Epac as a novel regulator of airway smooth muscle phenotype and function
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Chapter 10
General discussion and summary
Epac as a novel cAMP effector in airway functioning

The most common and versatile second messenger cyclic adenosine 3',5'-monophosphate (cAMP) controls a wide range of physiological processes [1] (chapter 1). Elevation of intracellular cAMP levels is initiated upon ligand binding to G protein-coupled receptors (GPCRs) and subsequent activation of adenyl cyclases (ACs). Intracellular cAMP levels are tightly regulated by the coordinated action of ACs and phosphodiesterases (PDEs), the latter known to terminate cAMP signaling upon cAMP hydrolysis [2]. Moreover, association of GPCRs and ACs to caveolae and lipid rafts controls cAMP signaling by forming spatially and functionally discrete complexes within the surface of the plasma membrane [3-5]. Recent updates in the field show that the specific outcomes of cAMP elevations are also dictated by the activation of distinct downstream effectors [6-9].

The discovery of exchange proteins directly activated by cAMP (Epac’s) in 1998 [10], tremendously altered our insights into the cellular signaling of cAMP, previously solely assigned to protein kinase A (PKA). Thus, Epac has been shown to mediate a variety of cAMP-induced effects on cell adhesion, secretion, cell proliferation, cell differentiation, inflammation and gene expression, which may be independent of PKA (for details see chapter 2). Actions of Epac are mediated via GDP/GTP exchange on small Ras-like GTPases, such as Rap1, and their subsequent activation [7, 11]. Epac has been shown to signal to a plethora of downstream effectors, including Rho GTPases, phospholipase C-ε (PLC-ε), mitogen-activated protein kinase family members, such as extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), protein kinase B (PKB)/Akt, and regulators of the actin-microtubule network (chapter 2). Cell-type specific factors, such as cell-cell contacts, abundance of effectors and localization seem to direct Epac to distinct cellular outcomes and might explain apparent controversial effects of cAMP on, for example, inflammation and cell proliferation [6, 8, 9]. In fact, Epac may act pro-inflammatory by enhancing integrin-mediated monocyte adhesion to the extracellular matrix (ECM) proteins laminin [12] and fibronectin [13], and by promoting chemotaxis of immune cells [13]. On the other hand, Epac, via its effector Rap1, may also have anti-inflammatory effects by enhancement of the endothelial barrier function [14] and by inhibition of cytokine release from immune cells [15, 16]. Moreover, Epac has been shown to inhibit as well as enhance the phosphorylation of ERK1/2, and these responses seem to correlate with anti- and pro-proliferative effects, respectively [17]. Epac may act alone, or either synergize or antagonize PKA-induced effects [18-21], raising questions about the specificity of cAMP to integrate diverse extracellular signals by eliciting the appropriate functional response. Signaling compartmentalization has emerged as a potential explanation to distinguish between the roles of Epac and PKA, and to give insights into their specific biological functions. Distinct subcellular targeting of Epac and PKA may reflect their multi-functionality, and tethering of these proteins to caveolae and/or A-kinase anchoring
protein (AKAP) complexes [22, 23] (chapter 6) may offer dual control of cAMP actions and contribute to signal specificity. For example, co-localization of PKA and Epac to distinct AKAPs has been associated with opposing biological effects of the two cAMP effectors in cardiomyocytes and neurons [22, 23]. In neuronal cells, AKAP79 tethers Epac2 and PKA, providing a dual control of the phosphorylation status of PKB/Akt, which may be of relevance for the treatment of Alzheimer’s disease [23]. In the heart, muscle-specific mAKAP harbors Epac1, Rap1, PKA, PDE4 and ERK5, thus sensing and transmitting local cAMP alterations in the control of cardiac hypertrophy [22]. Activation of ERK5 leads to PKA activation and subsequent Ca\(^{2+}\) mobilization, responsible for the nuclear translocation of prohypertrophic nuclear factor of activated T cells (NFAT) [22]. By contrast, activation of Epac1 and Rap1 reduces the development of cardiac hypertrophy through inhibition of ERK5 and PKA signaling [22]. Recent findings in cardiomyocytes revealed that Epac may increase excitation-contraction coupling via direct interaction with type II ryanodine receptors and Ca\(^{2+}\)-ATPases in the sarcoplasmic reticulum [24, 25]. Epac-driven PLC-ε activation and subsequent increase in intracellular Ca\(^{2+}\) have also been reported in cardiomyocytes [24, 26, 27]. Moreover, Epac may trigger accumulation of connexin 43, a process known to be required for gap junction formation, proper ion transfer and generation of contractile force in the heart [28]. Interestingly, the biological outcome of Epac activation in cardiomyocytes corresponds to the ionotropic and chronotropic action of cAMP-elevating β-agonists (reviewed in 8, 29).

It has been hypothesized that Epac, as part of the signaling cascade induced by β2-adrenoceptor activation, is also involved in the regulation of specific cellular functions in the lung [20, 30, 31]. This may be of importance for the therapy of chronic inflammatory airway diseases, like asthma and chronic obstructive pulmonary disease (COPD). Inhaled β2-agonists are therapeutically used to relieve bronchoconstriction in patients with asthma and COPD, by inducing cAMP-mediated airway smooth muscle (ASM) relaxation. However, these medications are not preventive nor curative towards these diseases [32]. This may partially be explained by the fact that β2-agonists are not effective to inhibit airway inflammation, which is presumably involved in the development of irreversible structural changes in the airways, known as airway remodeling, that contribute to airway hyperresponsiveness and a decline in lung function [33, 34] (for details see chapter 1). In addition to inflammatory cells, different structural cells and extracellular compartments of the airway wall are thought to be importantly involved in the development of airway remodeling [33]. Among the structural cell types, ASM cells have been recognized as key players in the pathophysiology of obstructive airway diseases. Thus, growth factors and cytokines released during the inflammatory response may cause a (reversible) phenotypic shift of these cells from a contractile to a proliferative, synthetic phenotype [35, 36]. This phenotype plasticity is postulated to cause thickening of the ASM layer and secretion of ECM...
proteins and inflammatory cytokines by ASM cells, altogether contributing to the airway remodeling and changes in airway function [33, 34] (chapter 1). The ineffectiveness of β₂-agonists to inhibit airway inflammation in vivo may in part be explained by the controversial role of β₂-agonists in the modulation of cytokine release in vitro. In fact, studies indicate that β₂-agonists may either inhibit or promote cytokine production and/or secretion in inflammatory and ASM cells [37-39]. Moreover, dysfunctional β₂-adrenoreceptor signaling such as receptor desensitization - leading to a loss of β₂-agonist-induced cAMP production - may impair the beneficial effects of β₂-agonists and may even be responsible for adverse effects such as increased hyperresponsiveness [40, 41]. Desensitization of the β₂-adrenoceptor is the result of uncoupling and/or down-regulation of the receptor, and may involve PKA and G-protein receptor-kinase (GRK)-induced phosphorylation of the receptor and binding of β-arrestin after acute or chronic exposure to β₂-agonists (homologous desensitization) [42-44]. Moreover, inflammatory cytokines, contractile agonists and prostanoids may also cause β₂-adrenoceptor desensitization (heterologous desensitization), either by enhanced cAMP production or by protein kinase C-induced phosphorylation of the receptor [44]. After internalization, the receptor can either be degraded in clathrin-coated pits or recycled, a process known as resensitization [44]. Interestingly, AKAP250 (gravin), a unique AKAP protein that specifically binds PKA and PKC, has been shown to play a role in β₂-adrenoceptor resensitization in A431 cells, by forming a complex with GRK2, β-arrestin and clathrin [45, 46]. Hence, activation of post-β₂-adrenergic receptor mechanisms and particularly of Epac, could be advantageous to maintain the beneficial effects of β₂-agonists without the risk of receptor desensitization. Despite the increasing number of studies regarding Epac signaling, however, our knowledge of the impact of Epac on airway functioning is still limited. Therefore, in chapter 3, we started to unravel signaling properties of Epac in human ASM and bronchial epithelial cells, and in chapter 6 we addressed the issue of cAMP compartmentalization in ASM cells to better elucidate the regulation of Epac signaling and its interaction with PKA. In chapter 3, we report that Epac1 and Epac2 are expressed in both human ASM and bronchial epithelial cells. Only in ASM cells, Epac activation enhanced Rap1-GTP loading, whereas in both ASM and bronchial epithelial cells we observed Epac-dependent activation of ERK1/2, JNK and matrix metalloproteinase-9 (MMP-9). These data indicate cell-type specific orchestration of Epac signaling, which might be associated with the distinct functional roles of ASM and epithelial cells in the lung. As described in chapter 2, Rap1 represents the best characterized Epac effector and its activation allows Epac to control several cellular processes including integrin-mediated cell adhesion, endothelial barrier functioning, and cytokine release. Moreover, Rap1 often acts as a mediator between Epac and ERK1/2 to regulate cell proliferation and differentiation (chapter 2). Epac-dependent activation of JNK has also been described, but the functional role of this interaction remains unknown [47]. Although cross-talk...
between cAMP and MMP family members has been reported [48, 49], we were the first to connect Epac with the activation of MMP-9 (Chapter 3). MMP-9 represents an important player in both airway structural remodeling and inflammation, upon degradation of ECM components and release of cytokines and growth factors [50]. Indeed, in COPD MMP-9 contributes to the destruction of the lung parenchyma (emphysema), which importantly contributes to lung function decline [51]. However, increased activity of MMP-9 in large airways, such as bronchial epithelial and smooth muscle cells may also play a regulatory role in wound-healing after lung injury. Moreover, in ASM cells Epac activated the phosphoinositide 3-kinase (PI3-K) effector PKB/Akt (Chapter 3), which has been shown to mediate survival effects for example in macrophages [52]. Finally, activation of Epac in ASM cells induced the phosphorylation of transcription factors, such as p53 and c-Jun, and actin/microtubule cytoskeleton-related proteins, including tubulin and β-catenin, confirming previous observations that Epac may potentially regulate gene transcription and cytoskeleton-mediated processes [14, 53] (Chapter 3). Collectively, given the potential signaling capacities of Epac in ASM and bronchial epithelial cells, our data further strengthen the hypothesis that Epac may contribute to several aspects of lung (patho)physiology.

In Chapter 6, we characterized compartmentalization of cAMP signaling in ASM cells by studying Epac subcellular (re)distribution and potential clustering of cAMP effectors to caveolae microdomains or AKAP-dependent complexes. We found that Epac1 and Epac2 exhibit distinct expression patterns in ASM: Epac1 is ubiquitously distributed, whereas Epac2 is mainly located to membrane and cytosolic fractions. Moreover, upon activation only Epac1 translocated from the cytosol to membranes and nucleus, suggesting that differential functions of Epac1 and Epac2 may be dictated upon distinct subcellular targeting to distinct pools of cellular effectors. Importantly, in ASM cells Epac1, Epac2, Rap1, PKA and AKAP79 were found to localize in caveolae microdomains. Previous studies have shown that caveolae act as important regulators of ASM phenotype and functions by sequestering important central effectors of contractile, proliferative and synthetic responses, including bradykinin receptors [36, 54, 55]. In support, we demonstrated that caveolae play a prominent role in bradykinin-induced release of the pro-inflammatory cytokine interleukin (IL)-8 from ASM cells. Hence, association of cAMP-related effectors to caveolae may provide functional control of such processes by cAMP signaling (see Chapter 6).

As discussed above, some of the signaling molecules activated by Epac in ASM regulate cellular functions involved in the pathogenesis of asthma and COPD. For example, ERK1/2 and PI3K effectors represent central mitogenic signals for ASM cells [56]. Thus, prolonged activation of ERK1/2 and PI3K triggers a phenotypic change of intact ASM, characterized by increased ASM cell proliferation as well as reduced ASM contractile force development and contractile protein expression [60, 61] (Chapter 8). Moreover, JNK, which can be activated by Epac as well, may also
Contribute to the airway structural changes observed in asthma [62]. Next to proliferation, ERK1/2 and JNK actively participate in chronic airway inflammation by promoting the release of cytokines from structural cells [63, 64] (chapter 4). At this regard, Rap1 activation by Epac acts anti-inflammatory by inhibiting the release of macrophage inflammatory protein-1α (MIP-1α or CCL3), MIP-1β (CCL4) and tumor necrosis factor-α (TNF-α) and the expression of interferon-β (IFN-β) from activated dendritic cells and macrophages [15, 16, 63-65]. Moreover, in pulmonary artery endothelial cells Epac and Rap1 counteract the pro-inflammatory effect of thrombin by activating Rac1 and inhibiting Rho [14]. Such mechanisms result in a decreased permeability of the endothelial barrier and may therefore reduce the transmigration of leukocytes to the site of inflammation. In addition, RhoGTPases contribute to airway functions due to their ability to regulate phosphorylation of myosin light chain (MLC), a necessary step in ASM contraction [66], and Rho-kinase has recently emerged as a potential target for airway hyperresponsiveness [67]. Therefore, having established that Epac proteins are expressed in the airways and regulate the activity of a number of proteins potentially involved in asthma and COPD pathogenesis, we further investigated the effects of Epac - either alone or in concert with PKA - on ASM phenotype modulation and functional responses known to contribute to pathological features of these diseases, particularly inflammation, contraction and proliferation.

Role of Epac and PKA in airway inflammation

Asthma and COPD are characterized by a massive infiltration and activation of inflammatory cells in the airways, caused by the release of a variety of cytokines, chemokines and growth factors by immunologically active cells that reside in the airway wall, including airway epithelial and ASM cells [68] (chapter 1). IL-8 represents one of the best characterized members of the family of chemokines known to attract neutrophils and eosinophils [69, 70], that have been implicated in inflammatory airway diseases [71]. Increased levels of the cytokine IL-8 have been found in blood, bronchial mucosa [70] and bronchial epithelial cells of patients with asthma [72] as well as in sputum [73], broncho-alveolar lavage fluid and bronchiolar epithelium [72, 74] from COPD patients. Interestingly, in addition to neutrophils, macrophages and the airway epithelium, ASM cells are also a rich source of IL-8, and its production is believed to be regulated at the transcriptional level upon activation of distinct intracellular signaling pathways [63, 75]. The promoter region of the IL-8 gene contains sequences responsive to nuclear factor-kappa B (NF-κB) and ERK1/2-induced transcription factors [63, 76]. cAMP has been shown to either inhibit [39] or increase [77] the release of IL-8 from ASM cells, but the mechanisms of such effects have not been elucidated yet. Hence, in chapters 4 and 5 we investigated the effects of Epac and PKA on IL-8 release from ASM cells, induced by distinct mediators known to be involved in asthma or COPD, and we unraveled the underlying molecular mechanisms. In chapter 4, we used the asthma-associated
pathogenic factor bradykinin, which is known to induce IL-8 release from ASM cells upon activation of ERK1/2 [75]. We found that the effect of bradykinin was potentiated upon selective activation of Epac and PKA. By using specific pharmacological tools, we discovered that these responses were mediated by signaling to Rap1 and ERK1/2. Moreover, we confirmed previous observations by others [78], that bradykinin directly activates cAMP signaling, identifying a novel pro-inflammatory mechanism of action of these pluripotent molecules. The inflammatory effects of Epac and PKA were confirmed by specific pharmacological inhibition or down-regulation of the cAMP effectors, showing that both Epac1 and Epac2 are involved. In addition, we revealed the interconnectivity between Epac and PKA with regard to the modulation of bradykinin-induced IL-8 release. As we found AKAP79 to be expressed in ASM cells (chapter 6), we investigated the potential role for AKAPs in Epac- and PKA-mediated functions and their interconnectivity, by using the PKA-AKAP binding disrupting peptide Ht31 (chapter 6). Importantly, Ht31 treatment reduced augmentation of IL-8 release by Epac and PKA, suggesting that bradykinin-induced IL-8 release may involve an AKAP-PKA-Epac complex.

In chapter 5, we investigated the regulatory role of cAMP effectors in the release of IL-8 by cigarette smoke extract. As cigarette smoke is an important risk factor in COPD, unravelling such mechanisms might provide insights into the treatment of inflammation in COPD patients. In line with previous observations [79, 80], cigarette smoke extract markedly increased IL-8 release from ASM cells. However, in contrast to our previous findings using bradykinin (chapter 4), activation of Epac proteins and/or PKA inhibited cigarette smoke extract-induced IL-8 from both immortalized and primary human ASM cells. Specific down-regulation/inhibition of Epac or PKA confirmed the inhibitory role of the two cAMP effectors on cigarette smoke extract-induced IL-8 release. In line with previous reports [81], the pro-inflammatory effect of cigarette smoke extract was driven by activation of ERK1/2 and NF-κB. Importantly, the inhibitory role of Epac on cigarette smoke extract-induced IL-8 release was mediated via inhibition of NF-κB activation, whereas PKA inhibited the phosphorylation of ERK1/2. In support of the distinct roles of the two cAMP effectors in regulating IL-8 release by bradykinin and cigarette smoke extract, we did not find connectivity between Epac and PKA-driven responses on cigarette smoke extract-induced effects. This suggests that distinct pools of Epac and PKA might contribute to the specificity of cAMP effects in the modulation of cytokine release by inflammatory stimuli. Interestingly, both in immortalized and primary human ASM cells cigarette smoke extract reduced Epac1 protein and mRNA levels, leaving Epac2 and PKA unaffected. The down-regulation of Epac1 could account for the lower anti-inflammatory potential of Epac activation compared to PKA. Importantly, a reduction of Epac1 expression was also observed in lung samples from COPD patients. These findings point at Epac and PKA as potential endogenous targets for anti-inflammatory therapy in COPD, although Epac1 sensitivity towards
cigarette smoke may suggest a different contribution of the cAMP effectors in the immuno-suppressive potential of cAMP. Importantly, the effects of Epac and PKA on bradykinin- and cigarette smoke extract-induced IL-8 release corresponded to the effect of the β₂-agonist fenoterol (chapters 4 and 5). Our results are in line with previous observations [77, 82, 83] showing that, depending on the inflammatory stimulus, β₂-agonist and cAMP signaling may exert either pro-(chapter 4) or anti-(chapter 5) inflammatory effects on ASM cells. Hence, this may give an additional explanation to the lack of efficacy of β₂-agonists in the treatment of inflammation in asthma and COPD, where the ASM is continuously stimulated with an array of pro-inflammatory cues. Moreover, Epac1 down-regulation by inflammatory stimuli such as cigarette smoke may account for the variable anti-inflammatory capacities of cAMP-elevating agents in the treatment of COPD. Although difficult to interpret, in vitro settings represent a fundamental way to study the inflammatory signaling pathways and their potential interactions with the cAMP signaling and may help to find new strategies to reduce airway inflammation and the progression of asthma and COPD.

Role of Epac in ASM contraction

Dysfunctional regulation of ASM tone is another feature of obstructive airway diseases and results in airway hyperresponsiveness to a variety of stimuli [84]. cAMP-elevating β₂-agonists are the most potent inhibitors of ASM contraction and for this reason the treatment of airway obstruction is focused on cAMP elevations. Although cAMP-relaxant properties have been attributed to PKA-dependent effects on contractile proteins, K⁺ channels, Na⁺/K⁺ ATPases, Ca²⁺ sequestration, sensitivity of myosin and IP₃ formation (reviewed in [85]), additional PKA-independent mechanisms have been recently postulated [32, 86]. Epac may be a potential candidate to mediate β₂-agonist effects in ASM, as it also mediates β₂-agonist effects in cardiomyocytes, upon modulation of calcium homeostasis and actin-cytoskeletal dynamics [87, 88]. Moreover, Epac modulates ASM tone in aortic smooth muscle cells [89], via yet unknown mechanisms. In chapter 7, we demonstrated that the relaxant effect of the β₂-agonist isoproterenol on pre-contracted guinea pig and human tracheal preparations is largely independent of PKA and specific activation of Epac induced ASM relaxation independent of PKA. The underlying mechanisms of Epac-mediated ASM relaxation were unraveled using cultured guinea pig and human ASM cells. ASM contraction is directly associated with changes in the phosphorylation of MLC [66], which is increased by Rho and decreased by Rac [14, 90]. Contractile agonists, such as methacholine, activate Rho, thereby enhancing MLC phosphorylation [40, 66] and ASM contraction [67]. Recently, Epac has been shown to modulate the Rho/Rac balance in favour of Rac, a process resulting in decreased MLC phosphorylation and enhancement of pulmonary artery endothelial barrier function [14, 90, 91]. As expected, treatment of ASM cells with methacholine induced activation of RhoA and inhibition of Rac1, leading to a subsequent increase
in MLC phosphorylation. All these responses were counteracted upon stimulation of Epac. Epac-driven inhibition of both methacholine-induced muscle contraction by the GTPases inhibitor Toxin B-1470 and MLC phosphorylation by the Rac1-inhibitor NSC23766 were significantly attenuated, confirming the importance of Rac1 in Epac-mediated relaxation. Collectively, these results elucidate a completely new pathway by which cAMP affect ASM tone and point at Epac as a novel target for the treatment of airway obstruction in asthma and COPD.

**Role of Epac in regulating ASM phenotype**

Increased ASM mass is particularly caused by ASM hyperplasia (increase in cell number) and hypertrophy (increase in cell size) [92], and contributes to thickening of the airway wall, airway hyperresponsiveness and impaired lung function in asthma [34]. Increased ASM mass is likely the result of mitogenic stimulation by growth factors, cytokines, GPCR agonists, and extracellular matrix proteins (ECM) which are able to modulate ASM phenotype and function [33-35, 57, 93]. Under normal conditions, ASM cells are generally characterized by low proliferative rates, relatively high contractile function and expression of contractile proteins, such as smooth muscle myosin heavy chain and smooth muscle α-actin [60, 94]. However, in vitro exposure of ASM cells or tissue to growth factors, inflammatory mediators or ECM proteins results in a switch from a contractile to a proliferative phenotype, characterized by increased proliferative rates, decreased expression of contractile markers and decreased contractility [58, 60, 94]. Due to their innate phenotypic plasticity, ASM cells may return to their contractile phenotype upon removal of mitogenic stimuli or even develop a hypercontractile phenotype in the presence of insulin or transforming growth factor-β (TGF-β) [95-97]. This hypercontractile phenotype is characterized by increased contractility, high expression of contractile markers, and an elongated morphology of the cells [33, 95-97]. The Rho/Rho kinase, ERK1/2 and PI3K pathways play an important role in the phenotypic transitions [60, 97].

cAMP regulates cell proliferation in a cell-type and stimulus-dependent manner upon activation of Epac and/or PKA [19-21, 98]. In ASM cells, cAMP drives anti-proliferative signals, mainly appointed to PKA-driven phosphorylation of mitogenic signaling elements and cell-cycle-dependent proteins [17, 40]. However, Epac has recently emerged as an important regulator of cAMP-mediated inhibition of cell proliferation in human ASM cells [31], although with an a yet unknown mechanisms. Moreover, Epac exerts anti-mitogenic effects in lung fibroblasts, a response being insensitive to PKA activation [20, 99]. In chapter 8, we addressed the role of cAMP and its effectors Epac and PKA on proliferative responses in bovine tracheal smooth muscle (BTSM) cells stimulated with platelet-derived growth factor (PDGF). Moreover, we related this response with the effect of PDGF on BTSM tissue contractile function to investigate the capacity of the cAMP effectors to regulate ASM phenotype. In line with previous findings [94], PDGF increased BTSM cell
proliferation and reduced smooth muscle contractility and expression of contractile proteins. Importantly, these responses were all inhibited by co-treatment with the cAMP-elevating prostaglandin E2 (PGE2), and with specific activators of Epac and PKA, without affecting basal proliferation and contractility. Moreover, Epac and PKA prevented ERK1/2 phosphorylation by PDGF. However, only PKA was able to additionally reduce PDGF-induced phosphorylation of the pro-proliferative PI3K effector molecule p70S6K [100]. These findings indicate that cAMP signaling may be important in the maintenance of a normal contractile ASM phenotype and give insight in the mechanisms underlying the anti-proliferative effects of cAMP in ASM. Indeed, cAMP elevating agents such as β2-agonists and PGE2 have been reported to act anti-mitogenic in cultured ASM cells [101, 102], but their effects in vivo are still lacking. Our data confirm the potential beneficial role of PGE2 in inhibiting ASM proliferation, and suggest that stimulation of cAMP effectors may be exploited to treat ASM mass increase in airway remodeling.

In chapter 9, the effects of activation of Epac and PKA on PDGF-induced ASM phenotypic modulation were studied in human ASM cells and tissue. It has previously been shown that long-term treatment with serum induces a phenotypic modulation of human bronchiolar smooth muscle, characterized by decreased contractile responses and contractile protein expression [103]. We found that long-term treatment with PDGF modulates human ASM phenotype by decreasing tissue contractility and contractile protein expression, responses being associated with increased ASM cell proliferation. Importantly, co-treatment of human ASM strips with the PKA activator 6-Bnz-cAMP or the Epac activator 8-pCPT-2'-O-Me-cAMP strongly inhibited PDGF-induced ASM cell proliferation and prevented PDGF-induced hypcontractility and the reduction of contractile protein expression by the growth factor.

The maintenance of a contractile phenotype by cAMP-effectors may appear paradoxical considering the bronchodilatory effect of cAMP and the results shown in chapter 7. However, it must be noted that these observations rely on completely different mechanisms and experimental settings. Thus, the results presented in chapter 7 derive from acute stimulation of Epac and PKA in the presence of a contractile agonist and are based on rapid cellular processes at the level of the contractile machinery. On the other hand, the data in chapters 8 and 9 are the result of chronic stimulation of the cAMP effectors (4 days in culture) that inhibits the growth factor-induced reduction of contractile protein expression by modulating transcriptional and translational processes, while the Epac and PKA stimuli are not present during the contraction experiments. Importantly, we not only confirmed the importance of cAMP signaling in the acute treatment of bronchoconstriction, but we additionally provided a novel potential approach to treat the long-term feature of asthma, due to the chronic alterations of ASM phenotype and functions which eventually results in the permanent structural airway remodeling.
Epac and implications for disease therapy

During the last decades, our insight into cAMP signaling has dramatically changed and the discovery of Epac has opened new avenues in the understanding of biological functions of cAMP. As mentioned above, cAMP elevation by β2-agonists is widely used to treat obstructive airways diseases such as asthma and COPD, even though these drugs are not curative or preventive and the efficacy of their action is reduced in severe disease states. The limited effectiveness of β2-agonists is mainly attributed to the β-adrenoceptor desensitization and subsequent reduced cAMP elevations due to the receptor internalization (reviewed in [44]). Although this response minimally affect the bronchodilatory capacities of β2-agonists on ASM cells, it has been postulated that receptor desensitization on inflammatory and structural cells may limit the anti-inflammatory and anti-proliferative effects of β2-agonists in vivo [104]. Hence, alternative strategies have been considered. PGE2 may hypothetically represents a good candidate as its actions are also driven by cAMP-elevating GPCRs abundantly expressed in ASM. In vitro, PGE2 has been reported to have anti-proliferative effects in ASM [101] and our studies have proven that this effects are associated with a regulation of ASM phenotype (chapter 8). In addition, PGE2 receptors do not undergo a similar rapid desensitization in ASM as observed for β2-adrenoreceptors [43]. However, both relaxant and contractile effects of PGE2 have been reported on ASM [105]. Moreover, this prostanoid also modulates the immune response by regulating the functions of cells such as macrophages and T and B lymphocytes, leading to pro- and anti-inflammatory effects [106]. Such contractictory responses are the consequence of the activation of four receptors designated as EP1 to EP4, which are coupled to a multitude of intracellular signaling pathways. In particular, EP2 and EP4 are Gs-coupled receptors and increase cAMP, probably contributing to the beneficial effects on contraction, inflammation and cell proliferation, whereas EP1 and EP3 are coupled to Go and Ca2+ signaling and have been involved in smooth muscle contractility [107]. Moreover, PGE2 causes heterologous desensitization of the β2-adrenoreceptor [42], a process recently being associated with activation of PKA and ERK1/2, which also induce expression of PDEs and subsequently decrease intracellular cAMP levels [108]. Hence, activation of cAMP-regulated pathways distal from the receptor may represent a better approach in order to exploit the beneficial effects of cAMP elevation and at the same time to avoid the problems due to receptor desensitization.

PDE inhibitors possess anti-inflammatory and anti-immunomodulatory effects in the airways [109, 110], due to their capacity to increase cAMP levels. In the airways, PDE4 represents the most abundant PDE isoform and therefore, selective PDE4 inhibitors, such as rolipram and roflumilast, have undergone clinical trials to determine their usefulness/efficacy in the treatment of asthma and COPD [109]. However, these compounds are only weak bronchodilators and their usage is additionally limited by systemic side effects, especially nausea and vomiting [109].
Hence, a more targeted approach such as the modulation of cAMP downstream effectors, including Epac, may represent a promising alternative for potential therapeutic intervention in airways diseases. Collectively, the findings presented in this thesis show that Epac proteins are expressed in ASM and bronchial epithelial cells and potentially able to drive important cAMP responses in ASM (chapter 3), including modulation of cytokine release (chapters 4 and 5), bronchodilation (chapter 7) and inhibition of ASM cell proliferation (chapters 8 and 9). Most functions are also driven by PKA, via identical (chapter 4), similar or distinct (chapters 5, 8 and 9) mechanisms. The cross-talk between the two cAMP effectors may be dictated by their specific compartmentalization (chapter 6). However, Epac-driven relaxation of methacholine-induced contraction in both guinea pigs and human intact ASM appeared independent of PKA (chapter 7), supporting the importance of this novel cAMP effector molecule in ASM function. Studies of Epac and PKA expression and function in disease models of asthma and COPD in vivo are warranted to elucidate the therapeutic potential of specific activators of these two cAMP effectors in asthma and COPD.

Based on our observations, it is reasonable to assume that, in addition to PKA, Epac drives the downstream effects of β2-agonists and their biological outcome. At this moment, however, there are limited tools available to prove this theory and we can only speculate on the contribution of Epac and/or PKA to the effects of β2-agonists in health and disease. Thus, as PKA knock-out mice are not vital, the functional roles of PKA have so far been studied by pharmacological approaches using different PKA inhibitors. However, such compounds have raised doubts with respect to their efficacy and specificity [111, 112]. In addition, studies of the physiological functions of Epac proteins are also limited by the current lack of (isoform)-selective activators and inhibitors. The guanine nucleotide exchange factor brefeldin A has been shown to inhibit Epac-induced responses [113], but there is no evidence of its direct attenuation of Epac activity. In vitro, suppression of Epac protein expression using silencing RNA has been successfully utilized to elucidate several Epac-related functions [20, 114] (chapters 4 and 5). However, such strategies have considerable limitations for whole organ and in vivo studies due to transfection toxicity and scarce construct permeability in tissue. Importantly, Epac knock-out mice have recently been developed and have already demonstrated the role of Epac2 in pancreas organogenesis and function [115]. Mice lacking specific Epac effector proteins have also been developed to gain insights into Epac-regulated signaling and its biological outcomes [27]. Hence, development of such tools is warranted to study the issue of cAMP signaling specificity, and to better approach the in vivo effects in animal models. Finally, understanding cAMP spatio-temporal regulation in ASM cells remains a future challenge to explain the link between Epac and PKA.

In conclusion, the study of Epac in the lung holds promise as Epac controls various properties of ASM and may fulfill a multifunctional role in airway physiology and pathophysiology. Importantly, however, beneficial effects of targeting Epac and/or
their effectors in airway diseases need to be weighed against potential side-effects, as virtually all cells express these proteins. Selective targeting of Epac1- and/or Epac2-regulated signaling in the airways by inhalation may help to reduce systemic side effects.

**Fig. 1.** Epac as a novel regulator of airway smooth muscle phenotype and function. β2-Agonists and/or Prostaglandin E2 elevate cAMP, which in turn activate Epac and PKA. Upon orchestration of specific intracellular pathways, Epac dynamically regulates ASM phenotype and functions, including contraction, proliferation and cytokine release. Such processes might contribute to pathological features of airway diseases, such as airway hyperresponsiveness, airway remodeling and inflammation. **Acutely,** Epac relieves methacholine-induced ASM contraction by skewing the balance between Rac1 and RhoA towards Rac1, resulting in a net diminishment of MLC phosphorylation. **Chronically,** Epac and PKA differentially contribute to restore the normo-contractile phenotype of ASM by inhibiting PDGF-induced activation of mitogenic signaling such as ERK and p70S6K. This also results in inhibition of ASM proliferation. Epac and PKA both act pro- and anti-inflammatory with regard to IL-8 release from ASM, depending on the stimulus. An AKAP-dependent multiprotein complex probably coordinates the interconnectivity of Epac and PKA in the potentiation of bradykinin-induced IL-8 release, a process dependent on Rap1-mediated activation of ERK. In contrast, cigarette smoke-induced IL-8 release is inhibited by Epac and PKA via inhibition of NF-κB and ERK, respectively. Clustering of cAMP effector molecules to caveolae may also affect cellular functions. Hypothetical interactions are indicated by dotted lines. See text for further details.
Taken together, the studies presented in this thesis have revealed the following main findings:

- Both Epac proteins are expressed in guinea pig, bovine and human ASM as well as in human bronchial epithelial cells (chapters 3, 7 and 8). In resting ASM cells, Epac1 is ubiquitously distributed and translocates to the membranes and the nucleus upon activation, whereas Epac2 localized at cytosolic and membrane fractions in both resting and stimulated ASM cells (chapters 4 and 6).

- Epac differentially activates a subset of effectors proteins including Rap1 and ERK1/2 in both ASM and epithelial cells (chapters 3-5 and 8). In ASM cells, Epac phosphorylates peptides involved in inflammation, cell proliferation and contraction, pointing at Epac as a novel key effector in lung (patho)physiology and as a regulator of ASM function (chapter 3).

- Epac1, Epac2, Rap1, PKA and AKAP79 associate to caveolae in ASM cells and this complex regulates bradykinin-induced IL-8 release (chapter 6).

- In ASM cells, an AKAP-dependent multiprotein complex probably coordinates the interconnectivity of Epac and PKA in the potentiation of bradykinin-induced IL-8 release (chapter 6), a process dependent on Rap1-mediated activation of ERK (chapter 4). Epac-and PKA-induced release of IL-8 may limit the anti-inflammatory properties of β2-agonists in asthma.

- Epac and PKA inhibit cigarette smoke extract-induced IL-8 release from immortalized and primary human ASM cells. Epac effects are mediated by inhibition of the NF-κB signaling, whereas PKA inhibits ERK1/2 signaling (chapter 5).

- Cigarette smoke reduces expression of Epac1, but not Epac2 or PKA, in human ASM cells, resulting in a different anti-inflammatory potential of Epac and PKA. The expression of Epac1 is also reduced in lung tissue from COPD patients (chapter 5).

- Epac relaxes methacholine-induced ASM contraction and reduces MLC phosphorylation by shifting the balance between Rho and Rac1 towards Rac1. These findings highlight a potential role of Epac in the treatment of exaggerated airway obstruction in asthma and COPD (chapter 7).

- In bovine ASM, Epac and PKA maintain a normo-contractile phenotype in the presence of PDGF and inhibit PDGF-induced cell proliferation by inhibiting the activation of ERK1/2 (both Epac and PKA) and p70S6K (PKA) by this growth factor (chapter 8).
Epac and PKA inhibit PDGF-induced phenotypic modulation of human ASM by inhibiting the mitogenic increase in ASM proliferation and normalizing ASM contractility and contractile protein expression (chapter 9).

References


52. Misra UK, Pizzo SV: Coordinate regulation of forskolin-induced cellular proliferation in macrophages by protein kinase A/cAMP-response element-binding protein (CREB) and...


Chapter 10


