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Phosphodiesterases link the Aryl Hydrocarbon Receptor Complex to Cyclic Nucleotide Signaling

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Abstract

The aryl hydrocarbon receptor (AHR) is a major transcription factor regulated by different mechanisms. The classical view of AHR activation by xenobiotics needs to be amended by recent findings on the regulation of AHR by endogenous ligands and by crosstalk with other signaling pathways. In the cytosol the AHR recruits a large number of binding partners, including HSP90, p23, XAP2 and the ubiquitin ligases cullin 4B and CHIP. Furthermore, XAP2 binds the cyclic nucleotide phosphodiesterases PDE2A and PDE4A5. PDE2A inhibits nuclear translocation of AHR suggesting an important regulatory role of cyclic nucleotides in AHR trafficking. Signaling involving cAMP is organized in subcellular compartments and a distinct cAMP compartment might be required for proper AHR mobility and function. We conclude that the AHR complex integrates ligand binding and cyclic nucleotide signaling to generate an adequate transcriptional response.

Keywords: ARA9, AIP, PDE, cAMP, cGMP, dioxin
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1. Introduction

The aryl hydrocarbon receptor (AHR) is a transcription factor that regulates expression levels of many genes. The pattern of cellular functions under control of AHR is quite diverse including detoxification of xenobiotics, inflammatory responses, tissue development and regeneration [1-5]. The AHR can be activated by exogenous compounds such as dioxins, however, endogenous ligands appear to exist and the AHR might be active in the absence of exogenous substances [6-8]. The AHR shares many of its mechanisms of action and regulation with other nuclear receptors or ligand-activated transcription factors. In this article we will focus on the proteins binding to the cytosolic AHR protein. We will describe the functional role of these AHR binding proteins and we will highlight recent findings on the interplay between cyclic nucleotide phosphodiesterases and the AHR protein complex.

2. The arylhydrocarbon receptor

2.1. AHR domain organization

The aryl hydrocarbon receptor (AHR) is encoded by a single gene and no splice variants have been described, so far. It is a ligand-activated transcription factor known to mediate the cellular effects elicited by xenobiotic compounds, including a wide variety of hydrophobic environmental pollutants such as TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxin). The translated protein exhibits an N-terminal basic helix-loop-helix domain (bHLH) required for DNA-binding and heterodimerization. The N-terminus also contains nuclear localization and nuclear export signal sequences. The following two PER-
ARNT-SIM (PAS) domains mediate ligand binding, interaction with other proteins such as HSP90 and might contribute to DNA-binding [9]. The C-terminus holds a transactivation domain (TAD), responsible for the induction of gene transcription. AHR shares this basic structural organization with other transcriptional regulators including the hypoxia inducible factors (HIF) involved in hypoxia signaling, the single-minded (SIM) proteins involved in neurogenesis, and the circadian rhythm proteins CLOCK, PER and BMAL [10]. The AHR is the only member of the bHLH/PAS family known to bind and be activated by small chemical ligands.

2.2. Control of AHR functions

AHR function is controlled at different levels. One level of regulation is constituted by the binding of ligand. Interacting proteins form a second level of control and mediate crosstalk between the AHR and other signaling pathways. Normally, AHR resides in the cytoplasm, in a dormant state, associated with HSP90, XAP2, and p23. The classical pathway for AHR activation is initiated by binding of membrane-permeable ligands, such as TCDD, to AHR. Ligand binding to AHR probably exposes the nuclear localization sequence and the ligand-activated AHR complex translocates into the nucleus and forms a heterodimer with the closely related protein ARNT (AHR nuclear translocator, also known as HIF-1β), with the concurrent loss of HSP90, XAP2, and p23 from the complex [11, 12]. The AHR/ARNT heterodimer forms the functional transcription factor complex that recruits other co-activator molecules and chromatin remodelling enzymes [10, 13].
The AHR/ARNT complex binds to xenobiotic-inducible transcriptional control elements, XREs, on the DNA resulting in the initiation of gene transcription.

A number of studies suggest that exogenous high affinity ligands like TCDD are not necessarily required for AHR function [14-16]. There is evidence for nucleocytoplasmic shuttling of AHR in the absence of exogenous ligand [17-19]. Studies using AHR knockout mice suggest that the developmental role of AHR does not require addition of ligands [20] and TCDD-independent functions of AHR have been observed in different species [21]. Oesch-Bartlomowicz et al. (for additional reading see Oesch-Bartlomowicz 's review elsewhere in this issue) showed that cAMP, in a similar way to TCDD, is able to induce translocation of AHR to the nucleus [22]. Though in this case nuclear AHR did not dimerize with ARNT, but formed a complex with other unidentified proteins. Moreover, cAMP did not induce CYP1A1 reporter gene transcription in an in vitro assay. 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 [22]. These results corroborate the idea that in the absence of TCDD, endogenous ligands might be attached to AHR and alternative cyclic nucleotide regulated pathways might regulate AHR function. Recently, an alternative association of AHR with the nuclear transcription factor RelB was described. Vogel et al. showed that the nuclear AHR, activated by TCDD as well as forskolin, associates with RelB and drives the transcription of pro-inflammatory genes like IL-8, suggesting a new mechanism of crosstalk between AHR, cyclic nucleotides and RelB pathways [23]. The AHR signaling pathway can be down-regulated by two main mechanisms: 1) through the
AHR-induced transcription of a dominant negative bHLH/PAS protein, known as AHR Repressor (AHRR) that seems to compete with AHR for ARNT binding, in that way lowering AHR binding to DNA and AHR-regulated gene transcription [24-26]; 2) through the ligand dependent degradation of AHR protein by the proteasome [27, 28] and possibly other proteases [29].

The cytosolic AHR binds to a number of different proteins that affect nuclear translocation and crosstalk with other signaling pathways. Formation of distinct protein complexes is an important general principle in cellular organization. Protein complexes can help stabilize individual protein components and enable coordination and optimization of cellular processes simply by putting proteins that need to communicate into close proximity of each other, as has been shown extensively for the family of A-kinase anchoring proteins (AKAPs) [30].

3. AHR binding proteins

3.1. HSP90, p23 and E3 ubiquitin ligases

In the cytosol the AHR is attached to a dimer of HSP90 (heat shock protein 90) molecules. This interaction is mediated by the bHLH and PAS domains of AHR [31, 32]. HSP90 belongs to a group of molecular chaperones managing protein folding [33] and appears to be required for proper folding and stability of AHR [34]. HSP90 thus contributes to efficient ligand binding conformation of AHR. Furthermore, HSP90 can repress the ability of AHR to bind to ARNT [35]. The role of HSP90 for AHR stability is comparable to the function of HSP90 for other ligand activated transcription factors such as the steroid receptors [36]. HSP90 associates with p23, a co-chaperone protein
that binds to the ATP-bound form of HSP90 [33] and p23 takes part in the AHR complex [37]. Recently, the E3 ubiquitin ligase CHIP (C-terminal of HSP70 interacting protein) was detected as component of the AHR protein complex [28]. CHIP is a known HSP90 binding protein proposed to mediate ubiquitination leading to proteasomal degradation of other HSP90 binding proteins [33]. Accordingly, CHIP might be involved in the regulation of AHR protein levels. Another E3 ubiquitin ligase detected in the AHR protein complex is the cullin 4B protein, which can mediate degradation of the estrogen receptor alpha after binding of ligand to the AHR [38]. Addition of a high-affinity AHR ligand was observed to induce the binding of damaged-DNA-binding protein 1, and Rbx1, both parts of the cullin 4B-based ubiquitin ligase complex, as well as subunits of the proteasome to AHR [38].Ubiquitination of estrogen receptors is probably facilitated through direct interactions of AHR and the estrogen receptor [38-40].

3.2. XAP2

Another component of the AHR complex is XAP2 (also known as ARA9 or AIP), a 38-kDa protein that was initially identified as a protein binding to the hepatitis B virus X protein [41] and in parallel also as binding partner of AHR [42-44]. XAP2 shares sequence identity with FKBP52, an immunophilin of the family of FK506 binding proteins and an established component of the glucocorticoid receptor complex. Although XAP2 contains an immunophilin homology domain within its N-terminal region, no binding to immunosuppressant drugs was found [42]. The C-terminus of XAP2 contains multiple tetratricopeptide repeat (TPR) motifs that mediate protein-protein
interactions [45]. Thus, XAP2 might serve to regulate the composition of the AHR complex. The TPR motifs and the extreme C-terminus of XAP2 mediate binding to both the AHR and HSP90 [46]. In terms of function, it has been shown that XAP2 enhances the stability of the AHR and that XAP2 can retain AHR in the cytoplasm [47-51]. In addition, XAP2 competes with p23 for binding to the AHR/HSP90 complex [52] and protects AHR from being ubiquitinated, at least in vitro [28]. Association of XAP2 with transcription factors appears to be a rather general phenomenon. XAP2 interacts with hepatitis B virus protein X [41], EBNA-3 (Epstein-Barr-virus encoded nuclear antigen 3) [53] and thyroid hormone receptor [54] besides other molecules, like survivin, an anti-apoptotic molecule that has been implicated not only in preservation of cell viability but also in essential regulatory circuitries of cell division [55]. These versatile roles of XAP2 are reflected in the observation that deletion of the XAP2 gene in mice causes embryonic lethality associated with gross defects in the development of the cardiovascular system [56].

Recently, type 2A and type 4A5 phosphodiesterases (PDE) were identified as new binding partners of XAP2 providing an unexpected molecular link between the AHR and the cyclic nucleotide signaling system [57, 58]. From this point we shall describe some important new aspects of cyclic nucleotide signaling including main cellular functions and crosstalk with the AHR pathway.

4. Role of cyclic nucleotides and phosphodiesterases in AHR function

4.1. Compartments of cyclic nucleotide signaling
The second messengers cAMP and cGMP play central roles in signaling pathways that regulate many physiological responses [59]. The cyclic nucleotides are generated in the cytosol through the action of adenylyl or guanylyl cyclases. Intracellular concentrations of cAMP and cGMP are strictly regulated by the rate of cyclase mediated biosynthesis and PDE mediated degradation. The cAMP formed activates specific effector proteins in the cell. The main cAMP effector is the cAMP-dependent protein kinase holoenzyme (PKA), which phosphorylates substrates in the cytoplasm [60] or migrates to the nucleus to phosphorylate transcription factors such as the cAMP-response element binding protein CREB [61]. Besides PKA, cAMP can exert its cellular effects by binding cAMP-dependent ion channels [62] and EPACs (exchange proteins directly activated by cAMP) that activate the small guanine-nucleotide-binding protein Rap1 [63]. The specificity of cAMP action is conditioned by the organization of protein complexes involved in the signaling pathway. In fact, early reports of subcellular compartmentmentation of cAMP signaling showed that insulin preferentially activated membrane-associated, not cytosolic, cAMP PDE in rodent adipocytes [64] and that different G protein-coupled receptors could selectively activate different PKA subtypes in cardiomyocytes [65]. This hypothesis was consolidated with the discovery of AKAPs (A-kinase-anchoring proteins) as signaling scaffolds that interact with PKA, phosphatases, PDEs and other proteins and that anchor these complexes in defined subcellular regions [30, 66, 67].

PDEs catalyze the hydrolysis of cyclic nucleotides generating the corresponding nucleotides AMP and GMP. The PDE superfamily consists of 11 different subfamilies (see Table 1) distinguished by their unique regulation,
enzymatic characteristics, structure and pharmacological inhibitory profiles, as well as by their tissue, cellular and subcellular expression [68]. Each subfamily encompasses 1 to 4 distinct genes and each gene encodes multiple protein products generated by alternative splicing and/or the use of multiple promoters, resulting in more than 50 different PDE proteins identified in mammalian cells. This multiplicity of PDE proteins may allow specific intracellular localization of PDEs in the vicinity of various protein effectors inducing compartmentation and fine-tuning of cAMP and cGMP signals [69, 70]. So far, all the phosphodiesterases identified consist of a modular architecture, with a highly conserved catalytic core located in the C-terminal part of the protein and distinct regulatory domains located in the N-terminal portion [68].

4.2. Phosphodiesterase types 2 and 4

As mentioned before, crosstalk between AHR and cyclic nucleotide pathways was recently identified by different research groups. Bolger et al. [57] observed for the first time the interaction of a type 4 PDE with XAP2. In their work the authors showed that specifically the PDE4A5 isoform could bind XAP2. Later, de Oliveira and colleagues [58] discovered that the type 2A PDE interacts with XAP2 and actively regulates AHR function. Since phosphodiesterases constitute the only pathway for cyclic nucleotide degradation they act as key enzymes in the regulation of all processes mediated by cAMP and/or cGMP.

A single gene encodes three PDE2 splice variants (2A, 2B and 2C). Human PDE2A, also known as cGMP-stimulated PDE, is a homodimer with a
molecular mass of about 213-kDa. Each monomer of PDE2A contains an N-terminal domain of unknown function, two tandem GAF domains (GAF A and GAF B) and a catalytic domain at the C-terminus. PDE2A is able to breakdown both cAMP and cGMP, with a slightly lower apparent $K_m$ for cGMP. However, when cGMP binds to the allosteric GAF B domain, the enzyme undergoes a conformational change that results in a 20-fold lower $K_m$ for cAMP [71], an important event for several pathways that PDE2A has been shown to regulate. GAF domains are found in many different proteins in nearly all organisms and serve to bind cyclic nucleotides and other small molecules [72]. The GAF acronym derives from the names of the first three proteins identified to contain them: cGMP-phosphodiesterases, Adenylyl cyclase and Fh1A (a transcription factor of *E. coli*). Five of the 11 PDE families contain regulatory segments consisting of one or two GAF-domain modules (PDE2, 5, 6, 10 and 11). The x-ray crystal structure of murine PDE2A regulatory segment shows that the GAF A domains from each monomer form the dimer interface, while the GAF B domains are well separated and responsible for cGMP binding [73]. PDE2A is highly expressed in brain, heart, platelets, adrenal medulla and endothelial cells, but it is also found in lung, kidney and liver. The subcellular distribution of PDE2 varies according to the tissue so that it can be localized in the cytosol or associated to functional membrane structures like plasma membrane, sarcoplasmic reticulum, Golgi complex, as well as the nuclear envelope [69]. Compartmentation of cyclic nucleotides pathway components and the distinct expression-profile of PDEs in different tissues are fundamental characteristics that determine the specificity of action and the physiological relevance of
PDEs. For example, one of the first specific functions attributed to PDE2A was blood pressure control. Under high blood pressure condition the heart secretes atrial natriuretic peptide that acts on zona glomerulosa cells in the adrenal cortex increasing cGMP concentration through stimulation of the particulate guanylyl cyclase. cGMP stimulates PDE2A catalytic activity, which in turns degrades cAMP resulting in an inhibition of aldosterone secretion, a hormone involved in water and salt retention [74, 75]. In endothelial cells and platelets, PDE2A (cGMP-stimulated) along with PDE3 (cGMP-inhibited) play a coordinate role in the regulation of intracellular cAMP concentration and, consequently, in the modulation of vascular permeability [76, 77] and platelet aggregability [78, 79]. Moreover, compartmentalized PDE2A activity has a pivotal role in regulating cardiac contractility. Upon activation of β3-receptors, intracellular NO-cGMP levels rise, which serves to activate PDE2 activity anchored to the plasma membrane, thereby selectively attenuating spatially confined pools of intracellular cAMP that, ultimately, affect contractile function [80].

Four genes (PDE4A, B, C and D) encode more than 20 forms of type 4 PDEs. The PDE4 enzymes form dimers and exhibit a C-terminal catalytic domain that selectively recognizes and hydrolyzes cAMP. The N-terminal regulatory regions of type 4 PDEs contain the so-called upstream conserved regions (UCR1 and UCR2 domains) that may function as binding domains for acidic phospholipids [81]. The PDE4A5 isoform is characterized by an extended N-terminal region involved in subcellular targeting [82]. Numerous binding proteins have been identified for the PDE4D enzymes, many of which are AKAPs. For example, PDE4D3 can be found in a complex with muscle
mAKAP, PKA, EPAC1, the ryanodine receptor, the phosphatases PP2A and calcineurin, and the MAP kinase ERK5, thus creating a microdomain of cAMP signaling that communicates with other signaling pathways [83]. In this manner, the assembly of cAMP-activated and cAMP-degrading enzymes determines a sophisticated way of spatio-temporal control of cAMP signaling. The PDE4A gene is expressed in a wide variety of tissues including the lung, various regions of the brain and in leukocytes [57, 84, 85]. Membrane as well as cytosolic distribution of PDE4A proteins has been observed [82]. In contrast to PDE2A and others PDE4 isoforms, only a few specific functions of PDE4A have been established, so far. For example, PDE4A5 was found to interact with AKAP3 and might modulate sperm motility [86].

4.3. XAP2 interacts with PDE2A and PDE4A5

By yeast two-hybrid screening using PDE2A as bait and a human brain cDNA library as prey, our group recently identified the interaction between PDE2A and XAP2 [58]. Other binding partners for PDE2A may well exist, as revealed by the results of our yeast two-hybrid screening; however, these potential interactors await further characterization. Moreover, Bentley et al. showed by a co-immunoprecipitation approach that several phosphoproteins can bind to epitope-tagged PDE2A in PC12 cells, however, the identity of these phosphoproteins is still unknown [87]. Because the yeast two-hybrid technique tends to generate considerable numbers of false-positive clones [88], the interaction between PDE2A and XAP2 was confirmed by GST pull-down and co-immunoprecipitation experiments in COS-1 and Hepac1c7 cells [58]. To verify the interaction of PDE2A and XAP2 at the level
of endogenous proteins, co-immunoprecipitation experiments in whole brain lysate were done. Both proteins, PDE2A and XAP2, were present in total lysate (Fig. 1, first lane). After precipitation of XAP2 with a specific antibody endogenous PDE2A could be detected in the precipitate by immunoblotting (Fig. 1, third lane). In a reverse experiment using anti-PDE2A we could verify the existence of a PDE2A/XAP2 complex (Fig.1, fourth lane). Further biochemical analyses clearly showed that the GAF-B domain is necessary and sufficient to mediate binding of PDE2A to XAP2 [58]. Among PDEs, cAMP and cGMP are the only ligands known to bind this domain [73] and our findings were the first description of the binding of proteins to the GAF domains of PDEs. Mapping studies revealed that the TPR containing carboxyl terminus of XAP2 is solely responsible for PDE2A binding. At present, the relationship between the number and arrangement of TPR repeats and their affinity and specificity for target proteins such as the various PDE subtypes is still unclear [45, 46, 89]. Initial experiments from our group indicate that XAP2 can bind simultaneously to PDE2A and HSP90 (unpublished data).

Bolger et al. [57] identified XAP2 as a specific binding partner for the cAMP-specific PDE4A5. This interaction was also mediated by the TPR domain containing C-terminus region of XAP2 and it seems to be specifically restricted to the A5 isoform of PDE4, since PDE4 isoforms A1, A8, B2, B3, D3, D4, and D5 did not bind XAP2. A careful mapping study revealed that the amino-terminal region of PDE4A5 holding an Glu-Glu-Leu-Asp (EELD) amino acid sequence motif in the upstream conserved region-2 exposes the major binding site of XAP2 [57]. This segment has no apparent similarity to GAF-B of PDE2A, suggesting different modes of XAP2 binding among PDE isotypes.
The binding of XAP2 to PDE4A5 drastically reduced the catalytic activity of this isoform in vitro. Furthermore, XAP2 increased the sensitivity of PDE4A5 toward the inhibitor rolipram and attenuated the ability of PKA to phosphorylate PDE4A5 [57]. Unlike the effect on PDE4A5, the binding of XAP2 to PDE2A did not affect the catalytic activity of the enzyme. This finding is not trivial because the target segment of XAP2 in PDE2A, i.e. the GAF-B domain, binds cGMP, thereby enhancing enzymatic activity.

4.4. Impact of phosphodiesterases on AHR

Because cAMP has been shown to induce nuclear translocation of the AHR [22] we investigated PDE2A effects on nuclear translocation of AHR. We transfected PDE2A into Hepa1c1c7 cells, which are known to express AHR and XAP2 endogenously [44]. Then we incubated the cells with TCDD (obtained from Sigma, Taufkirchen, Germany) or forskolin (an activator of adenylyl cyclase, Sigma) and monitored the subcellular localization of AHR by indirect immunofluorescence of fixed cells. Untreated cells exhibited a diffuse localization of AHR in the cytosol and occasionally involving the nucleus. As expected, TCDD induced an enrichment of AHR in the nucleus with a concurrent depletion of AHR from the cytosol (Fig. 2, A versus D). In PDE2A expressing cells (Fig. 2, E and F) AHR staining remained diffuse without significant nuclear accumulation of AHR in most cells. In line with previous findings [22] forskolin (cAMP) could also induce nuclear translocation of AHR (Fig. 2, G). Compared with TCDD some degree of cytosolic AHR staining remained after forskolin treatment. In PDE2A-expressing cells again forskolin-induced nuclear translocation of AHR was strongly reduced (Fig. 2, H and I.
versus G). Quantitative evaluation of these experiments showed that XAP2 binding to PDE2A inhibited nuclear translocation of AHR induced by TCDD by about 50% and in forskolin treated cells by about 70%. The reduced nuclear translocation of the AHR interfered with its function in the nucleus as shown by reporter gene expression assay [58]. Treatment with TCDD increased the expression of a CYP1A1 reporter gene, however, expression of PDE2A resulted in a moderate but significant reduction of TCDD-dependent induction of the reporter, suggesting that inhibition of nuclear translocation of AHR by PDE2A correlates with reduced AHR function. Recruitment of PDE2A to the AHR complex is likely to induce a reduction of local cAMP levels. This could block a potential permissive action of cAMP on the translocation of AHR into the nucleus (Fig. 3). The possible effect of PDE4A5 binding to XAP2 on AHR function has not been investigated. Since XAP2 inhibits the catalytic activity of PDE4A5, at least in vitro, cAMP concentrations in the vicinity of the AHR complex might remain elevated and PDE4A5 might enhance nuclear localization of AHR. However, the exact mechanism of cAMP-mediated nuclear translocation of AHR is unknown. PDE2A might also have a chaperone-like effect and participate indirectly, through its interaction with XAP2, in the stabilization of AHR in the cytosol. It is unclear how PDE2A could impact on TCDD-mediated activation of AHR. Cyclic nucleotides might play a role in the activation mechanism of AHR by TCDD. It has been reported that TCDD leads to a slight but significant elevation of cAMP levels, with consequent increase of PKA activity that up-regulates C/EBPβ gene transcription, leading to an inhibitory effect on adipocyte differentiation [90]. It would be reasonable to extrapolate this idea to TCDD-induced AHR
activation, since the PKA inhibitor H89 blocks the nuclear effects of cAMP-activated AHR [22]. However, it is rather intriguing that neither PKA nor PKG were capable of phosphorylating AHR in vitro ([58] and unpublished data). It needs to be taken into account that cAMP effects on AHR might be mediated by EPAC, an important effector of cAMP (Fig. 4) [63]. Interestingly, cAMP/EPAC appear to play a role in regulation of the circadian clock suggesting crosstalk between EPAC and the CLOCK, PER and BMAL members of the bHLH/PAS family of transcription factors [91]. Present findings on the possible roles of PDEs in AHR function raise a number of questions that should be addressed in future experiments. First, the effects of PDE2A and PDE4A5 on AHR need to be compared directly. Different activation patterns and substrate specificities of both PDEs are likely to result in different effects on cAMP and cGMP levels leading to specific changes in AHR trafficking. It is also unclear, if both PDEs are attached to XAP2 and to the AHR complex simultaneously, and if specific signals can trigger this association. Second, the effects of specific inhibitors of PDE2A and PDE4A5 on AHR complex formation and function should be studied in cell lines expressing all proteins endogenously. Third, it might be worthwhile to investigate the presence of other components of cAMP signaling compartments such as AKAPs, PKA, EPAC and other PDEs in the AHR complex.

Further evidence for a functional connection between the AHR pathway and cAMP signaling stems from recent findings in the field of cancer development. The AHR binding protein XAP2 acts as tumor suppressor and mutations in its gene disrupt this function and also disrupt protein-protein
interaction between XAP2 and its interacting partner PDE4A5 [92]. Germline mutations in XAP2 gene cause a condition called pituitary adenoma predisposition. In an international series of familial isolated pituitary adenomas, germline XAP2 mutations were identified in 15% of the families. Since the identification of XAP2 as a tumor susceptibility gene, mutations spanning the whole coding region have been reported in familial and sporadic settings. So far, there are 33 different mutations reported in the XAP2 gene [93-96]. Furthermore, cortisol-producing adrenocortical tumors have been associated with abnormalities of the cAMP signaling pathway. Recently, Horvath et al. have reported a genomewide search for genes conferring a predisposition to micronodular adrenocortical hyperplasia, leading to Cushing's syndrome in childhood and they identified a genetic locus harboring PDE genes as those most likely to be linked to the disease. Inactivating mutations of PDE11A gene were associated with adrenocortical lesions, mostly micronodular hyperplasia [97, 98]. Furthermore, the chromosomal locus harboring the gene encoding PDE8B was associated with a predisposition to micronodular adrenocortical hyperplasia. Horvath et al. [99] identified the mutation of a single base in the PDE8B gene in a patient with micronodular adrenocortical hyperplasia. That mutation affects a residue (His305Pro) highly conserved along the evolution, located at the end of the PAS domain, which seems to impair the catalytic activity of the enzyme. The mechanisms by which XAP2 and PDEs exert their tumor-suppressive action in the pituitary and the adrenal gland remain to be determined. Possible interactions of PDE8B and PDE11A with XAP2 have not been studied as yet. One might speculate that the AHR could in some way be involved in these
processes and in fact links between AHR and tumorigenesis have been made before [100, 101].

5. Conclusions

We conclude that various factors impact on the regulation of gene transcription by AHR. On one hand ligand binding appears to be required for AHR activation and on the other hand interacting proteins and input from other signaling pathways influence the subcellular localization of AHR. Studies using high affinity ligands such as TCDD might not reflect the physiological situation [14-16, 20] since these ligands might force the AHR into an activation state that does not exist in the normal cell. In contrast, emerging findings on the regulation of AHR by cAMP emphasize the impact of other signaling pathways on AHR function [22, 23, 58]. The discovery of cAMP-degrading PDE2A and PDE4A5 enzymes as XAP2 interacting proteins suggests a role for compartmentalized cAMP signaling in the control of AHR. PDE2A appears to regulate cAMP as well as TCDD induced activation of AHR by affecting the subcellular localization of the AHR complex. The possible role of other PDE family members and specific cAMP effector proteins such as PKA and EPAC in the formation of a cAMP/AHR microdomain remains to be determined. Taken together current data indicate that the AHR complex is capable of integrating diverse types of information derived from exogenous and endogenous ligands as well as cyclic nucleotide and other signaling pathways. This high degree of connectivity places the AHR transcription factor in a key position to regulate many different cellular functions.
References


Pongratz I, Mason GG, Poellinger L. Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. J Biol Chem 1992;267:13728-34.

Grad I, Picard D. The glucocorticoid responses are shaped by molecular chaperones. Mol Cell Endocrinol 2007;275:2-12.


Bell DR, Poland A. Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein. The role of hsp90. J Biol Chem 2000;275:36407-14.


Kaupp UB, Seifert R. Cyclic nucleotide-gated ion channels. Physiol Rev 2002;82:769-824.


Pryzwansky KB, Madden VJ. Type 4A cAMP-specific phosphodiesterase is stored in granules of human neutrophils and eosinophils. Cell Tiss Res 2003;312:301-11.


### Table 1

Overview of mammalian PDE families (for reference please see reviews on PDEs mentioned in the text)

<table>
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<tr>
<th>PDE Family</th>
<th>No. of genes</th>
<th>Substrate(s)</th>
<th>Regulation</th>
<th>Some suggested specific function(s)</th>
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<tr>
<td>PDE1</td>
<td>3</td>
<td>cAMP/cGMP</td>
<td>(+) Ca2+/CaM (-) PKA</td>
<td>Neuronal regulation, olfaction, smooth muscle regulation, sperm development</td>
</tr>
<tr>
<td>PDE2</td>
<td>1</td>
<td>cAMP/cGMP</td>
<td>(+) cGMP</td>
<td>Aldosterone secretion, regulation of cardiac myocytes and endothelial cells</td>
</tr>
<tr>
<td>PDE3</td>
<td>2</td>
<td>cAMP/cGMP</td>
<td>(-) cGMP (+) PKA, PKB</td>
<td>Regulation of platelets and adipocytes</td>
</tr>
<tr>
<td>PDE4</td>
<td>4</td>
<td>cAMP</td>
<td>(+) PKA, ERK</td>
<td>Inflammation, regulation of immune cells, airway smooth muscle contractility</td>
</tr>
<tr>
<td>PDE5</td>
<td>1</td>
<td>cGMP</td>
<td>(+) cGMP, PKG</td>
<td>Vascular smooth muscle cell relaxation</td>
</tr>
<tr>
<td>PDE6</td>
<td>3</td>
<td>cGMP</td>
<td>(+) transducin</td>
<td>Light reaction</td>
</tr>
<tr>
<td>PDE7</td>
<td>2</td>
<td>cAMP</td>
<td></td>
<td>Regulation of lymphocytes</td>
</tr>
<tr>
<td>PDE8</td>
<td>2</td>
<td>cAMP</td>
<td></td>
<td>Thyroid function, Leydig cell function</td>
</tr>
<tr>
<td>PDE9</td>
<td>1</td>
<td>cGMP</td>
<td></td>
<td>Neuronal regulation</td>
</tr>
<tr>
<td>PDE10</td>
<td>1</td>
<td>cAMP/cGMP</td>
<td></td>
<td>Neuronal regulation</td>
</tr>
<tr>
<td>PDE11</td>
<td>1</td>
<td>cAMP/cGMP</td>
<td></td>
<td>Skeletal muscle regulation, sperm function</td>
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Figure legends

Figure 1: Endogenous PDE2A and XAP2 bind to each other. Brain tissue was lysed, and PDE2A or XAP2 proteins were precipitated using specific antibodies (obtained from FabGenix, Frisco, TX and Abcam, Cambridge, UK) and protein A or G bound to Sepharose beads. Precipitated proteins were solubilized, separated by SDS-PAGE, and immunoblotted with PDE2A and XAP2 antibodies (IB). As control nonspecific antibodies (IgG) were used (second lane). In the first lane, expression of PDE2 and XAP2 in total brain lysate is shown. This figure was reproduced with permission from the Journal of Biological Chemistry, Vol 282, page 13659, 2007.

Figure 2: PDE2A inhibits nuclear translocation of AHR. Hepa1c1c7 cells grown on glass coverslips were transfected with VSV-tagged PDE2A. One day post-transfection, cells were treated with 5 nM TCDD (D and E, F) or 20 µM forskolin (G and H, I) for 1 h at 37 °C, fixed, permeabilized, and stained with goat anti-AHR (M20, Santa Cruz Biotechnology, Heidelberg, Germany) to visualize endogenous AHR, mouse anti-VSV (Sigma) to detect transfected PDE2A and with appropriate secondary antibodies (Cy3-anti-goat, Cy2-anti-mouse, Jackson ImmunoResearch, Newmarket, England). PDE2A transfected cells are shown as merge of AHR (red) and PDE2A (green) stainings (B, E, H) or with the AHR staining alone (C, F, I). Untreated cells are presented as controls (A and B, C). Arrows point to cells that exhibit a reduced nuclear AHR staining in the untreated state and to cells that have lost the capacity for AHR translocation under the respective treatment. The figure
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Figure 3: Possible role of phosphodiesterases and cAMP microdomains in nuclear translocation of AHR. The cytosolic AHR core complex is composed of the AHR, two molecules of HSP90, p23 and XAP2. XAP2 recruits PDE4A5 to the AHR complex and inhibits the catalytic activity of the enzyme. It is still unclear if PDE4A5 modulates AHR function. On the other hand, binding of PDE2A to XAP2 inhibits AHR translocation to the nucleus, probably by lowering the cAMP concentration in the vicinity of the AHR protein complex.

Figure 4: Expanded modes of AHR activation. In the classical pathway exogenous ligands like TCDD enter the cytosol and activate the AHR to translocate into the nucleus to induce transcription. The second messenger cAMP has emerged to play a role in TCDD effects on AHR. cAMP can also activate AHR translocation in the absence of TCDD. This process might involve binding of ligand to an as yet unknown G-protein coupled receptor resulting in cAMP production by adenylyl cyclase. The effects of cAMP could be mediated by the cAMP-dependent protein kinase (PKA) or the exchange protein directly activated by cAMP (EPAC) or both. The cAMP-activated AHR appears to interact with nuclear proteins such as RelB inducing a specific transcriptional response.
Figure 1
Figure 2

A B C

non-transfected PDE2A transfected untreated

D E F

TCDD

G H I

forskolin