Published in final edited form as:

Horm Metab Res. 2012 September ; 44(10): 790–794. doi:10.1055/s-0032-1321861.

Regulation of Adrenal Steroidogenesis by the High-affinity Phosphodiesterase 8 Family

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Abstract

The main function of cyclic AMP phosphodiesterases (PDEs) is to degrade cAMP, a ubiquitous second messenger. Therefore, PDEs can function as prime regulators of cAMP/PKA-dependent processes such as steroidogenesis. Until recently, the roles of the PDE8 family have been largely unexplored, presumably due to the lack of a selective inhibitor. This review focuses on recent reports about the regulatory roles of the PDE8 family in adrenal steroidogenesis, as well as the inhibitory properties and specificity of a new PDE8-selective inhibitor, PF-04957325. We also describe a method of measuring urinary corticosterone levels in vivo as a minimally invasive way of monitoring the stress level in a mouse.

Keywords

adrenal cortex; glucocorticoids; androgen

Introduction

Unlike humans, corticosterone is the predominant glucocorticoid in many species, including rodents. Glucocorticoids mediate the classical stress response (also known as fight-or-flight response), glucose, and fat metabolisms, and they also influence immune and behavior responses, among many other functions [1–4]. The synthesis of corticosterone is controlled by the pituitary hormone, adrenocorticotrophic hormone (ACTH) [5]. High circulating levels of corticosterone feedback then inhibit corticotropin-releasing factor (CRF) release from the hypothalamus as well as ACTH production from the pituitary gland forming a regulatory feedback loop known as the hypothalamic-pituitary-adrenal (HPA) axis. This feedback circuit maintains glucocorticoid homeostasis [6].

In adrenocortical cells, activation of the cAMP/PKA pathway is regarded as a major mechanism for promoting steroidogenesis. Upon ACTH stimulation cAMP is produced and cAMP-dependent protein kinase (PKA) is subsequently activated, which in turn promotes steroidogenesis by both acute and chronic mechanisms [7, 8]. Acutely, PKA promotes steroidogenesis via phosphorylation and activation of key regulators, such as the

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Conflict of Interest The authors report no conflict of interest.

steroidogenic acute regulatory (StAR) protein. Activation of StAR protein increases the transport of free cholesterol to the mitochondria [9]. These processes stimulate steroid biosynthesis by making substrate available to the mitochondrial cytochrome p450s therefore allowing the conversion of cholesterol into steroid intermediates and eventually into the final steroid products. In some conditions, such as ACTH or angiotensin II stimulation, increased cAMP/PKA also increases cholesterol availability by increasing the activity of cholesterol ester hydrolase (also known as hormone sensitive lipase) [10, 11]. In addition to short-term stimulatory effects, activation of the cAMP/PKA pathway also increases mRNA transcript levels of several of the key steroidogenic genes via cAMP/PKA mediated activation of transcription factors, including SF-1 and DAX-1 [12–15]. Besides modulating steroidogenic genes, transcription factors like WT-1 and PBX-1 also play a critical role in adrenal cell growth and proliferation during development [16]. All of these regulatory processes are controlled by cAMP, although possibly by different cAMP compartments. The level of cAMP in each of these compartments is determined by its rates of synthesis by adenylyl cyclases (ACs) and degradation by cyclic nucleotide phosphodiesterases (PDEs). Therefore, PDEs play a key, modulating role in regulating cAMP-dependent steroidogenesis.

The PDE8 Family

In mammals, there are 11 families of PDEs, and each PDE family has its own unique kinetic, regulatory partners, as well as inhibitor sensitivity [17]. The PDE8 family is one of the more recently discovered PDE families, and it consists of 2 distinct genes – *PDE8A* and *PDE8B*. *PDE8A* and *PDE8B* have very high affinity towards cAMP and hydrolyze cAMP with a $K_m \approx 0.15 \mu\text{M}$ [18]. Neither of the PDE8s 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+} [19, 20]. However, no other small molecule or protein 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 [21]. Comparable to what is found in humans, high levels of mRNA transcripts of *Pde8a* are also found in testes, heart, liver, and kidney of a mouse as shown by Northern blot analysis [20]. Nevertheless, the expression of *PDE8B* appears to be more restricted than most other PDEs. Northern blot analysis revealed a high level of *PDE8B* expression in the human thyroid, and lower but significant levels in brain, spinal cord, and placenta [22]. *PDE8B* expression was validated in a recent study using quantitative real-time PCR in various human tissues [23]. Recently, the expression of *PDE8B* was also demonstrated in human adrenal glands [24]. Interestingly, *Pde8b* mRNA expression in rats and mice differs slightly from that in humans. For example, mRNA transcripts of *Pde8b* were only expressed at very low level in the thyroid of rat [25], and the same observation was made in thyroid of mouse. Nevertheless, some similarities are preserved; for example *Pde8b* transcripts were also found highly enriched in steroidogenic tissues [26, 27] and brain (unpublished data) of mice.

Unlike other cAMP-specific PDEs, PDE8s are insensitive to a commonly used nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), but can be inhibited by a high

concentration of dipyridamole ($IC_{50} = 23 \mu M$) [20]. Only recently, Pfizer, Inc. has developed a series of small molecules that selectively inhibit PDE8s [28]. The availability of this new PDE8-selective inhibitor enabled us to carefully investigate possible roles for PDE8s. A part of this review therefore describes characteristics of PF-04957325 (structure published in [29]).

Only a few PDE8-modulated physiological processes have been described since the discovery and characterization of this PDE family. PDE8A has been suggested to regulate chemotaxis of activated mouse lymphocytes demonstrated by using a semi-selective inhibitor (dipyridamole) [30], and PDE8A also modulates effector T cell functions shown by treatment with dipyridamole and PF-04957325 [29]. PDE8A also modulates testosterone production in mouse Leydig cells [31, 32] as well as androgen production from ovarian theca cells [33]. Furthermore, genetic ablation of *Pde8a* causes a potentiation of β -adrenergic receptor induced Ca^{2+} release in mouse ventricular cardiomyocytes [34]. Compared to PDE8A, the physiological role of PDE8B is less well understood. Genetic studies have shown a correlation between a SNP of the human *PDE8B* gene and thyroid function [35]. Mutations in human *PDE8B* gene also have been linked to adrenal hyperplasia [24] and autosomal-dominant striatal degeneration [36]. Finally, the Nesher group demonstrated that siRNA knockdown of *Pde8b* sensitized the insulin response to glucose in pancreatic β -cells of rats [37]. The *Pde8b* knockout mice exhibit hyperactive adrenal glands; however other possible abnormalities have not been extensively studied. This review focuses on the current findings on the roles of PDE8 family in regulation of adrenal steroidogenesis.

Both Isoforms of the PDE8 Family Regulate Basal Steroid Production in Adrenocortical Cells

Messenger RNA transcripts for both *PDE8A* and *PDE8B* isoforms can be detected in both human and mouse adrenal glands [23, 24]. In mice, *Pde8a* mRNA transcripts appear to be largely restricted to a small population of fasciculata cells adjacent to the glomerulosa layers. In contrast, the *Pde8b* mRNAs are highly expressed throughout the fasciculata layers [27]. Since, *Pde8a* and *Pde8b* have different staining patterns in the adrenal, it is unclear if both isoforms of PDE8 are expressed in the same cells in vivo. In isolated adrenal and Leydig cell lines both PDEs can be found in the same cell [27].

Regulation of PDE8s in adrenal steroidogenesis was demonstrated by increased pregnenolone secretion from primary adrenal cells isolated from *Pde8b* KO and *Pde8a/b* double KO mice (Fig. 1). Adrenal cells with both *Pde8a* and *8b* disrupted showed significantly higher steroid production in the basal state, and cells with only *Pde8b* disrupted also showed a smaller increase in steroid secretion suggesting that PDE8A and 8B isoforms both play a modulating role in adrenal steroidogenesis. Similarly, a PDE8-selective inhibitor that inhibits both PDE8A and PDE8B, PF-04957325, also greatly potentiates steroidogenesis in WT adrenal cells (Fig. 1), demonstrating the involvement of Pde8s.

Mechanistically, we have shown that acute treatment with PF-04957325 increases PKA activity, which was determined by increased intensity of multiple bands recognized by a

pan-PKA substrate antibodies in Western analysis [27]. Furthermore, chronic treatment with PF-04957325 also activated cAMP-dependent transcription as shown by increased mRNA transcripts of *StAR* protein and *p450_{scc}* [27]. It is unclear if an individual PDE8 isoform is responsible for controlling these cAMP-dependent mechanisms, or both isoforms are working in concert. However, PDE8B alone is in part responsible for the modulation of cAMP-dependent transcription as evidenced by an increased in mRNA transcripts of *StAR* protein found in *Pde8b* knockout adrenal glands [27].

The involvement of PDE8B was further confirmed by performing acute ablation of PDE8B enzyme via an shRNA-based RNA interference in Y-1 cells, a commonly used adrenal tumor line. The shRNA construct effectively reduced the level of *Pde8b* mRNA transcripts in Y-1 cells, and as a result *Pde8b*-reduced cells secreted more steroid basally compared to control cells transfected with a nonselective shRNA construct [27].

Measurement of Urinary Corticosterone Reveals *Pde8b*-Ablation Increased Corticosterone Levels in both Basal and Stimulated States

In order to determine the consequence of genetically ablating *Pde8b* on corticosterone secretion in vivo, we adapted a minimally invasive method of measuring urinary corticosterone [38], which enabled us to measure levels of this stress hormone close to its basal state. The reasoning here is that the level of corticosterone measured in the urine, which is collected in the bladder of a mouse prior to any handling, reflects an average of circulating corticosterone over the previous few hours before handling. We obtained urine samples by gently pressing on the bladder to encourage urination if urination did not happen spontaneously upon initial handling. Then the animals were placed individually in holding chambers (as illustrated in Fig. 2) to collect urine samples that reflect the corticosterone level due to the stress of handling, presumably a rather mild-stress state. Using this method, we showed that genetically ablated *Pde8b* mice exhibited elevated urinary corticosterone in both basal and stress-stimulated states in face of reduced circulating ACTH [27], attesting to the regulatory role of PDE8B in adrenal steroidogenesis. We also interpreted the decrease in circulating ACTH as a consequence of chronic elevation of corticosterone and as an attempt of the HPA-axis to correct for this abnormality.

Characterization of a New PDE8-Selective Inhibitor (PF-04957325) in vitro and in Intact Cell Systems

Pfizer Inc. developed a PDE8-selective inhibitor, PF-04957325, with a reported IC₅₀ of 0.7 nM against PDE8A, 0.2 nM against PDE8B, and > 1.5 μM against all other PDE isoforms [27, 29]. We verified the IC₅₀ of PF-04957325 by constructing PF-04957325 inhibition curves on immunoprecipitated PDE8A and PDE8B from MA-10 cells (a mouse Leydig cell line) [32], and recombinant PDE4D enzymes as shown in Fig. 3. We showed that PF-04957325 has ≈ 1 000-fold selectivity toward PDE8s in vitro. The specificity of this compound was further demonstrated by the observations that PF-04957325 showed only a partial stimulation of steroid production in *Pde8b* knockout cells and no stimulation in *Pde8a/8b* double knockout cells (Fig. 1). These results indicate that PF-04957325-elicited

steroid productions in adrenal cells [27] and Leydig cells [32] are in fact due to inhibition of PDE8s and not some other off-target effect.

Furthermore, we also observed that PF-04957325 exhibits qualities of a conventional competitive inhibitor. For example, the effectiveness of this PDE8-selective inhibitor is greatly affected by concentrations of substrate of PDE8, cAMP. PDE activity assay revealed a rightward shift and an increase in IC_{50} of PF-04957325 when cAMP concentration was increased from 12 nM to 1 μ M (Fig. 4a) as would be expected for a competitive inhibitor. A similar rightward shift in PF-04957325 dose-response curve and an increase in EC_{50} were observed in Y-1 cells in presence of 10 pM ACTH (Fig. 4b). These observations suggest that PF-04957325 is likely competing for the same binding site on PDE8B as cAMP, or a remote possibility that the cAMP-bound PDE8B adapts to a lower affinity toward the drug. It is also noteworthy that the apparent EC_{50} of PF-04957325 in an intact cell system is at least 10-fold higher than its IC_{50} . This phenomenon likely can be attributed to the inherently high basal cAMP concentration in cells and/or possibly to poor permeability and rapid degradation of PF-04957325.

Concluding Remarks

The ability of both members of the PDE8 family to regulate steroid production has been demonstrated in different steroidogenic cell types, including testicular Leydig cells and adrenal fasciculata cells. In Leydig cells, PDE8A modulates testosterone production in concert with PDE8B and PDE4 [31, 32]. In adrenal cells, PDE8B and PDE8A both modulate corticosterone synthesis under basal to submaximal stimulation [27]. Interestingly, PDE8A and PDE8B showed distinct immunostaining patterns and appeared to localize to different organelles. PDE8A appears to localize near mitochondria while PDE8B appears more cytosolic and closely associated with the Golgi apparatus [32]. The question remains, “are PDE8A and 8B subserving different functional and/or physical pools of cAMP in those cell types?”. Also, which aspects of cAMP-stimulated steroidogenesis is each member of the PDE8 family modulating?

Regardless if PDE8A and PDE8B are functionally distinct, it is clear that both PDE8A and PDE8B modulate steroid production most effectively under low cAMP conditions in all tested cell types. This is likely due to the intrinsic kinetics of the PDE8 family, a low K_m and V_{max} enzyme. Its unique characteristics make the PDE8 family a perfect candidate for regulating cAMP levels in basal state, but they become insufficient to keep up when cAMP level is rising rapidly under stimulated conditions. Therefore, additional PDEs, such as PDE4, are employed to regulating the same process (steroidogenesis) under different conditions (high cAMP). From a therapeutic perspective, inhibition of multiple PDEs (PDE8s and PDE4s) may be necessary if the goal is to achieve maximum steroidogenesis.

Acknowledgments

We thank Dr. Masami Shimizu-Albergine and Stephen Kraynik for their contribution in generating Fig. 3. Supported by NIH Grant GM 083296.

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Fig. 1.

Steroid from adrenal cells isolated from WT, *Pde8b* KO and *Pde8a/8b* and double KO mice. Pregnenolone levels were measured in presence of a 3β HSD inhibitor (10 μ M trilostane) as an indicator of steroidogenic capability. The involvement of the PDE8 family was demonstrated by the increase in basal pregnenolone secretion from *Pde8b* KO and *Pde8a/b* dKO cells compared to WT control, and the potentiating effect of a PDE8-selective inhibitor, PF-04957325 when given to WT cells. The specificity of PF-04957325 was demonstrated by a partial loss of steroid potentiation in *Pde8b* KO cells and a complete loss of effect in *Pde8a/b* dKO cells. The data are reported as means $\pm$ SEM, and the p values were obtained with Dunnett post hoc test: n.s.: no significance; **p < 0.01, using WT vehicle as control; #p < 0.5 using WT treated with PF-04957325 as control.

Fig. 2.

Urine collection holding chamber set-up. The holding chambers for urine collection were constructed from transparent sheets (as walls) and 96-well strip well frames (as floors). The holding chambers were also wrapped in aluminum foils to minimize outside disturbance during the holding period. Each chamber then was placed on top of a 96-well plate for urine collection with a 2 mm mash in-between to separate feces from urine.

Fig. 3.

Inhibitory properties of PF-04957325 against PDE8B, PDE8A, and PDE4D.

Immunoprecipitated PDE8A (Santa Cruz PDE8A C-15 antibody) and PDE8B (Santa Cruz PDE8B I-16 antibody) from MA-10 cells lysate, and a crude lysate of PDE4D expressing *E. coli* were used in PDE activity assays. With 12–14 nM of cAMP as substrates, we obtained IC₅₀ values of 3.05 ± 2.9 nM against PDE8A, and 0.44 ± 0.4 nM against PDE8B (reported as means $\pm$ SD, n = 3) [32]. These IC₅₀ values agreed with the reported values. We also verified the IC₅₀ value of PF-04975325 against PDE4D to be approximately 1 μ M.

Fig. 4.

Effect of cAMP concentrations on IC_{50} of PF-04957325. **a** PDE8B was purified from the lysate of striatal region of mouse brains with a polyclonal goat antibody (PDE8B I-16) from Santa Cruz Biotechnology, Inc. The inhibitory properties of PF-04957325 toward PDE8B were determined under cAMP concentration of either 12 nM or 1 μ M. A rightward shift in the inhibition curve was observed under higher cAMP concentration. **b** Similarly, the ability of PDE8-selective inhibitor (PF-04957325) to potentiate pregnenolone production in Y-1 cells was also affected by intercellular cAMP levels. Under basal condition, PF-04957325 had an EC_{50} of approximately 3 nM in Y-1 cells; the EC_{50} was shifted over 10-fold to the right ($\approx$ 60 nM) when Y-1 cells were stimulated with 10 pM ACTH. The data are reported as means $\pm$ SD ($n = 2$).