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RESEARCH PAPER

Protein kinase A and the exchange protein directly activated by cAMP (Epac) modulate phenotype plasticity in human airway smooth muscle

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BACKGROUND AND PURPOSE

Platelet-derived growth factor (PDGF) modulates the airway smooth muscle (ASM) 'contractile' phenotype to a more 'proliferative' phenotype, resulting in increased proliferation and reduced contractility. Such phenotypic modulation may contribute to airway remodelling in asthma. We have previously shown that the cAMP effector molecules, protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac) inhibited PDGF-induced phenotypic modulation in bovine ASM. Here, we investigated these mechanisms in human ASM strips and cells.

EXPERIMENTAL APPROACH

ASM strips were incubated with PDGF in the absence or presence of the activators of Epac (8-pCPT-2'-*O*-Me-cAMP) or of PKA (6-Bnz-cAMP) for 4 days. Strips were mounted for isometric contraction experiments or analysed for the expression of contractile markers. Cell proliferation was measured and proliferative markers were analysed under similar conditions.

KEY RESULTS

Activation of Epac and PKA prevented PDGF-induced ASM strip hypocontractility, and restored the expression of smooth muscle actin, myosin and calponin, which had been markedly diminished by PDGF. Epac and PKA activation inhibited the PDGF-induced ASM cell proliferation and G_1/S phase transition and the expression and phosphorylation of cell cycle regulators.

CONCLUSIONS AND IMPLICATIONS

Epac and PKA maintain a normally contractile ASM phenotype in a mitogenic environment, suggesting that specific activators of Epac and PKA may be beneficial in the treatment of airway remodelling in asthma.

Abbreviations

α-SMA, α-smooth muscle actin; ASM, airway smooth muscle; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; Epac, exchange protein directly activated by cAMP; FBS, foetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KH, Krebs-Henseleit; PDGF, platelet-derived growth factor; PFA, paraformaldehyde; PI, propidium iodide; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; Rb, retinoblastoma tumor suppressor protein; RIPA, radioimmunoprecipitation assay; *sm*-MHC, smooth muscle myosin heavy chain; TBS(T), Tris buffered saline with Tween

Introduction

Asthma is a chronic inflammatory disease characterized by exaggerated bronchoconstriction (airway hyper-responsiveness) and permanent structural changes in the airways (airway remodelling). Airway smooth muscle (ASM) cells participate importantly in lung physiology and airway disease pathogenesis because of their ability not only to contract, but also to proliferate and to synthesize several inflammatory and contractile mediators. Such multi-functionality is reflected by ASM phenotypic plasticity, which refers to the existence of distinct ASM phenotypes with unique morphological and functional properties, and the ability to switch between the different phenotypes (Halayko and Amrani, 2003). Thus, exposure of ASM cells to mitogenic stimuli and extracellular matrix components *in vitro* modulates ASM phenotype to a proliferative, hypocontractile phenotype, characterized by increased proliferation, decreased expression of contractile proteins – such as smooth muscle α-actin (α-SMA), smooth muscle myosin heavy chain (*sm*-MHC) and calponin – and decreased contractile capability (Hirst *et al.*, 2000a,b; Gosens *et al.*, 2002; Dekkers *et al.*, 2007; Roscioni *et al.*, 2011). After serum deprivation, or in the presence of either insulin or transforming growth factor-β (TGF-β), the proliferative ASM cells can return to their contractile state or even a hypercontractile state, indicating that phenotypic alterations are highly dynamic processes (Ma *et al.*, 1998; Schaafsma *et al.*, 2007; Dekkers *et al.*, 2009). Furthermore, studies have proved the functional significance of ASM phenotype modulation in intact human ASM tissue (Moir *et al.*, 2003). Although *in vivo* evidence of ASM phenotypic plasticity is still lacking, these processes may participate in airway disease pathogenesis (Halayko and Stephens, 1994; Hirst, 1996; Hirst *et al.*, 2000b; Hirota *et al.*, 2009). In fact, increased ASM proliferation could explain the accumulation of ASM within the airway wall (Ebina *et al.*, 1993), a striking feature of chronic asthma which may also contribute to airway hyper-responsiveness and the decline in lung function in asthmatics (Lambert *et al.*, 1993; Bousquet *et al.*, 2000; Oliver *et al.*, 2007). Therefore, identification of mechanisms that regulate ASM phenotypic plasticity is essential in order to target pharmacologically any dysregulated ASM proliferative and contractile responses.

β_2-Adrenoceptor agonists that raise cAMP are widely used in the treatment of exaggerated bronchoconstriction in asthma (Scheid *et al.*, 1979; Torphy, 1994; Giembycz and Newton, 2006). Intracellular cAMP levels are tightly controlled by the activities of adenylyl cyclases (Bogard *et al.*, 2011) and phosphodiesterases (PDEs) (Houslay *et al.*, 2007). cAMP Elevation of cAMP results in the activation of protein kinase A (PKA) (Pidoux and Tasken, 2010), and the exchange protein directly activated by cAMP (Epac), which triggers a wide range of biological responses by favouring the GDP/GTP exchange on small GTPases (Gloerich and Bos, 2010; Grandoch *et al.*, 2010). In ASM, activation of PKA and Epac induce relaxation (Scheid *et al.*, 1979; Giembycz and Newton, 2006; Roscioni *et al.*, 2010). G_s-protein-coupled receptor agonists, as well as direct activators of PKA and Epac, also inhibit mitogen-induced proliferation of cultured ASM (Tomlinson *et al.*, 1994; Kassel *et al.*, 2008; Misior *et al.*, 2009; Yan *et al.*, 2010; Roscioni *et al.*, 2011) and vascular smooth muscle cells (VSMCs; Mayer *et al.*, 2010; Hewer *et al.*, 2011). In VSMCs, such effects were associated with a diminished expression of G_1/S phase cell cycle regulators, including cyclin D, and diminished phosphorylation of the retinoblastoma tumour suppressor protein (Rb) resulting in G_1 arrest (Hewer *et al.*, 2011). Moreover, cAMP-elevating agents prevented the shift from a contractile/quiescent VSMC phenotype to a synthetic/activated one, which is important in vascular remodelling (Maurice *et al.*, 2003). Recently, we have demonstrated that activation of Epac and/or PKA inhibits mitogen-induced proliferation of bovine ASM (Roscioni *et al.*, 2011). In addition, activation of the two cAMP effectors also prevented platelet-derived growth factor (PDGF)-induced hypocontractility of bovine ASM strips, a result paralleled by restoration of contractile protein expression (Roscioni *et al.*, 2011). However, the role of cAMP effectors in the regulation of phenotypic modulation of human ASM has not been explored yet. Therefore, using intact human ASM strips and primary and immortalized ASM cells, we have investigated the effects of Epac and PKA activation on contractility, contractile protein expression, cell proliferation and cell cycle progression.

Methods

Tissue preparation and organ culture

Human tracheal sections from anonymized lung transplantation donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen. After dissection of the smooth muscle layer and careful removal of the mucosa and connective tissue, human tracheal smooth muscle strips of identical length and width were prepared as described for bovine tissue (Gosens *et al.*, 2002; Dekkers *et al.*, 2007). Tissue strips were transferred to serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45 $\mu g \cdot mL^{-1}$), penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$), amphotericin B (1.5 $\mu g \cdot mL^{-1}$), apo-transferrin (human, 5 $\mu g \cdot mL^{-1}$) and ascorbic acid (100 μM). The strips were cultured for 4 days in an Innova 4000 incubator shaker (37°C, 55 r.p.m.). The Epac activator 8-pCPT-2′-O-Me-cAMP (30 μM), the PKA activator 6-Bnz-cAMP (500 μM) and/or PDGF (10 $\mu g \cdot mL^{-1}$) were present during the entire incubation period. PDGF was added 30 min after the other stimuli. After culture, strips were thoroughly washed and mounted for isometric tension measurements or snap frozen for Western blot analysis.

Isometric tension measurements

Isometric contraction experiments were performed as described previously (Gosens *et al.*, 2002; Dekkers *et al.*, 2007; Roscioni *et al.*, 2011). Briefly, human ASM strips were thoroughly washed and mounted for isometric recording in organ-baths, containing Krebs-Henseleit (KH)-buffer (composition in mM: 117.5 NaCl, 5.6 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 1.28 NaH_2PO_4, 25 $NaHCO_3$ and 5.5 glucose) at 37°C. During a 90 min equilibration period with wash-outs every 30 min, resting tension was adjusted to 0.5 g, followed by pre-contractions with 20 and 40 mM KCl. Following wash-out, maximal relaxation was established by the addition of

(-)-isoprenaline (0.1 μM). Tension was readjusted to 0.5 g, immediately followed by two changes with KH buffer. After another equilibration period of 30 min, cumulative concentration–response curves were constructed to KCl (5.6 mM–50 mM) or methacholine (1 nM–0.1 mM). When maximal tension was reached, strips were washed several times and maximal relaxation was established by using (-)-isoprenaline (10 μM). Contractions were expressed as percentage of maximal contraction induced by KCl or methacholine in vehicle-treated control strips. After the experiment, strips were snap frozen for Western blot analysis.

Cell culture

After the removal of mucosa and connective tissue, human ASM tissue was chopped using a McIlwain tissue chopper, three times at a setting of 500 μm and three times at a setting of 100 μm (Gosens *et al.*, 2002). Tissue slices were washed with DMEM containing 10% foetal bovine serum (FBS), placed in 25 cm^2 culture flasks to allow adherence. ASM cells were cultured in DMEM containing 10% serum and passages 1–5 were used. Alternatively, human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT-ASM cells) (Roscioni *et al.*, 2009; 2011) were used for cell proliferation measurements. Cells were grown in DMEM supplemented with 50 U·mL^{-1} streptomycin, 50 μg·mL^{-1} penicillin, 10% (v/v) FBS and 3 mL fungizone (1.5 μg·mL^{-1}).

[^{3}H]thymidine incorporation and cell count

Primary and immortalized ASM cells were grown to subconfluence in 24-well plates. The next day, cells were washed with PBS and made quiescent by incubation with free-serum medium supplemented with antibiotics and insulin, transferrin and selenium for 72 h. Subsequently, cells were incubated with 8-pCPT-2′-*O*-Me-cAMP (30 μM) and/or 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 28 h, the last 24 h in the presence of [^{3}H]thymidine (0.25 μCi·mL^{-1}). After incubation, cells were washed twice with PBS at room temperature and subsequently with ice-cold 5% trichloroacetic acid on ice for 30 min and the acid-insoluble fraction was dissolved in 1 mL NaOH (1 M). Incorporated [^{3}H]thymidine was quantified by liquid-scintillation counting using a Beckam LS1701 β-counter as described for bovine ASM cells (Roscioni *et al.*, 2011).

Alternatively, following serum deprivation, primary ASM cells were treated with 8-pCPT-2′-*O*-Me-cAMP (30 μM) and/or 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (10 ng·mL^{-1}). After 4 days, cells were counted using a Bürker-Türk counting chamber (Marienfeld, Germany).

Flow cytometric cell cycle analysis

Primary and immortalized ASM cells were grown to subconfluence in six-well plates. The next day, cells were washed with PBS and made quiescent by incubation with free-serum medium supplemented with antibiotics and insulin, transferrin and selenium for 72 h. Subsequently, cells were incubated with 8-pCPT-2′-*O*-Me-cAMP (100 μM) and/or 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (30 ng·mL^{-1}) for 24 h, and were harvested and washed with warm PBS. Cell were then resuspended in ice-cold PBS and fixed by transfer to ice-cold 70% ethanol, before finally being resuspended in a solution containing 10 μg·mL^{-1} propidium iodide (PI), 20 mM EDTA, 0.05% Tween20 and 50 μg·mL^{-1} RNAse. Cell cycle analysis was performed on a BD FACSCalibur (Becton, Dickinson and Company, BD, Franklin Lakes, NJ, USA). Fluorescence histograms were collected for at least 10 000 cells, satisfying light scatter and doublet discrimination criteria as previously described (Nunes *et al.*, 2008). The cell cycle distributions were analysed by using ModFit LT flow cytometry modelling software (ModFit LT, version 3.2) obtained from Verity Software House (Topsham, ME, USA).

Western blot analysis

ASM strip homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonication in radioimmunoprecipitation assay buffer (composition in mM: 50 mM Tris·HCl, 150.0 NaCl, 1.0 EDTA, 1.0 phenylmethylsulphonyl fluoride, 1.0 Na_3VO_4, 1.0 NaF, pH 7.4, supplemented with 10 μg·mL^{-1} leupeptin, 10 μg·mL^{-1} aprotinin, 10 μg·mL^{-1} pepstatin, 0.25% sodium and 1% Igepal). Protein content was determined using the Pierce BCA protein assay. Equal amounts of protein were separated on a 10% for GAPDH, α-smooth muscle actin (α-SMA), calponin and cyclin D1; on a 8% gel for phospho-Rb and on a 6% gel for *sm*-MHC. Proteins were transferred onto nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline plus Tween20 and incubated overnight with primary antibodies. After washing, the membranes were incubated with horseradish peroxidase-labelled secondary antibody. Protein bands were visualized using Western lightning plus-ECL and quantified using TotalLab software (Nonlinear Dynamics, Newcastle, UK). All protein levels were normalized to GAPDH.

Immunofluorescence

Primary ASM cells were plated on 8-well Lab-Tek™ Chamber Slides (Thermo Scientific, Pittsburgh, PA, USA) at a density of 5000 cells per well. Cells were treated with 8-pCPT-2′-O-Me-cAMP (30 μM) and/or 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. After stimulation, cells were fixed with 3% paraformaldehyde (PFA) for 15 min at room temperature, followed by 3% PFA + 0.3% Triton X100 for 5 min at room temperature and washed twice with cold cytoskeletal buffer (composition in mM: 10 2-(*N*-morpholino) ethanesulphonic acid, 150 NaCl, 5 ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA), 5 $MgCl_2$, 5 glucose, pH 6.1) After fixing, the cells were blocked overnight at 4°C with a blocking buffer solution containing 1% bovine serum albumin and 2% donkey serum. The following day, cells were incubated overnight at 4°C with the primary antibody α-SMA (dilution 1:100) in CytoTBST (composition: 20 mM Tris base, 0.154 M NaCl, 2 mM EGTA, 2 mM $MgCl_2$ and 1 mL Tween-20). The day after, cells were washed three times for 10 min with CytoTBST. Cells were incubated with the secondary antibody donkey anti-mouse Cy3 (Red) (dilution 1:50 in CytoTBST) for 3 h at room temperature. After the incubation, cells were washed and phalloidin Alexa Fluor 488 was added for 15 min, followed by four washing steps of 15 min with CytoTBST. After that, nuclei were stained with Hoechst (dilution 1:10 000 in CytoTBST) for 5–10 s, immediately followed by two quick and 4–10 min washing steps with

water. After staining, coverslips were mounted using ProLong Gold antifade reagent (InVitrogen) and analysed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens, Olympus Corporation, Tokyo, Japan).

Data analysis

Data represent means ± SEM from *n* individual experiments. The statistical significance of differences between means was determined by the Student's *t*-test for paired or unpaired observations. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Methacholine hydrochloride was from ICN Biomedicals (Costa Mesa, CA, USA). 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP were from BIOLOG Life Science Institute (Bremen, Germany). Human PDGF-AB (-)-isoprenaline hydrochloride, protease inhibitors, apo-transferrin, anti-β-actin, anti-α-SMA, Triton X-100, Tween20, RNAse and secondary antibodies were from Sigma-Aldrich (St. Louis, MO, USA). FBS was obtained from Hyclone Thermo Scientific (Waltham, MA, USA) and propidium iodide was from Fluka (Buchs, Switzerland). Anti-GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and *sm*-MHC and calponin antibodies were from Neomarkers (Fremont, CA, USA). [^{3}H]-thymidine was from Amersham (Buckinghamshire, UK). Western lightning plus-ECL was from PerkinElmer Inc. (Waltham, MA, USA) and Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA). Prolong Gold Antifade reagent, Hoechst and CY3 were purchased from InVitrogen (Carlsbad, CA, USA). All other used chemicals were of analytical grade.

Results

Activation of Epac and PKA inhibit PDGF-induced ASM hypocontractility

Four days of treatment with PDGF (10 ng·mL^{-1}) resulted in a significant decrease of both KCl-induced (Figure 1A) and methacholine-induced (Figure 1B) ASM strip contractions ($P < 0.01$, Table 1). We previously reported that such responses in bovine tracheal smooth muscle (BTSM) were inhibited by co-treatment with activators of the cAMP effector Epac and PKA (Roscioni *et al.*, 2011). Here, we studied the occurrence of these mechanisms in human ASM. As described in BTSM strips (Roscioni *et al.*, 2011), activation of Epac or PKA did not affect basal ASM contractility (not shown). Importantly, co-incubation with the Epac activator 8-pCPT-2′-*O*-Me-cAMP completely prevented the PDGF-induced decrease in KCl-induced ($P < 0.01$, Figure 1A, Table 1) and methacholine-induced ($P < 0.05$, Figure 1C, Table 1) contractions. Similarly, co-treatment with the PKA activator for 4 days significantly prevented the PDGF-induced decrease in methacholine-induced ($P < 0.05$, Figure 1D, Table 1) contractions and largely counteracted the decrease in KCl-induced (Figure 1B, Table 1) contraction by PDGF.

In BTSM strips, the reduced contractility induced by PDGF has been shown to be accompanied by a reduced expression of contractile proteins, including α-SMA, *sm*-MHC and calponin (Dekkers *et al.*, 2007; Roscioni *et al.*, 2011). In line with these findings, long-term treatment with PDGF also significantly reduced the basal expression of α-SMA in human ASM cells and tissue, as measured by immunofluorescence (Figure 2A) and Western blot ($P < 0.01$, Figure 2B). Moreover, phalloidin-stained filamentous actin was also reduced by PDGF treatment (Figure 2A). Interestingly, as shown by both immunofluorescence staining (Figure 2A) and Western blot analysis ($P < 0.01$ both; Figure 2B), these effects of PDGF were largely inhibited by 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP. Both cAMP analogues alone did not affect the basal expression of filamentous actin (not shown) and α-SMA (Figure 2). Similar to the effect on α-SMA expression, PDGF also reduced the expression of *sm*-MHC and calponin ($P < 0.05$, Figure 3), which was also prevented by activators of Epac and PKA ($P \leq 0.05$, Figure 3).

Activation of Epac and PKA inhibits PDGF-induced ASM cell proliferation

To assess whether alterations in ASM contractility by PDGF, 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP are associated with changes in ASM proliferative responses, cell proliferation assays were performed using primary (Figure 4) and immortalized (not shown) ASM cells. DNA synthesis was significantly increased by PDGF (10 ng·mL^{-1}) treatment ($P < 0.01$, Figure 4A). This response was partially prevented by

Table 1

Contractile responses of human ASM strips to KCl or methacholine after 4 days of culturing without (control) or with 8-pCPT-2′-*O*-Me-cAMP (30 μM) or 6-Bnz-cAMP (500 μM) in the presence of PDGF (10 ng·mL^{-1})

Treatment	KCl E_{max} (%)	EC_{50} mM	Methacholine E_{max} (%)	pE_{50} (–logM)
Control	100 ± 0	27.3 ± 1.9	100 ± 0	6.32 ± 0.22
PDGF	62.0 ± 9.8**	29.9 ± 0.8	56.6 ± 12.1**	6.18 ± 0.14
+ 8-pCPT-2′-*O*-Me-cAMP	110.9 ± 14.2##	27.0 ± 0.9	112.1 ± 18.6#	6.22 ± 0.20
+ 6-Bnz-cAMP	112.0 ± 29.0	28.4 ± 0.9	93.1 ± 13.2#	6.04 ± 0.30

The values shown in the Table were derived from the concentration-response curves shown in Figure 1. Data represents means ± SEM of three to six independent experiments, performed in duplicate. Results are expressed as % of corresponding values for Control-treated strips due to intervariability between strips. **$P < 0.01$ compared with Control; #$P < 0.05$, ##$P < 0.01$, compared with PDGF.

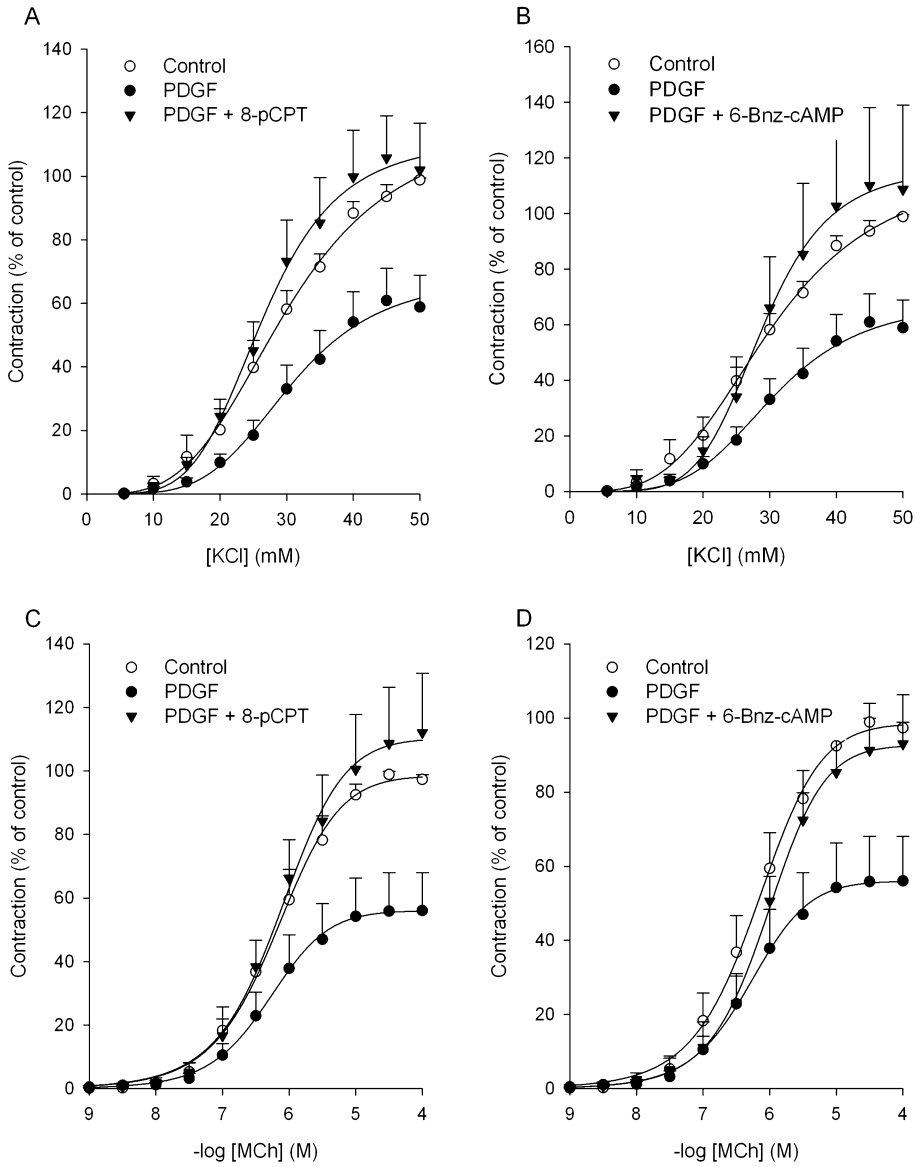

Figure 1

8-pCPT-2′-*O*-Me-cAMP (8-pCPT) and 6-Bnz-cAMP prevent PDGF-induced hypocontractilityof human ASM strips. Concentration–response curves of KCl-induced (upper panel) and methacholine(MCh)-induced (lower panel) contractions of human ASM strips treated without (control) or with PDGF (10 ng·mL^{-1}) in the absence or presence of (A and C) 8-pCPT-2′-*O*-Me-cAMP (8-pCPT, 30 μM) and (B and D) 6-Bnz-cAMP (500 μM) for 4 days. Data represent means ± SEM of three to six experiments, performed in duplicate.

co-treatment with 8-pCPT-2′-*O*-Me-cAMP (30 μM) or 6-Bnz-cAMP (500 μM) ($P < 0.01$, Figure 4A), whereas no significant effects were observed with the compounds alone (Figure 4A). To evaluate potential synergism between the two cAMP effectors, cells were stimulated with the combination of 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP. Such strategy appeared more potent than the single treatments in inhibiting PDGF-induced DNA synthesis ($P < 0.01$, Figure 4A). However, no synergistic or additive effects of the two cAMP analogues were observed (Figure 4A). Similarly, PDGF treatment resulted in an increased cell number ($P < 0.01$, Figure 4C). This response was completely inhibited by 8-pCPT-2′-*O*-Me-cAMP and by 6-Bnz-cAMP ($P < 0.05$, Figure 4C).

Activation of Epac and PKA inhibits PDGF-induced ASM cell cycle progression

Epac and PKA have been recently shown to provoke G_1 arrest of the cell cycle in VSMCs by inhibition of cyclin D expression and reduction of Rb phosphorylation (Hewer *et al.*, 2011). In view of their anti-mitogenic properties in human ASM, we tested whether the Epac and PKA activators inhibited cell cycle progression induced by PDGF, by using flow cytometry in combination with PI staining of DNA (Figure 5). We performed our experiments in hTERT-ASM cells, in which Epac and PKA similarly inhibited PDGF-induced DNA synthesis compared with the primary human ASM cells (not shown). Upon treatment with PDGF (30 ng·mL^{-1}), a higher

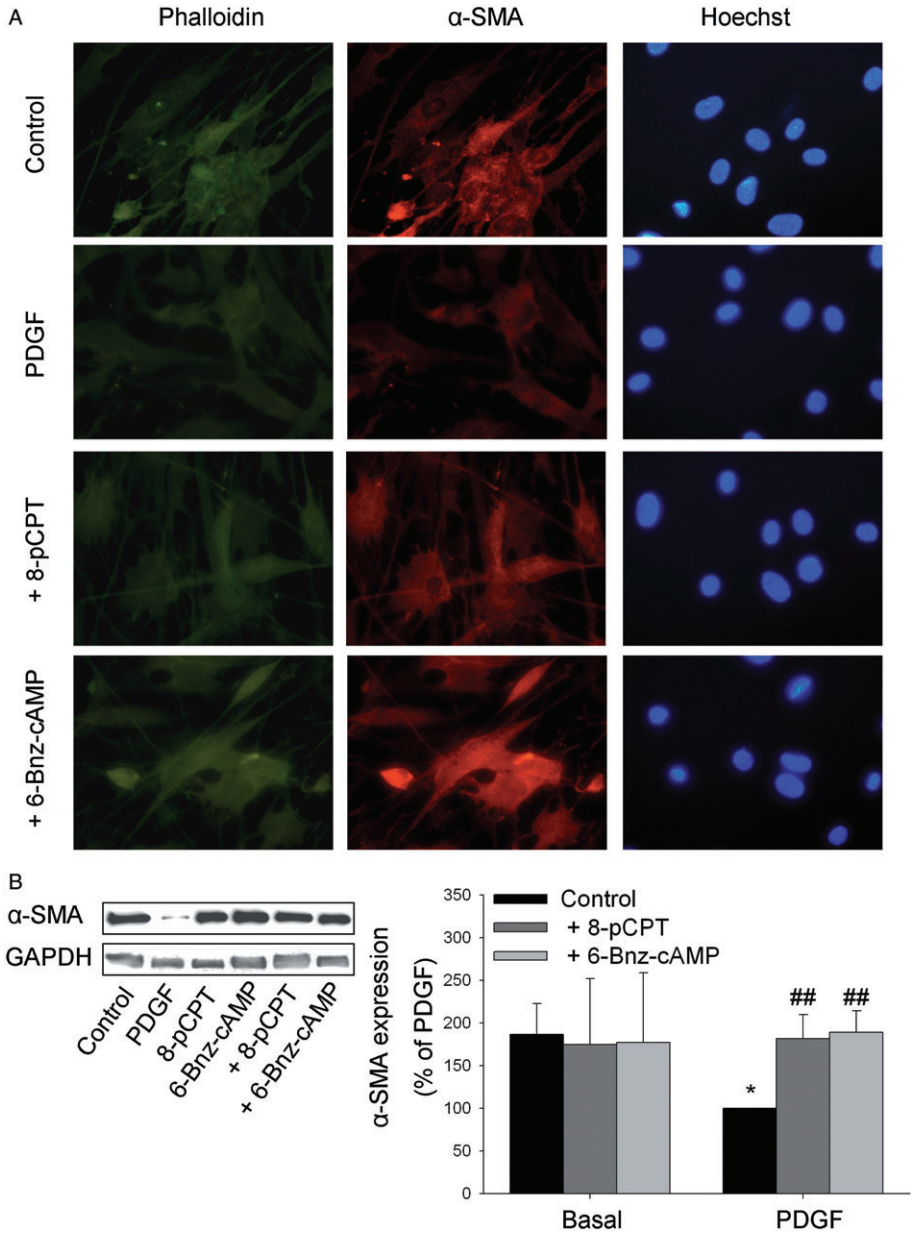

Figure 2

8-pCPT-2′-*O*-Me-cAMP (8-pCPT) and 6-Bnz-cAMP prevent PDGF-induced decrease of actin expression. Immunofluorescent staining of filamentous actin (by phalloidin, green, left panel), α-smooth muscle actin (α-SMA; red, middle panel) and nuclei (blue, right panel) in primary human ASM cells treated without (control) or with 8-pCPT-2′-*O*-Me-cAMP (30 μM) and 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. Western analysis of α-SMA expression in human ASM strips treated without (control) or with 8-pCPT-2′-*O*-Me-cAMP (30 μM) and 6-Bnz-cAMP (500 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. Means ± SEM of three to five experiments are shown with representative immunoblots. α-SMA levels were normalized to GAPDH. *$P < 0.05$, **$P < 0.01$ compared with basal control; ##$P < 0.01$ compared with PDGF.

percentage of cells entered the S phase (Figure 5), in agreement with the data on DNA synthesis. Co-treatment with 8-pCPT-2′-*O*-Me-cAMP (100 μM) or 6-Bnz-cAMP (500 μM) reduced this G_1/S phase transition (Figure 5). To identify the molecular mechanisms behind the inhibition of DNA synthesis, the expression of the cell cycle regulators cyclin D1 and the phosphorylation of Rb were analysed. As shown in Figure 6, PDGF induced cyclin D1 expression ($P < 0.05$), which was associated with a higher Rb phosphorylation ($P < 0.01$). Activation of Epac ($P < 0.01$) and PKA ($P < 0.001$) prevented both responses (Figure 6).

Discussion

In the present study, we demonstrated that prolonged exposure of intact human ASM tissue to PDGF induces a functionally hypocontractile, proliferative phenotype, which is

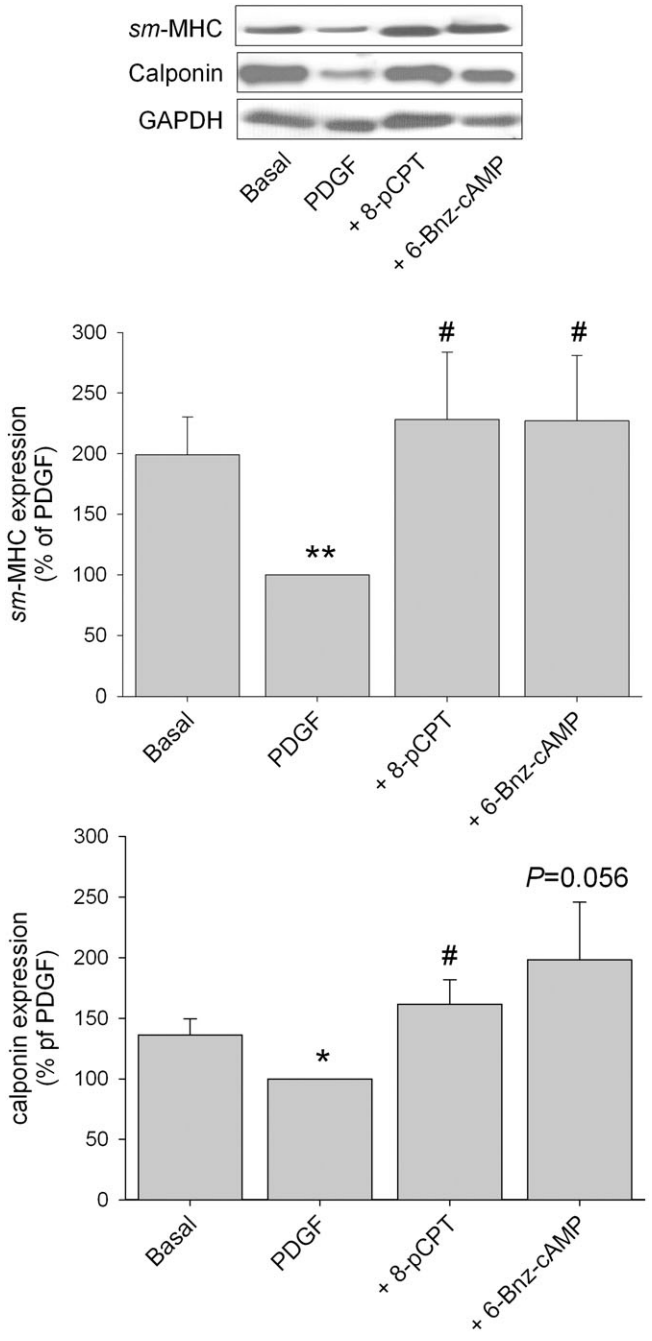

Figure 3

8-pCPT-2′-*O*-Me-cAMP (8-pCPT) and 6-Bnz-cAMP prevent PDGF-induced decrease of smooth muscle myosin heavy chain (*sm*-MHC) and calponin expression. Western analysis of *sm*-MHC and calponin expression in human ASM strips treated without (basal) or with 8-pCPT-2′-*O*-Me-cAMP (30 μM) and 6-Bnz-cAMP (500 μM) in the presence of PDGF (10 ng·mL^{-1}) for 4 days. Means ± SEM of five to eight experiments for *sm*-MHC and 3–4 for calponin are shown with representative immunoblots. Protein levels were normalized to GAPDH. *P < 0.05, **P < 0.01 compared with basal; #P < 0.05 compared with PDGF.

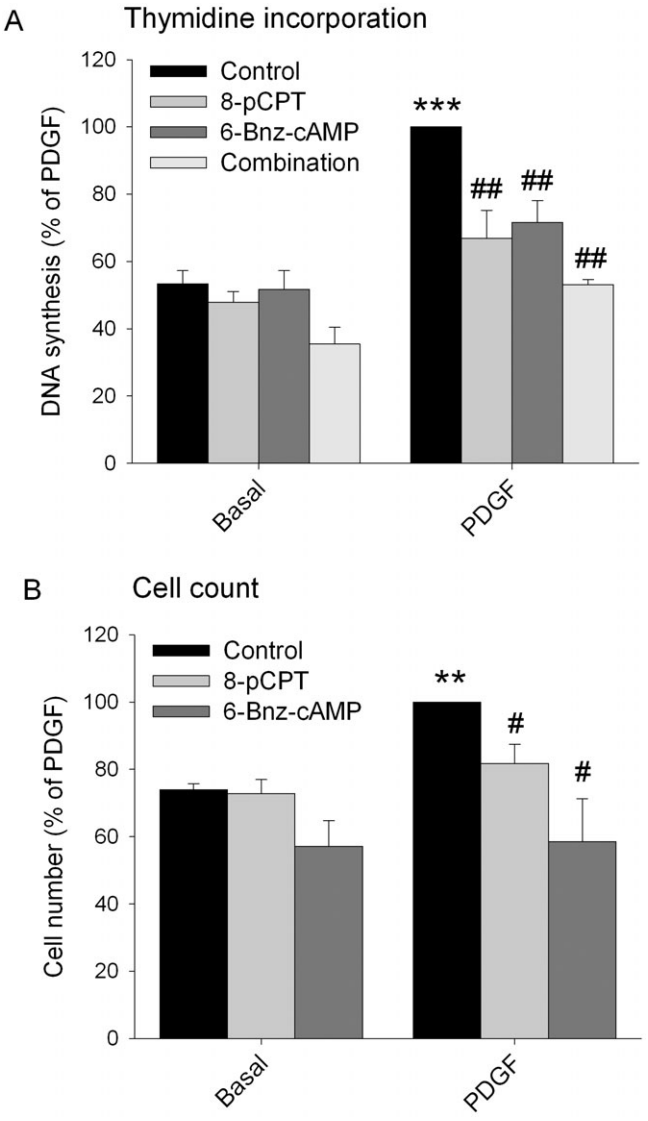

Figure 4

8-pCPT-2′-*O*-Me-cAMP (8-pCPT) and 6-Bnz-cAMP prevent PDGF-induced primary human airway smooth muscle cell proliferation. DNA synthesis (A) and cell number measurements (B) in non stimulated (basal) or PDGF (10 ng·mL^{-1}, 4 days)-stimulated primary human ASM cells in the absence (control) or presence of 8-pCPT-2′-*O*-Me-cAMP (30 μM), 6-Bnz-cAMP (500 μM) or their combination. Data represent means ± SEM of three to five experiments. **P < 0.01, ***P < 0.001 compared with basal control; #P < 0.05, ##P < 0.01 compared with PDGF.

largely prevented by activation of Epac and PKA. Thus, the selective Epac and PKA activators counteracted PDGF-induced primary human ASM cell proliferation via inhibition of cyclin D1 expression and Rb phosphorylation, resulting in cell cycle arrest in the G_0/G_1 phase. Such response was paralleled by the restoration of contractile marker expression and normalization of human ASM strip contractility, which were reduced by PDGF.

Our findings are in line with previous studies showing that mitogenic stimuli differentially regulate ASM phenotype

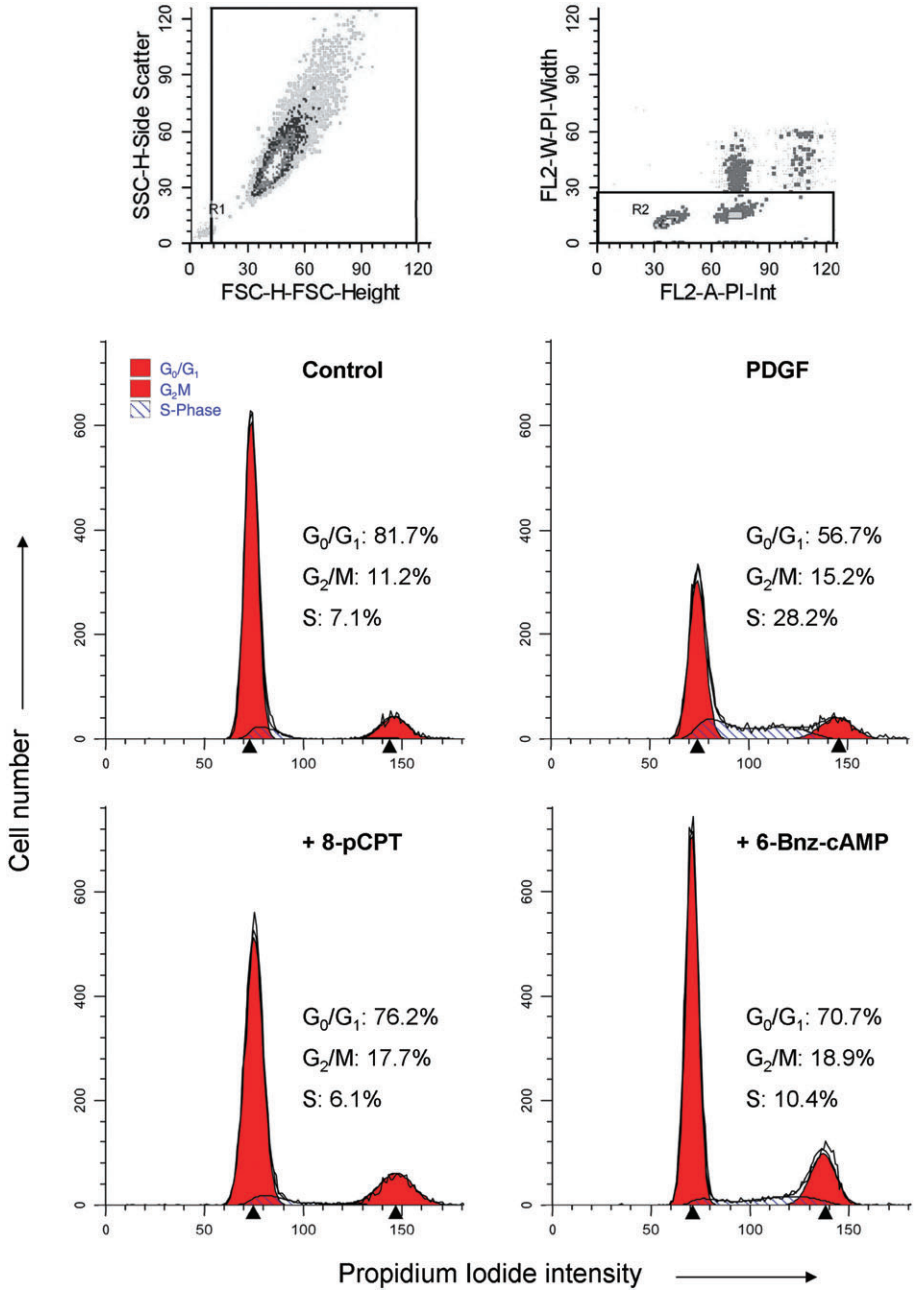

Figure 5

8-pCPT-2′-*O*-Me-cAMP(8-pCPT) and 6-Bnz-cAMP inhibit PDGF-induced ASM cell cycle progression. Sub-confluent immortalized hTERT-ASM cells were treated without (control) or with PDGF (30 ng·mL^{-1}) in the absence or presence of 8-pCPT-2′-*O*-Me-cAMP (100 μM) or 6-Bnz-cAMP (500 μM). For each stimulation, fluorescence histograms were collected for at least 10 000 cells, satisfying light scatter and doublet discrimination criteria (top). After 24 h, cells were harvested, stained with propidium iodide and analysed for DNA content by flow cytometry. The percentages of the cells in the respective phases of the cell cycle (G_0/G_1: first peak; G_2/M: second peak; and S: intermediate distribution) for each stimulation are depicted in the figure. Experiments were performed in triplicate. Representative histrograms from a single experiment are shown.

(Ma *et al.*, 1998; Hirst *et al.*, 2000a,b; Gosens *et al.*, 2002; Dekkers *et al.*, 2007; Schaafsma *et al.*, 2007; Roscioni *et al.*, 2011). Studies have shown that ASM cells exhibit a 'contractile' phenotype, characterized by low proliferative rates, high expression of contractile markers and relatively high contractile capability (Halayko *et al.*, 1996; Halayko and Amrani, 2003). Upon exposure to *pro*-proliferative compounds, ASM cells shift to a more 'synthetic/proliferative' phenotype, with lower contractile function and higher proliferative rates and expression of organelles responsible for increased synthesis of bioactive molecules (Halayko *et al.*, 1996; Halayko and Amrani, 2003; Dekkers *et al.*, 2009). Removal of growth factors or addition of either insulin or TGF-β results in the opposite process, pushing ASM to a hypercontractile pheno-

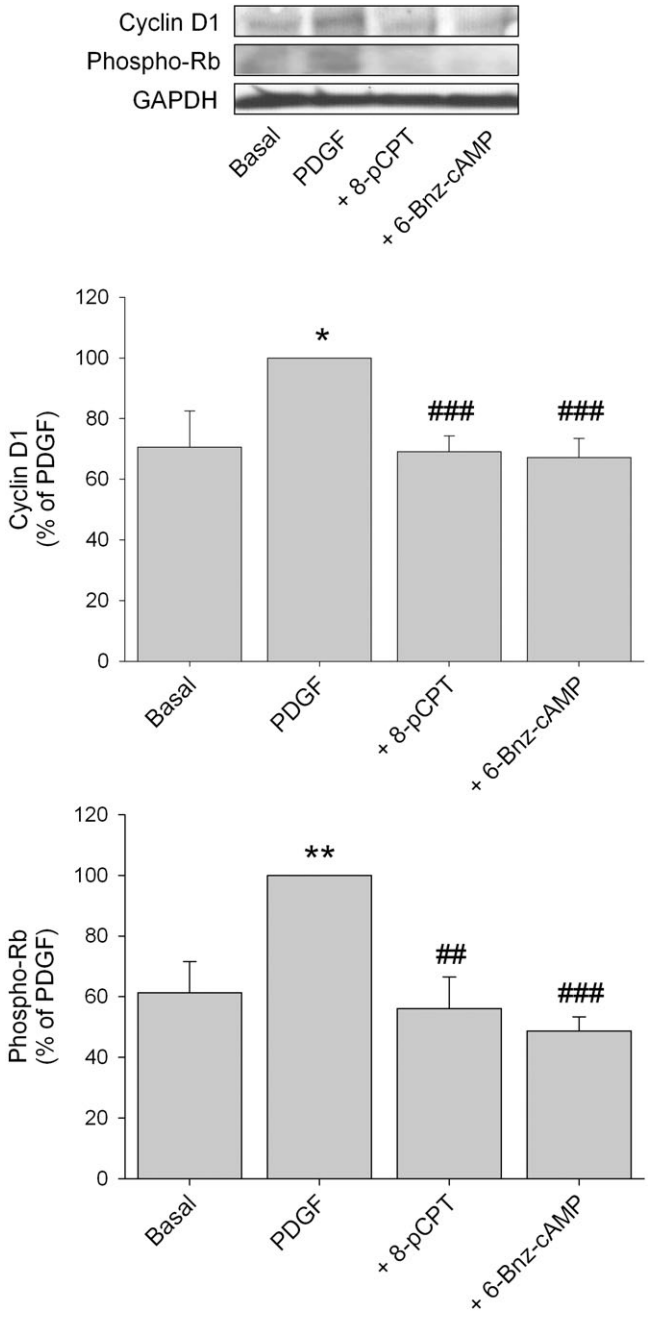

Figure 6

8-pCPT-2′-*O*-Me-cAMP (8-pCPT) and 6-Bnz-cAMP inhibit PDGF-induced mitogenic signaling. Western blot analysis of cyclin D1 and phospho-Rb in human ASM strips treated without (basal) or with PDGF (10 ng·mL^{-1}) in the absence or presence of 8-pCPT-2′-*O*-Me-cAMP (30 μM) and 6-Bnz-cAMP (500 μM) for 24 h. Means ± SEM of six experiments are shown with representative immunoblots. Protein levels were normalized to GAPDH. *P < 0.05, **P < 0.01 compared with basal; ##P < 0.01, ### P < 0.001 compared with PDGF.

type (Ma *et al.*, 1998; Schaafsma *et al.*, 2007; Dekkers *et al.*, 2009), indicating the highly dynamic nature of phenotypic alterations. ASM phenotypic plasticity may be important in physiological responses, including growth and repair mechanisms. However, these processes may also play a role in the pathogenesis of asthma, as the airways are continuously stimulated with an array of pro-proliferative stimuli (Halayko and Stephens, 1994; Hirst *et al.*, 2000b; Hirota *et al.*, 2009). Different phenotypic states may coexist *in vivo* as cultured ASM cells obtained from asthmatics proliferate faster (Johnson *et al.*, 2001) and have a higher velocity and maximal shortening capacity (Ma *et al.*, 2002; Leguillette *et al.*, 2009). Hence, phenotypic plasticity of ASM may contribute to the increased ASM mass and exaggerated bronchoconstriction which occur in asthmatics (Halayko and Stephens, 1994; Hirst, 1996; Hirst *et al.*, 2000b), and studying the mechanisms of its regulation may offer novel therapeutic strategies in asthma.

Inhaled β_2-adrenoceptor agonists are widely used to relieve acute bronchoconstriction in asthma by elevating cAMP (Scheid *et al.*, 1979; Giembycz and Newton, 2006). Although beneficial effects of cAMP were previously solely associated to PKA, we have recently shown that activation of Epac also induces ASM relaxation (Roscioni *et al.*, 2010). (Subcellular expression of Epac and its biological partners supports numerous cell-type-specific functions, including ASM proliferation (Gloerich and Bos, 2010; Grandoch *et al.*, 2010; Roscioni *et al.*, 2011). Despite these novel insights, the effects of β_2- adrenoceptor agonists and cAMP signalling on ASM phenotype regulation are still largely unknown. As observed for ASM, switching from the contractile/quiescent phenotype to the synthetic phenotype of VSMCs participates in the vascular remodelling, which occurs during vascular diseases (Owens *et al.*, 2004). Interestingly, such process appears to be inhibited by cAMP-elevating agents (Maurice *et al.*, 2003).

Studies in both airway and VSMCs have demonstrated that cAMP-elevating agents act anti-proliferatively (Tomlinson *et al.*, 1994; Scott *et al.*, 1996; Indolfi *et al.*, 1997; Mayer *et al.*, 2010), and identified both Epac and PKA as potential mediators of these effects (Kassel *et al.*, 2008; Yan *et al.*, 2010; Hewer *et al.*, 2011; Roscioni *et al.*, 2011). In particular, the anti-mitogenic properties of cAMP in VSMCs have been associated with inhibition of the expression of G_1/S phase regulatory proteins (Wu *et al.*, 2006), such as cyclin D1 and its downstream target Rb (Vadiveloo *et al.*, 1997), which accounts for the G_1 arrest and reduction in neointima development (Wu *et al.*, 2009). Importantly, Hewer *et al.* (2011) showed that these responses in vascular smooth muscle are regulated by both Epac and PKA. Our data show for the first time that Epac and PKA inhibited PDGF-induced cyclin D1 expression and Rb phosphorylation in human ASM cells as well. Together with the results obtained by flow cytometry and thymidine incorporation, these findings indicate that activation of PKA and/or Epac inhibited ASM proliferation by arresting the cells in G_0/G_1 phase.

Mitogen-activated protein kinases such as extracellular signal-regulated kinases (ERKs) are recognized as active players in growth factor-induced cell proliferation (Zhou and Hershenson, 2003). It has been shown that increasing intracellular levels of cAMP can block activation of Raf-1 and the subsequent activation of ERKs in Rap1-dependent and independent mechanisms (Houslay and Kolch, 2000; Stork and Schmitt, 2002). Based on our previous observations in bovine and human ASM that Epac and PKA activate Rap1 and inhibit PDGF-induced ERK phosphorylation (Roscioni *et al.*, 2009;

2011), it is tempting to speculate on the role of these effectors in the anti-mitogenic properties of Epac and PKA in human ASM. However, several other routes may also be involved in cAMP-induced growth arrest (Gosens *et al.*, 2008).

By blocking ASM cell cycle progression, cAMP signalling may also prevent phenotype switching after exposure to proliferative signals. Indeed, the induction of a proliferative phenotype in bovine ASM cells by PDGF was paralleled by the induction of a hypocontractile phenotype in ASM strips (Roscioni *et al.*, 2011). This induction of a hypocontractile phenotype by PDGF is in line with previous observations that the exposure of human bronchiole ring segments to mitogenic stimuli reduced contractile agonist-induced force generation (Moir *et al.*, 2003). Our current findings in human ASM are highly reminiscent of these observations, and reveal the importance of Epac and PKA in regulating phenotypic plasticity in intact human ASM. In line with the findings on ASM strip contractility, activation of Epac and PKA also prevented the down-regulation of contractile proteins by PDGF.

Hence, our findings suggest that beside their bronchodilating properties, cAMP-elevating agents might be used therapeutically to inhibit the proliferative capacity of ASM cells in disease. However, evidence of such an effect of β_2- adrenoreceptor agonists in asthmatic patients is still lacking, probably due to the rapid desensitization of the β_2-adrenoreceptor (Ammit *et al.*, 2009). Post-receptor strategies have been used to circumvent this problem and PDE4 inhibitors have been recently approved for the treatment of respiratory diseases (Houslay *et al.*, 2005; Giembycz and Field, 2010). Despite their beneficial effects on inflammation, the effects of PDE4 inhibitors on airway remodelling and ASM contractility remain uncertain (Giembycz and Field, 2010), probably due to the differential (sub)cellular expression and distribution of PDE4 isoforms, which in turn generate distinct functional cAMP gradients within the cells (Houslay, 2010). Moreover, the use of PDE4 inhibitors has been associated with unwanted adverse events (Houslay *et al.*, 2005; Giembycz and Field, 2010). Based on our findings, direct activation of Epac and/or PKA may represent a more specific approach to exploit the multi-functional properties of cAMP in ASM.

In conclusion, our results indicate that activation of Epac and PKA results in the maintenance of a *normo*-contractile, *normo*-proliferative ASM phenotype in a mitogenic environment, and it may represent a novel therapeutic option in the treatment of airway diseases.

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Conflicts of interest

The authors declare no conflicts of interest.

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