A-kinase anchoring proteins: Cyclic AMP compartmentalization in neurodegenerative and obstructive pulmonary diseases.

Chapter 2

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Abstract

The universal second messenger cyclic AMP (cAMP) is generated upon stimulation of Gs protein-coupled receptors, such as the β2-adrenergic receptor (β2-AR), and leads to the activation of protein kinase A (PKA), the major cAMP effector protein. PKA oscillates between an on and off state and thereby regulates a plethora of distinct biological responses. The broad activation pattern of PKA and its contribution to several distinct cellular functions lead to the introduction of the concept of compartmentalization of cAMP. A-kinase anchoring proteins (AKAPs) are of central importance due to their unique ability to directly and/or indirectly interact with proteins that either determine the cellular content of cAMP, such as β2-AR, adenylyl cyclases and phosphodiesterases, or are regulated by cAMP such as the exchange protein directly activated by cAMP (Epac).

We report on lessons learned from neurons indicating that maintenance of cAMP compartmentalization by AKAP5 is linked to neurotransmission, learning and memory. Disturbance of cAMP compartments seem to be linked to neurodegenerative disease including Alzheimer’s disease. We translate this knowledge to compartmentalized cAMP signalling in the lung. Next to AKAP5, we focus here on AKAP12 and Ezrin (AKAP78). These topics will be highlighted in the context of the development of novel pharmacological interventions to tackle AKAP-dependent compartmentalization.

Introduction

G protein-coupled receptors, such as the Gs-coupled β2-adrenergic receptor (β2-AR), currently represent one of the largest groups of drug targets (1). After receptor binding of β2-agonists such as isoprenaline and fenoterol, elevation in the cellular content of cyclic adenosine monophosphate (cAMP) is catalysed by membrane-bound adenylyl cyclases (ACs) (2-4), a process known to be shaped by cAMP-degrading phosphodiesterases (PDEs) (5-9). Among the PDE superfamily members, PDE4, PDE7 and PDE8 exhibit substrate specificity towards cAMP (7, 8).

The best known effector of cAMP is protein kinase A (PKA). The PKA holoenzyme consists of two catalytic (C) subunits, which exist in three isoforms (Cα, Cβ and Cγ), and two regulatory (R) subunits. There are two major isoforms of PKA, designated as PKA(I) and PKA(II), which differ exclusively due to the RI and RII subunits, each again subdivided in an α and β isoform (RIα, RIβ, RIIα, RIIβ). Upon binding of two cAMP molecules to each R subunit, the dimer releases the C subunits and thereby initiates target protein phosphorylation. PKA is known to oscillate between an on and off state and thereby regulates a plethora of cellular responses (10). With the
discovery of the exchange factor directly activated by cAMP (Epac) (11, 12), the subset of biological functions driven by cAMP started to become even more diverse (13-16), and thereby further supported the concept of compartmentalization of cAMP. Tough cyclic nucleotide gated ion channels represent another cAMP targeted group, a detailed description of this is beyond the scope of our current review and we would like to refer the reader to recent review (17).

**Concept of compartmentalization of cAMP**

The localisation of the different PKA isoforms and of the Epac proteins as well as of cAMP generating and degrading enzymes is strictly regulated. Indeed, PKA was already some time ago found activated in either particulate or soluble cellular fractions (18, 19). Clustering of PKA to lipid rafts and caveolae further support the existence of subcellular regions specialised in cAMP signalling that are characterized by a rather dynamic composition of a specific subset of signalling molecules which include G	extsubscript{i}-coupled receptors, ACs, PDEs and Epac (20-24).

About 40 years ago, studies primarily performed in heart tissue reported that the two prototypical G	extsubscript{i}-coupled receptor agonists isoprenaline and prostaglandin E	extsubscript{1} elevated both the cellular content of cAMP, while only isoprenaline increased cardiac contractility (18, 19, 25, 26). Based on these early studies, the concept of compartmentalization of cAMP signalling was introduced, which ignited a new surge of cAMP-related research. Since then, several studies have provided further insights into the diversity of cellular strategies to compartmentalize intracellular signalling, a concept currently believed to enable a tightly and fine-tuned control of biological functions. Of particular interest is a recent study from Feinstein et al. (27). Combining mathematical modelling and experimental measurements, the authors demonstrated that the microvascular endothelial barrier strictly relies on subtle local changes in cellular cAMP. Cytosolic produced cAMP disrupted the microvascular endothelial barrier integrity whereas cAMP produced at the plasma membrane increased pulmonary microvascular endothelial barrier integrity (27). Thus, studies on compartmentalization of cellular cAMP emerged as a theme of central importance to unravel the multiple facets of cAMP signalling and its effect in physiological and pathophysiological situations. Such cAMP gradients may display high spatial resolution, as cAMP signalling often occurs within one protein complex orchestrated by a scaffold protein; the most studied family of scaffold proteins coordinating cAMP signalling is the A-kinase anchoring protein (AKAP) family, outlined in the next paragraph.
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A-kinase anchoring proteins
Microtubule-associated protein 2 (MAP2) was the AKAP that tether PKA together with microtubules (28). Members of the AKAP family represent important scaffolding proteins and thereby determine the specificity of cellular cAMP signalling. AKAPs control the spatio-temporal activity of the main cAMP effector PKA and some AKAPs have been shown to bind Epac (29-31).

Through their association with cAMP-elevating receptors, ACs and/or cAMP-degrading PDEs, AKAPs are able to create and maintain local cAMP pools (4, 32-34). To date, over 50 members and splice variants of the AKAP family have been identified (35-40).

A-kinase anchoring proteins: PKA-RI and PKA-C
Differentiation between AKAPs is based on their ability to bind exclusively PKA-RI, PKA-RII subunits or in the case of dual specific AKAP members both PKA-R subtypes. Most of the AKAP superfamily members bind the PKA-RII subunit (35). In 2010, however, sphingosine kinase interacting protein (SKIP) was identified as the first mammalian AKAP specific for the binding of PKA-RI (41-43). In Rlα−/− mouse embryonic fibroblasts, SKIP was unable to bind any PKA thereby strongly supporting the notion that SKIP specifically binds PKA-RII (42). SKIP is also one of the few AKAPs shown to sequester two PKA holoenzymes thereby leading to their sequestration at the inner mitochondrial membrane (42). Most AKAPs bind with the R subunits and thereby interact also indirectly with the catalytic (C) subunit of PKA. This is distinctly different from the scaffolding proteins A-kinase interacting protein (AKIP1) (44) and caveolin-1 (45) which directly interact with the C subunit. Upon binding to both the C subunit of PKA and the p65 subunit of NFkB, AKIP1 seems to act as a molecular switch in PKA driven NFkB signalling (46, 47). In cardiomyocytes AKIP1 protected against ischemia/reperfusion damage by decreasing reactive oxygen species generation, a process requiring the mitochondrial localization of AKIP1 (48). As both SKIP and AKIP1 seem to exert their primary biological functions in close proximity to mitochondria, it is tempting to speculate that AKAP scaffolding mechanisms via the PKA-RII subunit and/or PKA C subunit most likely represent novel molecular mechanisms to unravel yet undefined cellular roles of AKAP-dependent compartmentalization of cAMP.
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A-kinase anchoring proteins: Functional diversity and oligomerization

Utilization of distinct combinations of broad-spectrum signalling proteins, such as PKA, protein kinase C (PKC) and protein phosphatase 2B/calcineurin (PP2B/CaN), on the same AKAP, namely AKAP5, modulated the activity of the two distinct neuronal ion channels: 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA)-type glutamate receptor and M-type potassium channels, thereby triggering precise localized cellular responses (49). With this notion, it is meanwhile generally accepted that AKAPs act as a Swiss army knife that seem to execute differential cellular tasks upon subtle changes in their interacting proteins. Together with the huge number of different members of the AKAP family, the multitude of cellular tasks being performed in different cellular compartments is largely increased.

Even further complexity is added with the finding that AKAPs form homo- (50-52) and hetero-dimers (53), a process initially described for AKAP-Lbc (50). For example, overexpression of AKAP12 in cells that endogenously express AKAP5, such as HEK293 or A431 cells, potentiates AKAP5-mediated phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in response to the β2-agonist isoprenaline (53). Interestingly, however, AKAP12 mediated recycling of the β2-AR was unaffected upon AKAP5 overexpression (53) (Figure 1). Thus, oligomerization of AKAP family members may regulate a distinct subset of signalling properties. However, mechanisms involved in AKAP oligomerization, and how such dimer formation is triggered by molecular cues still remain obscure.

For the purpose of this review, we will summarize the most important features of AKAP5, AKAP12, and Ezrin (AKAP78) (Table 1). Neuronal key discoveries will be recapitulated to introduce paradigm shifts that illustrate the general spatio-temporal nature of the compartmentalized cAMP signalling. Our goal is to translate the lessons learned from neurons to the lung as our current knowledge about cAMP compartmentalization in the airways is rather limited. Before that, we will focus in the next section on cAMP compartmentalization via AKAPs acting alone with PKA or in concert with Epac, starting in the following section with the different tools currently available or under development.

Tools to study compartmentalization of cAMP

In the following section, we will highlight novel tools used to study the effect of AKAP-bearing multiprotein complexes on a diverse subset of biological functions. As some AKAPs bind to Epac in addition to PKA, we will briefly discuss some tools that are used to interfere at the level of PKA or Epac. For further details about Epac, we would like to...
Refer the reader to recent reviews on this topic (13-16). Our main focus is the tools that interfere with AKAP-bearing multiprotein complexes.

Figure 1. Member of the AKAP family and $\beta_2$-AR functioning. Left: AKAP5 has been shown to constitutively associate with the $\beta_2$-AR receptor (164, 200, 204). Upon $\beta_2$-AR activation, AKAP5 bound PKA phosphorylates the receptor, facilitates the switch of $G_i$ to $G_o$ and thereby permits signaling to ERK (164, 200, 204). In addition, AKAP5 bound PKA phosphorylates G-protein coupled receptor kinase 2 (GRK2), enhances the affinity of GRK2 for $G_{\text{cy}}$ subunits and subsequent interaction with the $\beta_2$-AR (207). Middle: Receptor bound GRK2 bears the ability to interact with Ezrin (AKAP78), the latter known to be required for the internalization of the $\beta_2$-AR (208). Right: $\beta_2$-AR activation leads also to phosphorylation of AKAP12 via bound PKA and increases the association of AKAP12 with the $\beta_2$-AR receptor, a process known to be essential for the recycling of the $\beta_2$-AR (201, 241).
Table 1: Subset of AKAP family members known to regulate biological functions in the lung and brain. The most important AKAP interactions are highlighted, except of their primary binding partner PKA. Text between parentheses, AKAP synonym using the HUGO gene nomenclature or name of a certain orthologue. For further details and references, see text.

<table>
<thead>
<tr>
<th>AKAP</th>
<th>Interactions</th>
<th>Processes involved</th>
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<tbody>
<tr>
<td>AKAP5 (HUGO)</td>
<td>AKAP12, AKAP5</td>
<td>β2-AR switching to ERK</td>
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<tr>
<td>AKAP79 (Human)</td>
<td>β1-AR, β2-AR</td>
<td>β2-AR desensitization</td>
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<td>AKAP150 (Murine)</td>
<td>AC5, Epac2</td>
<td>Cell cycle progression</td>
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<tr>
<td>AKAP75 (Bovine) H21</td>
<td>PKB/Akt, PKC, PP2A/B, Calcinurin, Calmodulin PSD-95, MAGUK, SAP97, PIP2, F-actin E-/N-Cadherin AMPA/NMDA receptor Cav1.2</td>
<td>Synaptic plasticity</td>
</tr>
<tr>
<td>AKAP12 (HUGO)</td>
<td>AKAP5, AKAP12</td>
<td>β2-AR resensitization</td>
</tr>
<tr>
<td>AKAP250</td>
<td>β2-AR</td>
<td>Cell cycle progression</td>
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<td>Gravin (Human)</td>
<td>PKC</td>
<td>Synaptic plasticity</td>
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<td>SSeCKS (Murine)</td>
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<td>AI317366</td>
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<tr>
<td>Ezrin</td>
<td>EBP50 (NHERF1), GRK2, RhoGDI, Rho, Rac, Epac</td>
<td>β2-AR internalisation</td>
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<tr>
<td>AKAP78</td>
<td></td>
<td>Actin-binding linker protein</td>
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<td>Cytovillin</td>
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<td>p81</td>
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<td>Villin-2</td>
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Epac and PKA

To distinguish between PKA and Epac, cell membrane-permeable cyclic nucleotide analogues have been developed, such as N\(^\text{6}\)-benzyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP) for PKA or 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT-2'-O-Me-cAMP) for Epac (15, 54, 55). In addition, inhibitors of PKA have also been synthesized, such as Rp-8-CPT-cAMPS, and have been shown to abolish the dissociation of PKA-C subunits from the PKA-R subunits (54). These compounds seem to provide more specificity compared to PKA inhibitors known to act on the ATP binding site, such as H-89 (56). Inhibition of PKA can also be achieved with the PKA inhibitor (PKI) (57). Very recently, pharmacological inhibitors of Epac have been identified, which seem to act primarily on Epac1 (CE3F4) or Epac2 (ESI-05) (58-60). Even though researchers worldwide use the novel compounds to gain insight into the contribution of Epac1 and/or Epac2 to biological functions (61), their mode of action and specificity warrants further studies (62). Specific activators for Epac1 and Epac2 are still lacking.

A-kinase anchoring proteins: Genetically modified mice

To address the physiological importance of specific AKAPs in vivo, mice deficient for a specific AKAP gene e.g. AKAP5\(^{-/-}\), or for a specific AKAP-protein interaction, for example by introducing a truncation e.g. AKAP5\(\Delta\)36 for AKAP5-PKA interactions, have been developed. Ablation of AKAP members has led to several phenotypes such as decreased fertility (e.g. AKAP1, AKAP4), cardiac arrhythmias (e.g. AKAP10 (D-AKAP2)), developmental (e.g. mAKAP (AKAP6), WAVE-1) and neuronal defects (e.g. AKAP5, MAP2) (35, 63). Based on these findings, it has been suggested that drug targets interfering at the level of AKAPs might have the ability to disturb signalling driven by cAMP and might, therefore, represent a novel layer of pharmacological interventions (39, 63, 64).

A-kinase anchoring proteins: Dynamics of PKA and AKAP

To assess the dynamics of the primary AKAP interaction partner PKA in vivo, several fluorescence resonance energy transfer (FRET) tools have been developed taking advantage of genetically encoded A-kinase activity reporters (65-70). The addition of cellular localization signals permits the recruitment of these tools to subcellular compartments, including the cytosol, the nucleus, the sarcoplasmic reticulum, the mitochondria (using an AKAP based localization), the plasma membrane (68, 71) and...
even the raft or non-raft domains of the cell membrane (69). Interestingly, the PKA based biosensors have been transferred to AKAP research by combining them with AKAP12 (72) and AKAP5 (73). Using this novel approach, distinct dynamics of PKA bound to either AKAP12 or AKAP5 at the membrane compared with cytosolic/perinuclear regions were identified (72, 73). Currently, several novel insights into the subcellular dynamics of AKAP bound PKA are based on cell transduction with PKA defined AKAP reporters and studies in genetically modified mice.

A-kinase anchoring proteins: Pharmacological Tools
Novel pharmacological tools have been developed to overcome the technical limitations and to study the biological effect of AKAP-based multiprotein complexes in vivo. A conserved amphipathic helix represents a well-defined domain structure present in all AKAP superfamily members which is required for the interaction with the primary AKAP binding partner PKA (74, 75). The amphipathic helix is inserted into the hydrophobic pockets formed by the dimer of the PKA-R subunits (76, 77). It is this amphipathic helix that provided the first basis for the design of dominant interfering peptides able to disrupt the interaction between PKA and AKAP, such as Ht31 (Figure 2A). The stearated form of Ht31, st-Ht31, exhibits an improved membrane permeability (35). It is important to note that the generation of such PKA-AKAP interfering peptides has enabled the research community to gain insights into the contribution of AKAP–PKA interactions to a diverse subset of cellular functions in physiology and pathophysiology (35, 38, 39, 63). The original peptides, however, provided little, if any, distinction between PKA-RI and PKA-RII subtypes and members of the AKAP family. Through bioinformatics RI (AKB-RI, RIAD) (78, 79) and RII-specific (AKB-RII, (Super)-AKAP-IS) (77, 78, 80) were designed to discriminate between different type of PKA-AKAP interactions, PKA-RI or PKA-RII subunits. In attempts to overcome the central limitation in the current AKAP research field, a recent study from Scott and colleagues reported on the design of Rselect peptides, based on the RII subunits of PKA, that seem to exhibit selective affinity for certain members of the AKAP family (81). Intriguingly, using phage selection procedure combined with high-resolution structural bioinformatics AKAP2 (AKAP-KL) and AKAP7 (AKAP18) selective Rselect peptides were validated by biochemical and cell-based experiments (81). The AKAP5 (AKAP79, AKAP150) Rselect peptide, however, not only interfered with the binding of PKA to AKAP5, but also its binding to AKAP7 and AKAP11 (81). Functional data for these new tools have yet to come, however, the importance of

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this development is evident as for the first time it is possible to distinguish between the individual PKA compartmentalizers without genetic modifications.

Figure 2. Strategies to disrupt AKAP complexes. Schematic illustration of the different ways to disrupt AKAP complexes. A, using PKA-AKAP dominant interfering peptides, such as Ht31, to displace PKA as the archetypical AKAP interaction partner. B, using dominant interfering peptides to disrupt interactions between proteins and AKAPs, such as GSK3Ptide to remove GSK3 from AKAP complexes. C, Similar strategies are now applied by using small molecules such as FMP-API-1. Further details, see text.
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In addition, recent studies intend to facilitate a distinction between different AKAPs based on their ability to interact with a discrete interaction partner and/or on mechanisms distinct from the AKAP–PKA interaction outlined earlier. The dominant interfering peptide GSKIPptide, structurally based on the glycogen synthase kinase 3β (GSK3β) binding site of GSK3β interaction protein (GSKIP), competes with AKAP members known to bind to GSK3β, including GSKIP, AKAP11 and MAP2D (in rat) and thereby to disrupt the compartmentalization of GSK3β (82) (Figure 2B). Meanwhile, similar peptides were designed, such as a phospholamban peptide, which is able to prevent the interaction with AKAP7δ (83) and EBP50 (also known as NHERF1, SLC9A3R1) peptide which prevents the interaction with Ezrin (AKAP78) (84) (Table 1). Also of particular interest are peptides that specifically inhibit the interaction between mAKAP and the AC isoform 5 (AC5), leaving the interaction between AKAP5-AC5 unaltered (85). Recently, a disruptor for the Hsp20-PDE4 interaction has been described that liberates PDE4 from the AKAP-Lbc based complex (86).

Most tools being developed thus far, however, are still peptide based and might therefore exert some unknown interactions. For example, it has been reported that st-Ht31P, generated from st-Ht31 by two proline substitutions believed to render the molecule incapable of disrupting the AKAP–PKA interaction (35), seems to inhibit PKA (87). The aim of current research is to design small molecule inhibitors for AKAP–PKA interactions (88, 89). Intriguingly, it has been reported that the small molecule 3,3′-diamino-4,4′-dihydroxydiphenylmethane (FMP-API-1) and its derivatives inhibit AKAP–PKA interactions in vitro and in cultured cardiomyocytes (88) (Figure 2C). As FMP-API-1, however, also activates PKA (88), synthesis of additional small molecules is still warranted. Indeed, new terpyridine scaffolds has been recently synthesized (89), representing the non-peptidic compounds which might exert less unwanted biological side effects.

Relation to disease
Disturbance of AKAPs either at the level of their expression profile or biological functions has been associated with a variety of diseases (35, 38, 39, 63). For example, AKAP12, also known as AKAP250 or Gravin, was first identified as an auto-antigen in myasthenia gravis (90). Down regulation of AKAP12 is associated with prostate hyperplasia (91) and several types of cancer (92), including gastric cancer (93). It is tempting to speculate that down regulation of AKAP12 might be mediated by promoter hypermethylation, a mechanism described before in the context of oesophageal and colon cancer (94-96).
Such a mechanism for the promotion of cancer cell invasiveness by AKAP12 (97). In line with this, AKAP12 inhibits cell proliferation (92, 98). In addition to AKAP12, other members of the AKAP family such as AKAP4 and AKAP9 are discussed as cancer markers (99-104).

In the following sections we will first focus on the compartmentalization of cAMP maintained by AKAPs in the context of neuronal learning and memory processes related to neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, Huntington's disease, multiple sclerosis and Wallerian degeneration. Then, we will highlight our current knowledge about compartmentalized cAMP signalling networks in the context of obstructive pulmonary diseases such as chronic obstructive pulmonary disease and asthma, and whenever appropriate we will emphasize the effect of AKAP-based multiprotein complexes.

Lessons from neurons and neurodegenerative diseases
In the following sections we will discuss the most recent findings on compartmentalized cAMP signalling to maintain proper neuron functions and to alleviate symptoms of neurodegenerative disease. In particular, we will highlight studies that focus on members of the AKAP family.

Concept of neuronal cAMP compartmentalization: PKA and Epac
Neurons represent highly polarized structures, displaying short, tapered dendrites and long, thin axons (105-107). In primary rat hippocampal neurons, Poo and colleagues demonstrated that LKB1 phosphorylation by PKA represents an early event in axonal differentiation, whereas Smurf1 phosphorylation by PKA directs selective neuronal degradation of Par6 or RhoA (106, 108). In rat dorsal root ganglion neurons, local cAMP levels regulate axonal guidance through the attraction and repulsion of axons, a process involving netrin-1 and myelin-associated glycoprotein (MAG) (109, 110). High cAMP levels during the embryonic stage regulate axonal guidance by Epac, whereas low cAMP levels during the postnatal stage result in growth cone repulsion by PKA (110).

Local changes in cAMP determine hippocampus-dependent learning and memory stages such as acquisition, consolidation, retrieval, reconsolidation and extinction (111, 112). Since the pioneering work in Aplysia 30 years ago (113, 114),
several studies link cAMP and PKA to locally defined synaptic plasticity, learning and different memory stages (30, 112, 115-117).

In addition, several recent genetic and pharmacological studies report on the role of Epac in a context-dependent fear-conditioning paradigm (118-125).

**Concept of compartmentalization of cAMP: AKAP5**

As outlined earlier, both PKA and Epac seem to sense local changes in cAMP to control neuronal development and differentiation, learning and memory. Compartmentalization of cAMP in the brain seems to be maintained primarily by AKAP5 (126). AKAP5 is regulated during neuronal development (127), and provides a platform to integrate neuronal cAMP signalling networks (30). Thus, AKAP5 most likely coordinates the fine tuning of cAMP by regulating the temporal and spatial events controlling cAMP levels. Indeed, a neuronal cAMP-sensing multiprotein complex maintained by AKAP5, PKA, Epac2 and PKB/Akt, controlled the survival protein kinase B/Akt pathway (30).

**AKAP5: Neurotransmission, learning and memory**

Binding of PKA to AKAPs alters synaptic protein phosphorylation and thereby controls synaptic plasticity and memory consolidation (126). In hippocampal neurons, AKAP5 acts as a postsynaptic scaffold protein that also binds to phosphatase 2B/calcineurin (PP2B/CaN; also PPP3) (128) and PKC next to PKA protein (129, 130) (**Figure 3**). The postsynaptic AKAP5 localization is dependent on its association with the actin cytoskeleton, acidic phospholipids, and cadherins (131, 132). Binding of AKAP5 with membrane-associated guanylate kinase (MAGUK) is required for maturation of dendritic protrusions into large, dendritic spines with an increased density of synaptic AMPA receptors (127). The functional relation between AKAP5 and AMPA receptors may also be linked to the binding of AKAP5 to the MAGUK family member SAP-97 (133). AKAP5 can also bind the postsynaptic density protein PSD-95 to regulate N-Methyl-D-aspartic acid (NMDA) receptors (134, 135) (**Figure 3**). Next to the interaction of AKAP5 with several members of the scaffold protein PSD family, binding of AKAP5 to cadherins may also influence synaptic plasticity mechanisms, a process implicated in the regulation of NMDA receptors (136) (**Figure 3**).
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AKAP5 directly interacts with the neuronal L-type calcium channel subunit Cav1.2 (137), and thereby forms a complex with AC, PKA and PP2A, and is, therefore, able to modulate Ca²⁺ signalling downstream of the β₂-AR (138). Anchoring of PP2B/CaN to AKAP5 regulates internalization and rapid dephosphorylation of the AMPA

Figure 3. Compartmentalization of cAMP in neurons in relation to neurodegenerative diseases. A, Illustration of cAMP compartmentalization with emphasize on AKAP5 and selected adaptor proteins in neurons and their alterations under pathological conditions including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, multiple sclerosis and Wallerian degeneration. B, An example of disrupted cAMP compartmentalization as it was shown that AKAP5 coordinated calcineurin (CaN) was required for AMPA receptor internalization and LTD, removal of the AKAP5 caused an impairment of this LTD. For further details, see text.
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receptor, and most likely reflects a form of molecular and cellular memory associated with long-term depression (LTD) (Figure 3B) (134). Indeed, brain slices derived from adult AKAP5 knock-out mice display normal basal hippocampal spine density and synaptic transmission, but exhibit deficiency in LTD, learning and memory (127). Malenka and colleagues (139) reported that AKAP5 modulates LTD most likely through binding of AKAP5 to PSD-95, causing the release of PP2B/CaN, and subsequently enhances endocytosis of synaptic AMPA receptors. As a consequence, AKAP5 may leave the spine, and thereby contribute to the shrinkage of spines that accompanies LTD (139). Currently, the best genetic models for studying AKAP5 function are the Δ36 mice, which lack the PKA binding site at the C-terminus of AKAP5 (140), and AKAP5-deficient mice (141). Δ36 mice display both long-term potentiation and LTD defects. In contrast, the AKAP5 deficient mice exhibit only LTD defects. Such differences suggest that the most critical function of AKAP5 is most likely related to its interaction with PKA, to control the formation and/or maintenance of dendritic spines (142). It is clear that regulation of PKA signalling by AKAP5 is necessary to facilitate neurotransmission, learning and defined stages of the memory.

Throughout the mouse brain, AKAP5 is widely distributed in regions linked to learning and memory in rodents, such as the cortex, the hippocampus and the amygdala (126, 143-145). Using contextual fear conditioning in mice, the expression of AKAP5 protein was increased in the hippocampus in a late phase of memory consolidation of associative memory (117). Disruption of hippocampal AKAP–PKA interactions by st-Ht31 or st-superAKAP-IS facilitates extinction and impairs the consolidation of contextual fear memories, whereas acquisition and retrieval remain unchanged (30) (Figure 3). Disruption of AKAP–PKA interactions by st-Ht31 in the rat lateral amygdala impaired memory consolidation in auditory fear conditioning (126). Using the Morris water maze to study learning and spatial memory, AKAP5-deficient mice exhibit deficits in spatial memory retention most likely caused by delocalization of PKA and subsequent alterations in the local environment of cAMP signalling in the hippocampus (130). Taken together, the results from several recent studies illustrate the importance of AKAP5 for maintaining neuronal compartmentalized cAMP signalling to coordinate learning and memory.

**AKAP5: Lessons from Alzheimer’s disease**

As discussed earlier, cAMP in neurons is crucial for learning, memory and physiological events but it is not known how this system is altered under pathological...
neurodegenerative circumstances. Elucidating this is likely to provide mechanistic insights that may give some clues for the development of novel pharmacological tools. Ample evidence suggests that perturbation of local cAMP signalling contributes to the development and progression of neurodegenerative diseases. Here we focus on the role of the players discussed previously in the context of Alzheimer’s disease.

Alzheimer’s disease is a neurodegenerative disease characterized by the progressive decline of cognitive function and memory, and is the fourth largest cause of death for people over 65 years of age (146). The disease is characterized by extracellular β-amyloid plaques, intracellular neurofibrillary tangles, cholinergic transmission defects and neuronal loss preferentially in the entorhinal cortex and hippocampus (146). As several inflammatory markers are up-regulated in Alzheimer’s disease, it is generally assumed that inflammation is linked to the pathogenesis of Alzheimer’s disease. Indeed, amyloid plaques seem to trigger inflammatory processes (147-150).

Chronic infusion of lipopolysaccharide has been used as an experimental model to mimic certain aspects of Alzheimer’s disease (151). Chronic lipopolysaccharide infusion into the fourth ventricle of young rats induces brain inflammation and subsequently activation of microglia, a process accompanied by a reduction in the expression of adenosine A<sub>2B</sub>-receptors and cAMP (152). Mengod and colleagues (153) using in situ hybridization showed that at early stages of Alzheimer’s disease PDE4, in particular PDE4B, and PDE7 are up-regulated, while at later stages of Alzheimer’s disease PDE8 is upregulated (153). Both studies imply that progression of Alzheimer’s disease is associated with a limitation in cellular cAMP.

Accumulating evidence suggests that Aβ-induced neurotoxicity alters NMDA receptor signalling through the cAMP response element-binding protein (CREB), a transcription involved in learning and memory processes (154). Moreover, CREB phosphorylation was reduced in the hippocampus of Alzheimer’s post-mortem brains (155). Intriguingly, Shelanski and colleagues showed that treatment of rat hippocampal neurons with Aβ peptides decreases the dissociation of PKA C and R subunits and thereby phosphorylation of downstream targets such as CREB (156). The PDE4 inhibitor rolipram promotes the dissociation of PKA’s C and R subunits and reverses inhibitory effects of Aβ peptides on CREB phosphorylation (156-158). As PKA-dependent signalling studied by CREB phosphorylation in the hippocampus of Alzheimer’s post-mortem brains was reduced (155), Arima and colleagues proposed that CREB
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phosphorylation may serve as a molecular biomarker of ageing-related pathological processes (159), in particular of Alzheimer's disease.

In addition to PKA, recent studies indicate that Epac may also be linked to Alzheimer's disease. Lezoualc'h and colleagues show that the Epac effector Rap1 promotes the activation of Rac, and subsequently leads to the cleavage of the amyloid precursor protein (APP) and production of secreted APPα (sAPPα) (160). Rap1 can directly interact with STEF, a specific guanine exchanging factor (GEF) for Rac1, and this association is involved in the secretion of the sAPPα (161). Moreover, activation of the serotonin receptor of the subtype 4 increases sAPPα through Epac1/Rap1/Rac (162). It has been postulated that sAPPα acts as a memory-enhancer and neuroprotector (160, 162). Thus, production of sAPPα by Epac may reduce symptoms of Alzheimer's disease. Indeed, in human brain regions associated with Alzheimer’s disease, Epac1 mRNA is up-regulated, which is accompanied by a down regulation of Epac2 mRNA (163).

Next to Alzheimer’s disease, cAMP and its players are associated with others neurodegenerative disease such as Parkinson’s disease, Huntington’s disease, multiple sclerosis and Wallerian degeneration (Table 2). Several lines of evidence indicate that alterations in local cAMP dynamics might be caused by inhibition of PKA, up-regulation of a specific PDE subset, up-/down-regulation of Epacs, or a combination of these events. Persistent limitations in the cellular cAMP level, due to either defects in the cAMP-producing receptors and/or elevations of the cAMP-degrading PDEs, such as PDE4, seem to underpin the development and progression of neurodegenerative diseases (Table 2). Even though not yet being studied in detail in the context of neurodegenerative diseases, a central role for the AKAP family member AKAP5 might be envisaged due to its ability to interact with the β2-AR and/or PDE4 (164), and due to its ability to maintain neuronal cAMP compartmentalization.

Table 2: cAMP compartmentalization in neurodegenerative diseases.

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Airway smooth muscle and obstructive pulmonary diseases

Chronic obstructive pulmonary disease (COPD) and asthma are both obstructive inflammatory airway diseases characterized by chronic inflammation, airway obstruction and airway remodelling, albeit with different aetiology and specific pathological features (165, 166). COPD is predicted to be the third-leading cause of death by disease.
phosphorylation may serve as a molecular biomarker of ageing-related pathological processes (159), in particular of Alzheimer's disease. In addition to PKA, recent studies indicate that Epac may also be linked to Alzheimer's disease. Lezloualc'h and colleagues show that the Epac effector Rap1 promotes the activation of Rac, and subsequently leads to the cleavage of the amyloid precursor protein (APP) and production of secreted APPα (sAPPα) (160). Rap1 can directly interact with STEF, a specific guanine exchanging factor (GEF) for Rac1, and this association is involved in the secretion of the sAPPα (161). Moreover, activation of the serotonin receptor of the subtype 4 increases sAPPα through Epac1/Rap1/Rac (162).

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### Airway smooth muscle and obstructive pulmonary diseases

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worldwide in 2020 (167). Airflow limitation in asthma is reversible with bronchodilators and associated with airway hyperresponsiveness, whereas airway obstruction in COPD is largely irreversible and lung function decline is progressive (166, 168-170). Airway smooth muscle cells contribute to disease symptoms in both asthma and COPD due to their multifunctional behavior that supports airway remodeling and airway obstruction, causing the limitation of airflow (171-173).

Different classes of bronchodilators are used in practice: $\beta_2$-AR agonists ($\beta_2$-agonists), muscarinic receptor antagonists (anticholinergics), individually or in combinations, with or without the addition of anti-inflammatory glucocorticosteroids (174-178). The main targets for the therapeutic treatment of obstructive pulmonary diseases have a direct or indirect link to G protein-coupled receptor signalling, mainly to the $\beta_2$-AR and the $M_3$ muscarinic receptor. In obstructive airway diseases, increase in smooth muscle mass and hypercontractility cause severe limitations in the airflow. Airway smooth muscle cell growth is inhibited by several $\beta_2$-agonists such as fenoterol and salbutamol (57, 179). Increased smooth muscle mass is believed to reduce the lumen size of the airways, a process associated with aberrant $\beta_2$-AR signalling (180). Despite the fact that $\beta_2$-agonists are generally well tolerated (181, 182) long term use of $\beta_2$-agonists caused variations in the treatment outcome in asthma and COPD patients, being either less efficacious in COPD patients or even leading to an increased incidence of asthma exacerbations and other markers of morbidity and mortality (183-186).

Another treatment option in obstructive airway diseases is represented by PDE inhibition, for instance the selective PDE4 inhibitors rolipram and roflumilast (187-189). PDE inhibitors increase the cellular level of cAMP by preventing its degradation. Although both $\beta_2$-agonists and PDE inhibitors show anti-inflammatory properties in vitro (190-192), a notable difference is seen in vivo. PDE4 inhibitors show anti-inflammatory properties in vivo, but largely lack airway smooth muscle relaxing properties. In contrast, $\beta_2$-agonists show bronchorelaxing properties in vivo, but lack anti-inflammatory properties (187, 193). Possible explanations for this discrepancy are most likely $\beta_2$-AR desensitization and/or biased signalling of the $\beta_2$-AR towards ERK signalling (194, 195), features largely absent with PDE4 inhibitors due to their post-receptor mode of action. Both the process of $\beta_2$-AR desensitization and biased signalling seem to be facilitated by scaffolding proteins such as AKAP5 and AKAP12 (196, 197) (Figure 1). Subcellular localized cAMP pools seem to cause differential biological effects upon scaffolding protein mediated targeting of either the $\beta_2$-AR or PDEs.
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An innovative alternative is, therefore, urgently required to safeguard long term treatment of obstructive lung disorders. Compartmentalized cAMP signalling may provide a novel opportunity for pharmacological interventions. For example, targeting downstream of the β²-AR will most likely circumvent receptor desensitization. One might also expect that such strategies will increase treatment specificity, and thereby minimize unwanted side effects, by targeting only the desired cAMP pool. In the following section, the potential effect of compartmentalized cAMP signalling in the lung for further improvement of obstructive airway diseases will be discussed.

A-kinase anchoring proteins: Signalling in the airway smooth muscle

In the airway smooth muscle, the main signalling pathways that determine its functionality are receptors coupling to G₃ or G₄ proteins. The G₃ protein-coupled receptor family is the M₃ muscarinic receptor known to be activated by acetylcholine, and to be inhibited by anticholinergics such as tiotropium (178). After agonist binding, the G₃₉ subunit activates phospholipase C (PLC), thereby leading to the elevation in cellular calcium and activation of calcium/calmodulin-dependent myosin light chain (MLC), a process known to result in airway smooth muscle contraction (198, 199). Activation of PKC by diacylglycerol alters also the (de)phosphorylation of the MLC through several pathways and thereby contributes to the airway smooth muscle tone (198). Activation of the G₄ protein-coupled receptors by drugs targeting the β²-AR, causes elevation of cAMP production via G₄ and subsequent activation of ACs (Figure 4).

Two members of the AKAP superfamily are known to interact with the β²-AR, AKAP5 and AKAP12. Whereas the association of AKAP5 with the β²-AR is constitutive (164, 200), agonist binding to the β²-AR increases the interaction of the receptor with AKAP12 (201). Despite the fact that AKAP5 and AKAP12 share many common features, no redundancy is seen between them with regard to this cellular response (197). AKAP5 has been reported to switch the coupling of the β²-AR from G₃ to G₁, a process most likely facilitated by a PKA-mediated phosphorylation of the receptor (164, 200, 202, 203) (Figure 1). It has been reported that coupling of the β²-AR to G₁ leads to activation of ERK signalling (204). The ERK pathway is known to be linked to both proliferative and cytokine production pathways in airway smooth muscle (15, 16, 205, 206). In the context of obstructive pulmonary diseases, it is worthwhile to emphasize reports indicating that AKAP5 seems to determine the cell surface expression of the β²-AR by increasing the affinity of G-protein coupled receptor kinase 2 (GRK2) for βγ subunits of the G-proteins, causing their translocation to the membrane, leading to the desensitization and
internalization of the β2-AR (207) (Figure 1). In contrast, after desensitization, AKAP12 is essential for the dephosphorylation, resensitization and recycling of the β2-AR back to the cell membrane (197, 201, 204). In addition, interaction of GRK2 with Ezrin (AKAP78) determines the β2-AR internalization (208) (Figure 1).

Based on these findings, it is reasonable to assume that β2-AR functions are determined by the balance between AKAP5, AKAP12 and Ezrin (AKAP78) (Figure 1). Indeed, a recent study from Penn and colleagues reported on the expression of AKAP5, AKAP12 and Ezrin (AKAP78) in human airway smooth muscle cells (209). Penn and colleagues did not observe effects of Ht31 or AKAP inhibitors in this system (209). Using a combination of st-Ht31 and a PDE inhibitor cocktail, the authors showed that disruption of PKA-AKAP interactions resulted in sustained AC activity (209). Mathematical models predicted that tethering of PKA to AKAP should cause a threefold increase in PKA at the β2-AR compartment, thereby decreasing input of the β2-AR acting as a negative feedback for AC and PDE activity (209). Indeed, direct inhibition of PKA with the protein kinase inhibitor (PKI) completely blunted the rapid
decay of the cAMP signal over time (209). With multiple AKAPs possibly involved to create such PKA pool, utilization of tools recently described by Gold et al. (81) would be necessary to assess the individual contribution of each AKAP.

In the following sections we will discuss the role of cAMP compartmentalization in some of the important features of chronic obstructive pulmonary diseases; contraction, inflammation and remodelling. Herein we will keep the focus on studies performed in airway smooth muscle.

A-kinase anchoring proteins: Airway smooth muscle contraction

Elevation of cAMP leads to the activation of both PKA and Epac and thereby modulates airway smooth muscle responses (15, 16). It is well established that PKA on its own deactivates PLC kinase and desensitizes the IP3 receptor, thereby functionally counteracting the PLC-PKC pathway. In our research group and by others, Epac has been identified as a novel factor being involved in the regulation of airway smooth muscle relaxation. Epac, acting most likely via its main effector Rap1, deactivates RhoA and up-regulates Rac1 activation, causing the balance to shift from phosphorylated MLC to non-phosphorylated MLC and thus to airway smooth muscle relaxation (210, 211) (Figure 4). Interestingly, Ezrin (AKAP78) is phosphorylated by Rho-regulated Rho-kinase and binds via its ezrin-radixin-moesin domain the Rho inhibitor Rho guanine-nucleotide-dissociation inhibitor (RhoGDI) (212). Airway smooth muscle cells express both Epac and Ezrin (AKAP78) (209, 213). Thus deactivation of Rho by Epac might involve mechanisms driven by Ezrin (AKAP78) and RhoGDI.

In a Madin-Darby canine kidney cell line, activated Ezrin (AKAP78) binds in a calcium dependent manner to Rac and thereby delayed membrane localization of E-cadherin (214). Calcium underlies also cellular compartmentalization and cross-talk with cAMP, a process being facilitated by members of the AKAP family. For example, AKAP5, known to be involved in β2-AR desensitization as outlined earlier (Figure 1), interacts with calcineurin (215, 216) and calmodulin (217). Calmodulin competes with PKC in a Ca2+-dependent manner for binding to AKAP5 (218). More recently, AKAP12, known to be involved in β2-AR sensitivity (Figure 1), rapidly redistributes from the plasma membrane to the cytosol upon stimulation with calcium-elevating agents such ionomycin or thapsigargin (219). Moreover, it has been reported that AKAP12 displace PKA-RII from the membrane (219).

A striking example of cooperativity between cAMP and calcium facilitated by AKAPs is shown for AKAP11 upon assembly of a complex that includes IQGAP1, GSK3β and PKA. It has been shown that binding of AKAP11 and IQGAP2 requires high
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intracellular calcium levels (220, 221). At lower intracellular calcium, AKAP11-anchored PKA phosphorylates IQGAP2 and thereby leads to an increase in Rac binding. In the presence of inactive GSK3β, however, AKAP11 serves as a platform for the assembly of a complex between IQGAP and CLASP2 a plus-end microtubule tracking protein involved in microtubule polymerization. PKA phosphorylation of GSK3β and elevations in calcium cooperatively drive the formation of an IQGAP1-CLASP2. Both the IQGAP1-Rac and IQGAP1-CLASP2 complexes have been suggested to be involved in microtubule dynamics and cell motility (220, 221). AKAP11 was found to be expressed in airway smooth muscle (209). In addition, Epac not only interacts with AKAP5, but also with the microtubule network and with the calcium-elevating phospholipase C-epsilon (15). Future studies should point out if similar mechanisms contribute to airway smooth muscle contraction.

A-kinase anchoring proteins: Airway smooth muscle inflammation

Recently, we reported in human airway smooth muscle cells that direct pharmacological activation of PKA and Epac synergistically enhances Gα protein-coupled receptor-induced release of the neutrophil chemoattractant interleukin-8 (IL-8) (213). Silencing of Epac expression decreased not only IL-8 release in response to Epac activation but also in response to PKA activation, and vice versa PKA inhibition by Rp-8-CPT-cAMPS reduced IL-8 release induced by both PKA and Epac (213). Using st-Hi31 to disrupt PKA-AKAP interactions (Figure 2A), preliminary results of our group suggest that PKA and Epac regulate the IL-8 release in an AKAP dependent manner.

Results from our research groups and others implicate that such close interconnectivity requires the presence of spatial regulation. AKAP5 was shown to be present in the same AKAP–PKA -Epac complex described before in neuronal cells (30). In a related study, we showed that induction of IL-8 release by cigarette smoke extract (CSE) was attenuated by the β2-agonist fenoterol, seemingly via Epac and PKA (222). Disturbance of AKAP-based multiprotein complexes might be expected due to the down regulation of Epac1 and members of the AKAP family by CSE (222, 223). Indeed, AKAP12 is down regulated in lung cancer (224). With AKAP5 and AKAP12 known to determine β2-AR functions (Figure 1), an important role for PKA and Epac localization close to G protein-coupled receptors in asthma and COPD could be imagined. This could explain the varying treatment outcomes seen for these bronchodilators in COPD (184-186).
The underlying molecular mechanisms of the attenuation of IL-8 release by cAMP seem to be coordinated via parallel routes. Epac was shown to inhibit the NFkB translocation to the nucleus caused by CSE, and PKA counteracts CSE-induced ERK phosphorylation, both known to underlie IL-8 production (222, 225). Although limited knowledge is currently available on Epac compartmentalization, both NFkB and ERK are known to interact with proteins that anchor catalytic and/or regulatory PKA subunits, respectively (46, 47, 226, 227). Thus, it is tempting to speculate that a distinct subset of AKAP members mediate the anti-inflammatory properties of both PKA and Epac, a research topic open for future investigation.

Our current knowledge implicates AKAPs as important factors of both inflammation and contraction. The question that remains: what role AKAPs play in airway remodelling?

A-kinase anchoring proteins: Airway smooth muscle remodelling

Another important functional feature of airway smooth muscle cells encompasses the existence of multiple phenotypes, a process reported to involve both PKA and Epac. Upon chronic exposure to stimuli such as growth factors, airway smooth muscle cells switch between a contractile and proliferative (synthetic) phenotype (172). Some researchers have suggested that airway smooth muscle proliferation is primarily inhibited by Epac, but not by PKA (228), while others state a more prominent role for PKA (57, 179). Recently, our research group demonstrated that pharmacological activation of either Epac or PKA prevented platelet-derived growth factor-induced hypocontractility of airway smooth muscle strips and airway smooth muscle proliferation, a process being accompanied by the inhibition of ERK1/2 (205, 206), suggesting a possible synergism between PKA and Epac. Our findings were strengthened by other studies in vascular smooth muscle cells (229). Here a concerted action of PKA and Epac inhibited serum-induced BrdU incorporation, Rb phosphorylation and the expression of cell cycle progression proteins, in a Rap1a-independent fashion (229).

Several signalling pathways have been shown to be involved airway smooth muscle cell proliferation, including ERK1/2 (230) and phosphoinositide 3-kinase/Akt (179, 231). Until now, molecular interactions between the cAMP effectors PKA and Epac have been studied in great detail in non-pulmonary systems pointing to compartmentalization of both cAMP effectors via muscle specific mAKAP (29), via β2-AR associated AKAP5 (30) and via the cytoskeletal scaffolding-AKAP11 complex (232). Interestingly, AKAP11 was found to be expressed in airway smooth muscle using real-time PCR (209). AKAP11 is not only able to bind PKA, but also GSK3, a kinase shown...
to be involved in expression of contractile proteins in airway smooth muscle (233), their proliferation (234, 235) and profibrotic signalling (236). Thus, AKAP11 driven cAMP compartmentalization may regulate airway smooth muscle remodelling.

In summary, several lines of evidence point towards the logical conclusion that AKAP family members are most likely of key importance for cAMP compartmentalization and thereby signalling to maintain a fine-tuned control over structural lung cell responses. Future studies will surely add additional insights into our current knowledge of signal compartmentalization and perhaps cross-talk between calcium and cAMP in the lung.

**Outlook and Future perspectives**

Compartmentalization of cAMP by AKAP family members represents a highly specialized and dynamic process to fine-tune intracellular signalling. Disturbance of cAMP compartmentalization, either due to alterations in AKAP expression or complex composition with a variety of tools outlined herein, seems to profoundly regulate biological functions and thereby to contribute to neurodegenerative and obstructive lung diseases.

Aging of the worldwide population will require further improvement of the management of chronic diseases. Notably, cAMP and its effectors seem to be critical in regulating several processes both in chronic brain and lung diseases. Next to PKA, Epac seems to act as a novel pharmacological target in both groups of diseases; however, the impact of Epac compared with PKA might be diverse and sometimes even conflicting. Members of the AKAP superfamily maintain cellular compartmentalization of cAMP primarily via direct interaction with PKA, a process now also linked to Epac. As AKAP bearing multiprotein complexes regulate receptor desensitization and are able to target simultaneously cAMP and calcium, the AKAP superfamily most likely represent an interesting novel pharmacological concept. In chronic obstructive pulmonary disease, targeting calcium-mediated bronchoconstriction and cAMP-mediated bronchorelaxation by one AKAP related drug might give an additional benefit above the current combination therapy with anticholinergics and β₂-agonists (237).

As outlined herein, the design of small molecule inhibitors seems to represent one of the most recent key findings in the field of AKAP research (88, 89). The AKAP–PKA interaction has been also used as a template for drug design based on the 'Dock-and-Lock Method’ (238, 239). Here, a trivalent drug is created upon conjugation of two identical (pro-)drugs (e.g. interferon-alpha 2b (240)) to the PKA-RII dimer and another drug-(targeting) antibody to an AKAP peptide derived from the amphipathic helix (such
as AKAP-I(S) (238-240), a process being stabilized by cysteine residues allowing covalent ‘locking’ of the subunits via disulphide bridges. In theory it should be possible to combine any RII module with any AKAP-module (238, 239), the benefit of this method most likely should be envisaged for the creation of a diverse set of potential pharmacological drugs.

Within the AKAP research field, pharmacological tools focus on PKA-AKAP interactions and disruption of other interaction partners from the AKAP complexes. Until now, however, no reports focus on the disruption of AKAP-Epac complexes. Even though an increasing amount of evidence indicates that Epac interacts with AKAPs, and other scaffolds independently of PKA (15). These Rap-GEF interacting proteins might add another dimension to the concept of subcellular compartmentalization of cAMP, in particular in the context of the physiology and pathophysiology of biological functions.

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Authors Contributions

Author contributions: WJP, PM-L, CGB, and MS conception and design of the review; WJP prepared figures and tables; WJP and MS edited and revised the manuscript; MS approved final version of manuscript.
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