General discussion and conclusions.
Chapter 7

Objective and main conclusion

In the current thesis we aimed to unravel roles of AKAPs in the lung. AKAPs are scaffolding proteins crucial for locating enzymes to the proper location in the cell. In particular, we focused on their potential involvement in the regulation of the bronchodilatory action of β2-agonists and in inflammation in relation to chronic obstructive pulmonary disease (COPD).

One of the main causes of COPD is the exposure to cigarette smoke. We found that after cigarette smoke exposure AKAP5 and AKAP12, both important in β2-adrenoceptor regulation, are decreased in airway smooth muscle (ASM) cells. We also found that AKAP5 and AKAP12 are expressed at a lower level in COPD patients compared to non-COPD patients. Inhibiting protein kinase A (PKA)-AKAP interactions with the dominant interfering peptide stHt31 increased cigarette smoke extract (CSE)‐induced release of the neutrophil attractant interleukin-8 (IL-8) from human ASM cells. Deficiency of AKAP5 enhanced cigarette smoke‐induced neutrophilia in mice, demonstrating the importance of proper AKAP functions in regulating airway inflammation. Furthermore, deficiency of AKAP12 in mice leads to decreased airway responsiveness to β2-agonists, and also to an increase in pulmonary inflammation, most notably neutrophilia. Thus, the lower expression of AKAP12 in COPD patients may also have important clinical consequences. Indeed, single nucleotide polymorphisms (SNPs) in AKAP12 are associated with altered reversibility of lung function by β2-agonists.

Implications of AKAP functions for COPD

AKAPs are a family of scaffolding proteins that share the ability to bind the cyclic AMP (cAMP) effector PKA to different areas of the cell. Importantly certain AKAPs also bind to receptors, including the β2-adrenoceptor (Chapter 2). Coordination of cAMP signaling is crucial to prevent disorganization of its signaling properties inside the cell and disturbance of AKAP-driven coordination of cAMP signaling has been suggested to lead to various diseases, including cardiovascular diseases, cancer (1-5) and neurodegenerative diseases (Chapter 2). In this thesis we unraveled the role for altered AKAP functions in the pathophysiology and pharmacological treatment of chronic obstructive pulmonary disease (COPD).

In order to identify the potential impact of compartmentalized cAMP signaling in the ASM, we first assessed the expression profile of AKAPs and its potential alteration upon exposure to cigarette smoke. In chapter 3 we showed that ASM expresses
General discussion and conclusions

Objective and main conclusion

In the current thesis we aimed to unravel roles of A-kinase anchoring proteins (AKAPs) in the lung. AKAPs are scaffolding proteins crucial for locating enzymes to the proper location in the cell. In particular, we focused on their potential involvement in the regulation of the bronchodilatory action of $\beta_2$-agonists and in inflammation in relation to chronic obstructive pulmonary disease (COPD). One of the main causes of COPD is the exposure to cigarette smoke. We found that after cigarette smoke exposure AKAP5 and AKAP12, both important in $\beta_2$-adrenoceptor regulation, are decreased in airway smooth muscle (ASM) cells. We also found that AKAP5 and AKAP12 are expressed at a lower level in COPD patients compared to non-COPD patients. Inhibiting protein kinase A (PKA)-AKAP interactions with the dominant interfering peptide st-Hi31 increased cigarette smoke extract (CSE)-induced release of the neutrophil attractant interleukin-8 (IL-8) from human ASM cells. Deficiency of AKAP5 enhanced cigarette smoke-induced neutrophilia in mice, demonstrating the importance of proper AKAP functions in regulating airway inflammation. Furthermore, deficiency of AKAP12 in mice leads to decreased airway responsiveness to $\beta_2$-agonists, and also to an increase in pulmonary inflammation, most notably neutrophilia. Thus, the lower expression of AKAP12 in COPD patients may also have important clinical consequences. Indeed, single nucleotide polymorphisms (SNPs) in AKAP12 are associated with altered reversibility of lung function by $\beta_2$-agonists.

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In order to identify the potential impact of compartmentalized cAMP signaling in the ASM, we first assessed the expression profile of AKAPs and its potential alteration upon exposure to cigarette smoke. In chapter 3 we showed that ASM expresses
AKAP5, AKAP8, AKAP9, AKAP12 and Ezrin. In line, Penn and colleagues have also demonstrated the expression of these as well as other AKAPs in ASM cells (6). We demonstrated that the AKAP expression profile in ASM cells changed upon exposure to CSE, with AKAP5, AKAP12 and AKAP9 being decreased and Ezrin being increased (Chapter 3). We also found both AKAP5 and AKAP12 to be lower expressed in lung tissue of COPD patients compared to non-COPD patients (Chapter 3). As AKAP5 and AKAP12 have been implicated in β2-adrenoceptor responsiveness (Chapter 2) (7-9), our findings suggest that AKAP expression might be linked to β2-agonist responsiveness.

We confirmed the downregulation AKAP5 and AKAP12 in the lung upon cigarette smoke exposure using an acute murine model of cigarette smoke-induced pulmonary inflammation (Chapter 4). Although AKAP5 and AKAP12 are also highly expressed in the airway epithelium, we previously demonstrated that CSE exposure did not affect AKAP5 and AKAP12 expression in human bronchial epithelial cells (10). Histological staining showed that Ezrin expression, although increased in CSE-treated ASM cells, was lower in COPD – compared to non-COPD control lung tissue (Chapter 3). However, it must be noted that Ezrin was not detected in the ASM in either non-COPD or COPD tissue, whereas Ezrin was highly expressed in the airway epithelium (Chapter 3). In contrast, others found Ezrin to be higher expressed in the epithelium of COPD – compared to control subjects (11). In the airway epithelium it is required for mucus production (11). Recently, genome-wide gene expression profiling in lung tissue samples has confirmed that Ezrin is higher expressed in lungs from COPD patients compared to control subjects (12). Vitamin D receptor knock out mice were characterized by an increased Ezrin expression (13) Together these data may provide an additional explanation for a higher Ezrin expression found in COPD, although reduced vitamin D signaling in COPD patients is still under debate (14, 15).

Taken together, findings from our group and others put forward several AKAPs as potential candidate drug targets in COPD. We focused on AKAP5 and AKAP12 as known interactors with β2-adrenoceptors, and their beneficial effects on the regulation of smooth muscle tone and inflammation. We hypothesized that lower AKAP5 and AKAP12 expression, as observed in COPD, affects the therapeutic efficacy of β2-agonists to induce bronchorelaxation as well as the anti-inflammatory properties of cAMP.

**β2-agonist treatment in COPD**

Patients suffering from COPD are often treated chronically with β2-adrenergic bronchodilator drugs. Chronic β2-adrenergic stimulation can lead to decreased receptor
functioning, either due to a decreased expression of the receptor at the cell membrane or due to disturbed signaling (16, 17). In addition, β₂-adrenoceptor activation has been associated with different signaling pathways such as the canonical adenylyl cyclase or the non-canonical extracellular signal-regulated kinase (ERK) pathway. The latter can be activated either by G₃ signaling or after G-protein coupled receptor kinase (GRK)/arrestin-mediated desensitization of the β₂-adrenoceptor (18, 19) and is associated with detrimental effects of β₂-agonists for patients, which may also result in reduced efficacy (20). Thus far this has been mainly studied in the context of asthma, but might hold true also in the treatment of the symptoms in COPD patients.

Based on findings in the literature (7-9), we postulated that AKAPs play a role in β₂-agonist responsiveness by modulating the expression of β₂-adrenoceptor at the membrane (Chapter 2). We demonstrated that treatment of ASM cells with st-Ht31 increased activation of the ERK pathway in the absence of a β₂-agonist (Chapter 3). Non-canonical GRK-mediated β₂-adrenoceptor signaling does not appear to be mediated via AKAP5 or AKAP12 as GRK-mediated (Ser355/356) β₂-adrenoceptor phosphorylation is rather reduced than increased in AKAP12⁻/⁻, and also reduced to some extent in AKAP5⁻/⁻ mice (Chapter 5). Ser355/356 phosphorylation of the β₂-adrenoceptor only occurs in the presence of a ligand (21), and is shown to mediate non-canonical ERK signaling (22). Taken together the data suggest that AKAP12 is necessary for GRK/arrestin-mediated signaling. However, we only investigated Ser355/356 phosphorylation of the β₂-adrenoceptor in the absence of a β₂-agonist and thus far it has been shown in vitro that downregulation of AKAP12 does not change β₂-adrenoceptor-mediated ERK signaling (23). In vivo, AKAP12-mediated ERK signaling still remains an option as multiple routes potentially activate ERK signaling downstream of the β₂-adrenoceptor and biased signaling properties of certain ligands (24, 25). Removal of AKAP5 or disturbing its interaction with the β₂-adrenoceptor does decrease β₂-adrenoceptor-mediated ERK signaling (23, 26). However, AKAP5 has been related to G₁-mediated ERK activation, not GRK/arrestin-mediated ERK signaling (26, 27). These are two independent signaling pathways downstream of the β₂-adrenoceptor (28). Therefore, if AKAP5 and AKAP12 influence β₂-agonist responsiveness through activation of non-canonical β₂-adrenoceptor signaling the involvement of separate signaling pathways is rather likely. We found an increased ERK signaling in ASM cells after treatment with st-Ht31, and ERK signaling has been shown to increase IL-8 release (29-31). However, whether the increased neutrophilia found in AKAP12⁻/⁻ mice depends on this ERK signaling remains to be studied, as there was no basal increase in KC
release (Chapter 4). Similarly, for the AKAP5/− mice an enhanced KC release was only seen after cigarette smoke treatment (Chapter 4). A potential explanation might be that we measured cytokine release in the bronchoalveolar lavage fluid, which contains mediators released from more cell types than just ASM cells. Similarly, we studied the β2-adrenoceptor phosphorylation in whole lung lysates. Therefore, we cannot exclude an increased ERK signaling at the level of ASM cells. However, our data suggest that the contribution of increased ERK signaling in the ASM might have only minor implications on IL-8/KC release in vivo.

β2-Adrenoceptor activation leading to ASM relaxation is perhaps the most important bronchodilatory pathway in the lung. Here we found that treatment with st-Ht31 reduced β2-agonist-induced relaxation of guinea pig ASM (Chapter 5). As discussed in chapter 2, we expected AKAP12 and AKAP5 to be involved in β2-agonist-induced ASM relaxation. AKAP5 was found in vitro to be involved in processes preceding internalization of the β2-adrenoceptor (7, 23), whereas AKAP12 bound to the β2-adrenoceptor was found to be necessary for β2-adrenoceptor recycling (8, 9, 23). Therefore, lower expression of either AKAP5 or AKAP12, as we found in COPD patients compared to non-COPD patients (Chapter 3), was expected to have an effect on β2-agonist effectiveness. Indeed, AKAP12+/− mice showed decreased ASM relaxation induced by the β2-agonist isoprenaline, however, AKAP5+/− showed no difference compared to wildtype mice (Chapter 5; Figure 2).

Surprisingly AKAP12+/− mice showed a decrease in the phosphorylation of Ser355/Ser356 of the β2-adrenoceptor, known to be targeted by GRK (Chapter 5). GRK-mediated phosphorylation of the β2-adrenoceptor is known to precede desensitization and internalization, and until now AKAP12 was primarily implicated in resensitization (Chapter 2). Our results suggest that AKAP12 is more important in β2-adrenoceptor regulation than thought previously, although to what extent remains to be explored (Figure 2).

We reported here on a link between AKAP12 and β2-agonist responsiveness and a decreased AKAP12 expression after cigarette smoke exposure, the question remains if β2-agonist responsiveness, smoking and AKAP12 are interconnected. Using existing databases from smoking asthmatics we showed that SNPs in the AKAP12 gene are associated with β2-agonist responsiveness in smokers, but not in non-smokers (Chapter 6). Although the exact mechanism is unclear, rs13212161 and rs12201388, which cause a change in amino acid sequence, are interesting (Chapter 6). Changes in the amino acid composition of AKAP12 may lead to altered protein structure and
compromise protein-protein interactions that are important for its functions, including the regulation of β₂-adrenoceptor function. Our observations at least suggest that the above mentioned SNPs may affect the function of AKAP12. For the AKAP5 gene we found the SNP rs745686 to affect β₂-agonist responsiveness. However, this SNP only had an effect in the never-smoking population (Chapter 6). With cigarette smoke being a leading cause of COPD, it would be of more interest to look at SNPs affected in smoking subjects, as for the AKAP12 SNPs, instead of never-smoking subjects, as for the AKAP5 SNPs.

**Pulmonary inflammation**

COPD has a strong inflammatory component, particularly neutrophilia which are attracted to the lungs by the release of particularly IL-8. Previously, we and others have shown that CSE exposure of ASM cells caused the production of IL-8 (30, 31). CSE-induced IL-8 release was reduced by a β₂-agonist, via parallel stimulation of PKA and exchange protein directly activated by cAMP (Epac), and subsequent reduced activation of ERK1/2 and NFκ-B, respectively (29). CSE exposure decreased Epac expression in ASM cells (29), a process involving the induction of miRNA-7 (32). Since AKAPs are known to interact with both PKA and Epac proteins, the role of AKAPs in regulating these processes was subsequently studied.

In chapter 3 we confirmed that CSE-induced IL-8 release by ASM is reduced by the β₂-agonist fenoterol as well as direct PKA activation. Interestingly, we also showed that disruption of AKAP-PKA complexes using the dominant interfering peptide st-Ht31 caused a small increase in basal IL-8 release from ASM cells and augmented CSE-induced IL-8 release. Disrupting AKAP-PKA complexes using st-Ht31 prevented both the β₂-agonist-induced inhibition of CSE-induced ERK1/2 activation and the subsequent reduction of IL-8 release (Chapter 3). These findings demonstrate that anti-inflammatory effects mediated by the β₂-adrenoceptor depend on proper AKAP-PKA interactions (Figure 1). The anti-inflammatory effect of direct activation of PKA by the cAMP analogue 6-Bnz-cAMP - thereby bypassing the β₂-adrenoceptor - was insensitive to treatment with st-Ht31, providing further support for the importance of AKAPs in coordinating the signal from the β₂-adrenoceptor to PKA (Chapter 3). In vivo, β₂-agonists exert anti-inflammatory effects in the presence of PDE4 inhibitors (33). However, β₂-agonists are not used for anti-inflammatory purposes due to the rapid desensitization of the β₂-adrenoceptor combined with the relatively low expression on inflammatory cells (34). To investigate a role for AKAPs in the inflammatory response in
vivo studies we did not need β₂-agonists however, as st-HI31 already increased basal IL-8 release from ASM cells (Chapter 3). Our results from chapter 3 suggest a role for AKAPs in the regulation of inflammation independent of β₂-adrenoceptor signaling.

Therefore, we focused on the basal roles of AKAP5 and AKAP12 in cigarette smoke-induced inflammation. In chapter 4, we confirmed that KC, the murine equivalent of IL-8, was induced in the lung by cigarette smoke as shown previously (35) (Figure 1). Cigarette smoke-induced KC release was increased in AKAP5⁻/⁻ mice compared to wildtype mice and accordingly the number of neutrophils was higher in the lung of AKAP5⁻/⁻ mice compared to wildtype mice. Our findings indicate that AKAP5 is able to dampen cigarette smoke-induced inflammation in the lung (Figure 1). AKAP12⁻/⁻ mice already showed an increased pulmonary neutrophilia without exposure to cigarette smoke and without an increased KC release (Figure 1). The AKAP12⁻/⁻ mice also had an increase in macrophages and a trend for increased lymphocytes in the lung. Together these findings indicate that AKAP12 has an anti-inflammatory role, in the absence of an inflammatory signal. However, the increased infiltration of inflammatory cells seen in the AKAP12⁻/⁻ mice is independent of an increase in KC release. It might be that in the lung, AKAP12 positive cells are important for resolving ongoing inflammation as was found in the brain (36). Therefore, AKAP12 deficient mice may be unable to remove inflammatory cells once present. In addition, inflammatory cells may proliferate more due to the loss of AKAP12, as AKAP12 is known as an inhibitor of proliferation (2, 37). However, the processes being involved in the recruitment of these inflammatory cells to the lung still remains to be characterized. In conclusion, both AKAP5 and AKAP12 possess anti-inflammatory capabilities, particularly with respect to neutrophilia. Therefore, the lower expression of AKAP5 and AKAP12 found in lung tissue of COPD patients compared to non-COPD patients as described in chapter 3 could contribute to increased pulmonary inflammation (Figure 1).
vivo studies we did not need β\textsuperscript{2}-agonists however, as st-Ht31 already increased basal IL-8 release from ASM cells (Chapter 3). Our results from chapter 3 suggest a role for AKAPs in the regulation of inflammation independent of β\textsuperscript{2}-adrenoceptor signaling. Therefore, we focused on the basal roles of AKAP5 and AKAP12 in cigarette smoke-induced inflammation. In chapter 4, we confirmed that KC, the murine equivalent of IL-8, was induced in the lung by cigarette smoke as shown previously (35) (Figure 1). Cigarette smoke-induced KC release was increased in AKAP5\textsuperscript{-/-} mice compared to wildtype mice and accordingly the number of neutrophils was higher in the lung of AKAP5\textsuperscript{-/-} mice compared to wildtype mice. Our findings indicate that AKAP5 is able to dampen cigarette smoke-induced inflammation in the lung (Figure 1). AKAP12\textsuperscript{-/-} mice already showed an increased pulmonary neutrophilia without exposure to cigarette smoke and without an increased KC release (Figure 1). The AKAP12\textsuperscript{-/-} mice also had an increase in macrophages and a trend for increased lymphocytes in the lung. Together these findings indicate that AKAP12 has an anti-inflammatory role, in the absence of an inflammatory signal. However, the increased infiltration of inflammatory cells seen in the AKAP12\textsuperscript{-/-} mice is independent of an increase in KC release. It might be that in the lung, AKAP12 positive cells are important for resolving ongoing inflammation as was found in the brain (36). Therefore, AKAP12 deficient mice may be unable to remove inflammatory cells once present. In addition, inflammatory cells may proliferate more due to the loss of AKAP12, as AKAP12 is known as an inhibitor of proliferation (2, 37). However, the processes being involved in the recruitment of these inflammatory cells to the lung still remains to be characterized. In conclusion, both AKAP5 and AKAP12 possess anti-inflammatory capabilities, particularly with respect to neutrophilia. Therefore, the lower expression of AKAP5 and AKAP12 found in lung tissue of COPD patients compared to non-COPD patients as described in chapter 3 could contribute to increased pulmonary inflammation (Figure 1).

**Figure 1:** Cigarette smoke-induced reduction of AKAP5 and AKAP12 expression results in exaggerated neutrophilia. Exposure to cigarette smoke increases the release of the neutrophil attractant IL-8/KC, a process involving activation of ERK1/2. This process can be reduced by activating the cAMP/PKA pathway either by activating the β2-adrenoceptor (β\textsubscript{2}-AR) or inhibiting phosphodiesterases (PDE). AKAP5\textsuperscript{-/-} mice exhibit an increase in cigarette smoke-induced KC release, suggesting that this process is inhibited in the presence of functional AKAP5. AKAP12\textsuperscript{-/-} mice exhibit an increase in neutrophils independent of KC release, suggesting an effect downstream of KC. Our findings indicate that cigarette smoke-induced reduction in AKAP5 and AKAP12 expression may further amplify cigarette smoke-induced neutrophilia. Activation of ERK1/2 is inhibited by β\textsubscript{2}-agonist-induced PKA in an AKAP-dependent manner, a process potentially hampered in vivo by receptor desensitization and PDE4-dependent degradation of cAMP.
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Taken together, ASM expresses several AKAPs and the β2-adrenoceptor associated AKAP5 and AKAP12 are decreased by cigarette smoke (Chapters 3 & 4) and are lower expressed in COPD patients compared to non-COPD patients (Chapter 3). Reduction in AKAP12 increases pulmonary inflammation and reduces β2-agonist responsiveness, at least in the ASM. It remains to be studied whether this pulmonary inflammation and β2-agonist responsiveness is linked and can be influenced by AKAP12. It is known that β2-adrenoceptor-mediated anti-inflammatory effects in vivo are limited when PDE4 is not simultaneously inhibited. AKAP12 has no direct protein-protein interaction with PDE4; however there are PDE4 isoforms that are recruited to the β2-adrenoceptor following GRK-mediated phosphorylation (27), which is reduced in AKAP12−/− mice (Chapter 5). We speculate that in the absence of AKAP12, there is potentially less PDE4 in the proximity of the receptor to degrade cAMP, and also less PKA activation due to the lack of AKAP12-PKA interactions, causing reduced anti-inflammatory signaling and potentially reducing the additive effect of PDE4 inhibitors. However, what the actual functional outcomes are needs further investigation.

Reduction in AKAP5 increases the sensitivity to cigarette smoke-induced neutrophilia suggesting that the lower expression of AKAP5 observed in COPD patients compared to non-COPD patients (Chapter 3) may lead to an increase in cigarette smoke-induced inflammation in the lung (Figure 2). The role of AKAP5 in inflammation is thus far unstudied, so no clear mechanism can be put forward at this point, but a role for Epac, suggested to bind AKAP5 in neurons (38), can be excluded because Epac knock out mice did not show the same increased sensitivity to cigarette smoke-induced neutrophilia (39).

For COPD patients, cAMP signaling in AKAP5 and AKAP12 compartments may be crucial to maintain a healthy lung and the cigarette smoke-induced loss of these proteins will exaggerate pulmonary inflammation and reduce therapeutic efficacy of β2-agonist. Therefore, an approach to ameliorate symptoms and perhaps even progression of disease, improving AKAP5− and AKAP12-mediated signaling could provide a novel therapeutic potential.
Figure 2: AKAP12 mediated β₂-agonist-induced ASM relaxation. Activation of β₂-adrenoceptors (β₂-AR) induces airway smooth muscle relaxation in an AKAP12-dependent manner. After agonist binding, the receptor undergoes recycling a process involving AKAP12 and GRK-mediated phosphorylation of the β₂-adrenoceptor. Receptor recycling is required to maintain long term functioning for β₂-agonist-induced airway smooth muscle relaxation. As cigarette smoke induced reduction of AKAP12 expression our findings also imply that the loss of AKAP12 may impair β₂-agonist responsiveness.

Translational aspects of AKAPs

Based on the novel findings presented in this thesis, AKAP research potentially opens an entire new field of drug discovery. Current drugs mainly target receptors or enzymes irrespective of their location in the cell. The potential benefit of specific targeting of scaffolding proteins is that one would be able to target only a specific subset of receptors and/or enzymes, thereby limiting side effects due to inhibition of potential beneficial effects of these effectors. In combination with existing drugs (e.g. PDE4 inhibitors) such compounds could even provide improved efficacy, as AKAPs can also shield enzymes from selective drugs, as shown for PKC (48).

Although therapeutic outlooks for the use of AKAPs have been reviewed before in detail elsewhere (5, 49-51), here the potential in the context of this thesis will be discussed. The most widely used dominant interfering peptide used in AKAP research, (st-)Ht31, was discovered to inhibit sperm mobility (52) and subsequently a patent was applied for male contraception (US 6011013 A). Although far from therapeutic
application, quinones demonstrated to cause alkylation of AKAP3 and AKAP4 selectively in spermatozoa, thereby reducing their motility. In addition, these compounds act against Chlamydia by alkylation a major chlamydial membrane protein, providing these drugs with a dual function against pregnancy and the transmission of sexually transmitted diseases (53). No male contraception has thus far come to market; however, a precedent has been made for AKAPs to be used as drug target, but also for drug design as described at the end of this section (Chapter 2).

Effects of non-selective PKA-AKAP interaction inhibitors such as st-Ht31 have been as diverse as the described functions of PKA itself. In sensory neurons for example, st-Ht31 could prevent forskolin-induced potentiation of heat-activated ion channels through the VR1 channel and could therefore potentially inhibit inflammatory hyperalgesia (54). The data suggest that st-Ht31 may be beneficial to use in the context of disease. However, in chapters 3 and 5 we show that st-Ht31 increases cytokine release and in chapters 3 and 5 we show that st-Ht31 decreases β-agonist responsiveness. The data indicate that in the ASM st-Ht31 or other non-selective AKAP-PKA disruptors would not be viable drug candidates, perhaps even harmful. By contrast, in airway epithelium we could find beneficial effects of st-Ht31 by promoting epithelial barrier function (10). To prevent side effects, there is a need for more selective compounds, targeting only one compartment. A step towards this aim has been done with the development of the Rslect compounds (55). These peptides, created using a phage display technique are dominant interfering peptides that can remove PKA from selective AKAPs, although thus far only available for AKAP2 and AKAP7 complexes (55).

Besides affecting protein-protein interactions, there is an option to reverse pathological changes in AKAP expression. Although for AKAP5 there is little known about its transcriptional cues, for AKAP12 there are known transcriptional inducers. Most relevant may be glucocorticoids (56). AKAP12 expression can also be pharmacologically induced by COX-2 inhibition (57), lysophosphatidylcholine (58) and angiotensin II (59). Furthermore, AKAP12 mRNA can be induced pharmacologically by the phorbol ester phorbol-12-myristate-13-acetate and by retinoids. The induction by phorbol-12-myristate-13-acetate and retinoids is enhanced by the addition of the protein synthesis inhibitor cycloheximide, suggesting that both mRNA transcription and stability contribute to the steady-state control of mRNA expression (60-64).

In addition to classical pharmacological induction, AKAP12 expression is strongly regulated at an epigenetic level by both DNA methyltransferases and histone deacetylases, although there appears to be an isoform specific regulation at this level.
The AKAP12 gene produces three proteins derived from 2 transcription variants, 305 kDa AKAP12α representing Gravin/SSeCKS and present in human, rat and mouse, 287 kDa AKAP12β, only found in humans, and 250 kDa AKAP12γ, found in human, rat and mouse (65, 66). The lung expresses AKAP12α, AKAP12β but not AKAP12γ (66). AKAP12 is reduced in many cancers (37) and treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC) can restore AKAP12 expression in most cancer cell lines (67, 68), demonstrating the reversible nature of this epigenetic regulation in these cells. In cell lines unable to restore AKAP12 expression after 5-Aza-dC treatment, AKAP12β expression can be restored after treatment with the histone deacetylase inhibitor trichostatin A, whereas restoration of AKAP12α expression required a combination of both 5-Aza-dC and trichostatin A (67). Similar results were found for lung cancers where only 50% of the lung cancer cell lines could restore AKAP12α expression with 5-Aza-dC alone (69, 70). Therefore, it is possible to restore AKAP12 expression, even isoform specifically, although it is currently unclear what the role of the different isoforms is in the lung and how they are regulated.

Although the use of AKAP complex disruptors might still seem far in the future, the knowledge obtained about the protein-protein interactions has been used in drug design. A method of drug design, named the Dock-and-Lock method, uses the dimerization and docking domain found in the regulatory subunit of PKA and the anchoring domain of an interactive AKAP, each attached to a biological entity (Chapter 2) (71). The Dock-and-Lock method created an unlimited amount of combinations to be made by mixing and matching dimerization and docking domains with anchoring domains with different biological entities. The Dock-and-Lock method has subsequently led to several patents in cancer (US 7901680 B2), HIV (US 8481041 B2), siRNA delivery (US 8491914 B2) and several other applications.

From this thesis, the following conclusions can be drawn:

- ASM cells express several members of the scaffolding protein AKAP family, including AKAP5, AKAP12 and Ezrin (Chapter 3). These AKAP family members are known to regulate the recycling of the β2-adrenoceptor, and may therefore be important for airway β2-adrenoceptor functioning (Chapter 2).
- Cigarette smoke reduces AKAP5 and AKAP12 expression in cultured ASM cells, this could contribute to the lower AKAP5 and AKAP12 expression found in lung tissue from COPD patients compared to non-COPD patients (Chapter 3).
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- AKAP-PKA interactions are important to reduce basal and cigarette smoke-induced IL-8 release from ASM cells as well as to mediate the anti-inflammatory effect of β2-agonists hereon (Chapter 3).
- *In vivo*, AKAP12 and AKAP5 control basal and cigarette smoke-induced pulmonary inflammation, respectively, in particular neutrophilia (Chapter 4).
- AKAPs, and specifically AKAP12, mediates a normal β2-agonist-induced ASM relaxation (Chapter 5).
- AKAP12 regulates GRK-mediated phosphorylation of the β2-adrenoceptor in ASM (Chapter 5).
- The AKAP12 gene carries SNPs that associate with β2-agonist-induced improvement of lung function and that affect the amino acid sequence - but not the gene expression - of AKAP12, suggesting that there is a need to study protein-protein interaction domains of AKAP12, such as the β2-adrenoceptor binding domain, more closely in COPD (Chapter 6).

Given the anti-inflammatory roles of AKAP5 and AKAP12, the observed decrease in the expression of AKAP5 and AKAP12 in patients with COPD could contribute to the pulmonary inflammation, particularly neutrophilia, observed in these patients. In addition, since AKAP12 mediates β2-agonist-induced ASM relaxation, loss of AKAP12 in COPD may hamper bronchodilation in response to treatment with β2-agonists. Therefore, therapeutic options that can restore functional AKAP5 and/or AKAP12 complexes could possibly enhance existing pharmacological approaches and decrease disease progression in COPD.
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