Epac as a novel regulator of airway smooth muscle phenotype and function
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cAMP inhibits airway smooth muscle phenotype modulation via Epac and PKA

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Abstract

Airway smooth muscle (ASM) phenotype changes may contribute to the pathogenesis of airways diseases. Platelet-derived growth factor (PDGF) switches ASM phenotype from a contractile to a proliferative, hypocontractile phenotype, a process which requires activation of extracellular signal-regulated kinase (ERK) and p70S6K Kinase (p70^S6K). The impact 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 cAMP effectors protein kinase A (PKA) and exchange protein activated by cAMP (Epac) on PDGF-induced bovine tracheal smooth muscle (BTSM) phenotype switching.

The 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 hypocontractile, proliferative BTSM phenotype by PDGF as well as PDGF-induced activation of ERK and p70^S6K.

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. These effects may be due to activation of both Epac and PKA, as activation of both cAMP effectors similarly prevented PDGF-induced phenotype switching. PDGF-induced activation of ERK was inhibited by both effectors. Interestingly, only PKA activation resulted in inhibition of PDGF-induced p70^S6K phosphorylation.

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

Introduction

Phenotypic plasticity refers to the capacity of cells to exhibit distinct phenotypes in response to mitogenic stimuli, like for example growth factors, extracellular matrix proteins, G-protein coupled receptor agonists and inflammatory mediators [1, 2]. 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 \(\alpha\)-actin (\(\alpha\)-SMA) [1, 3, 4]. ASM cells may change their phenotype in response to mitogenic stimuli and modulate to a proliferative, hypocontractile phenotype, characterized by increased expression of proliferative markers, increased proliferation, decreased expression of contractile proteins and decreased contractile capability [3-5]. Phenotypic changes are dynamic as ASM cells in a proliferative phenotype can return to a contractile or even a hypercontractile phenotype, by for example by serum-deprivation or in the presence of insulin or TGF-\(\beta\) [6-8]. Regulation of ASM growth and proliferation by growth factors, including platelet-
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derived growth factor (PDGF) [4, 9], involves activation of extracellular signal-regulated kinase (ERK) and p70S6 kinase (p70^{S6K}) [10, 11], and these mechanisms play an important role in ASM phenotype switching [4].

ASM phenotype plasticity may contribute to the pathogenesis of airways diseases, including chronic asthma [2, 9, 12, 13]. Chronic asthma is an inflammatory airways disease, which is characterized by early and late bronchial obstructive reactions, airway hyperresponsiveness and structural changes in the airway wall (airway remodelling), which include increased ASM mass, due to cellular hypertrophy and/or hyperplasia [14, 15]. Mathematical modeling studies have shown that increase ASM mass may contribute substantially to AHR and decline in lung function [16, 17]. Therefore, identification of mechanisms that prevent ASM phenotypic plasticity is important to pharmacologically target derailed ASM proliferative and contractile responses.

Prostaglandin E$_2$ (PGE$_2$) has been shown to inhibit ASM proliferation, presumably via a mechanism involving cAMP elevation [18, 19]. Subsequently, cAMP transduces its effects in ASM cells via activation of protein kinase A (PKA) and/or exchange protein directly activated by cAMP (Epac). Recent publications have demonstrated that both PKA and Epac are involved in cAMP-mediated contractile [20-23] (chapter 7), proliferative [24-26] and inflammatory [27-29] (chapters 4 and 5) 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 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 PDGF-induced phenotypic modulation of bovine tracheal smooth muscle (BTSM) strips and cells. We investigated also the effects of the compounds on PDGF-induced activation of ERK and p70^{S6K}. Our data show that cAMP and its effectors prevent 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.

Material and 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$_2$PO$_4$, 25.00 NaHCO$_3$, and 5.50 glucose), pre-gassed 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 of Eagle’s medium (DMEM), supplemented with sodium pyruvate (1 mM), nonessential 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 rpm). When used, the PGE2 analogue 16,16-dimethyl PGE2 (1.5 and 15 µM), the Epac activator 8-pCPT-2′-O-Me-cAMP (5 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 analysis.

Isometric tension measurements. Isometric contraction experiments were performed as described previously [3, 4]. 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 wash-out, maximal relaxation was established by the addition of (-)-isoproterenol (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 (-)-isoproterenol (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 DMEM, supplemented with sodium pyruvate (1 mM), nonessential amino-acid mixture (1:100), gentamicin (45 µg ml⁻¹), penicillin (100 U ml⁻²), streptomycin (100 µg ml⁻¹), amphotericin B (1.5 µg ml⁻¹), and fetal bovine serum (FBS, 0.5%). Enzymatic digestion was performed in the same medium, supplemented with collagenase P (0.75 mg ml⁻¹), papain (1 mg ml⁻¹) and soybean trypsin inhibitor (1 mg ml⁻¹). During digestion, the suspension was incubated in the Innova incubator shaker at 37 °C, 55 rpm for 20 min, followed by a 10-min period of shaking at 70 rpm. After filtration over 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.

[^H]-thymidine incorporation. BTSM cells were plated on 24-well plates at a density of 40,000 cells/well. The next day, cells were washed with phosphate buffered saline (PBS) and made quiescent by incubation with free-serum medium supplemented with antibiotics and insulin, transferrin and selenium for 72 hrs. Subsequently, cells were incubated with 16,16-dimethyl PGES1, 8-pCPT-2′-O-Me-cAMP and/or 6-Bnz-cAMP in the absence or presence of PDGF for 28 h, the last 24 hrs in the presence of [³H]-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).
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Incorporated [³H]-thymidine was quantified by liquid-scintillation counting using a Beckam LS1701 β-counter.

Alamar blue assay. BTSM cells were plated as described for the [³H]-thymidine incorporation protocol above. Following serum deprivation, cells were treated with 16,16-dimethyl PGE₂, 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 pre-treated for 30 min with the EP₃ antagonist AH6809 (1 μM), 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) [30]. 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 analysis. BTSM strip homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonication in RIPA buffer (composition in mM: 50 mM Tris·HCl, 150.0 NaCl, 1.0 EDTA, 1.0 PMSF, 1.0 Na₃VO₄, 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). For preparation of BTSM cell homogenates, cells were treated with 16,16-dimethyl PGE₂, 8-pCPT-2’-O-Me-cAMP, 6-Bnz-cAMP in the absence or presence of PDGF for 30 min or 2 hrs. 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 10mM Tris·HCl; pH 7.4) and heated to 95°C for 5 min and resuspended with a 25-gauge needle for 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 Epacl and Epac2 and 10% for VASP, GAPDH, α-SMA, phospho-ERK and phospho-p70S6K. 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-p70S6K, VASP, Epacl and Epac2, 1:500). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (dilution 1:2000). Protein bands were visualized using western lightning plus-ECL and quantified using TotalLab software (Nonlinear Dynamics, Newcastle, United Kingdom). All protein levels were normalized to GAPDH.

Chemicals. 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 PGE₂ was from Cayman Chemical (Ann Arbor, MI, USA). Human PDGF-AB, (-)-isoproterenol hydrochloride, AH6809, H89 dihydrochloride hydrate, protease inhibitors, apo-transferrin, 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-p70S6K 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). [3H]-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.

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 observations or one-way ANOVA followed by a Bonferroni post-hoc test, as appropriate. Differences were considered to be statistically significant when \( P<0.05 \).

Results

16,16-dimethyl-PGE2 inhibits PDGF-induced BTSM phenotypic modulation.

To determine the effect of cAMP elevation on ASM phenotypic modulation, we studied the effect of the stable PGE2 analogue 16,16-dimethyl PGE2 on PDGF (10 ng ml\(^{-1}\))-induced decreases in BTSM contractility. Fully in line with previous findings [3, 4], pretreatment of BTSM strips with PDGF for 4 days reduced maximal contraction in response to KCl and methacholine (\( P<0.05 \), Fig. 1A, Tables 1 and 3) compared to vehicle treated control strips. At a concentration of 15 µM, but not 1.5 µM, the PGE2 analogue significantly inhibited the PDGF-induced decrease in both KCl- and methacholine-induced contractions (\( P<0.05 \); Fig. 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 PGE2 reduced the PDGF-induced down-regulation of α-SMA expression (\( P<0.05 \); Fig. 1B).

To assess whether the inhibition of PDGF-induced hypococontractility was associated with changes in BTSM cell proliferation, cell number was measured. 16,16-dimethyl PGE2 strongly inhibited PDGF-induced increases in cell number (\( P<0.05 \); Figs 1C and 1D and 1E), without affecting basal proliferative responses (not shown).
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Fig. 1. Activation of the Gs-protein coupled EP2 receptor inhibits PDGF-induced phenotypic modulation. Concentration-response curves of methacholine-induced contractions (A) and western blot analysis of α-SMA expression (B) in BTSM strips pre-treated with 16,16-dimethyl (dm) PGE2 (1.5 and/or 15 μM) in the absence or presence of PDGF (10 ng ml⁻¹) for 4 days. α-SMA expression was normalized to GAPDH. Representative immunoblots are shown. Data represent mean ± SEM of 3-10 experiments. Effects of 16,16-dm PGE2 on basal and PDGF-induced increase in BTSM cell number in the absence (white bars) or presence (black bars) of the EP2-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 experiments. Measurement of VASP phosphorylation (F) after 15 min treatment with 16,16-dm PGE2 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 was normalized to GAPDH. *P<0.05, **P<0.01, ***P<0.001 compared to basal control; #P<0.05, ###P<0.001 compared to PDGF; §§P<0.01; †P = 0.02.
Table 1. Activation of Gs-coupled EP2 receptor inhibits PDGF-induced BTSM strips hypotcontractility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KCI Emax, g</th>
<th>EC50 mM</th>
<th>Methacholine Emax, g</th>
<th>pEC50 (-logM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.8±3.3</td>
<td>20.2±1.6</td>
<td>35.0±3.4</td>
<td>7.03±0.30</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>19.0±1.2</td>
<td>17.6±0.4</td>
<td>25.5±1.6</td>
<td>7.20±0.26</td>
</tr>
<tr>
<td>16,16 dimethyl PGE2 1.5 μM</td>
<td>18.8±1.7</td>
<td>21.1±1.7</td>
<td>27.0±1.0</td>
<td>7.11±0.08</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>16.7±2.7</td>
<td>19.8±1.9</td>
<td>28.4±5.4</td>
<td>7.30±0.06</td>
</tr>
<tr>
<td>Control</td>
<td>16.8±0.6</td>
<td>25.0±1.2</td>
<td>30.8±4.4</td>
<td>7.21±0.13</td>
</tr>
</tbody>
</table>

Contractile responses to KCl and methacholine of BTSM strips pre-treated with 16,16 dimethyl-PGE2 (1.5 and 15 μM) in the absence or presence of PDGF (10 ng ml⁻¹) for 4 days. Data represents means ± SEM of 3-4 experiments. Emax, maximal contraction; EC50, concentration of agonist eliciting half-maximal response; pEC50, negative logarithm of the EC50. #P<0.05 compared to PDGF-stimulated control.

To evaluate the role of EP receptor subtypes in this response, AH6809 (1 μM) was used [24]. This compound has been reported to antagonize both EP1 (Gq-coupled) and EP2 (Gs-coupled) receptors with similar affinities (Ki of about 1 μM) [31, 32]. AH6809 reduced the inhibitory effect of 16,16-dimethyl PGE2 on PDGF-induced BTSM cell proliferation (P<0.05; Fig. 1C). The involvement of PKA was demonstrated by treatment with the PKA inhibitor H89 which partially reduced the inhibitory effect of 16,16-dimethyl PGE2 on PDGF-induced cell proliferation (P<0.05; Fig. 1D). Because reported non-specific effects of H89 might render interpretation of the results difficult [33], we also used a combination of two selective PKA antagonist, Rp-cAMPS and Rp-8-Br-cAMPS [30]. Combined treatment with Rp-cAMPS and Rp-8-Br-cAMPS (500 μM; each) fully inhibited the effect of the PGE2 derivative (P<0.05; Fig. 1E). To further prove the activation of PKA by 16,16-dimethyl PGE2, we tested phosphorylation of the PKA downstream target vasodilator-stimulated phosphoprotein (VASP). Western analysis was performed using an antibody which recognizes both phosphorylated VASP (phospho-VASP) and non-phosphorylated VASP (VASP). As expected, treatment with 16,16-dimethyl PGE2-induced a strong phosphorylation of VASP (P<0.05; Fig. 1F, Table 2), which was partially inhibited by H89 (Table 2) and significantly...
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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). Collectively, these data indicate that 16,16-dimethyl PGE2 exert its effects upon activation of the Gs-coupled EP2 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 [27], both Epac1 and Epac2 were expressed in BTSM tissue (Fig. 2A).

The specificity of both cAMP analogs 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, Fig. 2B, Table 2), whereas no significant changes were observed after treatment with 8-pCPT-2'-O-Me-cAMP. Treatment with H89 hardly reduced 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 significantly both DNA synthesis and cell number (P<0.05; Figs 2C, 2D and 2E). Both 8-pCPT-2'-O-Me-cAMP (Figs 2C and 2D, left panels) and 6-Bnz-cAMP (Figs 2C and 2D, 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, Fig. 2E and P<0.05 for the combination of Rp-cAMPS and Rp-8-Br-cAMPS, Fig. 2F). Importantly, the effect of the highest concentration of the Epac activator was not altered by PKA inhibition (Figs 2E and 2F).
Fig. 2. Activation of Epac and PKA decreases PDGF-induced BTSM cell proliferation. Expression of Epac1 and Epac2 from BTSM strips homogenates obtained from two cows (A). Measurement of VASP phosphorylation (B) 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 was normalized to GAPDH. Data represent means ± SEM of 3-9 experiments.

Effects of the indicated concentrations of 8-pCPT and 6-Bnz-cAMP on basal (white bars) and PDGF (10 ng ml⁻¹)-induced (black bars) 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 (white bars) or presence (black bars) 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 experiments. *P<0.05, **P<0.01, ***P<0.001 compared to basal control; #P<0.05, ##P<0.01, ###P<0.001 compared to PDGF; §P<0.05, §§P<0.01.
Table 2. Specificity of PKA activation by 16,16 dimethyl PGE$_2$ and 6-Bnz-cAMP measured by VASP phosphorylation in BTSM cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phospho-VASP % of 16,16-dm PGE$_2$</th>
<th>n</th>
<th>Treatment</th>
<th>Phospho-VASP % of 6-Bnz-cAMP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48±6</td>
<td>9</td>
<td>Control</td>
<td>40±6</td>
<td>9</td>
</tr>
<tr>
<td>+ H89</td>
<td>54±7</td>
<td>9</td>
<td>+ H89</td>
<td>52±7</td>
<td>9</td>
</tr>
<tr>
<td>+ AH6809</td>
<td>58±12</td>
<td>5</td>
<td>6-Bnz-cAMP</td>
<td>100±0***</td>
<td>9</td>
</tr>
<tr>
<td>16,16-dm PGE$_2$</td>
<td>100±0***</td>
<td>9</td>
<td>+ H89</td>
<td>93±3</td>
<td>9</td>
</tr>
<tr>
<td>+ H89</td>
<td>84±9</td>
<td>9</td>
<td>8-pCPT</td>
<td>66±15</td>
<td>6</td>
</tr>
<tr>
<td>+ AH6809</td>
<td>74±7***</td>
<td>5</td>
<td>+ H89</td>
<td>62±14</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>55±4</td>
<td>3</td>
<td>Control</td>
<td>41±3</td>
<td>3</td>
</tr>
<tr>
<td>+ PKA inhibitors</td>
<td>48±15</td>
<td>3</td>
<td>+ PKA inhibitors</td>
<td>38±7</td>
<td>3</td>
</tr>
<tr>
<td>16,16-dm PGE$_2$</td>
<td>100±0**</td>
<td>3</td>
<td>6-Bnz-cAMP</td>
<td>100±0**</td>
<td>3</td>
</tr>
<tr>
<td>+ PKA inhibitors</td>
<td>67±5§</td>
<td>3</td>
<td>+ PKA inhibitors</td>
<td>79±2§</td>
<td>3</td>
</tr>
</tbody>
</table>

VASP phosphorylation in BTSM cells after treatment with 16,16-dm PGE$_2$ (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$_2$-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 $n$ experiments. $^*$P<0.01, $^{**}$P<0.001 compared to respective control; $^#P<0.05$, $^{###}P<0.001$ compared to 16,16-dm PGE$_2$; $^\$P<0.05 compared to 6-Bnz-cAMP.

8-pCPT-2’-O-Me-cAMP and 6-Bnz-cAMP reverse PDGF-induced hypocontractility.

Interestingly, co-incubation of BTSM strips with PDGF and 8-pCPT-2’-O-Me-cAMP or 6-Bnz-cAMP at the same concentrations which inhibited PDGF-induced pro-mitogenic properties, completely normalized the PDGF-induced decrease in both KCl- and methacholine-induced contractions ($P<0.05$ both; Figs 3A and 3B, 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 [3], PDGF treatment significantly reduced α-SMA and sm-MHC expression by about 50%, as compared to control ($P<0.001$ both; Figs 3C and 3D). 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; Fig 3B), whereas both cAMP analogues alone did not affect basal expression of α-SMA.

**Fig. 3.** Activation of Epac and PKA normalizes PDGF-induced hypocontractility of 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. α-SMA (C) and sm-MHC (D) expression from BTSM strips homogenates obtained after the same treatment. Contractile protein levels were normalized to GAPDH. Representative immunoblots are shown. Graphs represent means ± SEM of 3-10 experiments. ***\(P<0.001\) compared to (basal) control; \(#P<0.05\) compared to PDGF.
**Table 3.** Activation of Epac and/or PKA inhibits PDGF-induced BTSM strips hypocontractility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>KCl</th>
<th>Methacholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$, $g$</td>
<td>$EC_{50}$, mM</td>
</tr>
<tr>
<td>Control</td>
<td>24.9±1.9</td>
<td>20.8±1.0</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>14.6±2.4*</td>
<td>19.3±0.5</td>
</tr>
<tr>
<td>8-pCPT-2'-O-Me-cAMP 30 μM</td>
<td>29.5±4.7</td>
<td>21.2±1.3</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>25.5±3.1*</td>
<td>22.1±1.1</td>
</tr>
<tr>
<td>6-Bnz-cAMP 500 μM</td>
<td>22.6±2.9</td>
<td>16.9±2.0</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>24.6±2.5*</td>
<td>17.3±0.8*</td>
</tr>
<tr>
<td>Control</td>
<td>30.0±2.6</td>
<td>20.0±1.2</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>20.7±1.8*</td>
<td>17.8±0.4</td>
</tr>
<tr>
<td>8-pCPT-2'-O-Me-cAMP 3 μM + PDGF</td>
<td>24.5±1.7</td>
<td>19.6±1.6</td>
</tr>
<tr>
<td>6-Bnz-cAMP 100 μM + PDGF</td>
<td>26.4±2.1</td>
<td>19.2±1.2</td>
</tr>
<tr>
<td>8-pCPT-2'-O-Me-cAMP 3 μM + 6-Bnz-cAMP 100 μM + PDGF</td>
<td>25.0±1.4</td>
<td>19.7±0.8</td>
</tr>
<tr>
<td>Control</td>
<td>24.2±2.6</td>
<td>20.0±0.8</td>
</tr>
<tr>
<td>+ PDGF</td>
<td>18.1±2.0**</td>
<td>19.1±0.8</td>
</tr>
<tr>
<td>8-pCPT-2'-O-Me-cAMP 30 μM + 6-Bnz-cAMP 500 μM + PDGF</td>
<td>22.2±2.1</td>
<td>17.4±1.2</td>
</tr>
</tbody>
</table>

Contractile responses to KCl and methacholine of BTSM strips pre-treated with 8-pCPT-2'-O-Me-cAMP (3 and 30 μM) or 6-Bnz-cAMP (100 and 300 μM) or their combination in the absence or presence of PDGF (10 ng ml⁻¹) for 4 days. Concentration-response curve corresponding to these values are depicted in Figs 3A, 3B and 4C. Data represents means ± SEM of 3 series of 3-5 experiments. $E_{\text{max}}$, 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 to unstimulated control. #$P<0.05$ compared to PDGF-stimulated control.

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; Fig 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 Fig. 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 (Figs 4A and 4B, left panels) and contractile (Fig 4C, left panel) responses.

Combined pretreatment with the highest concentrations of the two cAMP analogs significantly inhibited the PDGF-mediated increase in BTSM DNA synthesis and cell number ($P<0.001$, Figs 4A and 4B, right panels) and the reduction in KCl- and methacholine-induced maximal contractions ($P<0.05$, Fig. 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...
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compared to the singular treatment ($P<0.01$ compared to 6-Bnz-cAMP and $P<0.001$ compared to 8-pCPT-2'-O-Me-cAMP, Fig. 4A, right panel). No additional effects of combined treatment were observed on other parameters assessed.

The effects of Epac and PKA activation PDGF-induced ERK and p70S6K phosphorylation.

BTSM phenotypic modulation by PDGF has previously been shown to be dependent on activation of ERK [4]. Moreover, Epac and PKA have been shown to regulate different biological functions by ERK modulation [27, 34] (chapters 4 and 5).

**Fig. 5.** Differential regulation of ERK and p70S6K upon activation of Epac, PKA and the Gs-protein coupled EP2 receptor. Western blot analysis of phospho-ERK (A) and phospho-p70S6K (B) expression in BTSM cells treated for 30 min and 120 min with 8-pCPT (30 μM), 6-Bnz-cAMP (500 μM) or 16,16-dimethyl-PGE2 (15 μM) in the absence (control) or presence of 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 to basal controls at 30 min and 120 min; †$P<0.05$, ‡$P<0.01$, ††$P<0.001$ compared to PDGF-treated condition.

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-PGE2.
In agreement with our previous data in human ASM [27], 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 ($P<0.05$; Fig. 5A). Importantly, after 30 min, this response was reduced of approximately 30% by co-treatment with 8-pCPT-2’-O-Me-cAMP ($P<0.001$), 6-Bnz-cAMP ($P<0.001$) and 16,16-dimethyl-PGE$_2$ ($P<0.01$; Fig. 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 and 16,16-dimethyl-PGE$_2$ (Fig. 5A).

Phosphorylation of p70$^{S6K}$ also underpins the mitogenic properties of PDGF in ASM [11]. In BTSM cells, PDGF increased phosphorylation of p70$^{S6K}$ after 30 min ($P<0.001$) and after 120 min ($P<0.01$; Fig. 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% (Fig. 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$^{S6K}$ is inhibited by activation of PKA, but not 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, hypocontractile 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 (Fig. 6).

Changes in the ASM phenotype are considered to contribute to airway hyperresponsiveness and airway remodeling in asthma [9, 12, 13]. Long term treatment of ASM with growth factors, like PDGF, results in modulation from a normocontractile to a proliferative, hypocontractile phenotype, characterized by reduced maximal contractions in response to receptor-dependent and –independent stimuli, reduced expression of contractile proteins and increased proliferative responses [3, 4]. cAMP exerts anti-proliferative effects in ASM from different species, including cells from bovine and human origin [11, 35-37]. In the present study, we demonstrated that 16,16-dimethyl-PGE$_2$ not only inhibited ASM proliferation, but also prevents modulation to a hypocontractile, 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 [38]. However, in human ASM, EP$_1$ expression remains under detection limit [39, 40]. 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 mediator of these effects. The EP$_2$ and EP$_4$ receptors are G$_s$-coupled and activation of these receptors elevates cAMP [41]. Compared to β$_2$-adrenoceptors, EP$_2$ receptors are less susceptible to desensitization and their activation results in a more efficient cAMP elevation and activation of PKA [39, 42]. 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 [30] 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$_2$ receptor [39] and via increasing cAMP levels [18, 19]. 16,16-dimethyl-PGE$_2$ also fully inhibited the PDGF-induced hypocontractility of BTSM strips and normalized the PDGF-induced reduction in contractile protein expression, thereby preventing growth factor-induced ASM phenotypic modulation. This may also be of relevance in vivo as a recent publication has shown that allergen-induced vascular smooth muscle cell hyperplasia and in the thickness of intrapulmonary vessels was reversed by 16,16-dimethyl PGE$_2$ in a mouse model of airway inflammation [43].

PKA 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 [24-26]. 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. Activation of Epac and PKA also prevented the induction of a hypocontractile 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 ionic transportation across the cell membrane via affecting voltage operated calcium channel-mediated Ca$^{2+}$ influx and
the K⁺ equilibrium potential [44] upon PKA activation. Taken together, these findings clearly show that elevation of intracellular cAMP levels prevents PDGF-induced phenotypic modulation of ASM via activation of Epac as well as PKA.

Fig. 6. Mechanisms of Epac- and PKA-mediated inhibition of 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 p70S6 kinase (p70S6K). 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 EP2 subtype Gs-coupled receptor (Gs-PCR) for PGE₂ inhibits the PDGF-induced phenotypic modulation. See text for detailed description.

The mitogen activated protein (MAP) kinase family is known to regulate a variety of cellular responses, including proliferation, cell cycle progression and differentiation [45]. Moreover, PDGF induces ASM cell proliferation via the ERK pathway [10] and inhibition of ERK prevents PDGF-induced phenotype modulation in intact BTSM strips [4]. 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 [46]. Long-term activation of ERK is critical for mitogenic signals [47-49], which probably requires prolonged stimulation of the transcription machinery, whereas non-mitogenic stimuli provide only a transient activation of ERK [27, 50]. In the present study, PDGF induced a strong and sustained phosphorylation of ERK. Interestingly, treatment with 16,16-dimethyl-PGE₂, 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 which may be involved in ASM phenotypic modulation.

Another potential downstream effector in ASM phenotypic modulation is p70\textsuperscript{S6K}. This kinase has been shown to drive growth factor-induced ASM cell enlargement [51] and proliferation [11], although the role of p70\textsuperscript{S6K} in contractile protein expression remains contradictory [51, 52]. Sustained activation of p70\textsuperscript{S6K} is required for PDGF-induced BTSM proliferation, which is inhibited by treatment with the adenylyl cyclase activator forskolin [11]. In line with these observations, PDGF-induced activation of p70\textsuperscript{S6K} was fully inhibited by 6-Bnz-cAMP, whereas activation of Epac did not significantly alter this process. Treatment with 16,16-dimethyl-PGE\textsubscript{2} only partially inhibited the activation of p70\textsuperscript{S6K}. Hence, we can conclude that activation of p70\textsuperscript{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\textsuperscript{S6K} phosphorylation.

In conclusion, cAMP elevation inhibits 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 a normocontractile ASM phenotype. Activation of Epac and PKA inhibited the activation of ERK by PDGF, whereas p70\textsuperscript{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 ASM accumulation as a long-term feature of asthma pathogenesis.

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Chapter 8

References


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