New avenues for Epac in inflammation and tissue remodeling in COPD

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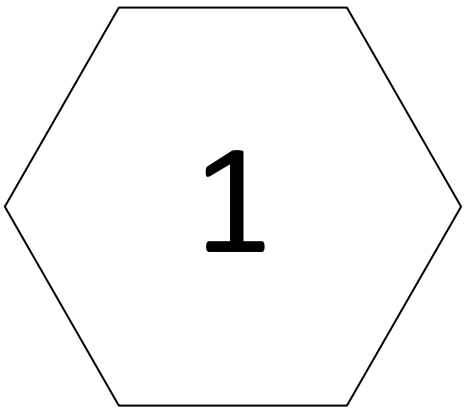
General introduction



COPD

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disorder of the airways and the lung parenchyma (1). Currently, COPD is the third leading cause of death worldwide (2). Up to 20% of smokers develop COPD, and more than 80% of all COPD patients are cigarette smokers (3). These numbers clearly identify smoking as the major health risk for the development of COPD but other factors such as environmental pollution are also important risk factors (3). The severity of COPD is subdivided in Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages, GOLD stage I is an indication for mild COPD, while the most severe COPD is indicated by GOLD stage IV (4). Due to the high death rate and the great risk of developing COPD by smoking and environmental pollution, current research focuses on disease prevention by cessation of smoking and on drug development for COPD.

COPD is characterized by a progressive and irreversible decline in lung function caused by inflammation and tissue remodeling, including loss of epithelial barrier small airway fibrosis, mucus hypersecretion, increased airway smooth muscle mass and parenchymal destruction (emphysema) (1; 5-7). In particular, the accelerated, not fully reversible decline in lung function in COPD is characterized by infiltration and activation of inflammatory cells, including macrophages, lymphocytes and neutrophils (Figure 1), which promote the release of proteases and inflammatory cytokines, such as interleukin-8 (IL-8), IL-1β, IL-6 and tumor necrosis factor (TNF) (1; 5-7). Small airways and lung parenchyma in COPD patients are predominantly affected by inflammation and this contributes to the airway obstruction and progressive loss of lung function (8; 9).

In COPD, several pro-inflammatory cytokines, including IL-8, IL-1β, IL-6 and TNF (Figure 1), are increased and seem to create an amplified expression of multiple inflammatory genes, partly through the activation of the transcription factor nuclear factor (NF)-κB (1; 5). Activation of epithelial cells and macrophages lead to the release of IL-1β, TNF and IL-8. Whereas IL-1β attract monocytes (10), IL-8 is a potent neutrophil chemoattractant and activator (11). In addition, the abundance of IL-8 correlates with neutrophil counts in COPD, which are increased in COPD (12). Epithelial cells can secret in addition IL-9, IL-10 and IL-11 which leads to the

recruitment of lymphocytes such as T helper (Th) 1 cells and type 1 cytotoxic T (Tc1) cells, both of which release interferon-γ (IFN-γ) (5; 13). IFN-γ has an effect on both epithelial cells as well as macrophages. Together these processes create a persistent inflammation in COPD patients (Figure 1) (5).

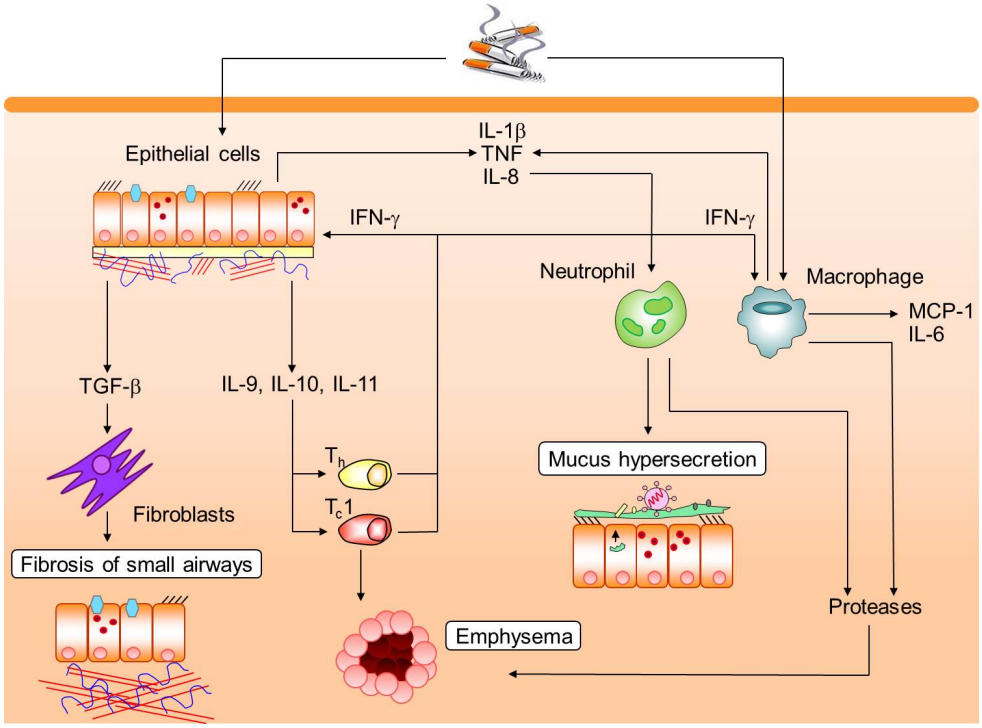


Figure 1: Inflammatory processes in COPD. Cigarette smoke, the main risk factor for COPD, activates epithelial cells and macrophages and subsequently induces IL-1β, IL-8, TNF release. In addition macrophages will secret IL-6 and monocyte chemoattractant protein-1 (MCP-1). IL-8 attracts neutrophils to the site of infection. Neutrophil activation contributes to mucus hypersecretion and emphysema by production of proteases. Both macrophages and neutrophils activate proteases that contribute to emphysema. TGF-β secreted by epithelial cells promotes fibroblast proliferation leading to airway fibrosis. Activated epithelial cells secrete a diverse set of cytokines (IL-9, IL-10, IL-11), which recruit lymphocytes such as T helper (T_h) cells and type 1 cytotoxic T (T_c1) cells, both of which release IFN-γ which has an effect on both epithelial cells and macrophages and is known to contribute to emphysema. Figure adapted from (5; 13).

Increased permeability of the epithelial barrier may contribute to the inflammatory response in COPD (14; 15). The epithelial barrier normally acts as a

physical barrier against unwanted particles, including cigarette smoke, and regulates the transport of molecules across the barrier (16). Goblet cells, specialized epithelial cells, and submucosal glands are responsible for mucus secretion in healthy persons enabling mucociliary clearance to remove particles from the lung (17; 18). Cigarette smoke induces a cycle of injury and repair in the epithelium (19), leading to alterations in the barrier function, goblet cell and submucosal gland hyperplasia resulting in loss of barrier function and mucus hypersecretion. The airway lumen is reduced by thickening of the epithelial barrier which is likely the result of chronic irritation initiated by cigarette smoke (20; 21). Another factor in this respect is the increase in size and activity of goblet cells, induced by inflammation and cigarette smoke, both leading to mucus hypersecretion in COPD patients (22-24). Mucus hypersecretion contributes to the morbidity and mortality of COPD, particularly in those patients with more severe disease (8; 25; 26). Mucus hypersecretion from goblet cells and mucus glands further reduces the airway lumen and lung function in COPD, particularly in patients with chronic bronchitis (22).

Fibrosis and emphysema represent two remodeling processes, with opposing effects on the extracellular matrix (ECM), together leading to progressive and irreversible decline in lung function of COPD patients (14; 27). Fibrosis around the small airways is considered to be the result of abnormal repair mechanisms, involving the recruitment and activation of (myo)fibroblasts, which produce ECM proteins like collagens, fibronectin and proteoglycans (6; 28). These processes stiffen the airway walls and prevent airway relaxation, causing an even more pronounced airway narrowing (20; 29). In contrast, emphysematous destruction of alveoli, the gas-exchanging region of the parenchyma but also forming the peribronchial attachments, causes loss of elasticity and ultimately loss of recoil of the airways (30).

In addition to goblet cell hyperplasia and submucosal gland hypertrophy, airway remodeling also includes an increase in airway smooth muscle mass, which contributes to airway obstruction in COPD patients limiting their lung function (20). Airway smooth muscle mass increases significantly in the small airways in smokers (31; 32), and this increase is believed to be a main contributor to airway

hyperresponsiveness (33; 34). Increased airway smooth muscle mass may result from hyperplasia as well as hypertrophy (35; 36). Various stimuli including growth factors, certain ECM proteins, inflammatory mediators and cigarette smoke have been shown to increase airway smooth muscle proliferation, which could explain airway smooth muscle hyperplasia (37-43).

A proper balance in the production and degradation of ECM proteins is mediated by the interaction between matrix metalloproteases (MMPs), which degrade ECM proteins(44), and tissue inhibitors of metalloprotease (TIMPs), which are endogenous inhibitors of MMPs (45). Members of the MMP family differ in their substrate specificity: MMP9 degrades gelatin and all types of collagens, while MMP12 degrades elastin, gelatin and collagen I (46). An imbalance between MMP and TIMP activities may underpin small airway fibrosis (increased production/reduced degradation of ECM) and emphysema (reduced production/increased degradation of ECM). Indeed, an increase in MMP9 and 12 has been reported in COPD patients (47), a process supposed to be associated with an enhanced degradation of ECM proteins most likely contributing to emphysema (47). However, the main inhibitor of MMP9, TIMP1 was also elevated in COPD patients (48) making the net effect of increased MMP9 expression on ECM turnover difficult to predict. Next to ECM degradation, MMP9 seems to modulate inflammatory cytokine release and thereby to regulate both repair and destruction processes (49).

Taken together, inflammation and remodeling processes importantly contribute to the observed decline in lung function in COPD. Since most of these features cannot effectively be reversed by currently available drugs, COPD is as yet incurable and pharmacotherapy is therefore primarily aimed at reducing the symptoms associated with COPD.

Pharmacotherapy in COPD

Currently, there is no cure for COPD and symptoms are treated with (combinations of) anticholinergics, β_2-agonists, phosphodiesterase (PDE)-4 inhibitors and to a lesser extent with glucocorticosteroids (50-53). The primary goal of these therapies is to induced bronchodilation and to reduce inflammation.

Therapy with anticholinergics, such as the long-acting muscarinic receptor antagonist tiotropium, inhibits bronchoconstriction by antagonism of muscarinic M_3 receptors (52). Glucocorticosteroids are effective in reducing airway inflammation in most asthma patients (54), but COPD patients only marginally benefit from glucocorticosteroids treatment and show a relative glucocorticosteroid insensitivity (55; 56). This may be the result of the different nature of the inflammatory response. Eosinophils are the major inflammatory cells in asthma, whereas in COPD levels of neutrophils are particularly increased (57). Neutrophilic inflammation in general responds poorly to glucocorticosteroid treatment (58), and the steroid insensitivity may be enhanced by smoking (51). In the GLUCOLD study it has been reported that ex-smoking COPD patients treated with glucocorticosteroid show a reduction in CD8+ lymphocytes and in neutrophils as compared to smoking COPD patients (51). Some studies indicate that β_2-agonists can augment the anti-inflammatory effect of glucocorticosteroids (59), as is the case for asthma (60; 61). Others did not observe an increased anti-inflammatory effect when a combination of inhaled corticosteroids and β_2-agonists was used (53). Nevertheless an improved forced expiratory volume in one second (FEV_1), as a measure of lung function, was found when using the glucocorticosteroid fluticasone and the β_2-agonist salmeterol (53). These findings indicate that combination of inhaled corticosteroids and β_2-agonists may improve pharmacotherapy.

Both β_2-agonists and PDE4 inhibitors are known to elevate the intracellular levels of the second messenger 3'-5'cyclic adenosine monophosphate (cAMP) by inducing its production by adenylyl cyclase (AC) and by preventing its breakdown into the inactive 5'-AMP, respectively (18; 62). Notably, though both β_2-agonists and PDE4 inhibitors elevate cAMP, they modulate distinct cellular functions. It has been reported that β_2-agonists effectively reduce airflow obstruction by inducing bronchodilation (63; 64); however, their effect on airway inflammation is not very promising (50-52).

Thus, although it has been reported that β_2-agonists reduces inflammatory processes such as cytokine release *in vitro* (65-67), evidence for their anti-inflammatory properties *in vivo* is still lacking (63). This contradiction might be

explained by the risk of rapid β_2-adrenergic receptor desensitization, particularly in inflammatory cells (68-70). In contrast, in inflammatory cells PDE4 inhibitors maintain the beneficial effects of β_2-agonists without the risk of receptor desensitization due to their capacity to elevate cAMP by preventing its breakdown, and thereby control inflammation in the airways (71). Selective inhibitors of PDE4, such as rolipram iscurrently tested in clinical trials for the treatment of COPD (72). PDE4 inhibitors such as roflumilast are already used in the clinic to treat COPD (73). Compared to β_2-agonists, these PDE4 inhibitors only marginally reduce airflow obstruction, but reduce airway inflammation and thereby possibly the frequency of exacerbations (8; 72; 74).

Current therapy with β_2-agonists and PDE4 inhibitors is beneficial in various stages of the disease and is mainly symptomatic. Although receptor desensitization could account for the difference between PDE4-inhibitors and β_2-agonists with respect to their impact on inflammation, it does not explain their difference in reversing airflow obstruction. In addition to differential expression of PDE4 in inflammatory cells and airway smooth muscle, it is tempting to speculate that compartmentalization of the cAMP pathway may account for differences between PDE4 inhibitors and β_2-agonists, and may therefore be a topic for further drug development.

cAMP and its effectors PKA and Epac

The second messenger cAMP is known to be involved in a large variety of biological functions, including calcium handling, smooth muscle relaxation, secretion, memory, metabolic processes, gene transcription and immune function. The importance of cAMP as a signaling molecule is underscored by the number of Nobel prizes that have already been assigned to research linked to cAMP (18; 75; 76). Even though cAMP is now studied for more than 50 years, novel findings are still presented, indicating how complex and versatile this pathway is.

The cAMP pathway is initiated by ligand binding to G_s protein-coupled receptors, such as the β_2-adrenergic receptor (Figure 2). In turn, the G_s protein activates ACs which subsequently produce cAMP from adenosine triphosphate (77). Cellular levels of cAMP are controlled by the balance between the activities of AC and PDE.

PDEs hydrolyze cAMP to pharmacologically inactive 5'-AMP and thereby reduce intracellular cAMP levels (78). cAMP can activate three effectors: cyclic nucleotide gated ion channels, protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) (75; 76; 79). The best studied cAMP effector is PKA, a tetrameric molecule consisting of two regulatory (R) subunits and two catalytic (C) subunits. Binding of cAMP to the two binding sites on the regulatory subunits of PKA results in the activation of the catalytic subunits, which phosphorylates threonine and serine residues (80). Distinction between the two PKA isoforms PKAI and PKAII is made based on the presence of the regulatory subunits, RI and RII, respectively (80).

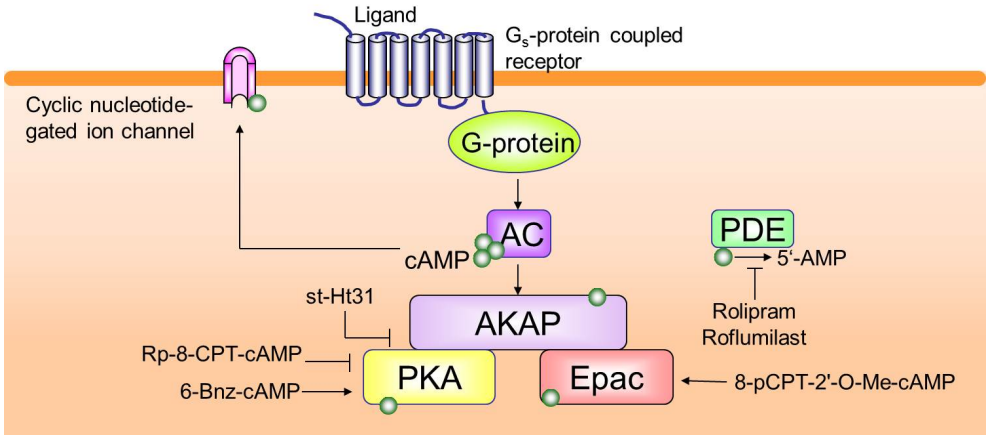


Figure 2: Ligands activate G protein-coupled receptors. The α_s subunit activates adenylcyclase (AC) which results in the production of cAMP. Phosphodiesterases (PDE) hydrolyse cAMP into inactive 5'-AMP. cAMP is able to activate 3 effectors: protein kinase A (PKA), exchange protein directly activated by cAMP (Epac) and cyclic nucleotide-gated ion channels. Compartmentalization of the cAMP pathway is achieved by A-kinase anchoring proteins (AKAPs) which bind cAMP effectors, including PKA and Epac. Pharmacological tools to specifically inhibit or activate certain parts of the cAMP pathway are mentioned in the figure. Rolipram and roflumilast are examples of PDE4 inhibitors. Epac is activated by 8-pCPT-2-*O*-Me-cAMP, and PKA is activated by 6-Bnz-cAMP. Rp-8-pCPT-cAMP inhibits PKA, whereas st-Ht31 inhibits the interaction between AKAPs and PKA.

Most of the biological effects mediated by cAMP were initially assigned to PKA (76; 79). The discovery of Epac in 1998 offered answers to effects known to be PKA-

independent (81). Epac is a guanine-nucleotide exchange factor (GEF) which exchanges GDP for GTP on small GTPases, such as Rap1 and Rap2, leading to their activation (Figures 2 and 3) (79). There are two isoforms of Epac, Epac1 and Epac2, both capable to interact with several proteins (79; 82). The expression of the two Epac isoforms differs between tissues (83). Although the expression of Epac1 is ubiquitous, it is most abundant in the heart, kidney and leukocytes (83). In contrast, Epac2 is mainly expressed in the brain and the adrenal glands (83). The expression profiles of both Epac1 and Epac2 seem to alter during development (83). The difference in localization of Epac1 and Epac2 enhances the specificity of their effectors present at a certain location. Thereby Epac seems to regulate many biological functions, primarily through its canonical GEF activity signaling to a variety of cellular effectors (Figure 3), acting either alone or in concert with PKA (79).

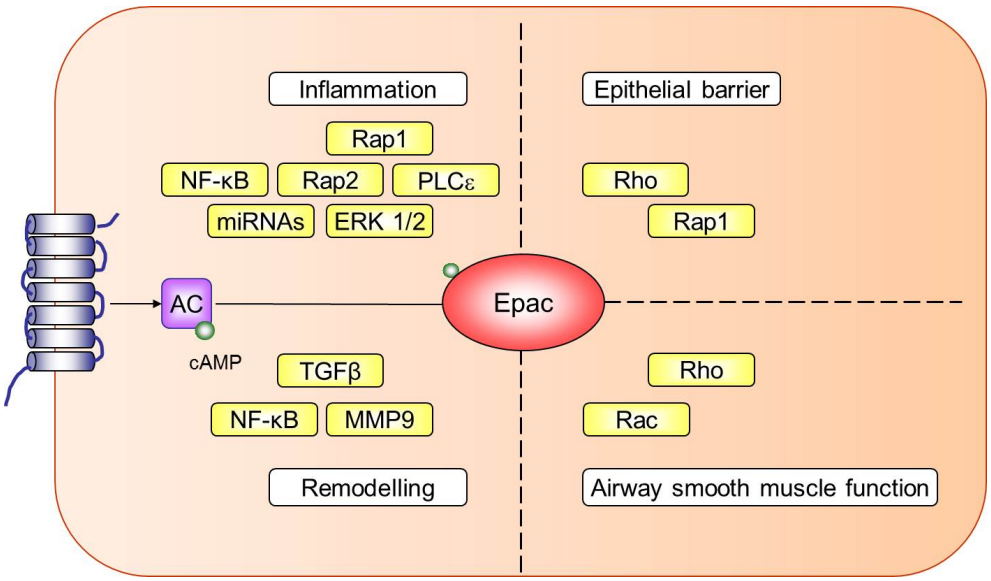


Figure 3: Activation of a G_s protein-coupled receptor increases adenylyl cyclase (AC)-mediated cAMP production, leading to activation of exchange protein directly activated by cAMP (Epac). Epac is able to interact with a diverse set of proteins such as Rap1, Rap2, Rho, ERK 1/2, NF-κB, TGFβ, Rho and Rac which may depend on the tissue. Recently, miRNAs have also been linked to Epac. Upon such mechanisms Epac might be capable to regulate central features of COPD such as the epithelial barrier, inflammation, remodeling processes and airway smooth muscle function.

To study the role of either Epac or PKA in cAMP mediated biological functions different tools have been developed. Specific activators of Epac and PKA, 8-pCPT-2'-*O*-Me-cAMP and 6-Bnz-cAMP, respectively, are useful to assign biological functions to either Epac or PKA (Figure 2) (84-86). The newer Epac activator, Sp-8-pCPT-2'-*O*-Me-cAMP is a more potent Epac activator as it is resistant to PDE-mediated hydrolysis (85). However, these activators do not discriminate between Epac1 and Epac2 as both subtypes are activated to a similar extent by these compounds. In addition to the specific activator of PKA, specific inhibitors for PKA are available: Rp-8-CPT-cAMPs, Rp-cAMPs and Rp-8-Br-cAMPs (Figure 2) (85; 87), which are more selective than the classic inhibitor H89 (88). For a long time, no specific inhibitors were known for Epac. However, specific knockouts for Epac1 and Epac2 were developed in mouse strains, which enables studies on the specific

role of the two different Epac subtypes (89; 90). Very recently, specific inhibitors of Epac were developed, which also show subtype-selectivity (91-93). This opens new possibilities to study the specific role of Epac in cellular processes induced by cAMP elevation in the near future.

The presence of multiple effectors of cAMP and their signaling pathways as well as differences in expression pattern create the challenge to unravel the exact mechanisms of cAMP and its effectors involved in the different aspects of the pathophysiology and pharmacotherapy of COPD.

Compartmentalization of cAMP

Another layer of complexity of the cAMP pathway is added due to the fact that Epac and PKA are localized at different sites and organelles in the cell, including the plasma membrane, perinuclear regions and mitochondria (79; 83). In addition, cAMP is unevenly distributed throughout the cell most likely due to a rather localized distribution of ACs and PDEs (18). To enable a distinct regulation of several diverse biological responses by both cAMP and its effectors, compartmentalization of cAMP has been emerged as an important concept. Compartmentalization of cAMP is maintained primarily by expression of ACs, PDEs and complex formation by A-kinase anchoring proteins (AKAPs), which play the major role in the compartmentalization of cAMP (94).

Local cAMP levels are generated and maintained by ACs and PDEs by increasing cAMP only near certain targets (95). As most ACs are membrane bound, cAMP levels are increased in close proximity to G protein-coupled receptors or ion channels (Figure 2). Hydrolysis of cAMP by PDEs reduces cAMP levels and prevents diffusion of cAMP to other cellular compartments and signaling pathways. Thus, compartmentalization of both PDEs and ACs results in a diminished diffusion of cAMP throughout the entire cell and enables cAMP to localize to specific organelles. Importantly, AKAPs further support compartmentalization of cAMP upon formation of multi-protein complexes comprized of G protein-coupled receptors, ACs, PDEs, PKAs and Epacs (Figure 2) (79; 95). In humans, more than 50 AKAP members belong to the AKAP family (95). All members of the AKAP family bind to PKA, some also interact with Epac1 and

Epac2 (79; 96). Upon formation of multi-protein complexes bearing PKA and/or Epac, AKAPs direct the activity of a distinct subset of cAMP effectors to different subcellular compartments and effector proteins (95). In addition, AKAP family members differ in their subcellular localization, their size and their protein interactions. Most AKAPs bind PKA-RII, but some specifically bind PKA-RI, whereas others can bind both PKA isoforms (96). Examples of AKAP members are AKAP9/Yotiao, AKAP12 and AKAP5, which are downregulated in lung tissue of COPD patients and might therefore play a role in the pathophysiology and pharmacotherapy of this disease (97).

Indeed, AKAPs can integrate differential coupling of cAMP to specific cellular responses (95). Concerning cAMP signaling in general, it is known that cAMP enhances barrier properties in endothelial cells (98; 99), which is regulated by activation of Rac1 by AKAP-anchored PKA (99). One of the AKAPs which is important in regulating barrier properties in endothelial cells is AKAP9. A multiprotein complex with Epac1 and AKAP9 enhances microtubule growth in endothelial cells, an indirect parameter for an improved endothelial barrier function (100). Multiple splice variants are known for AKAP9, such as Yotiao, and AKAP9 is also known to interact with ACs and to increase the phosphorylation of PKA effectors (101; 102). The largest splice variant of AKAP9 is AKAP450 which interacts with PDE4 (18; 95). Interestingly, AKAP9 is found in multiple cell types present in the lung, such as bronchial epithelial cells, pulmonary fibroblasts, inflammatory cells and airway smooth muscle cells (18). Certain lessons about the epithelial barrier can be learned from endothelial cells. The epithelial barrier protects against inhaled agents by secretion of antioxidants, protease inhibitors and antimicrobial factors, maintains mucociliary clearance and regulates the passage of solutes into the intracellular space by forming a physical barrier (16). It has been reported that cigarette smoke increases the permeability of the epithelial cell layer (15), and causes aberrant epithelial repair (24; 103). This loss of epithelial properties may play a role in COPD progression (24). The importance of compartmentalization of Epac and Rac by AKAP9 has been reported in the endothelial barrier, however, it is currently unknown whether this complex also regulates the epithelial barrier.

AKAP5 is localized at the plasma membrane in airway smooth muscle cells and forms a complex with certain ACs and with the β_1- and β_2-adrenergic receptor (95; 104). AKAP12 (aka Gravin) interacts with PDE4D and the β_2-adrenergic receptor (95). The interaction between a certain AKAP and its effectors may be of potential benefit in drug development to target AKAP-bearing multiprotein complexes to specific cellular locations.

To study the role of AKAPs in biological processes, Ht31 is used, which represents a peptide encompassing the AKAP-RII interaction domain thereby competing for the binding of PKA-RII to all AKAP members. (Figure 2) (105). The disadvantages of a peptide are that it cannot enter the cell in an easy way and is not stable which makes it less efficient in cell or animal studies and also for drug development (106). A steared form for Ht31 was developed (st-Ht31) which enhances cellular uptake (107). Non-peptide helix mimetics (for example terphenyl and terpyridine scaffolds) are being developed to overcome the disadvantages of the peptides (106) .

Epac in the context of COPD

Epac is involved in a broad range of biological processes including those which are features of COPD. Part of these biological processes are regulated by additional proteins which interact with Epac (79; 81).

Inflammation

Inflammation in COPD is characterized by an increase in cytokine release and of inflammatory cells. In different studies a role for Epac has been described in the regulation of inflammatory processes. In alveolar macrophages, pharmacological activation of Epac inhibited phagocytosis (108). Since alveolar macrophages express Epac1, but not Epac2, the functional responses have been assigned to Epac1 (108). In the murine macrophage-like cell line J774A.1, pharmacological activation of Epac inhibited lipopolysaccharide-induced IFN-γ production (109). In the murine macrophage cell line RAW 264.7 Epac increases the expression of IL1-β and IL-6 mRNA (110). Overall these studies clearly show that Epac acts as an inflammatory modulator and that Epac can either be anti-inflammatory or pro-inflammatory.

Epac regulates several signaling pathways, including the extracellular signal-regulated kinases (ERK1/2) (111) and the NF-κB pathways (112). Both pathways alter gene expression. Activation of NF-κB leads to nuclear translocation of the p65 subunit of NF-κB and thereby induces inflammatory gene expression and inflammation (113; 114). Epac activation is known to be involved in activation of NF-κB signaling (115) which could explain the role of Epac in inflammation. Activation of the ERK1/2 pathway by Epac has also been described in different cell types (83; 116; 117). ERK1/2 is involved in inflammatory cytokine release and inflammatory cell recruitment (118).

Members of the Ras superfamily of GTPases are known to interact with Epac. Two members, Rap1 and Rap2, are both activated by Epac, but share only 60% sequence homology and are involved in different downstream signaling pathways (119). Rap 1 seems to be involved in inflammation via interaction with Epac (120), assigning the Epac-Rap1 pathway to inflammatory processes in COPD. In contrast, the role of Rap2 in biological processes is rather limited, but an effector has been assigned to Rap2, namely phospholipase Cε (PLCε) (121).

PLCε, like all other PLCs, hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (122). Due to a Ras association homology domain, Ras family members can directly bind to PLCε, which distinguishes PLCε from the other PLCs (122). The role of PLCε has particularly been established in the heart (122), and little is known about its role in the lung. Interestingly, Takenaka and colleagues demonstrated that PLCε elevates the expression of IL-6 mRNA in a skin inflammation model (123), pointing towards a pro-inflammatory role. Together with the notification that PLCε plays a role in the production of keratinocyte derived chemokine (KC), the mouse equivalent of the neutrophil attractant IL-8 (124), PLC may act directly or via the Epac-Rap2 pathway as a possible regulator of inflammation in the lung.

Recent studies indicate that Epac may also interact with microRNAs (miRNAs) (125; 126). MiRNAs are small noncoding functional RNA molecules of 18-25 nucleotides (127) that bind to mRNA, thereby inducing degradation of the mRNA and blocking

translation (128). An abnormal expression of miRNAs seems to underlie various disease features, including inflammation (129; 130). In COPD, miRNAs have been implicated in the regulation of inflammatory processes (128) and miRNA-7 is increased in serum of COPD patients (129). *In silico* analysis indicates that Epac1 is a putative target of miRNA-7 (targetscan.org). The above studies suggest that there is indeed a relation between Epac and miRNA, but the interaction between these two proteins has not been studied yet in the lung.

Taken together, Epac may regulate inflammation via a variety of different pathways. However, the specific role of Epac in regulating airway inflammation is not known. Moreover, there is currently no report on the role of Epac1 and Epac2 in inflammation *in vivo*.

Epithelial barrier

Another feature of COPD is an increase in epithelial permeability and a loss of epithelial barrier properties (131). Via Rap1, Epac may play an important role in integrin-mediated cell adhesion and formation of cell junctions via E-cadherin in endothelial cells (132; 133). Rho, another member of the Ras superfamily, is also involved in the epithelial barrier function via actin dynamic dependent processes (134; 135). Rho is involved in stress fiber formation and focal adhesion formation. Both processes are involved in cell migration (136), which is an important feature of epithelial barrier repair (137). The role of Epac on the epithelial barrier is not known, but in endothelial cells a stabilizing role on the barrier is observed when Epac is activated (98). Via currently unknown pathways Epac1 stabilizes the endothelial barrier by increasing growth of micotubuli (138). Therefore, Epac could be similarly important in the regulation of the barrier function of airway epithelium.

Remodeling

As mentioned above, fibrosis, mucus hypersecretion, increased airway smooth muscle mass and emphysema all belong to the tissue remodeling features observed in COPD patients. Concerning fibrosis, both PKA and Epac inhibit the proliferation of fibroblasts and the production of ECM proteins such as collagen I and III (139). In addition, the pro-fibrotic cytokine transforming growth factor

(TGF)-β_1 decreases the cellular level of Epac1 (139). Taken together, downregulation of the anti-fibrotic Epac by TGF-β_1 may contribute to the fibrotic processes observed in airways in COPD. In support, Yokoyama and colleagues showed that adenoviral overexpression of Epac1 inhibits TGF-β1 induced synthesis of collagen (140). These anti-fibrotic actions of Epac might be mediated via Rap1 (140).

Interestingly, elevation of cAMP following PDE4 inhibition reduces cigarette smoke induced IL-13 levels (141), the main inducer of mucus secretion (142). Mucus secretion induced by cigarette smoke is regulated via the Epac effector ERK1/2 (22; 143; 144). Therefore, activation of Epac by cAMP may underlie the inhibitory effect on mucus hypersecretion, presumably by interacting with the ERK1/2 pathway.

Growth factors are able to transform a contractile phenotype of airway smooth muscle (ASM) into a proliferative, hypo-contractile phenotype (62; 79). Interestingly, previous studies of our group demonstrated that Epac, next to PKA, prevented the growth factor-induced reduction of ASM strip contractility and contractile protein expression (α-smooth muscle actin), and thereby reversed the phenotypic modulation of ASM (145; 146). Activation of both PKA and Epac inhibit the growth factor-induced modulation of ASM upon inhibition of ERK1/2 (145; 146).

Also emphysema might be regulated by Epac. Previous studies have shown that cAMP has the ability to alter MMP expression and activity (147-150). However the involvement of Epac in relation to altered MMP expression has not been described yet. Since the NF-κB signaling pathway, which is also regulated by Epac (115), induces the expression of MMP9 (151), a regulatory role for Epac in MMP9 expression in emphysema can be envisaged.

Airway smooth muscle function

In COPD, β_2-agonists induce bronchodilation of the obstructed airways. Airway obstruction is partly caused by increased phosphorylation of the myosin light chain (MLC) and subsequent interaction with actin polymers in the airway smooth

muscle (152). The balance between phosphorylated MLC and non-phosphorylated MLC is regulated via both Rac and Rho. Rho enhances the activity of the MLC phosphatase, leading to increased levels of phosphorylated MLC and airway smooth muscle contraction (153). Reduction in the phosphorylation of MLC due to inhibition of MLC kinase by Rac will lead to airway smooth muscle relaxation (62; 79). Recent studies from our laboratory demonstrated that Epac shifts the balance between Rho and Rac towards Rac, thereby inducing relaxation of airway smooth muscle (154). By contrast, it is also known that the Epac-Rap-PLCε pathway releases calcium from the sarcoplasmic reticulum and thereby induces cardiac muscle contraction (155). However, since Epac activators actually induce relaxation of airway smooth muscle, it is tempting to speculate that compartmentalization of Epac in combination with activators and (distinct) effectors determines the overall effect on muscle tone: contraction in cardiac muscle and relaxation in airway smooth muscle.

Scope of the thesis

Since Epac and its interplay with PKA, AKAPs and effector proteins may play a major role in a diverse set of cellular processes involved in the pathophysiology of COPD, particularly inflammation and remodeling (Figure 3), analyzing the role of Epac in these processes and underlying mechanisms and signaling routes may be a step forward in the drug development for COPD. In this thesis, we aimed to study the specific role of particularly Epac in COPD in these processes, both *in vitro* and *in vivo*.

In **chapter 2** we describe the importance of compartmentalized cAMP signaling in cellular functions. In this chapter, we highlight the different proteins involved in cAMP compartimentalization, such as PDEs, ACs and AKAPs. We focus specifically on the interaction between the cAMP pathway and the epithelial barrier function in COPD and the possible molecular mechanisms involved. Compartmentalized cAMP signaling regulates important cellular processes that are important for a proper lung function and may be used as a therapeutic target.

The potential role of the cAMP effectors Epac and PKA on airway inflammation in COPD is described in **chapter 3**. As a model for airway inflammation in COPD, human airway smooth muscle cells have been exposed to cigarette smoke extract (CSE) and levels of IL-8 levels have been analyzed. The pro-inflammatory cytokine IL-8 recruits neutrophils to the site of damage or infection (156) and its levels are increased during acute COPD exacerbations contributing to the high numbers of neutrophils present at that time (157). The effects of the β_2-agonist fenoterol, the Epac activator 8-pCPT-2-*O*-Me-cAMP and the PKA activator 6-Bnz-cAMP on CSE-induced IL-8 transcription and release have been determined using PCR and ELISA, respectively. We also have analyzed the impact of PKA and Epac on CSE-induced activation of the NF-κB pathway - by studying the degradation of the NF-κB inhibitor IκBα and the nuclear translocation of the transcriptionally active NF-κB subunit p65, - and the ERK1/2 pathway, by studying ERK phosphorylation. Moreover, the effects of CSE on the mRNA and protein expression of PKA, Epac1 and Epac2 in human airway smooth muscle have been measured. Lastly, we also have determined the protein expression of PKA, Epac1 and Epac2 in lung tissue obtained from COPD patients and non-COPD controls.

To investigate the potential mechanisms underlying the downregulation of Epac1 after CSE exposure in airway smooth muscle cells and in lung tissue from COPD patients (**chapter 3**), the link between miRNA-7 and Epac is described in **chapter 4**. In silico analysis identified Epac1 as a putative target for miRNA-7. Human bronchial epithelial cells, human lung fibroblasts and human airway smooth muscle cells have been exposed to CSE and analyzed for miRNA-7 expression. To study whether miRNA-7 is able to reduce the expression of Epac1 and Epac2, miRNA-7 has been ectopically overexpressed in airway smooth muscle cells and Epac1 and Epac2 expression was determined. We also have studied the expression of miRNA-7 in lung tissue from COPD patients as well as in bronchial smooth muscle from COPD patients obtained by laser dissection of lung sections.

The potential role of PKA and Epac in fibrotic processes and emphysema is described in **chapter 5.** Since cAMP alters MMP9 and TIMP1, we studied the potential role of PKA and Epac on MMP9 and TIMP1 expression and activity (**chapter 5**). The human bronchial epithelial cell line 16HBE14o- was exposed to

CSE with or without the β_2-agonist fenoterol, the Epac activator 8-pCPT-2'-*O*-Me-cAMP or the PKA activator 6-Bnz-cAMP. Supernatant was collected and analyzed for the presence of active MMP9 and pro-MMP9 by zymography, whereas MMP9 and TIMP1 mRNA expression was analyzed in cell homogenates. We also analyzed the expression of MMP9 and TIMP1 in lung tissue obtained from COPD patients.

In **chapter 6** we describe the impact of compartmentalization of cAMP signaling by AKAP proteins on the epithelial barrier function. The effect of CSE on the functional epithelial barrier and on the protein expression of cell-junction proteins, such as E-cadherin and ZO-1, in human bronchial epithelial cells was analyzed. In addition, the effect of st-Ht31 was analyzed on these parameters. Moreover, AKAP and E-cadherin expression was analyzed in the human bronchial epithelial cell line 16HBE14o- exposed to CSE, primary epithelial cells from smokers and non-smokers and lung tissue of control and COPD patients.

To study the specific roles of Epac1 and Epac2 in airway inflammation and remodeling *in vivo*, wild type, Epac1-/-, Epac2-/- and PLCε-/- mice were exposed to filtered air or cigarette smoke, as described in **chapter 7**. Aspects of cigarette smoke-induced inflammation, such as the levels of the inflammatory cytokines KC (the murine equivalent of human IL-8), IL-6 and IL-1β, and the infiltration of neutrophils, macrophages and lymphocytes were studied. In addition, to study remodeling parameters, differences in mucus secretion and deposition of ECM proteins induced by cigarette smoke were determined by measuring the expression of mucin-5AC and SPDEF (SAM pointed domain ETS fraction), and collagen I and fibronectin, respectively, mucus hypersecretion and deposition of ECM proteins. The effect of cigarette smoke exposure on the expression of components of the cAMP pathway, including Epac1, Epac2, PKA, ACs and PDEs, was evaluated in the wild type animals. In **chapter 8** all results are summarized and discussed and a future perspective is given.

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