Published in final edited form as:

Horm Metab Res. 2014 November ; 46(12): 863–868. doi:10.1055/s-0034-1389916.

Phosphodiesterases and Adrenal Cushing in Mice and Humans

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Abstract

The majority of benign adrenal cortex lesions leading to Cushing syndrome are associated to one or another abnormality of the cAMP/cGMP-phosphodiesterase signaling pathway. Phosphodiesterases (PDEs) are key regulatory enzymes of intracellular cAMP/cGMP levels. These second messengers play important regulatory roles in controlling steroidogenesis in the adrenal. Disruption of PDEs has been associated with a number of adrenal diseases. Specifically, genetic mutations have been associated with benign adrenal lesions, leading to Cushing syndrome and/or related adrenal hyperplasias. A Genome Wide Association study, in 2006, led to the identification of mutations in 2 PDE genes: *PDE8B* and *PDE11A*; mutations in these 2 genes modulate steroidogenesis. Further human studies have identified PDE2 as also directly regulating steroidogenesis. PDE2 decreases aldosterone production. This review focuses on the most recent knowledge we have gained on PDEs and their association with adrenal steroidogenesis and altered function, through analysis of patient cohorts and what we have learned from mouse studies.

Keywords

PDE2A; PDE8B; PDE11A; Cushing syndrome; cAMP; bilateral adrenal hyperplasia

Introduction

Molecular defects in the 3',5'-cyclic nucleotide phosphodiesterase (PDE) superfamily, as well as various components of the cAMP signaling pathways, predispose to certain types of adrenocortical tumors [1]. This is not surprising given that PDEs main function is in the regulation of the integral intracellular second messengers: adenosine 3',5'-cyclic monophosphate (cAMP) and/or guanosine 3',5'-cyclic monophosphate cGMP. Importantly, the majority of benign adrenal lesions, leading to Cushing syndrome (CS) and/or related adrenal hyperplasias, are linked to one or another abnormality of the cAMP signaling pathway.

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Conflict of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Cushing syndrome describes the clinical consequences of chronic exposure to excess glucocorticoids, irrespective of the underlying cause [2]. There occur 3 rare, albeit potential endogenous causes of CS: (A) presence of a cortisol-producing adrenal tumor, which may be benign or malignant [this is also referred to as adrenocorticotrophic hormone (ACTH)-independent CS], (B) excess secretion of ACTH from a pituitary tumor (or ACTH-dependent, also referred to as Cushing's disease), or (C) an ectopic ACTH-producing tumor (ectopic Cushing's syndrome) [3, 4]. Cortisol-producing adrenal tumors are grouped in with bilateral adrenal hyperplasia (BAH), and, unlike adrenal adenomas and cancer, BAH can be inherited or result from a de novo mutation [5–7]. BAHs can be characterized into 2 groups based on their epidemiology, description and gene involvement: (A) massive macronodular adrenocortical disease [ACTH-independent macronodular adrenal hyperplasia (MAH)], characterized by lesions > 1 cm (predominant occurrence in older adults) [3, 8], and (B) micronodular BAH (MAD), characterized by multiple nodules < 1 cm [9], and is further subdivided into (a) the relatively more frequent pigmented variant (primary pigmented nodular adrenocortical disease; PPNAD) which often leads to CS and is frequently associated with Carney complex (CNC) [10], and (b) the recently documented isolated micronodular adrenocortical disease (iMAD), bearing similarity with PPNAD [11, 12].

A Genome Wide Association study in 2006 led to the identification of mutations in PDEs in iMAD, specifically *PDE8B* and *PDE11A* [13–17]. Our goal here is to bring together the current, and up to date, research of the PDEs associated with the adrenal and their dysregulation that results in CS and other BAHs, in humans and mice. This review is not intended as a systematic account of the properties of all PDEs; for excellent reviews on all PDEs please refer to [18–20].

PDE Family

There are 11 major classes of mammalian PDEs (PDE1–11), encoded by 21 distinct genes, characterized by distinctive biochemical and kinetic properties, subcellular localization, and mechanisms of regulation [18, 20]. Their main and unique function is in their ability, as intracellular metabolic isozymes, to compartmentalize, consequently degrading the second messenger cAMP and/or cGMP [21, 22]; this makes them the integral regulators of intracellular signaling. PDEs exert their functions via their downstream effector proteins that include the cAMP-dependent protein kinase (PKA) [23], cGMP-dependent protein kinase (PKG) [24], as well as cyclic nucleotide-gated ion channels (CNGCs) [25], and cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), also known as exchange proteins directly activated by cAMP (or Epacs) [26]. Thus, PDEs control numerous physiological and pathophysiological processes that are under the control of cyclic nucleotides.

The cAMP/cGMP Signaling Pathway: Adrenal Hyperplasias and Tumors

The cAMP/PKA-signaling pathway has been identified as the main molecular route that, when defective, leads to benign adrenocortical tumors and hyperplasias [1]. Genetic mutations in several components of the cAMP/PKA-signaling pathway have been linked to genetic forms of cortisol excess leading to CS, as well as different types of adrenocortical

tumors [5, 6, 27–29]. Under normal physiological conditions, the biosynthesis of cortisol is regulated, primarily, by adrenocorticotropin hormone (ACTH, a peptide hormone), which, upon binding to the melanocortin 2 receptor (MC2R) activates the cAMP/PKA-signaling pathway [22, 30–32]. Ligand binding triggers the Gs α -subunit to exchange GDP for GTP. The activated Gs α -subunit stimulates the membrane-associated adenylyl cyclase to convert ATP into cAMP thereby activating PKA, EPAC, or CNGCs [25, 26, 33, 34]. These intracellular levels of cAMP are hydrolyzed exclusively by PDEs.

PKA, a tetrameric serine/threonine kinase consisting of a dimer of 2 regulatory (R) and 2 catalytic (C) subunits, occurs in 2 holoenzyme forms: type I PKA and type II PKA [35]. When intracellular levels of cAMP increase, 2 cAMP molecules bind to each R subunit of PKA (RI α , RI β , RII α , and RII β) causing dissociation of active C subunits (C α , C β , C γ , and Prkx), and triggering, at the same time, A-kinase anchoring proteins (AKAPs) to bind to the R subunit dimer – this process enables cellular compartmentalization in the vicinity of its substrates [36, 37]. Consequently, this reduces the affinity of the R subunits for binding to C subunits thereby enabling free C subunits to transfer ATP terminal phosphates to protein substrates at serine/threonine residues. This phosphorylation frequently results in substrate activity alterations, such as that which occurs by the transcription factor cAMP response element-binding protein (CREB), which is involved in regulating the expression of many genes related to cell metabolism and proliferation. Similarly, when intracellular levels of cGMP increase PKG is activated, in turn catalyzing the phosphorylation of downstream proteins involved in several physiologic functions, such as glycogenolysis, ion channel conductance, and apoptosis [37].

PDE Mutations and Adrenocortical Hyperplasias

Each PDE gene possesses a unique peripheral and central expression pattern at the organ, tissue, and cellular level [38]. For the purpose of this review only the PDEs known to be associated with adrenal CS and/or BAH will be explored: PDE2A, PDE8B and PDE11A [17, 38–40]; however it is pertinent to make note that other PDEs are expressed in the adrenal, yet their relation to human disease remain(s) unknown (Table 1).

PDE2A

PDE2A, dual-substrate enzyme capable of degrading both cGMP and cAMP (depending on the cell type in which it is expressed), encodes for the single gene PDE2, and is expressed as 3 splice variants (PDE2A1–3). Uniquely, PDE2A is one of the 5 PDE family members that contain a regulatory segment containing GAF (cGMP-phosphodiesterases, Anabaena adenylyl cyclases and *Escherichia coli* Fh1A) domains known to bind cGMP [41–43]; the binding of cGMP to the GAF domain triggers activation of the catalytic activity leading to increased hydrolysis of cAMP [44]. This is the unique mechanism that provides PDE2A the ability to mediate cross talk between cGMP and cAMP signaling systems [45]. Importantly, this event has been shown to regulate several key pathways [46–50].

In the adrenal, PDE2A is predominant in the zona glomerulosa [39, 51–53]. In bovine adrenal zona glomerulosa cells, atrial natriuretic peptide (ANP) stimulates an elevation of cGMP, in turn decreasing, dose-dependently, intracellular cAMP, and results in the

attenuation of aldosterone secretion [52, 54, 55], thereby mediating the effect of ANP on blood pressure [52]. While PDE2A plays a role in regulating aldosterone secretion no human mutations have been identified nor has this gene been linked to BAH and/or CS. Additionally, in rat and human glomerulosa cell lines, an ACTH-induced increase in intracellular cAMP is observed [54]. Interestingly, in β -catenin (CTNNB1)-mutated adrenocortical tumors, PDE2A has been identified as upregulated [56]. Patients with PPNAD have been found with somatic mutations of the *CTNNB1* [57, 58]. Interestingly, a Pde2a knockout mouse has been developed, but limited data are available, as these mice do not survive past 17–18 days of gestation [53]. Nonetheless, expression analysis of PDE2A has been possible, by examining embryonic mice at 15 days of gestation. Analysis has revealed strong immunoreaction within the zona glomerulosa of mice as well as in rat, dog, cynomolgus monkey, and human [53]. Thus, PDE2A appears to be strongly involved in cAMP regulation with its activity controlling the production of cAMP, induced by ACTH.

PDE8A/B

The PDE8 family, encoded for by 2 highly homologous genes PDE8A and PDE8B, are exclusively single-substrate enzymes capable of degrading only cAMP, with the highest affinity amongst all other PDEs. PDE8A is expressed in a variety of tissues as 5 splice variants (PDE8A1–5) [59]. PDE8B is also expressed as 5 splice variants (PDE8B1–5). The biological significance of these alternative spliced transcripts remains unclear. Both PDE8A and PDE8B play an essential role in steroidogenesis, in addition to being localized to adrenal zona fasciculata cells [15, 39], both transcripts have also been found in human and mouse testis [59–61]. Pde8a mRNA transcripts, in mouse adrenal, are located in a small population of zona fasciculata cells bordering the zona glomerulosa; whereas Pde8b is highly expressed throughout the zona fasciculata layer [62]; PDE8B was first described in the thyroid [63] with PDE8B1 being the dominant variant expressed. The expression pattern of these enzymes important because the cells located here are major regulators of steroidogenesis. Limited studies on Pde8a in the mouse adrenal exist because the focus is largely on its important role in the testis and ovary [64, 65].

PDE8B has been identified as the key molecule in the predisposition to adrenal tumorigenesis and genetic forms of adrenal hyperplasia [15, 62]. The first reported association of PDE8B with adrenal tumors was the identification of a germ-line-mutation in a 2 year-old young girl with CS [15, 16]. There has been recent work on *PDE8B* and its association with adrenal tumor has broadened. The first systematic screen for *PDE8B* genetic defects in a heterogeneous cohort of patients with adrenocortical tumors, including benign and malignant, secreting and silent, unilateral and bilateral tumors, and nodular and hypertrophic adrenal lesions [40] identified 6 *PDE8B* coding sequence alterations. These alterations were found in a heterozygous state on germ-line DNA, in 8 unrelated individuals from a cohort of 216 patients with adrenal tumors [40]. Of these, 3 novel missense substitutions were identified: p.H391A, p. P660L, and p.V697I, and the c.1 365-5 g/a splice variant [40]. Gene expression profiles associated with the autonomous and excessive cortisol secretion of adrenocortical adenomas have also been examined, where the aim of the work was to identify the molecular alterations contributing to the autonomous and excessive cortisol secretion of adrenal adenomas [66]. Here, 22 unilateral adrenocortical adenomas (5

nonsecreting, 6 subclinical cortisol producing, 11 cortisol producing) were studied by correlating cortisol secretion levels of adrenocortical adenomas with transcriptome profiles. Unexpectedly, *PDE8B* showed the strongest positive correlation with cortisol secretion [66].

Pde8b inactivation in transgenic mice has shown its major function is in glucocorticoid secretion regulation [62]. To date, this is the first report of a functional mouse model describing that *Pde8b* is an effective modulator of adrenal steroid production. The *Pde8b* ablated mouse model was generated under contract to Pfizer, Inc. (Sandwich, UK) and were generated by targeting and replacing a critical region in the catalytic domain (exon 14–15) with DNA sequence encoding a lacZ reporter gene and a neomycin resistance gene followed by a stop codon [62]. Interestingly, these mice do not develop an adrenal hyperplasia phenotype, nor are the adrenals increased in size, as seen in one patient with a point mutation in *PDE8B* [15, 16]. This observation may be due to species variation. Nonetheless, *Pde8b* inactivation in transgenic mice does sensitize adrenal zona fasciculata to ACTH resulting in an in vivo increase in corticosterone production. This mouse data conclude that *Pde8b* is a major regulator of one or more pools of cAMP involved in promoting steroidogenesis via both short- and long-term mechanisms. Interestingly, although *PDE8A* and *PDE8B* regulate distinct overlapping cAMP pools that control basal steroidogenesis rates, maximal steroid production requires collective inhibition of *PDE8A* and *PDE8B* (with PF-04957325), as well as inhibition of *PDE4* [62, 67].

PDE11

PDE11 encodes for the gene *PDE11A*, of which there are 4 different isoforms (*PDE11A1–4*), each having distinct transcriptional start sites [68–70]. Exhibiting dual substrate specificity, *PDE11A* hydrolyzes both cAMP and cGMP with similar affinities. GAF domains are also present and affect catalytic activity [71]. *PDE11A* function has been linked to adrenocortical tumors predisposition [72, 73]. As mentioned previously, the genome wide association-study linked *PDE11A* to micronodular adrenocortical hyperplasia, leading to CS in childhood [17]. Thus, inactivating *PDE11A* mutations can be associated with micronodular adrenocortical lesions and elevated cAMP levels, implicating that *PDE11A* mutations lead to aberrant cAMP signaling, which can be associated with development of BAH [17]. The most recent study examined the frequency of *PDE11A* variants and the associated predisposition to MAH, in a large cohort of patients with MAH [72]. All functional variants that were found were contained in the catalytic domain, except for one localized in the exon containing the start codon. The presence of these variants leads to an increase in cAMP levels, in turn activating cAMP signaling. Thereby, the stimulation of cAMP by *PDE11A* variants is a genetic predisposing factor for MAH development. Interestingly, inactivation of *PDE11A* appears to also predispose to testicular germ cell and prostatic tumors [74, 75]. A high frequency of *PDE11A* variants in a large cohort of CNC patients with *PRKARIA* mutations has been demonstrated. Men with CNC due to *PRKARIA* mutation carrying *PDE11A* variants develop significantly more PPNAD as well as large-cell calcifying Sertoli cell tumors.

Mice deficient in *Pde11a* have been generated, however no effects on the adrenal have been reported [76]. These mice were developed to examine the role of *Pde11a* in sperm

physiology; as it is highly expressed in the male reproductive tract, including prostate, testes and developing spermatozoa and Leydig cells [68]. Although mouse data has been uninformative with respect to the role of *Pde11a* on adrenal function, a selective PDE11A inhibitor has been identified, using a yeast-based growth assay [77]. Here, the authors identified a Pde11a-inhibitor compound that significantly elevated cAMP and cortisol levels in H295R cells. These results are very promising in that they pave the way to developing compounds suitable for whole-animal studies of Pde11 function and may be a better alternative to knock-out mouse studies due to the likelihood that compensation of activity by other PDEs or developmental alterations caused by an early loss of PDE11 activity may be interfering in the whole animal approach.

Summary

Significant progress has been made toward the identification of genes leading to CS. Until recently, cAMP/PKA pathway activation was the only known signaling alteration that had been related to abnormal cortisol secretion. It is not surprising that PDEs play an important role in regulating adrenal steroidogenesis [82, 83]. However, PDEs are complex. Therefore, it is important to note that all PDEs are closely related to the regulation of each specific transduction signal. Consequently, multiple PDEs are likely to have important roles in controlling each cellular function. As outlined herein, the 3 important PDEs linked with adrenal CS and/or BAH (*PDE2A*, *PDE8B*, and *PDE11A*) are functionally distinct, whereby *PDE8B* has sole and very high specificity for cAMP only. Interestingly, *PDE8B* together with *PDE8A*, contains a PAS (Per, Arnt, and Sim) and a REC (receiver) domain, both of which have been observed in many signal transduction proteins. Although much interest has stirred over the involvement of the PDE8 family in adrenal steroidogenesis, little information exists on the exact molecular mechanisms regulating PDE8 activity. Given PDE8s significant role in adrenal CS/BAH it will not take long for us to understand these mechanisms. Furthermore, PDEs make excellent drug targets, some have been successfully used in nonendocrine diseases, and thus the development of new therapeutic approaches targeting adrenal steroidogenesis is conceivable. Together, the data presented here support the possibility that *PDE2A*, *PDE8B*, and *PDE11A*, alone or likely acting with other factors are involved in the predisposition to adrenocortical tumors associated with CS and BAH.

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Table 1

Biochemical characteristics of PDEs expressed in human adrenal and associated mouse models.^a

Gene Family	Gene	Chromosomal Location	Human	Mouse	Substrate	Human Adrenal or other Disease	Mutation(s) (dbSNP)	Mouse Model(s)	Mouse phenotype	Reference
PDE1	<i>PDE1B</i>	12q13	15 F3	15 F3	cAMP/cGMP	NI	NI	<i>Pde1^{tm1Cvv}</i>	Mice homozygous for disruptions in this gene display increased exploratory behavior. Learning deficits and hyperactivity are also observed in some situations.	[78]
PDE2	<i>PDE2A</i>	11q13.4	7 E3	7 E3	cAMP/cGMP	NI	NI	<i>Pde2^{tm1Dgen}-Pde2^{tm1Dna}</i>	Mice homozygous for a knock-out allele exhibit lethality between E17 and E18	[53]
PDE4	<i>PDE4B</i>	1p31	4 C6	4 C6	cAMP	NI	NI	<i>Pde4^{tm1Dgen}-Pde4^{tm1Mci}</i>	Mice homozygous for disruptions in this gene produce significantly less TNF- α in response to lipopolysaccharide stimulation. One mutation resulted in brain and spinal cord vacuoles.	[79]
	<i>PDE4C</i>	19p13.11	8 B3.3	8 B3.3	NI	NI	NI	NA		
PDE5	<i>PDE5A</i>	4q25-q27	3 G1	3 G1	cGMP	NI	NI	NA		
PDE6	<i>PDE6B</i>	4p16.3	5F	5F	cGMP	NI	NI	<i>Pde6^{tm1Whae}</i>	No abnormal phenotype observed.	[80, 82]
PDE7	<i>PDE7A</i>	8q13	3 A2	3 A2	cAMP	NI	NI		Homozygous inactivation of this locus does not impair T cell function but affects the humoral immune response.	[81, 83]
	<i>PDE7B</i>	6q23.3	10 A3	10 A3	NI	NI	NI		Homozygous inactivation of this locus does not impair T cell function but affects the humoral immune response.	
PDE8	<i>PDE8B</i>	5q13.3	13 D1	13 D1	cAMP	PPNAD: MAH; Striatal degeneration	PPNAD: HIS305PRO (rs121918360); MAH: H391A; P660L; Striatal degeneration: 94G-C and 1-BP DEL, 95T.	<i>Pde8^{tm1Dgen}</i>	Mice homozygous for a null allele exhibit increased urine corticosterone, decreased serum adrenocorticotropin and decreased sensitivity to a PDE8-selective inhibitor.	[15, 40, 62]
PDE11	<i>PDE11A</i>	2q31.2	2	2	cAMP/cGMP	PPNAD, AIMAH	PPNAD: ARG307TER (rs76308115); 1-BP DEL, 171T	<i>Pde11^{tm1Lex}</i>	Mice homozygous for a null allele have enlarged reduced sperm concentration and display increased premature/spontaneous capacitation. No adrenal phenotype has been observed.	[17, 74, 76]

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Based on combined microarray data that were acquired from the ArrayExpress database (www.ebi.ac.uk/arrayexpress; under the accession numbers E-GEOD-30352, E-AFMX-5, A-AFFY-44, and E-MTAB-513); the PDEs expressed in the adrenal are represented

NI: None identified, to date; NA: No available published mouse strain