Epac as a novel regulator of airway smooth muscle phenotype and function
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General Introduction
Cyclic AMP

Cyclic adenosine monophosphate (cAMP) is a universal second messenger which is produced in response to a wide range of extracellular signals and regulates pivotal processes in distinct cell systems [1]. cAMP modulates a variety of cellular functions, including metabolism, gene expression, cell growth, apoptosis and secretion (reviewed in [1]) upon engagement of diverse intracellular signaling pathways. Formation of cAMP is initiated by the stimulation of Gs-protein-coupled receptors (Gs-PCR), such as β2-adrenergic and prostanoid receptors (Fig. 1).

**Fig. 1. Model of cAMP signaling in airway smooth muscle.** Stimulation of Gs-coupled receptors for example by β2-agonists or prostanoid E2, activates adenylyl cyclase (AC), which in turn produces cAMP from ATP. Intracellular cAMP levels are regulated by phosphodiesterase (PDE), which degrades cAMP in 5′-AMP. cAMP regulates cellular functions, including contraction, inflammation and cell proliferation, presumably via activation of protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). Membrane clustering of Gs-PCR, AC and PDE to caveolae seems to play a role in cAMP signaling regulation. cAMP signaling specificity is mediated by the formation of A-kinase anchoring protein (AKAP)-dependent complexes, which bind Epac and PKA, and target them to specific cellular effectors. See text for further details.

After receptor ligand binding, the α subunit of the Gs-protein activates adenylyl cyclases (ACs) which results in the generation of cAMP from adenosine triphosphate (ATP) (Fig. 1) [2]. Cellular levels of cAMP are tightly controlled by the action of phosphodiesterases (PDEs), which degrade cAMP to 5′-AMP (Fig. 1) and thereby terminate signaling by cAMP [3]. Membrane clustering of ACs and PDEs to
l lipid rafts and caveolae (Fig. 1) [4, 5] and (co)localization of distinct cAMP effectors to specific cellular compartments [6] causes a dynamic regulation of cAMP signaling [7]. Indeed, spatiotemporal organization of cAMP signals in the heart has provided new insights for the development of specific therapeutic strategies [8]. Until recently, most cAMP effects have been attributed to the activation of protein kinase A (PKA) [9]. However, several cAMP-mediated cellular events appeared to be insensitive to PKA inhibition, and have prompted investigators to look for novel cAMP targets [10, 11].

In 1998, exchange protein activated by cAMP (Epac) was identified upon a database search aimed to unravel cAMP-dependent, but PKA-independent activation of the small GTPase Rap1 [12]. Epac is a cAMP-regulated guanine nucleotide exchange factor (GEF) that favors GDP/GTP exchange on small Ras-like GTPases, thereby inducing their activation [12]. Although our knowledge of Epac signaling properties has substantially increased over the last decade, studies on the functional role of Epac in organ systems such as the respiratory system are lacking and require further investigation.

cAMP effectors: Epac and PKA

PKA

PKA is composed of two catalytic (C) and two regulatory (R) subunits, the latter known to sequester the C subunits in an inactive heterotetrameric holoenzyme [13]. Based on their regulatory subunits (RI and RII), PKA is subdivided into two classes: type I and type II [14]. The R subunits bear two cAMP-binding domains (CBDs): A and B [15]. Upon cAMP binding to the B site, an intramolecular steric change allows the domain A to interact with cAMP, causing the dissociation of the enzyme into an R subunit dimer and two C subunit monomers [16]. Once released, the C subunit is fully active and affects a wide range of cellular processes upon phosphorylation of cytoplasmatic and nuclear enzymes and transcription factors, on serine and/or threonine residues [17]. Distinct genes encode for three catalytic subunits (α, β and γ) and four regulatory subunits (RIα, RIβ, RIIα, and RIIβ) which are differentially expressed in tissues and exhibit distinct subcellular localization [18, 19]. Whereas RIα and RIIα subunits are ubiquitously expressed, RIIβ is primarily expressed in endocrine tissues, brain, fat and reproductive tissues, and RIβ is mainly found in the brain (Reviewed in [20]).

Epac

Two isoforms of Epac (Epac1 and Epac2) encoded by two distinct genes have been identified. Moreover, Epac2 exists in two splice variants, Epac2A and Epac2B [21]. Epac is a multi-domain protein consisting of an N-terminal regulatory region and a C-terminal catalytic region. The N-terminal region contains a Dishevelled - Egl-10 - Pleckstrin (DEP) domain responsible for membrane association, and a CBD site
homologous to the B domain of PKA, which is required for Epac activation by cAMP. Only Epac2A contains an additional low-affinity CBD site, which confers plasma membrane localization and has been associated with Epac2A-mediated hormone secretion [21]. A Ras-exchange motif domain localizes between the regulatory and the catalytic region and may confer stability to the CDC25 homology catalytic domain, which in turn promotes the GDP/GTP exchange on GTPases. A Ras association domain allows specifically Epac2 to directly interact with Ras [22]. Crystallographic studies showed that the CBD domains exert auto-inhibitory effects by maintaining the protein in a closed structure, thereby hindering GTPases-binding to the catalytic site [23, 24]. Binding of cAMP relieves the catalytic domain from the inhibitory constraints, allowing GTPases interaction and activation [23, 24].

Epac1 and Epac2 exhibit distinct tissue and cellular expression patterns. Epac1 is ubiquitously expressed in all human tissues as well as peripheral blood cells (reviewed in [25]); Epac2A is mainly – but not exclusively - expressed in pancreatic islets and the cerebral cortex, whereas Epac2B is restricted to adrenal glands [21]. Alterations of Epac1 and Epac2 expression in the heart, brain, kidneys and lungs of mice at different stages of development [26], suggest that Epac proteins differentially contribute to fetal and adult organ function. Distinct subcellular pools of Epac1 are present at the plasma membrane, the nucleus and nuclear membrane, the microtubule cytoskeleton, the mitochondria and the cytoplasm (reviewed in [25]). Epac2A is mainly localized at the plasma membrane, the cytosol, the Golgi apparatus and the actin cytoskeleton [25], whereas Epac2B is predominantly cytosolic [21]. In particular, studies in HEK293 and COS1 cells demonstrated that subcellular distribution of Epac1 is subject to cell-cycle and cytoskeleton-dependent dynamics [27]. Recently, Epac-based fluorescence resonance energy transfer cAMP sensors visualized the dynamic nature of cAMP elevations and Epac activation [28, 29]. In addition, redistribution of Epac1 to specific cellular compartments upon cAMP-binding seems necessary for its activation and the maintenance of its specific functions [30].

Although Epac and PKA can act independently [31, 32], most cAMP-dependent processes driven by Epac are also modulated via PKA, suggesting interconnectivity between these two effectors. Indeed, Epac and PKA have been shown to antagonize [33] as well as synergize with each other [34, 35]. As Epac and PKA exhibit similar affinities for cAMP (Kd~2.9 µM) [36], cellular compartmentalization of cAMP formation and relative abundance, distribution and localization of its effector proteins may contribute to Epac and/or PKA activation in response to moderate cellular cAMP increases. In this regard, A-kinase anchoring proteins (AKAPs) have been identified as cAMP-responsive multiprotein complexes, able to locate cAMP signaling to specific cellular microdomains by binding distinct enzymes such as ACs and PDEs as well as cAMP effector proteins, including Epac and PKA (Fig. 1) [20, 37-40]. Such tightly controlled spatio-temporal regulation of cAMP formation allows cAMP to integrate specific signals into unique cellular responses.
Tools to study Epac and PKA signaling

Cell-permeable cAMP analogs, such as 8-pCPT-2'-O-Me-cAMP and 6-Bnz-cAMP, are generally accepted as specific pharmacological tools to study Epac- and PKA-driven biological effects. Thus, N6-derivatives of cAMP, such as the 6-Bnz-cAMP, have been shown to selectively activate PKA (Table 1) [41]. On the other hand, structural studies showed that substitution of the oxidrilic group in the 2' position of the cAMP ribose structure with a methoxy group increases nucleotide selectivity towards Epac. Introduction of a phenylthio group to the 8' position of the purine ring further increases the affinity for Epac and also renders these cAMP derivatives more membrane permeable (Table 1) [42]. These considerations resulted in the development of 8-pCPT-2'-O-Me-cAMP, nowadays the most widely used Epac activator [23, 43]. As a negative control for 8-pCPT-2'-O-Me-cAMP, a cGMP analog with identical substitutions, namely 8-pCPT-2'-O-Me-cGMP, has been developed (Table 1), which neither activates PKG, PKA nor Epac [31]. To further increase membrane permeability, an acetoxymethyl (AM)-ester was introduced, generating 8-pCPT-2'-O-Me-cAMP-AM (Table 1) [44]. As recent studies indicated that 8-pCPT-2'-O-Me-cAMP may act via its metabolic products generated by PDE hydrolysis [45], the PDE-resistant and cell permeable Epac activator Sp-8-pCPT-2'-O-Me-cAMP has been developed (Table 1) [41, 45].

Studies on Epac-driven signaling suffer from evident limitations as the currently available Epac activators do not differentiate between Epac1 and Epac2. In addition, the read-outs for Epac activation measuring GTP-loading of Ras-like GTPases using pull-down techniques are still characterized by technical limitations when studying primary cell cultures and whole organ systems. Moreover, a recent publication showed that various cyclic nucleotides, including 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP, might cause secondarily elevations of cAMP or cGMP upon inhibition of PDEs [41]. Hence, caution should be taken into consideration in the interpretation of dissected Epac- and PKA-driven signaling by solely using cyclic nucleotides and such strategy should be accompanied by additional specific approaches to selectively inhibit the two cAMP-routes. Indeed, PKA inhibitors such as Rp-8-CPT-cAMPS, Rp-cAMPS and Rp-8-Br-cAMPS allowed to demonstrate that Epac analogs act independently of PKA (Table 2) [41, 46, 47].
### Table 1. Cyclic nucleotides used to study Epac- and PKA-driven signaling

<table>
<thead>
<tr>
<th>Compound</th>
<th>Characteristics</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><img src="image" alt="N6-benzyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP), selective and membrane-permeable PKA activator" /></td>
<td>N6-benzyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP), selective and membrane-permeable PKA activator</td>
<td>[41, 42, 51, 186]</td>
</tr>
<tr>
<td><img src="image" alt="8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP), selective and membrane-permeable Epac activator" /></td>
<td>8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP), selective and membrane-permeable Epac activator</td>
<td>[41, 51, 69, 186]</td>
</tr>
<tr>
<td><img src="image" alt="Acetoxymethyl 8-pCPT-2'-O-Me-cAMP (8-pCPT-2'-O-Me-cAMP-AM), see above" /></td>
<td>Acetoxymethyl 8-pCPT-2'-O-Me-cAMP (8-pCPT-2'-O-Me-cAMP-AM), see above</td>
<td>[44]</td>
</tr>
<tr>
<td><img src="image" alt="8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-8-pCPT-2'-O-Me-cAMPS), selective and membrane-permeable Epac activator, insensitive to PDEs" /></td>
<td>8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-8-pCPT-2'-O-Me-cAMPS), selective and membrane-permeable Epac activator, insensitive to PDEs</td>
<td>[41, 45]</td>
</tr>
<tr>
<td><img src="image" alt="8-(4-chlorophenylthio)-2'-O-methyl-cGMP (8-pCPT-2'-O-Me-cGMP), negative control for 8-pCPT-2'-O-Me-cAMP" /></td>
<td>8-(4-chlorophenylthio)-2'-O-methyl-cGMP (8-pCPT-2'-O-Me-cGMP), negative control for 8-pCPT-2'-O-Me-cAMP</td>
<td>[31]</td>
</tr>
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**Table 2. Cyclic nucleotides used to antagonize PKA-driven signaling**

<table>
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<th>Compound</th>
<th>Characteristics</th>
<th>References</th>
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<tbody>
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<td><img src="https://example.com/image1.png" alt="Image" /></td>
<td>8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-CPT-cAMPS), site selective inhibitor of PKA type I and type II</td>
<td>[41, 42, 47]</td>
</tr>
<tr>
<td><img src="https://example.com/image2.png" alt="Image" /></td>
<td>Adenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS), competitive inhibitor of PKA type I and type II</td>
<td>[46]</td>
</tr>
<tr>
<td><img src="https://example.com/image3.png" alt="Image" /></td>
<td>8-Bromoadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS), site selective inhibitor of PKA type I, 2 times more lipophilic compared to Rp-cAMPS</td>
<td>[47]</td>
</tr>
</tbody>
</table>

*Site-selective inhibitors occupy cAMP binding sites on PKA regulatory subunits and prevent the kinase holoenzyme from dissociation and thus from activation.

Unfortunately, pharmacological inhibitors of (individual) Epac isoforms are not available. In vitro, suppression of Epac protein expression using silencing RNA has been successfully utilized to elucidate several Epac-related functions [31, 32]. In addition, Epac1 and Epac2 knock-out mice have recently been developed and revealed a novel crucial role for Epac2 in the regulation of insulin release and the development of pancreatic β-cells [48]. Future investigations in this direction would definitely help to elucidate Epac-specific functions in vivo and to address the contribution of Epac in organ development, physiology and pathophysiology.
Biological functions of Epac and PKA

Since its discovery in 1998, numerous Epac-mediated pathways and targets have been identified and Epac has been associated with several PKA-dependent and -independent functions induced by cAMP [25, 43, 49-52]. In particular, novel Epac-driven signals are described to mediate the cAMP-induced regulation of inflammation, contractility, cell proliferation and differentiation in several cell types.

Inflammation

cAMP modulates several steps of the inflammatory process, including vascular permeability, immune-cell adhesion, inflammatory mediator release and immune defence-cell phagocytosis [25, 52]. Vascular permeability depends on the formation of cell-cell junctions and on the contractile force generated by the actin cytoskeleton, and is essential in the regulation of extravasation of leukocytes during inflammation [53]. cAMP strengthens the endothelial barrier by activating PKA, which inhibits actin-myosin contractility by inhibiting Rho [54]. Recent evidence suggest that Epac signaling may also regulate endothelium integrity upon recruitment of junctional protein to sites of cell-cell contacts and suppression of vascular cadherins-mediated adhesion and redistribution in response to inflammatory stimuli [55-57]. Moreover, in the presence of permeability-increasing agents, Epac reduces endothelial permeability upon activation of the small GTPase Rac and inhibition of Rho [58]. As the actin cytoskeleton functionally interacts with the microtubule network, the described interplay between Epac and microtubules may also account for its effects on endothelial permeability [59].

Besides regulating the vascular permeability, cAMP also modulated the release of inflammatory cytokines, a response being previously solely assigned to PKA [60, 61]. This response appear cell-type and stimulus-dependent and similar actions have been reported for Epac. In fact, in vascular endothelial cells, Epac1-Rap1 inhibited activation of the interleukin-6 (IL-6) receptor trans-signaling complex [62]. Moreover, Epac seems to trigger the prostaglandin E$_2$ (PGE$_2$)-mediated inhibition of lipopolisaccaride (LPS)-induced interferon-$\beta$ (IFN-$\beta$) expression from macrophages and the PGE$_2$-mediated inhibition of cytokine expression and production from activated dendritic cells [63, 64]. On the other hand, activation of Epac resulted in the up-regulation of interleukin 1-$\beta$ (IL-1$\beta$) and IL-6 transcripts in a murine macrophage cell line [65].

Epac1 and Rap1 are also involved in the recruitment and migration of leukocytes and in their adhesion to the endothelium and subsequent transmigration towards the site of inflammation [66, 67]. Leukocyte transmigration relies on the stimulation of integrin-mediated cell-adhesion, which in turn occurs through increased affinity and avidity of integrins [68]. Recent studies reported that activation of Epac enhances integrin-mediated monocyte adhesion to the extracellular matrix components (ECM)
laminin [69] and fibronectin [66], and promotes chemotaxis in a human promonocytic cell line and primary monocytes [66]. Finally, elevation of cAMP inhibits the activation and phagocytic activity of distinct leukocytes, a process which requires cell-type specific activation of Epac and/or PKA [60, 70]. In human monocytes, PKA inhibits Fc receptor-mediated phagocytosis and subsequent respiratory burst activity [70]. Differentiation into macrophages, however, was paralleled by increased Epac expression and suggest a contribution of Epac in macrophage responses [70].

**Contractility**

Elevations in cAMP dramatically alter contractile responses of cardiomyocytes and smooth muscle (ASM) cells. Until recently, cAMP-dependent positive ionotropic/chronotropic effects in the heart and relaxant effects in the airways have been assigned to activation of PKA. In the heart, cAMP effects are driven by PKA-dependent increase in intracellular Ca^{2+} upon phosphorylation of L-type Ca^{2+} channels, the type II ryanodine receptor, myofilament proteins and phospholamban, an inhibitor of Ca^{2+} reuptake in the sarcoplasmatic reticulum (reviewed in [71]). Recent findings in cardiomyocytes revealed that Epac may affect excitation-contraction coupling via direct interaction with the type II ryanodine receptors and the Ca^{2+}-ATPases on the sarcoplasmatic reticulum [72, 73]. Epac-driven phospholipase C-ε (PLCε) activation and subsequent increase in intracellular Ca^{2+} have also been reported in cardiomyocytes [72, 74, 75]. Furthermore, Epac may trigger accumulation of connexin 43, a process known to be required for gap junction formation, proper ion transfer and for the generation of contractile force [76].

Contraction of the ASM depends on Ca^{2+}, which activates myosin light chain (MLC) kinase. [77]. MLC kinase-dependent phosphorylation of the 20 kDa regulatory MLC is crucial for smooth muscle contraction, and this process is counterbalanced by MLC phosphatase (MLCP) [78]. Inhibition of MLCP by the small GTPase Rho and subsequent MLC phosphorylation promotes ASM contraction [79]. cAMP-relaxant properties have been attributed to PKA-dependent effects on K+ channels, Na+/K+ ATPases, Ca^{2+} sequestration, sensitivity of myosin and IP_3 formation (reviewed in [80]). However, a study in intact guinea pig ASM reported on cAMP relaxant properties independent of PKA [11]. Given these observations, Epac might represent a novel cAMP relaxant factor in the airways.

**Cell proliferation**

cAMP plays an important role in cell proliferation. Several studies demonstrated the ability of cAMP to stimulate as well as inhibit cell division, depending on the cellular context [81]. Cell proliferation is triggered by Ras-mediated activation of mitogen activated protein kinase (MAPK) family members, such as extracellular
signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and phosphoinositide-3-kinase (PI3K) effectors protein kinase B (PKB)/Akt and p70S6 kinase (p70S6K) [82]. The anti-proliferative role of PKA is underpinned by inhibition of the Ras/Raf1/ERK1/2 pathway, a process being both dependent and independent of Rap1 [83, 84]. In addition, PKA inhibits mitogen-induced JNK and p70S6K phosphorylation [85, 86]. Furthermore, cAMP and PKA interfere with more distal signaling targets, such as cell-cycle regulators. Thus, PKA inhibits cyclin D1 expression by acting on its promoter region and by enhancing its proteosomal degradation [87, 88] and in Jurkat-T cells, PKA increases expression of the cell-cycle inhibitor p27Kip1 and favors cell-cycle arrest in the G1 phase [89]. Recent evidence suggests that at least some of the cAMP-mediated effects also involve activation of Epac. Rap1 appears to be the key effector of Epac in these responses, due to its ability to modulate ERK activity [84]. Rap1 can inhibit and activate ERK1/2 via Raf-1 and B-raf, respectively [84]. Epac modulates ERK1/2 phosphorylation and cell proliferation in a cell-type specific manner, probably due to the availability of distinct effectors and the targeting to distinct cellular compartments. In particular, Epac drives pro-proliferative processes in thyroid cells, macrophages, endothelial cells and osteoblasts (reviewed in [25]), whereas it inhibits proliferation of ASM cells and lung fibroblasts [31, 32, 90]. Activation of pro-mitogenic signals including Ras and ERK1/2 by Epac has also been associated with Rap2-dependent activation of PLCε in HEK293T and N1E-115 neuroblastoma cells [91]. In addition, Epac has been associated with the microtubule network in interphase and mitotic cells, suggesting a role for Epac in microtubule-dependent cell division [27].

Cell differentiation and phenotypic regulation

Cell differentiation plays an important role in development, but also contributes to pathogenic alterations of cells and such processes are often accompanied by characteristic cellular phenotypical and functional changes. cAMP is required for normal development and differentiation of the Drosophila eye disc, ovary and embryo in vivo and this process seems to involve Epac and Rap1 [92, 93]. In addition, activation of PLCε mediated by the Epac effector Rap2 participates in Wnt-β-catenin signaling pathway in the early development of Xenopus [94]. Importantly, Rap1 is able to reverse morphology and hyperproliferative phenotype of a Ras-transformed subline of NIH/3T3 cells [95] and cAMP/Epac/Rap1 activation seems to drive similar actions in pancreatic cancer cells [96]. cAMP stimulates specialized functions in thyroid cells, melanocytes, neuronal cells and adipocyte (reviewed in [49]). Recent work on the adipose conversion of a pre-adipocytic cell line has demonstrated a role for Epac in this process [35]. In neuronal PC12 cells, Epac converts the cAMP effect from proliferation to differentiation, resulting in neurite outgrowth [97]. Furthermore, the novel Epac effector Rit regulates neurite outgrowth and terminal differentiation in pheochromocytoma PC6 cells [98].
Given the substantial contribution of Epac to pivotal cAMP-dependent processes, it is not a surprise that attention has been focused on Epac as a potential effector in lung physiology and as a possible future therapeutic target of intervention in airway diseases.

Asthma and COPD

Asthma and chronic obstructive pulmonary disease (COPD) are among the leading causes of morbidity and mortality worldwide and their prevalence is expected to increase due to lifestyle factors and occupational or environmental exposure to various pollutants [99, 100]. Both diseases are chronic inflammatory disorders. Although distinctive patterns of inflammation are associated with the two diseases, the degree of inflammation correlates with decline in lung function and disease severity in both asthma [101] and COPD [102, 103]. Asthma is further characterized by variable and reversible airway obstruction, whereas in COPD airway obstruction is only partially reversible and lung function progressively declines. Both diseases are associated with increased responsiveness of the airways to environmental stimuli (airway hyperresponsiveness) as well as structural changes in the airways (airway remodeling) [103, 104].

Chronic inflammation in asthma and COPD is the result of massive infiltration of the airways and/or peripheral lung tissue by distinct activated inflammatory cells, which in turn amplify inflammation by releasing inflammatory mediators such as cytokines and growth factors [105]. Beside inflammation, cytokines and growth factors affect other cellular functions, such as contraction and proliferation of structural cells, and contribute to airway remodeling [106]. Airway remodeling encompasses epithelial changes, goblet cell and submucosal gland hyperplasia and increased airway smooth muscle mass [103]. Although the nature and the anatomic site of the alterations differ between asthma and COPD, remodeling in both diseases eventually impairs lung function and sensitizes the system to react to external insults [107, 108]. Indeed, structural alterations - such as increased ASM mass - play an important role in the severity of the disease and in the development of airway hyperresponsiveness (AHR) [109]. Although with distinct mechanisms and expression, AHR is present in both asthmatics and COPD patients, defined as exaggerated responsiveness of the airways to non-specific stimuli, resulting in airway obstruction [110]. AHR has been associated with the development of respiratory symptoms and is considered an important risk factor in subjects with established asthma and COPD [110, 111]. Severe AHR is associated with a more rapid decline in lung function in asthma [112, 113] and in both asthma and COPD a relationship exists between the degree of airway inflammation and the severity of AHR [110, 114].
Pathogenic features of asthma

Asthma symptoms encompass wheezing, cough, chest tightness and dyspnea, which are intermittent and variable and caused by exposure to environmental allergens, cold or exercise. Indeed, IgE-mediated response to common allergens represent the most widespread form of asthma [115]. Asthma is characterized by an early onset (often in childhood) and shows potential inheritance patterns [116, 117].

**Inflammation**

Allergen-induced asthma attacks are underpinned by an acute inflammatory response which encompasses an early-phase and a late-phase reaction. The early stage is initiated by activation of mast cells, bearing allergen-specific IgE and by the release of pro-inflammatory mediators, which cause ASM contraction, mucus production and vascular leakage with exudation of plasma in the airways [118], thereby contributing to airway obstruction. The late phase involves the recruitment and activation of eosinophils from the peripheral blood [119]. Eosinophils amplify inflammation, and together with other inflammatory and structural cells, contribute to airway obstruction and airway remodeling by releasing cytokines and growth factors [107, 120, 121]. In addition, CD4 T lymphocytes are believed to play a pivotal pathogenic role in asthma. They elicit a Th2-driven inflammatory response with up-regulation of cytokines such as IL-4, IL-5, IL-13, eotaxin and monocyte chemoattractant protein 1, which collectively result in allergic sensitization and inflammatory cell recruitment [122].

**Airway obstruction**

In asthma, airway narrowing is due to the exaggerated contraction of the muscle, and is mostly associated with eosinophilic airway inflammation [105]. In fact, the presence of enhanced eosinophilic inflammation in the asthmatic airways correlates with the increased airway resistance and increased airway responsiveness [123]. Moreover, the walls of the conducting airways in asthma are significantly thicker compared to normal and this contributes to the airflow limitation, further compromised by excessive mucus admixed with inflammatory exudates [103]. Increased ASM mass is believed to be the most important abnormality responsible for the increased airflow resistance observed in response to bronchoconstricting stimuli [109] and correlates with the severity of AHR [124] and the need of therapy [125].

**Airway remodeling**

In asthma, airway remodeling may start early in the childhood [103, 126] and involves both large and small airways. Remodeling encompasses mucus cell hyperplasia, deposition of ECM underneath the airway epithelium (subepithelial
General introduction

fibrosis) and within the smooth muscle layer, increased ASM mass, and angiogenesis [103, 127]. The increased ASM mass is caused by both hypertrophy and hyperplasia [128]. The main consequence of remodeling are airway wall thickening, mucus hypersecretion and airway smooth muscle hypercontractility, which profoundly affects airway function [129].

Pathogenic features of COPD

In COPD, respiratory symptoms such as wheezing, cough, chest tightness and dyspnea are slowly progressive and partially reversible, not necessarily associated with inhalation of noxious particles [130]. COPD has a very slow progressive onset, and afflicts middle-aged and elderly people [131], who usually bear a history of heavy smoking [132, 133]. Indeed, long-term exposure to smoke (especially cigarette smoke) represents the main risk factor in the development of COPD although less than 25% of smokers develops COPD and at least 15% of COPD-related mortality occurs in never-smokers, suggesting that other factors may be important as well [132-134].

Inflammation

The pathogenesis of COPD is related to a chronic abnormal inflammatory response to inhaled toxic particles and gases [102]. Inflammation in COPD patients predominantly affects small airways and lung parenchyma [105]. The main inflammatory cells in COPD are neutrophils, macrophages, natural killer cells, dendritic cells as well as CD4/CD8 T cells [102], which promote release of an impressive array of pro-inflammatory cytokines, including IL-8, IL-1, tumor necrosis factor-α (TNF-α), leukotriene B4 and interferon γ (IFN γ). Thus, inflammation leads to vascular leakage, mucus hypersecretion, ASM contraction, epithelial damage, AHR and airway structural alterations [105, 120].

Airway obstruction

In COPD, airway obstruction is mostly driven by the ongoing structural changes in the small airways and disruption of the alveolar attachments (emphysema), which result in increased airway resistance and air trapping [135]. Moreover, the reduced expiratory flow that defines COPD [114], results from reduction of the lumen by peribronchiolar fibrosis, thickening of the small-airway walls and occlusion of the lumen of the small conducting airways by exudates containing mucus [102]. Although the occurrence of AHR in COPD is debated, a considerable amount of COPD patients have been shown to exhibit higher responsiveness to contractile stimuli and the severity of AHR appears to be a good predictor of the rapid decline in lung function in patients with COPD [110, 136, 137]. ASM mass increases significantly in the small airways in COPD [138-141] and this increase is believed to be a main contributor to AHR [109]. Data from animal models and humans also
support the hypothesis that in COPD, changes at the level of cholinergic parasympathetic control of the smooth muscle result in increased bronchoconstriction in response to vagal stimulation, leading to AHR [142].

**Airway remodeling**

In COPD, airway remodeling appears later in adult life [103, 126] and predominantly affects small airways and lung parenchima [103, 126]. Airway remodeling in COPD is inextricably linked to the inflammatory cell infiltration into lung tissue and encompasses emphysema, enlargement of the bronchial mucus glands, increased mucus content in the airway lumen, fibrosis, increased epithelial cell proliferation, increased mucous, squamous cell metaplasia and increased ASM mass [102]. In COPD, there is excessive activity of proteases and an imbalance between proteases and endogenous antiproteases [143]. Several proteases, including matrix metalloproteinases (MMPs) are likely to be involved in the development of emphysema [144, 145], which seriously contributes to the airflow limitations by decreasing the elastic recoil pressure available to drive air out of the lung during forced expiration. This tissue destruction begins in the respiratory bronchioles in very close proximity to the small conducting airways that indeed become the major site of obstruction in COPD [102]. Fibrosis around the small airways is also believed to play a major role in the irreversible airway narrowing in COPD [146]. Moreover, in surgically resected lung tissues, increased accumulation of inflammatory exudates with mucus in the small airways was noted to correlate with the severity of disease [147]. In COPD, ASM mass increases only in the small airways [103, 127] but may represent an important determinant of disease severity [109]. Vascular remodeling due to inflammatory infiltration of the vessels, is also a characteristic feature of COPD and may generate pulmonary hypertension [148].

**Airway smooth muscle phenotype and function**

Due to their intrinsic contractile capability, ASM cells regulate the airway luminal diameter and respiratory function and represent the main players of AHR in asthma and COPD (Fig. 2) [149]. New evidence suggests that next to contraction, ASM cells may also exert synthetic and proliferative functions, thereby participating in inflammation and development of airway remodeling in airways diseases (Fig. 2) [150, 151]. Regulation of ASM functions is provided by cytokines and growth factors secreted from inflammatory cells. Alternatively, cellular and structural components of the airway wall, including the airway epithelium, the airway nerves and the ECM, interact with the ASM bundle and control its functions under inflammatory conditions (Reviewed in 152). ASM cells are multifunctional cells capable of expressing dynamic phenotypic plasticity. This term refers to the (co)existence of distinct phenotypic states characterized by specific cellular functions, such as contraction, proliferation and synthesis of pro-inflammatory
molecules (Fig. 2) [153]. The effect of contractile agonists, inflammatory mediators, growth factors and ECM components on ASM phenotype has been extensively studied in vitro and ex-vivo.

**Fig. 2. Airway smooth muscle phenotype plasticity.** In a healthy state, airway smooth muscle cells have a contractile phenotype, which is involved in the regulation of airway tone. Chronic stimulation by inflammatory mediators and growth factors in airway diseases may reversibly shift airway smooth muscle cell phenotype to a more proliferative and synthetic phenotype, causing increased airway smooth muscle mass, inflammation and fibrosis. Hence, such phenotypic plasticity may underpin the role of airway smooth muscle cells in the development of airway hyperresponsiveness, airway remodeling and inflammation. See text for further details.

Thus, mitogenic stimuli shift the ASM contractile phenotype to a proliferative/synthetic phenotype, characterized by high proliferative rates and low contractile abilities, accompanied with low contractile marker and caveolae expression [154]. In agreement with the reversibility of such processes, removal of growth factors results in the formation of a contractile phenotype of ASM cells with elongated morphology, higher shortening capability and increased expression of contractile markers and caveolae [155]. Moreover, ASM cells can even be pushed towards a hypercontractile phenotype upon prolonged serum deprivation or in the
presence of insulin or laminin-2 [155-157]. Although phenotypic plasticity has not yet been demonstrated in vivo, it is likely to play an important role in physiological processes, such as development, tissue repair and inflammation, as well as in the pathogenesis of asthma and COPD [158]. These phenotypic states (contractile, synthetic and proliferative) may coexist in vivo and such heterogeneity of ASM cells might contribute to the altered airway responsiveness and inflammation (Fig. 2) [159]. In fact, several studies have indicated that differences in contractile and proliferative responses exist between ASM cells from asthmatic compared to healthy subjects. Thus, asthmatic ASM cells contract with greater velocity and maximum shortening capacity compared with healthy ASM [160], probably due to the higher expression of contractile markers, which also correlates with methacholine responsiveness in subjects with asthma [161]. Asthmatic ASM cells also proliferate faster compared with healthy control cells [162], but the exact mechanisms which regulate these responses are still not completely defined.

Obviously, ASM cells importantly participate in the development of asthma and COPD and therefore represent a key therapeutic target for both diseases.

cAMP-elevating agents in the treatment of airway diseases

Given the ability of cAMP to regulate crucial cellular functions (Fig. 1), it is not a surprise that pharmacological manipulation of cAMP levels has been therapeutically exploited in a wide range of diseases. Based on their ability to induce ASM relaxation, β2-agonists represent central medications in the management of asthma and COPD [114]. However, responses to β2-agonists vary among patients and this is partially associated with the severity of the disease [163]. Multiple mechanisms may modulate clinical responses to β2-agonists. In general, asthmatic airways have a reduced capacity to dilate following administration of β2-agonists both in vitro and vivo [164]. Decreased responsiveness of the β2-adrenergic receptor (β2-AR) – known as receptor desensitization – is mediated by phosphorylation of the receptor with subsequent uncoupling from signal transduction, internalization of cell-surface receptor and down-regulation of the production of new receptor (reviewed in [165]). These mechanisms have been shown to occur in human ASM and are initiated by agonist-binding to the β2-AR (homologous desensitization) as well as by substances which (in)directly enhance cAMP intracellular levels, such as inflammatory cytokines, contractile agonists and prostanoids (heterologous desensitization) (reviewed in [165]). Mechanistic studies have shown that β2-AR desensitization in ASM is mediated by PKA- and/or G-protein coupled receptor-kinases (GRKs)-dependent phosphorylation on serine/threonine residues on the receptor [166, 167]. In addition, a variety of inflammatory and contractile agonists counteract β2-AR response by activating the inhibitory G-protein subunit G\textsubscript{i}α, which inhibits adenylyl cyclase and impairs cAMP formation and signaling [168]. Furthermore, genetic and/or epigenetic factors that affect the β2-agonist receptor function and/or its downstream signaling may account for altered responsiveness. Single nucleotide
polimorphisms (SNPs) in the coding region of the β2-AR may impact asthma severity and response to therapy and influence receptor desensitization [165, 169, 170]. Genetic alterations of cAMP signaling regulatory molecules such as PDEs, may also contribute to altered β2-agonists responsiveness [170]. The downstream molecular mechanisms by which β2-agonists elicit their effects are still not completely defined and recent studies in vitro indicate that activated β2-AR might couple to Gi-proteins, resulting in inhibition of ACs and subsequent cAMP production and stimulation of the MAPK pathway [171]. Next to their bronchodilatory properties, β2-agonists have been reported to have anti-proliferative and anti-inflammatory effects in vitro, mainly associated with PKA-dependent phosphorylation of cAMP responsive element binding protein (CREB) and subsequent regulation of gene transcription [165]. However, only minimal effects of β2-agonists on airway remodeling and inflammation in vivo have been reported so far [172, 173], probably due to the cytokine-dependent desensitization of the β2-AR. β2-agonists may also cause pharmacologically predictable side effects including mild tachycardia, rhythm disturbances, tremor and metabolic effects mainly observed with prolonged high-level dosing and due to activation of secondary targets (reviewed in [174]). Moreover, several studies have confirmed that chronic use of β2-agonists can result in increased AHR [174-176] and frequency of exacerbations, which are related to the epidemics of morbidity and mortality associated with β2-agonists therapy [177]. Treatment with β2-agonists for 7 days has also been shown to have adverse effect on the late asthmatic response to allergen, with increased sputum eosinophilic content [178, 179], suggesting that β2-agonists may potentiate allergic inflammation in the airways. Hence, alternatives have been considered for the therapeutic treatment of airway diseases. Some of the beneficial effects of β2-agonists are mimicked by other cAMP-elevating agents, such as PDE inhibitors and prostaglandin E2 (PGE2) [180, 181]. However, PGE2 has been shown to cause ASM contraction at low dose and to enhance release of some inflammatory cytokines [182, 183]. On the other hand, selective PDE inhibitors have entered clinical trials to determine their efficacy/usefulness in the treatment of asthma and COPD although their usage is limited by their systemic side effects [184, 185]. Hence, given the importance of cAMP signaling in the (patho)physiology and treatment of airway diseases, investigations into its mechanisms of action is of scientific and clinical relevance.

Epac in the lung

As a newly discovered effector in the cAMP signaling cascade able to modulate universal pivotal processes [186], researchers have started to speculate on the importance of Epac in lung physiology and its contribution to disease development. In the last years, expression of Epac1 and Epac2 in several lung cell types has been successfully evaluated. Epac1 and Epac2 mRNA expression was found in lung mesenchyme and airway epithelium of mouse embryos at different developmental
In human pulmonary fibroblasts, mRNA and protein expression for Epac1, but not Epac2, was detected [31, 32]. Epac1 is also expressed in human and rat alveolar macrophages and mice dendritic cells [187]. Studies of Epac expression under pathophysiological conditions are limited although the COPD-associated fibrogenic factor transforming growth factor-β (TGF-β) was shown to down-regulate Epac1 expression in human lung fibroblasts, which leads to the induction of fibrosis [188]. Importantly, recent studies have shown an anti-proliferative effect of Epac in both ASM cells and pulmonary fibroblasts, via yet unknown mechanisms [31, 32, 90].

Furthermore, Epac positively regulates endothelial barrier function in pulmonary artery endothelial cells via Rap-induced activation of Rac and inhibition of Rho [58]. Epac/Rap also regulates cell adhesion to the ECM protein laminin-5 [69], which acts as an adaptor for growth factor-induced invasive growth in lung cancer [189]. In models of inflammation, Epac was shown to mediate the anti-inflammatory effects of the Gs-coupled A2a adenosine receptor [190]. This response involved Epac-dependent inhibition of nuclear factor-κB and modulation of gene expression upon activation of the ECM protein hyaluronan [190]. Finally, activation of Epac suppressed phagocytosis in alveolar macrophages and inhibited the release of inflammatory chemokines by lipopolisaccaride upon activation of PKB/Akt and subsequent inhibition of glycogen synthase kinase-3 (GSK3) [60, 63, 64]. Importantly, activation of Epac in a mouse macrophage cell line increased the production of pro-inflammatory mediators [65], indicating that effects of Epac might be cell type-dependent.

Despite the growing body of data on Epac signaling in the lung, only few reports have addressed the role of Epac in β2-agonists/cAMP mediated responses such as regulation of inflammation and cell proliferation. Moreover, no studies have unraveled the potential role of Epac in ASM contraction. In fact, although Spicuzza et al. described the relaxant effect of the β2-agonist isoproterenol in intact guinea pig ASM as PKA-independent, the authors did not address the contribution of Epac in this effect [11]. Compartmentalization and/or clustering of Epac to cAMP-signaling complexes in the airways has not been studied yet and the role of Epac in ASM phenotype and function remains mostly unexplored. Hence, such studies are warranted to identify novel pathways of cAMP signaling which may mediate β2-agonists effects in the lung, and might eventually help to obtain a safer and more targeted intervention in airway diseases.
Scope of the thesis

Based on the above-mentioned observations, we started to characterize Epac signaling in ASM, its role in ASM phenotype and function and its interconnectivity with PKA. To this aim, we used a combination of in vitro and ex-vivo settings. Cellular and tissue effects of Epac and PKA in ASM contractile, proliferative and secretory functions and phenotypic regulation in different species including human were compared to the effects of intracellular or extracellular cAMP-elevating agents. Dissection of Epac and PKA-driven signaling was achieved upon specific activation/inhibition of the two effectors, by using various pharmacological and molecular tools. The distinct roles of Epac and PKA in the regulation of ASM responses were embedded into physiological and pathophysiological settings using human primary ASM cells and tissues and airway samples from COPD patients.

Chapter 2 highlights the multi-faceted effectors and diverse biological functions driven by Epac proteins that might explain certain controversial signaling properties of cAMP in inflammation and cell proliferation.

Chapter 3 provides the characterization of Epac expression and signaling properties in human ASM and bronchial epithelial cells. Activation of Rap GTPases and other downstream effectors, such as ERK1/2, JNK, and MMP-9, by Epac was also evaluated in both cell types, and phosphorylation of PKB/Akt and GSK-3 was analyzed in ASM cells. Moreover, the capability of Epac to signal to regulators of inflammatory, proliferative and contractile responses was evaluated by kinome peptide microarray analysis. These findings delineate the potential contribution of Epac to lung physiology and pathophysiology.

Potential contributions of Epac and PKA to airway inflammation in asthma and COPD are described in chapters 4 and 5, respectively. To this aim, human ASM cells were stimulated with the asthma-associated inflammatory mediator bradykinin or with with the COPD-associated pathogenic factor cigarette smoke, both known to induce IL-8 from ASM cells [191, 192]. The role of the two cAMP effectors was investigated by pharmacological inhibition (PKA) and by silencing RNA (Epac), and the effects of Epac and PKA were assessed by using selective activators. Moreover, the potential signaling mechanisms involved were investigated as well as the interconnectivity between the two cAMP effectors. The effect of cigarette smoke on mRNA and protein expression of Epac and PKA in immortalized and primary human ASM cells was also assessed (chapter 5). Importantly, these latter findings were translated into a pathophysiological context, by using lung samples derived from COPD patients and asymptomatic smokers.

In chapter 6, the issue of cAMP-signal compartmentalization in human ASM cells was addressed. Subcellular localization and (re)distribution of Epac was characterized in resting and stimulated ASM cells. The involvement of AKAPs and
caveolae in cAMP compartmentalization and potential functional consequences on IL-8 release from ASM cells were investigated.

Chapter 7 describes the anti-spasmogenic role of Epac in pre-contracted guinea pig tracheal preparations. Molecular mechanisms of this effect were investigated by studying the activation of Rac1 and RhoA and the phosphorylation of MLC upon methacholine treatment in the absence or presence of the Epac activator 8-pCPT-2'-O-Me-cAMP. Importantly, the functional role of Epac in regulating ASM tone and the molecular mechanisms involved were also studied using human ASM strip preparations and cultured immortalized and primary human ASM cells.

Chapters 8 and 9 address the roles of Epac and PKA in regulating PDGF-induced modulation of ASM phenotype and function, characterized by reduced contractility and contractile protein expression and increased cell proliferation [154]. In chapter 8, the effects of Epac and PKA on PDGF-induced phenotypic modulation of bovine tracheal smooth muscle (BTSM) were evaluated. To this aim, BTSM strips were cultured for 4 days with or without PDGF in the presence of selective Epac and PKA activators, and contractility and contractile protein expression were determined. In addition, cultured BTSM cells were treated under similar conditions, to study the impact of Epac and PKA on mitogen-induced cell proliferation. Moreover, potential signaling mechanisms involved (ERK1/2 and p70S6K) in the phenotypic changes were investigated. In chapter 9, some of the findings observed in bovine preparations were translated into human preparations. Thus, the effect of prolonged treatment of isolated human tracheal smooth muscle preparations with the selective Epac and PKA activators in the presence of PDGF was evaluated, and contractile and proliferative functions were assessed.

References


Chapter 1


General introduction


General introduction


