Epac as a novel effector of airway smooth muscle relaxation

**Sara S. Roscioni [a], *, #, Harm Maarsingh [a], #, Carolina R.S. Elzinga [a], Janke Schuur [a],
Mark Menzen [a], Andrew J. Halayko [b], Herman Meurs [a], Martina Schmidt [a]**

[a] *Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands*
[b] *Department of Physiology and Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada*

Abstract

Dysfunctional regulation of airway smooth muscle tone is a feature of obstructive airway diseases such as asthma and chronic obstructive pulmonary disease. Airway smooth muscle contraction is directly associated with changes in the phosphorylation of myosin light chain (MLC), which is increased by Rho and decreased by Rac. Although cyclic adenosine monophosphate (cAMP)-elevating agents are believed to relieve bronchoconstriction mainly *via* activation of protein kinase A (PKA), here we addressed the role of the novel cAMP-mediated exchange protein Epac in the regulation of airway smooth muscle tone. Isometric tension measurements showed that specific activation of Epac led to relaxation of guinea pig tracheal preparations pre-contracted with methacholine, independently of PKA. In airway smooth muscle cells, Epac activation reduced methacholine-induced MLC phosphorylation. Moreover, when Epac was stimulated, we observed a decreased methacholine-induced RhoA activation, measured by both stress fibre formation and pull-down assay whereas the same Epac activation prevented methacholine-induced Rac1 inhibition measured by pull-down assay. Epac-driven inhibition of both methacholine-induced muscle contraction by Toxin B-1470, and MLC phosphorylation by the Rac1-inhibitor NSC23766, were significantly attenuated, confirming the importance of Rac1 in Epac-mediated relaxation. Importantly, human airway smooth muscle tissue also expresses Epac, and Epac activation both relaxed pre-contracted human tracheal preparations and decreased MLC phosphorylation. Collectively, we show that activation of Epac relaxes airway smooth muscle by decreasing MLC phosphorylation by skewing the balance of RhoA/Rac1 activation towards Rac1. Therefore, activation of Epac may have therapeutical potential in the treatment of obstructive airway diseases.

Keywords: cAMP ● protein kinase A ● Rho ● Rac ● myosin light chain ● asthma ● chronic obstructive pulmonary disease ●
stress fibres ● actin-myosin dynamics

Introduction

Airway hyper-responsiveness is generally associated with exaggerated bronchoconstriction, a key feature of a number of obstructive airway diseases, including asthma and chronic obstructive pulmonary disease [1, 2]. Airway smooth muscle tone is regulated by actin-myosin interactions through the phosphorylation of the 20 kD regulatory myosin light chain (MLC) [3], which in turn results from the dynamic balance between MLC kinase and MLC phosphatase activities [3, 4]. Actin-myosin dynamics in fibroblasts, epithelial and airway smooth muscle cells are tightly regulated by the Rho GTPase family members RhoA and Rac1 [5–7]. In particular, RhoA is involved in the formation and maintenance of stress fibres, which are contractile structures consisting of bundles of actin and myosin filaments that regulate tension development [8]. Moreover, activation of RhoA leads to airway smooth muscle tissue contraction [9, 10] mainly *via* the inhibition of MLC phosphatase, leading to increased MLC phosphorylation [11, 12]. On the other hand, Rac1 activates p21-activated kinase, which phosphorylates and inhibits MLC kinase, leading to reduced MLC phosphorylation [13, 14]. Hence, given their opposing effects on MLC phosphorylation, cross-talk between and complementary regulation of RhoA and Rac1 is likely an important determinant of airway smooth muscle tone.

cAMP-elevating agents, such as β_2-adrenoceptor agonists, are potent inhibitors of bronchial constriction. Although used clinically for many years to induce airway smooth muscle relaxation, their precise mechanism of action is not yet completely clear [15, 16].

[#]These authors contributed equally to this work.
*Correspondence to: Sara S. ROSCIONI,
Antonius Deusinglaan 1, 9713 AV Groningen,
The Netherlands.
Tel.: +31 50 363 3304
Fax: +31 50 363 6908
E-mail: s.s.roscioni@rug.nl

Classically, activation of cAMP-mediated effector protein kinase A (PKA) is believed to be involved in airway smooth muscle relaxation [16]. In fact, PKA phosphorylates several target proteins that regulate airway smooth muscle tone, including MLC kinase and potassium channels [17–19]. However, using selective inhibitors of PKA, Spicuzza *et al.* [20] demonstrated that β_2-adrenoceptor-mediated relaxation of guinea pig airway smooth muscle is PKA independent. Importantly, alternative mechanisms have not been identified yet. A better understanding of these mechanisms would help to define more specific targets for pharmacological treatment of obstructive airway diseases. In this regard, exchange proteins directly activated by cAMP (Epac) have recently been discovered and identified as novel cAMP sensors able to elicit, alone or in concert with PKA, several biological effects attributed to intracellular cAMP [21–25]. Two isoforms of Epac (Epac1 and Epac2) have been described and partly characterized with respect to their subcellular localization, structure, and function [21, 26]. Although initially characterized as Rap1-specific guanine nucleotide exchange factors (GEFs) [21, 22], Epac proteins are also able to activate a number of small GTPases that affect a variety of biological processes [26]. Recent studies using human pulmonary artery endothelial cells show that Epac regulates endothelial integrity and permeability by inhibiting Rho and stimulating Rac [27, 28]. We have recently shown that both Epac1 and Epac2 are expressed in human airway smooth muscle cells [29]. Moreover, using the selective Epac activators in combination with PKA activators and inhibitors, we also demonstrated that both Epac and PKA modulate cytokine release by human airway smooth muscle cells *in vitro* [29].

In the present study, we assessed whether Epac proteins represent a functional effector pathway for cAMP-mediated airway smooth muscle relaxation. We show that Epac induces airway smooth muscle relaxation in guinea pig and human tracheal tissue by shifting the relative balance of pro-contractile RhoA and contraction-suppressing Rac1 in favour of Rac1, thereby reducing the phosphorylation of MLC.

Material and methods

Materials

Methacholine hydrochloride was from ICN Biomedicals (Costa Mesa, CA, USA). NSC23766 was from Tocris Biosciences (Bristol, UK). 6-Bnz-cAMP, 8-pCPT-2′-O-Me-cAMP (CPT, chlorophenylthio), Rp-8-CPT-cAMPS and Sp-8-pCPT-2′-O-Me-cAMPS were from BIOLOG Life Science Institute (Bremen, Germany). The (–)-isoproterenol hydrochloride, histamine dihydrochloride and protease inhibitors and secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). foetal bovine serum (FBS) was from Hyclone Thermo Scientific (Waltham, MA, USA). Other components of the cell culture medium were obtained from Gibco BRL Life Technologies (Paisley, UK). The antibodies against vasodilator-stimulated phosphoprotein (VASP) and Rac1 were obtained from Cell Signaling Technology (Beverly, MA, USA) and Millipore (Billerica, MA, USA), respec-

tively. The antibodies against RhoA and phospho and total MLC were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Epac1 and Epac2 were kindly provided by Dr. J. L. Bos (University Medical Center Utrecht, The Netherlands) [30]. *Clostridium difficile* toxin B-1470 was kindly provided by Drs. C. von Eichel-Streiber and H. Genth (University of Mainz, Germany). Alexa Fluor 488 phalloidin was from Invitrogen (Eugene, OR, USA). All chemicals used were of analytical grade.

Guinea pig tissue and cell isolation

Outbred specific pathogen-free male Dunkin Hartley guinea pigs (Harlan-Hillcrest, UK) (800–1200 g) were used. The animals were killed by experimental concussion followed by rapid exsanguinations. The trachea was removed from the larynx to the bronchi and rapidly placed in a Krebs–Henseleit (KH) solution (composition in mM: NaCl 117.50; KCl 5.60; $MgSO_4$ 1.18; $CaCl_2$ 2.50; NaH_2PO_4 1.28; $NaHCO_3$ 25.0 and **D**-glucose 5.50; pH 7.4) at 37°C, gassed with 95% O_2 and 5% CO_2. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation. The trachea was prepared free of serosal connective tissue in gassed KH solution. For the relaxation studies, single tracheal open-ring preparations were mounted for isometric recording. For Western blot analysis of VASP and Rac1, guinea pig tracheae were pulverized under liquid nitrogen after stimulation, followed by sonication in Ripa buffer (composition: 50 mM Tris HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Igepal and 0.25% Na-deoxycholate, pH 7.4) supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF and 1 mM PMSF. For the isolation of guinea pig tracheal smooth muscle cells, the epithelium-denuded airway smooth muscle layer was removed from the trachea, chopped by using a McIlwain tissue chopper and transferred to a culture flask. Cells were grown on DMEM + 10% FBS.

Human airway smooth muscle tissue and cell isolation

Human tracheal sections from anonymized lung transplantations donors were obtained from the Department of Cardiothoracic Surgery, University Medical Centre Groningen. Human airway tissue was rapidly transported to the laboratory in pre-gassed KH solution. After careful removal of mucosa and connective tissue and dissection of the smooth muscle layer, tracheal smooth muscle strips were prepared with macroscopically identical length and width and mounted for isometric recording. Airway smooth muscle strips were also pulverized and used for Western blot analysis of Epac expression. In addition, primary human tracheal smooth muscle cells were isolated from the epithelium-denuded airway smooth muscle layer using the same protocol described for guinea pig airway smooth muscle samples.

Isometric tension measurements

Single guinea pig tracheal open-ring preparations or human airway smooth muscle strips were connected to an isometric force-displacement transducer (Grass FT03) using surgical wire and the resting tension was adjusted to 0.5 g. After a 60 min. equilibration period with three washes, the preparations were contracted with cumulative administration of methacholine (0.1, 1, 10 μM), followed by washout. Thereafter, basal tone was

assessed using isoproterenol (1 μM) and tension was re-adjusted to 0.5 g. Subsequently, the preparations were contracted with methacholine (0.1, 1, 10 and 100 μM). After three additional washouts, tracheal preparations were pre-incubated for 30 min. with the selective PKA inhibitor Rp-8-CPT-cAMPS (100 μM) or vehicle (KH buffer) and pre-contracted with methacholine (0.3 μM for guinea pig tissue, 50% effect of 100 μM for human tissue) or histamine (3 μM). Cumulative concentration-response curves were constructed using the β_2-adrenoceptor agonist isoproterenol (10^{-9}–10^{-6} M), the specific Epac activators 8-pCPT-2'-O-Me-cAMP and Sp-8-pCPT-2'-O-Me-cAMPS (10^{-8}–$3 \cdot 10^{-4}$ M; both) or the PKA activator 6-Bnz-cAMP (10^{-8}–$3 \cdot 10^{-4}$ M). All cumulative concentration-response curves were constructed in 0.5 log increments. After washout, basal tone was re-assessed using isoproterenol (10 μM). To study the role of small GTPases in Epac-mediated effects, guinea pig open-ring preparations were incubated overnight with *Clostridium difficile* toxin B-1470 (0.1 ng/ml) or vehicle (0.1% FBS) in DMEM. The next day, the rings were thoroughly washed with fresh KH solution and mounted for isometric contraction. Toxin-induced glucosylation of Ras-like GTPases was monitored by immunoblotting using a specific anti-Rac1 antibody [29].

Cell culture

Guinea pig and human primary airway smooth muscle cells and the immortalized human airway smooth muscle cell line (hTERT) were grown in DMEM supplemented with streptomycin, penicillin and 10% FBS. hTERT airway smooth muscle cells were prepared as described previously [29, 31]. All procedures were approved by the human Research Ethics Board of the University of Manitoba [32]. For all experiments, cells were grown to 100% confluency and serum deprived for 24 hrs. Medium was refreshed every 48–72 hrs. Upon reaching confluency, cells were passaged by trypsinization. In the present study, passages 1–5 were used for primary guinea pig and human airway smooth muscle cells and passages 1 to 30 for hTERT airway smooth muscle cells.

Rac1 and RhoA pull-down, phosphorylation of VASP/MLC and immunoblot analysis

The amount of activated Rac1 or RhoA was measured with the pull-down technique by using glutathione S-transferase tagged PAK1 or Rhothekin, respectively, as previously described [33, 34]. The level of activated GTPases was normalized to Rac1 or RhoA content in total cell lysates, respectively. For the measurement of the phosphorylation of VASP and MLC, cells or tissue strips were stimulated with methacholine (100 μM), 8-pCPT-2'-O-Me-cAMP (300 μM) or their combination in the absence or presence of the PKA inhibitor Rp-8-CPT-cAMPS (100 μM) or the Rac1 inhibitor NSC23766 (100 μM). Subsequently, cells were lysed in supplemented Ripa buffer, and tissue rings and strips were pulverized under liquid nitrogen followed by sonication in Ripa buffer. Protein concentration was determined by Pierce measurement. Equal amounts of protein were loaded on 8–15% polyacrylamide gels and analysed for the protein of interest by using the specific primary antibody (dilution anti-Epac1, anti-Epac2, anti-Rac1 and anti-VASP, 1:500 all; anti-phospho-MLC, total MLC 1:200 each; anti Rho-A, 1:100; anti-β-actin, 1:2000) and the secondary HRP-conjugated antibody (dilution 1:2000 anti-rabbit, 1:3000 antimouse and 1:10,000 anti-goat). Protein bands were subsequently visualized on film using western lightning plus-ECL (PerkinElmer Inc., Waltman, MA, USA) and quantified by

densitometry using TotalLab software (Nonlinear Dynamics, Newcastle, UK). Results were normalized to specific control proteins.

Fluorescence staining

Guinea pig airway smooth muscle cells were plated on Lab-Tek™ Chamber Slides (Thermo Scientific) and treated with methacholine (100 μM) and/or 8-pCPT-2'-O-Me-cAMP (300 μM), fixed in 3% formaldehyde solution in Hank's Buffered Salt Solution (HBSS) for 15 min. at room temperature (RT), washed and permeabilized with 0,05% Triton X-100 in HBSS for 2 min. at RT. Stress fibres were stained with Alexa Fluor 488 phalloidin (15 min. at RT). After staining, cover slips were mounted using ProLong Gold antifade reagent (Invitrogen) and they were analysed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens; Olympus, Essex, UK).

Data analysis

The effects of isoproterenol and the cAMP analogues were expressed as percentages of methacholine-induced tone. The effect of the highest concentration of methacholine used was defined as Emax. Using this Emax, the sensitivity to the different substances was evaluated as pEC$_{50}$ (−log EC$_{50}$). All data represent means $\pm$ S.E.M. from n separate experiments. The statistical significance of differences between data was determined by a paired or unpaired Student's t-test when appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Results

Epac induces relaxation of guinea pig tracheal smooth muscle preparations

To activate PKA and Epac, we used two well-described cAMP analogues, known for their selectivity towards the distinct cAMP effectors [29, 35]. 6-Bnz-cAMP is a selective activator of PKA, which has been used to unravel PKA signalling in several systems including the airways [36, 37]. An index of PKA activation is the phosphorylation of its specific substrate VASP [38]. As expected, treatment of guinea pig tracheal rings with the PKA selective activator 6-Bnz-cAMP ($P < 0.01$) or with the β_2-receptor agonist, isoproterenol ($P < 0.01$), induced significant phosphorylation of VASP compared to basal levels (Fig. 1A). Moreover, VASP phosphorylation by direct PKA activation or *via* β_2-receptor was prevented by Rp-8-CPT-cAMPS ($P < 0.05$ for both, Fig. 1A), a selective inhibitor of both type I and type II of PKA [39]. Similar results were observed in isolated guinea pig airway smooth muscle cells (not shown). These data both confirm that isoproterenol-induced cAMP activates PKA, and the specificity of PKA-mediated VASP phosphorylation.

We next investigated the functional importance of isoproterenol-induced PKA activation in regulating airway smooth muscle tone.
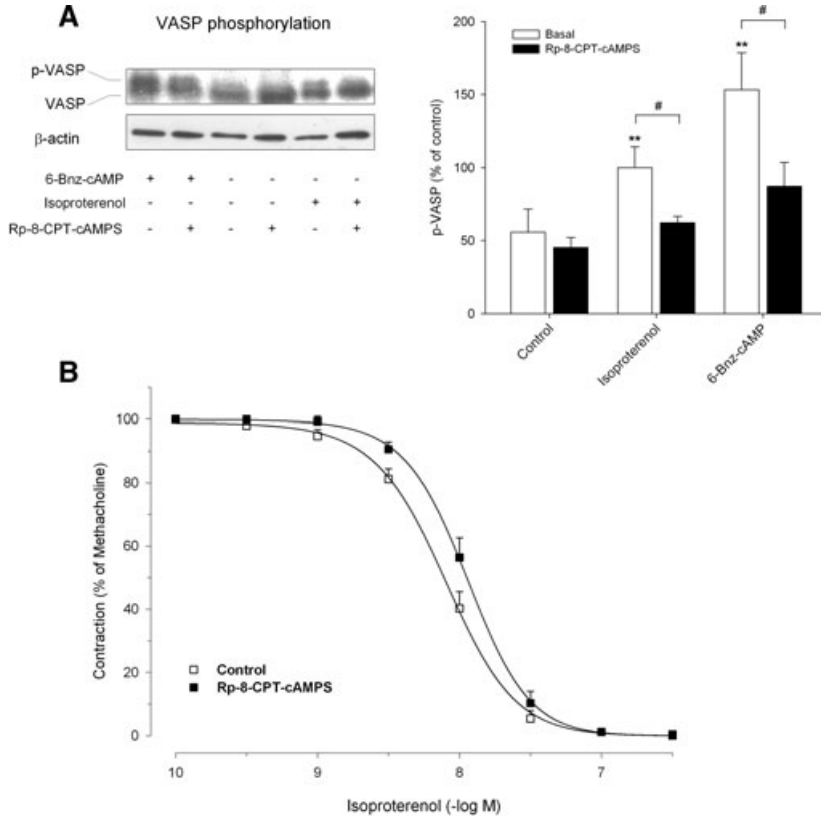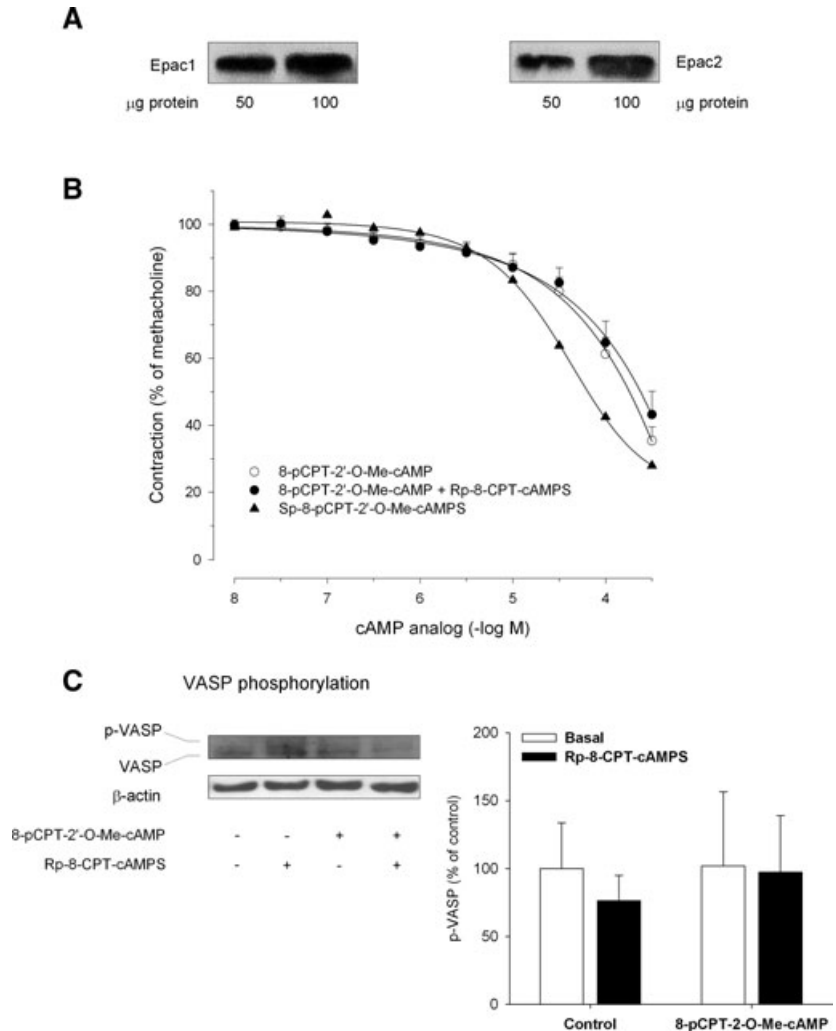
Fig. 1 Isoproterenol-induced relaxation of guinea pig tracheae. Impact of PKA. (**A**) Guinea pig tracheal open rings were treated without (Control) and with 100 μM Rp-8-CPT-cAMPS for 30 min., followed by addition of 1 μM isoproterenol or 500 μM 6-Bnz-cAMP for 15 min. Phosphorylation of the PKA effector VASP was evaluated by using a VASP-specific antibody which recognizes both phospho-VASP (p-VASP, upper band) and non-phospho-VASP (VASP, lower band). Representative blots are shown. Graphs depict the ratio between phospho-VASP and total VASP (phospho- + non-phospho-VASP) expressed in percentage of isoproterenol-treated conditions. Equal loading was verified by analysis of β-actin. Results are mean ± S.E.M. of seven to nine independent experiments. (**B**) Cumulative concentration response curves of isoproterenol-induced relaxation of guinea pig tracheal open ring preparations pre-contracted with 0.3 μM methacholine in the absence (Control) and presence of 100 μM Rp-8-CPT-cAMPS. Results are means ± S.E.M. of six independent experiments. $**P < 0.01$ compared to basal control; $^{\#}P < 0.05$; compared to basal.

Table 1 Effects of isoproterenol and specific analogues of cAMP on pre-contracted guinea pig and human tracheal preparations with or without treatment with Rp-8-CPT-cAMPS or toxin B-1470

	Treatment	Emax (% of methacholine)	pEC$_{50}$-log (M)	n
Guinea pig trachea				
Isoprenaline	Control	99.6 ± 0.40	8.10 ± 0.05	6
	Rp-8-CPT-cAMPS	101.3 ± 1.50	7.95 ± 0.06*	6
8-pCPT-2'-O-Me-cAMP	Control	64.6 ± 4.20	4.18 ± 0.08	8
	Rp-8-CPT-cAMPS	56.8 ± 7.00	4.20 ± 0.08	5
	Vehicle	72.3 ± 2.50	4.50 ± 0.06	5
	Toxin B-1470	57.4 ± 2.40	4.29 ± 0.07	5
Sp-8-pCPT-2'-O-Me-cAMPS	Control	72.1 ± 3.90	4.58 ± 0.13$^{\#}$	3
6-Bnz-cAMPS	Control	77.3 ± 6.80	3.90 ± 0.04	4
Human trachea				
8-pCPT-2'-O-Me-cAMP	Control	63.9 ± 3.65	5.26 ± 0.29	4
	Rp-8-CPT-cAMPS	55.8 ± 7.20	5.60 ± 0.41	3

The Emax (in% relaxation) and the pE$_{50}$-values for isoproterenol, 8-pCPT-2'-O-Me-cAMP, 6-Bnz-cAMP and Sp-8-pCPT-2'-O-Me-cAMP in guinea pig and/or human tracheal preparations pre-contracted with methacholine in the absence or presence of the PKA inhibitor Rp-8-CPT-cAMPS (100 μM) or after overnight treatment with vehicle or toxin B-1470 (0.1 ng/ml). Data represent means ± S.E.M. of n experiments.

Fig. 2 Impact of Epac activation on methacholine-induced contraction of guinea pig tracheae. (**A**) Expression of Epac1 and Epac2 in guinea pig tracheal homogenates. (**B**) Cumulative concentration response curves of 8-pCPT-2′-*O*-Me-cAMP and Sp-8-pCPT-2′-*O*-Me-cAMPS on methacholine (0.3 μM) pre-contracted guinea pig tracheal open ring preparations in the absence (Control) or presence of 100 μM Rp-8-CPT-cAMPS. Results are means ± S.E.M. of three to eight independent experiments. (**C**) Effects of 1 μM isoproterenol, 500 μM 6-Bnz-cAMP or 300 μM 8-pCPT-2′-*O*-Me-cAMP on the phosphorylation of VASP in guinea pig tracheal open rings in the absence and presence of 100 μM Rp-8-CPT-cAMPS. Equal loading was verified by analysis of β-actin. Representative immunoblots are shown together with densitometric quantifications of the percentage of phospho-VASP compared to control. Results are means ± S.E.M. of five independent experiments.

Treatment of guinea pig tracheal open ring preparations with isoproterenol resulted in a dose-dependent relaxation of metha-choline-induced tone (Fig. 1B, Table 1). Interestingly, pre-treatment of the same preparations with the PKA inhibitor Rp-8-CPT-cAMPS induced only a small rightward shift of the isoproterenol concentration-response curve (Fig. 1B, Table 1), indicating only a modest role for PKA in β2-receptor-mediated airway smooth muscle relaxation. Nonetheless, we confirmed that direct activation of PKA with 6-Bnz-cAMP did induce airway smooth muscle relaxation of guinea pig open rings pre-contracted with methacholine (Table 1). Collectively, these data strongly suggest that though PKA can suppress contractile tone of airway smooth muscle, it may not be the principal effector pathway engaged during β2-receptor-mediated relaxation.

Because isoproterenol-induced airway smooth muscle relaxation appears to occur largely independent of PKA, other cAMP activated pathways could be involved. We suggested an important role for Epac in airway smooth muscle relaxation. We observed that both Epac1 and Epac2 proteins are constitutively expressed in guinea pig tracheae (Fig. 2A). To examine the role of Epac in regulating airway smooth muscle tone, we used the cAMP analogue 8-pCPT-2′-*O*-Me-cAMP, which is a well-recognized Epac selective activator [35, 40]. By means of this compound, novel intriguing functions of Epac in the airways have been unravelled [24, 29, 36, 37]. The methylated structure of 8-pCPT-2′-*O*-Me-cAMP makes it an extremely poor PKA activator and allows for specific discrimination between both cAMP-activated signalling pathways [35, 40]. Treatment with 8-pCPT-2′-*O*-Me-cAMP relaxed guinea pig tracheal open-rings pre-contracted with methacholine (Fig. 2B, Table 1) or histamine (not shown) in a dose-dependent fashion. The effect of Epac on muscle tone was selective as the PKA inhibitor Rp-8-CPT-cAMPS had no impact on Epac-induced airway smooth muscle relaxation (Fig. 2B, Table 1). The specificity of 8-pCPT-2′-*O*-Me-cAMP was
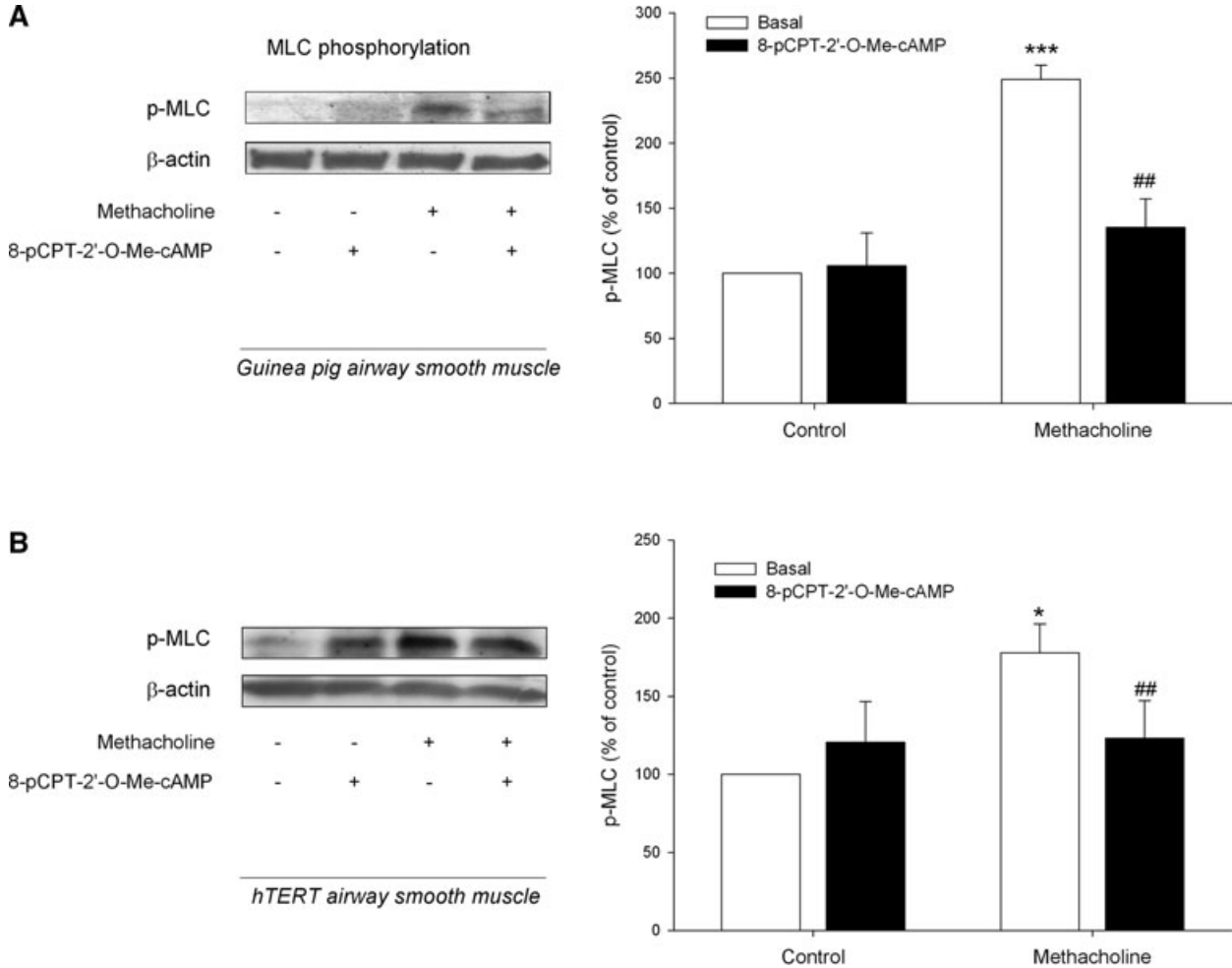
Fig. 3 Activation of Epac inhibits methacholine-induced phosphorylation of MLC. (**A**) Guinea pig and (**B**) hTERT-airway smooth muscle cells were stimulated for 30 min. with 100 μM methacholine, 300 μM 8-pCPT-2′-O-Me-cAMP or their combination and MLC phosphorylation (p-MLC) was evaluated by Western blot. p-MLC levels were normalized to β-actin. Representative blots for p-MLC and β-actin are shown. Graphs represent the results of four to seven independent experiments. $*P < 0.05$; $***P < 0.001$ compared to basal control; $^{\#\#}P < 0.01$ compared to methacholine.

further confirmed by the inability of the Epac activator to increase phosphorylation of VASP (Fig. 2C). To further confirm the importance of Epac in regulating airway smooth muscle tone, we used Sp-8-pCPT-2′-O-Me-cAMPS, a selective Epac activator which is more resistant to phosphodiesterase hydrolysis, compared to 8-pCPT-2′-O-Me-cAMP [41]. Indeed we observed that Sp-8-pCPT-2′-O-Me-cAMPS dose-dependently reduced methacholine-induced tone of guinea pig tracheal open rings (Fig. 2B, Table 1). Of note, sensitivity (pEC$_{50}$) to phosphodiesterase-resistant Sp-8-pCPT-2′-O-Me-cAMPS was significantly greater than that we measured for 8-pCPT-2′-O-Me-cAMPS (Table 1). Taken together, these findings clearly show that activation of Epac induces guinea pig airway smooth muscle relaxation independent of PKA.

Epac reduces the phosphorylation of MLC by modulating the Rac1/RhoA balance

Actin-myosin dynamics and airway smooth muscle tone are regulated by phosphorylation of MLC. Therefore, we studied the effect of Epac stimulation on (agonist-induced) MLC phosphorylation in both guinea pig and hTERT airway smooth muscle cells. As expected, treatment with methacholine increased the phosphorylation of regulatory MLC in both guinea pig airway smooth muscle cells (Fig. 3A) ($P < 0.001$) and hTERT airway smooth muscle cells (Fig. 3B) ($P < 0.01$). Importantly, 8-pCPT-2′-O-Me-cAMP completely prevented the induction of MLC phosphorylation by methacholine in both cell types ($P < 0.01$ for guinea pig and

$P < 0.05$ for hTERT airway smooth muscle cells), whereas the Epac activator did not affect basal phospho-MLC levels (Fig. 3A and B). No differences were observed in the levels of total MLC for the different treatments (not shown). These observations suggest a regulatory role for Epac in contractile agonist-induced actin-myosin activation, but a modest role in resting (basal) conditions.

The phosphorylation of MLC is partly regulated by the balance between RhoA and Rac1 activation. Therefore, we next studied the effect of methacholine on the activation of RhoA and Rac1, and assessed the effect of co-activation of Epac with 8-pCPT-2′-O-Me-cAMP on these responses in guinea pig and hTERT airway smooth muscle cells. The activation of RhoA was evaluated by two independent indices: the formation of stress fibres, and GTP-loading of RhoA. Rac1 activation was similarly evaluated by measuring GTP-loading of Rac1. Methacholine induced stress fibre formation ($P < 0.001$, Fig. 4A) and increased GTP-loading of RhoA ($P < 0.01$, Fig. 4B), whereas Rac1 activation was suppressed, as GTP-loading of Rac1 was reduced compared to basal levels ($P < 0.05$, Fig. 4C). Importantly, concomitant activation of Epac with 8-pCPT-2′-O-Me-cAMP diminished methacholine-induced stress fibre formation ($P < 0.001$, Fig. 4A) as well as GTP-loading of RhoA ($P < 0.01$, Fig. 4B), whereas basal RhoA activity was not affected by the Epac activator (Fig. 4A). On the other hand, the impaired Rac1 activity after methacholine treatment was completely prevented by co-treatment with 8-pCPT-2′-O-Me-cAMP ($P < 0.05$, Fig. 4C).

Taken together, these findings indicate that Epac stimulation relaxes methacholine-induced airway smooth muscle tone *via* shifting the relative activation of RhoA and suppression of Rac1 in such a way that an equilibrium favouring Rac1 is established, which in turn results in a reduced MLC phosphorylation. To confirm that activation of Rac1 by Epac is indeed important for its relaxant properties, guinea pig tracheal rings were incubated overnight with *Clostridium difficile* toxin B-1470. Toxin B-1470 is known to inhibit Epac substrate GTPases such as Rac1 by monoglucosylation, but not RhoA [42], and therefore can provide insight concerning the relative importance of Rac1 and RhoA regulation by Epac in the methacholine-induced contraction. The effectiveness of toxin B-1470 in inhibiting GTPases in guinea pig tracheae was studied by immunoblotting analysis using a specific antibody against the small GTPase Rac1 that only recognizes the non-glucosylated epitope of Rac1 [29]. Overnight treatment of guinea pig tracheal smooth muscle tissue with toxin B-1470 inactivated approximately 50% of total Rac1 ($P < 0.05$), as illustrated by the reduced expression of non-glycosylated Rac1 (Fig. 5A). Incubation of guinea pig smooth muscle preparations with toxin B-1470 did not alter the active tension generated by 0.3 μM concentration of methacholine (1.37 ± 0.17 g of control *versus* 1.35 ± 0.23 g for toxin B-1470). Importantly, the same treatment significantly reduced the relaxant effect of the Epac activator 8-pCPT-2′-O-Me-cAMP on methacholine-induced airway smooth muscle contraction (Fig. 5B, Table 1) ($P < 0.05$), demonstrating that Epac-mediated airway smooth muscle relaxation relies, in part, on the ability of 8-pCPT-2′-O-Me-cAMP to activate Rac1.

To confirm the importance of Rac1 in the Epac-mediated effects the selective Rac1-inhibitor NSC23766, was used. NSC23766 prevents Rac1 activation by Rac-specific GEFs TrioN and Tiam1, without interfering with the activation of the other Rho-like GTPases (Rho and Cdc42) and has been used to study the functional role of Rac1 [43]. Inhibition of Rac1 augmented basal MLC phosphorylation ($P < 0.05$, Fig. 5C) which was not further increased by methacholine (Fig. 5C), in line with the inhibitory effect of methacholine on Rac1 activity (Fig. 4C). Importantly, in the presence of NSC23766, 8-pCPT-2′-O-Me-cAMP was unable to reduce MLC phosphorylation (Fig. 5C). Collectively, these findings indicate that activation of Rac1 by Epac plays an important role in Epac-mediated inhibition of MLC phosphorylation and relaxation of airway smooth muscle.

Epac reduces airway smooth muscle tone and MLC phosphorylation in human tracheae

We confirmed our key findings in guinea pig in freshly isolated human airway smooth muscle strips and cells. Western analysis revealed that both Epac1 and Epac2 are constitutively expressed in human tracheal smooth muscle strips (Fig. 6A). Importantly, treatment with the Epac activator 8-pCPT-2′-O-Me-cAMP dose-dependently reduced methacholine-induced contraction of human tracheal strip preparations (Fig. 6B, Table 1). Treatment with the PKA inhibitor Rp-8-CPT-cAMPS did not affect 8-pCPT-2′-O-Me-cAMP-mediated relaxations (Fig. 6A, Table 1), confirming a PKA-independent effect for Epac proteins. Accordingly, activation of Epac did not induce VASP phosphorylation by PKA in human tracheal preparations (Fig. 6C). As expected, isoprotenerol and PKA activation with 6-Bnz-cAMP did induce phosphorylation of VASP in human airway smooth muscle cells (Fig. 6C and D) ($P < 0.01$), which was largely inhibited by co-treatment with selective PKA inhibition using Rp-8-CPT-cAMPS (Fig. 6D) ($P < 0.05$ both). Importantly, methacholine induced a ~3-fold increase of phosphorylation of MLC ($P < 0.001$), effect that was completely reversed by co-treatment with 8-pCPT-2′-O-Me-cAMP to activate Epac (Fig. 6E) ($P < 0.05$). As we observed for guinea pig cells, basal levels of phosphorylated MLC were not affected by 8-pCPT-2′-O-Me-cAMP and no differences were observed in the levels of total MLC for the different treatments (Fig. 6E). Taken together, these finding demonstrate that activation of Epac proteins induce relaxation of human airway smooth muscle, independent of PKA, by inhibiting agonist-induced MLC phosphorylation.
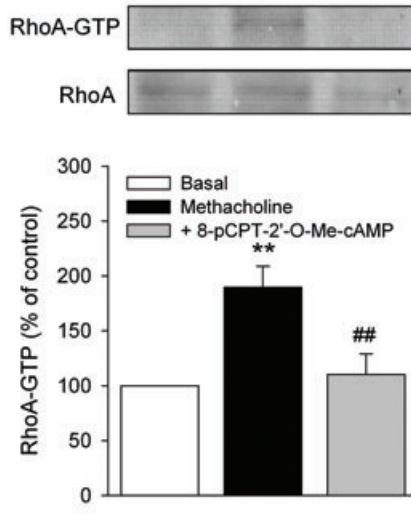
Discussion

Our study is the first to show activation of cAMP-dependent Epac as a novel signalling effector that promotes relaxation of airway smooth muscle. Here, we demonstrate that Epac activation inhibits contraction of airway smooth muscle from guinea pig and human by a process that is largely independent of PKA. Importantly, we
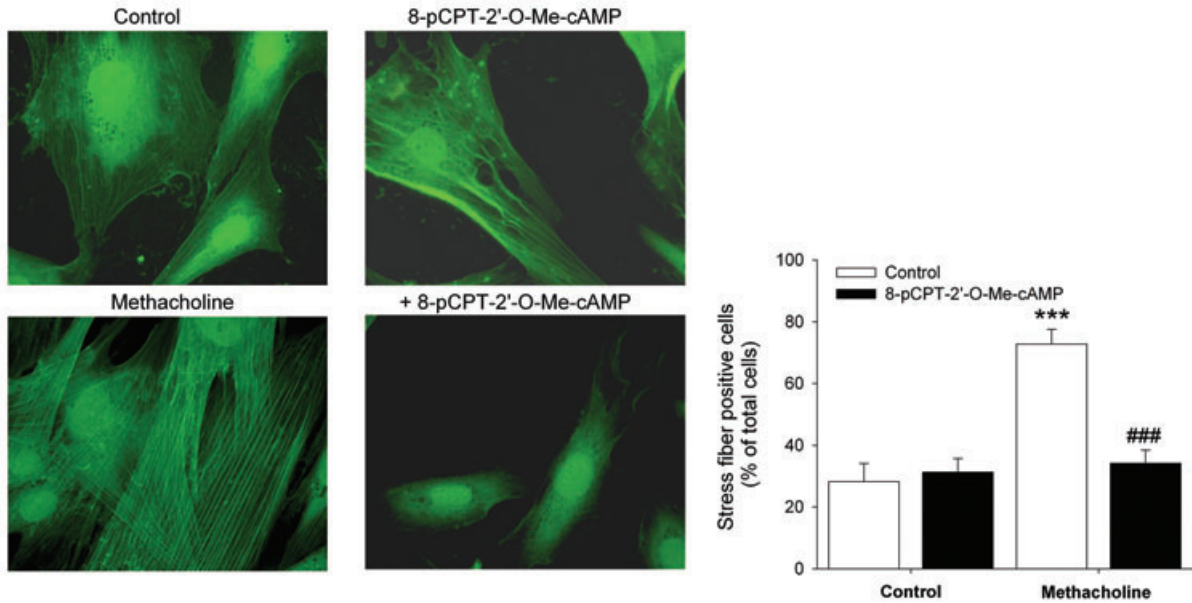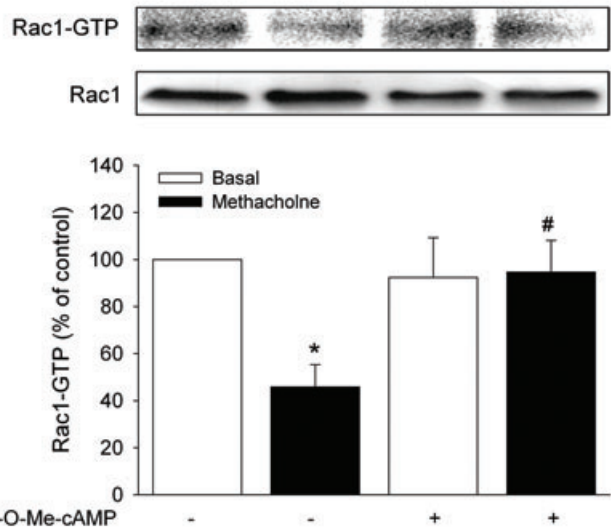
Fig. 4 Activation of Epac shifts the balance between RhoA and Rac1 towards Rac1. Guinea pig or hTERT-airway smooth muscle cells were stimulated for 30 min. with 100 μM methacholine, 300 μM 8-pCPT-2′-O-Me-cAMP or their combination. (**A**) Stress fibre formation was measured by phalloidin staining in guinea pig airway smooth muscle. Results are expressed as percentage of stress fibre-positive cells relative to the total number of cells. Representative images of five experiments are shown. (**B**) In hTERT-airway smooth muscle cells, GTP-loaded RhoA and total RhoA, and (**C**) GTP-loaded Rac1 and total Rac1 were determined as described in 'Materials and Methods'. Representative blots of three to five independent experiments are shown. Densitometric quantifications depicts GTP-loaded RhoA and Rac1 in percentage of control. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared to basal control; $^{#}P < 0.05$; $^{##}P < 0.01$; $^{###}P < 0.001$ compared to methacholine alone.
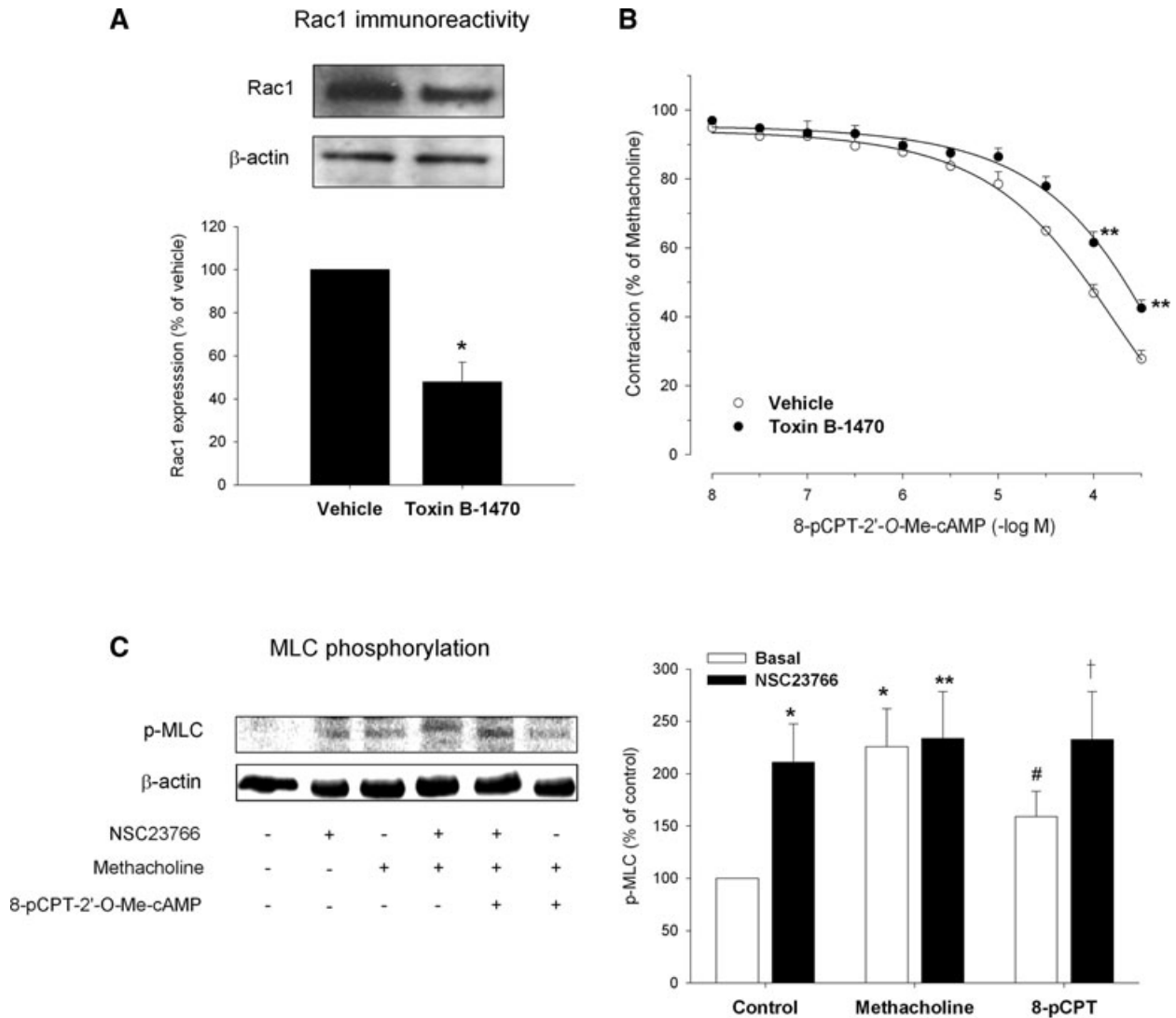
Fig. 5 Impairment of relaxant properties of the Epac activator 8-pCPT-2′-*O*-Me-cAMP by Rac1-inhibition. (**A**) Immunoreactivity of Rac1 in guinea pig trachea homogenates after overnight treatment with vehicle or with 0.1 ng/ml toxin B-1470 was measured by Western blot. Equal loading was verified by analysis of β-actin. Representative immunoblots are shown with the quantifications of four independent experiments. (**B**) Guinea pig tracheal ring preparations were incubated overnight with vehicle or 0.1 ng/ml toxin B-1470. Cumulative concentration response curves of 8-pCPT-2′-*O*-Me-cAMP-induced relaxation of guinea pig tracheal ring preparations pre-contracted with 0.3 μM methacholine are shown. Results are means ± S.E.M. of five independent experiments. (**C**) hTERT-airway smooth muscle cells were pre-treated with the Rac1 inhibitor NSC23766 (100 μM) for 30 min. and then stimulated for additional 30 min. with methacholine (100 μM) alone or in combination with 8-pCPT-2′-*O*-Me-cAMP (300 μM). MLC phosphorylation (p-MLC) was evaluated by Western blot and normalized to β-actin. Representative blots are shown. Graphs represent the results of six to eight independent experiments. *$P < 0.05$; **$P < 0.01$ compared to basal control; #$P < 0.05$ compared to methacholine.

found that Epac proteins shift the balance between Rac1 and RhoA activation during agonist-induced contraction in favour of Rac1, resulting in reduction of MLC phosphorylation and subsequent inhibition of airway smooth muscle contraction (Fig. 7).

Abnormal regulation of airway smooth muscle tone causes airway hyper-responsiveness, a main feature of obstructive airway diseases such as asthma and chronic obstructive pulmonary dis-ease [2]. The complex mechanisms of airway tone regulation rely on the intrinsic capacity of the airway smooth muscle to contract and to relax providing control of airway lumen diameter and respiratory capacity. The relaxant properties of cAMP-elevating β2-agonists are well established [15], but there is surprisingly little precise knowledge about the molecular mechanisms of cAMP action. Spicuzza *et al.* [20] have reported on the existence
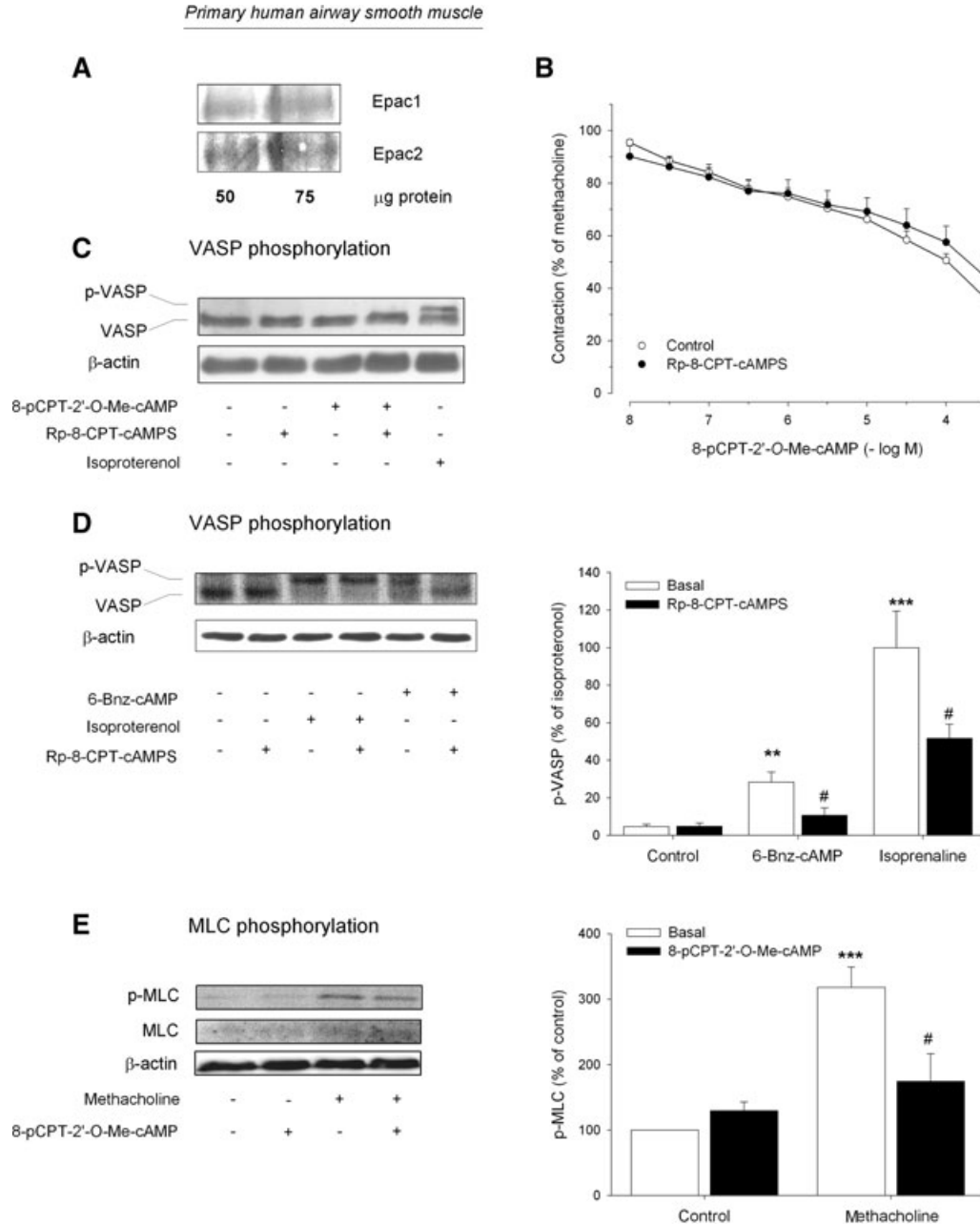
Fig. 6 Activation of Epac by 8-pCPT-2′-O-Me-cAMP induces relaxation of human tracheal smooth muscle strips. (**A**) Expression of Epac1 and Epac2 in human tracheal smooth muscle homogenates using two protein contents. (**B**) Cumulative concentration response curves of 8-pCPT-2′-O-Me-cAMP-induced relaxation of human tracheal smooth muscle strips pre-contracted with 0.1 μM methacholine in the absence and presence of 100 μM Rp-8-CPT-cAMPS. Results are means ± S.E.M. of three independent experiments. (**C**) and (**D**) Effects of 1 μM isoproterenol, 500 μM 6-Bnz-cAMP or 300 μM 8-pCPT-2′-O-Me-cAMP on phosphorylation of VASP in primary human tracheal smooth muscle cells in the absence and presence of 100 μM Rp-8-CPT-cAMPS. Results are mean ± S.E.M. of five to seven independent experiments. Graphs depict the ratio between phospho-VASP and total VASP (phospho- + non-phospho-VASP) expressed in percentage of isoproterenol-treated conditions. Equal loading was verified by analysis of β-actin. (**E**) Effect of 300 μM 8-pCPT-2′-O-Me-cAMP on methacholine-induced phosphorylation of MLC in primary human tracheal smooth muscle cells. p-MLC levels were normalized to β-actin. Representative blots of p-MLC, total MLC and β-actin are shown. Graphs represent the results of three to six independent experiments. $**P < 0.01$; $***P < 0.001$; compared to basal control; $^{\#}P < 0.05$ compared to methacholine.
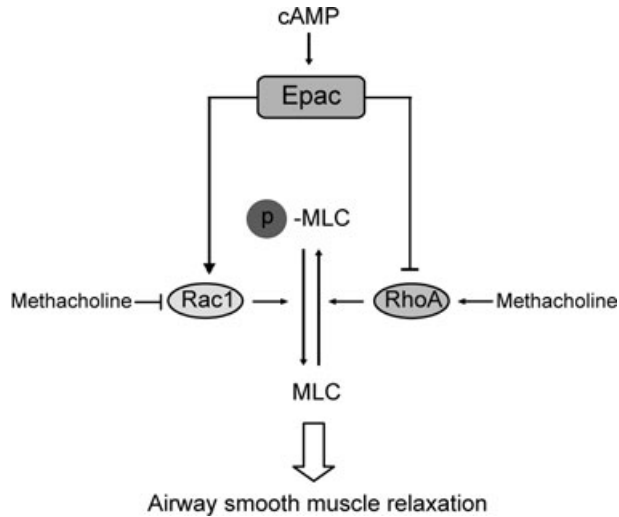
Fig. 7 Model of Epac-induced airway smooth muscle relaxation. Activation of Epac by cAMP shifts the balance of RhoA and Rac1 towards Rac1. A potential mechanism by which Epac affects airway relaxation is represented by inhibition of MLC phosphorylation. See text for further details.

of PKA-independent mechanisms of an unknown nature in the isoproterenol-induced relaxation of guinea pig tracheae [20]. In this study, we confirm that although isoproterenol activates PKA, isoproterenol-induced airway smooth muscle relaxation occurs largely independent of PKA, being refractory to the PKA inhibitor Rp-8-CPT-cAMPS. Unfortunately, the exact role of Epac in β₂-agonists-induced airway smooth muscle relaxation remains difficult to assess due to the lack of specific Epac inhibitors. Although PKA does have anti-spasmogenic effect, as shown by the capacity of the specific PKA activator 6-Bnz-cAMP to relax airway smooth muscle, our study identifies the novel cAMP-regulated Epac as a novel mediator of airway smooth muscle relaxation.

This conclusion is consistent with a growing body of data focused on Epac, showing these proteins to be significant contributors to responses previously solely associated with PKA activation [21, 26]. Interestingly, recent reports have indicated that Epac proteins modulate contractile function in cardiac and vascular muscle tissues [44, 45]. Moreover, they seem able to regulate contractile myofilament function [44] and potassium channels [46, 47], processes which may be also important in the contractile response of airway smooth muscle as well. Indeed, by selective activation or inhibition of PKA and Epac signalling, we have recently elucidated the relative contribution of the two cAMP-mediated effectors in the synthetic function of airway smooth muscle [29]. Here, we show a role for Epac in the regulation of airway smooth muscle tone in both guinea pigs and human beings. The Epac-specific cAMP analogues 8-pCPT-2′-*O*-Me-cAMP and Sp-8-pCPT-2′-*O*-Me-cAMPS cause relaxation of guinea pig and human airway smooth muscle preparations pre-contracted with histamine or methacholine. Importantly, the relaxant properties of 8-pCPT-2′-*O*-Me-cAMP on methacholine-induced contraction

were not affected by the PKA inhibitor Rp-8-CPT-cAMPS, suggesting there is no cross-talk involved in the effects of Epac. To ensure the rigor of our studies and reliability of our findings, we assessed and confirmed the specificity of the different cAMP analogues we used *in vitro* and applied this knowledge in designing functional *ex-vivo* contraction studies. Treatment of guinea pig and human airway smooth muscle cells with the highest functionally effective concentration of 8-pCPT-2′-*O*-Me-cAMP did not result in the activation of PKA, as confirmed by its inability to activate the specific PKA downstream effector VASP. Hence, our data indicate Epac as an important cAMP effector contributing to relaxation of guinea pig and human airway smooth muscle tissue.

We also investigated downstream molecular mechanisms that may underlie Epac-mediated airway smooth muscle relaxation. Airway smooth muscle tone is controlled by the phosphorylation level of regulatory MLCs, which permits subsequent actin-myosin interactions [3]. Interestingly, the methacholine-induced phosphorylation of MLC in guinea pig and human airway smooth muscle cells was completely prevented by concomitant Epac activation. This is in line with recent findings by Birokuva *et al.* [27, 28, 48] in human pulmonary artery endothelial cells showing that activation of Epac regulates endothelial barrier functioning *via* inhibition of MLC phosphorylation and activation of Rac1. Moreover, the authors described that the reduced phosphorylation of MLC following Epac activation was the result of a shift in the balance of activity of Rho and Rac towards Rac [28]. Whereas RhoA and RhoA-activated kinases increase the phosphorylation of MLC either directly or by inhibition of MLC phosphatase [12, 49], Rac reduces MLC phosphorylation *via* activation of p21-activated kinases and subsequent inhibition of MLC kinase [13]. Interestingly, we demonstrate here that activation of Epac also shifts the RhoA/Rac1 balance towards Rac1 in airway smooth muscle cells, as 8-pCPT-2′-*O*-Me-cAMP inhibited methacholine-induced activation of RhoA in cultured guinea pig and human airway smooth muscle cells, whereas it prevented methacholine-induced suppression of Rac1 activation. Taken together, these findings strongly suggest that Epac-mediated reduction of MLC phosphorylation is the result of inhibition of methacholine-induced RhoA activation and the preservation of Rac1 activity. The importance of Rac1 activity in Epac-mediated airway smooth muscle relaxation was confirmed by toxicological and pharmacological approaches using the *Clostridium difficile* Toxin B-1470 and the Rac1 inhibitor NSC23766, respectively. Overnight treatment of guinea pig tracheal open rings with toxin B-1470 reduced the immunoreactivity of Rac1 and impaired the relaxant effect of Epac towards methacholine-induced contraction. Although methacholine can reduce Rac1 activity *in vitro*, the treatment of guinea pig airway smooth muscle tissue with toxin B-1470 did not influence the methacholine-induced tone, probably due to alternative calcium-dependent mechanisms underpinning contraction. In addition, inhibition of Rac1 by NSC23766 completely blocked the inhibitory effect of 8-pCPT-2′-*O*-Me-cAMP on methacholine-induced phosphorylation of MLC.

Previous studies by Birukova *et al.* demonstrated an important role for Rap1 in Epac-mediated activation of Rac1 and subsequent inhibition of MLC phosphorylation [28]. As we reported recently

that activation of Epac induced GTP-loading of Rap1 in hTERT airway smooth muscle cells [29], Epac-dependent activation of Rap1 might contribute to the relaxant properties of Epac on guinea pig and human airway smooth muscle tone. Importantly, our current data indicate that Epac-mediated inhibition of methacholine-induced airway smooth muscle contraction relies on the enforcement of Rac1, which results in impaired MLC phosphorylation. Of note, Carzola *et al.* [44] have recently reported that Epac enhances cardiac contractile function *via* phosphorylation of contractile proteins in freshly isolated adult cardiomyocytes. Thus, it is tempting to speculate that Epac-dependent cAMP signals contribute to tissue specific β_2-agonist functions such as positive ionotropic properties in the heart and relaxant properties in airway smooth muscle. We also cannot exclude that Epac signalling to calcium-dependent potassium channels, calcium channels or ATP-sensitive potassium channels [46, 47] might also partially contribute to the relaxant signalling properties of Epac in airway smooth muscle. However, this does not preclude the strength of our data collected from a combination of *ex vivo* and *in vitro* experiments in airway tissues of different species. As similar results were obtained in both guinea pig and human airway smooth muscle, it is tempting to assume that Epac-mediated shift of the RhoA/Rac1 balance towards Rac1 and subsequential reduction of MLC phosphorylation represents a primary effector mechanism in airway smooth muscle relaxation. In conclusion, our present findings show that the cAMP-dependant effector Epac is an important regulator of airway smooth muscle tone. Given the relevance of cAMP-driven signalling in the airway pharmacology, the cAMP-activated Epac-mediated pathway may open new therapeutic strategies in the treatment of obstructive airways diseases.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflicts of interest.

References

1. **Opazo Saez AM, Seow CY, Pare PD.** Peripheral airway smooth muscle mechanics in obstructive airways disease. *Am J Respir Crit Care Med.* 2000; 161: 910–7.
2. **Postma DS, Kerstjens HA.** Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1998; 158: S187–92.
3. **de Lanerolle P, Paul RJ.** Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. *Am J Physiol.* 1991; 261: L1–14.
4. **Sanderson MJ, Delmotte P, Bai Y, et al.** Regulation of airway smooth muscle cell contractility by Ca2+ signaling and sensitivity. *Proc.Am.Thorac.Soc.* 2008; 5: 23–31.
5. **Gerthoffer WT.** Actin cytoskeletal dynamics in smooth muscle contraction. *Can J Physiol Pharmacol.* 2005; 83: 851–6.
6. **Sander EE, ten Klooster JP, van DS, et al.** Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. *J Cell Biol.* 1999; 147: 1009–22.
7. **Zondag GC, Evers EE, ten Klooster JP, et al.** Oncogenic Ras downregulates Rac activity, which leads to increased Rho activity and epithelial-mesenchymal transition. *J Cell Biol.* 2000; 149: 775–82.
8. **Ridley AJ, Hall A.** The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 1992; 70: 389–99.
9. **Gosens R, Schaafsma D, Nelemans SA, et al.** Rho-kinase as a drug target for the treatment of airway hyperresponsiveness in asthma. *Mini Rev Med Chem.* 2006; 6: 339–48.
10. **Schaafsma D, Roscioni SS, Meurs H, et al.** Monomeric G-proteins as signal transducers in airway physiology and pathophysiology. *Cell Signal.* 2008; 20: 1705–14.
11. **Janssen LJ, Wattie J, Lu-Chao H, et al.** Muscarinic excitation-contraction coupling mechanisms in tracheal and bronchial smooth muscles. *J Appl Physiol.* 2001; 91: 1142–51.
12. **Kimura K, Ito M, Amano M, et al.** Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science.* 1996; 273: 245–8.
13. **Sanders LC, Matsumura F, Bokoch GM, et al.** Inhibition of myosin light chain kinase by p21-activated kinase. *Science.* 1999; 283: 2083–5.
14. **Goeckeler ZM, Masaracchia RA, Zeng Q, et al.** Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2. *J Biol Chem.* 2000; 275: 18366–74.
15. **Giembycz MA, Newton R.** Beyond the dogma: novel beta2-adrenoceptor signalling in the airways. *Eur Respir J.* 2006; 27: 1286–306.
16. **Torphy TJ.** Beta-adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. *Trends Pharmacol Sci.* 1994; 15: 370–4.
17. **Billington CK, Penn RB.** Signaling and regulation of G protein-coupled receptors in airway smooth muscle. *Respir Res.* 2003; 4: 2–24.

18. **Kume H, Hall IP, Washabau RJ, *et al.*** Beta-adrenergic agonists regulate KCa channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J Clin Invest.* 1994; 93: 371–9.

19. **Scheid CR, Honeyman TW, Fay FS.** Mechanism of beta-adrenergic relaxation of smooth muscle. *Nature.* 1979; 277: 32–6.

20. **Spicuzza L, Belvisi MG, Birrell MA, *et al.*** Evidence that the anti-spasmogenic effect of the beta-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase. *Br J Pharmacol.* 2001; 133: 1201–12.

21. **Bos JL.** Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci.* 2006; 31: 680–6.

22. **de Rooij J, Zwartkruis FJ, Verheijen MH, *et al.*** Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature.* 1998; 396: 474–7.

23. **Hochbaum D, Hong K, Barila G, *et al.*** Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. *J Biol Chem.* 2008; 283: 4464–8.

24. **Huang SK, Wettlaufer SH, Chung J, *et al.*** Prostaglandin E2 inhibits specific lung fibroblast functions *via* selective actions of PKA and Epac-1. *Am J Respir Cell Mol Biol.* 2008; 39: 482–9.

25. **Petersen RK, Madsen L, Pedersen LM, *et al.*** Cyclic AMP (cAMP)-mediated stimulation of adipocyte differentiation requires the synergistic action of Epac- and cAMP-dependent protein kinase-dependent processes. *Mol Cell Biol.* 2008; 28: 3804–16.

26. **Roscioni SS, Elzinga CR, Schmidt M.** Epac: effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol.* 2008; 377: 345–57.

27. **Birukova AA, Zagranichnaya T, Fu P, *et al.*** Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement *via* PKA- and Epac1/Rap1-dependent Rac activation. *Exp Cell Res.* 2007; 313: 2504–20.

28. **Birukova AA, Zagranichnaya T, Alekseeva E, *et al.*** Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. *J Cell Physiol.* 2008; 215: 715–24.

29. **Roscioni SS, Kistemaker LE, Menzen MH, *et al.*** PKA and Epac cooperate to augment bradykinin-induced interleukin-8 release from human airway smooth muscle cells. *Respir Res.* 2009; 10: 88–104.

30. **Kooistra MR, Corada M, Dejana E, *et al.*** Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett.* 2005; 579: 4966–72.

31. **Gosens R, Stelmack GL, Dueck G, *et al.*** Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol.* 2006; 291: L523–34.

32. **Gosens R, Dueck G, Gerthoffer WT, *et al.*** p42/p44 MAP kinase activation is localized to caveolae-free membrane domains in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol.* 2007; 292: L1163–72.

33. **Lutz S, Freichel-Blomquist A, Yang Y, *et al.*** The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. *J Biol Chem.* 2005; 280: 11134–9.

34. **Roscioni SS, Elzinga CR, Schmidt M.** Epac: effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol.* 2008; 377: 345–57.

35. **Poppe H, Rybalkin SD, Rehmann H, *et al.*** Cyclic nucleotide analogs as probes of signaling pathways. *Nat Methods.* 2008; 5: 277–8.

36. **Haag S, Warnken M, Juergens UR, *et al.*** Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. *Naunyn Schmiedebergs Arch Pharmacol.* 2008; 378: 22–7.

37. **Kassel KM, Wyatt TA, Panettieri RA Jr, *et al.*** Inhibition of human airway smooth muscle cell proliferation by beta 2-adrenergic receptors and cAMP is PKA independent: evidence for EPAC involvement. *Am J Physiol Lung Cell Mol Physiol.* 2008; 294: L131–8.

38. **Smolenski A, Bachmann C, Reinhard K, *et al.*** Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation *in vitro* and in intact cells using a phosphospecific monoclonal antibody. *J Biol Chem.* 1998; 273: 20029–35.

39. **Gjertsen BT, Mellgren G, Otten A, *et al.*** Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture.

Basal cAMP-kinase activity modulates interleukin-1 beta action. *J Biol Chem.* 1995; 270: 20599–607.

40. **Enserink JM, Christensen AE, de RJ, *et al.*** A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol.* 2002; 4: 901–6.

41. **Laxman S, Riechers A, Sadilek M, *et al.*** Hydrolysis products of cAMP analogs cause transformation of Trypanosoma brucei from slender to stumpy-like forms. *Proc Natl Acad Sci USA.* 2006; 103: 19194–9.

42. **Schmidt M, Voss M, Thiel M, *et al.*** Specific inhibition of phorbol ester-stimulated phospholipase D by Clostridium sordellii lethal toxin and Clostridium difficile toxin B-1470 in HEK-293 cells. Restoration by Ral GTPases. *J Biol Chem.* 1998; 273: 7413–22.

43. **Gao Y, Dickerson JB, Guo F, *et al.*** Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc Natl Acad Sci USA.* 2004; 101: 7618–23.

44. **Cazorla O, Lucas A, Poirier F, *et al.*** The cAMP binding protein Epac regulates cardiac myofilament function. *Proc Natl Acad Sci USA.* 2009; 106: 14144–9.

45. **Sukhanova IF, Kozhevnikova LM, Popov EG, *et al.*** Activators of Epac proteins induce relaxation of isolated rat aorta. *Dokl Biol Sci.* 2006; 411: 441–4.

46. **Purves GI, Kamishima T, Davies LM, *et al.*** Exchange protein activated by cAMP (Epac) mediates cAMP-dependent but protein kinase A-insensitive modulation of vascular ATP-sensitive potassium channels. *J Physiol.* 2009; 587: 3639–50.

47. **Ster J, De BF, Guerineau NC, *et al.*** Exchange protein activated by cAMP (Epac) mediates cAMP activation of p38 MAPK and modulation of Ca2+-dependent K+ channels in cerebellar neurons. *Proc Natl Acad Sci USA.* 2007; 104: 2519–24.

48. **Birukova AA, Burdette D, Moldobaeva N, *et al.*** Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. *Microvasc Res.* 2010; 79: 128–38.

49. **Amano M, Ito M, Kimura K, *et al.*** Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem.* 1996; 271: 20246–9.