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The roles of cyclic nucleotide phosphodiesterases (PDEs) in steroidogenesis

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Abstract

The second messenger, cAMP, is one of the most important regulatory signals for control of steroidogenesis. This review focuses on current knowledge about regulation of cyclic nucleotides by phosphodiesterases (PDEs) in steroidogenic tissues. The first PDE known to directly regulate steroidogenesis was PDE2, the cGMP-stimulated PDE. PDE2 mediates ANP/cGMP-induced decreases in aldosterone production. Recently, the PDE8 family has been shown to control steroidogenesis in two tissues. Specifically, PDE8A regulates testosterone production by itself and in concert with additional IBMX-sensitive PDEs. PDE8B modulates basal corticosterone synthesis via acute and chronic mechanisms. In addition to cAMP-dependent pathways, cGMP signaling also can promote steroidogenesis, and PDE5 modulates this process. Finally, PDE mutations may lead to several human diseases characterized by abnormal steroid levels.

Introduction

Steroid hormones serve many essential roles in mammalian physiology, ranging from promoting development to regulation of metabolism. Two of the major steroidogenic tissues in mammals include the adrenal glands and gonads. The adrenal cortex contains three distinct steroidogenic layers: 1) the zona glomerulosa, a thin layer of cells that synthesizes aldosterone (a mineralocorticoid); 2) the zona fasciculata, the thickest layer of the cells in the cortex that produces glucocorticoids; and 3) the zona reticularis, the inner layer that makes the sex steroid precursor, 5-dehydroepiandrosterone (5-DHEA) [1]. Each of these layers can be regulated by changes in cAMP. Adrenal steroid biosynthesis responds to stimulation with adrenocorticotrophic hormone (ACTH) that is secreted by the pituitary gland. Unlike the adrenal steroids, the sex steroids (e.g. testosterone and estrogen) are secreted by gonads upon luteinizing hormone (LH) stimulation, which is made in the pituitary. In males, testicular Leydig cells produce the majority of testosterone, while in females, estrogen production initiates in theca cells and intermediary precursors are further converted into estrogen by granulosa cells both of which are located in the ovaries.

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In these each of these steroidogenic tissues, cytochrome P450s (p450_{sc}, p450_{c21}, p450_{c17}, p450_{c11}, etc.) and hydroxysteroid dehydrogenases (3 β HSD and 17 β HSD) are utilized as catalysts of the biosynthesis pathway [2]. These enzymes catalyze a cascade of reactions converting cholesterol ultimately into final steroid products. The relative expression levels of these steroidogenic enzymes in the different cell types dictate the type of steroids that are synthesized. For instance, aldosterone-producing glomerulosa cells differ from other steroidogenic cells in that they express aldosterone synthase, also known as P450_{c18}. This enzyme is able to further convert corticosterone into aldosterone.

Despite differences in the expression profiles of steroidogenic enzymes, steroidogenesis in each cell type is stimulated by the same cAMP/PKA-signaling pathway [3,4]. Both the luteinizing hormone receptor (LHR) and ACTH receptor, also known as melanocortin 2 receptor (MC2R), are coupled to G-protein regulated adenylyl cyclases (ACs). Upon hormone binding, activated AC converts ATP into the second messenger, cAMP. Cyclic AMP then activates cAMP-dependent protein kinase (PKA), which in turn promotes steroidogenesis by both acute and chronic mechanisms [3,5]. Acutely, PKA promotes steroidogenesis via phosphorylation and activation of key enzymes, for example hormone sensitive lipase (HSL) [also known as cholesterol ester hydrolase] and the steroidogenic acute regulatory (StAR) protein. Activation of HSL increases the availability of free cholesterol to be transported into the mitochondria by activated StAR protein [6]. These processes stimulate steroid biosynthesis by transporting substrate to the sites where the initial steps of steroid biosynthesis occur, thereby allowing the cytochrome p450 enzymes to convert cholesterol into steroid intermediates and eventually into final steroid products. In addition, PKA activation also has a longer term lasting influence on steroidogenesis. In the chronic phase of steroid production, mRNA transcripts of several of the key steroidogenic genes increase due to cAMP/PKA mediated activation of transcription factors, including SF-1 and DAX-1. [7-9]. All of these regulatory processes are controlled by cAMP, although possibly by different pools or functional compartments.

The level of cAMP in each of these compartments is determined by its rates of synthesis by ACs and degradation by PDEs. In mammals, there are 11 families of PDEs, and each PDE family has its own unique kinetics, regulatory partners, as well as inhibitor sensitivity [10]. Current theory holds that one or a combination of PDEs can subserve different compartment(s) of cAMP and regulate different biological processes. This review focuses on the documented findings on the roles of PDEs in regulation of steroidogenesis. Some of these roles are outlined in figure 1.

PDE2A mediates ANP/cGMP-induced decreases of steroidogenesis in adrenal glomerulosa cells

PDE2A is a dual substrate PDE, capable of hydrolyzing both cAMP and cGMP at comparable K_m s (35 μ M for cAMP and 11 μ M for cGMP). Both substrates show positive cooperative kinetics. PDE2A, also known as cGMP-stimulated PDE, exists as a homodimer of 103 kDa subunits, each of which contains a cGMP-binding regulatory GAF domain [11]. At low substrate concentrations PDE2 activity is increased up to 30 fold when the GAF B domain is occupied by cGMP [11,12]. PDE2A often serves as a point of convergence

between cAMP and cGMP signaling in situations where the effects of the two cyclic nucleotides are opposing, and its role in attenuating aldosterone production is a case in point.

Aldosterone serves a number of important physiological roles including maintenance of salt and water balance by promoting sodium and water reabsorption in the kidney [13-15]. In addition to ACTH, angiotensin II also effectively promotes aldosterone synthesis [16]. Aldosterone production is however attenuated by atrial natriuretic peptide (ANP). ANP is a vasodilating peptide secreted by the atria in response to elevated blood pressure. Upon binding to its receptor (particulate guanylyl cyclase-A), ANP induces the conversion of GTP into cGMP [17]. ANP-induced cGMP can then cause a decrease in aldosterone production mainly via lowering of cAMP by activation of PDE2A.

The activation of PDE2A in zona glomerulosa cells has been shown by a dose-dependent decrease in intracellular cAMP upon ANP stimulation, which is followed by attenuation of aldosterone secretion [18,19]. Furthermore, this ANP-attenuated aldosterone production is blocked by a PDE2A-selective inhibitor EHNA (erythro-9-[2-hydroxy-3-nonyl]adenine) [19]. More recently, the ANP-stimulated PDE2A decrease in cAMP has been confirmed by real-time imaging in bovine glomerulosa cells [20]. Surprisingly, the inhibitory effect of ANP on ACTH-stimulated aldosterone production is slightly blunted in PKGII^{-/-} animals compared to WT [21]. However, it remains unclear if PKGII directly modulates this inhibitory effect on aldosterone production. In contrast to this whole animal study, direct stimulation of PKG with a cGMP analog (8-pCPT-cGMP), which does not activate PDE2A, is reported to potentiate aldosterone production in rat glomerulosa cells [22]. This finding suggests that cGMP also may have the ability to promote steroidogenesis in addition to attenuating cAMP signaling, at least in some species.

PDE5 modulates cGMP/PKG-stimulated androgen production in Leydig cells

PDE5A, also known as the cGMP-binding PDE, is a cGMP-specific PDE. Its activity can be stimulated by approximately 10-fold by phosphorylation and cGMP binding to its GAF A domain [23]. Rat Leydig cells show immuno-reactivity toward a PDE5A-specific anti-body, suggesting the expression of PDE5A in this cell type [24].

Testosterone is synthesized by Leydig cells to maintain a high levels in testes, which is essential for normal spermatogenesis [25]. mRNA transcripts for guanylyl cyclases (GC), protein kinase G (PKG), and PDE5A have been detected in rat Leydig cells [26]. Moreover, stimulation of the cGMP pathway causes an accumulation of cGMP followed by an increase in androgen production. This potentiating effect was blocked by a PKG inhibitor (KT5823) but not a PKA inhibitor (H89) [26]. These authors also demonstrated that treatment with a PDE5-selective inhibitor (T-0156 [27]) raises intracellular cGMP level as well as potentiates androgen production [26]. These findings suggest that a cGMP pathway also may regulate steroid production in Leydig cells, and that PDE5 modulates this pool of cGMP. Furthermore, chronic treatment (28-30 days) with a PDE5-selective inhibitor (sildenafil) also potentiates testosterone production in mice [28,29]. Interestingly, this increase in

circulating testosterone level was attributed to alteration of mRNA transcripts of components of both the cAMP and cGMP pathways. Namely, a significant decrease in mRNA transcripts of PDE4A was detected resulting in an increase in basal cAMP level. Increases in mRNA transcripts of cGMP-hydrolyzing PDEs (PDE6C and PDE1A) and GC also were reported possibly causing hypersensitivity toward NO donor stimulation [29]. These findings suggest that chronic treatment with sildenafil increases testosterone secretion *in vivo* via transcriptional mechanisms. However, this pathway remains to be validated by *in vitro* experiments.

PDE8A modulates testosterone production in Leydig cells

The PDE8 family is one of the more recently discovered PDE families. It consists of two distinct genes - *PDE8A* and *PDE8B*. *PDE8A* and *PDE8B* have very high affinity towards cAMP and hydrolyze cAMP with a $K_m \approx 0.15 \mu\text{M}$. Both PDE8s do not hydrolyze cGMP, nor are they regulated by cGMP. Like many other PDEs, the activity of *PDE8A* and *PDE8B* are dependent on the binding of divalent cations such as Mg^{2+} [30,31]. However, no binding partners that regulate the activity of PDE8s have been identified to this day. High levels of mRNA transcripts for the human *PDE8A* gene are found in testes, spleen, colon, small intestine, ovary, placenta, and kidney [32]. Comparable to what is found in humans, mRNA transcripts of *pde8a* are also expressed in testes of a mouse as shown by Northern blot analysis [31].

Using immunohistochemistry, Vasta et al. showed that isolated mouse Leydig cells have reactivity for both PDE8A-specific and P450_{scc}-specific antibodies [33]. P450_{scc} (cytochrome P450 side chain cleavage) is regarded as a marker for steroidogenic cell types, confirming that PDE8A is expressed in the steroidogenic cells of the testes. These authors also showed that Leydig cells from PDE8A-null mice secrete approximately 4-fold higher testosterone under basal conditions compared to Leydig cells isolated from wild-type mice. Furthermore, PDE8A-null Leydig cells were also more responsive to LH stimulation as demonstrated by a small leftward shift in the LH dose-response curve compared to PDE8A containing Leydig cells. Interestingly, IBMX treatment of wild-type Leydig cells also caused only a small leftward shift in the dose response curve. However IBMX treatment of PDE8A-null Leydig cells caused a dramatic 13-fold shift in the EC₅₀ for the LH dose-response curve, demonstrating that PDE8A and at least one other IBMX-sensitive PDE can regulate this pool of cAMP [33]. We have recent evidence to suggest that this IBMX-sensitive PDE is PDE4 (unpublished data).

PDE8B modulates corticosterone secretion in adrenal fasciculata cells

In the mouse, corticosterone is the major adrenocortical steroid hormone known to mediate the stress response (also known as fight-or-flight response), including glucose and fat metabolism. In humans this glucocorticoid is cortisol. The glucocorticoids also influence immune and behavior responses, among many other functions [34-37].

mRNA transcripts for both the PDE8A and PDE8B isoforms of the PDE8 family can be detected in both human and mouse adrenal glands [38,39]. In mice, *pde8a* mRNA transcripts are enriched in a small population of fasciculata cells adjacent to the glomerulosa layers; in

contrast, the *pde8b* isoform is highly expressed throughout the fasciculata layers [40]. Treatment with a PDE8-selective inhibitor (PF-04957325) alone is sufficient to potentiate basal steroid secretion, while treatment with a non-selective PDE inhibitor, IBMX, has little to no effect on basal adrenal steroidogenesis [40]. However, IBMX treatment greatly potentiates ACTH-stimulated steroid production under maximally stimulated conditions (figure 2). Mechanistically, acute inhibition of PDE8 increases the phosphorylation states of many PKA substrates including hormone sensitive lipase (HSL) [40]. Furthermore, chronic ablation of PDE8B increases cAMP-dependent transcription of several key steroidogenic proteins, such as StAR protein and $p450_{\text{sc}}$ [40]. The importance of PDE8B in adrenal function has also been demonstrated *in vivo* by the fact that PDE8B knockout mice show significantly higher urinary corticosterone levels even in the face of lower circulating ACTH [40]. These findings suggest that PDE8B modulates steroidogenesis predominantly under low cAMP conditions, and additional IBMX-sensitive PDEs become increasingly important when cAMP levels are raised and the low K_m and low V_{max} PDE8s are overwhelmed.

Genetic variants of PDEs as contributors to steroidogenic diseases

Horvath et al. have implicated mutations in PDE8B enzymes as a contributing factor to predisposition of adrenal hyperplasia and Cushing's syndrome. These authors report a point mutation (c.914A to C, p.His305Pro mutation) in the *PDE8B* gene from a patient with a severe form of adrenal hyperplasia. The authors suggest that this PDE8B mutant retains only partial cAMP hydrolyzing ability since when overexpressed in HEK 293 cells, increased basal cAMP level is observed [41]. However, it is not yet clear if this mutation in the PDE8B gene can be causal for the disease. For example, PDE8B knockout mice do not develop enlarged adrenal glands compared to their littermate WT controls suggesting that additional mutations, perhaps another PDE and/or other elements of cAMP/PKA signaling pathway, may be required for development of the disease state in humans.

In addition to PDE8B, another high-affinity dual specificity PDE, PDE11A also has been implicated in adrenal diseases. A genome-wide scan of chromosome region 2q31-2q35 showed an increased number of polymorphisms in patients having micronodular adrenocortical hyperplasia. This region encodes several genes including the PDE11A gene [42]. The authors further showed adrenal tumor lysates from patients harboring PDE11A mutations contain elevated cGMP and cAMP levels, suggesting these mutations affect the hydrolytic activity of PDE11A.

A similar genomic analysis was also performed in patients with polycystic ovary syndrome (PCOS). PCOS is characterized by increased androgen production by ovarian theca cells. In this study, several single nucleotide polymorphisms (SNPs) in PDE8A (R136Q, L55V, and N401S) were identified and characterized [43]. Interestingly, these SNP do not appear to affect the enzymatic activity of PDE8A nor protein localization when the mutated PDE8A genes were overexpressed in COS-1 cells [43]. Therefore the mechanism by which alteration of PDE8A might contribute to the manifestation of PCOS remains unclear.

Concluding remarks

It is clear that inhibition of the right combination of PDEs promotes steroidogenesis, a highly cAMP-dependent process. As demonstrated in both adrenal and testicular cells, inhibition of PDE8 alone can be sufficient to potentiate basal steroid synthesis. More importantly, inhibition of multiple PDEs is necessary to achieve maximum steroid production. Vasta et al. showed that PDE8A-ablated Leydig cells exhibit a 5.7-fold decrease in the EC₅₀ for LH, but a 13-fold decrease in EC₅₀ if IBMX is also added [33]. Similarly, in adrenocortical cells both PDE8 isoforms appear to regulate steroid production under low cAMP conditions and other, IBMX-sensitive PDEs are most important when cellular cAMP synthesis is fully stimulated. Regardless of the conditions dual inhibition of PDE8 and one or more IBMX-sensitive PDEs is required to elicit maximal steroidogenesis [40], as demonstrated in figure 2. These observations suggest that different PDEs have overlapping roles for regulation of steroidogenesis.

The current theory of compartmentalized cyclic nucleotide signaling postulates that multiple PDEs but not all PDEs control any given functional pool of cAMP. However, in the design of most of the studies that led to this conclusion combinations of PDE inhibitors usually were not tested. It is therefore quite possible that an important regulation of any cAMP dependent process by any given member of the PDE family can be missed due to the overlapping role of PDEs. As illustrated by the synergistic roles of PDE2, PDE4, PDE8A and PDE8B described in this review, this is clearly the case for cAMP regulation of steroidogenesis. By analogy it is quite likely that we may well have been too quick to dismiss the involvement of any individual PDE before based solely on lack of an effect of a selective inhibitor of this PDE. A better approach should be to identify what PDE partner(s) it may have in the regulation of the process being measured. From a drug development perspective, it is quite possible that targeting multiple rather than single PDEs will allow us to achieve more effective therapeutic outcomes, possibly at lower, safer doses of each inhibitor.

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Highlights

> The cGMP-stimulated PDE2 mediates cGMP-induced decrease in aldosterone production. > PDE8A regulates testosterone production by itself and in concert with other PDEs. > PDE8B modulates basal corticosterone synthesis via acute and chronic mechanisms. > cGMP signals also can potentiate steroidogenesis, and PDE5 modulates this process.

Figure 1. PDE regulation of steroidogenesis

Steroidogenic cell types respond to pituitary hormones, such as ACTH and LH. Upon binding to these G_{α_s} -protein coupled receptors, cAMP is synthesized by adenylyl cyclase (AC) from ATP. The cAMP/PKA pathway then promotes steroidogenesis via acute PKA activation and long-term activation of cAMP-dependent transcription. The low K_m and low V_{max} PDE8 family is shown to modulate this pool of cAMP under basal to minimally stimulated conditions. Other IBMX-sensitive PDEs, like PDE4, modulate cAMP when cells are more highly stimulated. Additionally, the cGMP/PKG pathway also promotes steroidogenesis in some cell types, such as Leydig cells. PDE5 is reported to modulate this pool of cGMP in Leydig cells. However, in adrenal glomerulosa cells cGMP attenuates aldosterone production via activation of PDE2A. PDE2A, also known as cGMP-stimulated PDE, attenuates steroid production via increasing cAMP hydrolysis upon atrial natriuretic peptide (ANP) activation of guanylyl cyclase (GC).

Figure 2. The effects of PDE inhibitors on steroid production

Isolated primary mouse adrenal cells were used to establish this ACTH dose-response curve. In all conditions, the conversion of pregnenolone to progesterone is blocked by 10 μ M trilostane (a 3β HSD inhibitor). Therefore, pregnenolone secreted into the culture media is a reflection of the initial steps of steroid production. The same preparation of adrenal cells was pretreated for 30 min with vehicle, 50 μ M pan-PDE inhibitor IBMX, 100 nM PDE8-selective inhibitor PF-04957325, or a combination of both PDE8 and pan-PDE inhibitors. All data points were normalized to vehicle treated maximum pregnenolone as 100%, and curves were drawn using the sigmoidal dose-response fit on Graphpad Prism®. Under basal and submaximal stimulated conditions, PDE8 inhibition significantly potentiates pregnenolone production while IBMX treatment is less effective. However, IBMX treatment increases pregnenolone production most effectively under higher ACTH-stimulation. Furthermore, under all conditions inhibition of PDE8 plus at least one other IBMX-sensitive PDE is necessary to achieve maximum steroidogenesis.

Table 1

PDE Isoform	Tissue type	Functions Modulated
PDE2A	Adrenal glomerulosa cells	ACTH-stimulated aldosterone production in response to ANP-elicited cGMP [18].
PDE4	Leydig cells	LH-stimulated testosterone secretion [in submission].
PDE5	Leydig cells	cGMP/PKG-mediated potentiation in androgen production [26].
PDE8A	Adrenal fasciculata cells	Part of basal corticosterone production [40].
	Leydig cells	Testosterone production in conjunction with at least one other IBMX-sensitive PDE [33].
PDE8B	Adrenal fasciculata cells	Corticosterone production via both acute and chronic mechanisms [40].
	Leydig cells	A pool of cAMP distinct from that regulated by PDE8A and PDE4 [in submission].