The high affinity cAMP-specific phosphodiesterase 8B (PDE8B) controls steroidogenesis in the mouse adrenal gland†

Li-Chun Lisa Tsai, Masami Shimizu-Albergine, and Joseph A. Beavo

Department of Pharmacology, University of Washington, Seattle, Washington 98195, USA
Running title page

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Corresponding author: Joseph A. Beavo

Address: University of Washington, Department of Pharmacology, Box 357280, Seattle, WA 98195-7280, USA

E-mail: beavo@u.washington.edu

Tel: 206 543-4006

FAX: 206 685-3822

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Abbreviations: AZF, adrenal zona fasciculate; ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor; HPA axis, hypothalamic-pituitary-adrenal axis; MC2R, melanocortin 2 receptor; PKA, cAMP-dependent kinase; HSL, hormone sensitive lipase; StAR protein, steroidogenic acute regulatory protein; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; shRNA, short-hairpin RNA; RNAi, RNA interference; GFP, green fluorescent protein; ANP, atrial natriuretic peptide.
Abstract

The functions of the PDE8 family of phosphodiesterases have been largely unexplored due to the unavailability of selective pharmacological inhibitors. Here we report a novel function of PDE8B as a major regulator of adrenal steroidogenesis using a genetically ablated PDE8B mouse model as well as cell lines treated with either a new PDE8-selective inhibitor or an shRNA construct against PDE8B. We demonstrate that PDE8B is highly enriched in mouse adrenal fasciculata cells, and show that PDE8B knockout mice have elevated urinary corticosterone due to adrenal hypersensitivity toward ACTH. Similarly, acute ablation of PDE8B mRNA transcripts by an shRNA construct potentiates steroidogenesis in the commonly used Y-1 adrenal cell line. We also observed that the PDE8-selective inhibitor (PF-04957325) acutely potentiates ACTH stimulation of steroidogenesis by increasing PKA activity in both primary isolated adrenocortical cells and Y-1 cells. Interestingly, PDE8s have their greatest control under low ACTH-stimulated conditions while other higher $K_m$ PDE(s) modulate steroidogenesis more effectively when cells are fully stimulated. Finally, both genetic ablation of PDE8B and chronic pharmacological inhibition of PDE8s cause increased expression of steroidogenic enzymes. We conclude that PDE8B is a major regulator of one or more pools of cAMP that promote steroidogenesis, via both acute and chronic mechanisms. These findings further suggest PDE8B as a potential therapeutic target for the treatment of several different adrenal diseases.
Introduction

Glucocorticoids serve a number of important roles in mammalian physiology as they regulate glucose and fat metabolism, mediate stress responses, and influence the immune system, among other functions (Aguilera et al, 2007; Macfarlane et al, 2008; McCann et al, 2000; Vinson, 2009). The major murine glucocorticoid is corticosterone. Corticosterone is synthesized by adrenal zona fasciculata (AZF) cells, found in the thickest layer of the adrenal cortex. Steroidogenesis by these cells is regulated by the pituitary hormone, adrenocorticotropic hormone (ACTH) (Vinson, 2003). Among the many functions of corticosterone, high circulating levels can inhibit corticotropin-releasing factor (CRF) release from the hypothalamus as well as ACTH production from the pituitary gland. These effects on the hypothalamus and pituitary form an efficient feedback regulatory loop known as the hypothalamic-pituitary-adrenal (HPA) axis which maintains glucocorticoid homeostasis (Jacobson, 2005).

ACTH binding to its receptor, the melanocortin 2 receptor (MC2R), stimulates production of the second messenger cyclic adenosine monophosphate (cAMP) in AZF cells. cAMP then activates cAMP-dependent protein kinase (PKA), which in turn promotes steroidogenesis by at least three mechanisms (Manna et al, 2009; Stocco et al, 2005). First, PKA can regulate the availability of free cholesterol, the initial substrate for corticosterone biosynthesis. This occurs by phosphorylation and activation of hormone sensitive lipase (HSL) [also known as cholesterol ester hydrolase] which catalyzes the hydrolysis of
stored cholesterol esters into free cholesterol and a fatty acid (Arakane et al., 1997; Kraemer & Shen, 2002). Second, PKA can phosphorylate and activate the steroidogenic acute regulatory protein (StAR), a key regulator of free cholesterol transfer from stores to the mitochondrial membrane (Stocco, 2001). This process then allows a steroidogenic cytochrome p450 enzyme, p450scc, to convert cholesterol into pregnenolone, initiating corticosterone synthesis. This synthetic pathway includes the enzymes 3βHSD, p450c21, and p450c11 that catalyze a cascade of reactions ultimately leading to production of corticosterone. It is important to mention that the transport of cholesterol to mitochondria by StAR protein is usually regarded as the rate-limiting step of this pathway. However, under some conditions, HSL activity can acutely limit synthesis.

Finally, PKA activation also has a long-term 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. (Davis & Lau, 1994; Sewer & Waterman, 2003; Simpson & Waterman, 1988). All of these regulatory processes are controlled by cAMP, although possibly by different pools.

The level of cAMP in each of these pools is determined by its rate of synthesis by adenylyl cyclases and rate of degradation by phosphodiesterases (PDEs) (Conti & Beavo, 2007). Of these PDEs, the PDE8 family is one of the more recently discovered. The PDE8 family consists of two distinct genes - Pde8a and Pde8b. Both PDE8A and 8B hydrolyze cAMP with a very high affinity.
(Km ~0.15 µM). Unlike other cAMP-specific PDEs, PDE8s are insensitive to a common non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), but can be inhibited by a high concentration of dipyridamole (Soderling et al, 1998). Until recently, no truly selective PDE8 inhibitor has been available. However Pfizer recently has reported on a small molecule that selectively inhibits PDE8 (Vang et al, 2010). This new selective PDE8 inhibitor (PF-04957325) has a reported in vitro IC<sub>50</sub> of 0.7nM against PDE8A, 0.2nM against PDE8B, and >1.5 µM against all other PDE isoforms (Vang et al, 2010). In this study, we have used this pharmacological tool with isolated primary adrenal cells as well as with a commonly used adrenocortical cell line, Y-1 cells, to demonstrate that PDE8 inhibition potentiates steroid production under subsaturating levels of ACTH stimulation.

Additionally, we have taken a genetic approach by utilizing a global PDE8B knockout mouse model to investigate the long-term consequence of PDE8B ablation on steroidogenesis. In 2006, our laboratory reported modulation of testosterone secretion by PDE8A in Leydig cells using a similar PDE8A KO (Vasta et al, 2006). These studies showed that PDE8A plays an important role in regulating a pool of cAMP that promotes testicular steroidogenesis. Here we report that the other member of the PDE8 family, PDE8B, regulates ACTH-stimulated AZF steroidogenesis by both acute and chronic mechanisms. Finally, a mutation in PDE8B has also been implicated in a single severe case of adrenal
hyperplasia in humans (Horvath et al, 2008a). Together, these data suggest the general importance of PDE8s in cAMP regulation of steroid production.
Materials and Methods

\textbf{\(\beta\)-gal activity staining} -

Adrenal glands were fixed in 4% (wt/vol) paraformaldehyde on ice for 6 hours. The fixed glands were sequentially rinsed with 10% sucrose, soaked in 20% sucrose for 8 hours, and then soaked in 30% sucrose overnight to remove paraformaldehyde. The tissue was embedded in Tissue-Tek O.C.T. compound and sectioned on a cryostat into 20 \(\mu\)m thick floating slices in PBS (pH 7.4). \(\beta\)-galactosidase activity was evaluated by staining the sections with 5-bromo-4-chloro-3-hydroxyindole (X-gal), as adapted from a protocol described previously (Duffield et al, 2005). Briefly, after mounting the floating sections on slides, the slides were placed in an X-gal staining mixture (2 mM MgCl\(_2\), 0.01% Na-deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, 20 mM K\(_3\)Fe(CN)\(_6\), 20 mM K\(_4\)Fe(CN)\(_6\)\(\cdot\)H\(_2\)O, and 1 mg/mL X-gal in PBS pH 7.4) at 37\(^\circ\)C overnight. After the incubation with X-gal, the slides were washed three times with PBS pH 7.4, once with water, and then counterstained with Eosin and mounted with Permount\textsuperscript{®} (Fisher Scientific).

\textbf{Real-Time PCR} -

Whole mouse adrenal glands were disrupted with a dounce homogenizer in RTL buffer from a Qiagen RNeasy mini kit (Qiagen Inc.). Total RNA was isolated using Qiashredder columns and the RNeasy mini kit according to the manufacturer’s protocol. Then cDNA samples were generated with Qiagen Omniscript RT kits using 2\(\mu\)g of total RNA for each reaction. Relative gene
expression was determined by performing real-time PCR on a MX3000P QPCR system (Stratagene) and analyzed with Mx-Pro® software. The primers for adrenal p450s have been previously reported and verified (Cooray et al, 2008; Otawa et al, 2007). RT-PCR reactions were run with iTaq SYBR supermix (Biorad) with the following thermal profile: denaturing at 95°C for 15 sec, annealing at 58°C for 1 min, extension at 72°C for 1 min, for 40 cycles.

**Immunoprecipitation and PDE activity assay -**

Immunoprecipitation of PDE8B was performed with goat poly-clonal antibodies to PDE8B [PDE8B (I-16) from Santa Cruz Biotechnology, Inc.] in tissue lysates made in 0.5% Triton® X-100, 1mM EDTA, 1:100 diluted protease inhibitor cocktail (Sigma) in pH 7.4 phosphate buffered saline (PBS). The immunopellet was re-suspended and washed three times with lysis buffer before PDE activity was measured. PDE activity was measured as previously described (Hansen & Beavo, 1982; Soderling et al, 1998). Briefly, the activity assay was carried out at very low substrate conditions (10-20 nM [³H]-cAMP, ~100,000 cpm/reaction) in 40mM MOPS (pH 7.5), 15mM Magnesium acetate, 2mM EGTA, and 0.2 mg/mL bovine serum albumin (BSA) in a final volume of 250 mL. The reaction time and amount of lysate were maintained so that less than 30 percent of the substrate was hydrolyzed.

**Animals –**
The PDE8B KO mice utilized in these studies were generated on a 129 genetic background by Deltagen, Inc. (San Carlos, CA) under contract to Pfizer, Inc. (Sandwich, U.K.). The animals were then backcrossed with C57BL/6 mice obtained from Charles River or Jackson Laboratory for 12-15 generations. All animal usage and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Washington, in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All experiments were performed on PDE8B KO animals between the ages of 4-12 months, using their WT littermates as controls.

The double PDE8A/8B knockout mice were established in the laboratory by crossing the PDE8B KO with the previously described PDE8A KO (Vasta et al, 2006). Deltagen, Inc generated the PDE8A KO with a similar knockout strategy as the PDE8B KO.

**Urinary corticosterone measurements** -

All corticosterone measurements were carried out between 6PM and 12AM during the dark cycle when the animals were most active. First, animals were provided with 4mM Saccharin water *ad libitum* in their home cages to increase their water consumption 2 hours prior to an experiment or any handling (at 4PM). Urine from an individual mouse reflecting the basal, non-stressed, state was collected at 6PM. Usually the animals urinated spontaneously during handling. Occasionally, gentle pressure was applied to the bladder to encourage urination. Animals were then injected intraperitoneally with 1mL isotonic saline to increase
frequency of urination. The combination of handling and saline injection provided a mild stress. Mice were then placed individually in urine collection cages (constructed with a disposable 96-well plate and a 96-well strip well frame, with 2mm mesh above the wells to separate feces from urine. Transparent plastic sheets were used as walls). All mice were handled and injected for two days prior to the actual urine sample collections. On the third day their urine samples collected at 3 hours and 6 hours post intraperitoneal injection, these were regarded as post-stimulated state samples.

**Primary mouse adrenal cell culture and pregnenolone measurement**

A primary adrenal cell isolation protocol was adapted as previously described (Enriquez de Salamanca & Garcia, 2003; Supornsilchai et al, 2005). Briefly, mouse adrenal glands were removed and the attached fat tissue was trimmed off. Whole adrenal glands were minced with a pair of scissors and digested in a buffer (90mM HEPES, 120mM NaCl, 5mM KCl, 1mM CaCl2, 4.5mM Glucose, and 15mg/mL BSA) containing freshly prepared Collagenase type IV (2 mg/mL) and DNAse (0.1 mg/mL) (Worthington biochemical Corp., Lakewood, NJ). The minced adrenals were incubated with digestion buffer for 1 hour at 37°C with agitation. The digested tissue was then triturated 15-20 times with a 1mL Eppendorf pipette and filtered once into a polystyrene tube fitted with a cell-strainer cap (Falcon, 352235). Cells were collected by brief centrifugation (~300g, 10 min) and washed twice with oxygenated HBSS + 1 mg/mL BSA (further purified fraction V and γ-globulin free, Sigma). Cells were then re-
suspended in DMEM/F12 (1:1) media with 1mg/mL BSA and plated in 96-well plates. The cells then were allowed to recover for 3 hours in the incubator at 37°C. After recovery, the cells were pre-incubated with 10µM trilostane \[\{(4\alpha,5\alpha,17\beta)-4,5-Epoxy-3,17-hydroxyandrost-2-ene-2-carbonitrile\}\] (Sanofi-synthelabo research, Malvern, PA) for 30 minutes to inhibit the conversion of pregnenolone to progesterone. Synthetic full length ACTH peptide (1-39) (AnaSpec, San Jose, CA) was used on some cells to stimulate steroidogenesis. The amount of pregnenolone in the media was determined by pregnenolone ELISA (Diagnostics, Biochem Canada Inc., Ontario, Canada).

**Y-1 cell culturing and reagents –**

Y-1 cells were cultured in F12K media with 20% FBS. Stocks of Y-1 cells were frozen in media with 5% DMSO according to ATCC guidelines. The non-selective PDE inhibitors used in the cell culturing models, dipyridamole and IBMX, were purchased from Sigma-Aldrich. A low PDE2A Y-1 expression clone was established in our laboratory by clonal selection and used in all reported study. Y-1 cells were originally obtained from ATCC.

**shRNA ablation of PDE8B –**

Y-1 cells were transfected with either an shRNA construct (3’-aatcctcatcaacgcatgat-5’) or control shRNA plasmid (SureSilencing™ shRNA plasmids, SA Biosciences) using the Nucleofector® technology (Lonza Walkersville Inc., Walkersville, MD). The control shRNA plasmid had all the
same elements as shRNA plasmid such as a green fluorescent protein (GFP) marker and a 21-nucleotide shRNA sequence (3'-ggaatctcatcagctcatac-5') with no known target. The Y-1 cells were transfected according to the manufacturer’s instruction. Briefly, Y-1 cells (2.5 million cells per transfection) were resuspended in buffers provided with the Cell Line Nucleofector® Kit V along with 2 µg of plasmid DNA. Y-1 cells were electroporated with a Nucleofector® Device (program T-32). The transfected Y-1 cells were plated and allowed to recover for 48 hours before testing. Transfection efficiency was monitored by determining the percentage of cells expressing measurable GFP plasmid marker. The knockdown efficiency was measured by real-time PCR probing for PDE8B mRNA (detecting exon 8-9). Steroid secretion of Y-1 cells containing shRNA plasmids was determined using the same pregnenolone kit described earlier.
Results

PDE8B is highly expressed in adrenal zona fasciculata (AZF) cells.

In the PDE8B KO animals utilized in these studies, a critical region in the catalytic domain (exon 14-15) was replaced with DNA sequence encoding a lacZ reporter gene and a neomycin resistance gene followed by a stop codon (figure 1A). The exogenous lacZ gene also contained a nuclear targeting sequence that directs the β-galactosidase activity to the nucleus of cells having active endogenous PDE8B promoter. The enzymatic activity of the gene product (β-galactosidase) therefore can be used as an indirect measurement of PDE8B mRNA expression. Using this method we found that the PDE8B promoter was highly active in the adrenal gland. Moreover, we observed that over 80% of the adrenocortical cells contained blue (X-gal stained) nuclei (figure 1B). These results indicate that the PDE8B gene is transcribed in most of the adrenocortical cells, and that PDE8B mRNA is primarily made in the AZF cells.

As shown in figure 1C, disruption of PDE8B gene was verified by real-time PCR. We observed that the amplicons of the deleted exon 14-15 region and the 3’ region downstream of this region were either very low or undetectable in the PDE8B KO. However, all of the 5’ PDE8B mRNA amplicons were increased in relation to WT, perhaps due to lack of functional PDE8B enzymes. Using Western blotting with commercially available antibodies (with either C-terminal or N-terminal epitope), we did not detect any truncated PDE8B proteins in the PDE8B KO adrenal glands. To further demonstrate that no active PDE8B
protein was produced in the PDE8B KO, we performed immunoprecipitation with an antibody [Santa Cruz, PDE8B (I-16)] against an epitope in PDE8B that is upstream of the disrupted exon 14-15. The results show that IBMX-insensitive PDE8B activity can be precipitated with this antibody from the WT but not PDE8B KO adrenal lysate (figure 1D). In addition, we also demonstrated that the new selective PDE8 inhibitor (PF-04957325) blocked the immunoprecipitated PDE activity at 10nM. Finally, a lack of PDE activity in the peptide blocking control indicated antibody specificity for PDE8B. Overall these data verify the absence of functional PDE8B activity in the PDE8B KO mice used in these studies.

**PDE8B KO mice exhibit adrenal hypersensitivity in vivo.**

The general importance of cAMP/PKA signaling in steroidogenesis plus the high expression of PDE8B in the adrenal cortex lead us to hypothesize that PDE8B is a modulator of one or more pools of cAMP stimulated by ACTH in AZF cells. Additionally, because PDE8A modulates testosterone production in Leydig cells (Vasta et al, 2006), we speculated that PDE8B might play a similar role in AZF cells. Up to now, specific functions of PDE8B have remained relatively unexplored due to the unavailability of a selective inhibitor. Therefore, the PDE8B KO mice provided us with a good tool to test for possible role(s) of PDE8B in adrenal steroidogenesis.
These PDE8B KO mice did not exhibit any obvious reproductive or developmental defects. They appeared healthy and maintained a normal weight under regular chow diet (figure 2A). However, we were interested to see if complete ablation of the PDE8B gene in the mouse could induce an adrenal hyperplasia phenotype as reported for one individual patient who had a point mutation in the PDE8B gene (Horvath et al, 2008a). However, by comparing the adrenal weight and morphology of serial cross-sections of the adrenal glands, we did not observe any obvious increase in the size of the PDE8B KO adrenal glands (supplementary figure 1).

We then tried to determine if the PDE8B KO mice had any adrenal hypersensitivity to ACTH in vivo. As one test of this idea, we determined urinary corticosterone under basal and mild stress conditions as a measure of adrenal steroid production. As shown in figure 2B and 2C, we found that PDE8B KO mice had elevated levels of urinary corticosterone in both basal and stressed conditions compared to their littermate WT controls. These data suggest that PDE8B KO mice exhibit adrenal hypersensitivity toward ACTH. However, it also was possible that the loss of PDE8B might be causing an increase in ACTH. Therefore, we measured serum ACTH levels by ELISA and found that the PDE8B KO mice had a lower, not higher, circulating ACTH level compared to the WT (figure 2D). This result is consistent with the idea that the suppressed ACTH level is due to a chronic elevation of corticosterone negatively feeding back to the HPA-axis. Overall, these findings suggest that PDE8B is an important regulator
of adrenal steroidogenesis in vivo. Therefore ablation of the PDE8B gene causes adrenal hypersensitivity toward ACTH and an altered HPA-axis as a result of abnormal corticosterone production.

**PDE8B gene ablation increases mRNA expressions of StAR protein and MC2R.**

We then investigated the molecular mechanisms by which PDE8B ablation might increase adrenal steroid production. Since the expression of several key steroidogenic enzymes are known to be transcriptionally regulated by cAMP, we hypothesized that the ablation of PDE8B might chronically increase the expression of these genes, namely StAR protein, several cytochrome p450s, and MC2R (Hammer et al, 2005; Manna et al, 2009; Mountjoy et al, 1994; Sewer & Waterman, 2003). As shown in figures 3A and 3B, PDE8B KO adrenal glands expressed mRNA for these cAMP-regulated steroidogenic genes at a higher level than WT. There was an approximately two-fold increase in mRNA levels of StAR protein and MC2R in the KO adrenal glands. However, the mRNA levels of several of the other cAMP-regulated enzymes (P450_{scc}, P450_{21}, P450_{c11b}, and 3βHSD) showed no statistically significant increase in the PDE8B KO. Therefore it seems that an increase in mRNA levels of StAR protein and MC2R may explain at least part of the adrenal ACTH hypersensitivity seen in the PDE8B KO animals in vivo.
PDE8 inhibition with PDE8 selective inhibitors (dipyridamole and PF-04957325) increases acute adrenal steroid production in Y-1 cells.

In addition to the chronic effects on gene transcription mentioned above, it seemed possible that loss/inhibition of PDE8B activity also might have acute effects on corticosterone production. To test this idea, we used two adrenal cell culture models, the mouse adrenocortical Y-1 cell line and primary isolated mouse adrenal cells, to study the effects of PDE8 inhibition on more acute phases of steroidogenesis. In both cases, we measured pregnenolone secreted into the media as the readout of steroid production because it is an early immediate product of the rate-limiting step of steroidogenesis. In all experiments, cells were pretreated with a 3βHSD inhibitor (10µM trilostane) (Bhattacharyya et al, 1995; Chaffin et al, 2000). At this concentration, trilostane effectively prevents most conversion of pregnenolone to corticosterone. Moreover, the Y-1 cells still respond to ACTH-stimulation in the presence of trilostane, and their activation was reflected by a dose-dependent increase in pregnenolone production (supplementary figure 2).

We first showed that a semi-selective PDE8 inhibitor, dipyridamole (IC₅₀ ~20µM), potentiated ACTH-induced pregnenolone production in Y-1 cells. Interestingly, dipyridamole appeared to be more effective than the non-selective PDE inhibitor, IBMX, which does not inhibit PDE8 (figure 4A). However, since dipyridamole can also inhibit several other PDEs, we also tested a new selective PDE8 inhibitor (PF-04957325, Lot #: 0006) to see if it had a similar potentiating
effect on pregnenolone production in Y-1 cells. As expected, treatment with the selective inhibitor (PF-04957325) potentiated pregnenolone production in Y-1 cells, while IBMX only slightly increased steroid production (figure 4B). Together these findings suggest that one or more PDE8s are important modulators of the pool(s) of cAMP that promote steroidogenesis in Y-1 cells. Since these cells contain both PDE8A and 8B, and since neither inhibitor distinguishes between these two gene products it was still unclear from these experiments if one or both PDE8 isoforms are important regulators of steroidogenesis in the Y-1 cells.

**Short hairpin RNA (shRNA) against PDE8B also potentiates steroidogenesis in Y-1 cells.**

To address the issue of Y-1 cells containing both PDE8 isoforms, we used short hairpin RNA (shRNA) induced RNA interference (RNAi) against PDE8B to convincingly demonstrate the effect of PDE8B in steroidogenesis. Using an electroporation system, we were able to transfected the majority of Y-1 cells with the shRNA containing plasmids shown in supplementary data 3. As presented in figure 5A, transfection with this shRNA construct significantly reduced the amount of PDE8B mRNA transcripts. More interestingly, this acute ablation of PDE8B caused an increase in basal steroid production (figure 5B) similar to the effect observed when Y-1 cells were treated with the PDE8-selective inhibitor (PF-04957325) in figure 4B. We interpret this result to support that the potentiation in steroid production is caused by the ablation of PDE8B and not due to unintended non-sequence-specific effect because the control shRNA plasmid
containing shRNA sequence lacking a specific target had no effect on steroidogenesis in Y-1 cells. Secondly, it is highly unlikely that any toxic effect of introducing an exogenous plasmid would elicit a gain of function such as an increase in steroid production. These data further support the idea that PDE8B is a major modulator of adrenal steroidogenesis.

**PDE8 inhibitor also potentiates steroidogenesis in primary isolated adrenal cells.**

To demonstrate that the Y-1 cell line was a representative model for adrenocortical cells, we also used primary isolated adrenal cells from wild type animals to determine if PDE8B can modulate acute steroid synthesis. Under a relatively short ACTH-stimulating condition (1hr), WT primary adrenal cells appeared to be more responsive to ACTH when PDE8s were inhibited. As presented in figure 6A, IBMX, a non-selective PDE inhibitor that does not inhibit PDE8s, potentiated pregnenolone production most effectively under the condition of saturating ACTH concentration, while the PDE8 inhibitor (PF-04957325) did not increase pregnenolone production under maximum stimulation. However, the PF-04957325 did potentiate pregnenolone production of both basal and low doses of ACTH. These in vitro experiments strongly suggest that one or more PDE8s are also effective modulators of acute steroidogenesis, in addition to regulating cAMP-dependent gene expression in the chronic phase (Figure 3).

Secondly, other PDEs are probably controlling some additional aspects of the cAMP-dependent pathway, since the non-selective PDE inhibitor, IBMX, causes
an increase in steroidogenic capacity (higher maximum effect), which is distinct from the increased in responsiveness (lower EC$_{50}$) observed due to PDE8 inhibition.

*The effect of the PDE8 inhibitor (PF-04957325) is only due to blocking of PDE8 activity.*

In order to verify the specificity of PF-04957325 in the cell, we tested the selectivity of this compound using primary adrenal cells from the PDE8B KO and PDE8A/8B double KO animals. As presented in figure 6B, this PDE8 inhibitor had only a partial effect to potentiate basal steroid production in PDE8B KO adrenal cells. More importantly, this compound had no effect in the PDE8A/B double KO cells. Since PF-04957325 fully blocks the activity of both PDE8A and 8B at 100nM *in vitro*, the partial effect observed from the PDE8B KO is likely due to PDE8A inhibition. This finding also provides evidence in intact cells that PF-04957325 is working by selectively inhibiting only PDE8A and 8B, and not other PDEs, in these cells. This observation also confirms the hypothesis that both PDE8s are important modulators of pool(s) of cAMP promoting adrenal steroidogenesis. However, it is still unclear if PDE8A and 8B are regulating the same or distinct pools of cAMP in these cells.

PDE8 inhibition increases basal PKA activity and also increases mRNAs of steroidogenic enzymes in Y-1 cells.
Next, we tried to determine the mechanisms by which PDE8B modulates acute steroidogenesis. We found that the PF-04957325 increased basal PKA activity in Y-1 cells, while IBMX treatment did not (figure 7A). For instance, treatment with the PDE8 inhibitor increased the phosphorylation state of a number of proteins under basal and sub-maximal stimulated condition, as shown by western blot analysis with a phospho-PKA substrate (RRXS/T) antibody (Cell Signaling Technology Inc.). Additionally, we identified one of the proteins to be HSL by probing with a phospho-HSL antibody (Ser660) (Cell signaling technology Inc.). We observed that PF-04957325 treatment potentiated the basal phosphorylation state of HSL while IBMX treatment did not (figure 8A and 8B). Furthermore, PF-04957325 treatment as well as IBMX treatment potentiated the phosphorylation state of HSL when cells were stimulated with a sub-maximal dose of ACTH (figure 8A and 8B). This observation is consistent with the previous finding that IBMX does potentiate steroid production but only under the stimulated condition in figure 7A. These results suggest that one role for PDE8(s) is to control substrate availability by lowering basal cAMP levels and keeping PKA inactive in the vicinity of stored cholesterol esters (the substrate for HSL/CEH). This finding is also consistent with the observation that PDE8 inhibition raises basal steroid level while IBMX has little to no effect in the Y-1 cells.

To determine if the PDE8 inhibitor also produced an increase in cAMP-dependent transcription similar to that produced by the global ablation of PDE8B seen in the KO mice, we treated Y-1 cells with PF-04957325 chronically. After Y-
1 cells were pre-treated with PDE8 inhibitor for 16 hours, and then stimulated with 100pM ACTH for 2 hours, we observed a significant increase in mRNA expression of StAR protein and p450_{ss}c (figure 7B). This finding further verifies the observation in the PDE8B KO that PDE8B modulates steroidogenesis in part via increases in cAMP-dependent transcription.
Discussion

Due to the unavailability of selective pharmacological inhibitors and the lack of knowledge about regulating partners, functional studies for the PDE8 family of phosphodiesterases have been difficult and until recently have remained largely unexplored. We adopted a genetic approach that utilized a global PDE8B KO mouse model and a new PDE8-selective inhibitor (PF-04957325) to determine what role if any PDE8B might play in adrenal steroidogenesis. Using an X-gal staining method based on the insertion of β-galactosidase into the PDE8B knock-out cassette (Duffield et al, 2005), we were able to show that PDE8B transcripts are highly enriched in a majority of the adrenocortical zona fasciculata cells.

Generally the PDE8B knockout mice develop normally as evidenced by a normal body weight and fertility. However, when examined more closely for an adrenal-related phenotype substantial differences from WT animals can be seen. PDE8B KO mice have elevated urinary corticosterone levels in the face of suppressed levels of circulating ACTH. This chronic elevation of glucocorticoid levels is likely due to an adrenal hypersensitivity to ACTH, which would be expected if PDE8B has an important role in modulating steroidogenesis. Notably, we observed no detectable increase in adrenal size as reported by Horvath, et al. for one patient with severe adrenal hyperplasia (Horvath et al, 2008b). This difference may be due to either species variation or perhaps more likely to an additional mutation present in the patient that allows for hyperplasia of
the adrenal gland. Nevertheless, the ablation of functional PDE8B enzyme in the mouse is sufficient to sensitize the adrenal AZF cells to ACTH and cause an increase in corticosterone production in vivo. This observation is consistent with the idea that the activity of PDE8B normally acts as a negative modulator of adrenal steroidogenesis. To our knowledge, this is the first report of a functional phenotype for a PDE8B KO mouse.

We then became interested in the mechanisms by which ablation of functional PDE8B can cause an increase in corticosterone production. We found that PDE8B KO mice had increased expression of several key steroidogenic genes in the adrenal compared to their littermates, including transcripts for StAR protein and MC2R. Similarly, when PDE8 was chronically inhibited in Y-1 cells, we also observed that cAMP-dependent transcription was activated. Therefore we suggest that ablation of functional PDE8B leads to a chronic increase in basal cAMP levels in specific compartments that control cAMP-dependent gene regulation. This cAMP elevation is capable of increasing the transcript levels of several key steroidogenic enzymes and therefore increasing urinary corticosterone in the PDE8B KO mice.

In addition to ACTH sensitization in the whole animal model, we also observed a similar sensitization effect on steroid production in cell culture models of both Y-1 cells and isolated primary adrenal cells. Using a semi-selective PDE8 inhibitor (dipyridamole) and a selective PDE8 inhibitor (PF-04957325), we
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showed that acute PDE8 inhibition potentiates basal as well as submaximal
ACTH-stimulated pregnenolone. Moreover, PF-04957325 had no additive effect
under the maximal ACTH-stimulation. In other words, PDE8 inhibition by PF-
04957325, increased the responsiveness of adrenal cells toward ACTH by
shifting the EC$_{50}$ for ACTH to the left without increasing the maximum effect. We
further demonstrated that this potentiation in pregnenolone production by PDE8
blockade is due at least in part to an increase in substrate availability. Treatment
with PF-04957325 increased PKA activity and the phosphorylation state of HSL
in Y-1 cells. As shown in figure 7A, PDE8A/B inhibition substantially increased
the phosphorylation state of several other proteins while a high-dose of IBMX did
not. These results suggest that PDE8A/B solely control the pool of cAMP/PKA
stimulated by ACTH in Y-1 cells.

Due to the fact that the new PDE8-selective inhibitor does not distinguish
between PDE8A and 8B, it remains unclear if PDE8A and PDE8B have distinct
or overlapping roles in Y-1 cells. However, the ability of PDE8B to modulate
acute steroidogenesis was demonstrated by 1) an increase in steroid production
elicted by acute ablation of PDE8B mRNA transcript via an shRNA induced RNA
interference as shown in figure 5B and 2) a significant decrease in the
potentiating effect of the PDE8 inhibitor on steroidogenesis in PDE8B KO adrenal
cells (figure 6B). Furthermore, the PDE8A/8B double KO adrenal cells were
completely insensitive toward the PDE8 inhibitor, which further validates the
selectivity of this compound. From X-gal staining experiments, we also observed
that PDE8A transcripts are expressed at low levels in a sub-population of AZF. Therefore, we conclude that both PDE8A and PDE8B inhibition can modulate acute steroidogenesis. However, it is still not clear if PDE8A and 8B regulate steroid production by the same mechanisms or even in the same cell types.

The ability of PDE8B to modulate steroidogenesis is not surprising due to the general importance of cyclic nucleotide signaling in this process and the relatively high expression levels of PDE8B mRNAs in the adrenal cortex. However, it is known that other PDEs also can modulate this process. For example PDE2A is known to regulate the effect of atrial natriuretic peptide (ANP) on aldosterone production from adrenal glomerulosa cells (MacFarland et al, 1991). Here we report a seemingly distinct mechanism by which PDE8B modulates acute steroid production as shown in figure 8C. PDE8s appear to be regulating basal steroidogenesis while other IBMX-sensitive PDEs modulate steroidogenesis more effectively when cells are stimulated. The low $K_m$ ($\sim 0.15\mu M$) of the PDE8s would seem to make them ideal modulators of the low cAMP level present in the basal state, while higher $K_m$ PDEs are better suited to regulate cAMP in a fully stimulated state. Furthermore, we also observed an additive effect of PF-04957325 and IBMX on basal pregnenolone production from Y-1 cells. Taken together these findings suggest that both PDE8s as well as at least one additional IBMX-sensitive PDE, such as PDE2A, can regulate one or more pools of cAMP that in turn control steroidogenesis. Furthermore, pharmacological inhibition of all of these PDEs is needed to achieve a maximum
potentiating effect on adrenal steroid production. Many questions still remain
with regard to the roles of different PDEs in modulating adrenal steroidogenesis.
However, PDE8B gene ablation clearly elicits increased corticosterone
production, which is at least partially due to an increased number of
steroidogenic enzymes in the adrenal glands. Also, acute inhibition of PDE8s
can increase basal PKA activity, thereby promoting acute steroid production.

In addition to adrenal glands, we also screened for other peripheral
tissues that expresses PDE8B gene using the X-gal staining technique, and
found that PDE8B expression is rather limited. Secondly, we demonstrate that
PDE8B is a effective modulator of adrenal steroid production. Therefore, we
believe that PDE8B might be a good therapeutic target to treat various adrenal
diseases. For instance, a PDE8-selective inhibitor might be used to correct
adrenal insufficiency, and a PDE8 activator might be used to treat Cushing's
syndrome.
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Authorship Contributions

Participated in research design: Tsai, Shimizu-Albergine, and Beavo.

Conducted experiments: Tsai and Shimizu-Albergine.

Performed data analysis: Tsai, Shimizu-Albergine, and Beavo.

Wrote or contributed to the writing of the manuscript: Tsai, Shimizu-Albergine, and Beavo.
References


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Footnotes

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Figure Legends

Figure 1. PDE8B is highly expressed in AZF cells. (A) The catalytic domain (exon 14-15) of PDE8B gene was disrupted by a construct containing a lacZ reporter gene, a neomycin resistance gene, and premature stop codons. (B) X-gal staining of PDE8B KO adrenal gland showed that the promoter of PDE8B gene was active, and PDE8B was expressed in the AZF cells. (C) The full length PDE8B mRNA was not transcribed in the PDE8B KO adrenal. However, the 5' mRNA region of PDE8B was up regulated perhaps due to the absence of functional PDE8B (N=3). (D) The absence of functional PDE8B enzyme in PDE8B KO adrenals was shown by immunoprecipitating PDE8B from the PDE8B KO in comparison to the WT control (N=3). The data are reported as means ± SEM, and the data were analyzed by student's t-test (unpaired, two-tailed): no significance (ns), **, p<0.01, ***, p<0.001.

Figure 2. PDE8B KO mice exhibit adrenal hypersensitivity in vivo. PDE8B KO mice had no gross developmental defects compared to their WT littermates. (A) The PDE8B KO mice maintained normal body weight under standard lab chow diet (N=3-7). (B) PDE8B KO mice had elevated basal urinary corticosterone (N=26-27). (C) PDE8B KO mice also exhibited increased stimulated corticosterone levels, when mice were mildly stressed via an i.p. saline injection (N=5-11). (D) The circulating ACTH level of the PDE8B KO was not higher than WT control (N=10-13). The data are reported as means ± SEM,
and the data were analyzed by student’s t-test (unpaired, two-tailed): *, p<0.05, **, p<0.01.

Figure 3. PDE8B gene ablation increases mRNA expressions of StAR protein and MC2R. The chronic phase of steroidogenesis elicits cAMP-dependent transcriptional activation to increase steroid production. (A) PDE8B KO adrenals had an increase of mRNA level of steroidogenic enzyme, StAR protein (N=7-8). (B) The mRNA of the ACTH receptor (MC2R), which is a known cAMP activated transcript, was also elevated in PDE8B KO adrenals (N=8). The data are reported as means ± SEM, and the data were analyzed by student’s t-test (unpaired, two-tailed): no significance (ns), *, p>0.05, **, p<0.01.

Figure 4. PDE8 inhibition with inhibitors increases acute adrenal steroid production in Y-1 cells. The commonly used Y-1 adrenal cell line was used for acute steroid measurements. (A) Treatment with the semi-selective PDE8 inhibitor, dipyridamole, potentiated basal steroid production in Y-1 cells, while IBMX only slightly increased steroid production (N= 3). Pregnenolone secreted from Y-1 no inhibitor control cells averaged 0.824 ng/100,000 cells/hr. (B) The more PDE8 selective inhibitor (PF-04957325) showed similar results (N=3 or 4). Maximum pregnenolone secreted (in ng/100,000 cells/hr) from Y-1 cells averaged 1.37 under no ACTH stimulation, and 0.782 at 10pM ACTH, and 1.37 at 1000pM ACTH. The data are reported as means ± SEM, and data were analyzed with one-way ANOVA with Dunnett post hoc test: *, p<0.05.
Figure 5. shRNA against PDE8B also increases acute adrenal steroid production in Y-1 cells. shRNA induced RNAi was utilized to verify the modulation of PDE8B in adrenal steroidogenesis. (A) PDE8B mRNA transcripts were greatly attenuated by the shRNA construct (N=3). (B) Furthermore, this reduction of PDE8B mRNA expression elicited an increase in basal steroid production in Y-1 cells similar to that seen in the PDE8 inhibitor treated Y-1 cells shown in figure 4A (N=3). Pregnenolone secreted from Y-1 cells transfected with control shRNA averaged 0.2 ng/million cells/hr. The data are reported as means ± SEM, and the data were analyzed by student’s t-test (unpaired, two-tailed): **, p>0.01, ***, p<0.001.

Figure 6. Treatment with PDE8 inhibitor increases acute adrenal steroid production in primary isolated adrenal cells. Primary isolated adrenal cells were also used for acute steroid measurements. (A) These cells responded to 100nM selective PDE8 inhibitor (PF-04957325) treatment with an elevated basal steroid production. Interestingly, these primary adrenal cells became insensitive to PDE8 inhibitor treatment when the cells were fully stimulated with ACTH. IBMX-sensitive PDEs became the predominate PDEs regulating the pool(s) of cAMP generated upon ACTH stimulation (N= 3-5). (B) The effect of the PDE8 inhibitor on steroidogenesis in the 8B and 8A/8B double KO cells was also tested. The effect of PF-04957325 was partially but not completely abrogated in adrenal cells from PDE8B KO. However, the effect of the PDE8 inhibitor was
entirely abolished in isolated adrenal cells from double PDE8A/B KO adrenals. This strongly suggests that the drug inhibits only PDE8s at 100nM in WT adrenal cells (N= 5). Basal pregnenolone secreted (in ng/10,000 cells/hr) by primary isolated adrenal cells averaged 0.65 for WT cells, 0.84 for PDE8B KO cells, and 1.28 for PDE8A/B KO cells. The data are reported as means ± SEM, and data were analyzed with one-way ANOVA with Dunnett post hoc test: *, p<0.05, **, p<0.01, $$$, p<0.001.

**Figure 7. PDE8 inhibition increases basal PKA activity and also mRNAs of steroidogenic enzymes.** Here we tested two mechanisms by which PDE8 might regulate steroidogenesis. (A) Acute treatment with PF-04957325 increased the phosphorylation state of multiple proteins in Y-1 cells, as shown by western blotting with a general phospho-PKA substrate selective (RRXS/T) antibody (N=4). (B) Chronic treatment with the selective PDE8B inhibitor elicited an increase in mRNA levels of steroidogenic enzymes in Y-1 cells (N= 3). The data are reported as means ± SEM, and the data were analyzed by student’s t-test (unpaired, two-tailed): *, p<0.05, **, p<0.01.

**Figure 8. PDE8 inhibition increases the phosphorylation state of HSL.** (A) Acute treatment with PF-04957325 increased the phosphorylation state of HSL under both basal and sub-maximal ACTH stimulation, as shown by western blot analysis with a phospho-HSL antibody (Ser660). (B) The phospho-HSL bands were quantified by image J, and analyzed by one way ANOVA analysis and p...
values obtained with Dunnett post hoc test (N=3): no significance (ns), **, p<0.01. (C) A diagram demonstrates the current model for modulation of adrenal steroidogenesis by PDEs. The low $K_m$ and $V_{max}$ of the PDE8s are depicted as modulating the basal state of cAMP thereby keeping PKA. However, under higher ACTH stimulation, PDE8s are overwhelmed by the higher level of cAMP and PKA becomes activated. Under this elevated cAMP condition, other higher $K_m$ PDEs become predominate in modulation of steroidogenesis as shown by the experiment in figure 4C.
Figure 1

A. PDE8B gene:

B. WT adrenal gland: PDE8B KO adrenal gland:

C. mRNA levels

D. PDE8B Activity - adrenal gland IP

% WT activity

No inhibitor IBMX (50 μM) PF-4957325 (10nM) Blocking peptide
**Figure 2**

**A.**

PDE8B KO body weight

**B.**

Basal Urinary Corticosterone

**C.**

Stimulated Urinary Corticosterone

**D.**

Plasma ACTH level
Figure 3

A. Steroidogenic enzymes mRNA Expression

B. ACTH Receptor (MC2R) mRNA Expression

**WT (N=7)**

**8B KO (N=8)**

Relative Expression (Gene/GAPDH)

**MC2R/GAPDH**

Relative Expression

WT (N=8)

8B KO (N=8)
Figure 4

A. Y-1 Cells

B. Y-1 Cells

Pregnenolone (fold over control)

Pregnenolone (% Maximum)

ACTH (pM)

ACTH (pM)
Figure 5

A. shRNA treated Y-1 Cells

Relative mRNA level (PDE8B/GAPDH)

Control shRNA  PDE8B shRNA

B. shRNA treated Y-1 Cells

Pregnenolone (% control)

Control shRNA  PDE8B shRNA

**  ***
Figure 6

A. Primary Adrenal Cells

- No Inhibitor control
- PF-04957325 (100nM)
- IBMX (50 μM)

B. Primary Adrenal Cells

- No Inhibitor control
- PF-04957325 (100nM)
- IBMX (50 μM)
Figure 7

A. Basal 100pM ACTH

No Inhibitor
PF-04957325
IBMX
No Inhibitor
PF-04957325
IBMX

B. Chronic PDE8 inhibition in Y-1 cells

Relative mRNA Expression (Gene/GAPDH)

No Inhibitor
PF-04957325

kDa

170
130
95
72
55
34
26
Figure 8

A. Molecular mass of p-HSL (Ser660) with and without ACTH stimulation.

B. Basal and stimulated pHSL/GAPDH intensity with various inhibitors.

C. Diagram illustrating the basal and stimulated state of cAMP signaling.

Low p-HSL and low StAR and p450 levels in the basal state.

High p-HSL and high StAR and p450 levels in the stimulated state.

MC2R, PDE8s, Other PDEs, PKA.