Pulmonary artery smooth muscle cells from normal subjects and IPAH patients show divergent cAMP-mediated effects on TRPC expression and capacitative Ca\(^{2+}\) entry

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Pulmonary vascular remodeling due to overgrowth of pulmonary artery smooth muscle cells (PASMC) is a major cause for the elevated vascular resistance in patients with idiopathic pulmonary arterial hypertension (IPAH). Increased cytosolic Ca\(^{2+}\) concentration, resulting from enhanced capacitative Ca\(^{2+}\) entry (CCE) and upregulated transient receptor potential (TRP) channel expression, is involved in stimulating PASMC proliferation. The current study was designed to determine the impact of cAMP, a second messenger that we hypothesized would blunt aspects of PASMC activity, as a possible contributor to IPAH pathophysiology. Short-term (30 min) pretreatment with forskolin (FSK; 10 \(\mu\)M), a direct activator of adenylyl cyclase, in combination with the cyclic nucleotide phosphodiesterase inhibitor isobutyl methylxanthine (IBMX; 200 \(\mu\)M), attenuated CCE in PASMC from normal subjects, patients without pulmonary hypertension (NPH), and patients with IPAH. The FSK-mediated CCE inhibition was independent of protein kinase A (PKA), because the PKA inhibitor H89 negligibly affected the decrease in CCE produced by cAMP. By contrast, longer (4 h) treatment with FSK (with IBMX) attenuated CCE in normal and NPH PASMC but enhanced CCE in IPAH PASMC. This enhancement of CCE was abolished by PKA inhibition and associated with an upregulation of TRPC3. In addition, cAMP increased TRPC1 mRNA expression in IPAH (but not in normal or NPH) PASMC, an effect blunted by H89. Furthermore, iloprost, a prostacyclin analog that increases cAMP, downregulated TRPC3 expression in IPAH PASMC and FSK-mediated cAMP increase inhibited IPAH PASMC proliferation. Although a rapid rise in cellular cAMP decreases CCE by a PKA-independent mechanism, sustained cAMP increase inhibits CCE in normal and NPH PASMC but increases CCE via a PKA-dependent pathway in IPAH PASMC. The divergent effect of cAMP on CCE parallels effects on TRPC expression. The results suggest that the combined use of a PKA inhibitor and cAMP-elevating drugs may provide a novel approach for treatment of IPAH.

idiopathic pulmonary arterial hypertension; adenosine 3',5'-cyclic monophosphate; transient receptor potential channel; pulmonary vascular remodeling

PULMONARY VASCULAR MEDIAL HYPERTROPHY due to increased proliferation and inhibited apoptosis of pulmonary artery

Smooth muscle cells (PASMC), and sustained pulmonary vasoconstriction contribute to the elevated pulmonary vascular resistance and arterial pressure in patients with idiopathic pulmonary hypertension (IPAH). Ca\(^{2+}\) channel blockers (e.g., nifedipine and diltiazem) and infusion of prostacyclin (e.g., epoprostenol) or oral intake of its analogs (e.g., iloprost) can reduce pulmonary vascular resistance and arterial pressure in some IPAH patients, but it remains unclear why only a minority of patients respond to these drugs (2, 30).

In vitro experiments have demonstrated that removal of extracellular Ca\(^{2+}\) abolishes agonist-mediated pulmonary vasoconstriction and inhibits PASMC proliferation (34, 48). Blockade of Ca\(^{2+}\) channels in the plasma membrane of PASMC also attenuates agonist-mediated pulmonary vasoconstriction and growth factor-mediated proliferation (34). Such results suggest that a rise in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) due to Ca\(^{2+}\) influx plays a critical role in pulmonary vasoconstriction and PASMC proliferation. Human PASMC express multiple Ca\(^{2+}\)-permeable channels that contribute to regulating [Ca\(^{2+}\)\(_{cyt}\)] and excitation-contraction coupling (26, 49): 1) voltage-dependent Ca\(^{2+}\) channels, 2) receptor-operated Ca\(^{2+}\) channels (ROC), and 3) store-operated Ca\(^{2+}\) channels (SOC), which are opened by depletion of Ca\(^{2+}\) from intracellular stores (e.g., the sarcoplasmic or endoplasmic reticulum).

Our studies of PASMC isolated from IPAH patients have shown that the amplitude of store-operated Ca\(^{2+}\) entry through SOC, or capacitative Ca\(^{2+}\) entry (CCE), is enhanced and the protein expression of canonical type transient receptor potential channels (e.g., TRPC3 and TRPC6) is upregulated relative to expression in PASMC from normal subjects and normotensive patients or patients who do not have pulmonary hypertension (NPH) (47). Since TRPC channel subunits participate in forming functional SOC and ROC, upregulated TRPC gene expression in IPAH PASMC would be predicted to increase the number of functional SOC and ROC, enhance vasoconstrictor- and mitogen-mediated increases in [Ca\(^{2+}\)\(_{cyt}\)], stimulate vasoconstriction, and promote cell growth.

The second messenger cAMP activates cAMP-dependent protein kinase A (PKA) and/or directly binds to target proteins, thereby regulating cell contraction, proliferation, apoptosis, and gene expression (36). Binding of cAMP to the regulatory (R) subunits of PKA facilitates dissociation of its catalytic (C) subunits, thereby promoting phosphorylation of substrates that...
include the cAMP-response element-binding (CREB) protein. Phosphorylated CREB recruits the CREB-binding protein (CBP), a transcriptional coactivator, and stimulates the transcription of genes that contain the CREB-binding sequences, CRE, in their promoter region (25, 52). Increased cytosolic cAMP also can affect membrane channel functions by PKA-independent pathways (11, 50).

A rise in cellular cAMP or activated PKA can promote pulmonary vasodilation (1, 17) and exert antiproliferative and proapoptotic effects (35, 50); however, the precise mechanisms are unclear. In this study we sought to study 1) whether TRPC channels are regulated by cAMP differently in PASMC from normal subjects, NPH patients, and IPAH patients and 2) whether short- and long-term treatment with cAMP-elevating compounds differentially affects TRPC channel activity and expression in normal, NPH, and IPAH PASMC.

METHODS

Cell preparation and culture. PASMC were prepared from explanted lung tissues of two IPAH patients (a 57-yr-old woman and a 31-yr-old man with mean pulmonary arterial pressures of 51 and 53 mmHg, respectively) undergoing lung/heart transplantation (47, 51) and two normotensive patients (a 49-yr-old man and a 77-yr-old woman). The IPAH patients were treated with Flolan (prostacyclin), warfarin, digoxin, and furosemide before lung transplantation. There was no record showing that the normotensive patients were on Flolan treatment. Peripheral pulmonary arteries isolated from the explanted lung were first incubated in Hanks’ solution (20 min) containing 2 mg/ml collagenase to remove adventitia and endothelium and then digested with collagenase (2.25 mg/ml) and elastase (0.5 mg/ml) to make a PASMC suspension. Cells were incubated at 37°C in the smooth muscle growth medium (SmGM) composed of smooth muscle basal medium (SmBM) supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Human PASMC (Cambrex) from normal subjects were also used in the experiments at the fourth to sixth passages.

Measurement of [Ca2+]\textsubscript{cyt}. Cells were loaded with fura-2-AM (3 μM) for 30 min at 22–24°C and then superfused for 20 min with a standard bath solution that contained (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl\textsubscript{2}, 1.2 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose (pH 7.4 with 5 M NaOH). Fura-2 fluorescence from the cells and background fluorescence were collected at 32°C using Nikon UV-Fluor objectives. The fluorescence signals were monitored continuously using an Intracellular Imaging fluorescence microscopy system and recorded on a computer for later analysis. [Ca2+]\textsubscript{cyt} was calculated from the fura-2 fluorescence emission at 340 and 380 nm (F\textsubscript{340}/F\textsubscript{380}) using a ratiometric method (16, 52).

Western blot analysis. PASMC were gently washed twice in cold PBS, scraped into 0.3 ml of radioimmunoprecipitation assay buffer, sonicated, and centrifuged at 14,000 rpm for 15 min at 4°C. Total protein was extracted from the supernatant and separated electrophoretically on a 10% acrylamide gel. Protein bands were transferred phoretically on a 10% acrylamide gel. Protein bands were transferred to nitrocellulose membranes by electroblotting in a Mini Trans-Blot apparatus (Bio-Rad). After 1 h of incubation in a blocking buffer, membranes were incubated overnight at 4°C with affinity-purified rabbit polyclonal antibody against TRPC3 (Alomone) and then washed and exposed to anti-rabbit horseradish peroxidase-conjugated IgG for 90 min at room temperature. Bound antibody was detected with an enhanced chemiluminescence detection system (Amersham). Monoclonal anti-α-actin antibody (Upstate) was used as a control.

Synthesis and transfection of small interfering RNA. The 21-nucleotide small interfering RNA (siRNA) sequence targeting TRPC3 (sense, 5′-uuggaucugacacccggcaccctg-3′; antisense, 5′-ccaagacggugga-3′) (Invitrogen) and a scrambled sequence (sense, 5′-ugugcuacgucucuagggcctt-3′; antisense, 5′-ccagggagccuagacaact-3′) were synthesized. PASMC grown in SmGM to ~80% confluence were transfected with either the scrambled siRNA or TRPC3 siRNA (20 nM) using the Gene Porter 2 transfection reagent kit (Gene Therapy Systems). After transfection (for 6 h), cells were incubated at 37°C in fresh SmBM; protein was extracted 72 h after siRNA treatment. The efficiency of siRNA transfection was determined using fluorescence-labeled siRNA; fluorescence was visible only in siRNA-transfected cells (9).

Real-time PCR analysis of TRPC gene expression. Total RNA was isolated from PASMC using the RNeasy mini kit. First-strand cDNA synthesis was performed using random hexamers on 2 μg of total RNA. The concentration of cDNA was determined and adjusted to 50 ng/μl for subsequent real-time PCR analysis, which was performed on a MJ Research Opticon 2 using the QPCR Mastermix Plus for SYBR green kit (Eurogentec) with 100 ng cDNA and 0.5 μM forward/ reverse primer mix in a 20-μl final reaction volume. Specific primers were as follows: TRPC1, forward-5′-ctgggtgaggaattgagaag-3′ and reverse-5′-ggtcgtgctgctgctgcac-3′; and TRPC3 forward-5′-gacttcggtgcctaaata-3′ and reverse-5′-cctctggacgtctctctctgc-3′. PCR products were confirmed by melt curve analysis and agarose gel electrophoresis. Analysis of cycle threshold (C\textsubscript{T}) was performed using Opticon 2 Analysis Software (MJ Research); normalized values were obtained for each group by subtracting matched glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ct values.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using unpaired Student’s t-tests or ANOVA as indicated. Differences were considered to be significant when P < 0.05.

RESULTS

cAMP treatment inhibits CCE in normal and NPH PASMC but enhances CCE in IPAH PASMC. In PASMC isolated from normal subjects, NPH patients, and IPAH patients, short-term (0.5 h) treatment with forskolin (FSK; 10 μM) in the presence of the nonselective cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; 200 μM) attenuated the amplitude of CCE induced by passive store depletion with cAMP-elevating agents. Treatment with FSK (10 μM) for 4 h in the presence of IBMX (200 μM) reduced CPA-mediated CCE in normal and NPH PASMC (302 ± 44 nM, n = 33 cells; P < 0.001) and IPAH PASMC (302 ± 4 nM, n = 33 cells; P < 0.01) (Fig. 1C). The percentage of short-term FSK-mediated inhibition of CCE was comparable in normal (31%, from 302 ± 19 to 209 ± 6 nM), NPH (52%, from 302 ± 4 to 145 ± 4 nM), and IPAH PASMC (46%, from 460 ± 35 to 249 ± 14 nM) (Fig. 1, A and B). In PASMC from normal subjects and IPAH patients, short-term (0.5 h) treatment with the membrane-permeable cAMP analog 8-(chlorophenylthio)-cAMP (CPT) also attenuated the amplitude of CCE (data not shown). The percentage of CPT-mediated inhibition of CCE was similar in normal (33%) and IPAH (39%) PASMC. From these results we conclude that increases in cAMP levels acutely block the Ca2+-permeable channels responsible for CPA-mediated CCE in normal and hypertensive PASMC.

By contrast, long-term (4 h) increase in cAMP caused divergent effects on CCE in normal and IPAH PASMC. Treatment with FSK (10 μM) for 4 h in the presence of IBMX (200 μM) reduced CPA-mediated CCE in normal and NPH PASMC but enhanced this response in IPAH PASMC (Fig. 1, A and B). Thus the inhibitory effect of a longer term of cAMP...
increase in cAMP on CCE is not present in IPAH PASMC, an effect that may contribute to sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling in IPAH patients.

cAMP can inhibit CCE by directly affecting SOC activity and expression or indirectly through activation of PKA (23). As shown in Fig. 2A, short-term treatment of IPAH PASMC with FSK + IBMX or CPT attenuated CPA-mediated CCE; the PKA inhibitor H89 negligibly affected the inhibition by short-term treatment with CPT. By contrast, H89 abolished the augmenting effect of long-term FSK + IBMX treatment on CCE in IPAH PASMC (Fig. 2B). Downregulation of TRPC3 with siRNA eliminated the long-term enhancement of CCE by FSK + IBMX in IPAH PASMC (Fig. 2B). These data suggest that, in IPAH PASMC, increases in cAMP levels acutely inhibit CCE or SOC/TRPC channel function via a PKA-independent pathway, whereas longer exposure to elevations in cAMP enhances CCE by a PKA-dependent pathway that depends on expression of TRPC3.

cAMP treatment upregulates TRPC3 expression in IPAH-PASMC. A possible mechanism for the enhancement in CCE in response to FSK + IBMX in IPAH-PASMC would be a cAMP-promoted increase in expression of TRPC channels, several of which are expressed in human and rat PASMC (21, 27, 42, 47, 48). We found that expression of TRPC3, a TRPC isoform that participates in forming SOC channels, was upregulated in IPAH PASMC compared with normal and NPH PASMC (Fig. 3A), an increase in expression that may contribute to enhanced CCE in IPAH PASMC (Fig. 1A) (47). Consistent with the effect on CCE, 1-h treatment with FSK + IBMX negligibly influenced TRPC3 protein expression, whereas long-term (4 h) incubation with those agents downregulated TRPC3 protein expression in normal and NPH PASMC but upregulated TRPC3 protein expression in IPAH PASMC (Fig. 3B). Treatment with the TRPC3-specific siRNA abolished the upregulating effect of FSK + IBMX on TRPC3 protein expression in IPAH PASMC (Fig. 3B). Similar to its inhibitory effect on CCE (Fig. 2B), H89 abolished FSK + IBMX-mediated upregulation of TRPC3 mRNA expression in IPAH PASMC (Fig. 3C). The association of cAMP-mediated TRPC upregulation and CCE enhancement in IPAH PASMC and the dependence of the effects on PKA are consistent with the conclusion that cAMP/PKA-promoted enhancement of CCE results from increased TRPC3 gene expression.

**Divergent effect of cAMP on TRPC1 mRNA expression in normal and IPAH PASMC.** In addition to TRPC3, cAMP-mediated effects on TRPC1 were also different in normal, NPH, and IPAH PASMC. Treatment with FSK + IBMX for 1 h had little effect on mRNA expression of TRPC1 in normal and NPH PASMC but rapidly and transiently upregulated TRPC1 expression in IPAH PASMC in an H89-inhibitable
Treatment of IPAH PASMC with the prostacyclin analog iloprost (30 nM) significantly downregulated mRNA expression of TRPC3 (Fig. 5B). The iloprost treatment also caused a 14% decrease (from 155.5 ± 3.2 to 133.9 ± 3.6 nM; P < 0.05) in the rise in [Ca^{2+}]_{cyt} due to CPA-mediated Ca^{2+} leakage from intracellular stores. These results indicate that the vasodilating and antiproliferative effects of prostacyclin and its analogs (e.g., iloprost) may, at least partially, result from their inhibitory effect on TRPC expression in PASMC.

**Antiproliferative effect of cAMP in IPAH PASMC.** As shown in Fig. 6A, the cell proliferation rate, determined by measuring [3H]thymidine incorporation, in IPAH PASMC was significantly higher than in normal PASMC. Incubation of growth-arrested cells in SmGM (basal medium supplemented with 5% FBS and growth factors) for 24 h increased [3H]thymidine uptake by 52% in normal PASMC and 91% in IPAH PASMC (P < 0.05) (Fig. 6A). These data indicate that PASMC from IPAH patients grow faster, which is at least in part due to upregulated TRPC channels and enhanced CCE.

Incubation in SmGM with FSK significantly inhibited cell proliferation or [3H]thymidine incorporation in IPAH PASMC (Fig. 6B). The FSK-mediated antiproliferative effect on IPAH PASMC was further enhanced by inhibition of PKA with H89 (Fig. 6B). These data indicate that increasing intracellular cAMP inhibits PASMC proliferation; however, the antiproliferative effect of cAMP is attenuated in IPAH PASMC, perhaps because of its upregulating effect on TRPC expression via PKA. Inhibition of PKA with H89, thus, enhances the antiproliferative effect of cAMP on IPAH PASMC (Fig. 6B).

In addition to its antiproliferative effect, cAMP diminishes pulmonary vasoconstriction induced by a rise in [Ca^{2+}]_{cyt} due to CCE. In pulmonary arterial (PA) rings isolated from normal rats, treatment with 50 μM CPA in 0 Ca^{2+} solution induced vasoconstriction when extracellular Ca^{2+} was restored, whereas incubation with FSK + IBMX inhibited CPA-mediated PA contraction (data not shown). Consistent with the idea that the inhibitory effect of short-term cAMP treatment on CPA-mediated PA contraction may result from inhibition of TRPC channel activity, an increase in cAMP decreased PA contraction induced by the vasoconstrictive α-adrenergic receptor agonist phenylephrine. These data complement the findings in PASMC and are consistent with the conclusion that blockade of TRPC channels by acute increases in cellular cAMP inhibits agonist-mediated increase in [Ca^{2+}]_{cyt} and PA contraction in intact tissue.

**DISCUSSION**

Patients with IPAH have pulmonary vascular remodeling and excessive PASMC proliferation that is caused by numerous factors; these include increased production of vasoconstrictive and mitogenic agonists (5, 8, 13), endothelial cell apoptosis (31), decreased production of endothelium-derived relaxing factors (5, 12), and increased proliferation and/or decreased apoptosis of PASMC (7, 33, 51). Mitogen- or growth factor-mediated proliferation of PASMC depends in part on a rise in [Ca^{2+}]_{cyt} from Ca^{2+} influx through membrane TRPC channels (14, 21, 48, 52). Removal of extracellular Ca^{2+} inhibits cell growth, whereas blockade of TRPC channels with pharmacological agents (e.g., Ni^{2+}, La^{3+}, SK&F 96365) or downregulation of TRPC channel expression attenuates PASMC proliferation (14, 47, 48). In IPAH patients, upregulated TRPC channels with enhanced agonist-mediated Ca^{2+} influx contrib-
cAMP appears to have a “dose-dependent” effect on CCE and TRPC expression, and PASMC from normal subjects (or NPH patients) and IPAH patients differ in their cAMP-regulated responses in terms of CCE and TRPC expression. The PKA-dependent upregulation of TRPC3 in IPAH PASMC could blunt the therapeutic efficacy of drugs (e.g., prostacyclin and adenosine) that increase cAMP and activate PKA. Treatment of IPAH patients with drugs that increase intracellular cAMP might thus be more effective if a PKA inhibitor were used concurrently. Desensitization of prostacyclin receptors (10, 32) may paradoxically contribute to therapeutic efficacy of such drugs by helping to blunt their augmentation of CCE and TRPC expression in IPAH PASMC.

The mechanisms for cAMP-mediated acute inhibition of TRPC channels in PASMC are unclear. cAMP/cGMP can directly bind to the intracellular domains of cyclic nucleotide-gated (CNG) cation channels from photoreceptors and olfac-

Fig. 3. Distinct effects of treatment with cAMP on TRPC3 expression in PASMC from normal subjects, NPH patients, and IPAH patients. A: Western blot analysis of TRPC3 in PASMC from a normal subject (Nor), 2 IPAH patients (IPAH1 and IPAH2), and a NPH patient (NPH). B: Western blot analysis (a) and summarized data (b; means ± SE, normalized to α-actin, n = 4–6 experiments) showing TRPC3 protein levels in IPAH PASMC treated with vehicle (control), FSK (10 μM) + IBMX (200 μM) for 1 (FSK-1h) and 4 h (FSK-4h), or FSK + IBMX and TRPC3 siRNA for 4 h (FSK-4h/siRNA), as well as in normal and NPH PASMC treated with vehicle (control) or FSK + IBMX for 1 and 4 h. **P < 0.01 as indicated by brackets. C: real-time RT-PCR analysis showing changes in TRPC3 mRNA expression in IPAH PASMC before (0 h) and after treatment with FSK + IBMX in the presence (+H89; for 0.5–8 h) or absence (control; for 0.5–48 h) of H89.

Fig. 4. Distinct effects of cAMP on TRPC1 mRNA expression in PASMC from normal subjects, NPH patients, and IPAH patients. Real-time RT-PCR analysis shows the time course of changes in TRPC1 mRNA expression in normal (A), NPH (B) and IPAH PASMC (C, control) before (0 h) and after treatment with FSK (10 μM) + IBMX (200 μM) for 0.5, 1, 2, 4, 8, 12, and 48 h, respectively. The FSK + IBMX-induced transient increase in TRPC1 mRNA expression was abolished in IPAH PASMC treated with H89 (10 μM; C).
cAMP (and cGMP) can also modulate the activity of Ca\(^{2+}\)-activated K\(^+\) channels (1, 53), voltage-gated Ca\(^{2+}\) channels (46), and Ca\(^{2+}\) release channels (i.e., ryanodine receptors) (28). Such data suggest that cAMP can have a nonselective, PKA-independent inhibitory effect on channels that regulate [Ca\(^{2+}\)]\(_{cyt}\) in PASMC.

Other data, for example, in rabbit portal vein myocytes, show that cAMP can acutely modulate SOC activity through a PKA-dependent mechanism (23). Since SOC activation underlies, at least in part, CCE (27, 43, 48), the findings that increased cAMP can inhibit SOC currents parallel our finding that treatment with FSK + IBMX attenuates CCE in PASMC. Because prolonged cAMP elevation enhanced CCE only in IPAH PASMC, an effect that was attenuated by both PKA inhibition and “silenced” TRPC3 expression, TRPC3 upregulation by sustained cAMP may alter both the function of SOC channels (during acute cAMP exposure) and the expression of TRPC3 subunits, contributing to the cAMP-promoted enhancement of CCE in IPAH PASMC (Fig. 7). In this study, we used FSK and IBMX to increase intracellular cAMP. IBMX is a relatively nonspecific PDE inhibitor and, as such, could increase cGMP levels as well as cAMP levels. Therefore, the enhancement of CCE and upregulation of TRPC3 in IPAH PASMC after long-term treatment with FSK and IBMX might also be partially related to an increase in intracellular cGMP levels.

Future studies need to define the precise sequence of events that mediate the short-term cAMP-mediated blockade of SOC channels (i.e., ryanodine receptors) (4). Although their membrane topology is similar to that of TRPC channels (6 transmembrane domains, a pore domain, and intracellular NH\(_2\) and COOH termini), CNG channels generally have calmodulin binding sites in either the NH\(_2\)- or COOH-terminal regions, whereas cAMP/cGMP binding sites are on the COOH terminus (4). A similar cyclic nucleotide-binding domain is found at the COOH terminus of Kv1.10, a voltage-gated K\(^+\) channel (38). Interestingly, a decrease in [Ca\(^{2+}\)]\(_{cyt}\) potentiates the response of cGMP-gated channels to cGMP (4), a finding reminiscent of the ability of intracellular Ca\(^{2+}\) depletion to activate TRP-encoded SOC channels and CCE (29). In vascular smooth muscle cells,
function and the long-term cAMP-mediated upregulation of TRPC3 expression. Possibilities for the inhibitory effects of acute cAMP exposure on TRPC channel activity include: 1) direct binding of cAMP to the pore-forming region of TRPC channels and occlusion of the channels, 2) dissociation of the coupling mechanism required for the TRPC channel activation (e.g., G protein activation, PLC, and/or diacylglycerol synthesis), and 3) disruption of the caveolar microdomain that colocalizes mitogen receptors with TRPC channels (3).

A previous publication described the upregulation of TRPC4 (and TRPC3) mRNA and protein expression by the mitogen ATP in PASMC, with enhanced CCE and PASMC proliferation, effects mediated by a CREB-dependent pathway (52). Because the consensus binding sequences of CREB and AP-1, another transcription factor, are similar, it is conceivable that cAMP-mediated PKA activation also promotes the expression, phosphorylation, and nuclear translocation of AP-1 transcription factors (e.g., c-Fos and c-Jun), subsequent binding to the TRPC3 promoter (which contains AP-1 and CREB binding sequences), and activation of gene transcription. Therefore, upregulated and/or activated CREB and AP-1 transcription factors may be involved in the long-term cAMP/PKA-mediated upregulation of TRPC3 in IPAH PASMC.

There are multiple TRPC subunits expressed in vascular smooth muscle cells, including PASMC. The upregulated TRPC3 expression is associated with an increased CCE in PASMC from IPAH patients (Fig. 3A). It is however still unknown whether TRPC3 subunit contributes to the formation of functional SOC in human PASMC. There have been many reports showing that overexpressed TRPC3 channels appear to be activated in response to store depletion (e.g., by SERCA inhibitor or ionophores) (19, 20, 37, 40, 45, 55). However, there are equally many reports indicating that TRPC3 channels overexpressed in mammalian cells are activated by PLC-coupled receptors but are apparently not activated by store depletion in response to thapsigargin or CPA (18, 24, 39, 41, 54, 56). Functional TRP channels are homotetramers that are composed of the same TRP subunits or heterotetramers that are composed of different TRP subunits. It is thus possible that a heterotetramer composed of four TRPC3 subunits may function differently in response to store depletion or receptor activation compared with a heterotetramer composed of one or two TRPC3 subunits. Furthermore, whether TRPC3 heterotetramers can be activated by store depletion or PLC-coupled receptor activation may also depend on the other TRPC subunits (e.g., a heterotetramer composed of 2 TRPC3 and 2 TRPC1 subunits may respond to store depletion and PLC-coupled receptor activation differently from a heterotetramer composed of 2 TRPC3, 1 TRPC1, and 1 TRPC5 subunit). The results from our study indicate a correlation of upregulated TRPC3 expression and enhanced CCE amplitude in PASMC from IPAH patients; however, they do not rule out the possibility that TRPC3 may also participate in forming ROC in PASMC.

In the treatment of IPAH, prostacyclin and its analogs presumably act via increases in cellular cAMP and PKA activity, thereby leading to pulmonary arterial vasodilation and antiproliferative effects on PASMC (2, 6). However, the therapeutic effects on IPAH of prostacyclin and other cAMP-stimulating agents vary among different patients (44). The current data suggest that multiple effects of cAMP and PKA, including direct action on ion channels (1) and indirect effects on protein expression (15, 22), contribute to the inefficiency of cAMP-generating agents in the treatment of IPAH.
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