ATP-induced mitogenesis is mediated by cyclic AMP response element-binding protein-enhanced TRPC4 expression and activity in human pulmonary artery smooth muscle cells

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Zhang, Shen, Carmelle V. Remillard, Ivana Fantozzi, and Jason X.-J. Yuan. ATP-induced mitogenesis is mediated by cyclic AMP response element-binding protein-enhanced TRPC4 expression and activity in human pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 287: C1192–C1201, 2004. First published June 30, 2004; doi:10.1152/ajpcell.00158.2004.—Extracellular ATP and intracellular cyclic AMP response element-binding protein (CREB, a transcription factor) promote cell proliferation in many cell types. The canonical transient receptor potential (TRPC) channels, which play a role in in forming store- and receptor-operated Ca2⁺ channels, have been implicated in the pulmonary vascular remodeling processes. A link between extracellular ATP, CREB activation, and TRPC4 channel expression and activity has not been shown in human pulmonary artery smooth muscle cells (PASMC). Long-term (24–48 h) treatment of human PASMC with a low dose (100 μM) of ATP, which did not trigger a transient rise in free cytosolic Ca²⁺ concentration ([Ca²⁺]c) when applied acutely to the cells, caused marked increases in CREB phosphorylation and TRPC4 protein expression. The time course indicated that the ATP-mediated CREB phosphorylation preceded TRPC4 upregulation, whereas transfection of a non-phosphorylatable CREB mutant abolished ATP-mediated TRPC4 expression. Furthermore, treatment of human PASMC with ATP also enhanced the amplitude of capacitative Ca²⁺ entry (CCE) induced by passive store depletion, whereas the small interfering RNA specifically targeting TRPC4 attenuated ATP-mediated increases in TRPC4 expression and CCE amplitude and inhibited ATP-induced PASMC proliferation. These data suggest that low-dose ATP exerts part of its mitogenic effect in human PASMC via CREB-mediated upregulation of TRPC4 channel expression and activity and the subsequent increase in CCE and [Ca²⁺]c.

Capacitative Ca²⁺ entry; proliferation; vascular smooth muscle

NUCLEOTIDES SUCH AS ATP, ADP, AND UTP act as extracellular messengers and play an important role in regulating vascular tone and cell proliferation and differentiation (12, 18, 25, 41, 56). Intracellular ATP is an essential molecular energy source, while extracellular ATP, which can be released from vascular endothelial cells (44, 71), damaged vessel walls (4), hypoxic myocardium (15), and aggregating platelets (29), serves as a potent vasoconstrictor (20, 24) and smooth muscle and endothelial cell mitogen (7, 19). ATP also can be released from perivascular nerves as an excitatory neurotransmitter (6). The contractile and mitogenic effects of extracellular ATP on vascular smooth muscle cells (VSMC) are mediated by activation of a family of G protein-coupled receptors, P₂ purinoreceptors (including at least 6 subtypes: P₂X₁, P₂X₂, P₂X₇, P₂U, P₂Z, and P₂Dₙ), which are linked to phospholipases and adenylate cyclase (7, 65). Activation of P₂ purinoreceptors by ATP and UTP (24) in pulmonary artery smooth muscle cells (PASMC) causes pulmonary vasoconstriction and in fibroblasts promotes the progression of pulmonary vascular remodeling by stimulating fibroblast and SMC proliferation (19, 56).

While increased pulmonary vasoconstriction can underlie the increased pulmonary vascular resistance (PVR) in patients with pulmonary arterial hypertension, pulmonary vascular remodeling due to increased proliferation of PASMC in the tunica media, resulting in medial hypertrophy, also contributes greatly to the increased PVR. Increased extracellular or interstitial ATP level has been found to be involved in the progression of pulmonary hypertension (38, 56) as well as in the regulation of cell cycle progression and cell proliferation (19, 36, 56), all of which depend on elevations in cytosolic free Ca²⁺ concentration ([Ca²⁺]c). Investigators at our laboratory previously showed that store-operated Ca²⁺ (SOC) influx through canonical transient receptor potential (TRPC) channels may underlie part of the agonist-mediated Ca²⁺ response via capacitative Ca²⁺ entry (CCE) in PASMC (21, 61, 72). In all of these studies, enhanced CCE influenced either vasoconstriction or proliferation. Along with TRPC1 and TRPC6, TRPC4 is a TRPC isoform that has been implicated in forming receptor-operated Ca²⁺ (ROC) and/or SOC channels in many cell types (2, 9, 17, 30, 39, 51, 60, 62, 66, 72).

In this study, we hypothesized that extracellular ATP induces human PASMC proliferation via upregulation of TRPC4 expression and increase in CCE. Furthermore, we proposed that ATP-induced phosphorylation of the cyclic AMP response element-binding protein (CREB), a critical transcription factor in the regulation of cell survival (14, 49, 64), is involved in the increased transcription of TRPC4 induced by ATP.

MATERIALS AND METHODS

Cell preparation and culture. Human PASMC from healthy individuals were purchased from Cambrex and used at passages 4–6. PASMC were cultured as described previously (61). Briefly, PASMC were plated onto coverslips or petri dishes and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C in the smooth muscle growth medium (SMGM; Cambrex). The SMGM was composed of smooth muscle basal medium (SMBM) supplemented with 5% fetal bovine serum (FBS), 0.5 ng/ml human epidermal growth factor, 2
ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Cells were subcultured or plated onto 25-mm coverslips using trypsin-EDTA buffer when 70–90% confluence was achieved. The morphology of the cells was examined using an inverted phase-contrast microscope attached to a digital camera.

Rat PASMC were used in some experiments to compare the response to ATP with human PASMC. Rat PASMC were prepared from the left and right branches of the main pulmonary artery and the intrapulmonary arteries of male Sprague-Dawley rats (150–200 g). Briefly, the isolated pulmonary arteries were incubated for 20 min in Hank’s balanced salt solution containing 1.5 mg/ml collagenase (Worthington Biochemical). Adventitia and endothelium were carefully removed after the incubation. The remaining smooth muscle was then digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma) at 37°C. Approximately 45–50 min later, PASMC were sedimented by centrifugation, resuspended in fresh medium, and placed onto petri dishes or coverslips. The cells were cultured in high-glucose (4.5 g/l) DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (BioFluids) and incubated in 5% CO2 at 37°C in a humidified atmosphere.

**DNA synthesis determination and cell counting.** [3H]Thymidine incorporation was determined to evaluate DNA synthesis and cell proliferation. Briefly, human PASMC were seeded in 24-well microplates at a density of ~2 x 104 cells/well, cultured in SMGM for 48 h, and growth arrested in SMBM for 24 h. Treated cells were then incubated in 0.2% FBS-SMGM with 1 mM ATP for 24 h before [3H]thymidine incorporation was measured. For small interfering RNA (siRNA) experiments, cells were transfected with control siRNA with scrambled sequence (si-Cont) and TRPC4-siRNA sequences for 0, 24, 48, or 72 h in the continuous presence of 100 μM ATP before [3H]thymidine uptake measurements. The dose-response curve of [3H]thymidine uptake in response to ATP treatment was best fitted using the SigmaPlot program.

For cell-counting experiments, previously growth-arrested (with 24-h SMBM) human PASMC were cultured in 0.2% FBS-SMGM with ATP for 48 h. Cell viability after 48 h was determined using 0.45% trypan blue (Sigma). Cell number was determined using a hemocytometer. The cell counts in the four 1-mm2 corner squares of the hemocytometer were averaged to calculate the total number of PASMC (1 x 10^6/ml) in the cell suspension.

**Western blot analysis.** Human PASMC were gently washed twice in cold phosphate-buffered saline (PBS), scraped into lysis buffer [1% Nonidet P-4 (Amresco), 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, and 30 μl/μl aprotinin], and incubated on ice for 30 min. Cell lysates were then sonicated and boiled in SDS-PAGE sample buffer for 5 min. The protein concentration in the supernatant was determined using a bicinchoninic acid protein assay with bovine serum albumin (BSA) as the standard. Aliquots of protein were mixed with ATP for 48 h. Cell viability after 48 h was determined using a fluorescence microscopy system and recorded on a personal computer for later analysis. [Ca2+]i was calculated from fura 2 fluorescence signals emitted from the cells using the AdCREB1 vector as described by Tokunou et al. (63).

**Transfection of cells with the recombinant adenovirus.** A recombinant adenoviral vector containing a CREB mutant (AdCREBM1) in which the phosphorylation site at 133 Ser was mutated to an alanine (AdCREBM1-133AA) was used to transfect human PASMC (32). The recombinant adenovirus was used to transfect human PASMC (32). The recombinant adenovirus was used to transfect human PASMC (32). The recombinant adenovirus was used to transfect human PASMC (32). The recombinant adenovirus was used to transfect human PASMC (32).
RESULTS

Extracellular ATP causes cell proliferation in a dose-dependent manner. In human PASMC, extracellular application of ATP acutely induced [Ca\(^{2+}\)]\textsubscript{i} increases in a dose-dependent manner. Low doses (0.1–1 mM) of ATP did not affect resting [Ca\(^{2+}\)]\textsubscript{i} (~180 nM in this cell, average 105 ± 6 nM; n = 32), while a high dose (5 mM) of ATP caused a significant, albeit transient, increase in [Ca\(^{2+}\)]\textsubscript{i} (Fig. 1, A and B) from a resting value of 147 ± 2 nM to a peak value of 1,030 ± 13 nM (n = 36; peak value ~1,230 nM in this cell). In contrast to human PASMC, rat PASMC exhibited much higher sensitivity to ATP. Extracellular application of 100 μM and 1 mM ATP significantly increased [Ca\(^{2+}\)]\textsubscript{i} (Fig. 1, A and B, insets). These results indicate that in human PASMC, but not in rat PASMC, 100 μM ATP is not sufficient to cause a transient increase in global [Ca\(^{2+}\)]\textsubscript{i}, which serves as an important mechanism or signal transduction pathway for inducing PASMC contraction (24). Potential underlying causes of this species-specific difference in the [Ca\(^{2+}\)]\textsubscript{i} response after ATP treatment are discussed below.

By measuring [\(^{3}\)H]thymidine uptake in human PASMC, we confirmed the proliferative effect of low doses of ATP. After 24-h treatment with ATP, [\(^{3}\)H]thymidine incorporation had increased 1.2-fold (P < 0.05), 1.4-fold (P < 0.001), and 1.6-fold (P < 0.001) from control (day 0) levels in the presence of 1, 10, and 100 μM ATP, respectively (Fig. 1C). The number of cells (Fig. 1C, inset) also increased in the presence of 100 μM ATP (1.7-fold; P < 0.01) under similar conditions. These data show that, despite its lack of an acute effect on [Ca\(^{2+}\)]\textsubscript{i} levels, long-term treatment of human PASMC with 100 μM ATP is sufficient to induce PASMC proliferation. We therefore focused on this low dose of ATP (100 μM) and attempted to elucidate the mechanism by which it causes cell proliferation.

ATP upregulates protein expression of TRPC4 channels and enhances CCE in PASMC. Having previously established that TRPC channels and CCE are important in vascular smooth muscle cell proliferation (13, 61, 72), we first verified whether ATP could also affect CCE by regulating TRPC4 channel expression. Western blot analysis (Fig. 2A) showed that 100 μM ATP significantly increased TRPC4 expression over the period of 48 h, with an increase of 1.7-fold (P < 0.001 vs. control) (Fig. 2A, inset). In addition to the increased amplitude of CCE, the [Ca\(^{2+}\)]\textsubscript{i} in the sarcoplasmic reticulum ([Ca\(^{2+}\)]\textsubscript{SR}) was also increased in PASMC treated with 100 μM ATP for 48 h ([Ca\(^{2+}\)]\textsubscript{SR} rise due to Ca\(^{2+}\) mobilization was increased from 121 ± 4 to 164 ± 9 nM), while the change in resting [Ca\(^{2+}\)]\textsubscript{i}, was not significant (Fig. 2, A and B).

In addition to the augmenting effect on TRPC4 expression, we then determined the effect of low-dose ATP on SOC-mediated CCE. As predicted, the ATP-mediated upregulation of TRPC4 was accompanied by a marked enhancement of cyclopiazonic acid (CPA; 10 μM)-induced CCE in PASMC, from 381 ± 16 to 535 ± 29 nM [Ca\(^{2+}\)]\textsubscript{i} (P < 0.001 vs. control) (Fig. 2B; n = 68). In addition to the increased amplitude of CCE, the [Ca\(^{2+}\)]\textsubscript{i} in the sarcoplasmic reticulum ([Ca\(^{2+}\)]\textsubscript{SR}) was also increased in PASMC treated with 100 μM ATP for 48 h ([Ca\(^{2+}\)]\textsubscript{SR} rise due to Ca\(^{2+}\) mobilization was increased from 121 ± 4 to 164 ± 9 nM), while the change in resting [Ca\(^{2+}\)]\textsubscript{i}, was not significant (Fig. 2A and B).

We also considered the possibility that [Ca\(^{2+}\)]\textsubscript{i}, might be increased because of an alternate mechanism after CPA application in the presence of ATP and reintroduced Ca\(^{2+}\). More specifically, the combination of store depletion and loss of extracellular Ca\(^{2+}\) may elevate intracellular Na\(^{+}\) levels via TRPC4 and result in diminished Na\(^{+}\)/Ca\(^{2+}\) exchange activity, as was suggested previously regarding TRPC3 channels in carbachol-stimulated human embryonic kidney (HEK-293) cells (53). Reintroduction of extracellular Ca\(^{2+}\) could cause the exchanger to operate in a reverse mode, thereby causing Ca\(^{2+}\) influx rather than its “normal” Ca\(^{2+}\) extrusion. ATP-treated human PASMC exposed to 10 μM KB-R9743, a Na\(^{+}\)/Ca\(^{2+}\) exchange inhibitor, still exhibited a significant increase in both Ca\(^{2+}\) release [128 ± 7 nM control (n = 53), 246 ± 25 nM ATP (n = 66); P < 0.001] and CCE levels (145 ± 10 nM control, 291 ± 37 nM ATP; P < 0.001), with no change in resting Ca\(^{2+}\) levels (108 ± 4 nM control, 109 ± 7 nM ATP) (Fig. 2C). These data indicate that ATP-induced CCE is me-
mediated primarily by SOC channels with little involvement of the reverse-mode Na\(^+\)/Ca\(^{2+}\) exchanger.

**Downregulation of TRPC4 mRNA using siRNA inhibits CCE and cell proliferation in PASMC.** To confirm that the low-dose ATP-induced TRPC4 gene upregulation (Fig. 2A) is responsible for enhanced CCE (Fig. 2B), a critical question to address is whether TRPC4 is involved in CCE in human PASMC. The molecular identity of SOC responsible for CCE remains unclear. TRPC4 is a TRPC isoform that can form heterotetramers with TRPC1 and TRPC5 and is involved in forming native SOC in many cell types (39, 58). Therefore, we tested whether TRPC4 in human PASMC is involved in forming Ca\(^{2+}\) channels that are activated by passive store depletion using CPA.

Using a previously identified siRNA specifically targeting TRPC4 (si-TRPC4) (13) (Fig. 3A), we were able to inhibit ATP-mediated upregulation of TRPC4 protein expression by 77% (Fig. 3B). The control siRNA with scrambled sequence (si-Cont) had no effect on TRPC4 expression. Furthermore, in PASMC treated with 100 μM ATP for 48 h, inhibition of TRPC4 expression with si-TRPC4 markedly attenuated the CPA-mediated CCE (Fig. 3C), bringing the amplitude of CCE down to 137 ± 7 nM from a control (i.e., si-Cont) value of 447 ± 17 nM. These data suggest that TRPC4 is involved, at least in human PASMC, in forming heterotetrameric store depletion-activated SOC channels. In parallel, si-TRPC4 caused a significant decrease in ATP-induced PASMC proliferation (Fig. 3D) over a 72-h period compared with cells treated with si-Cont. These results suggest that ATP-mediated upregulation of TRPC4 protein expression contributes to the enhancement of CCE and proliferation in human PASMC.

**ATP-induced CREB activation upregulates TRPC4 expression in human PASMC.** CREB has been identified as an important signal transduction element in agonist-mediated VSMC proliferation (32, 63). Consistent with its mitogenic effect on human PASMC, low-dose ATP (100 μM) significantly increased phosphorylation of CREB (Fig. 4A). Blockade of P\(_2\) receptors with suramin (100 μM) markedly attenuated ATP-mediated CREB phosphorylation (Fig. 4A), suggesting that ATP-mediated CREB phosphorylation is due to activation of a suramin-sensitive P\(_2\)X or P\(_2\)Y receptor.

ATP induced CREB phosphorylation in a time-dependent manner. Short-term (1–2 h) treatment of quiescent human PASMC with 100 μM ATP had little effect on the level of phosphorylated CREB (pCREB) (Fig. 4B). However, pCREB levels were significantly elevated after 4 h of treatment; the increase in pCREB levels maximized at 8 h and persisted after 24 h of treatment (Fig. 4B). Compared with the time course of the ATP-mediated effect on TRPC4 protein expression, these results indicate that ATP-induced CREB phosphorylation precedes TRPC4 upregulation (Fig. 4B) and that phosphorylated CREB may be involved in enhancing TRPC4 gene expression. Indeed, transfection of a nonphosphorylatable CREB mutant

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**Fig. 2.** ATP upregulates canonical transient receptor potential TRPC4 expression and enhances capacitative Ca\(^{2+}\) entry (CCE) in human PASMC. A: Western blot analysis (top) showing the time-dependent increase in TRPC4 protein expression induced by 100 μM ATP over a 48-h period; bottom: summary of the time course of ATP-induced TRPC4 expression (n = 3 experiments). **P < 0.001 vs. ATP 0 h. B: CCE induced by cyclopiazonic acid (CPA; 10 μM) shown as a function of time in control cells and cells treated with 100 μM ATP for 48 h (ATP) (a). Summarized data show resting [Ca\(^{2+}\)]\(_i\) (b) as well as increases in [Ca\(^{2+}\)]\(_i\) via Ca\(^{2+}\) release and CCE (c) in cells treated with ATP and without (control) ATP for 48 h (n = 32 cells for each group). C: time course of CPA-induced CCE in human PASMC before (n = 66) or after (n = 53) treatment with 100 μM ATP in the presence of 10 μM KB-R9743, a Na\(^+\)/Ca\(^{2+}\) exchanger blocker (a). Summarized data show resting [Ca\(^{2+}\)]\(_i\) (b) as well as increases in [Ca\(^{2+}\)]\(_i\) via Ca\(^{2+}\) release and CCE (c) in cells treated with ATP + KB-R9743; n = 53) and without (control + KB-R9743; n = 66) ATP for 48 h. **P < 0.01 vs. control. ***P < 0.001 vs. control, 0Ca, Ca\(^{2+}\)-free solution.

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**Fig. 3.** Downregulation of TRPC4 mRNA using siRNA inhibits CCE and cell proliferation in PASMC. A: Summary of the time course of ATP-induced TRPC4 expression (n = 3 experiments). B: ATP-mediated CREB phosphorylation (Fig. 4A). Blockade of P\(_2\) receptors with suramin (100 μM) markedly attenuated ATP-induced CREB phosphorylation (Fig. 4A), suggesting that ATP-mediated CREB phosphorylation is due to activation of a suramin-sensitive P\(_2\)X or P\(_2\)Y receptor.
(AdCREB-M1) using an adenoviral vector to human PASMC precluded ATP-mediated increase in TRPC4 expression (Fig. 4C). This strongly suggests that CREB activation is required for ATP-induced TRPC4 expression to occur.

Upregulated TRPC4 expression by ATP involves protein kinase activation. As shown in Fig. 4A, the ATP-mediated increase in CREB phosphorylation was due mainly to activation of suramin-sensitive P2 receptors. At this stage, we cannot ascertain whether CREB phosphorylation occurs because of stimulation of P2x or P2y receptors. Suramin can block both P2x (42) receptors, which form ligand-gated channels, and P2y receptors (65), which belong to the superfamily of G protein-coupled receptors (GPCRs) (7, 65). GPCR are coupled to many signaling pathways, including those involved in protein kinase-mediated phosphorylation. CREB phosphorylation at S133 by protein kinase A (PKA) in response to cAMP and other kinases is sufficient to induce the transcription and expression of several genes. PKA in particular acts as a central processing hub not only in mediating but also in transmitting the effects of cAMP to different effector proteins, such as PKC, PKB, and mitogen-activated protein kinase (MAPK) (7, 50). Protein kinases may therefore be involved in the signal transduction pathway that occurs between plasmalemmal receptor binding by ATP and CREB phosphorylation before TRPC4 gene transcription.

We verified that PKA and PKG might be involved in the enhanced TRPC4 transcription and expression induced by 100 μM ATP. Inhibition of PKA or PKG with 10 μM H-89 or 2 μM KT-5823 (48-h treatments) significantly attenuated ATP-induced TRPC4 upregulation (Fig. 5). Taken together, these results suggest that ATP-mediated TRPC4 upregulation involves PKA- or PKG-induced phosphorylation of CREB.

**DISCUSSION**

Pulmonary vascular remodeling due to excessive smooth muscle and endothelial proliferation is a major cause of elevated PVR and pulmonary arterial pressure (PAP) in patients with pulmonary hypertension (57). Upregulation of TRPC channels is an important mediator of vascular smooth muscle and endothelial proliferation (13, 21, 61, 72). A potent mitogen, extracellular ATP, also plays an important role in the development of pulmonary vascular wall remodeling (19). Our results suggest that the increased PASMC proliferation induced by low-dose ATP is due, at least in part, to increased TRPC4 channel expression and activity via P2 receptor stimulation and increased CREB phosphorylation. Although the data do not fully establish a causal relationship between CCE via TRPC4-encoded SOC and PASMC proliferation, the data do provide evidence that TRPC4 and SOC are involved in PASMC proliferation.
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ATP upregulates TRPC4 expression by inducing cAMP response element-binding protein (CREB) phosphorylation. A: Western blot analysis (left) of phosphorylated CREB (pCREB) in human PASMC treated with (ATP) or without (Cont) 100 μM ATP for 8 and 12 h in the absence (−) or presence (+) of 100 μM suramin. Summarized data averaged from 3 experiments are shown at right. ***P < 0.001 vs. Cont (open bar). B: time course of ATP-induced pCREB expression. Western blot analysis of pCREB and α-actin levels (a) in cells treated with (ATP) and without (Cont) 100 μM ATP for 1, 2, 4, 8, 12, 24, and 48 h. Summarized data (a, bottom) from 3 experiments indicate a transient increase in pCREB levels between 4 and 24 h of ATP treatment. The time courses of ATP-mediated increases in pCREB (closed circles) and TRPC4 (open circles; same data shown in Fig. 2A) are compared in b. C: ATP-induced TRPC4 upregulation is abolished by a nonphosphorylatable CREB mutant. Western blot analysis of TRPC4 and α-actin levels (left) in human PASMC infected with an empty vector (C) or with an adenoviral vector containing the nonphosphorylatable CREB-M1 mutant (Ad-C) before (Cont) and after (ATP) 48 h of treatment with 100 μM ATP. Summarized data from 4 experiments are shown at right. *P < 0.05 vs. cells not treated with ATP. ***P < 0.001 vs. empty vector-infected cells treated with ATP.

Effect of ATP on [Ca2+]i, and CREB phosphorylation. In human PASMC, acute application of low doses (0.1–1 mM) of ATP did not evoke transient increases in [Ca2+]i (Fig. 1), while high-dose ATP caused a large, transient increase in [Ca2+]i. The former observation contrasts with reports showing that ATP at concentrations as low as 100 μM can cause cyclical Ca2+ oscillations in rat PASMC (present study), human coronary artery SMC, murine colonic SMC, and vascular endothelial cells (28, 31) mainly because of Ca2+ release from inositol-1,4,5-trisphosphate (IP3)-sensitive sarcoplasmic reticulum (SR) stores. Therefore, the difference in Ca2+ reactivity to low-dose ATP we observed may be attributed to the different expression levels of sarcolemmal purinoceptors and the different sensitivities of IP3 receptors in the SR membrane in human PASMC relative to the other preparations.

In spite of the inability to induce transient increases in [Ca2+]i, long-term treatment with the low dose of ATP markedly upregulated TRPC4 expression (Fig. 2A), enhanced CCE amplitude (Fig. 2B), and increased proliferation (Fig. 1C) in human PASMC. Furthermore, ATP-mediated CREB phosphorylation preceded the ATP-induced increases in TRPC4 expression (Fig. 4B). Inhibition of CREB phosphorylation by overexpressing a nonphosphorylatable CREB mutant in human PASMC abolished ATP-mediated upregulation of TRPC4 and significantly inhibited ATP-mediated cell proliferation (data not shown). Taken together, these results suggest that treatment of human PASMC with ATP, at concentrations insufficient to cause an acute increase in [Ca2+]i, can phosphorylate CREB, upregulate TRPC4 expression, enhance CCE, and stimulate proliferation (Fig. 6).

Fig. 5. Inhibition of protein kinase A (PKA) and PKG abolishes ATP-induced TRPC4 upregulation. A: Western blot analysis of TRPC4 and α-actin levels in human PASMC treated with 100 μM ATP for 48 h in the absence (Cont) and presence of 10 μM N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride (H-89; a PKA inhibitor) or 2 μM KT-5823 (KT, a PKG inhibitor). B: summarized data from 3 experiments showing the inhibitory effect of H-89 and KT-5823 on ATP-mediated TRPC4 expression. ***P < 0.001 vs. ATP control (open bar).
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Inducing a transient increase in \([\text{Ca}^{2+}]_i\) via \([\text{Ca}^{2+}]_i\) release and influx (8, 67). On the other hand, the change in \([\text{Ca}^{2+}]_i\) induced by low-dose ATP may occur at a more local level that does not influence global \([\text{Ca}^{2+}]_i\) levels. Lipp et al. (34) described the role of IP3 receptor-mediated \([\text{Ca}^{2+}]_i\) puffs in the regulation of perinuclear \([\text{Ca}^{2+}]_i\), which may then regulate key nuclear functions such as gene transcription. A similar case may also be made for ryanodine receptor-mediated \([\text{Ca}^{2+}]_i\) sparks, which have been described in rat PASMC (47) and may be involved in \([\text{Ca}^{2+}]_i\) cross signaling between IP3 and ryanodine receptors (73). Therefore, without more conclusive evidence, we cannot say with certainty that ATP-induced CREB activation occurs via a \([\text{Ca}^{2+}]_i\)-independent mechanism.

**Mechanisms involved in ATP-mediated CREB phosphorylation and TRPC4 upregulation.** Thrombin-induced aortic SMC proliferation and hypertrophy are mediated by CREB-dependent gene transcription of the c-fos gene (63). Inhibition of CREB phosphorylation attenuates neointimal proliferation in rat carotid artery by downregulating Bcl-2, an anti-apoptotic protein (64). Therefore, CREB is a transcription factor that plays an important role in the vascular remodeling process.

Various kinases are able to phosphorylate CREB, including PKA (22), PKG, PKC (69), Ca2+/calmodulin-dependent protein kinases (CaM) (8, 59), Akt/protein kinase B (45), MAPK (3), and Ras-dependent protein kinase (52). The phosphorylation of CREB on Ser133 enables CREB to bind to the cAMP response element (CRE) and to modulate transcription of the genes (e.g., bcl-2, c-fos) whose promoters contain a CRE binding sequence. In the present study, we have demonstrated that ATP-induced TRPC4 upregulation was attenuated when PKA and PKG were inhibited (Fig. 5), indicating that CREB was activated or phosphorylated by PKA/PKG-dependent pathways when human PASMC were treated with low-dose ATP for 4–24 h. The initial PKA/PKG-mediated CREB phosphorylation appeared not to be dependent on transient increases in \([\text{Ca}^{2+}]_i\), because acute application of 100 \(\mu\text{M}\) ATP had little effect on \([\text{Ca}^{2+}]_i\), in human PASMC (Fig. 1A).

The phosphorylated CREB, by binding to the CRE, can upregulate many proteins and factors that regulate cell proliferation and survival (3, 32, 48, 49, 63). Although our observations provide evidence that ATP-mediated upregulation of TRPC4 is related to phosphorylation of CREB, it is still unclear how CREB modulates TRPC4 gene transcription. In other words, we still do not know whether ATP-mediated TRPC4 upregulation is due directly to binding of CREB to CRE in the TRPC4 gene promoter or indirectly to a CREB-induced intermediate (e.g., c-Fos or c-Jun) that subsequently upregulates or facilitates TRPC4 gene transcription.

In contrast to our finding that CREB phosphorylation preceded TRPC4 upregulation in human PASMC treated chronically with ATP, a recent study (46) suggested that enhanced SOCE (but not \([\text{Ca}^{2+}]_i\) entry through L-type voltage-dependent \([\text{Ca}^{2+}]_i\) channels) led to CREB phosphorylation and c-fos transcription in rat and human cerebral arteries and rat aorta. In light of these new findings, it is apparent that upregulation of TRPC4 can initially be caused by \([\text{Ca}^{2+}]_i\)-dependent and -independent mechanisms. The subsequent enhancement of CCE and rises in \([\text{Ca}^{2+}]_i\), may further upregulate TRPC4 gene expression, forming a positive feedback mechanism to increase \([\text{Ca}^{2+}]_i\), required for cell cycle progression and PASMC proliferation. These observations also suggest that the physiological role of TRPC4 in the regulation of vascular contractility and VSMC proliferation is complex.
expression was unaffected by ATP treatments (data not shown). In parallel with the increased expression of TRPC4, CPA-induced CCE was also upregulated in PASMC treated with ATP (Fig. 2B). Both the ATP-induced TRPC4 upregulation and CCE enhancement were abolished by TRPC4-targeted siRNA application (Fig. 3, B and C), as was ATP-induced proliferation (Fig. 3D). These observations strongly suggest that ATP-induced human PASMC proliferation is partially mediated by the upregulation of tetrameric SOC comprising part of TRPC4 proteins.

A rise in [Ca\(^{2+}\)]_i is essential for cell proliferation (1, 35); removal or chelation of extracellular Ca\(^{2+}\) significantly inhibits PASMC proliferation in the presence of serum and growth factors (21). In the cell cycle, there are at least four CaM-sensitive steps: 1) the G0-to-G1 transition, 2) the G1-to-S transition, 3) the G2-to-M transition, and 4) the M phase (1, 35). Although low-dose ATP does not trigger a transient rise in [Ca\(^{2+}\)]_i, the upregulated TRPC4 channels and enhanced CCE resulting from long-term ATP treatment would amplify and help to maintain mitogen-induced increases in [Ca\(^{2+}\)]_i, accelerate transitions in the cell cycle, and stimulate PASMC proliferation (Fig. 6). Maintenance of sufficient Ca\(^{2+}\) within the SR is also required for cell growth, and depletion of the SR Ca\(^{2+}\) store induces growth arrest (27, 54) and triggers apoptosis (26). A recent study by Rosker et al. (53) also suggested that Na\(^+\) influx via TRPC3 channels might influence Na\(^+/\)Ca\(^{2+}\) exchanger activity in carbchol-treated HeLa cells, leading to enhanced Ca\(^{2+}\) influx via reverse-mode Na\(^+/\)Ca\(^{2+}\) exchange upon reintroduction of extracellular Ca\(^{2+}\). Our data (Fig. 2C) clearly demonstrate that Na\(^+/\)Ca\(^{2+}\) exchange plays no role in the ATP-mediated enhancement of CCE. Because CCE is critical in refilling SR Ca\(^{2+}\) stores and in maintaining [Ca\(^{2+}\)]_i, increased CCE amplitude due to enhanced TRPC4 expression may also participate in promoting cell proliferation by increasing [Ca\(^{2+}\)]_i in human PASMC.

**Physiological and pathophysiological implications.** In light of its mitogenic effects and its multiple sources, extracellular ATP may play a critical role in the development of pulmonary vascular disease (37, 38, 56). In hypoxia-mediated pulmonary hypertension, for example, extensive pulmonary vascular remodeling can occur in addition to increased vasoconstriction, with both elements contributing to increased PVR and PAP. While much of the mediial hypertrophy is due to PASMC hyperplasia and/or hypertrophy (11, 57), hypoxia also triggers the differentiation of adventitial fibroblasts and medial quiescent PASMC to synthetic phenotypes, leading to increased cell growth (10, 56). More recently, compelling evidence has indicated that, during hypoxia, ATP released from endothelial cells and fibroblasts induces fibroblast transdifferentiation to myofibroblasts, DNA synthesis, and proliferation of pulmonary adventitial fibroblasts (19, 56, 57). Our observations suggest that, under normoxic conditions, low-level ATP stimulation is sufficient to cause PASMC proliferation via a CREB and TRPC4-dependent pathway. Therefore, small increases in extracellular ATP concentration may play a significant role in PASMC proliferation. Our findings may be relevant to unraveling the pathophysiological basis of severe pulmonary hypertension.

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