cAMP-Specific Phosphodiesterases 8A and 8B, Essential Regulators of Leydig Cell Steroidogenesis

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ABSTRACT

Phosphodiesterase (PDE) 8A and PDE8B are high-affinity, cAMP-specific phosphodiesterases that are highly expressed in Leydig cells. PDE8A is largely associated with mitochondria, whereas PDE8B is broadly distributed in the cytosol. We used a new, PDE8-selective inhibitor, PF-04957325, and genetically ablated PDE8A(−/−), PDE8B(−/−) and PDE8A(−/−)/B(−/−) mice to determine roles for these PDEs in the regulation of testosterone production. PF-04957325 treatment of WT Leydig cells or MA10 cells increased steroid production but had no effect in PDE8A(−/−)/B(−/−) double-knockout cells, confirming the selectivity of the drug. Moreover, under basal conditions, cotreatment with PF-04957325 plus rolipram, a PDE4-selective inhibitor, synergistically potentiated steroid production. These results suggest that the pool(s) of cAMP regulating androgen production are controlled by PDE8s working in conjunction with PDE4. Likewise, PDE8A(−/−)/B(−/−) cells had higher testosterone production than cells from either PDE8A(−/−) or PDE8B(−/−) mice, suggesting that both PDE8s work in concert to regulate steroid production. We further demonstrate that combined inhibition of PDE8s and PDE4 greatly increased PKA activity including phosphorylation of cholesterol-ester hydrolase (CEH)/hormone-sensitive lipase (HSL). CEH/HSL phosphorylation also was increased in PDE8A(−/−)/B(−/−) cells compared with WT cells. Finally, combined inhibition of PDE8s and PDE4 increased the expression of steroidogenic acute regulatory (STAR) protein. Together these findings suggest that both PDE8A and PDE8B play essential roles to maintain low cAMP levels, thereby suppressing resting steroidogenesis by keeping CEH/HSL inactive and STAR protein expression low. They also suggest that in order for PDE inhibitor therapy to be an effective stimulator of steroidogenesis, both PDE8 isoforms and PDE4 need to be simultaneously targeted.

Introduction

The cAMP-dependent protein kinase (PKA) signaling pathway is an essential regulator of many different physiological processes, including hormone-stimulated steroidogenesis. The amplitude and duration of the hormone/cAMP/PKA signals are regulated by the activity and spatial distribution of the hormone receptors, adenylyl cyclases, and PKAs (Taskén and Aandahl, 2003). An equally important determinant of the response is the activity, levels, and localization of one or more cyclic nucleotide phosphodiesterases (PDEs) that terminate cAMP action by hydrolyzing it to inactive 5′-AMP (Conti and Beavo, 2007). The spatial localization and temporal activation of these PDEs contribute to the specificity and magnitude of cAMP availability to its effectors (Wong and Scott, 2004).

Testicular Leydig cells produce androgens that are essential for puberty, fertility, sexual motivation, and sexual performance in male organisms. The cAMP/PKA signaling pathway is a well established regulator of androgen production in Leydig cells. In these cells, testosterone production is predominantly regulated through interaction of luteinizing hormone (LH) with its receptor, resulting in increased intracellular cAMP and subsequent activation of PKA. PKA can then phosphorylate numerous proteins including those that facilitate cholesterol availability and transport into mitochondria (Manna et al., 2009). These proteins include cholesterol ester

ABBREVIATIONS: PKA, cAMP-dependent protein kinase; PDE, phosphodiesterase; LH, luteinizing hormone; CEH, cholesterol ester hydrolase; HSL, hormone-sensitive lipase; STAR, steroidogenic acute regulatory; IBMX, 3-isobutyl-1-methylxanthine; WT, wild type; BSA, bovine serum albumin; 3p-HSD, 3β-hydroxysteroid dehydrogenase; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PFA, paraformaldehyde; Bay 60-7550, 2(3,4-dimethoxybenzyl)-7-((1R)-1-{(1R)-1-hydroxyethyl}-4-phenylbutyl)-5-methyl imidazo[1,2,4]triazin-4-(3H)-one; MOPS, 3-(N-morpholino)propanesulfonic acid; GFP, green fluorescent protein; shRNA, short hairpin RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
hydrolase (CEH), known as hormone-sensitive lipase (HSL), that catalyzes the hydrolysis of stored cholesterol esters into fatty acids and free cholesterol (Kraemer and Shen, 2002). Another control point in this process is the amount and activity of the steroidalogenic acute regulatory (STAR) protein that facilitates delivery of cholesterol substrate to the steroidalogenic enzyme machinery inside of the mitochondria (Dyson et al., 2008; Poderoso et al., 2009;rone et al., 2009). Stimulation of the cAMP/PKA pathway leads to an increase in both the levels and activity of STAR protein (Arakane et al., 1997; Stocco et al., 2005; Manna et al., 2009). Overall, the levels of cAMP in response to stimulation by hormones are tightly correlated with the ultimate rate of steroid production by Leydig cells.

The PDE8 family consists of two distinct genes, Pde8a and Pde8b. Both PDE8A and PDE8B hydrolyze cAMP with a very high affinity (K_m <0.15 μM). Unlike other cAMP-specific PDEs, PDE8s are insensitive to the commonly used, nonselective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX) (Hayashi et al., 1998; Soderling et al., 1998). Moreover, these two isoforms have a relatively restricted tissue expression pattern compared with most other PDEs. PDE8A and PDE8B are expressed in most if not all steroidogenic cell types (Soderling et al., 1998; Vasta et al., 2006; Tsai et al., 2011). In addition, PDE8a mRNA and protein are abundant in cardiomyocytes (Patrucco et al., 2010). They also are induced in T cells upon activation of the T-cell receptor and are thought to affect T-cell function (Glavas et al., 2001; Dong et al., 2006; Vang et al., 2011). PDE8B is reported to be high in steroidogenic cells, both PDE8s might be critical regulators of steroid production by tightly controlling intracellular cAMP levels. However, with the exception of mouse adreno-cortical cells, little is known about which combination of PDEs can regulate steroidogenesis in other steroidogenic cell types. (Dov et al., 2008).

Using a PDE8A(−/−) knockout mouse model, we have reported previously that PDE8A can modestly regulate testosterone production in Leydig cells (Vasta et al., 2006). More recently, we found that steroid production in isolated adrenocortical cells from PDE8B(−/−) mice was significantly increased compared with wild-type (WT) cells. We further demonstrated that PDE8B(−/−) mice were more susceptible to stress-induced corticosterone release than WT animals (Tsai et al., 2011). These studies suggested that perhaps in all steroidogenic cells, both PDE8s might be critical regulators of steroid production by tightly controlling intracellular cAMP levels. However, with the exception of mouse adrenocortical cells, little is known about which combination of PDEs can regulate steroidogenesis in other steroidogenic cell types. In this study, we have examined potential combinatorial roles for PDE8A, PDE8B, and PDE4s in the regulation of testosterone synthesis by using Leydig cells isolated from PDE8A(−/−) and PDE8B(−/−) mice in conjunction with IBMX. We also have used a recently available PDE8-selective inhibitor, PF-04957325 (Supplemental Fig. 7). By comparing the responsiveness of steroid production in PDE8A(−/−), PDE8B(−/−), and PDE8A(−/−)B(−/−) double-knockout cells, we can now propose that PDE8A and PDE8B probably control distinct compartments of cAMP in the Leydig cells and that these PDEs in turn are major regulators of basal steroidogenesis. In addition, we show that PDE8A can work in a highly synergistic manner with one or more PDE4s to regulate this process. Finally, we report that simultaneous inhibition of both PDE8s and PDE4 is required for large increases in PKA-mediated phosphorylation of several proteins implicated in regulation of steroidogenesis, including CEH/HSL and also for increased STAR protein expression.

Materials and Methods

Animals. Both PDE8A(−/−) and PDE8B(−/−) mice used in these studies were generated on a SV129 genetic background by Deltagen, Inc. (San Carlos, CA) under contract to Pfizer, Inc. (Sandwich, UK). The animals were then backcrossed in our laboratory with C57BL/6 mice obtained from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME) for at least 12 to 15 generations as previously described (Vasta et al., 2006; Tsai et al., 2011). Both proteins were completely eliminated in the knockout animals as determined by lack of PDE8 activity and mRNA transscripts (Vasta et al., 2006; Tsai et al., 2011). PDE8A(−/−)/B(−/−) double-knockout mice were established in the laboratory by crossing the PDE8B(−/−) and PDE8A(−/−) mice. 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 animals between the ages of 9 to 14 weeks, using WT littermates from the PDE8A colony as controls.

Isolation of Mouse Leydig Cells and Testosterone Measurement. Isolation of Leydig cell-enriched preparations was performed as described previously (Vasta et al., 2006). In brief, mouse testes were decapsulated and incubated in Medium 199 buffered to pH 7.4 with 8.8 mM HEPES containing 0.25 mg/ml collagenase type IV (Worthington Biochemical Corp., Lakewood, NJ), 6 μg/ml DNase I (Sigma-Aldrich, St. Louis, MO), and 1 mg/ml bovine serum albumin (BSA) at 35°C for 20 min with agitation (200 rpm). The same volume of stopping solution [10 mg/ml BSA and 25 μg/ml soybean trypsin inhibitor (Sigma-Aldrich) in Medium 199] was added to terminate digestion. The digested tissue was then dispersed 10 times with a 1-ml Eppendorf pipette and seminiferous tubules were allowed to settle by gravity for 5 min. The supernatant containing interstitial cells was filtered once with a cell-strainer (70 μm, 15 min). The cell pellet was washed twice with Dulbecco’s modified Eagle’s medium/F-12 (without Phenol Red) containing 1 mg/ml BSA, then resuspended in the same media and plated in 48-well plates (150 μl/well). The cells were allowed to recover for 3 h in a 5% CO_2 incubator at 37°C. After recovery, the media was replaced with fresh media containing PF-04957325 (Pfizer, Groton, CT; the structure is shown in Supplemental Fig. 7) and/or IBMX (Sigma-Aldrich) or vehicle. The cells were incubated a further 3 h. In experiments of LH stimulation, after 30 min of preincubation with PDE inhibitors, cells were stimulated for 2.5 h by addition of a 5% volume of 20% concentrated LH solution. Recombinant human LH was obtained from the National Hormone and Peptide Distribution Program (A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA). After a 3-h incubation period, the media was collected and stored at −20°C until assayed for testosterone assay using an ELISA kit (Neogen, Lexington, KY). To correct for any differences in Leydig cell purity and yield between preparations, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was used to normalize the amount of steroid produced by each preparation of cells. This 3β-HSD activity assay was performed by incubating cells for 3 h in phosphate-buffered saline (PBS) containing 1.5 mM β-nicotinamide adenine dinucleotide hydrate, 0.25 mM nitro blue tetrazolium, 0.2 mM 5β-androstane-3β-ol,17-one (all obtained from Sigma-Aldrich), 0.1% BSA, and 0.5% Triton X-100 at 37°C for 4 h. The cells were then dissolved in dimethyl sulfoxide (100 μl) and the absorbance at 570 nm was measured.

MA10 Cell Culturing and Measurement of Progesterone. The MA10 mouse Leydig cell line was maintained in culture medium (RPMI 1640 medium supplemented with 15% horse serum). Stocks of MA10 cells were frozen in media with 10% dimethyl sulfoxide ac-
cording to American Type Culture Collection (Manassas, VA) guide-
lines. For experiments, the cells were plated in 24-well plates at
8.0 × 10^4 cells/well and grown in the culture medium. After 2 days,
the medium was removed, cells were washed twice with serum-free
RPMI 1640 medium, and incubated in serum-free RPMI 1640 me-
dium for 3 h at 37°C. The medium was then replaced with RPMI
1640 medium (300 μl) containing vehicle or PDE inhibitors [PP-
04957325 and/or IBMX or rolipram (Sigma-Aldrich)] and incubated
for 3 h. To stimulate with LH or forskolin (Sigma-Aldrich), 15 μl
of a concentrated solution of agonist or vehicle was added after
30-min preincubation with PDE inhibitors followed by 2.5-h of incu-
bation. After the incubation, the medium was collected for measure-
ment of steroid content. Because the MA10 cell line has a lesion in
17α-hydroxylase activity, progesterone was measured as the major
steroid secreted (Ascoli, 1981) using a progesterone ELISA kit (Neo-
gen). The protein concentration in each well was determined using
the bicinchoninic acid protein assay (Thermo Fisher Scientific,
Waltham, MA).

**Immunocytochemistry.** For immunostaining of β-galactosidase,
tests were dissected from WT and knockout mice and rapidly frozen
in Tissue-Tek O.C.T. (optical cutting temperature) compound
(Sakura Finetek, Torrance, CA) on dry ice. Sections (20 μm) were
cut on a cryostat, mounted on a Superfrost plus microslide (VWR
Scientific, West Chester, PA), and air-dried. After fixing in 4% (w/v)
paraformaldehyde (PFA) at room temperature for 10 min, the sec-
tions were incubated in a blocking solution (5% goat serum, 1 mg/ml
BSA, and 0.05% Triton X-100 in PBS, pH 7.4) for 1 h at room
temperature, and then incubated with an anti-β-galactosidase anti-
body (1:500; 5 PRIME, Gaithersburg, MD) in PBS containing 1 mg/ml
BSA and 0.05% Triton X-100 at 4°C overnight. The sections
were washed in a washing buffer (PBS containing 0.05% Tween 20)
three times for 20 min, incubated with goat anti-rabbit Alexa Fluor
546 (1:500; Invitrogen, Carlsbad, CA) in PBS containing 1 mg/ml
BSA and 0.05% Triton X-100 at room temperature for 2 h, and
washed. The sections were further washed with TO-PRO-3 (1:
1000; Invitrogen) for nuclear counterstaining and mounted in Slow-
Fade Gold anti-fade reagent (Invitrogen).

For immunostaining in isolated Leydig cells, enriched Leydig cell
suspensions obtained from WT testes were plated in a BioCoat eight-
well culture slide (BD Biosciences Discovery Labware) and main-
tained at 37°C for 3 h to allow attachment. MA10 cells were cultured
in the same culture slide for 2 days. These cells were fixed with 4% PF
for 10 min at room temperature and washed with PBS. The cells
were treated with blocking solution (with 5% donkey serum) for 1 h
and then incubated with an anti-PDE8A antibody (1:200; Santa Cruz
Biotechnology, Santa Cruz, CA) at 4°C overnight. After washing, the
PDE8A antibody was labeled by donkey anti-goat Alexa Fluor 488.
For double staining with PDE8B, the cells were incubated in block-
ing solution (with 5% goat serum) followed by incubation with an
anti-PDE8B antibody (1:1000; FabGennix Inc., Frisco, TX) for 2 h
at room temperature. After washing, the PDE8B antibody was labeled
by goat anti-rabbit Alexa Fluor 546 or Alexa Fluor 633. For double
staining with P450eec antibody, the cells were incubated with an anti-P450eec
antibody (1:1000; Millipore Bioscience Research Reagents, Te-
meula, CA) for 2 h at room temperature. After washing, the anti-
body was labeled with goat anti-mouse Alexa Fluor 546. The staining
was used for nuclear staining. All slides were mounted in SlowFade
Gold antifade reagent and observed using a confocal microscope
(Leica SL, Leica Microsystems Ins., Bannockburn, IL) at the Keck
Microscopy Facility, University of Washington.

**Treatments with Other PDE Inhibitors.** Different PDE inhibi-
tors were applied to Leydig cells isolated from WT or PDE8A(-/-)
mice. Inhibitors included SCH51866 (10 μM; Schering-Plough
Research Institute, Kenilworth, NJ), cibosamide (1 μM; Cul-
biochem/EMD Millipore, Billerica, MA), 2-(3,4-dimethoxybenzyl)-7-
((1R)-1-(1R)-1-hydroxyethyl)-4-phenylbutyl)-5-methyl imidazo[5,1-
/1,2,4]triazine-4-(3H)-one (Bay 90-7550) (1 μM; Axxora, San Diego, CA), or
sildenafil (100 nM; Pfizer, New York, NY) as well as PP-04957325 (300
nM), IBMX (50 μM), or rolipram (20 μM) for 3 h. The amount of secreted
testosterone was measured by a testosterone ELISA kit. MA10 cells were
recovered with each inhibitor in the presence or absence of PP-04957325 for
3 h. The amounts of secreted progesterone were measured using a proges-
terone ELISA kit.

**Western Blot Analysis.** After treatments with PDE inhibitors,
Leydig cells or MA10 cells were dissolved in an SDS lysis buffer.
Samples were separated on 10% SDS polyacrylamide gels made from
Protogel solutions (National Diagnostic, Atlanta, GA). The resolved
protein bands were then electrotransferred to nitrocellulose mem-
branes. After preblocking with either 5% BSA (for anti-phospho-
protein antibodies) or 5% milk in 20 mM Tris-buffered saline, the mem-
branes were incubated with either anti-phospho-PKA substrate
antibody (Cell Signaling Technology, Danvers, MA), anti-phospho-
HSL (Ser 660) antibody (Cell Signaling Technology, anti-HSL anti-
bodies (Santa Cruz Biotechnology), anti-StAR antibody (Santa Cruz
Biotechnology), or anti-GAPDH antibody (Zymed Laboratories,
Inc., Concord, MA). Detection was carried out with appropriate per-
oxidase-conjugated secondary antibody (Bio-Rad Laboratories, Her-
cules, CA) and chemiluminescent reagents (Thermo Fisher Sci-
tific). The blot was exposed to autoradiographic film for visualization
of detected bands. The intensity of the bands was analyzed using
Imager (http://rsweb.nih.gov/ij/).

**Immunoprecipitation and PDE Activity Assay.** Immunopre-
cipitation of PDE8A and PDE8B were performed with rabbit poly-
clonal antibodies to PDE8A (Scottish BioMedical, Glasgow, Scotland,
UK) or goat polyclonal antibodies to PDE8B (Santa Cruz Biotechnol-
ogy, respectively). MA10 cell homogenates made with lysis buffer
[150 mM NaCl, 2 mM NaF, 2 mM Na3VO4, 1 mM β-glycerol, 1%
Triton X-100, 1 mM EDTA, and protease inhibitor cocktail (Sigma-
Aldrich) in 50 mM Tris-HCl, pH 7.4] were incubated with the
antibody and Protein G Plus-Agarose (Calbiochem) at 4°C overnight.
The immunopellet was resuspended and washed three times with the
lysis buffer before PDE activity was measured. PDE activity was
measured as described previously (Hansen and Beavo, 1982; Soder-
ing et al., 1998). In brief, the activity assay was carried out at very
low substrate conditions ([10–20 nM [3H]cAMP, 10–5 cm3/minute) in
40 mM MOPS, pH 7.5, 15 mM magnesium acetate, 2 mM EGTA, and
0.2 mg/ml BSA in the presence or absence of drugs in a final
volume of 250 μl. The reaction time and amount of lystate were
maintained so that less than 30% of the substrate was hydrolyzed.
Dose-response inhibition curves of PP-04957325 were analyzed, and
IC50 values were calculated by Prism (GraphPad Software, San
Diego, CA). Three individual experiments were performed.

**shRNA Ablation of PDE8A.** MA10 cells were transfected with
either a short-hairpin RNA (shRNA) construct (5'-gattgtgcagtcg-
tcaat-3') or control shRNA plasmid (SureSilencing shRNA plas-
mid, SA Biosciences, Frederick, MD) using Lipofectamine 2000
(Invitrogen). The control shRNA plasmid had all the same elements
as the shRNA plasmid, including a green fluorescent protein (GFP)
marker and a 21-nucleotide shRNA sequence (5'-ggaacattttgattg-
tcaat-3') with no known target. The MA10 cells were transfected
according to the manufacturer's instructions and cultured for 48 h.
After the transfection, the cells were fixed with 4% PFA and stained
with anti-PDE8A antibody as described under Immunocytoche-
my. Transfected cells were determined by the presence of GFP
fluorescence. The knockdown efficiency of PDE8A shRNA was also
evaluated by real-time PCR probing for PDE8A mRNA.

**Statistical Analysis.** Statistical analysis was determined by Stu-
dent's unpaired t test when only two groups were being compared.
Statistical analysis of multiple groups was modeled by one-way
ANOVA. Densitometry data from Western blot for phospho-HSL in
PDE8(-/-) cells was normalized against loading control and
expressed as the mean fold change relative to WT, and significant
difference was determined by Mann-Whitney test. Statistical test
results were considered significant at p < 0.05.
Results

PDE8A and PDE8B Are Expressed in Mouse Leydig Cells. Both the PDE8A(-/-) and PDE8B(-/-) animals used in this study were generated by replacing regions in the catalytic domain [exon 17 in the PDE8A(-/-) or exon 14 to 15 in the PDE8B(-/-) animals, respectively] with DNA sequence encoding a lacZ reporter gene with a nuclear localization signal and a neomycin resistance gene followed by a stop codon as described previously (Vasta et al., 2006; Tsai et al., 2011). This allows detection of PDE8A or PDE8B promoter activity by measurement of β-galactosidase expression in the nucleus of PDE8-expressing cells. When immunocytochemistry for β-galactosidase was carried out on sections of mouse testis, the only cells strongly stained were the interstitial cells located between seminiferous tubules in both the PDE8A(-/-) and PDE8B(-/-) mice (Fig. 1). These same cells expressing β-galactosidase enzymatic activity also were labeled with an antibody to cytochrome P450scc (Cyp11A1), a Leydig cell marker (Vasta et al., 2006).

To determine the intracellular localizations of PDE8A and PDE8B in Leydig cells, we performed immunocytochemical analysis. In isolated Leydig cells, high-resolution confocal microscopy revealed that the PDE8A antibody colocalized with antibodies to cytochrome P450scc (PDE8B) and ATP synthase (Supplemental Fig. 2), each serving as a mitochondrial marker. These results indicated a selective enrichment of PDE8A to or very near to mitochondria. On the other hand, the immunoreactivity of PDE8B was evenly distributed throughout the cell in these isolated Leydig cells (Fig. 2A). In MA10 cells, the PDE8A was again colocalized with cytochrome P450scc (PDE8B) and ATP synthase (Supplemental Fig. 2). To verify specificity of the antibody staining for PDE8A, MA10 cells were transfected with a PDE8A shRNA construct or control construct. The percentage of transfected cells was assessed as 45 to 55% in both cases by observing GFP that is built into the shRNA plasmid as a marker. In PDE8A shRNA-transfected cells, reduction or complete ablation of staining for PDE8A was seen in all GFP-positive cells inspected, but not in GFP-negative cells (Fig. 2F). The negative control shRNA construct showed little or no change in PDE8A staining in any cell (Fig. 2G). In contrast to the PDE8A localization pattern, PDE8B immunoreactivity was seen broadly distributed in the MA10 cells (Fig. 2C). Clearly, these two PDE8 isozymes, PDE8A and PDE8B, had differential intracellular distribution patterns in the Leydig cells and Leydig cell lines.

Both PDE8A and PDE8B Regulate Basal Steroidogenesis in Isolated Mouse Leydig Cells. We have previously reported that PDE8A can be one regulator of testosterone production in mouse Leydig cells (Vasta et al., 2006) and that PDE8B, in conjunction with PDE8A, can regulate corticosterone production from adrenal fasciculata cells (Tsai et al., 2011). In this study, we took advantage of a new PDE8-selective inhibitor and PDE8A(-/-) and PDE8B(-/-) knockout mice to determine whether each PDE8 has a unique role in Leydig cell steroidogenesis. Leydig cell-enriched preparations were isolated from four distinct groups of animals: WT mice, PDE8A(-/-) mice, PDE8B(-/-) mice, or PDE8A(-/-)/B(-/-) double-knockout mice. Measuring testosterone production at 3 h, we first observed that basal testosterone production from the PDE8A(-/-)/B(-/-) double-knockout cells was the highest among these four populations. Basal testosterone production in either the PDE8A(-/-) or PDE8B(-/-) cells was less than that in the double knockout cells but significantly higher than that in WT cells (Fig. 3A); this suggests that both PDE8A and PDE8B are partially responsible for regulation of basal testosterone production and that their regulatory effects are independent of each other, at least in part.

As another method to assess the general role of PDE8s in the regulation of steroidogenesis, Leydig cell preparations isolated from WT mice, PDE8A(-/-) mice, PDE8B(-/-) mice, or PDE8A(-/-)/B(-/-) double-knockout mice were incubated with increasing concentrations of the PDE8-selective inhibitor PF-04957325 (10–500 nM) (Fig. 3B). Note that this inhibitor will competitively antagonize both PDE8A and PDE8B. In the WT, PDE8A(-/-), and PDE8B(-/-) cells, testosterone production was increased by PF-04957325 in a dose-dependent manner. The degree of this effect, however, differed among cell types. As shown in Fig. 3B, PF-04957325 stimulated testosterone production in the PDE8A(-/-) cells with an EC_{50} = 3.9 nM. Note that these cells contain only the PDE8B isofom of PDE8s. However, in PDE8B(-/-) cells,
PF-04957325 stimulated testosterone production than in the PDE8A(−/−) cells with an EC50 = 38 nM. These cells contain only the PDE8A isoform of PDE8s. This 10-fold difference in the EC50 values for PF-04957325 between the PDE8A(−/−) and PDE8B(−/−) cells probably reflects the approximately 10-fold intrinsic difference in affinity of the drug for PDE8A and PDE8B. In MA10 cell extracts, we observed in vitro IC50 values of 3.1 ± 0.68 nM (n = 3) for PDE8A inhibition and 0.44 ± 0.12 nM (n = 3) for PDE8B inhibition in immunoprecipitated fractions. Therefore, nearly the same rank-order potency for PF-04957325 occurs in the intact cells as seen in vitro studies. It is noteworthy that PF-04957325 had no effect on testosterone production in the PDE8A(−/−)B(−/−) cells (Fig. 3B; Supplemental Fig. 3), confirming the specificity of the drug as a selective inhibitor of PDE8s and the ability of PF-04957325 to increase resting levels of testosterone production that had been stimulated by LH (Fig. 3A). In the PDE8A(−/−) cells and the PDE8A(−/−)B(−/−) cells, IBMX could augment testosterone production independent of whether or not PF-04957325 was present. However, in WT and PDE8B(−/−) Leydig cells, the effects of IBMX were very small at lower concentrations of PF-04957325 (<30 nM) but more prominent at higher concentrations (>50 nM) (Fig. 3B and supplemental Fig. 3). Concentrations of more than 50 nM PF-04957325 were able to block PDE8A activity in WT and PDE8B(−/−) cells, therefore IBMX became effective at concentrations of PF-04957325 that block PDE8A.

**PDE8s Regulate Stimulation of Testosterone Production by LH.** To determine whether PDE8s also could regulate testosterone production that had been stimulated by LH, Leydig cell-enriched preparations isolated from WT mice, PDE8A(−/−) mice, PDE8B(−/−) mice, or PDE8A(−/−)B(−/−) double-knockout mice were treated with two different concentrations of LH (30 or 100 pg/ml) for 2.5 h (Fig. 4). These concentrations of LH were chosen from a dose/response curve for LH on testosterone production as moderate (30 pg/ml) and high (100 pg/ml) stimulatory concentrations. First, testosterone production stimulated by 30 pg/ml LH was higher in the PDE8A(−/−), PDE8B(−/−), and PDE8A(−/−)B(−/−) cells compared with WT cells. When the cells were treated with PF-04957325 (300 nM) before and during LH stimulation, the drug treatment further increased steroid production in WT, PDE8A(−/−), and PDE8B(−/−)
cells, suggesting that PDE8s are able to exert some regulatory effects on cAMP at this concentration of LH. However, PF-04957325 was no longer effective in cells stimulated with 100 pg/ml LH. In contrast with PF-04957325, when the cells were treated with IBMX (50 μM), testosterone production in response to 100 pg/ml LH was increased in WT and PDE8(--/-) cells as well as in the PDE8A(+/−) cells. Therefore, other IBMX-sensitive PDEs became effective regulators at the cAMP produced by the higher range of LH, although inhibition of them does not alter basal steroidogenesis.

**PF-04957325 Stimulated Progesterone Production in MA10 Leydig Cells.** Because the mouse MA10 Leydig cell line expresses both PDE8A and PDE8B, we tested whether or not total PDE8 inhibition was necessary to potentiate the basal steroidogenesis in these cells. In this case, increasing concentrations of PF-04957325 (30–1000 nM for 3 h) caused a modest increase (~6-fold) in progesterone production (Fig. 5A). PF-04957325-induced progesterone was greatly potentiated by adding either IBMX (~13-fold) or rolipram (~19-fold) to the cells. However, neither IBMX nor rolipram alone had an appreciable effect on the resting steroidogenesis (Fig. 5B and supplemental Fig. 4). These results emphasize that the PDE8s predominantly regulate the resting basal levels of at least one compartment of cAMP in these cells, one that controls basal steroidogenesis.

The MA10 cells also were stimulated with LH in the presence or absence of the PDE inhibitors. In the presence of...
either PF-04957325 or IBMX alone, the dose-response curves for LH-stimulated progesterone production were shifted slightly to the left (EC_{50} = 6.3 ng/ml; PF-04957325, EC_{50} = 3.6 ng/ml; IBMX, EC_{50} = 3.2 ng/ml). However, when the cells were cotreated with both PF-04957325 and IBMX, the curve was further shifted to the left (EC_{50} = 1.4 ng/ml), indicating that the responsiveness of LH in steroidogenesis can be controlled by each of these PDEs (Fig. 5C). Moreover, a much greater potentiating effect of PF-04957325 was observed under at the lower concentrations of LH (<1 ng/ml) compared with the higher concentrations (>3 ng/ml) (Supplemental Fig. 5A). To rule out that this effect might be due to altered regulation of the LH receptor or efficacy of G-protein coupling, the MA10 cells also were stimulated with forskolin (0.1–50 μM) in the presence of either PF-04957325, IBMX, or PF-04957325 plus IBMX. As with the LH studies, the dose-response curve for forskolin-stimulated progesterone production was greatly shifted to the left (Fig. 5D). Like LH stimulation, the effects of PF-04957325 on progesterone production were more prominent at the lower concentrations of forskolin (1 and 3 μM) than higher concentrations (10 and 50 μM) (Supplemental Fig. 5B).

**PDE4 Is the Dominant IBMX-Sensitive PDE in Regulation of Leydig Cell Steroidogenesis.** To further understand which of the IBMX-sensitive PDEs that are expressed in Leydig cells are able to regulate steroidogenesis in concert with PDE8s, we tested several different PDE isozyme selective inhibitors, including rolipram (PDE4), SCH51866 (PDE1), cilostamide (PDE3), Bay60-7550 (PDE2), and sildenafil (PDE5) for their effects on testosterone production. mRNA for each of these PDEs is expressed in Leydig cells (Supplemental Fig. 1). Only PF-04957325 significantly increased basal testosterone production in WT Leydig cells (Fig. 6). In PDE8A(-/-)/B(-/-) double-knockout cells, of the inhibitors tested, only rolipram significantly increased testosterone production. IBMX, of course, because it can inhibit PDE4, also potentiated progesterone production in the presence of PF-04957325 in both MA10 cells and PDE8A(-/-)/B(-/-) Leydig cells (Fig. 6). These results indicate that PDE4 is likely to be the most important IBMX-sensitive PDE for regulation of Leydig cell steroidogenesis. These data again strongly suggest that PDE8A and PDE8B each subserve functionally distinct compartments and each contributes to regulation of steroidogenesis. However, only one of these functional compartments, the PDE8A compartment, is substantially also regulated by PDE4.

**PDE8 Inhibition Enhances Basal PKA Activity.** To observe whether PDE8 inhibition activates PKA activity, MA10 cells were treated with PF-04957325 and/or the PDE4 inhibitor for 3 h and analyzed by immunoblotting to detect phosphorylated PKA substrates. As presented in Fig. 7A, PF-04957325 alone slightly increased the amount of PKA substrate phosphorylation. However, when either IBMX or rolipram was coinubated with PF-04957325, phosphorylation by PKA was greatly augmented. It is noteworthy that high IBMX alone had only a small effect on phosphorylation. Corresponding to this general PKA activation, phosphorylation of CEH/HSL at its Ser660 PKA site was increased by these inhibitors. Dose-dependent increases in phosphorylation of CEH/HSL elicited by PF-04957325 as shown in Fig. 7B were correlated closely with the amount of progesterone production. The phosphorylation of CEH/HSL also was slightly increased in PDE8A(-/-) and PDE8B(-/-) Leydig cells and much more so in PDE8A(-/-)/B(-/-) cells (Fig. 7, C and D). These increases in phosphorylation do not result
from increased protein levels in these cells because the intensity of total CEH/HSL showed little change (Fig. 7C).

The synthesis of STAR protein, a protein that controls steroidogenesis, A, MA10 cells were incubated with or without PF-04957325 (concentrations indicated in figure) in the presence or absence of IBMX (50 μM) or rolipram (20 μM) for 3 h. The cell lysates were immunoblotted with anti-phospho-PKA substrate antibody or anti-phospho-HSL (Ser660) antibody. GAPDH was used as a loading control. B, PF-04957325 stimulated a dose-dependent phosphorylation of HSL and induction of STAR protein in the presence of IBMX in MA10 cells. Immunoblot analysis of the cell lysates was carried out with anti-phospho-HSL (Ser660), anti-HSL, or anti-STAR antibody. Representative data were shown here. All experiments were repeated at least three times and produced nearly identical results. C, isolated Leydig cell-enriched preparations were obtained from WT, PDE8A(−/−), PDE8B(−/−), and PDE8A(−/−) B(−/−) cells. The cell lysates were used for immunoblot analysis of phospho-HSL (Ser660) or total HSL. GAPDH was used as a loading control. All immunoblots are representative set of three repeated experiments. D, a quantitative analysis of phospho-HSL content in isolated Leydig cell preparations. Phosphoantitobody-specific bands were scanned by densitometry and are represented as a ratio to GAPDH expression. The data are expressed as a fold increase in the ratio of phospho-HSL to GAPDH of WT and represent the mean ± S.D. Statistical analyses were performed by unpaired Student’s t test (two-tailed); **, p < 0.01 versus no inhibitor. The data are representative sets of four repeated experiments.

Discussion

To date, the physiological functions regulated by PDE8s have largely been limited to studies in a few steroidogenic cells and to a somewhat lesser extent in heart (Patrawoo et al., 2010). This has been due largely to the lack of a selective inhibitor for the PDE8s. Unlike other cAMP-specific PDEs, PDE8s are insensitive to the commonly used PDE inhibitor IBMX (Hayashi et al., 1998; Soderling et al., 1998). PDE8s are inhibited by dipryridamole, but this drug is best known as a relatively nonselective PDE5 inhibitor, and until recently,
there has been no PDE8-specific inhibitor available. However, two studies have described a newly available PDE8-selective inhibitor, PF-04957325 (Vang et al., 2010; Tsai et al., 2011).

Using this PDE8-selective inhibitor in isolated Leydig cells from WT mice, mice having the Pde8a and Pde8b genes ablated, and also in mouse MA10 Leydig cells, we tested for possible overlapping regulatory roles for each of PDE8 isoyme in the control of steroidogenesis. First, we found an increase in the resting basal state of steroid production in both PDE8A(−/−) and PDE8B(−/−) Leydig cells compared with WT (Fig. 3A). The facts that PDE8A−/−/B−/− double knockout cells showed additive increases in steroid production (Fig. 3A) and that these two isoymes seem to localize to different part of the cell (Fig. 2) suggest that PDE8A and PDE8B may control distinct cAMP pools.

Second, we found that incubation with PF-04957325 increased steroid production in both WT Leydig cells and MA10 cells (Figs. 3B and 5). The fact that PF-04957325 had no effect on steroid production in PDE8A(−/−) and PDE8B(−/−) cells (Fig. 3B) provides strong evidence that the stimulatory effect of this compound on steroidogenesis is due to inhibition of PDE8 and not to some off-target effect.

Third, in WT cells, inhibition of PDE8s by PF-04957325 potentiated both basal testosterone production and production stimulated by 30 pg/ml LH (moderate stimulation), but not stimulation by 100 pg/ml LH (high stimulation) (Fig. 4). This strongly suggests that in the cell, the high-affinity but relatively low- Vmax PDE8s can be “overwhelmed” by high rates of adenylyl cyclase activity. A similar result was found in MA10 cells in that PF-04957325 was more effective at lower ranges of LH or forskolin (Fig. 5; Supplemental Fig. 5). Overall, the data suggest that PDE8s are “designed” to control steroidogenesis at the resting basal state and at low-to-moderate agonist stimulation but fail to do so at high agonist stimulation. In the latter case, another higher Km and higher Vmax PDE becomes rate-limiting.

In support of this latter idea, IBMX, a PDE inhibitor that potently inhibits all other cAMP PDEs expressed in Leydig cells, increased steroidogenesis in both primary cells (Fig. 3) and MA10 cells (Fig. 5). It is most likely that the other major PDE involved in this process is a PDE4, because rolipram (a selective PDE4 inhibitor) mimics the effects of IBMX (Fig. 6). In these cells at lower levels of adenylyl cyclase activation, the effectiveness of a PDE4 inhibitor is almost completely dependent on concomitant PDE8 inhibition (Fig. 5B). This is outlined in the model presented in Fig. 8. In the absence of PDE8A (PDE8A(−/−) and PDE8A−/−/B−/− cells), IBMX significantly potentiated resting steroidogenesis. This indicates that PDE8A and a PDE4 (higher-Km PDE) can control the same “functional compartment(s)” of cAMP. Moreover, it would seem that PDE8A alone can “handle” the low level of cAMP synthesis present in the resting condition. Therefore, the PDE4(s) are most important for regulation of steroidogenesis under higher LH stimulation, because IBMX was more effective than PF-04957325 on the steroid production at high LH. This scheme also seems to hold true for steroid regulation in MA10 cells, because a synergistic effect of PF-04957325 and IBMX or rolipram on basal progesterone production was seen (Fig. 5). Upon stimulation with LH or forskolin, coinubcation with PF-04957325 and IBMX lowered the EC50 value for both agonists and potentiated maximum responsiveness.

Using a generic, anti-PKA phosphosubstrate-selective antibody, we found that treatment of cells with PF-04957325, especially when cotreated with a PDE4 inhibitor, caused multiple substrates of PKA to be phosphorylated (Fig. 7). This increased phosphorylation could be detected within 15 min of treatment and was maintained for at least 3 h (data not shown). These data suggest that PDE8 inhibition can cause sustainable activation of PKA, probably in multiple compartments, because many different phosphoprotein bands were seen (Fig. 7). One of the PKA substrates was CEH/HSL, phosphorylation of which could be seen on serine 660 in MA10 cells. CEH/HSL is known to be activated by PKA and can be a rate-limiting step in the hydrolysis of stored cholesterol (Anthonsen et al., 1998; Yeaman, 2004). CEH/HSL is responsible for nearly all of the neutral CEH activity in steroidogenic cells, and no detectable CEH activity is seen in the testis of CEH/HSL knockout mice (Osuga et al., 2000). Phosphorylation of CEH/HSL at serine660 was increased by treatment with PF-04957325 in the presence of the PDE4 inhibitor. The phosphorylation closely correlated with steroidogenesis in the MA10 cells (Fig. 5A). This study also demonstrated that the resting basal level of phosphorylated CEH/HSL was significantly higher in PDE8A−/−/B−/− Leydig cells than in WT cells, whereas total CEH/HSL levels did not differ between the two groups (Fig. 7, C and D). Presumably, this increase of CEH/HSL phosphorylation is one mechanism causing the increased resting steroid production observed in PDE8 ablated cells.

It is known that steroidogenesis also can be regulated at other steps in the synthetic pathway. For example, chronic activation of cAMP-PKA signaling can result in increased synthesis of several key steroidogenic pathway proteins (Rao et al., 2003; Li et al., 2004). The level and activity of StAR protein is usually considered the major rate-limiting step for steroid production, and transcription of this protein is highly regulated by cAMP/PKA (Jefcoate, 2002). Treatment of
MA10 cells with PF-04957325 plus IBMX increased the levels of StAR protein in a dose-dependent manner (Fig. 7B). This effect was well correlated with steroid production in these cells. These data therefore strongly indicate that the potentiation of steroidogenesis by PDE8 inhibition also is at least partially due to increased STAR protein production. We also have detected increased levels in Nur77, a nuclear receptor that is one of the transcription factors regulating STAR gene induction (Davis and Lau, 1994; Martin et al., 2008), in cells treated with PF-04957325 and IBMX (data not shown). The increase in both CEH/HSL activity and STAR protein as a result of PDE8 inhibition is interesting in light of the finding that CEH/HSL can be activated by direct interaction with STAR protein and together they facilitate cholesterol movement from lipid droplets to mitochondria (Shen et al., 2003).

PDE8A and PDE8B apparently control separate pools of cAMP that work together to regulate steroid production. For example, our immunocytochemical studies showed that PDE8A specifically localizes with or very near to mitochondria, whereas PDE8B broadly distributes in the cytosol (Fig. 2). It is therefore of interest to understand how such distinct localization of two PDE8 isoforms works together to regulate steroidogenesis. One possibility is that distinct pools of cAMP activate distinct PKA isozymes that in turn regulate different downstream targets. For example, Dyson et al. (2009) used PKA subtype-specific cAMP analogs to suggest that type I PKA is most capable of inducing STAR gene transcripts, whereas type II PKA may predominantly serve to regulate the phosphorylation of STAR protein in Leydig cells. Such compartmentalization is probably organized by one or more A-kinase anchoring proteins that serve to localize distinct PKA subtypes to specific locations within the cell (Li et al., 2001; Carlucci et al., 2008; Manna et al., 2009; Poderoso et al., 2009).

We looked for β-galactosidase activity in other organs of PDE8A(−/−) and PDE8B(−/−) knockout animals and found that theca cells in the ovary were positive, with this marker being expressed in both PDE8A(−/−) and PDE8B(−/−) tissues (data not shown). Like Leydig cells, ovarian theca cells are regulated by cAMP/PKA signaling upon LH stimulation. These cells are responsible for production of the androgen substrate required for ovarian estrogen biosynthesis (Magoffin, 2005). One other report suggests that a PDE8 activity seems to be expressed in bovine ovarian follicles (Sasseville et al., 2009). However, it remains to be elucidated how these PDE8s may be as regulators of theca cell steroidogenesis.

Given the multiple roles played by PDE8s in regulation of testosterone secretion, it would seem that PDE8s could be a promising target for treatment of some types of infertility in men or women. For example, short-term treatment with a selective PDE8 inhibitor, or perhaps more likely a combination PDE4/PDE8 inhibitor, would be expected to boost androgen production in patients with infertility associated with testicular or follicular hypoandrogenism. Likewise, a PDE8 activator, if one can be found, would be expected to greatly decrease cAMP-dependent testosterone production. Such an agent might be particularly effective if administered with an androgen agonist to decrease LH secretion as a male contraceptive agent.

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References


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