/untransfected adenosinovirus. Contractile experiments were conducted 24–48 h after the initial infection. Another set of PA rings, isolated from the same branches as those that were infected with Adv-hTRPC1, was incubated with Adv-empty under the same conditions as controls. Transfection of human TRPC1 in isolated PA rings using the Adv-hTRPC1 vector was determined by a fluorescent objective lens coupled to a digital camera in a Nikon microscope.
3 \mu M) for 30–40 min at room temperature (22–24°C) under an atmosphere of 5% CO2 in air. The fura-2 AM-loaded cells were then superfused with standard bath solution for 20 min at 34°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura-2 AM into active fura-2. The standard bath solution contained (in mM): 141 NaCl, 4.7 KCl, 1.8 CaCl2, 1.2 MgCl2, 10 HEPES, and 10 glucose (pH 7.4 with 5 mM NaOH). Fura-2 fluorescence (510 nm emission, 340 and 380 nm excitation) from the cells and background fluorescence were collected at 32°C. 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. [Ca2+]c was calculated from fura-2 fluorescence emission excited at 340 and 380 nm (F340/F380) using the ratio method based on the following equation: 

\[
[Ca^{2+}]_{c} = \frac{K_d \times (S_f/S_b) \times (R - R_{min})}{R_{max} - R}
\]

where \(K_d\) (225 nM) is the dissociation constant for Ca2+, \(S_f\) 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 \(R_{min}\) and \(R_{max}\) are minimal and maximal ratios, respectively (21). To quantitate changes in [Ca2+]c, multiple cells were imaged in a single field, and one arbitrarily chosen peripheral cytosolic area from each cell was spatially averaged.

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

**RESULTS**

**Transfection efficacy of hTRPC1 gene into rat PA rings.**

Incubation of HEK-293 cells (Fig. 1Aa) or rat PASMC (Fig. 1Ab) with the Adv-hTRPC1 for 18 h significantly increased the protein level of TRPC1 based on Western blot analysis. Incubation of isolated rat PA rings in media with the Adv-hTRPC1 vector markedly increased the mRNA level of human TRPC1 (Fig. 1B). In addition to the mRNA and protein levels of TRPC1, we also examined the distribution of transfected GFP. As shown in Fig. 1C, the GFP fluorescence appeared to be distributed in the whole vascular wall of the PA ring infected with the Adv-GFP vector. Infection with the adenoviral vector seemed to have no effect on the histological structure of the isolated PA rings (Fig. 1D). These results indicate that Adv-hTRPC1 was effective in transfecting the hTRPC1 gene into rat PASMC and isolated PA rings, and the transfected endogenous TRPC1 seemed to be distributed across the whole vascular wall (including fibroblasts, smooth muscle cells, and endothelial cells).

**Short-term culture of the isolated PA ring does not affect its contractility.**

Whether culture of the isolated PA rings in the media used for transfecting hTRPC1 affects contractility or active tension development was examined by comparing the high K+-mediated contraction in freshly isolated PA rings and cultured PA rings. As shown in Fig. 2, 40K-induced active tension in freshly isolated PA rings was comparable to that in PA rings cultured in DMEM for 48 h (Fig. 2A and B), whereas the contraction induced by 50 \mu M CPA-mediated CCE (see below) was slightly increased in cultured PA rings (Fig. 2A and C), as previously shown by other investigators (13). These results show that PA contraction induced by [Ca2+]cyt increases due to the membrane depolarization (40K)-mediated opening of voltage-dependent Ca2+ channels is not altered by short-term culture in DMEM. Furthermore, short-term culture...

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**Fig. 1. Transfection of the human canonical transient receptor potential 1 gene (hTRPC1) to rat pulmonary artery smooth muscle cell (PASMC) and pulmonary artery (PA) rings.**

A: Western blot analysis of hTRPC1 protein levels in wild-type HEK-293 (WT) and HEK-293 (a) infected with the adenovirus containing hTRPC1 (Adv-hTRPC1) vector (plaque number 1–4 and 15), as well as in rat PASMC (b) 2 days after infection with an empty adenoviral vector (Adv-empty) and the Adv-hTRPC1 vector. In rat PASMC, the dense upper band may represent endogenous rat TRPC1 rather than hTRPC1, pfu, Plaque-forming units. B: analyses of hTRPC1 mRNA levels in PA rings infected with Adv-empty (−hTRPC1) and the Adv-hTRPC1 vector (+hTRPC1, for 48 h). GAPDH was used as a positive control. M, 100-bp DNA ladder. C: phase-contrast (top) and fluorescence (bottom) images of isolated PA rings infected with Adv-empty and an adenoviral vector carrying a green fluorescence protein gene (Adv-GFP). D: histological examination of the infected PA ring showing the 3-layer structure (adventitia, media, and intima) of the PA [hematoxylin and eosin (H&E) stain, original magnification ×200].
In cultured PASMC, CPA, a reversible SR Ca\(^{2+}\) entry (CCE)-induced active tension, indicated by percentage of the maximal 40K-induced contraction in the same rings, in freshly isolated (gray bar, n = 16) and cultured (solid bar, n = 10) PA rings. No statistical significant difference between gray and solid bars.

Five to ten minutes after SR Ca\(^{2+}\) depletion by CPA, restoration of extracellular Ca\(^{2+}\) leakage (Release) or CCE.

**Induction of CCE-mediated contraction in isolated PA rings.** In cultured PASMC, CPA, a reversible SR Ca\(^{2+}\)-Mg\(^{2+}\) ATPase inhibitor, elicits CCE by depleting SR Ca\(^{2+}\) stores (22, 35). Application of 10 \(\mu\)M CPA in the absence of extracellular Ca\(^{2+}\) induced a transient \([Ca^{2+}]_{cyt}\) increase in rat PASMC due to passive Ca\(^{2+}\) leakage from the SR to the cytosol (Fig. 3Aa). Five to ten minutes after SR Ca\(^{2+}\) depletion by CPA, restoration of extracellular Ca\(^{2+}\) induced a rise in \([Ca^{2+}]_{cyt}\), presumably due to CCE through store depletion-activated SOC (Fig. 3Ab and b). Similar to its effect on \([Ca^{2+}]_{cyt}\) in single PASMC, superfusion of 50 \(\mu\)M CPA in the absence of extracellular Ca\(^{2+}\) induced a small transient contraction in isolated PA rings (Fig. 3Ba), whereas restoration of extracellular Ca\(^{2+}\) in CPA perfusate 5–10 min later caused a large contraction, due apparently to CCE (Fig. 3Bb and b). These results indicate that CPA-induced store depletion is able to trigger a sustained contraction due to Ca\(^{2+}\) influx through SOC in PA.

**Adenoviral overexpression of hTRPC1 enhances CCE-mediated PA contraction.** As mentioned above, 40K-mediated active tension in isolated rat PA rings was not affected by a short-term culture (Fig. 3, Aa and Ab). In PA rings infected with Adv-hTRPC1, the amplitude (703 ± 123 mg/mg) of 40K-induced active tension was similar to the amplitude (648 ± 88 mg/mg, P = 0.3) in PA rings infected with Adv-empty (Fig. 4, A and Ba). Furthermore, the 40K-induced active tension in Adv-empty- and Adv-hTRPC1-transfected PA rings was also similar to that in freshly isolated (634 ± 119 mg/mg) and cultured (622 ± 100 mg/mg) PA rings (cf. Fig. 4Ba with Fig. 4Bb).
TRPC1 enhances pulmonary vascular contractility.

Overexpression of hTRPC1 enhances CCE-mediated contraction in endothelium-denuded PA rings. All tension experiments to this point were conducted in endothelium-intact PA rings. Because the transfected hTRPC1 was distributed not only in PASMC but also in other cell types (e.g., endothelial cells) in the PA rings, the next set of experiments was designed to test 1) whether removal of endothelium affected the augmenting effect of hTRPC1 overexpression on CCE-induced PA contraction, and 2) whether overexpression of hTRPC1 affected endothelium-dependent PA relaxation. First, we conducted the same experiments (as shown in Fig. 4) in endothelium-denuded PA rings transfected with the Adv-empty or Adv-hTRPC1 vector. As shown in Fig. 5A, extracellular application of 10 μM ACh caused a ~50% reduction of active tension in endothelium-intact (+Endo) PA rings precontracted with 40K, whereas removal of the endothelium (-Endo) abolished the ACh-mediated PA vasodilation. Similar to the endothelium-intact PA rings, overexpression of hTRPC1 in the endothelium-denuded PA rings also significantly enhanced CCE-induced PA contraction but negligibly affected the Ca²⁺ leakage-induced transient contraction (Fig. 5, B and C). Therefore, overexpression of hTRPC1 appears to selectively enhance the amplitude of CCE-induced PA contraction. Because CCE is closely related to SOC activity and TRPC1 expression, these results further suggest that TRPC1 is an important subunit of functional native store depletion-activated SOC in PASMC.

Secondly, we compared ACh-induced PA relaxation in endothelium-intact PA rings infected with the Adv-empty and Adv-hTRPC1 vectors to determine whether overexpression of TRPC1 enhances endothelium-dependent PA relaxation. As shown in Fig. 6, the ACh-mediated relaxation appeared to be significantly diminished in cultured PA rings (regardless of Adv-empty or Adv-hTRPC1 transfection); 10 μM ACh induced a 50% reduction of active tension in freshly isolated PA PASMC responsible for CCE-induced pulmonary vasoconstriction.

2B). These results indicate that adenoviral infection per se did not significantly affect membrane depolarization-mediated PA contraction.

By blocking the Ca²⁺-Mg²⁺ ATPase in the SR, CPA induced a transient increase in [Ca²⁺]SR in single or dissociated PASMC superfused with Ca²⁺-free solution (Fig. 3Aa), which was mainly due to Ca²⁺ leakage from the SR to the cytosol. Five to ten minutes after treatment with CPA in the absence of extracellular Ca²⁺, leakage depletes SR Ca²⁺ and activates store-operated cation channels. Therefore, restoration of extracellular Ca²⁺ then causes a sustained increase in [Ca²⁺]SR due to CCE. In the absence of extracellular Ca²⁺, exposure of isolated PA rings to CPA thus caused a transient contraction (which was likely due to the CPA-induced Ca²⁺ leakage). The Ca²⁺ leakage-mediated small transient contraction was negligibly changed in PA rings infected with the Adv-hTRPC1 vector compared with rings infected with the Adv-empty vector (Fig. 4, A and Bb). However, the amplitude of CCE-induced PA contraction was significantly increased in PA rings infected with the Adv-hTRPC1 vector compared with the rings infected with Adv-empty (Fig. 4, A and Bc). These results provide compelling evidence that TRPC1 is a canonical TRP isofrom that is involved in forming functional cation channels in

![Fig. 5. Enhanced active tension following hTRPC1 overexpression is independent of endothelium. A: representative records of active tension in response to 40K and 10 μM ACh in endothelium-intact (+Endo) and -denuded (-Endo) rings. ACh was applied to the rings when 40K-mediated contraction reached a plateau. B: representative records of active tension induced by 40K and 50 μM CPA in endothelium-denuded PA ring 48 h after infection with Adv-empty (left) or the Adv-hTRPC1 (right) vector. C: summarized data showing the normalized active tension induced by CPA-mediated Ca²⁺ release (a) and CCE (b) in endothelium-denuded PA rings infected with the Adv-empty vector (gray bars, n = 10) and Adv-hTRPC1 vector (solid bars, n = 10). **p < 0.01 vs. gray bar.](http://ajplung.physiology.org/)

![Fig. 6. Overexpression of hTRPC1 does not affect endothelium-dependent pulmonary vasodilation. A: representative records of active tension in response to 40K and 10 μM ACh in cultured endothelium-intact PA rings infected with Adv-empty (left) and Adv-hTRPC1 (right). ACh was applied to the rings when 40K-mediated contraction reached a plateau. B: summarized data showing the ACh-induced decrease in active tension in PA rings infected with the Adv-empty vector (gray bar, n = 6) and the Adv-hTRPC1 vector (solid bar, n = 6). No statistical significant difference between the gray and solid bars.](http://ajplung.physiology.org/)
and 4.31/H11006
[Ca2+ by vasoconstrictors, is a critical pathway for elevating vasoconstriction, whereas a decrease in [Ca2+]cyt mediates relaxation results different cell types and conducted experiments involving overexpression of specific TRP proteins yielding conflicting results (9, 10, 48). Part of this variability relies on the fact that native TRP channels exist as homo- or heterotramers in cells. For example, for TRPC-based channels, a channel made up of one TRPC1, one TRPC4, and two TRPC5 may form an SOC, whereas a channel made of one TRPC3 and three TRPC6 may form an ROC. This suggests that the expression ratio of different TRP isoforms may likely vary among different cell types and the composition of functional TRP heterotrameric channels determines whether the channel can be opened by receptor activation, store depletion, or both (25, 26, 48, 49).

Among the various TRPC isoforms, TRPC1 is a channel that can form not only homotrameric channels but also heterotrameric channels with other TRPC channel subunits (e.g., TRPC4 and TRPC5) (25, 26, 33, 35, 53). Overexpression of TRPC1 would lead to upregulation of both homomeric TRPC1 channels and heterotrameric TRPC channels containing TRPC1 and thus enhance agonist-mediated Ca2+ influx through both ROC and SOC. Our results from this study demonstrate that, in isolated rat PA rings overexpressing hTRPC1, the CCE-induced contraction is much greater than in control (empty vector) PA rings. This suggests that TRPC1 is involved in forming functional SOC in the rat PA rings.

Both pulmonary vascular smooth muscle (21, 32, 35, 42, 54) and endothelial (6, 16, 34) cells express functional TRPC channels. Infection of isolated endothelium-intact PA rings with the Adv-hTRPC1 would increase hTRPC levels in both PA smooth muscle and endothelial cells. To demonstrate that the enhanced CCE-induced PA contraction was due to overexpression of hTRPC1 in PASMC, we removed the endothelium-intact PA rings before infecting them with hTRPC1. Overexpression of hTRPC1 in endothelium-denuded PA rings caused a significant increase in CCE-induced active tension, quantitatively comparable to that observed in endothelium-intact PA rings. These results further suggest that TRPC1 is involved in forming functional SOC in PASMC. Our observations with TRPC1 in PA are similar to that previously demonstrated for TRPC6 in cerebral arterioles (51). Observations in the latter study and our current study, coupled with knowledge that native TRPC channels are often heteromeric tetramers, suggest that TRPC isoforms are important components of heteromeric SOC channels in vascular smooth muscle cells.

To overexpress hTRPC1 in intact rat PA, we cultured the vessels in media containing Adv-empty or Adv-hTRPC1 for a short period of time to ensure the expression of the exogenous TRPC1. It has been demonstrated that the vasoconstrictive response to agonists (e.g., histamine, norepinephrine, serotonin, and high K+) and the vasodilative response to nitroprusside (a nitric oxide donor) and pinacidil (K+ channel opener) were all well preserved in the cultured rabbit mesenteric arterial rings compared with the freshly isolated mesenteric arterial rings (29). However, the endothelium-dependent vasodilative response to ACh was markedly inhibited in arterial rings cultured in fetal calf serum and DMEM (29). Consistent with the report on rabbit mesenteric arteries, our data also indicate that the ACh-mediated relaxation appeared to be significantly diminished in cultured rings (cf. Fig. 6 with Fig. 5A, left), although 40K-induced contraction was not different between freshly isolated and cultured PA rings (see Fig. 2, A and B). It remains unclear why the different tissues (i.e., PA smooth muscle and endothelium) were affected differently by

DISCUSSION

PVR is largely determined by the intralumen diameter of the pulmonary vascular bed. According to Poiseuille’s law, the inverse relationship between resistance (R) and the fourth power of radius (r) \( R \propto \frac{1}{r^4} \) predicts that a 50% decrease in the intralumen radius of a blood vessel leads to a 16-fold increase in vascular resistance to blood flow. Therefore, pulmonary vasoconstriction is an important contributor to the elevated PVR and PAP in patients with PA hypertension.

Pulmonary vascular contractility is mainly controlled by the level of [Ca2+]cyt in PASMC; a rise in [Ca2+]cyt causes vasoconstriction, whereas a decrease in [Ca2+]cyt mediates vasorelaxation (41). Because extracellular free [Ca2+] \( \sim 2 \) mM is much higher than cytoplasmic free [Ca2+] \( \sim 100 \) nM, opening of Ca2+-permeable channels in the plasma membrane, stimulated by vasocostrictors, is a critical pathway for elevating Ca2+ in PASMC to cause smooth muscle contraction and pulmonary vasoconstriction. There are at least three classes of Ca2+-permeable channels functionally expressed in vascular smooth muscle cells including PASMC: 1) voltage-dependent Ca2+ channels; 2) ROC that require agonist binding to sarcolemmal receptors for activation, and SMOC whose activity is regulated by receptor-mediated production of second messengers (e.g., DAG and IP3); and 3) SOC that are activated by intracellular Ca2+ store (SR) depletion (2, 40).

Opening of SOC when intracellular store is depleted by agonist-induced IP3 production is an important pathway for initiating agonist-induced smooth muscle contraction (22, 35, 40, 41). The molecular composition and structure of SOC in animal and human vascular smooth muscle cells have not been completely elucidated. Work in this area has focused on the TRP gene-encoded proteins, although it is still controversial whether and which TRP channels form functional SOC (10, 37). Thus far, the mammalian TRP family can be divided into three subfamilies: the TRPC (TRPC1–7), TRP vanilloid (TRPV1–6), and TRP melastatin (TRPM1–8) isoforms (9, 10, 33). Expression of TRP genes in mammalian cells and Xenopus oocytes leads to the formation of Ca2+-permeable cation channels activated by SR store depletion and by activation of G protein-coupled receptors and tyrosine kinase receptors (5, 20, 23, 27, 46, 57).

In attempting to correlate specific TRP isoforms with ROC and SOC properties, investigators have produced data from different cell types and conducted experiments involving overexpression of specific TRP proteins yielding conflicting results (5, 6, 10, 48). Part of this variability relies on the fact that native TRP channels exist as homo- or heterotramers in cells. For example, for TRPC-based channels, a channel made up of one TRPC1, one TRPC4, and two TRPC5 may form an SOC, whereas a channel made of one TRPC3 and three TRPC6 may form an ROC. This suggests that the expression ratio of different TRP isoforms may likely vary among different cell types and the composition of functional TRP heterotrameric channels determines whether the channel can be opened by receptor activation, store depletion, or both (25, 26, 48, 49).

Among the various TRPC isoforms, TRPC1 is a channel that can form not only homotrameric channels but also heterotrameric channels with other TRPC channel subunits (e.g., TRPC4 and TRPC5) (25, 26, 33, 35, 53). Overexpression of TRPC1 would lead to upregulation of both homomeric TRPC1 channels and heterotrameric TRPC channels containing TRPC1 and thus enhance agonist-mediated Ca2+ influx through both ROC and SOC. Our results from this study demonstrate that, in isolated rat PA rings overexpressing hTRPC1, the CCE-induced contraction is much greater than in control (empty vector) PA rings. This suggests that TRPC1 is involved in forming functional SOC in the rat PA rings.

Both pulmonary vascular smooth muscle (21, 32, 35, 42, 54) and endothelial (6, 16, 34) cells express functional TRPC channels. Infection of isolated endothelium-intact PA rings with the Adv-hTRPC1 would increase hTRPC levels in both PA smooth muscle and endothelial cells. To demonstrate that the enhanced CCE-induced PA contraction was due to overexpression of hTRPC1 in PASMC, we removed the endothelium-intact PA rings before infecting them with hTRPC1. Overexpression of hTRPC1 in endothelium-denuded PA rings caused a significant increase in CCE-induced active tension, quantitatively comparable to that observed in endothelium-intact PA rings. These results further suggest that TRPC1 is involved in forming functional SOC in PASMC. Our observations with TRPC1 in PA are similar to that previously demonstrated for TRPC6 in cerebral arterioles (51). Observations in the latter study and our current study, coupled with knowledge that native TRPC channels are often heteromeric tetramers, suggest that TRPC isoforms are important components of heteromeric SOC channels in vascular smooth muscle cells.

To overexpress hTRPC1 in intact rat PA, we cultured the vessels in media containing Adv-empty or Adv-hTRPC1 for a short period of time to ensure the expression of the exogenous TRPC1. It has been demonstrated that the vasoconstrictive response to agonists (e.g., histamine, norepinephrine, serotonin, and high K+) and the vasodilative response to nitroprusside (a nitric oxide donor) and pinacidil (K+ channel opener) were all well preserved in the cultured rabbit mesenteric arterial rings compared with the freshly isolated mesenteric arterial rings (29). However, the endothelium-dependent vasodilative response to ACh was markedly inhibited in arterial rings cultured in fetal calf serum and DMEM (29). Consistent with the report on rabbit mesenteric arteries, our data also indicate that the ACh-mediated relaxation appeared to be significantly diminished in cultured rings (cf. Fig. 6 with Fig. 5A, left), although 40K-induced contraction was not different between freshly isolated and cultured PA rings (see Fig. 2, A and B). It remains unclear why the different tissues (i.e., PA smooth muscle and endothelium) were affected differently by
the culture procedure, ACh causes vasodilation by activating M receptors in endothelial cells, which subsequently mediates synthesis and release of nitric oxide. The inhibited vasodilative response to ACh in cultured arterial rings might be due to downregulation of M₂ muscarinic receptors in endothelial cells as previously reported by other investigators (11, 15, 44). Accordingly, the inability of overexpressed TRPC1 (which appears to be distributed in both smooth muscle and endothelial cells) to affect ACh-mediated vasodilative response was probably due to potential downregulation of M receptors in endothelium.

Sustained pulmonary vasoconstriction is, at least in part, involved in the elevated PVR and increased PAP in patients and animals with hypoxia-induced PA hypertension (17, 43). Indeed, functional TRPC channels are expressed in rat distal PA, and chronic hypoxia increases mRNA and protein expression of several TRPC isoforms and increases the amplitude of CPA-mediated CCE in pulmonary vascular smooth muscle and endothelial cells (16, 50). The upregulated TRPC expression and enhanced CCE may play a role in the development of hypoxia-induced PA hypertension. We believe that opening of SOCs, formed by TRPC subunits, may play an important role in promoting sustained Ca²⁺ influx when PASMC are constantly stimulated because of a high level of circulating vasoconstrictors or mitogens (7, 8, 14, 19, 28, 31) in lung tissues from patients with PA hypertension. Understanding the molecular nature and the composition of the Ca²⁺ channels involved in mediating pulmonary vasoconstriction may lead to develop novel drug therapy for patients with PA hypertension.

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