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RESEARCH PAPER

cAMP inhibits modulation of airway smooth muscle phenotype via the exchange protein activated by cAMP (Epac) and protein kinase A

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BACKGROUND AND PURPOSE

Changes in airway smooth muscle (ASM) phenotype may contribute to the pathogenesis of airway disease. Platelet-derived growth factor (PDGF) switches ASM from a contractile to a proliferative, hypo-contractile phenotype, a process requiring activation of extracellular signal-regulated kinase (ERK) and $p70^{S6}$ Kinase ($p70^{S6K}$). The effects of cAMP-elevating agents on these processes is unknown. Here, we investigated the effects of cAMP elevation by prostaglandin E_2 (PGE_2) and the activation of the cAMP effectors, protein kinase A (PKA) and exchange protein activated by cAMP (Epac) on PDGF-induced phenotype switching in bovine tracheal smooth muscle (BTSM).

EXPERIMENTAL APPROACH

Effects of long-term treatment with the PGE_2 analogue 16,16-dimethyl-PGE_2, the selective Epac activator, 8-pCPT-2′-*O*-Me-cAMP and the selective PKA activator, 6-Bnz-cAMP were assessed on the induction of a hypo-contractile, proliferative BTSM phenotype and on activation of ERK and $p70^{S6K}$, both induced by PDGF.

KEY RESULTS

Treatment with 16,16-dimethyl-PGE_2 inhibited PDGF-induced proliferation of BTSM cells and maintained BTSM strip contractility and contractile protein expression in the presence of PDGF. Activation of both Epac and PKA similarly prevented PDGF-induced phenotype switching and PDGF-induced activation of ERK. Interestingly, only PKA activation resulted in inhibition of PDGF-induced phosphorylation of $p70^{S6K}$.

CONCLUSIONS AND IMPLICATIONS

Our data indicate for the first time that both Epac and PKA regulated switching of ASM phenotype via differential inhibition of ERK and $p70^{S6K}$ pathways. These findings suggest that cAMP elevation may be beneficial in the treatment of long-term changes in airway disease.

Abbreviations

ASM, airway smooth muscle; α-SMA, α-smooth muscle actin; BTSM, bovine tracheal smooth muscle; Epac, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; KH, Krebs-Henseleit; $p70^{S6K}$, $p70^{S6}$ kinase; PDGF, platelet-derived growth factor; PGE_2, prostaglandin E_2; PKA, protein kinase A; sm-MHC, smooth muscle myosin heavy chain; VASP, vasodilator-activated phosphoprotein

Introduction

Phenotypic plasticity refers to the capacity of cells to exhibit distinct phenotypes in response to mitogenic stimuli, such as growth factors, extracellular matrix proteins, G-protein coupled receptor agonists and inflammatory mediators (Halayko *et al.*, 2008; Hirota *et al.*, 2009). Contractile airway

smooth muscle (ASM) cells are characterized by low proliferative rates, normal contractile capabilities and high expression levels of contractile proteins such as smooth muscle myosin heavy chain (sm-MHC) and smooth muscle α-actin (α-SMA) (Gosens *et al.*, 2002; Dekkers *et al.*, 2007; Halayko *et al.*, 2008). ASM cells may change their phenotype in response to mitogenic stimuli and modulate to a proliferative, hypo-contractile phenotype, characterized by increased expression of proliferative markers, increased proliferation, decreased expression of contractile proteins and decreased contractile capability (Hirst *et al.*, 2000a; Gosens *et al.*, 2002; Dekkers *et al.*, 2007). Phenotypic changes are dynamic as ASM cells in a proliferative phenotype can return to a contractile or even a hyper-contractile phenotype, following, for example, serum deprivation in the presence of insulin or transforming growth factor-β (Ma *et al.*, 1998; Schaafsma *et al.*, 2007; Dekkers *et al.*, 2009b). Regulation of ASM growth and proliferation by growth factors, including platelet-derived growth factor (PDGF) (Hirst *et al.*, 2000b; Gosens *et al.*, 2002), involves activation of extracellular signal-regulated kinase (ERK) and p70^{S6} kinase (p70^{S6K}) (Scott *et al.*, 1996; Karpova *et al.*, 1997), and these mechanisms play an important role in ASM phenotype switching (Gosens *et al.*, 2002).

Airway smooth muscle phenotype plasticity may contribute to the pathogenesis of airway disease, including chronic asthma (Halayko and Stephens, 1994; Hirst, 1996; Hirst *et al.*, 2000b; Hirota *et al.*, 2009). Chronic asthma is an inflammatory airway disease, characterized by early and late bronchial obstructive reactions, airway hyperresponsiveness (AHR) and structural changes in the airway wall (airway remodelling), which include increased ASM mass, due to cellular hypertrophy and/or hyperplasia (Ebina *et al.*, 1993; Dekkers *et al.*, 2009a). Mathematical modelling studies have shown that increased ASM mass may contribute substantially to AHR and declining lung function (Lambert *et al.*, 1993; Oliver *et al.*, 2007). Therefore, identification of mechanisms that prevent ASM phenotypic plasticity is important to target, pharmacologically, the altered proliferative and contractile responses of ASM.

Prostaglandin E_2 (PGE_2) has been shown to inhibit ASM proliferation, presumably via a mechanism involving cAMP elevation (Florio *et al.*, 1994; Tomlinson *et al.*, 1995). Subsequently, cAMP transduces its effects in ASM cells via activation of protein kinase A (PKA) and/or the exchange protein activated by cAMP (Epac). Recent publications have demonstrated that both PKA and Epac are involved in cAMP-mediated contractile (Sukhanova *et al.*, 2006; Cazorla *et al.*, 2009; Metrich *et al.*, 2009; Roscioni *et al.*, 2010), proliferative (Haag *et al.*, 2008; Huang *et al.*, 2008; Kassel *et al.*, 2008) and inflammatory (Roscioni *et al.*, 2009; Scheibner *et al.*, 2009; Xing and Birukova, 2010) responses in several cell types, including ASM cells. The functional impact of cAMP and its downstream effectors on ASM phenotype changes are mostly unknown.

Therefore, we investigated the effects of a metabolically stable PGE_2 derivative (16,16-dimethyl-PGE_2) and specific and selective activators of Epac (8-pCPT-2′-*O*-Me-cAMP) and PKA (6-Bnz-cAMP) on the phenotypic modulation of bovine tracheal smooth muscle (BTSM) strips and cells, induced by PDGF. We investigated also the effects of the compounds on PDGF-induced activation of ERK and p70^{S6K}. Our data showed that cAMP and its effectors prevented PDGF-induced ASM phenotype modulation, presumably via Epac- and PKA-mediated inhibition of ERK phosphorylation, whereas inhibition of p70^{S6K} phosphorylation by PKA, but not Epac, may also be involved.

Methods

BTSM strip preparation

Bovine tracheae were obtained from local slaughterhouses and rapidly transported to the laboratory in Krebs-Henseleit (KH) buffer (composition in mM: 117.5 NaCl, 5.60 KCl, 1.18 $MgSO_4$, 2.50 $CaCl_2$, 1.28 NaH_2PO_4, 25.00 $NaHCO_3$, and 5.50 glucose), pregassed with 5% CO_2 and 95% O_2, pH 7.4. After dissection of the smooth muscle layer and careful removal of the mucosa and connective tissue, BTSM strips of identical length (1 cm) and width (2 mm) were prepared. Tissue strips were cultured in serum-free Dulbecco's modified Eagle's medium, supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45 μg·mL^{-1}), penicillin (100 U·mL^{-1}), streptomycin (100 μg·mL^{-1}), amphotericin B (1.5 μg·mL^{-1}), apo-transferrin (human, 5 μg·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.). When used, the PGE_2 analogue 16,16-dimethyl-PGE_2 (1.5 and 15 μM), the Epac activator 8-pCPT-2′-*O*-Me-cAMP (3 and 30 μM), the PKA activator 6-Bnz-cAMP (100 and 500 μM) and/or PDGF (10 μg·mL^{-1}) were present during the entire incubation period. PDGF was added 30 min after the other stimuli. After culture, strips were washed thoroughly and used 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). Briefly, BTSM strips were mounted for isometric recording in organ-baths, containing KH buffer at 37°C. During a 90 min equilibration period, resting tension was gradually adjusted to 3 g, followed by pre-contractions with 20 and 40 mM KCl. Following washout, maximal relaxation was established by the addition of (-)-isoprenaline (0.1 μM). Tension was readjusted to 3 g immediately followed by two changes with KH buffer. After another equilibration period of 30 min, cumulative concentration–response curves were constructed to KCl (5.6–50 mM) or methacholine (1 nM–1 mM). When maximal tension was reached, the strips were washed several times and maximal relaxation was established by using (-)-isoprenaline (10 μM).

Isolation of BTSM cells

After the removal of mucosa and connective tissue, BTSM tissue was chopped using a McIlwain tissue chopper. Tissue particles were washed twice with Dulbecco's modified Eagle's medium, 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 $\mu g \cdot mL^{-1}$), and fetal bovine serum (FBS, 0.5%). Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 $mg \cdot mL^{-1}$), papain (1 $mg \cdot mL^{-1}$) and soybean trypsin inhibitor (1 $mg \cdot mL^{-1}$). During digestion, the suspension was incubated in the Innova incubator shaker at 37°C, 55 r.p.m. for 20 min, followed by a 10 min period of shaking at 70 r.p.m. After filtration through a 50 μM gauze, cells were washed three times in medium supplemented with 10% FBS. In the current study, cells in passages 1–4 were used.

[^{3}H]-thymidine incorporation

Bovine tracheal smooth muscle cells were plated on 24-well plates at a density of 40 000 cells per well. The next day, cells were washed with phosphate buffered saline and made quiescent by incubation with serum-free medium supplemented with antibiotics and insulin, transferrin and selenium for 72 h. Subsequently, cells were incubated with 16,16-dimethyl-PGE_2, 8-pCPT-2′-*O*-Me-cAMP and/or 6-Bnz-cAMP in the absence or presence of PDGF for 28 h, the last 24 h in the presence of [^{3}H]-thymidine (0.25 $\mu Ci \cdot mL^{-1}$). After incubation, cells were washed twice with phosphate buffered saline 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.

Alamar blue assay

Bovine tracheal smooth muscle cells were plated as described for the [^{3}H]-thymidine incorporation protocol above. Following serum deprivation, cells were treated with 16,16-dimethyl-PGE_2, 8-pCPT-2′-*O*-Me-cAMP and/or 6-Bnz-cAMP in the absence or presence of PDGF for 4 days. In some experiments cells were pretreated for 30 min with the prostaglandin EP_2 receptor antagonist AH6809 (1 μM; receptor nomenclature follows Alexander *et al.*, 2009), H89, in a PKA-selective concentration (300 nM) or with a mixture of two PKA inhibitors (Rp-cAMPS and Rp-8-Br-cAMPS, 500 μM, each) (Jensen *et al.*, 2004). After 4 days, cells were washed twice with HBSS and incubated with a 5% vol/vol Alamar blue in HBSS for 45 min. Proliferation was assessed by conversion of Alamar blue into its reduced form by mitochondrial cytochromes and measured using a Wallac 1420 Victor 2TM at 590 nM.

Western blot analysis

Bovine tracheal smooth muscle strip homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonification in RIPA buffer (composition in mM: 50 mM Tris·HCl, 150.0 NaCl, 1.0 EDTA, 1.0 PMSF, 1.0 Na_3VO_4, 1.0 NaF, pH 7.4, supplemented with 10 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ pepstatin, 0.25% sodium and 1% Igepal). For preparation of BTSM cell homogenates, cells were treated with 16,16-dimethyl-PGE_2, 8-pCPT-2′-*O*-Me-cAMP, 6-Bnz-cAMP in the absence or presence of PDGF for 30 min or 2 h. When used, H89 or the other PKA inhibitors were added 30 min before the addition of the other stimuli. Cells were lysed using 200 μL of lysis buffer (composition: 1% SDS and 10 mM Tris/HCl; pH 7.4) and heated to 95°C for 5 min and resuspended by passage through a 25-gauge needle, 10 times. Protein content was determined using the Pierce BCA protein assay. Equal amounts of protein were separated on a 6% polyacrylamide gel for sm-MHC, 8% for Epac1 and Epac2 and 10% for the vasodilator-activated phosphoprotein (VASP), GAPDH, α-SMA, phospho-ERK and phospho-$p70^{S6K}$. Proteins were transferred onto nitrocellulose membrane, blocked with 5% milk in Tris-buffered saline + Tween (TBST) and incubated overnight with primary antibodies (sm-MHC 1:1000, α-SMA, 1:200; GAPDH, 1:400; phospho-ERK and phospho-$p70^{S6K}$, VASP, Epac1 and Epac2, 1:500). After washing, the membranes were incubated with horseradish

peroxidase-labelled secondary antibody (dilution 1:2000). 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.

Data analysis

Data are shown as means ± SEM from *n* individual experiments. The statistical significance of differences between means was determined by Student's *t*-test for paired observations or one-way ANOVA followed by a Bonferroni *post hoc* test, as appropriate. 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, 8-pCPT-2′-*O*-Me-cAMP and the two selective PKA inhibitors Rp-cAMPS and Rp-8-Br-cAMPS were from BIOLOG Life Science Institute (Bremen, Germany). 16,16-dimethyl-PGE_2 was from Cayman Chemical (Ann Arbor, MI, USA). Human PDGF-AB (-)-isoprenaline hydrochloride, AH6809, H89 dihydrochloride hydrate, protease inhibitors, apotransferrin, anti-β-actin, anti-α-SMA and secondary antibodies were from Sigma-Aldrich (St. Louis, MO, USA). FBS was obtained from Hyclone Thermo Scientific (Waltham, MA, USA). Collagenase P and papain were from Roche Diagnostics (Mannheim, Germany). Cell culture solutions were from Gibco BRL Life Technologies (Paisley, UK). Anti-VASP and anti-phospho-ERK antibodies were from Cell Signalling Technology (Beverly, MA, USA). Anti-phospho-$p70^{S6K}$ and anti-GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-sm-MHC was from NeoMarkers (Fremont, CA, USA). Antibodies against Epac1 and Epac2 were kindly provided by Dr J. L. Bos (University Medical Center Utrecht, the Netherlands). [^{3}H]-thymidine was from Amersham (Buckinghamshire, UK) and Alamar blue from Biosource (Camarillo, CA, USA). Western Lightning plus-ECL was from PerkinElmer Inc. (Waltham, MA, USA) and Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA). All other used chemicals were of analytical grade.

Results

16,16-dimethyl-PGE_2 inhibits PDGF-induced modulation of BTSM phenotype

To determine the effect of cAMP elevation on ASM phenotypic modulation, we studied the effect of the stable PGE_2 analogue 16,16-dimethyl-PGE_2 on PDGF (10 ng·mL^{-1})-induced decreases in BTSM contractility. Fully in line with previous findings (Gosens *et al.*, 2002; Dekkers *et al.*, 2007), pretreatment of BTSM strips with PDGF for 4 days reduced maximal contraction in response to KCl and methacholine ($P < 0.05$, Figure 1A, Tables 1 and 3), compared with vehicle-treated control strips. At a concentration of 15 μM, but not 1.5 μM, the PGE_2 analogue significantly inhibited the PDGF-induced decrease in both KCl- and methacholine-induced contractions ($P < 0.05$; Figure 1A, Table 1), without affecting contractile responses by itself. No changes in the sensitivity towards KCl or methacholine were observed (Table 1). In full agreement with the findings on contractility, 16,16-dimethyl-PGE_2 reduced the PDGF-induced down-regulation of α-SMA expression ($P < 0.05$; Figure 1B).

To assess whether the inhibition of PDGF-induced hypo-contractility was associated with changes in BTSM cell proliferation, cell numbers were measured. 16,16-dimethyl-PGE_2 strongly inhibited PDGF-induced increases in cell number ($P < 0.05$; Figure 1C–E), without affecting basal proliferative responses (not shown). To evaluate the role of EP receptor subtypes in this response, AH6809 (1 μM) was used (Haag *et al.*, 2008). This compound has been reported to antagonize both EP_1 (G_q-coupled) and EP_2 (G_s-coupled) receptors with similar affinities (K_i of about 1 μM) (Abramovitz *et al.*, 2000; Alexander *et al.*, 2009). AH6809 reduced the inhibitory effect of 16,16-dimethyl-PGE_2 on

Figure 1

Activation of G_s-protein coupled EP_2 prostaglandin receptors inhibits platelet-derived growth factor (PDGF)-induced phenotypic modulation. Concentration–response curves of methacholine-induced contractions (A) and Western blot analysis of smooth muscle α-actin (α-SMA) expression (B) in bovine tracheal smooth muscle (BTSM) strips pretreated with 16,16-dimethyl (dm) prostaglandin E_2 (PGE_2) (1.5 μM and/or 15 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. α-SMA expression obtained from BTSM strips homogenates was normalized to GAPDH. Representative immunoblots are shown. Data represent mean ± SEM of 3–10 independent experiments, performed in duplicate. Effects of 16,16-dimethyl-PGE_2 on basal and PDGF-induced increase in BTSM cell number in the absence or presence of the EP_2 receptor selective antagonist AH6809 (1 μM) (C), H89 (300 nM) (D) or the combination of Rp-cAMPS and Rp-8-Br-cAMPS (500 μM, each) (E). Data represent mean ± SEM of 4–10 independent experiments. Measurement of vasodilator-activated phosphoprotein (VASP) phosphorylation (F) in BTSM cells after 15 min treatment with 16,16-dm PGE_2 in the absence or presence of H89, AH6809 or the combination of Rp-cAMPS and Rp-8-Br-cAMPS. Representative immunoblots of 4–8 experiments are shown. VASP expression obtained from BTSM cell lysates was normalized to GAPDH. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with basal control; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with PDGF; $^{\S\S}P < 0.01$; $^{\dagger}P = 0.02$.

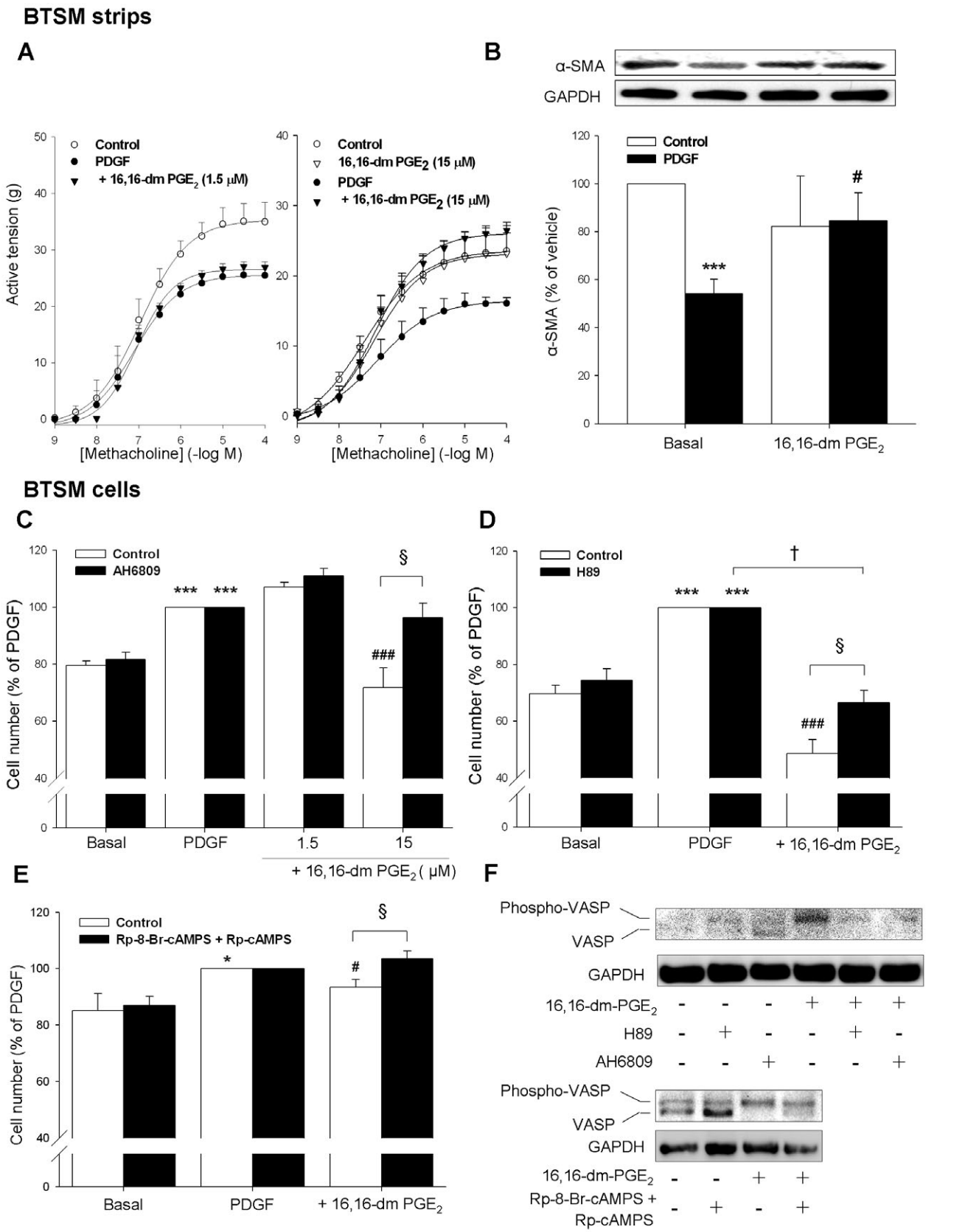
BTSM strips
A
Control
PDGF
+ 16,16-dm PGE2 (1.5 μM)
Active tension (g)
[Methacholine] (-log M)
Control
16,16-dm PGE2 (15 μM)
PDGF
+ 16,16-dm PGE2 (15 μM)
[Methacholine] (-log M)
B
α-SMA
GAPDH
Control
PDGF
α-SMA (% of vehicle)
***
#
Basal
16,16-dm PGE2
BTSM cells
C
Control
AH6809
Cell number (% of PDGF)
***
###
§
Basal
PDGF
1.5
15
+ 16,16-dm PGE2 (μM)
D
Control
H89
†
§
Basal
PDGF
+ 16,16-dm PGE2
E
Control
Rp-8-Br-cAMPS + Rp-cAMPS
*
#
§
Basal
PDGF
+ 16,16-dm PGE2
F
Phospho-VASP
VASP
GAPDH
16,16-dm-PGE2
H89
AH6809
Rp-8-Br-cAMPS + Rp-cAMPS

Table 1

Activation of G_s-coupled EP_2 receptors inhibited platelet-derived growth factor (PDGF)-induced hypo-contractility in bovine tracheal smooth muscle (BTSM) strips

	KCl		Methacholine	
Treatment	**E_{max}, g**	**EC_{50} mM**	**E_{max}, g**	**pEC_{50} (-logM)**
Control	28.8 ± 3.3	20.2 ± 1.6	35.0 ± 3.4	7.03 ± 0.30
+PDGF	19.0 ± 1.2	17.6 ± 0.4	25.5 ± 1.6	7.20 ± 0.26
16,16 dimethyl PGE_2 1.5 μM				
+PDGF	18.8 ± 1.7	21.1 ± 1.7	27.0 ± 1.0	7.11 ± 0.08
Control	16.7 ± 2.7	19.8 ± 1.9	28.4 ± 5.4	7.30 ± 0.06
+PDGF	12.8 ± 0.2	17.2 ± 1.8	17.4 ± 1.4	6.92 ± 0.27
16,16 dimethyl PGE_2 15 μM	18.4 ± 0.9	21.7 ± 0.4	32.5 ± 9.6	7.14 ± 0.04
+PDGF	16.1 ± 0.6$^{\#}$	25.0 ± 1.2	30.8 ± 4.4$^{\#}$	7.21 ± 0.13

Contractile responses to KCl and methacholine of BTSM strips pretreated with 16,16 dimethyl-PGE_2 (1.5 and 15 μM) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. Concentration–response curves corresponding to these values are shown in Figure 1A. Data represents means ± SEM of 3–4 independent experiments. E_{max}, maximal contraction; EC_{50}, concentration of agonist eliciting half-maximal response; pEC_{50}, negative logarithm of the EC_{50}. $^{\#}P < 0.05$ compared with PDGF-stimulated control.

PDGF-induced BTSM cell proliferation ($P < 0.05$; Figure 1C). The involvement of PKA was demonstrated by treatment with the PKA inhibitor H89, which partially reduced the inhibitory effect of 16,16-dimethyl-PGE_2 on PDGF-induced cell proliferation ($P < 0.05$; Figure 1D). Because reported non-specific effects of H89 might render interpretation of the results difficult (Davies *et al.*, 2000), we also used a combination of two selective PKA antagonist, Rp-cAMPS and Rp-8-Br-cAMPS (Jensen *et al.*, 2004). Combined treatment with Rp-cAMPS and Rp-8-Br-cAMPS (500 μM; each) fully inhibited the effect of the PGE_2 derivative ($P < 0.05$; Figure 1E). To further demonstrate the activation of PKA by 16,16-dimethyl-PGE_2, we tested phosphorylation of the PKA downstream target, VASP. Western blot analysis was performed using an antibody that recognizes both phosphorylated VASP (phospho-VASP) and non-phosphorylated VASP (VASP). As expected, treatment with 16,16-dimethyl-PGE_2 induced a strong phosphorylation of VASP ($P < 0.05$; Figure 1F, Table 2), which was partially inhibited by H89 (Table 2) and significantly reduced by AH6809 ($P < 0.01$; Table 2) and the combination of the PKA inhibitors Rp-cAMPS and Rp-8-Br-cAMPS ($P < 0.05$). Taken together, these data indicate that 16,16-dimethyl-PGE_2 exerted its effects by activation of the G_s-coupled EP_2 receptor and subsequent activation of PKA.

Activation of PKA and Epac reduces PDGF-induced BTSM cell proliferation

To determine the involvement of the cAMP effectors PKA and Epac in the inhibition of PDGF-induced phenotype modulation by cAMP elevation, the effects of the specific Epac activator 8-pCPT-2′-*O*-Me-cAMP and the PKA activator 6-Bnz-cAMP were assessed on ASM proliferative responses. As previously described for human ASM (Roscioni *et al.*, 2009), both Epac1 and Epac2 were expressed in BTSM tissue (Figure 2A). The specificity of both cAMP analogues was evaluated by measuring the phosphorylation of VASP, a substrate for PKA, but not for Epac. The PKA activator 6-Bnz-cAMP induced a strong VASP phosphorylation ($P < 0.001$, Figure 2B, Table 2), whereas no significant changes were observed after treatment with 8-pCPT-2′-*O*-Me-cAMP. Treatment with H89 did not inhibit VASP phosphorylation by 6-Bnz-cAMP whereas the combination of Rp-cAMPS and Rp-8-Br-cAMPS significantly reduced this response ($P < 0.05$, Table 2). PDGF treatment increased both DNA synthesis and cell number ($P < 0.05$; Figure 2C–E). Both 8-pCPT-2′-*O*-Me-cAMP (Figure 2C and D, left panels) and 6-Bnz-cAMP (Figure 2C and D, right panels) inhibited these responses in a concentration-dependent manner. Treatment with 8-pCPT-2′-*O*-Me-cAMP or 6-Bnz-cAMP did not significantly affect basal proliferative responses. To further confirm the anti-proliferative role of PKA, the effects of H89 and the combination of Rp-cAMPS and Rp-8-Br-cAMPS were assessed on the inhibitory effects of 6-Bnz-cAMP (500 μM) on PDGF-induced proliferation. As expected PKA inhibition dramatically reduced the effect of 6-Bnz-cAMP ($P < 0.001$ for H89, Figures 2E and $P < 0.05$ for the combination of Rp-cAMPS and Rp-8-Br-cAMPS, Figure 2F). Importantly, the effect of the highest concentration of the Epac activator was not altered by PKA inhibition (Figure 2E and F).

Table 2

Specificity of protein kinase A (PKA) activation by 16,16-dimethyl-PGE_2 and 6-Bnz-cAMP measured by vasodilator-activated phosphoprotein (VASP) phosphorylation in bovine tracheal smooth muscle (BTSM) cells

Treatment	Phospho-VASP % of 16,16-dm PGE_2	*n*	Treatment	Phospho-VASP % of 6-Bnz-cAMP	*n*
Control	48 ± 6	9	Control	40 ± 6	9
+H89	54 ± 7	9	+H89	52 ± 7	9
+AH6809	58 ± 12	5	6-Bnz-cAMP	100 ± 0***	9
16,16-dm PGE_2	100 ± 0***	9	+H89	93 ± 3	9
+H89	84 ± 9	9	8-pCPT	66 ± 15	6
+AH6809	74 ± $7^{\#\#\#}$	5	+H89	62 ± 14	6
Control	55 ± 4	3	Control	41 ± 3	3
+PKA inhibitors	48 ± 15	3	+PKA inhibitors	38 ± 7	3
16,16-dm PGE_2	100 ± 0**	3	6-Bnz-cAMP	100 ± 0**	3
+PKA inhibitors	67 ± $5^{\#}$	3	+PKA inhibitors	79 ± $2^{\S}$	3

VASP phosphorylation in BTSM cells after treatment with 16,16-dimethyl-PGE_2 (16,16-dmPGE_2; 15 μM), 6-Bnz-cAMP (500 μM) or 8-pCPT-2′-*O*-Me-cAMP (30 μM) in the absence and presence of the PKA inhibitor H89 (300 nM), the EP_2 receptor antagonist AH6809 (1 μM) or a combination of the PKA inhibitors Rp-cAMPS and Rp-8-Br-cAMPS (500 μM, each). Data represent means ± SEM from *n* independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with respective control; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with 16,16-dm PGE_2; $^{\S}P < 0.05$ compared with 6-Bnz-cAMP.

8-pCPT-2′-O-Me-cAMP and 6-Bnz-cAMP reverse PDGF-induced hypo-contractility

Interestingly, co-incubation of BTSM strips with PDGF and 8-pCPT-2′-*O*-Me-cAMP or 6-Bnz-cAMP at the same concentrations that inhibited PDGF-induced pro-mitogenic properties, completely normalized the PDGF-induced decrease in both KCl- and methacholine-induced contractions ($P < 0.05$ both; Figure 3A and B, Table 3). 8-pCPT-2′-*O*-Me-cAMP did not change maximal KCl- and methacholine-induced contractile force or sensitivity in the absence of the growth factor. Surprisingly, although 6-Bnz-cAMP did not affect maximal contractions, the sensitivity towards KCl was slightly increased after co-treatment with 6-Bnz-cAMP in the presence of PDGF ($P < 0.05$; Table 3). In line with previous reports (Dekkers *et al.*, 2007), PDGF treatment significantly reduced α-SMA and sm-MHC expression by about 50%, compared with control ($P < 0.001$ both; Figure 3C and D). Interestingly, the effect of PDGF on α-SMA expression was largely reduced by 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP ($P < 0.05$ both; Figure 3B), whereas both cAMP analogues alone did not affect basal expression of α-SMA. Similar effects of 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP were observed towards the PDGF-induced reduction of sm-MHC expression ($P < 0.05$ both; Figure 3C).

In order to elucidate the interaction between the cAMP effectors, BTSM cells and strips were treated with combinations of 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP. As shown in Figure 4 and Table 3, combined application of 3 μM 8-pCPT-2′-*O*-Me-cAMP and 100 μM 6-Bnz-cAMP only partially reduced the effects of PDGF on BTSM proliferative (Figure 4A and B, left panels) and contractile (Figure 4C, left panel) responses. Combined pretreatment with the highest concentrations of the two cAMP analogues significantly inhibited the PDGF-mediated increase in BTSM DNA synthesis and cell number ($P < 0.001$, Figure 4A and B, right panels) and the reduction in KCl- and methacholine-induced maximal contractions ($P < 0.05$, Figure 4C, right panel, Table 3), without affecting basal responses. Moreover, the effect of the combination of the highest concentrations of 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP on DNA synthesis was significantly higher, that the effects of treatment with either compound alone ($P < 0.01$ compared with 6-Bnz-cAMP and $P < 0.001$ compared with 8-pCPT-2′-*O*-Me-cAMP, Figure 4A, right panel). No additional effects of combined treatment were observed on other parameters assessed.

The effects of Epac and PKA activation on PDGF-induced ERK and $p70^{S6K}$ phosphorylation

Bovine tracheal smooth muscle phenotypic modulation by PDGF has previously been shown to be dependent on activation of ERK (Gosens *et al.*, 2002). Moreover, Epac and PKA have been shown to regulate different biological functions by

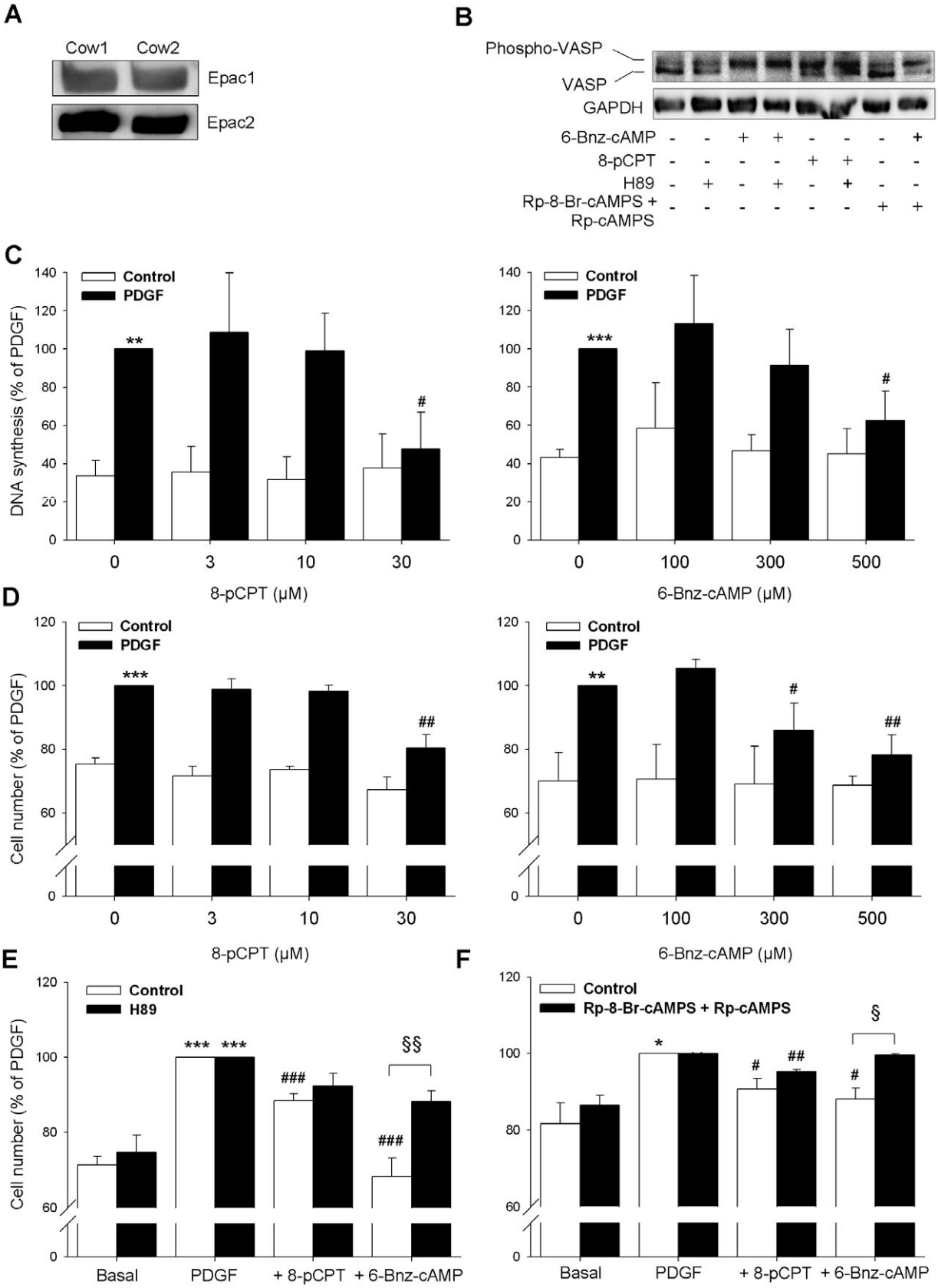
A
Cow1
Cow2
Epac1
Epac2
B
Phospho-VASP
VASP
GAPDH
6-Bnz-cAMP - - + + - - - +
8-pCPT - - - - + + - -
H89 - + - + - + - -
Rp-8-Br-cAMPS + Rp-cAMPS - - - - - - + +
C
Control
PDGF
DNA synthesis (% of PDGF)
8-pCPT (μM)
6-Bnz-cAMP (μM)
D
Cell number (% of PDGF)
E
H89
F
Rp-8-Br-cAMPS + Rp-cAMPS
Basal
PDGF
+ 8-pCPT
+ 6-Bnz-cAMP

Figure 2

Activation of Epac and protein kinase A decreases platelet-derived growth factor (PDGF)-induced bovine tracheal smooth muscle (BTSM) cell proliferation. Expression of Epac1 and Epac2 from BTSM strip homogenates obtained from two different animals (A). Measurement of vasodilator-activated phosphoprotein (VASP) phosphorylation (B) in BTSM cells after 15 min treatment with 8-pCPT-2′-*O*-Me-cAMP (8-pCPT, 30 μM) or 6-Bnz-cAMP (500 μM) in the absence or presence of H89 (300 nM) or the combination of Rp-cAMPS and Rp-8-Br-cAMPS (500 μM, each). VASP expression obtained from BTSM cell lysates was normalized to GAPDH. Data represent means ± SEM of 3–9 independent experiments. Effects of the indicated concentrations of 8-pCPT and 6-Bnz-cAMP on basal and PDGF (10 ng·mL^{-1})-induced increases in BTSM cell DNA synthesis (C) and cell number (D). Effects of 8-pCPT (30 μM) or 6-Bnz-cAMP (500 μM) on basal and PDGF-induced increase in BTSM cell number in the absence or presence of H89 (300 nM) (E) or the combination of Rp-cAMPS and Rp-8-Br-cAMPS (500 μM, each) (F). Data represent means ± SEM of 4–9 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with basal control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with PDGF; $^{\S}P < 0.05$; $^{\S\S}P < 0.01$.

modulation of ERK (Stork and Schmitt, 2002; Roscioni *et al.*, 2009). To address the contribution of ERK signalling in the Epac- and PKA-mediated effects, basal and PDGF-induced phosphorylation of ERK was evaluated in BTSM cells treated with 8-pCPT-2′-*O*-Me-cAMP, 6-Bnz-cAMP and 16,16-dimethyl-PGE_2. In agreement with our previous data in human ASM (Roscioni *et al.*, 2009), treatment with 8-pCPT-2′-*O*-Me-cAMP or 6-Bnz-cAMP induced a slight, but transient increase in basal ERK phosphorylation (not shown). PDGF increased phosphorylation of ERK by 50% after 30 min ($P < 0.001$) and 30% after 120 min ($P < 0.05$; Figure 5A). Importantly, after 30 min, this response was reduced by approximately 30% by co-treatment with 8-pCPT-2′-*O*-Me-cAMP ($P < 0.001$), 6-Bnz-cAMP ($P < 0.001$) or 16,16-dimethyl-PGE_2 ($P < 0.01$; Figure 5A). After 120 min PDGF-induced ERK activation was fully normalized by 6-Bnz-cAMP ($P < 0.05$) and ~50% decreased by 8-pCPT-2′-*O*-Me-cAMP or 16,16-dimethyl-PGE_2 (Figure 5A).

Phosphorylation of $p70^{S6K}$ also underpins the mitogenic properties of PDGF in ASM (Scott *et al.*, 1996). In BTSM cells, PDGF increased phosphorylation of $p70^{S6K}$ after 30 min ($P < 0.001$) and after 120 min ($P < 0.01$; Figure 5B). Treatment with 8-pCPT-2′-*O*-Me-cAMP, 6-Bnz-cAMP and 16,16-dimethyl-PGE_2 did not affect basal phosphorylation of $p70^{S6K}$ (not shown). Importantly, 6-Bnz-cAMP completely normalized PDGF-induced $p70^{S6K}$ phosphorylation at both time points ($P < 0.001$), whereas 16,16-dimethyl-PGE_2 only reduced this response by about 50% (Figure 5B). By contrast, treatment with 8-pCPT-2′-*O*-Me-cAMP did not significantly affect the PDGF-induced phosphorylation of $p70^{S6K}$, demonstrating that PDGF-mediated phosphorylation of $p70^{S6K}$ was inhibited by activation of PKA, but not by activation of Epac.

Discussion

In the current study, we show for the first time that cAMP elevation regulates ASM phenotype modulation via the activation of Epac and PKA. Thus, activation of the G_s-coupled EP_2-receptor and specific activation of Epac and PKA inhibited PDGF-induced modulation from a contractile towards a proliferative, hypo-contractile ASM phenotype. In addition, the PDGF-induced down-regulation of contractile protein expression was inhibited by the stable PGE_2 derivative 16,16-dimethyl-PGE_2 and by activation of Epac and PKA by 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP respectively. Mechanistically, activation of PKA inhibited activation of both ERK and $p70^{S6K}$ by PDGF, whereas activation of Epac only inhibited the activation of ERK (Figure 6).

Changes in the ASM phenotype are considered to contribute to AHR and airway remodelling in asthma (Halayko and Stephens, 1994; Hirst, 1996; Hirst *et al.*, 2000b). Long-term treatment of ASM with growth factors, such as PDGF, results in modulation from a 'normo-contractile' to a proliferative, hypo-contractile phenotype, characterized by reduced maximal contractions in response to receptor-dependent and independent stimuli, reduced expression of contractile proteins and increased proliferative responses (Dekkers *et al.*, 2007). cAMP exerts anti-proliferative effects in ASM from different species, including cells from bovine and human origin (Tomlinson *et al.*, 1994; Scott *et al.*, 1996; Billington and Penn, 2003; Gosens *et al.*, 2008). In the present study, we demonstrated that 16,16-dimethyl-PGE_2 not only inhibited ASM proliferation, but also prevented modulation to a hypo-contractile, proliferative phenotype, upon elevation of cAMP and activation of its downstream pathways. In the lung, all four PGE_2-receptor subtypes (EP_1 to EP_4) are expressed (Ushikubi *et al.*, 1995). However, in human ASM, expression of EP_1 receptors remains below limits of detection (Burgess *et al.*, 2004; Clarke *et al.*, 2005). In our experiments, the EP_1/EP_2 selective antagonist AH6809 significantly reduced the effects of the PGE_2 derivative on PDGF-induced ASM proliferation and VASP phosphorylation. Hence, our data strongly suggest the EP_2 receptor as the mediator of these effects. The EP_2 and EP_4 receptors are G_s-coupled and activation of these

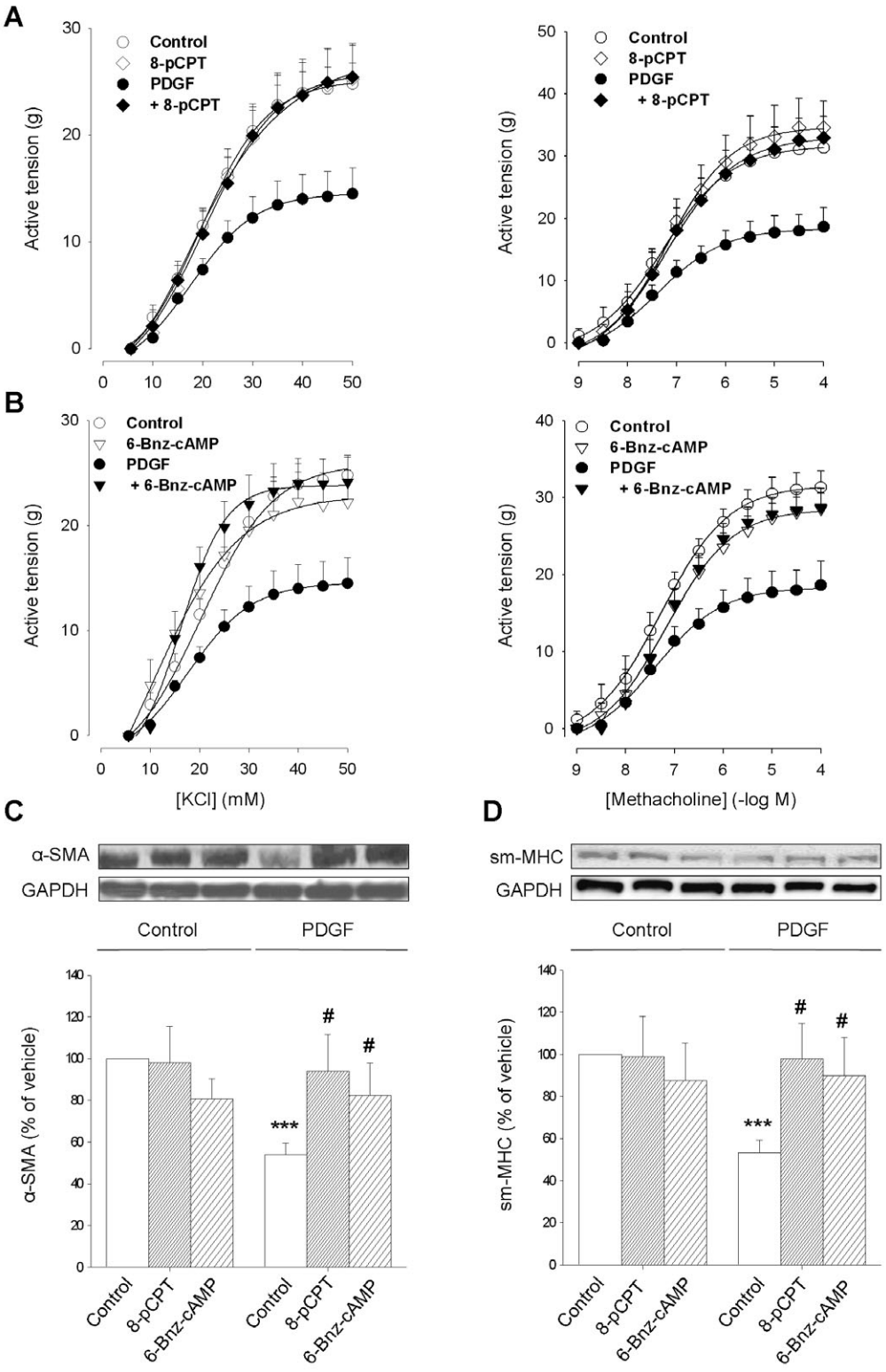

Figure 3

Activation of Epac and protein kinase A normalizes platelet-derived growth factor (PDGF)-induced hypo-contractility of bovine tracheal smooth muscle (BTSM) strips. Concentration–response curves of KCl- (left panels) and methacholine- (right panels) induced contractions in BTSM strips pretreated with 8-pCPT (30 μM) (A) or 6-Bnz-cAMP (500 μM) (B) in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. smooth muscle α-actin (α-SMA) (C) and smooth muscle myosin heavy chain (sm-MHC) (D) expression from BTSM strip homogenates obtained after the same treatments. Contractile protein levels were normalized to GAPDH. Representative immunoblots are shown. Graphs represent means ± SEM of 3–10 experiments. ***$P < 0.001$ compared with (basal) control; $^{\#}P < 0.05$ compared with PDGF.

Table 3

Activation of Epac and/or protein kinase A (PKA) inhibited platelet-derived growth factor (PDGF)-induced hypo-contractility in bovine tracheal smooth muscle (BTSM) strips

Treatment	KCl E_{max}, g	KCl EC_{50} mM	Methacholine E_{max}, g	Methacholine pE_{50} (-logM)
Control	24.9 ± 1.9	20.8 ± 1.0	31.3 ± 2.2	7.28 ± 0.15
+PDGF	14.6 ± 2.4*	19.3 ± 0.5	18.8 ± 3.0*	7.33 ± 0.13
8-pCPT-2′-*O*-Me-cAMP 30 μM	29.5 ± 4.7	21.2 ± 1.3	35.0 ± 4.6	7.09 ± 0.13
+PDGF	25.5 ± 3.1$^{\#}$	22.1 ± 1.1	33.0 ± 3.4$^{\#}$	7.09 ± 0.20
6-Bnz-cAMP 500 μM	22.6 ± 2.9	16.9 ± 2.0	28.5 ± 2.1	7.20 ± 0.23
+PDGF	24.6 ± 2.5$^{\#}$	17.3 ± 0.8*	29.1 ± 2.5$^{\#}$	7.14 ± 0.15
Control	30.0 ± 2.6	20.0 ± 1.2	36.4 ± 2.8	7.15 ± 0.25
+PDGF	20.7 ± 1.8*	17.8 ± 0.4	26.8 ± 1.8	7.26 ± 0.21
8-pCPT-2′-*O*-Me-cAMP 3 μM	24.5 ± 1.7	19.6 ± 1.6	31.5 ± 3.8	6.96 ± 0.14
+PDGF				
6-Bnz-cAMP 100 μM	26.4 ± 2.1	19.2 ± 1.2	32.7 ± 0.4	6.91 ± 0.31
+PDGF				
8-pCPT-2′-*O*-Me-cAMP 3 μM + 6-Bnz-cAMP 100 μM	25.0 ± 1.4	19.7 ± 0.8	31.1 ± 0.7	6.90 ± 0.18
+PDGF				
Control	24.2 ± 2.6	20.0 ± 0.8	32.0 ± 2.5	7.10 ± 0.12
+PDGF	18.1 ± 2.0**	19.1 ± 0.8	23.5 ± 2.4**	7.20 ± 0.11
8-pCPT-2′-*O*-Me-cAMP 30 μM + 6-Bnz-cAMP 500 μM	22.2 ± 2.1	17.4 ± 1.2	30.3 ± 0.7	7.10 ± 0.03
+PDGF	22.2 ± 2.2$^{\#}$	16.8 ± 1.1	31.1 ± 2.5$^{\#}$	7.16 ± 0.04

Contractile responses to KCl and methacholine of BTSM strips pretreated with 8-pCPT-2′-*O*-Me-cAMP (3 and 30 μM) or 6-Bnz-cAMP (100 and 500 μM) or their combination in the absence or presence of PDGF (10 ng·mL^{-1}) for 4 days. Concentration–response curves corresponding to these values are shown in Figures 3A and B and 4C. Data represents means ± SEM of 3 series of 3–5 independent experiments. E_{max}, maximal contraction; EC_{50}, concentration of agonist eliciting half-maximal response; pEC_{50}, negative logarithm of the EC_{50}. *P < 0.05; **P < 0.01 compared with unstimulated control. $^{\#}P$ < 0.05 compared with PDGF-stimulated control.

receptors elevates cAMP (Narumiya *et al.*, 1999). Compared with β_2-adrenoceptors, EP_2 receptors are less susceptible to desensitization and activation of these receptors results in a more efficient cAMP elevation and activation of PKA (Penn *et al.*, 2001; Burgess *et al.*, 2004). Indeed, 16,16-dimethyl-PGE_2 induced a strong VASP phosphorylation in BTSM cells, indicating activation of PKA. This response was partially attenuated by the PKA inhibitor H89 and largely inhibited by the combination of the PKA inhibitors Rp-cAMPS and Rp-8-Br-cAMPS (Jensen *et al.*, 2004) as well as by AH6809. Importantly, 16,16-dimethyl-PGE_2 also fully attenuated the PDGF-induced BTSM proliferation in a concentration-dependent manner via PKA. This is in line with previous studies, showing that PGE_2 inhibits growth factor-induced ASM proliferation presumably via EP_2 receptors (Burgess *et al.*, 2004) and via increasing cAMP levels (Florio *et al.*, 1994; Tomlinson *et al.*, 1995). 16,16-dimethyl-PGE_2 also fully inhibited the PDGF-induced hypo-contractility of BTSM strips and normalized the PDGF-induced reduction in contractile protein expression, thereby preventing growth factor-induced modulation of ASM phenotype. This may also be of relevance *in vivo* as a recent publication has shown that allergen-induced vascular smooth muscle cell hyperplasia and increased thickness of intrapulmonary vessels was reversed by 16,16-dimethyl-PGE_2 in a mouse model of airway inflammation (Lundequist *et al.*, 2010).

Protein kinase A is not the only effector involved in cAMP-mediated responses. Recently, activation of Epac was found to be crucial for the anti-mitogenic effect of PGE_2 in lung fibroblasts and ASM cells (Haag *et al.*, 2008; Huang *et al.*, 2008; Kassel *et al.*, 2008). The effect of activation of PKA and Epac on phenotypic modulation was assessed using selective activators of PKA and Epac, 6-Bnz-cAMP and 8-pCPT-2′-*O*-Me-cAMP respectively. Both compounds concentration dependently reduced PDGF-induced BTSM cell proliferation. As expected, the effects of 6-Bnz-cAMP was reduced by the PKA inhibitors, whereas the effect of 8-pCPT-2′-*O*-Me-cAMP was not. Similarly, 8-pCPT-2′-*O*-Me-cAMP did not phosphorylate VASP, whereas 6-Bnz-cAMP did.

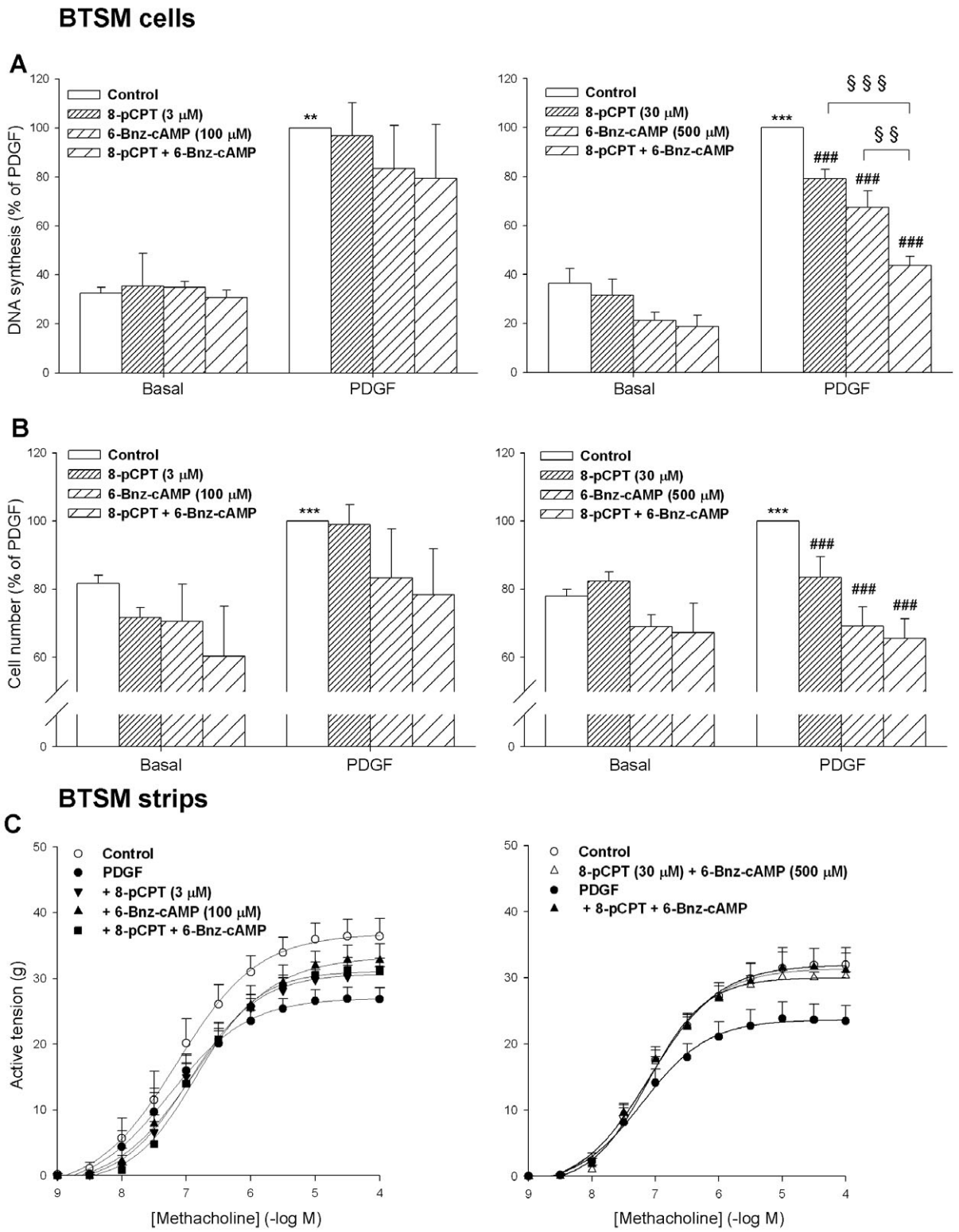

Figure 4

Combined activation of Epac and protein kinase A induces bovine tracheal smooth muscle (BTSM) phenotypic modulation. Effects of the combinations of the indicated concentrations of 8-pCPT (3 μM and 30 μM) and 6-Bnz-cAMP (100 μM and 500 μM) on basal and platelet-derived growth factor (PDGF) (10 ng·mL^{-1})-induced BTSM DNA synthesis (A) and cell number (B). Data represent mean ± SEM of 4–7 independent experiments. Concentration–response curves of methacholine-induced contractions (C) in BTSM strips pretreated with the combinations of 8-pCPT and 6-Bnz-cAMP in the absence or presence of PDGF for 4 days. Data represent mean ± SEM of 3–4 independent experiments, performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with basal control; $^{\#\#\#}P < 0.001$ compared with PDGF; $^{\S\S}P < 0.01$; $^{\S\S\S}P < 0.001$.

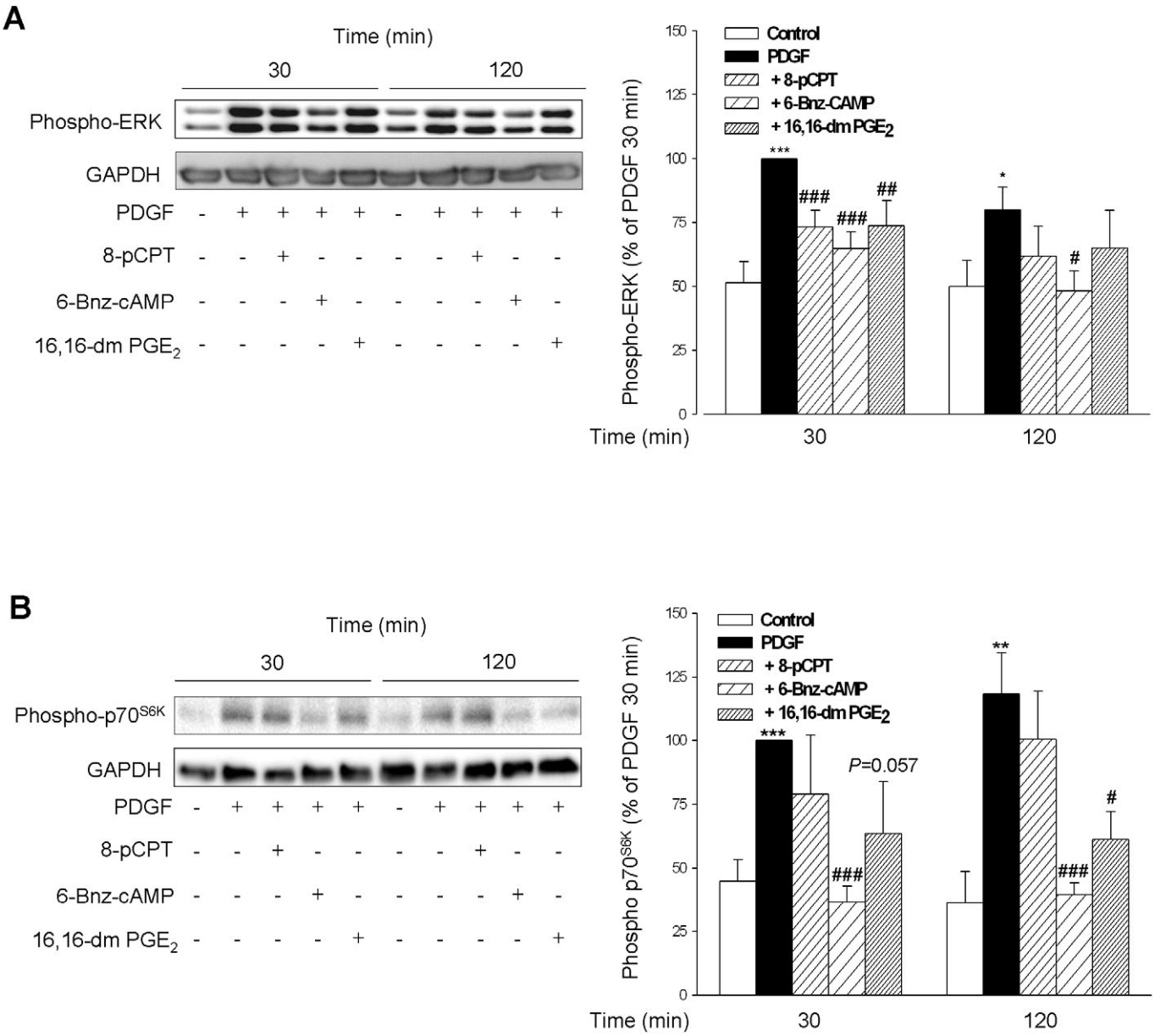

Figure 5

Differential regulation of ERK and p70^{S6K} upon activation of Epac, protein kinase A and the G_s-protein coupled EP_2 receptor. Western blot analysis of phospho-extracellular signal-regulated kinase (ERK) (A) and phospho-p70^{S6K} (B) expression in bovine tracheal smooth muscle cells treated for 30 min and 120 min with 8-pCPT (30 μM), 6-Bnz-cAMP (500 μM) or 16,16-dm PGE_2 (15 μM) in the absence (control) or presence of platelet-derived growth factor (PDGF) (10 ng·mL^{-1}). Results were normalized to GAPDH. Representative immunoblots are shown. Graphs represent means ± SEM of 4–12 experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with basal controls at 30 min and 120 min; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ compared with PDGF-treated condition.

Activation of Epac and PKA also prevented the induction of the hypo-contractile phenotype by PDGF, demonstrating the importance of these cAMP effectors in regulating ASM phenotypic modulation. Activation of Epac and PKA normalized the PDGF-induced reduction in maximal contractions in response to both receptor-dependent and independent stimuli, suggesting that changes occurred at the level of the contractile machinery. Indeed, 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP prevented the down-regulation of α-SMA and sm-MHC by PDGF. Of note, pretreatment with 6-Bnz-cAMP resulted in a left-ward shift in the KCl-induced concentration–response curve, which may be caused by changes in the ion flux across the cell membrane, such as effects on Ca^{2+} influx mediated by voltage operated calcium channels and the K^+ equilibrium potential (Ratz *et al.*, 2005), which in

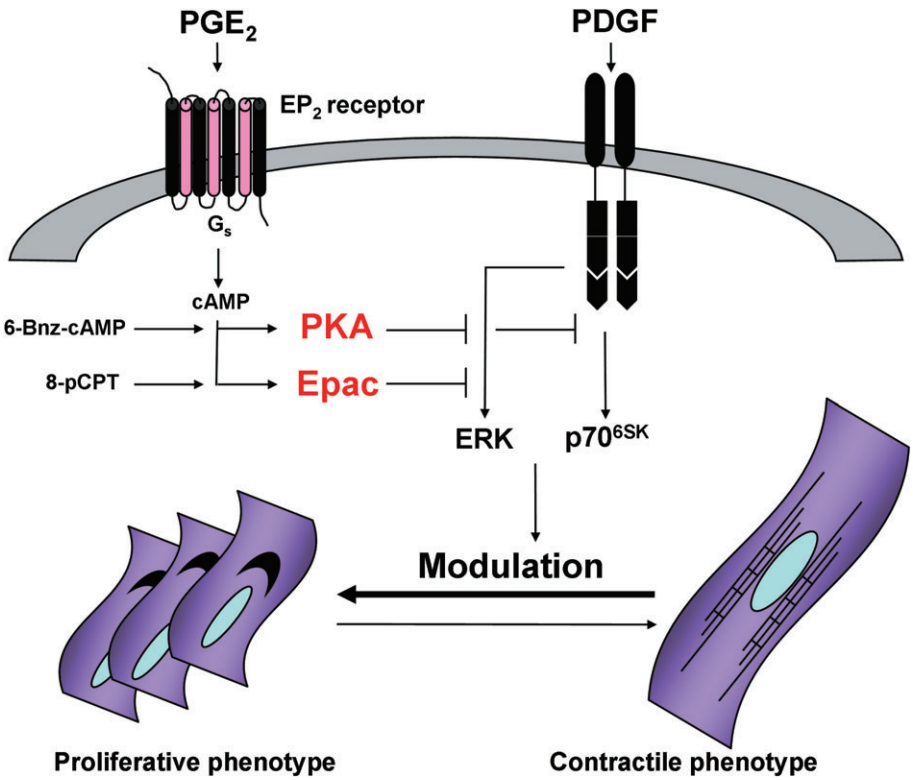

Figure 6

Mechanisms of Epac- and protein kinase A (PKA)-mediated inhibition of platelet-derived growth factor (PDGF)-induced phenotypic modulation. PDGF induces phenotypic modulation of airway smooth muscle from a contractile phenotype to a proliferative phenotype via a mechanism involving extracellular signal-regulated kinase (ERK) and $p70^{S6}$ kinase ($p70^{S6K}$). Stimulation of Epac and PKA, respectively, via the cAMP analogues 8-pCPT-2′-*O*-Me-cAMP (8-pCPT) or 6-Bnz-cAMP or via endogenous cAMP following activation of the G_s-coupled EP_2 receptor (G_s-PCR) for PGE_2 inhibits the PDGF-induced phenotypic modulation. See text for detailed description.

turn affect PKA activation. Taken together, these findings clearly show that elevation of intracellular cAMP levels prevented the PDGF-induced modulation of ASM phenotype, via activation of Epac as well as PKA.

The mitogen activated protein kinase family is known to regulate a variety of cellular responses, including proliferation, cell cycle progression and differentiation (Cowley *et al.*, 1994). Moreover, PDGF induces ASM cell proliferation via the ERK pathway (Karpova *et al.*, 1997) and inhibition of ERK prevents PDGF-induced phenotype modulation in intact BTSM strips (Gosens *et al.*, 2002). The duration of ERK activation determines the biological outcome. Acute activation of ERK (30 min) by PDGF underlies the down-regulation of contractile proteins, including α-SMA and sm-MHC (Wang *et al.*, 2004). Long-term activation of ERK is critical for mitogenic signals (Orsini *et al.*, 1999; Roy *et al.*, 2001; Kiermayer *et al.*, 2005), which probably requires prolonged stimulation of the transcription machinery, whereas non-mitogenic stimuli provide only a transient activation of ERK (Kelleher *et al.*, 1995; Roscioni *et al.*, 2009). In the present study, PDGF induced a strong and sustained phosphorylation of ERK. Interestingly, treatment with 16,16-dimethyl-PGE_2, 8-pCPT-2′-*O*-Me-cAMP and 6-Bnz-cAMP attenuated PDGF-induced ERK phosphorylation, although the inhibitory effects of 6-Bnz-cAMP appeared stronger than those of 8-pCPT-2′-*O*-Me-cAMP. This indicates that activation of Epac and PKA modulates ERK-dependent responses that may be involved in ASM phenotypic modulation.

Another potential downstream effector in ASM phenotypic modulation is $p70^{S6K}$. This kinase has been shown to drive growth factor-induced ASM cell enlargement (Deng *et al.*, 2009) and proliferation (Scott *et al.*, 1996), although the role of $p70^{S6K}$ in contractile protein expression remains contradictory (Halayko *et al.*, 2004; Deng *et al.*, 2009). Sustained activation of $p70^{S6K}$ is required for PDGF-induced BTSM proliferation, which is inhibited by treatment with the adenylyl cyclase activator forskolin (Scott *et al.*, 1996). In line with these observations, PDGF-induced activation of $p70^{S6K}$ was fully

inhibited by 6-Bnz-cAMP, whereas activation of Epac did not significantly alter this process. Treatment with 16,16-dimethyl-PGE_2 only partially inhibited the activation of $p70^{S6K}$. Hence, we can conclude that activation of $p70^{S6K}$ is not the only pathway by which cAMP elevation inhibits ASM phenotype switching, as activation of Epac inhibited PDGF-induced ASM phenotypic modulation without affecting $p70^{S6K}$ phosphorylation.

In conclusion, cAMP elevation inhibited PDGF-induced ASM phenotypic modulation through the activation of Epac and PKA, resulting in reduced PDGF-induced ASM cell proliferation and normalization of the PDGF-induced down-regulation of contractile protein expression and subsequent maintenance of the normally contractile ASM phenotype. Activation of Epac and PKA inhibited the activation of ERK by PDGF, whereas $p70^{S6K}$ was only inhibited by PKA. Collectively, our data indicate that besides acute alleviation of bronchoconstriction, cAMP elevation may be beneficial in the treatment of increased ASM mass as a long-term feature of asthma pathogenesis.

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Conflicts of interest

The authors declare no conflicts of interest.

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