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Review

# Role of cAMP in mediating AHR signaling

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ABSTRACT

Regulation of the nuclear import of many transcription factors represents a step in gene regulation which is crucial for a number of cellular processes. The aryl hydrocarbon receptor (AHR), a basic helix–loop–helix protein of the PAS (PER-ARNT-SIM) family of transcriptional regulators is a cytosol-associated and ligand-activated receptor. The environmental toxin dioxin binds with high affinity to AHR rendering it nuclear and leading to the activation of AHR sensitive genes. However, the fact, that the AHR mediates a large variety of physiological events without the involvement of any known exogenous ligand, including liver and vascular system development, maturation of the immune system, regulation of genes involved in cellular growth, cell differentiation and circadian rhythm, speaks for an important role of AHR in cell biology independent of the presence of an exogenous ligand. Different approaches were applied to study mechanism(s) which render AHR nuclear and design its function in absence of exogenous ligands. We found that AHR is sensitive to cAMP signaling mediated by cAMP-dependent protein kinase (PKA) which fundamentally differs from AHR signaling mediated by the exogenous ligand dioxin. It has been shown that PKA mediated signaling can be confined by compartmentalization of signaling components in microdomains conferring specificity to signaling by the ubiquitous second messenger cAMP. Moreover, A-kinase-anchoring proteins (AKAPs) and newly discovered cAMP receptors, Epac (*e*xchange *p*rotein directly *a*ctivated by *c*AMP), may give us a further chance to enter into new dimensions of cAMP signal transmissions that potentially may bring us closer to AHR physiology.



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## 1. Introduction

The AH receptor (AHR) has an ancient origin, dating back to the most primitive organisms evolved >550 million years ago, and is conserved in vertebrates and invertebrates, indicating its important function through evolution [1]. The *Drosophila spineless* (*ss*) gene, the closest known relative to the mammalian *AHR* gene, controls development of the antennae, bristles, tarsal regions of the legs [2] and the retinal photoreceptor pattern which is required for vision [3]. The vertebrate AHR took on functions to sense and transmit signals of certain classes of environmental toxicants. It binds many planar compounds [4–6], including polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene and many halogenated aromatic compounds including 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (interchangeably abbreviated as TCDD or dioxin; the abbreviation dioxin will be used in this review). Generally unfavorable environmental conditions e.g. stress factors, evoke an appropriate reaction in a living organism. In case where transcription is controlled by extracellular signals, at least one component of the signaling system has to be transported from cytoplasm to the nucleus in a signal-dependent fashion [7]. This is also the case for the ligand-regulated AHR which in response to the toxin dioxin translocates to the nucleus [8]. A peculiarity of this ligand/receptor system is the potential to – beside transmission of exogenous ligand induced signaling – be engaged in the regulation of life- and health important physiological processes e.g. liver and vascular system development and maturation of the immune system [9–11]. Evidence is available that cellular function of mammalian AHR, and thus also its nuclear localization is also controlled by endogenous systems which may be operating via low affinity ligands e.g. certain prostaglandins, ITE (an agonist from porcine lung), equilenin (an equine estrogen), heme metabolites or specific endogenous activator(s) e.g. metabolites of cytochrome P450(CYP)1A1, extensively reviewed in [12,13]. However, such signaling could also operate via low molecular weight molecules of second messenger systems.

According to the dynamic view of signal transduction known as a "softwired signaling concept" the cellular localization of signaling molecules can be discretely regulated and rapidly reversible [14]. In this concept the critical point in the signal transmission processes is, that signaling proteins translocate within the different cellular compartments and subcompartments, and undergo reversible protein interactions [14], both processes typically phosphorylation/dephosphorylation dependent [15]. One of the most spectacular examples for dynamic translocation has been observed for classical protein kinase C isoforms, which translocate to the plasma membrane within less then 1 s of an increase in calcium concentration and still rapidly exchange between cytosol and plasma membrane at elevated calcium concentrations [14].

A unique role in dynamic and spatial signal transduction processes is given to the universal second messenger cAMP. Its discovery by Earl Sutherland in 1957, and the elucidation of its role in hormone action [16] was a milestone in the field of signal transduction, and opened a new era for systems biology research [17]. The spatio-temporal dynamics of cAMP signaling rely on two processes: its synthesis by adenylate cyclases, and its inactivation through phosphodiesterases (PDEs) [18,19]. The observation of the existence of two distinct zones of adenylate cyclase localization led to the hypothesis that discrete cAMP microdomains exist, and that different pools of cAMP can be generated in response to extracellular or intrinsic signals [20–23 and references therein]. As an example: in cardiac myocytes, activation of β-adrenergic receptor correlated with activation of the particulate or membrane bound cAMP-dependent protein kinase (PKA), whereas prostaglandin E1 elevated cAMP in the soluble fraction leading to the activation of PKA in the soluble pool, which had no functional consequences [24]. Thus different subcellular sites appear to be crucial for specific cAMP actions [25]. For a long time it was thought that protein kinase A is the only effector for cAMP [26]. Now we know that cAMP mediates extracellular and intracellular signals by activation of various intracellular effectors

such as cyclic nucleotide-gated ion channels [27], and the guanine nucleotide exchange proteins activated by cAMP (Epac) [28,29]. It is amazing that via such limited numbers of effectors cAMP regulates a vast array of pleiotropic cellular activities and biochemical processes: proliferation, differentiation, apoptosis, neoplastic transformation, immune defence, migration, cardiac contractility, memory, circadian rhythm, insulin secretion, exocytosis, gene transcription and metabolism [30–33 and references therein]. One may ask the question: how is specificity achieved? The answer was given by the discovery that the second messenger cAMP is restricted in its diffusion and thus compartmentalized [33].

In this review we present a link between two old evolutionary highly conserved signaling pathways: first represented by the universal cAMP second messenger system that governs fundamental cell functions, and second, the AHR mediated signaling that shares some of these functions, and astonishingly has been selected by the evolution to accept as activators for its signaling, small-molecular mass planar pollutants such as dioxin.

## 2. Known functions of AHR

AHR signal transduction systems provide a fascinating array of numerous interacting components that sense changes caused by a variety of environmental and intracellular signals, and transmit them to different cellular supervisory systems resulting in modulation of physiology, behaviour or adaptive changes in metabolism of a cell [34–36].

A significant amount of mechanism-based data are available in respect to AHR in presence of exogenous ligands such as dioxin, and less about its signaling during development and in the cell homeostasis, implying a great complexity of cross-talk between various pathways modulated by AHR [37–39].

The point was made that lack of AHR agonists in invertebrates and its participation in developmental processes almost exclude the possibility that AHR was given us by the evolution to serve as an adaptor to environmental pollutions [13].

The principle evidence that AHR participates in important physiological functions is supported by the following observations: (i) high conservation through evolution; (ii) developmental abnormalities and pathology in mice missing AHR; (iii) function in ontogeny [13]. In the developing mouse embryo, nuclear localization of AHR and activation of an AHR target gene (*Cyp1a1*) during defined stages of embryonic development has been reported [40–43], and cross-talk of AHR with multiple developmental pathways was shown [37,39 and references therein]. Also in human tissues high expression of *CYP1A1* in the fetal adrenal, lung and liver has been reported [44]. All these findings support not only participation of AHR in developmental programs but also infer the existence of specific endogenous activator(s) or ligand(s) that provide AHR spatio-temporal signal(s) for its physiological duties, and indeed, much work was done searching for endogenous activator(s) and/or ligand(s) for AHR [12,13,45 and references therein].

An exciting feature of mammalian AHR is its functional diversity. Its important function in the regulation of liver and vascular system development, maturation of the immune system, regulation of genes involved in cellular growth and cell differentiation, nephrogenesis, retinoic acid and eicosanoids metabolism as well as participation in circadian rhythm and aging has been shown [9–11,45–51]. Such pleiotropic function requires high functional specificity which could be achieved by selective interactions with regulatory proteins of distinct signaling pathways reviewed in [37,39,48–51].

## 3. AHR/HSP90/XAP2/p23 complex and nuclear translocation of AHR

### 3.1. *Classical exogenous ligand-dependent AHR signaling*

The analysis of the molecular steps involved in the responses of AHR to a variety of stimuli has shown that a crucial event in the function of AHR is its nuclear translocation [52]. It has been shown that ligand free receptor shuttles between cytoplasm and nucleus [53]. In the absence of any added exogenous ligand or modulator the great majority of AHR is spread out in the cytoplasm, or depending on the cell type evenly distributed between the cytoplasm and the nucleus [54–55]. In the cytoplasm AHR is anchored with two molecules of chaperone HSP90, co-chaperone 23, an immunophilin-like protein also called ARA9, XAP2 or AIP1, and with the protein tyrosine kinase $pp60^{src}$, reviewed in [13 and references therein, 56].

The prevailing model holds that the constitutive association of AHR with the cytosolic compartment is due to XAP2 binding [57,58]. Ligand (dioxin) binding initiates conformational changes of AHR leading to dissociation of XAP2 and p23 from the AHR complex and activates the protein tyrosine kinase $pp60^{src}$ [58,59]. Multi-protein interactions are involved in keeping AHR cytoplasmic in the absence of ligand including binding to the actin filaments of the cytoskeleton via XAP2 [57,58]. Conformational changes in AHR which uncover the nuclear localization signal (NLS) result in rapid translocation of AHR to the nucleus [60,60a]. A short LxxLL motif (L is leucine and x is any amino acid) at AhR (50–54) also promotes the efficiency of nuclear localization in the absence of ligand without altering HSP90 and ARA9 binding or nuclear export activity [60b].

Once in the nucleus AHR forms a heterodimer with the bHLH-PAS protein ARNT (aryl hydrocarbon nuclear translocator), and binds to specific enhancer sequences called ''dioxin- or xenobiotic responsive element'' (DRE or XRE) adjacent to the promoter of target genes [8,61]. This association is essential for transcriptional activation of AH gene battery encoding several xenobiotic metabolizing enzymes. The top six genes activated in this way are: *Cyp1a1, Cyp1a2, Cyp1b1, glutathione S-transferase Ya, NADPH/quinone oxidoreductase and aldehyde dehydrogenase 3* [8]. Some of them, especially *Cyp1a1,* serve until now as a model to study AHR-dioxin signaling. In this way, the activation of the AHR by exogenous ligands, especially if they are long-lived and bind with high affinity such as dioxin, disturbs cellular homeostasis resulting in toxicity and health disorders [45,62,62a,b]. All components of the AHR nucleocytosolic shuttling system presented above are of prime importance for its intracellular mobility and

signaling. However transmission and modulation of AHR signaling can also be subject to exquisite control by reversible covalent modification e.g. phosphorylation versus dephosphorylation or other modulatory signals.

### 3.2. *Modulation of AHR signaling independent of exogenous ligand*

It has been reported that dioxin-free AHR (recombinant or native) is present in the nucleus after treatment with geldanamycin (a disruptor of the AHR/HSP90 association), MG132 (an inhibitor of 26S proteasome), or leptomycin (an inhibitor of AHR nuclear export). There is still some controversy whether such nuclear form of AHR activates or does not activate AHR/ARNT-dependent transcription of, e.g., *CYP1A1/CYP1A2* [63,63a–c]. With respect to proteasome activity the involvement of PKA has been shown. Proteolytic activity of 26S proteasome is ATP- and ubiquitin-dependent. O-GlcNAcylation of Rpt2, a proteasome-associated ATPase, shuts off the proteasome by the inhibition of ATPase activity and thus the function of proteasome is coupled to glucose metabolism. It has been found that another metabolic control of the proteasome is operating via PKA [64]. In contrast to O-Glc-NAcylation, PKA activates proteasomes both in vitro and in vivo in association with the phosphorylation at Ser(120) of Rpt6. Mutation of Ser(120) to Ala blocked proteasome function [64]. Thus compounds like MG132 could possibly interfere with PKA activity. Interestingly, for the glucocorticoid receptor-interacting protein (GRIP1) (nuclear receptor coactivator) subcellular localization and ubiquitin-proteasome-mediated degradation has been shown to be regulated by PKA [64a]. This study also has shown that the proteasome inhibitors MG132 and lactacystin abolished the PKA-mediated degradation of GRIP1. A very interesting observation came from the Puga laboratory studying the relationship between CYP1A1 activity and AHR activation using CYP1A1-deficient mouse hepatoma c37 cells and CYP1A1- and AHR-deficient African green monkey kidney CV-1 cells [65]. Chang and Puga have shown that c37 cells that had not been exposed to exogenous AHR ligands already contained transcriptionally active AHR-ARNT complexes. A similar finding was also observed in wild-type Hepa-1 cells treated with Ellipticine, a CYP1A1 inhibitor. In CV-1 cells, transient expression of AHR and ARNT resulted in high levels of AHR-ARNT-dependent luciferase gene expression even in the absence of an exogenous agonist. Moreover, elevated reporter gene expression correlated with constitutive nuclear localization of the AHR. These authors suggested that a CYP1A1 substrate, accumulating in cells lacking CYP1A1 enzymatic activity, is an AHR ligand responsible for endogenous activation of the AHR [65]. The Kawajiri group provided another example indicating a ligand-independent AHR nuclear translocation by showing that cell density (but not the cell cycle) influenced the subcellular localization of AHR in a keratinocyte cell line, HaCaT [66]. This study has shown that the AHR was predominantly nuclear at sparse cell densities, both nuclear and cytoplasmic at subconfluence, and predominantly cytoplasmic at confluence. Moreover, an association between XRE-mediated transcription and AHR relocalization has been shown. These authors also indicate that the p38 MAPK-mediated phosphorylation of the nuclear export signal and its dephosphorylation (sensitive to cell–cell contact signals) may play an important role in the AHR relocalization [66]. Sadek and Allen-Hoffmann have shown that suspension of wild-type Hepa 1c1c7 cells led to an induction of CYP1A1 mRNA, similar to that observed by treatment of adherent cells with dioxin [66a]. Mutants (defective in AHR signal transduction) of Hepa 1c1c7 cells showed negligible (Class I) or no (Class II) suspension-mediated induction of CYP1A1 mRNA. Gel mobility shift analysis of nuclear extracts from suspended or dioxin-treated wild-type Hepa 1c1c7 showed that both treatments resulted in identical shifts in the mobility of an XRE-containing probe, and that the AHR was present in the DNA–protein complex from suspended wild-type Hepa 1c1c7 cells [66a]. Similarly, a particular role of cell suspension in AHR ligand-independent nuclear translocation has also been reported by the Denison laboratory. It has been shown that CYP1A1 mRNA was induced in a variety of cultured rat epithelial cells by suspension, however the induction was transient. Remarkably, the suspension showed activated AHR, as detected in the mobility shift assays. The authors suggest that down-regulation appears to be mediated by a novel short-lived protein induced or activated by suspension [66b]. Another interesting point considering AHR ligand-independent nuclear localization came from the work by Ramadoss and Perdew [66c]. Their study has shown that the C-terminal half of the AHR, containing the transactivation domain, determines the cellular localization of the transiently transfected receptor and influences the ability of XAP2 to inhibit ligand-independent nuclear import of AHR. These authors conclude that the transactivation domain of the AhR influences important biochemical properties of the AHR (cellular localization, nucleocytoplasmic shuttling and their dependence on chaperone properties as well as relative ligand affinity). Moreover, Ramadoss and Perdew indicate that species-specific differences in AHR properties of mouse AHR and human AHR could be due to the degeneracy in the transactivation domain [66c].

In summary, the studies cited above allow for a new concept of intracellular localization of AHR and highlights the AHR as a sensitive target for signals originating from different transduction pathways.

The modulating properties of cAMP and flexibility of its signaling generate interactions between different pathways and integrate signals from distinct receptors. To understand the basis of cAMP-depending AHR biological functions it will be of paramount importance to gain insight into the network of cross communication between cAMP and AHR signals with other signaling pathways including second messenger systems [66d,e], which adds a further level of complexity. The biological relevance of such an enormous network remains to be fully explored.

## 4. Phosphorylation/dephosphorylation related to AHR/HSP90/XAP2 and to AHR signal transmission

### 4.1. *Protein kinase C*

Protein kinase C (PKC) dependent phosphorylation belongs to the most intensively studied signaling events regulating AHR functions, reviewed in [67]. In vivo, AHR was found to be a

phosphoprotein. In vitro, dephosphorylation of the ligand-activated AHR complex or dephosphorylation of either AHR or ARNT prior to assembly inhibited the XRE-binding [68 and references therein]. It has been demonstrated that PKC activates AHR to its DNA binding form and is necessary for *CYP1A1* transcriptional activation [68–72]. In line with this, treatment with dioxin plus the PKC inhibitor staurosporine almost completely blocked dioxin-initiated induction of *CYP1A1* transcription [71]. In contrast, it has also been reported that staurosporine (under conditions where no protein kinase activity was detectable in cytosol) did not inhibit translocation of the liganded AHR to the nucleus nor its binding to DNA [73]. These authors concluded that protein kinase activity does not appear to be necessary for dioxin-dependent AHR transformation and DNA bindig [73].

### 4.2. *Protein tyrosine kinases*

It has been reported that tyrosine phosphorylation on the AHR is required for its DNA binding activity [74]. Although tyrosine 9 is not a phosphoresidue, its presence augments PKC serine(s)/threonine(s) mediated phosphorylation of AHR and is essential for DNA binding of the AHR [75]. On the other hand, very intriguing is a role of the protein tyrosine kinase pp60src in the dioxin mediated signaling. It has been shown that $pp60^{src}$ protein tyrosine kinase cellular activity is markedly elevated after exposure to dioxin [76]. The same team demonstrated that c-Src protein kinase is associated in the cytosol with the AHR complex along with HSP90 and that upon ligand binding to the AHR, c-Src is activated and released from the complex. This in turn initiates the cascade of protein phosphorylations that ultimately alters functions of many proteins and thus represent a potentially important mechanism by which dioxin can exert rapid, pleiotropic effects through the AHR-associated kinase [59]. The finding that the AHR can be activated by omeprazole (a benzimidazole derivative clinically used as a gastric proton pump inhibitor) which does not bind AHR (but presumably a metabolite of it does bind) raised much interest [77,78]. It has been shown that omeprazole-dependent AHR activation was inhibited by protein tyrosine kinase inhibitors and abolished by mutation of a putative phosphorylation site, Tyr320 to Phe. However, the dioxin-dependent AHR signaling was neither affected by this mutation nor by tyrosine kinase inhibitors [79]. Most recently, Sasamori et al. identified tauCREM (cAMP responsive element modulator, tau-isoform) gene as a candidate gene for the gene responsive to omeprazole in HepG2 cells. The regulatory sequence of Cyp1a1 that responds to tauCREM shows region −60 to −52 bp relative to the transcription start site, and contain also basic transcription element (BTE). siRNA transfection against endogenous CREM inhibited the omeprazole induction of CYP1A1-mRNA in HepG2 cells [79a].

### 4.3. *Protein kinase CK2*

Protein kinase CK2 was identified to phosphorylate several serine residues in XAP2. Mutation of these phosphorylation sites did not affect interaction with AHR. However XAP2 S53A was unable to enter the nucleus what indicates a potential role of phosphorylation in nuclear translocation of XAP2 itself [80]. Another important component of AHR cytosolic complex, HSP90 has been found to be phosphorylated at Ser225 and Ser254 of HSP90beta and Ser230 of HSP90alpha. Such modifications of the HSP90 molecule modulate the formation of the functional cytosolic AHR complex [81]. Interestingly, it has been reported that HSP90s are substrates for several protein kinases, PKA and CK2 kinase were found to be the most effective [82].

### 4.4. *Protein kinase A and dioxin signaling*

The PKA is a paradigm for all kinases performing selective interactions with multiple protein substrates [26]. This kinase is activated by a second messenger, cyclic AMP and exerts its function on substrate/or effector proteins by amplifying cAMP signal [83].

It has been shown previously that dioxin interferes with cAMP/PKA signaling. Enan et al. have reported that the immediate effect of dioxin in the human luteinizing granulosa cells was to decrease facilitative glucose transport through a decrease in cAMP signaling [84]. Vogel et al. has shown in mouse embryonic fibroblasts (C3H10T) and Hepa 1c1c7 that induction of *C/EBPβ* (CCAAT/enhancer-binding protein) gene transcription by TCDD is associated with elevated levels of cAMP and stimulation of a PKA-dependent pathway [84a]. The fact that exposure to dioxin had an opposite effect on PKA activity in the studies reported by the two groups could be due to the different cellular context in the systems used: human versus rodent or steroidogenic versus non-steroidogenic or both, or even due to the diurnal rhythm influence. Highly interesting was the finding reported by Schibler and coworkers [84b] and by Pelkonen and coworkers [84c] groups that the expression of CYP2A5 (but not CYP1A1/2 and not CYP2B10) follows a day/night rhythm, being at the lowest level in the morning and peaking in the evening [84b,84c], and that diurnal clock controls cAMP fluctuation [84b and references therein]. In connection with these observations it is important to mention that recently the cAMP-dependent signaling was discovered as a core component of the mammalian circadian pacemaker [31].

The Matsumura laboratory reported, using 3T3-L1 cells as a model system, that dioxin inhibits adipocyte differentiation by disrupting the cAMP signaling pathway [84d]. Since severe wasting (hallmark of dioxin-induced toxicity in experimental animals) is striking in adipose tissue this finding brought us closer to the networks of signaling that should be expanded to study new interactions involved in disease. Dioxin also interferes with cAMP-dependent differentiation of rat C6 glial cells to astrocytes. This could give a possible link to adverse effects of dioxin on the development of the central nervous system [85]. Also dioxin reproductive toxicity has a link to PKA signaling. It has been found that dioxin changes the function of steroid-producing human luteinizing granulosa cells, and that the effect of doxin on steroid production is mediated through PKA [85a]. Support for involvement of cAMP/PKA in modulation the AHR and AHR-dependent signaling was provided by the finding that stimulation with cAMP results in positive or negative regulation of the expression of the AHR gene itself depending on tissue and cell type [85b]. C/EBPbeta (associated with toxic responses of dioxin such as wasting

syndrome, diabetes, and inhibition of adipocyte differentiation) is another example for dioxin and cAMP/PKA cross signaling. The above presented studies pointed out an active signaling role of cAMP/PKA for AHR. The second messenger cAMP could be involved in a switch mechanism that controls AHR function: on one hand relying on PKA-dependent signaling, and on the other hand shifting to and relying on ''ligand-like'' molecules potentially activating distinct signaling pathways depending on presence versus absence of exogenous or endogenous ligands, and on e.g. cell type or cell cycle phase [66d and references therein, 86 and references therein] thereby securing the dynamic behaviour of AHR [86a and references therein].

### 4.5. *Protein kinase A and modulation of Cyp1A1*

The classical mechanism for Cyp1A1 induction, as described in Section 1, requires ligand binding to the AHR with subsequent nuclear translocation of AHR, heterodimerization with ARNT and binding of AHR/ARNT to DRE in the enhancer region in *Cyp 1A1* gene and other Ah receptor battery genes [8]. The Jefcoate laboratory showed cell type specific and cAMP-dependent modulations of expression of CYP1B1. *CYP1B1* luciferase reporters responded cell type selectively to cAMP and TCDD: adrenal Y-1 cells (only cAMP), testis MA10 cells (cAMP > TCDD), and C3H10T1/2 mouse embryo fibroblasts (only TCDD). MA10 cells exhibited cAMP-dependent AHR down-regulation and AHR/ARNT complex formation. CYP1B1 was found to be unique among AHR-dependent CYPs in exhibiting induction responses mediated by both, the AHR and cAMP [87]. There also are other genes (not only CYPs) that have been found to be regulated by both AhR and cAMP such as COX2 [88,88a,b] and AREG (the EGF-like factor amphiregulin) [88c,d]. In our study [89] intracellular elevation of intracellular of cAMP led to the nuclear translocation of AHR (in absence of exogenous agonists). However, paradoxically, the subsequent step of the ''classical'' pathway, dimerization with ARNT, did not occur, and ARNT was also not present on DRE of *Cyp1A1* gene. Moreover, in the reporter assay cAMP did not influence the basal transcriptional activity of the *Cyp1A1* gene but clearly had an inhibitory effect on dioxin-induced transcriptional activation [89]. An opposite effect of cAMP on basal or inducible Cyp1A1 expression and activity have been also reported, however without investigating whether AHR translocates to the nucleus, and whether AHR and ARNT form a heterodimer. In the studies by Nemoto and Sakurai compounds that increase the intracellular level of cAMP, provoked a significant enhancement of both basal and methylcholanthrene (MCA)-induced 7-ethoxycoumarin-O-deethylase, and MCA-induced aryl hydrocarbon hydroxylase and 7-methoxyresorufin-O-demethylase activities in spheroid (multi-cellular aggregate) primary culture [90]. However, basal and MCA-induced CYP1A2 mRNA expression (also an Ah receptor battery regulated gene) were not affected by altering intracellular cyclic nucleotide content [90]. The Kaminsky group has reported that the treatment of intestinal cells with dbcAMP resulted in a two-fold increase in the extent of beta-naphthoflavone induction at both RNA and protein levels, with corresponding increases in CYP1A1 enzymatic activity. In conclusion this group suggests that PKA participates in AHR-mediated induction of CYP1A1 in the intestinal cells [90a]. The Prough laboratory has found that dbcAMP (and also forskolin), strongly inhibited both PAH induction and PKA inhibitor-dependent potentiation of PAH induction of aldehyde dehydrogenase 3 expression but had no effect on induction of CYP1A1 expression in cultured hepatocytes. The same group has demonstrated the existence of a negative regulatory region in the 5′-flanking region between −1057 and −991 base pairs most probably being responsible for the cAMP-dependent regulation of the aldehyde dehydrogenase 3 gene under both basal and PAH-induced conditions [90b]. In connection with the Prough study it should be mentioned that differential regulations of promoters (as response to PKA activation) show genes being under transcriptional control of steroidogenic factor 1 (SF 1), an integral components of the hypothalamic–pituitary–gonadal axis [90c and references therein]. Hackert has found that stimulation of the cAMP pathway differentially regulates SF-1 activation of the follicle-stimulating hormone receptor (FSHR) and equine luteinizing hormone (LH) beta promoters [90c]. This work also revealed that activation of PKA leads to inhibition of SF-1-stimulated transcription of FSHR, while it synergized with SF-1 to activate the equine LH beta. Importantly, for the activation of the FSHR promoter by SF-1 an E box element is essential that binds transcriptional regulators USF1 and USF2 (upstream stimulatory factors 1 and 2) but not SF-1 [90c]. Thus, it is conceivable that PKA may inhibit binding of USF1/USF2 to the E-box element of FSHR promoter. Interestingly, studies from the Fujii-Kuriyama laboratory have shown inhibition of the transcription of *Cyp1A1* gene by the USF1 in rabbits as a result of competitive binding of USF1 with AHR/ARNT complex to DRE [90d].

Although parts of the signaling pathways are clearly distinct, cAMP and dioxin signals converge at certain step/s to influence downstream functions such as *CYP1A1* expression and that point awaits further investigation.

## 5. Intracellular signaling via second messenger cAMP system

Appreciable amount of data indicates that many signal transduction processes utilize colocalization of sequentially acting signaling proteins for the selective activation of downstream functions. Activation of signaling proteins may occur by upstream activators, or activation of downstream effectors via interactions with adaptor complexes, cytoskeletal structures, and subcellular membranes or by direct interaction with the targets and activators [14,91]. These processes are tightly controlled and rapidly reversible allowing for a dynamic view of signal transduction which has been called ''softwired signaling concept''. According to this concept the translocation of signaling proteins and their reversible binding interactions are central to the selective signal transmission [14]. In this sense the question arose whether such universal molecules like cAMP operating in space and time can direct AHR to a specific subcellular localization using its cellular effector e.g. PKA.

### 5.1. *cAMP and cAMP-dependent kinase (PKA)*

The generation of cAMP from ATP occurs upon ligand (first messengers) hormones, neuromediators, odorants or mediators

binding to Gs protein-coupled receptors and the consequent activation of a family of transmembrane adenylyl cyclases (ACs) localized at the plasma membrane [92]. An alternative source of cAMP is soluble AC, that has been shown to localize in different subcellular compartments, the activity of which is independent of Gs protein-coupled receptors stimulation and is regulated by bicarbonate and calcium ions [93]. cAMP represents one of the most studied molecules in signal transduction. The fantastic feature of a second messenger system is that a wide array of first messengers is converted onto a single second messenger which transmits their signal further in the cytosol and nucleus [83]. PKA is composed of two functionally different subunits: the catalytic (C) and regulatory (R) subunit [26]. For the optimal catalytic activity the Thr197 in the activation loop of subunit C is required and needs to be phosphorylated [94]. cAMP activates PKA by binding to the R subunit, which results in a conformational change in the R subunit and dissociation of the R from the C subunit [26]. The free subunit C is active and phosphorylates a big variety of cytoplasmic and nuclear substrates [95]. The spatial organization of cAMP signaling in restricted areas (microdomains) is decisive for the specificity of PKA signaling [25]. Such domains include receptors, effectors, modulators and targets supporting interactions of components within the particular domain in response to cAMP [33]. A family of functionally related proteins known as A-kinase-anchoring proteins (PKA-anchoring proteins, AKAPs) play an indispensable role in the organization of PKA intracellular signaling ''microdomains'' [96]. They deliver and anchor PKA to distinct compartments of the cell (plasma membrane, nucleus, mitochondria, cytoskeleton) [97] in the vicinity of specific targets [33,96]. Moreover, AKAPs also interact with phosphodiesterases (PDE) which brake down cAMP and serve as a terminator of cAMP signaling. Compartment specific termination of cAMP signaling via cAMP specific PDE, contributes to a precisely controlled signaling system [33,96].

### 5.2. *Epac*

cAMP was thought for a long time to act by binding and activating solely PKA. Later on additional intracellular receptors for cAMP have been discovered including cyclic nucleotide-gated ion channels and PDEs [27]. However, discovery of Epac (*e*xchange *p*rotein directly *a*ctivated by cAMP) revolutionized the view about cAMP-dependent dynamic signal transduction leading to a new level for studying and understanding cAMP-mediated signal transduction [28,29]. Epac contains a cAMP-binding domain that is homologous to the R subunit of PKA and a guanine exchange factor domain, and mediates cAMP signaling using its cAMP-dependent guanine nucleotide exchange activity towards a small guanosine triphosphatase, Rap1 [28,29]. It is proposed that many cAMP functions previously associated with cAMP-dependent PKA are also Epac-dependent and that the net outcome of cAMP signaling is dependent on the dynamic abundance and distribution of intracellular Epac and PKA [98,99].

### 5.3. *Phosphodiesterases (PDEs)*

PDEs play a crucial role in the termination and spatial segregation of cyclic nucleotide signals and thus are important contributors to intracellular gradients of cAMP [100]. The PDE4 enzyme family consists of four subfamilies (4A, 4B, 4C, 4D). All PDE4 family members hydrolyze specifically cAMP [101,102]. The activity of the 'long' PDE4 isoforms (16 isoforms from four genes) is stimulated by PKA-dependent phosphorylation [101]. Specific PDE4 long isoforms and PKA can assemble with AKAPs in the perinuclear and centrosomal regions of cells to reduce the activation of cAMP targets [103]. Using cyclic nucleotide-gated channels to monitor cAMP levels it has been shown that under physiological conditions an AKAP/PKA/PDE4 complex controls the dynamic changes of the cAMP levels at the plasma membrane [100].

## 6. Modular function of protein interaction domains and cell signaling

For the regulation of many cellular processes regulatory proteins are often constructed in a cassette-like fashion that create interaction domains required to direct the interaction with other proteins, phospholipids, small molecules or nucleic acids. Interaction domains can target proteins to a specific subcellular location, provide means for recognition of protein posttranslational modifications or control the conformation and initiate the formation of multiprotein signaling complexes [104]. Intracellular signaling proteins often consist of a PAS or GAF sensor domain and various output domains [104].

### 6.1. *PAS and GAF domains*

PAS domains (named after their occurrence in the circadian protein *p*eriod, in the *a*ryl hydrocarbon receptor nuclear translocator and in the protein *s*ingle-minded) and GAF domains (named after their occurrence in c*G*MP-phosphodiesterases, *a*denylyl cyclases and *F*hlA [formate hydrogen lyase transcriptional activator]) are small-molecule binding domains present in many signal transduction proteins in organisms from all phyla [35,36,105,106]. They have similar structures, characterized by the presence of a ligand-binding pocket that can bind a variety of low molecular mass ligands [86,106,107]. PAS domains are important for conserved physiological processes like neurogenesis, myogenesis, circadian rhythm and cellular differentiation, homeostatic response to hypoxia, nuclear hormone receptor function [35,36 and references therein, 41,48], and in AHR also bind exogenous ligands such as dioxin and are important for toxin metabolism. PAS domains preferentially bind flat molecules including polycyclic aromatic hydrocarbons such as benzo[*a*]-pyrene, heme, flavin, cinnamic acid and the adenine moiety of ATP [86]. GAF domains bind formate, 2-oxoglutarate, some aromatic compounds including tetrapyrroles and photopigments, as well as cyclic nucleotides [105]. Importantly, GAF domains of both, adenylyl cyclase and phosphodiesterases bind cAMP. This could represent a feedback mechanism for maintaining cAMP levels [86,108]. The existence of GAF domains in cAMP stimulated adenylyl cyclase from Anabaena suggests a mechanism conserved across two billion years of evolution [106].

## 7. AHR signaling in presence of second messenger cAMP

We have shown that phosphorylation of several CYPs provides a very fast mean to profoundly modulate their activities (acting like a switch) with far-reaching consequences of CYP functions [109–115]. An especially interesting observation was that the inhibition of phosphoprotein phosphatases led to decreases of CYP-mediated mutagenicities by aromatic amines by more than 80% while in the CYPs known to be involved in metabolic activation of aromatic amines (CYP family 1) no incorporation of phosphate could be detected [111]. This implied a phosphorylation-mediated control not by posttranscriptional modification of these specific CYPs but in this case rather in their actual quantities possibly controlled by a phosphorylation-mediated modulation of the transcription factor AHR or of some of its associated proteins or of its transactivation potential.

### 7.1. Nuclear translocation

We found that in absence of exogenous ligand, an elevation of the intracellular second messenger cAMP, led to translocation of AHR from cytosol to the nucleus [89]. This was shown by treatment of Hepa 1c1c7 (''Hepa1'') cells, in which a fusion protein of AHR and green fluorescent protein (GFP) was expressed. The cells were treated with $N^6$-$O^{2'}$-dibutyryl-cAMP (db-cAMP), a membrane-permeating derivative of cAMP or forskolin, a potent activator of adenylate cyclase, known to rapidly increase intracellular cAMP level. Both treatments led to the nuclear translocation oft the GFP-tagged AHR. It was also shown that the constitutive AHR of the Hepa 1 cells visualized by indirect immunofluorescence migrated to the nucleus upon these treatments. Already at the shortest time point investigated, 30 min of treatment, the shift was pronounced and persisted during 60, 90 and 120 min of treatment, but started to fade at these later time points [89].

### 7.2. Protein–DNA interaction

The elevation of the intracellular cAMP led to binding of AHR to the mouse *Cyp1A1* enhancer, containing a dioxin-responsive element (DRE) with the AHR/ARNT-binding motif (5′-GCGTG-3′) [89 and references therein]. In an electrophoretic mobility shift assay the AHR complex generated by treatment with db-cAMP or forskolin migrated with a similar mobility as the dioxin-induced complex. Specificity of the binding was shown by the elimination of the binding to the radioactive oligonucleotide by a 50-fold molar excess of unlabeled DRE. However, a 50-fold molar excess of unlabeled oligonucleotide carrying a base mutation T to C in the AHR/ARNT consensus motif 5′-GCGCG-3′ (mDRE) only marginally reduced the intensity of the dioxin-dependent signal, while binding of db-cAMP- or forskolin-dependent complex was substantially reduced. When equivalent amounts of dioxin- or db-cAMP-induced nuclear proteins were probed with $^{32}$P-labeled DRE, this T-to-C mutation fully eliminated binding of the dioxin-dependent complex, whereas the binding of the db-cAMP-induced complex was reduced but clearly present [89]. These findings indicate that the binding site of cAMP-induced nuclear proteins on DRE is not identical with the binding site of dioxin-activated AHR/ARNT (5′-GCGTG-3′), the T being supportive but not a strict requirement for the binding of the cAMP-induced nuclear proteins while for the dioxin-activated AHR/ARNT complex it is a strict requirement. The mutated base is in the E-box half site (5′-GTG-3′) of the consensus sequence that binds ARNT within the dioxin-activated AHR/ARNT heterodimer [89]. Hence, one possibility is that the treatment with db-cAMP or forskolin leads to a higher affinity of the AHR for an ARNT-related protein other that ARNT itself, and that this partner of AHR has a similar but less stringent sequence specificity within the E-box half site of the DRE consensus sequence.

### 7.3. cAMP-dependent AHR binding to DNA without AHR/ARNT interaction

If cAMP-induced nuclear translocation of AHR is mediated by PKA, then PKA inhibition should block cAMP-dependent nuclear effects of AHR. Indeed, the PKA inhibitor H89 [*N*-(2-(*p*-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride] abolished the binding of forskolin-induced complex to the DRE-containing oligonucleotide. Immunoprecipitation experiments showed no ARNT (heterodimer essential for dioxin-dependent *Cyp1A1* induction) in the db-cAMP/forskolin-dependent nuclear form of AHR [89].

### 7.4. cAMP activated AHR and Cyp1A1 expression

In order to probe for possible functional consequences of the cAMP-dependent translocation of AHR to the nucleus we examined in Hepa1 cells the activity of the luciferase gene fused to the *Cyp1A1* natural promoter and the endogenous enhancer, containing six DRE copies and also a negative regulatory element (NRE) using the p1646P1Luc3 reporter plasmid [89,and references thererein]. In contrast to dioxin, db-cAMP or forskolin did not lead to activation of the reporter gene, but interfered with the activation of the reporter gene by dioxin: treatment with db-cAMP or forskolin (for 15 min before the treatment with dioxin) led to a clear and reproducible down-regulation of dioxin-dependent induction of the reporter gene transcriptional activity [89]. Thus, cAMP or an event downstream of cAMP may, although leading to nuclear translocation of AHR, act as a repressor rather than an activator of AHR-dependent gene expression. To determine whether the NRE of *Cyp1A1* is involved in cAMP-dependent reduction of luciferase activity, we used a second reporter construct, the pAhRDtkLuc3, lacking the NRE but containing three copies of DRE. Absence of NRE allowed for a much higher dioxin-dependent induction of the reporter gene activity. Again, this activation was reduced by db-cAMP or forskolin (by ≈30–50%) [89]. Thus intracellular elevation of cAMP generates a signal that is inhibitory to the AHR-mediated response to dioxin, and for this inhibitory modulation, the NRE is not required. Depending on the type of cellular signaling (cAMP vs. dioxin), the AHR–DNA complex may recruit different regulators, thereby mediating different effects on transcription. Possible candidates for operating within the repression mechanism of the cAMP-activated AHR could include the inducible cAMP early repressor (transcriptional repressor

ICER) which has been shown to operate in hormone receptor signaling e.g. FSHR [90c and references therein] or alternatively the AHR repressor (AHRR). The Fujii-Kuriyama group has reported that AHRR plays an important role in the regulation of AHR function by competing with AHR for dimerizing with ARNT, and binding to the XRE sequence [116]. The Hahn group has shown that repression of AHR-mediated transactivation by AHRR requires the N-terminal portion of AHRR. Moreover, they indicate that AHRR does not compete for ARNT, and does not require binding to XRE to fulfill its repressor function, although binding to XRE contributes to the repression. The Hahn group suggests a mechanism of AHRR action that involves ''transrepression'' of AHR signaling most probably via protein–protein interactions rather than by inhibition of AHR/ARNT heterodimer formation or binding to DNA. Interestingly, and rather unexpectedly, cAMP activates nuclear translocation of AHR that can inhibit to a certain extent the dioxin inducible CYP1A1 expression, however cAMP-activated AHR does not complex with ARNT [116a]. Thus, it is conceivable that cAMP signaling may involve AHR repressor at least with respect to *CYP1A1* gene regulation. Nevertheless, such relationship requires further studies.

In summary, there is here a clear functional separation between the AHR activation by dioxin and by cAMP. We suggest that cAMP-mediated activation of the AHR reflects the endogenous signaling of AHR essential for the cell physiology. Contrary, dioxin impedes that signaling by binding chronically to the AHR for days, weeks, or months, what might be central in dioxin toxicity. Importantly, the cAMP-activated AHR shows inhibitory effects on CYP1A1 induction by dioxin, probably by inducing a formation of new regulatory complexes with repressive function. The ability to forge such new interactions, and thus to reprogram dioxin-deviated cellular behaviour, could also be a strategy to counteract dioxin toxicity.

### 7.5. *Interaction of XAP2 with PDE and its potential relationship with cAMP-dependent AHR nuclear translocation*

Our finding that AHR responds to the second messenger cAMP by moving to the nucleus was exciting but at that time two points were missing: first, what mechanism is steering the cAMP-dependent AHR translocation; and second, what – if any – is the dimerization partner for the cAMP-dependent nuclear form of AHR because in our study ARNT apparently was not [89]. We have got a great support from subsequent studies of Smolenski and coworkers [117] and Matsumura and coworkers [118] groups investigating transmission of cAMP-dependent AHR signaling.

It has been found that the cyclic AMP-specific phosphodiesterase (PDE4) isoform PDE4A5 interacted with the co-chaperone XAP2 (essential unit of cytosolic AHR complex). The interaction was specific, in that PDE4A5 did not interact with the closely related immunophilins AIPL1, FKBP51, or FKBP52. XAP2 also did not interact with other PDE4A isoforms or typical isoforms from the other PDE4 subfamilies [119]. In respect to function, XAP2 reversibly inhibited the enzymatic activity of PDE4A5, increased the sensitivity of PDE4A5 to inhibition by the prototypical PDE4 inhibitor 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram) and attenuated the ability of PKA to phosphorylate PDE4A5 in intact cells [119]. Although the authors did not investigate a potential link with AHR signaling this finding is important with respect to the topic of this review since the PDE4A5 is a cAMP specific phosphodiesterase and XAP2 is important for the retention of AHR in the cytosol.

The Smolenski group tested the effects of PDE2A on cAMP-mediated nuclear translocation of the AHR [117]. In agreement with our finding [89] they found that forskolin (activator of adenylyl cyclase) and 8-Br-cAMP (a membrane-permeating cAMP analog) induced nuclear translocation of AHR in Hepa1c1c7 cells. They have shown that expression of PDE2A reduced both cAMP/forskolin and dioxin-dependent translocation of AHR, and that the observed effect is due to the interaction between XAP2 and PDE2A. The XAP2 binding site of PDE2A has been mapped to the GAF-B domain. This fascinating finding indicates that PDE2A may serve as a regulatory component of the AHR cytosolic complex. Although in vitro binding of purified XAP2 to purified PDE2A did not change PDE2A catalytic activity, the authors propose that this interaction could be responsible for AHR cytosolic retention by lowering the local cAMP concentrations under a level required for AhR complex translocation [117].

### 7.6. *RelB as a nuclear partner for cAMP-activated AHR*

A great complementation and support for our work was the identification by the Matsumura's laboratory of the RelB as a partner for AHR [118]. They found that activation of AHR by dioxin or forskolin results in the interaction of AHR with RelB and modulation of IL-8 gene transcription via a novel RelBAhR responsive element (RelBAhRE) in an ARNT independent way which is in agreement with our study that cAMP-activated AHR did not dimerize with ARNT [89]. Moreover, the enhanced recruitment of AHR and binding of RelB/AhR to RelBAhRE induced by forskolin or dioxin requires PKA activity for the full induction of IL-8. In an earlier report the same lab had shown that dioxin treatment is associated with an early increase of PKA activity leading to the induction of C/EBPβ [84a]. Matsumura and colleagues suggest that dioxin induces nuclear translocation of cytosolic AHR through an elevation of PKA activity, besides the classical well described ligand-dependent activation and nuclear translocation of the AHR, which forms heterodimers with ARNT. They propose further that increased AHR nuclear localization and DNA binding activity induced by forskolin or dioxin is not due to increased AHR synthesis but rather to a direct phosphorylation of the AHR by PKA. However, Smolenski et al. reported that AHR in their studies was not phosphorylated by PKA [117], and it has been shown that nuclear import of another bHLH transcription factor, important for myogenesis, MyoD requires PKA activity while MyoD itself is not phosphorylated [120]. Hence in principle, also for the control of AHR localization proteins other than AHR itself could be a target for PKA-dependent phosphorylation. Maybe in the context of AHR/RelB interaction and PKA-dependent phosphorylation an interesting point is that the Rel family of proteins (to which RelB belongs) are related through a highly conserved domain of approximately 300 amino acids, the Rel homology domain, that contains a consensus sequence for phosphorylation by PKA

(Arg-Arg-Pro-Ser). The insertion of two amino acids (Pro-Trp) within this sequence resulted in a shift in the localization of c-Rel from cytoplasmic to nuclear in chicken embryo fibroblast [121]. When the conserved Ser within the PKA recognition sequence was replaced by Ala, there was no significant effect on cytoplasmic retention of c-Rel. However, when this Ser was changed to Asp or Glu, c-Rel showed a diffuse nuclear and cytoplasmic localization [121]. This suggests that the cytoplasmic retention of c-Rel is dependent on the structure of the conserved PKA recognition motif, and that the phosphorylation at the conserved PKA site could induce nuclear translocation of c-Rel. The RelB could respond to PKA in a similar fashion so that the activation of the IL8 gene by AHR/RelB could be the sum of two PKA functions: its influence on AHR and on RelB. Matsumura and colleagues create an exciting model for cAMP-activated AHR as a partner of the NF-κB subunit RelB for cooperative regulation of inflammatory genes.

In our hand the binding of the dimer AHR/Partner X [89] as well as in Matsumura's studies the binding of the dimer AHR/RelB to the corresponding responsive element on DNA was diminished but not fully abolished by the PKA inhibitor H89 [118]. It may be speculated that the portion not blocked by H89 may be associated with Epac rather than related to PKA activity.

## 8. Conclusions and considerations

The components of the AHR multiprotein complex are targets for cAMP-dependent second messenger system signaling, providing a new and exciting insight into AHR-dependent signal transduction [89,117,118]. A new level of understanding of cAMP mediated signaling with far-reaching consequences is provided by recent findings on its spatio-temporal dynamics. By now it has been well documented that cAMP signals are compartmentalized to subcellular areas of distinct pools or microdomains [27,28], and thereby provide specificity of PKA signaling [19–22,29].

A crucial role in organization of microdomains play AKAPs which anchor PKA to distinct subcellular locations (e.g. plasma membrane, nucleus, cytoskeleton or mitochondria) in proximity to PKA-specific targets. Importantly, they also serve as scaffolding proteins that assemble PKA together with signal terminators such as phosphoprotein phosphatases and cAMP-specific PDEs, and facilitate cross-talk between signaling pathways [33,122].

In view of the broad PKA substrate specificity compartmentalization of receptors, cyclases and PKA by as well as creation of PDE-dependent local pools of cAMP within the cell are of obvious importance for the specificity of PKA-mediated signaling AKAPs [23,96,122]. The discovery of Epac allowed to further dissect the divergent roles of cAMP in different cell types. Interestingly, it is proposed that actually a great number of cAMP functions which previously had been associated only with PKA activity are in fact also Epac-dependent, and the net outcome of cAMP signaling relies on the dynamic abundance and distribution of Epac and PKA in the cell [98,123].

We propose that for proper cell homeostasis the interactions between AHR and cAMP signaling systems physiologically are under dynamic spatio-temporal control. Further on it is conceivable that cAMP microdomains upon receiving physiological stimuli (e.g. neurohormones, neurotransmitters, prostaglandins) attach dynamically and reversibly to AHR complex creating AHR/cAMP microdomains. Some components associated with cAMP microdomains have already been observed associated with AHR complexes (PKA and PDEA2), while further components such as phosphatases, Epacs and AKAPs may be interesting candidates for future studies. The signal evoked by exogenous AHR ligands such as dioxin may disrupt or convert smoothly operating AHR/cAMP microdomains into dioxin tagged AHR microdomains with sustained signaling that escapes from the control of physiological microdomain regulatory components perturbing cell homeostasis and leading to pathological responses which are not readily reversible. Interestingly, it has been shown that the cAMP microdomains imaged in cardiac myocytes in the presence of catecholamines were abolished in the presence of PDE inhibitors leading to PKA activation throughout the cell [23]. Thus non-physiological stimuli such as dioxin may lead to non-compartmentalized increases of intracellular cAMP activating PKA non-selectively throughout the cell. Such process could be evoked if dioxin directly or indirectly would inhibit non-compartmentalized PDE activity. In fact, an involvement of PDEA2 in cAMP-dependent signaling of AHR through its interaction with XAP2 has been shown [117]. In vitro the activity of this particular PDEA2 in purified form was not affected by this interaction. The in vivo situation may be different or other PDE(s) could be involved. A possible candidate could be the cyclic AMP-specific phosphodiesterase (PDE4) isoform PDE4A5. In fact, it has been demonstrated that PDE4A5 interacts specifically with XAP2 but not with closely related immunophilins. XAP2 also did not interact with other isoforms of PDE4 subfamilies. Importantly, XAP2 inhibited the enzymatic activity of PDE4A5 [119]. However, in these studies the AHR-dependent signaling was not investigated. If the activity of PDE4A5 is inhibited by dioxin, either by stabilizing PDE4A5/XAP2 binding or by any other mechanism then PDE4A5 would not be able to exert its function to inactivate cAMP and PKA activation would be expected. Future studies are expected to identify such AHR-specific cAMP microdomains, and clarify whether distinct individual cAMP microdomains exist for divergent AHR signaling and whether and how cross talks may be organized between them. The development of new tools for direct monitoring of cAMP in single living cells such as fluorescence resonance energy transfer-based imaging (FRET), allowed for detection of cAMP with submicrometer spatial resolution [124,125], and provided the first direct visualization of discrete microdomains of cAMP in cardiac myocytes in response to β-adrenergic receptor stimulation [23]. Such microdomains appeared to be as small as 1 μm and to contain a selected pool of AKAP-anchored activated PKA enzymes [30]. Additionally, based on individual PKA and Epac cAMP-binding domains the development of discriminatory novel fluorescent indicators for cAMP and its cell-and event specific spatio-temporal regulation has been reported [126]. These indicators could be ubiquitously applied to study cAMP-dependent spatio-temporal regulation. Thus with the help of the novel methodology, we envisage a great future to gain new insights into the complexities of this finely tuned cAMP messenger system, its dynamic, compartmentalized

interaction with AHR-depending signal transmission, and simultaneously monitoring spatio-temporal crosstalk with various pathways.

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