

# The role of A-kinase anchoring proteins (AKAPs) in the pathophysiology of chronic obstructive pulmonary disease (COPD)

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## **Abstract**

Chronic obstructive pulmonary disease (COPD) is an increasing global health problem and leading cause of disability and death. The disease is characterised by chronic inflammation due to increased levels of cytokines and chemokines as well as alveolar neutrophils, macrophages and T-lymphocytes. and will result in loss of lung elasticity and mucus hypersecretion. Also, due to exposure to noxious substances, like cigarette smoke extract, levels of reactive oxygen species (ROS) increase inducing damage to lipids, proteins and DNA. These responses originate from signal transduction mechanisms which are controlled by compartmentalization of cAMP-dependent protein kinase (PKA) which are bound to A-kinase anchoring proteins (AKAPs). The highly conserved members of the AKAP ensures spatial-temporal control of important signal transduction mechanisms. Dyregulation of AKAPs is related to a number of disorders like neurodegenerative disease, cardiovascular disease and COPD. This assay will focus on the role of AKAPs in the respiratory tract and the pathophysiology of COPD.

## Abbreviations

<b>AC</b>	Adenylyl cyclase
<b>ATP</b>	Adenosine triphosphate
<b>AKAP</b>	A-kinase anchoring protein
<b>cAMP</b>	Cyclic adenosine 3',5'-monophosphate
<b>COPD</b>	Chronic obstructive pulmonary disease
<b>DAG</b>	Diacylglycerol
<b>Epac</b>	Exchange protein activated by cAMP
<b>ERM</b>	Ezrin/Radixin/Moesin
<b>FEV<sub>1</sub></b>	Exhaled volume in the first second
<b>FVC</b>	Forced vital capacity
<b>GDP</b>	Guanosine diphosphate
<b>GOLD</b>	Global Initiative for Chronic Obstructive Lung Disease
<b>GTP</b>	Guanosine triphosphate
<b>IL-8</b>	Interleukin-8
<b>IP3</b>	Inositol triphosphate
<b>IP-10</b>	Interferon- $\gamma$ inducible protein
<b>I-TAC</b>	Interferon-induced T-cell $\alpha$ -chemo attractant
<b>LABA</b>	Long-acting $\beta$ -agonist
<b>LAMA</b>	Long-acting muscarinic antagonist
<b>LTB<sub>4</sub></b>	Leukotriene B <sub>4</sub>
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MMP</b>	Matrix metalloproteinase
<b>NE</b>	Neutrophil-derived elastolytic protease
<b>NF-<math>\kappa</math>B</b>	Nuclear factor-kappa B
<b>Nrf2</b>	Nuclear factor-erythroid 2-related factor-2
<b>PDE</b>	Phosphodiesterase
<b>PKA</b>	Protein kinase A
<b>PKC</b>	Protein kinase C
<b>PKI</b>	Protein kinase inhibitor
<b>ROS</b>	Reactive oxygen species
<b>TGF-<math>\beta</math></b>	Transforming growth factor- $\beta$

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## I. Introduction

Chronic obstructive pulmonary disease (COPD) is a slowly progressive lung condition and has become a rising global health problem with a high morbidity and mortality rate (Rahman, 2008). Currently, the disease affects more than 5% of the population and it is the fourth leading cause of death. The World Health Organization has announced that the disease will become the third leading cause of deaths by 2020 (Barnes, Shapiro and Pauwels, 2003; Mannino, 2006). COPD is associated with pulmonary obstructive bronchiolitis, emphysema causing loss of lung elasticity, increased mucous secretion, inflammatory components which ultimately lead to tissue damage and elevated levels of oxidative stress (Barnes et al., 2003; Fromer and Cooper, 2016). COPD is mainly caused by a prolonged exposure to noxious gases or particles such as cigarette smoke, the most common cause in well developed countries. Other risk factors are air pollution (indoor biomass fuels), poor diet and occupational exposure. COPD can also be caused by a genetic predisposition, like alpha-1 antitrypsin deficiency. Alpha-1 antitrypsin is a protease inhibitor protecting lung tissue by inactivating neutrophil elastase (Brode et al., 2012). As a result of alpha-1 antitrypsin deficiency some patients start to develop COPD at a young age (Taraseviciene-Stewart and Voelkel, 2008; Brode et al., 2012). For an authoritative diagnosis of COPD patients undergo spirometry testing, a standard instrument which is used for the assessment of the extent of airflow limitation and monitoring the progression of the disorder. During a spirometry test the FEV<sub>1</sub>, exhaled volume in one second, and the forced vital capacity (FVC), maximum volume that can be exhaled after full inhalation, is measured. The ratio of these two measurements (FEV<sub>1</sub>/FVC) provides insight into the lung volume of the patient.

COPD is classified into four stages (GOLD I –IV, mild to very severe). This classification is an initiative of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) started in 2006 and is used worldwide. Currently there are several approaches for the management of COPD. Most important are the long-acting  $\beta$ -agonist (LABA) and the long-acting muscarinic antagonist (LAMA), such as formoterol and salmeterol, used in stage I to stage IV COPD. These bronchodilators reduce symptoms, such as persistent cough and shortness of breath, and provide relaxation of smooth muscles in the airways (Carlin, 2016; Fromer and Cooper, 2016). Corticosteroids are used to reduce exacerbations in COPD stage III and IV but have limited effect on lung function (Barnes, 2005). Phosphodiesterase (PDE) inhibitors are another class of medicine used in the management of COPD. PDE 4 is an important regulator of cyclic adenosine monophosphate (cAMP) metabolism during inflammation. Research has shown that roflumilast, a PDE 4 inhibitor, causes reduction in exacerbations and improvement of FEV<sub>1</sub> (Giembycz, 2001; Rennard et al., 2011).

In the next chapters, this paper will focus on the injury and remodelling that occur in the lungs, A-kinase anchoring proteins (AKAPs) and their involvement in the pathophysiology of COPD.

### **Bronchiolitis and emphysema**

Continuous exposure of noxious substances to the lungs causes a persistent inflammatory reaction. Thereby, cytokines and chemokines are continuously released by the innate and adaptive immune system. These can stimulate epidermal growth factor receptors nearby which will activate transcription factors or inflammatory and mucin genes. By this activation there is an increased release of mucus by goblet cells. Mucus normally ensures clearance of the respiratory tract but during hypersecretion it has opposite effects. Cilia present in the lungs cannot function under excessive mucus production as well as mechanism that normally provide mucus degradation. A very devastating consequence of hypersecretion is the increased degranulation of elastase from neutrophils. These effect lead to persistent cough and chronic bronchiolitis (Burgel, 2004; Kim and Criner, 2015).

Persistent inflammation of the airways leads to disruption and damage of the alveolar structure and will eventually result in emphysema. The activity of protease and anti-protease are of great importance in the formation of emphysema. Proteases and anti-proteases play a major role in the intracellular and extracellular regulation of homeostatic, killing bacteria and regeneration of tissue. However, in case of persistent inflammation the balance between proteases and anti-proteases is disturbed resulting in an increase of proteases which causes damage (Sharafkhaneh et al., 2008; Greene and McElvaney, 2009; Abbout, 2012). An important serine protease in this process is neutrophil-derived elastolytic protease (NE) stored in the granules of neutrophils. NE is released during inflammation and causes degradation of elastin and collagen, matrix proteins important in the maintenance of interstitial tissue and airway structure (Nadel, 2000; Zhu et al., 2001; Dunsmore, 2008). Because of the degradation of elastin, the lungs loses their elasticity which reduces lung capacity and leads to enlargement of the airspace (Starcher, 1986; Taraseviciene-Stewart and Voelkel, 2008). NE and growth factors causes deposition of collagen and creating fibrosis in this way. All these processes result in airflow limitation in COPD.

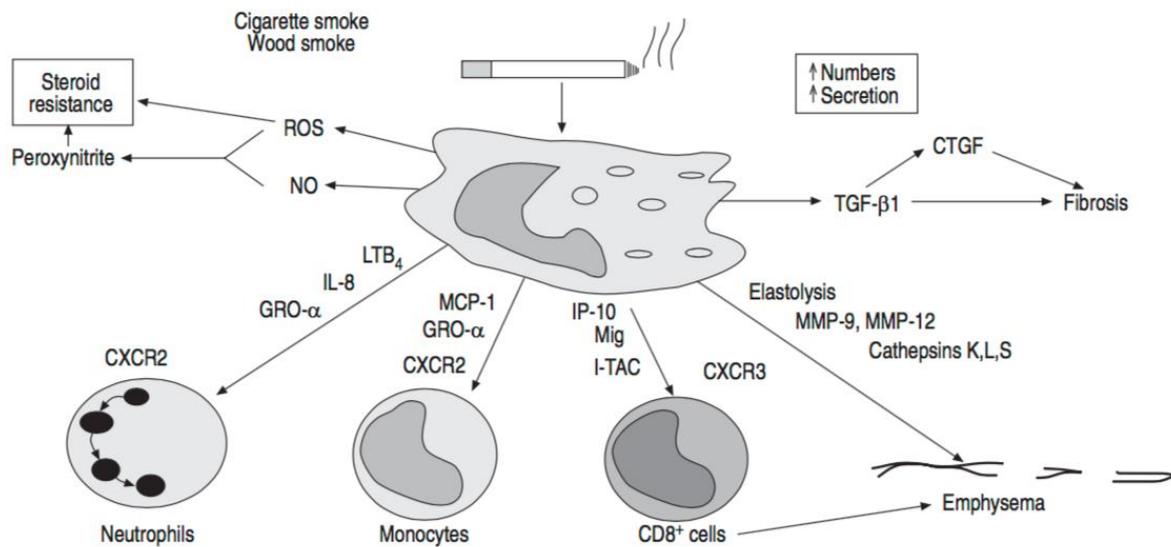
### **Inflammatory cells**

COPD is characterized as an inflammatory disease. Many different inflammatory cells and mediators derived from the innate and adaptive immune system, are involved in the pathophysiology of the disorder (Barnes, 2004; Rovina et al., 2013).

Neutrophils are highly involved in the inflammatory process in COPD and form the first defence in the airways. The cells are present in the bronchioles and parenchyma and are increased in COPD patients. The amount of neutrophils present is correlated to the progression of the disease and the decline of lung function (O'Donnell, 2006). Neutrophils secrete inflammatory cytokines and antimicrobial peptides such as NE, cathepsin G and matrix metalloproteinase (MMP) which contribute to mucus hypersecretion and causes alveolar destruction resulting in emphysema. Neutrophils are recruited by chemotactic signals: leukotriene B<sub>4</sub> (LTB<sub>4</sub>), interleukin-8 (IL-8) and CXC chemokines derived from alveolar macrophages and epithelial cells (Barnes, 2003).

Macrophages are of great importance in the pathophysiology of COPD. They provide the entire inflammatory reaction in response to cigarette smoke extract (**Fig. 1**). Macrophages recruit neutrophils by release of chemotactic signals and they secrete MMP, just like neutrophils, and together they ensure the recruitment of leukocytes to the site of inflammation (O'Donnell, 2006). Through the secretion of interferon- $\gamma$  inducible protein (IP-10) and interferon-inducible T-cell  $\alpha$ -

chemo attractant (I-TAC), CD8<sup>+</sup> T cells are attracted which contributes to emphysema. Also, the amount of macrophages correlates to the damage inflicted on parenchyma (Barnes, 2003).



**Figure 1** Macrophages provide inflammatory reaction in response to cigarette smoke. By the release of chemotactic signals such as interleukin-8 (IL-8) and leukotrien B<sub>4</sub> (LTB<sub>4</sub>) macrophages recruit neutrophils to the site of inflammation. Also through the secretion of interferon-γ inducible protein (IP-10) and interferon-inducible T-cell α-chemo attractant (I-TAC) CD8<sup>+</sup> cells are attracted which ultimately contribute to emphysema. Other cells are also recruited in response to signals. Oxidative stress due to inflammatory response increases reactive oxygen species (ROS) production. This triggers increased transcription of inflammatory genes (Barnes et al., 2003).

By an increased expression of IP-10 by epithelial cells in the respiratory tract, the number of T-lymphocytes raises, in particular CD8<sup>+</sup> compared with CD4<sup>+</sup>. CD8<sup>+</sup> cells cause destruction of lung parenchyma through the release of perforin and granzymes which leads to apoptosis or necrosis. Research showed that CD8<sup>+</sup> cells are increased in patients with COPD which do not smoke (Chung and Adcock, 2008; Freeman et al., 2010). This proves that chronic inflammation characteristic for COPD is continuously stimulated. The moderate elevated levels of CD4<sup>+</sup> cells consist of two types of cells, Th1 and Th17. Interferon γ is secreted by Th1 which ensures migration of leukocytes to the site of inflammation and promotes NK cells activity. Th17 cell secrete IL-17A and IL-17F that promote inflammation (Schroder et al., 2003).

The exact function of eosinophils is still unknown. As with asthma, eosinophils are increased in the airway of patients with COPD. However, it is known that these cells contribute to the production of reactive oxygen species (ROS) (Barnes, 20003; Chung and Adcock, 2008). The same applies to mast cells which are also increased in the airways of COPD patient but their function is unknown (Chung and Adcock, 2008).

### Oxidative stress

The lungs are continuously in contact with the external environment. Noxious substances or particles as well as bacteria and viruses can easily enter the airways which makes the lungs very vulnerable. Exposure to air pollutants but also the immune response of leukocytes and macrophages to bacteria en viruses increases oxidative stress in the airways (Langen et al, 2003; Kirkham and Barnes, 2013). Because of oxidative stress there is an increased attendance of exogenous ROS, free radicals derived from oxygen like superoxide anion ( $\cdot\text{O}_2^-$ ), hydroxyl radical ( $\cdot\text{OH}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and

singlet oxygen ( $O_2$ ) (Sharma et al., 2012). In addition, there is always endogenous ROS present derived from mitochondrial respiration. Elevated levels of ROS can cause damage to lipids, proteins and DNA (van Eeden and Sin, 2008). Because of the constant exposure to endogenous and exogenous sources of oxidative stress the lungs has expand anti-oxidative defence mechanisms that neutralize ROS. This anti-oxidative mechanisms consist of enzymatic and non-enzymatic components, including superoxide dismutase, enzymes of ascorbate-glutathione cycle, guaiacol peroxidase, carotenoid, etc. (Sharma et al., 2002). In patients with COPD the production of endogenous ROS by smooth muscle cells and the exogenous ROS are substantially increased. This is mainly the result of cigarette smoke which causes oxidative stress (Kohen and Nyska, 2002). Cigarette smoke contains a very high concentration of oxidants and free radicals and includes between 4000 and 4700 different chemicals (van Eeden and Sin, 2008; Langen et al, 2003).

ROS has a great effect on the respiratory system because it allows the release of cytokines and chemokines which activate molecular complexes. A well-known complex is nuclear factor-kappa B (NF- $\kappa$ B), a transcriptional factor. NF- $\kappa$ B activates a number of inflammatory genes which results in an immune response. In addition, ROS also activates a number of enzymes such as histone acetyltransferase, which alters histones resulting in an increased transcription of inflammatory genes, and transforming growth factor- $\beta$  (TGF- $\beta$ ). TGF- $\beta$  decreases the action of the anti-oxidative mechanism of superoxide dismutase whereby ROS can overcome this defence mechanism. Research has shown that the nuclear factor-erythroid 2-related factor-2 (Nrf2), a transcription factor that control antioxidant mechanisms, is decreased in patients with COPD and this is likely to be suppressed by similar mechanisms as TGF- $\beta$  (Barnes, et al., 2003; Kirkham and Barnes, 2013). Therefore, oxidative stress plays a major role in the pathophysiology of COPD.

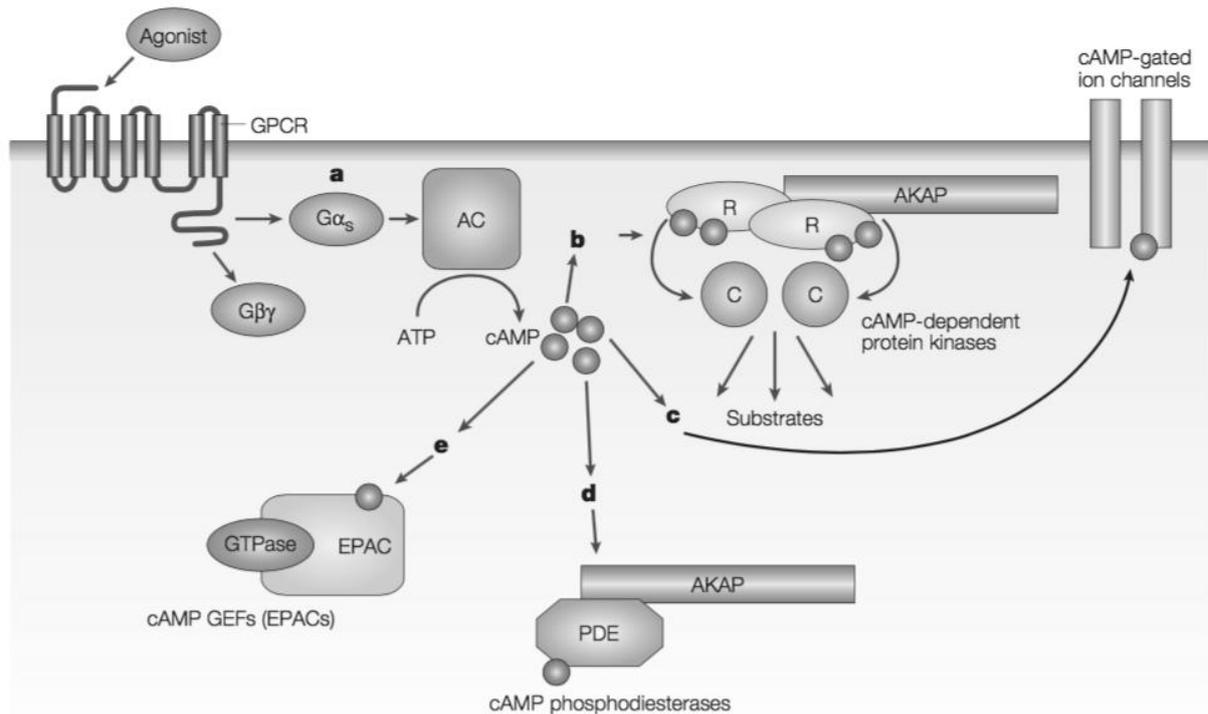
## II. A-kinase anchoring proteins

Numerous processes take place inside the cell which are crucial for survival such as proliferation, differentiation, metabolism and gene expression. Many of these processes are driven by extracellular signals like neurotransmitters and hormones. These signals are passed to intracellular transduction mechanisms via receptors. These transduction mechanisms are of great importance because the cell can engender an appropriate physiological response to the changing external environment. This response is partly achieved by compartmentalization of intracellular effectors by adapting of anchoring proteins, as will be described in this chapter.

### Cyclic adenosine monophosphate and protein kinase A

The first discovered and already more than 40 years familiar intracellular signal is the second messenger cyclic adenosine 3',5'-monophosphate (cAMP). The cAMP signal transduction mechanism is activated by binding of an extracellular ligand to a G protein-coupled receptor (GPCR) on a target cell causing conformational change (**Fig. 2**) (Chin et al., 2002). The intracellular heterotrimeric G protein (consisting of  $G\alpha$ ,  $G\beta$  and  $G\gamma$  subunits) which is attached to the receptor, exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP). As a result the G protein is activated and the  $G\alpha$  subunit dissociates from the  $G\beta\gamma$  subunit (Alto and Scott, 2004). The  $G\alpha$  (in this case  $G_s\alpha$ ) subunit interacts which ensures activation of adenylyl cyclase (AC), an enzyme that catalyses the turnover of adenosine triphosphate (ATP) to cAMP (Gloerich and Bos, 2010; Perion et al., 2012). cAMP is regulated by a balance between two types of enzymes, AC and phosphodiesterase (PDE) which catalyse the conversion of cAMP to 5'-AMP (Sassone-Corsi and Fimia, 2001; Pidoux and Tasken, 2010).

cAMP achieves its effect by binding to protein kinase A (PKA), but can also bind to exchange protein activated by cAMP (Epac), cyclic-nucleotide gated ion channels or PDEs, which ensures cAMP degradation (Sassone-Corsi and Fimia, 2001; Gloerich and Bos, 2010). PKA, also known as cAMP-dependent protein kinase, is a hetero tetramer composed of two catalytic (C) subunits, encoded by three genes ( $C\alpha$ ,  $C\beta$  and  $C\gamma$ ), and two regulatory (R) subunits, encoded by four genes ( $R1\alpha$ ,  $R1\beta$ ,  $R11\alpha$  and  $R11\beta$ ) (Sassone-Corsi and Fimia, 2001; Carnegie et al., 2009). Two types of PKA are known namely type I ( $R1\alpha$  and  $R1\beta$  dimer), which is mainly cytoplasmic, and type II ( $R11\alpha$  and  $R11\beta$  dimer) by which the sub cellular location is determined by an anchoring protein family (Alto and Scott, 2004; Di Benedetto et al., 2008; Scott, 1991). Normally, PKA is in an inactive state achieved by the association of the C and R subunit. Activation occurs by dissociation of these two subunits by binding of cAMP to the R subunit. The activity of PKA is administered by protein kinase inhibitors (PKIs), which phosphorylate serine and threonine residues and in this way regulating biological processes. The cAMP signalling transduction cascade via PKA is also controlled by another group of proteins called A-kinase anchoring proteins (AKAPs). AKAPs are complex but well-organized molecular complexes which determine the precise spatial-temporal signalling of the transduction mechanism after activation of GPCR (Pawson, 2003; Rababa'h et al., 2014).

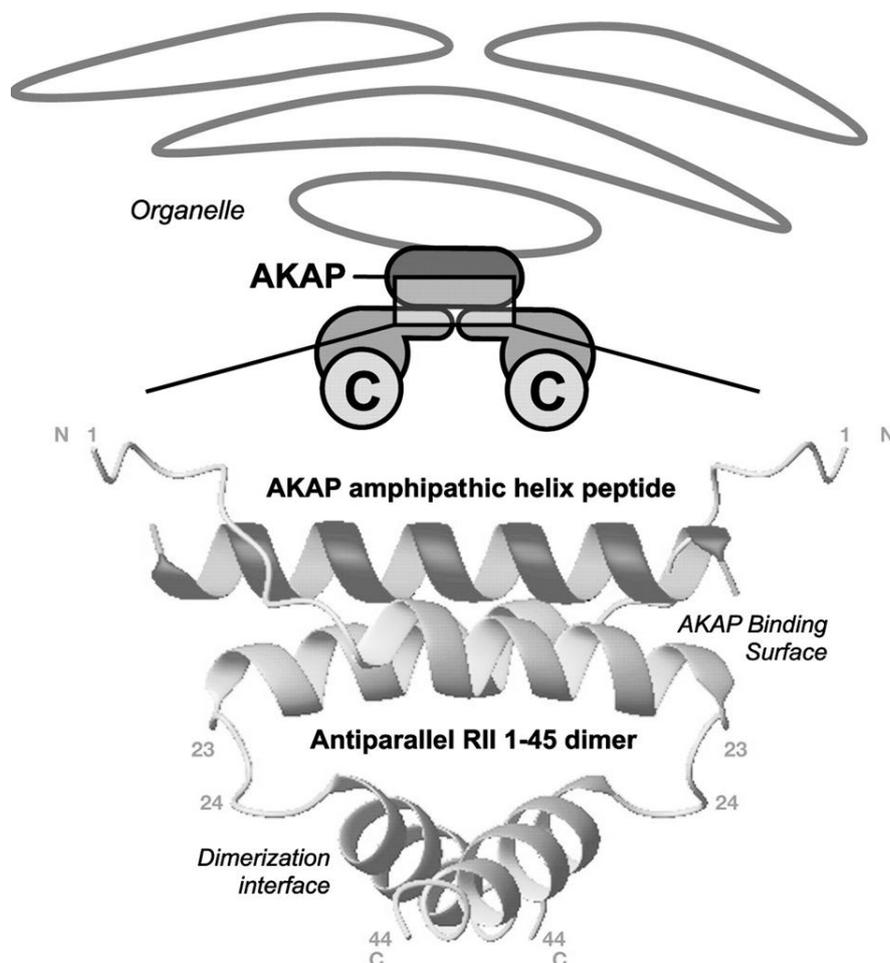


**Figure 2** The cAMP signal transduction mechanism. The mechanism is activated by an (a) extracellular ligand binding to G $\alpha_s$  G-protein coupled receptor (GPCR). This results in a conformation change and leads to the dissociation of the G $\alpha$  subunit from the G $\beta\gamma$  subunit. G $\alpha$  stimulates the conversion of adenosine triphosphate (ATP) to cyclic adenosine 3', 5'-monophosphate (cAMP) by adenylyl cyclase (AC). cAMP can bind to (b) protein kinase A (PKA) and are controlled by A-kinase anchoring proteins (AKAPs). Cyclic-nucleotide gated ion channels (c) can also be activated by cAMP, just like (d) phosphodiesterase (PDE) and (e) exchange proteins activated by cAMP (EPACs) (Wong and Scott, 2004).

### Regulatory role of A-kinase anchoring proteins

The specificity of intracellular signalling transduction cascade is controlled by the highly conserved AKAP family. AKAPs coordinate and regulate the compartmentalization of cAMP, phosphorylation and dephosphorylation and the gathering of signal transduction mechanism and their appropriate substrates by providing a framework (Wong and Scott, 2004). The highly conserved AKAP family has more than 50 members which all have their own function but have one thing in common, binding to PKA (Carnegie et al., 2009; Pidoux and Tasken, 2010). The R subunit of type II PKA provides a protein-protein interaction with AKAP and phosphorylation of substrates is arranged by the C subunit (**Fig. 3**). A special region on the N-terminal of the R subunit of type II PKA allows high affinity interaction. This region is known as the D/D domain because it is responsible for docking and dimerization of AKAP. The D/D domain comprises an anti-parallel dimeric X-type four-helix and allow docking with AKAP (Newlon, et al., 2001). AKAPs in turn can bind to an amphipathic helix of 14-18 amino acids (Carlisle and Scott, 2002). This amphipathic helix consists of hydrophobic residues, covered on one side of the helix, and charged residues covered on the other side of the helix. This helix structure is found in nearly all AKAPs (Pidoux and Tasken, 2010; Newlon, et al., 2001). All AKAPs so far known bind to the RII subunit of PKA but there are some exceptions. Some AKAPs can bind both RI and RII subunits (7).

AKAPs are present in different cellular compartments such as plasma membranes, mitochondria, centrosomes, cytoskeleton, endoplasmic reticulum, microtubules, Golgi apparatus, etc. (Pidoux and Tasken, 2010; Wong and Scott, 2004). AKAPs have the ability to form complexes with PKA and recruit enzymes nearby potential substrates. This will link upstream activators with downstream targets. The recruited enzymes will ensure signal transduction but also signal termination (Alto and Scott, 2004; Carlisle and Scott, 2002). Because of this AKAPs form a framework that can coordinate various signalling pathways and cellular locations. Different AKAPs achieve different responses for example AKAP5 (also known as AKAP79 in humans and AKAP150 in rodents) is essential for switching the  $\beta_2$ -adrenoceptor from  $G_s$  to  $G_i$  and thereby activating the mitogen-activated protein kinase (MAPK) pathway (Zhang et al., 2013). Another example is AKAP95 which controls chromosome condensation and is located at chromatin during mitosis and bound to the nuclear matrix during interphase (Le Guellec et al., 1999; Chen et al., 2016).



**Figure 3** Binding of the PKA holoenzyme to A-kinase anchoring protein (AKAP). AKAP bound to the PKA anchored to subcellular targeting domain. AKAP consist of a C and N terminus. The RII dimer of PKA consist of RII $\alpha$  and RII $\beta$  subunits. The C subunit of PKA phosphorylates substrates (Pidoux and Aandahl, 2003).

### III. AKAPs in the airways

Several AKAPs are expressed in the airway smooth muscle. The functional role of AKAPs in the airways and the lung disease COPD is not completely known. Therefore, we review the role and function of smooth muscle cells such as signalling, contraction, inflammation and remodelling and the involved AKAPs.

#### AKAP signalling

Important AKAPs in the signalling of airway smooth muscle cells are AKAP5 (AKAP79/150) and AKAP12 (Gravin). AKAP5 and AKAP12 share many common features in downstream signalling such as binding to type II PKA and protein phosphatase-2B. (Tao and Malbon, 2008). But most important is the interaction with the prototypic GPCR, the  $\beta_2$ -adrenoceptor (Fraser et al., 2000; Gao et al., 2011). When AKAP5 binds to the  $\beta_2$ -adrenoceptor,  $G_s$  will be activated. AKAP5 however, has the ability to change the protein  $G_s$  to  $G_i$  (Poppinga et al., 2014). This process is facilitated by PKA-mediated phosphorylation of the  $\beta_2$ -adrenoceptor.  $G_s$  normally ensures the activation of AC but due the switch  $G_i$  activates the MAPK (ERK) pathway. This pathway is involved in cell proliferation, differentiation, apoptosis, transformation, survival and gene expression (McCubrey et al., 2007; Kim and Choi, 2010). Also, the pathway is responsible for the activation of cytokine production (Scherle et al., 1998). AKAP5 has much influence on the  $\beta_2$ -adrenoceptor because it provides desensitization and internalization of the receptor. In this way, AKAP5 can regulate the precise expression of the receptor. This process occurs through the phosphorylation of the  $\beta_2$ -adrenoceptor by G protein-coupled receptor kinase-2 (GRK2) whereby the  $\beta\gamma$  subunit, like  $\beta$ -arrestin, is able to bind with high affinity which result in desensitization. Internalization of the receptor is a slower process and goes via endocytosis (Chen and Malbon, 2009). After desensitization of the receptor it is essential that receptor is expressed on the cell membrane again. AKAP12 ensures resensitisation, dephosphorylation and recycling of the  $\beta_2$ -adrenoceptor (Luttrell and Lefkowitz, 2002; Oldenburger et al., 2012). It is therefore important that AKAP5 and AKAP12 closely collaborate regarding to the signalling via the  $\beta_2$ -adrenoceptor important for downstream interactions such as smooth muscle relaxation and bronchodilation.

#### Contractile function of smooth muscle cells

A number of enzymes are important in the contraction of smooth muscle in the respiratory tract. Contraction occur in response to elevated cytosolic calcium ( $Ca^{2+}$ ) concentrations. By binding to a serpentine receptor, phospholipase C is activated. This enzyme ensures catalysis which result in the production of two second messengers, namely diacylglycerol (DAG) and inositol triphosphate (IP3). DAG is responsible for the activation of protein kinase C (PKC) which in turn can exert different biological functions such as receptor desensitization, cell growth, participation in immune responses, etc. (Choi et al., 2009). IP3 causes release of  $Ca^{2+}$  by binding to specific receptors present on the sarcoplasmic reticulum. The released  $Ca^{2+}$  binds to the intracellular  $Ca^{2+}$  receptor protein, calmodulin (CaM) ensures activation of myosin light-chain kinase (MLC kinase) and results in contraction of smooth muscle (Lincoln, 2007).

Smooth muscle relaxation is regulated by MLC phosphatase, together with a reduced level of cytosolic  $Ca^{2+}$ . Rho kinase, a serine/threonine kinase, is of great importance in the regulation of MLC phosphatase and is activated by RhoA. Rho kinase ensures phosphorylation of the myosin-binding subunit of MLC phosphatase (Wilson et al., 2001; Webb, 2003). The protein Epac also interact with this pathway. Epac binds to Rap1 which ensures the inhibition of RhoA and ultimately results in smooth muscle relaxation (Poppinga et al., 2014).

Ezrin (AKAP78) plays a central role in smooth muscle relaxation. Ezrin is part of the Ezrin/Radixin/Moesin (ERM) protein family and are cross link actin filaments associated with the plasma membrane. Rho-regulated Rho-kinase establish phosphorylation of ERM (Matsui et al., 1998). Because Epac and Ezrin are both involved in this process en also both found in smooth muscle, is it suggested that inhibition of RhoA by Epac is affected by Ezrin (Poppinga et al., 2014).

### **Inflammation and remodelling**

As described earlier, COPD is characterized as an inflammatory disease. Many chemokines and cytokines are released in response to cigarettes smoke extract. A cytokine that plays an important role is IL-8. IL-8 is elevated in the airways of patients with COPD and is release by smooth muscle cells in response to  $G_q$  protein. However, research has shown that PKA and Epac can reduce IL-8 in the airways. This is achieved through inhibition of ERK signalling which is influenced by a balance between AKAP5 and AKAP12 and also through the inhibition of NF- $\kappa$ B. Disruption of PKA-AKAP interaction by Ht31, a protein kinase A anchoring inhibitor, clearly display that PKA and Epac plays an important role in mediating the anti-inflammatory processes which are under mediation of AKAPs (Poppinga et al., 2014; Poppinga et al., 2015).

Chronic inflammation, emphysema and mucus hypersecretion are known to contribute to structural changes in lung tissue and airway remodelling, a major feature seen in patient with COPD (Dournes and Laurent, 2012; Grzela, 2015). There are a numbers of known pathways which lead to changes in structures of lung tissues such as the MAPK (ERK) pathway and phosphoinositide 3-kinase (PI3K) pathway. This pathway regulates cell proliferation and differentiation like MAPK (ERK) (Lui et al., 2009). PKA and Epac, which are fine-tuned and controlled by the AKAP family, have great influence on these two pathways and presumably also in airway remodelling. However, the exact mechanism and role of AKAP family mediated remodelling by PKA and Epac is not clear (Poppinga et al., 2014).

### **AKAP in relation to disease**

AKAPs act as spatial-temporal signalling, such as the compartmentalization of cAMP and associated transduction mechanisms like cell proliferation, differentiation, gene targeting, etc. It is therefore essential that these processes are precisely controlled. Disturbance of AKAPs function is associated with various disorders such as neurodegenerative diseases, cardiovascular disorders, and various types of cancers as well as lung disease such as COPD (Tröger et al., 2012; Smith et al., 2013).

Exposure of airway smooth muscle cells to cigarette smoke, the main cause of COPD, affects the expression of several AKAPs. mRNA levels of AKAP9 are decreased in lung tissue. AKAP9 interacts with E-cadherin. E-cadherin is an important transmembrane glycoprotein and found specific in cell-cell adhesion on smooth muscle cells (Tunggal et al., 2005; Van Roy and Berx, 2008). Down-regulation of AKAP9 by cigarette smoke reduces also E-cadherin. This ultimately results in dysfunction of the epithelial barrier. Also, mRNA levels of AKAP5 and AKAP12 are decreased in

response to cigarette smoke and will result ultimately in inflammation (Poppinga et al., 2014; Oldenburger et al., 2014; Poppinga et al., 2015). Taken together, dysregulation of AKAPs due to cigarette smoke extract are responsible for the pathophysiology of COPD.

## IV. Outlook

cAMP is an intracellular messenger which regulates important cellular and biological processes such as cell proliferation and differentiation, gene transcription, etc.. The specificity of intracellular signalling transduction mechanisms is controlled by the AKAP family. AKAPs act as spatial-temporal signalling by compartmentalization of cAMP (McConnachie et al., 2006). Disruption or malfunction of AKAPs is related to a number of disorders such as heart diseases, immune diseases, neurodegenerative disease and COPD (Zaccolo, 2011).

It is commonly known that average age of people increases and this will only increase over the years. With this increasing age, the number of chronic diseases will also raise. Therefore it is crucial to determine and understand the mechanisms that drives these diseases so it can be managed better. AKAPs play a major role in the pathophysiology of COPD but also in other chronic disease. Properly management of diseases by specific drugs, perhaps targeting of AKAPs, is of social importance.

There are many different types of medication for COPD patients such as anticholinergics,  $\beta_2$  agonisten, corticosteroids and combination therapie. However, these drugs are not always effective (Grimes et al., 2007). AKAPs are involved in different processes and are dysregulated in disease which makes them potential drug targets. A few experimental drugs are used to disrupt AKAP-PKA interactions such as peptides derived from PKA-binding domains and FMP-API-1 which are small disruption molecules. However these drugs lack selectivity (Tröger et al., 2012; Esseltine and Scott, 2013). One way to obtain selectivity for AKAP-PKA interaction might be via nanotechnology. Research has shown that nanoparticles act more efficiently than molecules and therefore these particles would be able to perform as effective drug delivery systems. However, for precise effect of nanosystems, AKAP-PKA interaction and there corresponding interactions in relation to cellular processes will need to be exactly indentified (Suri et al., 2007). Perhaps in the future, targeting AKAP-PKA interaction by nanosytems is an effective way for the management of COPD and because dysregulation occurs not only in COPD, nanosystems can serve as a broad spectrum.

So far medication only focus on disruption of AKAP-PKA interactions. But there is another important protein present in various biological and cellular processes which is also under spatial-temporal control of the AKAP family and that is Epac. Therefore, it is important not only to focus on pharmacological induced disruption of AKAP-PKA interaction but also on AKAP-Epac interactions (Cheng et al., 2008). In addition, cAMP is also of great interest in the previously mentioned processes. Increased concentrations of cAMP proved activation of PKA but excessively elevated levels of cAMP in the cytosol can lead to an increased activion of NF-kB resulting in hyper inflammation (Monterisi et al., 2012). Therefore it is of interest to pharmacological manage the components, cAMP, Epac and PKA, that are controlled by the AKAP family and contribute to the pathophysiology of COPD.

## V. References

- Abboud, T.** (2012). Pathogenic Mechanisms in Emphysema: From Protease Anti-Protease Imbalance to Apoptosis. INTEC\*H Open Access Publisher.
- Alto, N. and Scott, J.** (2004). The Role of A-Kinase Anchoring Proteins in cAMP-Mediated Signal Transduction Pathways. *CBB*, 40(3S), pp.201-208.
- Barnes, P.** (2004). Mediators of Chronic Obstructive Pulmonary Disease. *Pharmacological Reviews*, 56(4), pp.515-548.
- Barnes, P.** (2005). COPD: current therapeutic interventions and future approaches. *European Respiratory Journal*, 25(6), pp.1084-1106.
- Barnes, P., Shapiro, S. and Pauwels, R.** (2003). Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J*, 22(4), pp.672-688.
- Brode, S., Ling, S. and Chapman, K.** (2012). Alpha-1 antitrypsin deficiency: a commonly overlooked cause of lung disease. *Canadian Medical Association Journal*, 184(12), pp.1365-1371.
- Burgel, P.** (2004). Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. *Thorax*, 59(11), pp.992-996.
- Carlin, B.** (2012). COPD and Associated Comorbidities: A Review of Current Diagnosis and Treatment. *Postgraduate Medicine*, 124(4), pp.225-240.
- Carlisle, J., Scott, J.** (2002). AKAP mediated signal transduction. *Review of Pharmacology and Toxicology*. 42, pp 235-257.
- Carnegie, G., Means, C. and Scott, J.** (2009). A-kinase anchoring proteins: From protein complexes to physiology and disease. *IUBMB Life*, 61(4), pp.394-406.
- Chen, M. and Malbon, C.** (2009). G-protein-coupled receptor-associated A-kinase anchoring proteins AKAP5 and AKAP12: Differential trafficking and distribution. *Cellular Signalling*, 21(1), pp.136-142.
- Chen, X., Kong, X., Zhuang, W., Teng, B., Yu, X., Hua, S., Wang, S., Liang, F., Ma, D., Zhang, S., Zou, X., Dai, Y., Yang, W. and Zhang, Y.** (2016). Dynamic changes in protein interaction between AKAP95 and Cx43 during cell cycle progression of A549 cells. *Sci. Rep.*, 6, p.21224.
- Cheng, X., Ji, Z., Tsalkova, T. and Mei, F.** (2008). Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochimica et Biophysica Sinica*, 40(7), pp.651-662.
- Chin, K., Yang, W., Ravatn, R., Kita, T., Reitman, E., Vettori, D., Cvijic, M., Shin, M. And Iacono, L.** (2002). Reinventing the Wheel of Cyclic AMP. *Annals of the New York Academy of Sciences*, 968(1), pp.49-64.
- Choi, H., Allahdadi, K., Tostes, R. and Webb, R.** (2009). Diacylglycerol Kinase Inhibition and Vascular Function. *Current Enzyme Inhibition*, 5(3), pp.148-152.
- Chung, K. and Adcock, I.** (2008). Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *European Respiratory Journal*, 31(6), pp.1334-1356.
- David M Mannino, V.** (2006). Changing the burden of COPD mortality. *International Journal of Chronic Obstructive Pulmonary Disease*, 1(3), p.219.
- Di Benedetto, G., Zoccarato, A., Lissandron, V., Terrin, A., Li, X., Houslay, M., Baillie, G. and Zaccolo, M.** (2008). Protein Kinase A Type I and Type II Define Distinct Intracellular Signalling Compartments. *Circulation Research*, 103(8), pp.836-844.
- Dournes, G. and Laurent, F.** (2012). Airway Remodelling in Asthma and COPD: Findings, Similarities, and Differences Using Quantitative CT. *Pulmonary Medicine*, 2012, pp.1-8.

- Dunsmore, S. E.** (2008). Treatment of COPD: A matrix perspective. *International Journal of Chronic Obstructive Pulmonary Disease*, 3(1), 113–122.
- van Eeden, S. and Sin, D.** (2008). Chronic Obstructive Pulmonary Disease: A Chronic Systemic Inflammatory Disease. *Respiration*, 75(2), pp.224-238.
- Esseltine, J. and Scott, J.** (2013). AKAP signaling complexes: pointing towards the next generation of therapeutic targets?. *Trends in Pharmacological Sciences*, 34(12), pp.648-655.
- Fraser, I., Cong, M., Kim, J., Rollins, E., Daaka, Y., Lefkowitz, R. and Scott, J.** (2000). Assembly of an A kinase-anchoring protein– $\beta$ 2-adrenergic receptor complex facilitates receptor phosphorylation and signaling. *Current Biology*, 10(7), pp.409-412.
- Freeman, C., Han, M., Martinez, F., Murray, S., Liu, L., Chensue, S., Polak, T., Sonstein, J., Todt, J., Ames, T., Arenberg, D., Meldrum, C., Getty, C., McCloskey, L. and Curtis, J.** (2010). Cytotoxic Potential of Lung CD8+ T Cells Increases with Chronic Obstructive Pulmonary Disease Severity and with In Vitro Stimulation by IL-18 or IL-15. *The Journal of Immunology*, 184(11), pp.6504-6513.
- Fromer, L. and Cooper, C.** (2008). A review of the GOLD guidelines for the diagnosis and treatment of patients with COPD. *International Journal of Clinical Practice*, 62(8), pp.1219-1236.
- Gao, S., Wang, H. and Malbon, C.** (2011). AKAP5 and AKAP12 Form Homo-oligomers. *Journal of Molecular Signaling*, 6, p.3.
- Giembycz, M.** (2001). Cilomilast: a second generation phosphodiesterase 4 inhibitor for asthma and chronic obstructive pulmonary disease. *Expert Opinion on Investigational Drugs*, 10(7), pp.1361-1379.
- Gloerich, M. and Bos, J.** (2010). Epac: Defining a New Mechanism for cAMP Action. *Annu. Rev. Pharmacol. Toxicol.*, 50(1), pp.355-375.
- Greene, C. M., & McElvaney, N. G.** (2009). Proteases and antiproteases in chronic neutrophilic lung disease – relevance to drug discovery. *British Journal of Pharmacology*, 158(4), 1048–1058.
- Grimes, G., Manning, J., Patel, P., Marc, R.** (2007). Medication for COPD: A Review of Effectiveness. *Am Fam Physician*, 76(8), pp.1141-1148.
- Grzela, K., Litwiniuk, M., Zagorska, W. and Grzela, T.** (2015). Airway Remodeling in Chronic Obstructive Pulmonary Disease and Asthma: the Role of Matrix Metalloproteinase-9. *Arch. Immunol. Ther. Exp.*, 64(1), pp.47-55.
- Kim, E. and Choi, E.** (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1802(4), pp.396-405.
- Kim, V. and Criner, G.** (2015). The chronic bronchitis phenotype in chronic obstructive pulmonary disease. *Current Opinion in Pulmonary Medicine*, 21(2), pp.133-141.
- Kirkham, P. and Barnes, P.** (2013). Oxidative Stress in COPD. *Chest*, 144(1), pp.266-273.
- Kohen, R. and Nyska, A.** (2002). Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. *Toxicologic Pathology*, 30(6), pp.620-650.
- Langen, R., Korn, S., Wouters, E.** (2003). ROS in the local and systemic pathogenesis of COPD. *Free Radical Biology and Medicine*, 35(3), pp.226-235.
- Le Guellec, K., Tasken, K.** (1999). The A-Kinase-Anchoring Protein Akap95 Is a Multivalent Protein with a Key Role in Chromatin Condensation at Mitosis. *The Journal of Cell Biology*, 147 (6), pp.1167-1180.
- Lincoln, T.** (2007). Myosin Phosphatase Regulatory Pathways: Different Functions or Redundant Functions?. *Circulation Research*, 100(1), pp.10-12.
- Liu, P., Cheng, H., Roberts, T. and Zhao, J.** (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. *Nature Reviews Drug Discovery*, 8(8), pp.627-644.
- Luttrell, L., Lefkowitz, R.** (2002) The role of  $\beta$ -arrestins in the termination and transduction of G-protein-coupled receptor signals. *Journal of Cell Science*, 115, pp.455-465.

- Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S. and Tsukita, S.** (1998). Rho-Kinase Phosphorylates COOH-terminal Threonines of Ezrin/Radixin/Moesin (ERM) Proteins and Regulates Their Head-to-Tail Association. *J Cell Biol*, 140(3), pp.647-657.
- McConnachie, G., Langeberg, L. and Scott, J.** (2006). AKAP signaling complexes: getting to the heart of the matter. *Trends in Molecular Medicine*, 12(7), pp.317-323.
- McCubrey, J., Steelman, L., Chappell, W., Abrams, S., Wong, E., Chang, F., Lehmann, B., Terrian, D., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. and Franklin, R.** (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1773(8), pp.1263-1284.
- Monterisi, S., Favia, M., Guerra, L., Cardone, R., Marzulli, D., Reshkin, S., Casavola, V. and Zaccolo, M.** (2012). CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity. *Journal of Cell Science*, 125(5), pp.1106-1117.
- Nadel, J.** (2000). Role of Neutrophil Elastase in Hypersecretion During COPD Exacerbations, and Proposed Therapies. *Chest*, 117(5), pp.386-389.
- Newlon, M., Roy, M., Morikis, D., Carr, D., Westphal, R., Scott, J., Jennings, P.** (2001). A novel mechanism of PKA anchoring revealed by solution structures of anchoring complexes. *The EMBO Journal*, 20(7), pp.1651-1662.
- O'Donnell, R., Breen, D., Wilson, S., Djukanovic, R.** (2006). Inflammatory cells in the airways in COPD. *Thorax*, 61(5), pp.448-454.
- Oldenburger, A., Maarsingh, H. and Schmidt, M.** (2012). Multiple Facets of cAMP Signalling and Physiological Impact: cAMP Compartmentalization in the Lung. *Pharmaceuticals*, 5(12), pp.1291-1331.
- Oldenburger, A., Poppinga, W., Kos, F., de Bruin, H., Rijks, W., Heijink, I., Timens, W., Meurs, H., Maarsingh, H., Schmidt, M.** (2014). A-kinase anchoring proteins contribute to loss of E-cadherin and bronchial epithelial barrier by cigarette smoke. *Am J Physiol Lung Cell Mol Physiol*, 306, pp.C585-C597.
- Pawson, T.** (2003). Assembly of Cell Regulatory Systems Through Protein Interaction Domains. *Science*, 300(5618), pp.445-452.
- Perino, A., Ghigo, A., Scott, J. and Hirsch, E.** (2012). Anchoring Proteins as Regulators of Signaling Pathways. *Circulation Research*, 111(4), pp.482-492.
- Pidoux, G. and Tasken, K.** (2010). Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins. *Journal of Molecular Endocrinology*, 44(5), pp.271-284.
- Poppinga, W., Heijink, I., Holtzer, L., Skroblin, P., Klussmann, E., Halayko, A., Timens, W., Maarsingh, H. and Schmidt, M.** (2015). A-kinase-anchoring proteins coordinate inflammatory responses to cigarette smoke in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*, 308(8), pp.L766-L775.
- Poppinga, W., Muñoz-Llanca, P., González-Billault, C. and Schmidt, M.** (2014). A-kinase anchoring proteins: cAMP compartmentalization in neurodegenerative and obstructive pulmonary diseases. *British Journal of Pharmacology*, 171(24), pp.5603-5623.
- Rababa'h, A., Singh, S., Suryavanshi, S., Altarabsheh, S., Deo, S. and McConnell, B.** (2014). Compartmentalization Role of A-Kinase Anchoring Proteins (AKAPs) in Mediating Protein Kinase A (PKA) Signaling and Cardiomyocyte Hypertrophy. *IJMS*, 16(1), pp.218-229.
- Rahman, I.** (2008). Review: Antioxidant therapeutic advances in COPD. *Therapeutic Advances in Respiratory Disease*, 2(6), pp.351-374.

- Rennard, S., Calverley, P., Goehring, U., Bredenbröcker, D. and Martinez, F.** (2011). Reduction of exacerbations by the PDE4 inhibitor roflumilast - the importance of defining different subsets of patients with COPD. *Respiratory Research*, 12(1), pp.18.
- Rovina, N., Koutsoukou, A. and Koulouris, N.** (2013). Inflammation and Immune Response in COPD: Where Do We Stand?. *Mediators of Inflammation*, 2013, pp.1-9.
- Sassone-Corsi, P. and Fimia, G.** (2001). Cyclic AMP signalling. *Journal of Cell Science*, 114, pp. 1971-1972.
- Scherle, P., Jones, E., Favata, M., Daulerio, A., Covington, M., Nurnberg, S., Magolda, R., Trzaskos, J.** (1998). Inhibition of MAP Kinase Kinase Prevents Cytokine and Prostaglandin E<sub>2</sub> Production in Lipopolysaccharide-Stimulated Monocytes. *The Journal of Immunology*, 161(10), pp.5681-5686.
- Schroder, K., Hertzog, P., Ravasi, T., Hume, D.** (2003). Interferon- $\gamma$ : an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*, 75(2), pp.163-189.
- Scott, J.** (1991). Cyclic nucleotide-dependent protein kinases. *Pharmacology & Therapeutics*, 50(1), pp.123-145.
- Sharafkhaneh, A., Hanania, N. A., & Kim, V.** (2008). Pathogenesis of Emphysema: From the Bench to the Bedside. *Proceedings of the American Thoracic Society*, 5(4), 475-477.
- Sharma, P., Jha, A., Dubey, R. and Pessarakli, M.** (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, 2012, pp.1-26.
- Smith, F., Reichow, S., Esseltine, J., Shi, D., Langeberg, L., Scott, J. and Gonen, T.** (2013). Intrinsic disorder within an AKAP-protein kinase A complex guides local substrate phosphorylation. *eLife*, 2.
- Starcher, B.** (1986). Elastin and the lung. *Thorax*, 41(8), pp.577-585.
- Suri, S., Fenniri, H. and Singh, B.** (2007). Nanotechnology-based drug delivery systems. *Journal of Occupational Medicine and Toxicology*, 2(1), p.16.
- Tao, J. and Malbon, C.** (2008). G-protein-coupled receptor-associated A-kinase anchoring proteins AKAP5 and AKAP12: differential signaling to MAPK and GPCR recycling. *Journal of Molecular Signaling*, 3, p.19.
- Taraseviciene-Stewart, L. and Voelkel, N.** (2008). Molecular pathogenesis of emphysema. *Journal of Clinical Investigation*, 118(2), pp. 394-402.
- Tasken, K. and Aandahl, M.** (2004). Localized Effects of cAMP Mediated by Distinct Routes of Protein Kinase A. *Physiological Reviews*, 84(1), pp.137-167.
- Tröger, J., Moutty, M., Skroblin, P. and Klussmann, E.** (2012). A-kinase anchoring proteins as potential drug targets. *British Journal of Pharmacology*, 166(2), pp.420-433.
- Tunggal, J., Helfrich, I., Schmitz, A., Schwarz, H., Günzel, D., Fromm, M., Kemler, R., Krieg, T. and Niessen, C.** (2005). E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J*, 24(6), pp.1146-1156.
- van Roy, F. and Berx, G.** (2008). The cell-cell adhesion molecule E-cadherin. *Cell. Mol. Life Sci.*, 65(23), pp.3756-3788.
- Webb, R.** (2003). Smooth muscle contraction and relaxation. *APS*, 27(4), pp.201-206.
- Wilson, D., Sutherland, C. and Walsh, M.** (2001). Ca<sup>2+</sup> Activation of Smooth Muscle Contraction. *Journal of Biological Chemistry*, 277(3), pp.2186-2192.
- Wong. I. and Scott, J.** (2004). AKAP signalling complexes: focal points in space and time. *Nature Reviews Molecular Cell Biology*, 5, pp.959-970.
- Zaccolo, M.** (2011). Spatial control of cAMP signalling in health and disease. *Current Opinion in Pharmacology*, 11(6), pp.649-655.

**Zhang, M., Patriarchi, T., Stein, I., Qian, H., Matt, L., Nguyen, M., Xiang, Y., Hell, J.** (2013). Andenylyl Cyclase Anchoring by a Kinase Anchor Protein AKAP5 (AKAP79/150) Is Important for Postsynaptic- $\beta$ -Adrenergic Signalling. *The American Society for Biochemistry and Molecular Biology*, 288(24), pp.17918-17931.

**Zhu, Y., Liu, X., Sköld, C., Umino, T., Wang, H., Spurzem, J., Kohyama, T., Ertl, R. and Rennard, S.** (2001). Synergistic neutrophil elastase-cytokine interaction degrades collagen in three-dimensional culture. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 281(4), pp.868-878.