Modulation of Leydig cell function by cyclic nucleotide phosphodiesterase 8A

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Leydig cells produce testosterone in the testes under the pulsatile control of pituitary luteinizing hormone (LH). cAMP is the intracellular messenger for LH action on steroidogenesis, and pharmacological evidence indicates that the response to LH can be modulated by cyclic nucleotide phosphodiesterases (PDEs). However the types and roles of the PDEs present in Leydig cells have not been fully defined. We report here that PDE8A is expressed in Leydig cells, and using PDE8A knockout mice we provide evidence that PDE8A is a key regulator of LH signaling and steroidogenesis. A 4-fold increase in the sensitivity to LH for testosterone production was detected in Leydig cells isolated from PDE8A knockout mice. In Leydig cells from wild-type mice, 3-isobutyl-1-methylxanthine, a compound that inhibits all cAMP PDEs except PDE8A, elicited only a small increase in the sensitivity of testosterone production to LH. However, in the PDE8-null mice, the effect of this inhibitor is much more pronounced. These observations indicate that PDE8A and at least one other PDE control the same or a complementary pool of cAMP that mediates LH-regulated steroidogenesis. Overall, these results suggest that pharmacological manipulation of PDE8A, alone or in combination with other PDEs present in Leydig cells, may be exploited to modulate testosterone synthesis and possibly to treat various conditions where the local levels of this androgen need to be altered.

cAMP | testosterone | PDE8A | testis | steroidogenesis

The second messenger cAMP plays important roles in mediating the biological effects of a wide variety of first messengers. Increases in intracellular cAMP lead to activation of cAMP-dependent protein kinases, guanine nucleotide exchange factors, and cyclic nucleotide-gated channels, which in turn can regulate the activity of other signaling and metabolic pathways (1). cAMP signaling pathways are controlled through regulation of the synthesis of cAMP by adenylyl cyclases and degradation by phosphodiesterases (PDEs) (1, 2). The cyclic nucleotide-PDEs are now recognized to form a superfamily of 11 different, but homologous, gene-families that all contain highly homologous catalytic domains near their C termini (3). PDE-catalyzed cyclic nucleotide degradation provides an important mechanism for regulating signaling. Indeed, the PDE component of cAMP pathways ensures the proper intensity and spatiotemporal distribution of the signal (4, 5), as illustrated by many studies on different endocrine tissues (6–8).

Leydig cells are interstitial cells located adjacent to the seminiferous tubules in the testes. The best-established function of Leydig cells is to produce the androgen, testosterone, under the pulsatile control of pituitary luteinizing hormone (LH) (9). It has been demonstrated that cAMP is the major intracellular messenger for LH action on steroidogenesis and that most, if not all, of the signaling action of cAMP is due to cAMP-dependent protein kinase (PKA)-mediated effects on proteins regulating the steroid biosynthetic pathway (9, 10). Substantial evidence for the regulatory function of PDEs in Leydig cells has been reported, including a small stimulatory effect of nonselective PDE inhibitors on testosterone release by primary Leydig cells (11, 12). These observations indicated that one or more PDEs might be active in Leydig cells to modulate the intensity, duration, and perhaps the desensitization of the LH-stimulated hormonal response. We report here that PDE8A is expressed in mouse Leydig cells and provide evidence that it is one of the PDEs that regulate steroid production. Although some biochemical, pharmacological, and genetic characteristics of PDE8A have been elucidated, its biological functions still remain largely unknown (13–15). Although Northern analysis has shown that PDE8A mRNA is highly expressed in testis (13), the expression of PDE8A protein has to date only been reported for human $CD4^+$ T cells (16) and mouse sperm (17). Our data suggest that this enzyme plays an important role in setting the sensitivity to LH for testosterone production.

Results

The PDE8A knockout (KO) mice were generated by replacing 38 nucleotides in the PDE8A gene with a targeting cassette containing the LacZ gene encoding a modified β-galactosidase, including the simian virus 40 large tumor nuclear localization signal, and Neo resistance. The targeting cassette disrupted a portion of exon 17, which normally encodes the third helix of the catalytic domain (18) (Fig. 1*A*). All PDE catalytic domains studied so far have similar three-dimensional structures (19), and this N-terminal region of the catalytic core is crucial for the activity (20). Fig. 1*B* illustrates the conserved folding of PDE catalytic domains. Because the crystal structure of PDE8A is not known, the highly homologous PDE4B is shown. Highlighted in black is the third helix removed by the targeting cassette. Fig. 1*C* shows the reduction of PDE8A mRNA levels for the area downstream of the targeting cassette (exon 19–20 area) in PDE8A KO mice testis, as detected by real-time PCR.

To establish whether any activity of PDE8A could be detected from a cell type known to express high levels of PDE8A (17), PDE8A activity was immunoprecipitated from mouse sperm homogenates (10^6 sperm per aliquot). The activity of PDE8A was 23 ± 4 fmol cAMP hydrolyzed/min for immunoprecipitates from wild-type mice ($n = 3$), whereas it was undetectable over the background in immunoprecipitates from PDE8A KO mice ($n = 3$).

The testicular expression and localization of PDE8A mRNA was evaluated by *in situ* hybridization of mouse testis sections (Fig. 2). As expected from the real-time PCR data, the signal in KO mice testes was greatly reduced (Fig. 2*C*). The cellular localization of PDE8A mRNA confirmed our previous observations of the expression of PDE8A in germ cells in testis tubules (13) but also revealed a previously underestimated expression in the interstitial spaces corresponding to the location of Leydig cells (Fig. 2 *E* and *G*, rectangles).

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The authors declare no conflict of interest.

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; LH, luteinizing hormone; KO, knockout.

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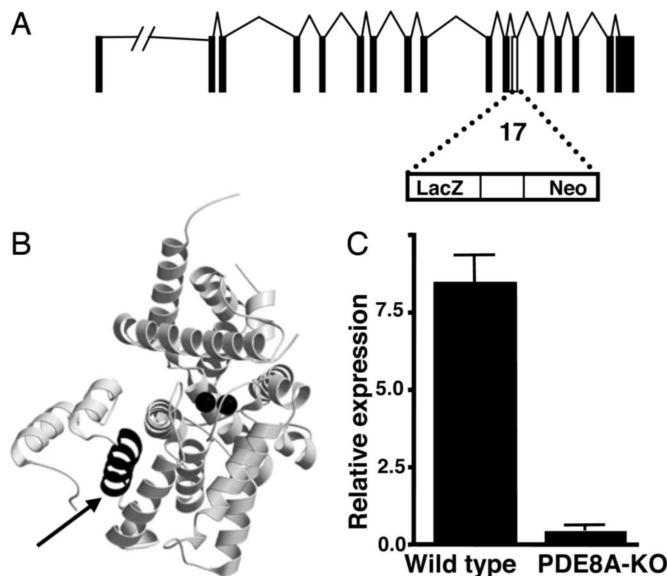

Fig. 1. Mouse PDE8A and targeting strategy. (*A*) Schematic representation of the mouse PDE8A gene and targeting cassette showing deletion of part of exon 17. (*B*) Conserved three-dimensional structure of the PDE4B catalytic domain (drawn from coordinates deposited in the Protein Data Bank under ID code ITB5) indicating in black the third helix corresponding to the area deleted in the PDE8A gene. (*C*) Relative abundance of PDE8A mRNA in wild-type ($n = 2$) and PDE8A KO ($n = 4$) mouse testis as determined by real-time PCR. The primers used detect the exon 19–20 junction of PDE8A mRNA. The data are reported as means ± SD, relative to hypoxanthine-guanine phosphoribosyltransferase expression.

The expression and localization of PDE8A protein was evaluated by immunostaining of mouse testis sections with a specific antibody. Immunofluorescent staining with a PDE8A-specific antibody showed a positive immunoreactivity in developing sperm tails, in agreement with a previous report (17) and with our ability to immunoprecipitate PDE8A activity from mature sperm, as well as in interstitial areas of the testis (Fig. 3 *A* and *E*). PDE8A-associated fluorescence was superimposed with staining for the Leydig cell marker CYP11A (Fig. 3 *C* and *E*). This staining was greatly diminished by preincubation of the antibody with a blocking peptide containing the epitope used to raise the antibody (Fig. 3*B*). No staining was detected by incubating the slides in the presence of secondary antibodies alone (Fig. 3*D*). Testis sections from PDE8A-null mice also were stained for β-galactosidase activity, because this enzyme is encoded in the targeting cassette with a nuclear-targeting N-terminal modification. X-gal nuclear staining could be detected (Fig. 3*G*) in the areas stained for the Leydig cell marker CYP11A in PDE8A KO mice but not in wild-type mice (Fig. 3*F*). Isolated Leydig cells were also stained for PDE8A (Fig. 3*H*), and the fluorescence could also be superimposed with staining for the Leydig cell marker CYP11A (Fig. 3*I*) as shown in Fig. 3*J*.

To study testosterone production Leydig cells isolated from wild-type and PDE8A KO mice were incubated in 96-well plates for 3 h in the presence of increasing concentrations of LH. Because many of the Leydig cells appeared to be clustered, they could not be counted with precision. Therefore, the values of testosterone released were normalized to the activity of 3β-hydroxysteroid dehydrogenase, a marker enzyme of Leydig cells. The basal testosterone production was on average ≈4-fold higher in Leydig cells isolated from PDE8A KO mice [8.48 ± 1.90 ng/ml testosterone released/relative cell number for wild-type mice (mean ± SD) ($n = 3$) versus 34.5 ± 14.6 ng/ml for PDE8A KO mice ($n = 3$); $P < 0.04$]. The maximal output of testosterone at high concentration of LH was not significantly different in wild-type and PDE8A-null Leydig cells (data not shown). LH induced testosterone production by Leydig cells from a wild-type and a PDE8A KO mouse is reported in Fig. 4*A*. The concentration dependence of the LH effect appears shifted to the left in the Leydig cells isolated from the PDE8A-null mice. When the concentration-dependence curves obtained with several Leydig cell preparations were fitted by nonlinear regression analysis to calculate the EC_{50} for LH (Fig. 4*B*), the average EC_{50} for cells from wild-type mice was 158 ± 38 pg/ml LH (mean ± SD; $n = 6$) and 39 ± 16 pg/ml ($n = 6$) for cells from PDE8A KO mice. The difference was determined to be statistically different by Student's *t* test analysis ($P < 0.0001$). These data strongly indicate that PDE8A regulates the cAMP transients induced by LH and thereby subsequent steroidogenesis.

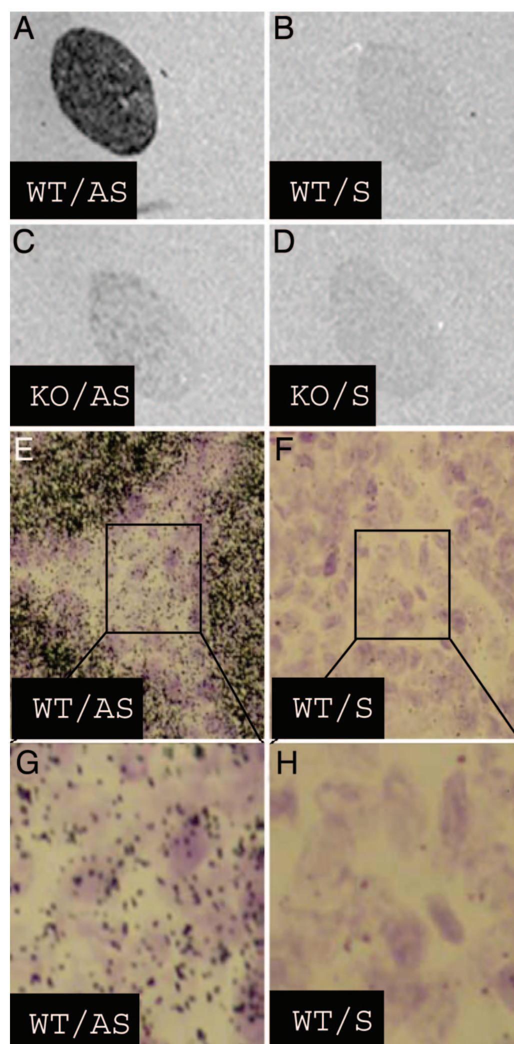

Fig. 2. *In situ* hybridization of mouse testes from wild-type (WT) and PDE8A KO mice with PDE8A riboprobes. Frozen testis sections (20 μm) of WT (*A*, *B*, and *E–H*) and PDE8A KO (*C* and *D*) were hybridized with ^{35}S-labeled cRNA probes for PDE8A, antisense (AS), or sense (S). The images in *E* and *G* (antisense) and *F* and *H* (sense) show probe signal (dark dots) on a hematoxyline-counterstained background. The rectangles in *E* and *F* show the interstitial areas surrounded by seminiferous tubules. The areas pictured within the rectangles are expanded in *G* and *H* to more easily see the specific signal.

To evaluate the potential role of other PDEs in this process, Leydig cells were preincubated 30 min in the presence or absence of 50 μM 3-isobutyl-1-methylxanthine (IBMX), a PDE inhibitor reported to act on all cAMP PDEs with the exception of the PDE8s (13, 14) and then stimulated 3 h with LH. Data from a

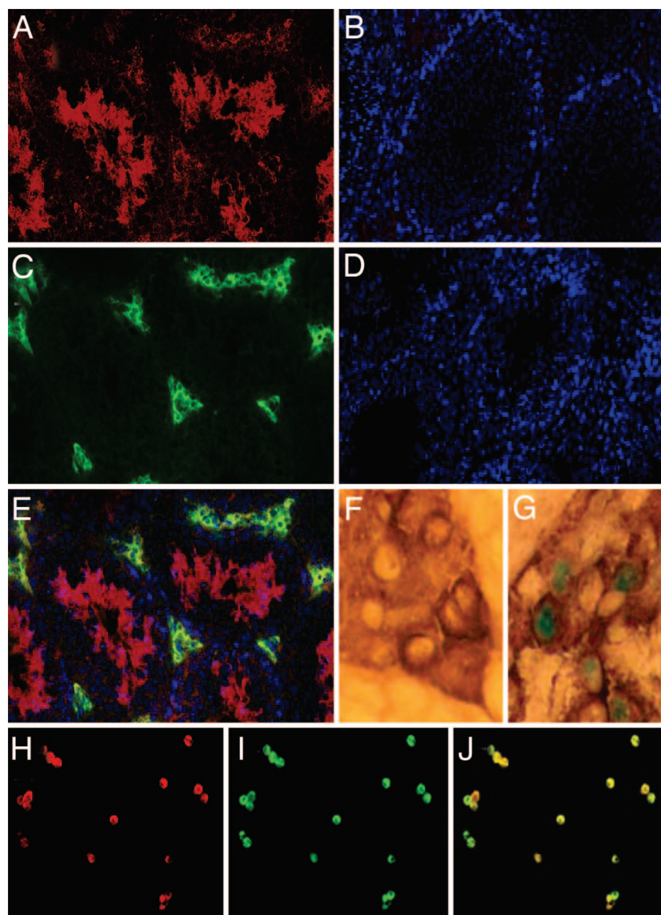

Fig. 3. Cellular localization of PDE8A in mouse testis. (*A* and *C*) Frozen sections of mouse testis were immunostained with the PDE8A-specific antibody (*A*) or with CYP11A antibody to label Leydig cells (*C*). (*E*) Merged image of PDE8A and CYP11A staining. (*B*) Staining with PDE8A antibody preincubated with the peptide used to raise the antibody and nuclear staining in blue. (*D*) Secondary antibody-only staining and nuclear staining. (*F* and *G*) CYP11A and β-galactosidase staining in a testis section from wild-type (*F*) and PDE8A KO (*G*) mice. (*H* and *I*) Isolated Leydig cells were immunostained with the PDE8A-specific antibody (*H*) or with CYP11A antibody (*I*). (*J*) Merged image of PDE8A and CYP11A staining. For each of these conditions, the analysis was repeated and showed similar staining pattern for three mice.

representative experiment, performed with cells from a wild-type mouse and a PDE8A KO mouse, are reported in Fig. 5*A*. The average EC_{50} values for LH in the absence and presence of IBMX, obtained with cells from several animals, are reported in Fig. 5*B*. IBMX preincubation decreased the average EC_{50} for LH 2.3-fold in cells from wild-type mice (from 137 ± 33 pg/ml LH, $n = 3$ to 59 ± 21 pg/ml, $n = 3$; $P < 0.03$) versus a 5.7-fold decrease for cells from PDE8A KO mice (from 51 ± 13 pg/ml LH, $n = 3$ to 9 ± 2 pg/ml, $n = 3$; $P < 0.0015$). Overall, the combination of PDE8A ablation and IBMX decreased the EC_{50} by 13-fold, indicating that PDEs are potent modulators of LH signaling.

Discussion

The stimulation of testosterone production by LH in Leydig cells is known to be mediated by an increase in the levels of the second messenger cAMP (9, 10). PDEs degrade cAMP and are thought to control its diffusion in cells so that the signal remains properly localized in subcompartments of the cell (4, 5). PDEs are also important in controlling the intensity and duration of the signal. Therefore, perturbation of the activity of a PDE endogenous to Leydig cells might be expected to modulate the response to LH.

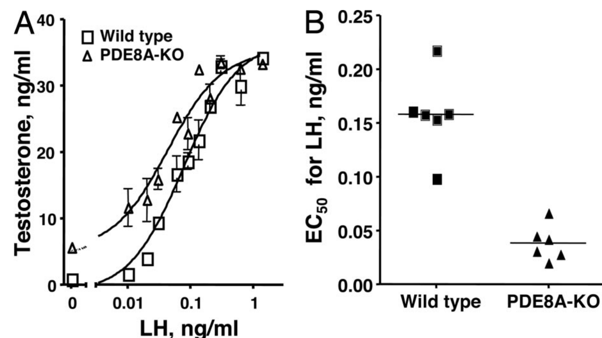

Fig. 4. LH concentration-dependence of testosterone production by Leydig cells. (*A*) Leydig cells isolated from one wild-type mouse and one PDE8A KO mouse were stimulated with various concentrations of LH. Testosterone released into the media after 3 h of incubation was measured by using an immunoenzymatic assay kit. Testosterone production was assayed in a duplicate cell sample for each LH concentration. Values are reported as mean ± SD. (*B*) EC_{50} for LH of testosterone production by Leydig cells isolated from six wild-type mice and six PDE8A KO mice. The EC_{50} for LH were calculated by nonlinear fitting of the LH concentration-dependence curves obtained for each mouse as described in *Materials and Methods*. Horizontal bars indicate the average EC_{50} for each group.

PDE8A was initially shown to be expressed in mouse testis by Northern blot analysis but the cellular expression and localization of the protein was not known (13). We now observe that PDE8A mRNA and protein are expressed in Leydig cells by *in situ* hybridization, by immunofluorescence analysis with a PDE8A-specific antibody, and by β-galactosidase staining of sections obtained from the PDE8A KO mice testes.

Considering the importance of cAMP in mediating the effect of LH on testosterone synthesis, it was of interest to analyze the potential role of PDE8A in modulating the hormonal response of Leydig cells. If important to this process, we expected that LH would be more effective in the absence of PDE8A. Therefore, a PDE8A KO mouse model was used to investigate the role of PDE8A in Leydig cell function. Basal release of testosterone was increased ≈4-fold in PDE8A-null Leydig cells. Moreover, a significant increase in the sensitivity to LH, measured as testosterone released into the media, was observed for cells isolated

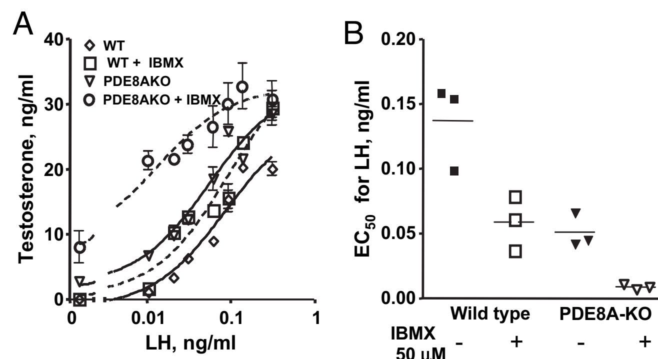

Fig. 5. Effect of IBMX on LH concentration-dependence of testosterone production. (*A*) Leydig cells isolated from one wild-type mouse and one PDE8A KO mouse were stimulated with various concentrations of LH in the absence (continuous line) or presence (dashed line) of 50 μM IBMX. Testosterone released into the media after 3 h of incubation was assayed for a duplicate cell sample at each LH concentration. Values are reported as mean ± SD. (*B*) EC_{50} for LH of testosterone production by Leydig cells isolated from three wild-type mice and three PDE8A KO mice, measured in the presence or absence of 50 μM IBMX. Horizontal bars indicate the average EC_{50} for each group.

from PDE8A KO mice, indicating that this PDE is normally controlling the pool of cAMP mobilized by LH.

Interestingly, in granulosa cells, another cell type responsive to LH, it has been proposed that PDE4D is responsible for the transient nature of LH signaling (4). Our observation that IBMX, a nonspecific PDE inhibitor that does not affect PDE8A (13), further potentiates LH action in PDE8A KO mice cells indicates that an additional PDE is also participating in the control of the relevant cAMP pool(s) in Leydig cells. However, the observation that IBMX alone does not by itself produce a large shift in the dose/response curve to LH suggests that the other PDE(s) alone is/are not sufficient to fully regulate this pool of cAMP. Future studies will address which other PDEs may be relevant in this regard.

Besides its action on spermatogenesis, testosterone plays key roles in health and well being in both males and females. Examples include enhanced libido, energy, immune function, and probably protection against osteoporosis (21–23). Several conditions are associated with a deficit in testosterone production including andropause, idiopathic male infertility, and metabolic syndrome (24–26). Many of these conditions can in principle be treated by androgen administration. Unfortunately, testosterone itself is difficult to administer orally in part because it causes liver toxicity (27). Moreover, treatment with testosterone usually does not improve spermatogenesis in idiopathic male infertility, because the exogenously administrated testosterone suppresses LH production by the pituitary gland and thereby testicular testosterone production (28). Conversely, many PDE inhibitors are amenable to an oral route of administration. Our data suggests that PDE8A inhibitors may represent an alternative route of pharmacological intervention to increase local production of testosterone. Although potent and selective PDE8A inhibitors are not yet available, the relative potency of dipyridamole (13), trequinsin (29), and cilomilast (30) on PDE8 can perhaps be initially exploited to inhibit the enzyme and increase testosterone production by Leydig cells. Also, based on the knowledge of the PDE8A nucleotide sequence, molecular drugs such as antisense RNA and siRNA could be developed. The observed potentiation of LH action by IBMX indicates that inhibition of PDE8A alone, or combined with inhibition of other PDEs present in Leydig cells, might be exploited to modulate testosterone levels pharmacologically.

Another possible clinical application suggested by these results may be the ability to reduce local production of testosterone by increasing cAMP degradation in Leydig cells through PDE activation. Indeed, considering the primary role of testosterone for spermatogenesis, agents that directly decrease testosterone production in Leydig cells could in theory be exploited as male contraceptives. It has been shown previously that transfection of MA-10 Leydig tumor cells with an expression vector encoding an active PDE4 results in a severely blunted steroidogenic response to hormonal stimulation (31). Therefore, activation of a PDE endogenous to Leydig cells could be expected to reduce testosterone release. Hence an activator of PDE8A should reduce testosterone production by degrading the cAMP required for the LH signaling that initiates steroidogenesis. Several current approaches for male contraception are based on androgen administration. For example, in clinical trials, testosterone or derivatives of it have been tested as a contraceptive to suppress the secretion of LH and follicle-stimulating hormone from the pituitary (32, 33). The idea is that by depriving the testes of the signals required for local production of testosterone, normal spermatogenesis would be blocked. However these agents have not yet achieved complete azoospermia in all individuals and therefore lack acceptable efficacy. The data in this manuscript suggest that a PDE8A activator would in theory decrease Leydig cell sensitivity to LH and local production of testosterone. Therefore, such an activator could be used in combination with exogenous testosterone to suppress pituitary function and still maintain the beneficial effects of the steroid hormone. The possibility of reducing testosterone pharmacologically might also present advantages for the treatment of prostate cancer as well, considering the dependence of the prostate on androgens for growth (34). Although no activators for PDE8A are known at this time, the domain structure of this PDE indicates that there is potential to modulate this enzyme activity (3). Indeed, PDE8A contains a PAS domain, which is known to have a regulatory role in other proteins (35, 36). For instance, compounds that bind to a PAS domain and activate PAS-K have been identified by screening of a chemical library (37). Therefore, it seems possible that such an activator could be isolated.

In summary, the present observations indicate that PDE8A is important for the regulation of steroidogenesis in Leydig cells and thus represents a potential pharmacological target for modulation of testosterone synthesis.

Materials and Methods

Mice. Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). PDE8A KO mice were initially produced by Deltagen, Inc. (San Carlos, CA) under contract to Pfizer, Inc. (Pfizer Global Research and Development, Sandwich, U.K.). They were subsequently bred to C57BL/6 mice at the University of Washington for 10 generations. For the experiments reported, age-matched mice between 6 and 8 weeks of age were used. All procedures used 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*.

Real-Time PCR. Testis cDNA was prepared from total RNA from wild-type and PDE8A-null mouse testis by using SuperScript III and Oligo dT (Invitrogen Corp., Carlsbad, CA). Real-Time PCR was performed by using Power SYBR green master mix (Applied Biosystems, Foster City, CA). Primers (IDT, Coralville, IA) for PDE8A, directed to the area downstream of the targeting cassette, were as follows: forward primer, GCCACAGAAATGACGAAGC (exon 19); reverse primer, ATGTCTTCCAGACTTCTGTCAGG (exon 20). The primers for hypoxanthine-guanine phosphoribosyltransferase were as follows: forward primer ATTATGCCGAGGATTTGGAA; reverse primer, CCCATCTCCTTCATGACATCT.

***In Situ* Hybridization.** The template for riboprobe synthesis was obtained by PCR by using a plasmid containing the mouse PDE8A sequence. In particular, the 3′ region of mouse PDE8A (exons 17–20) was amplified by using the following primers: forward primer, AATTAACCCTCACTAAAGGACGGCGTATTTCCTTTCCAG (underlined T3 phage RNA polymerase promoter sequence); reverse primer, TAATACGACTCACTATAGGGACACGTCGGCACACTTAAT (underlined T7 phage RNA polymerase promoter sequence). The PCR products were isolated from agarose gels and purified with a Gel Extraction Kit (Qiagen, Valencia, CA). ^{35}S-labeled riboprobes were synthesized by *in vitro* transcription with a MAXIscript T3/T7 kit (Ambion, Austin, TX) by using as template the PCR product containing T3 and T7 phage RNA polymerase promoter sites. *In vitro* transcription was carried out in a 20-μl reaction mixture containing 5 μl of [α-^{35}S]UTP (PerkinElmer, Boston, MA) and the T3 RNA polymerase or the T7 RNA polymerase to obtain the sense or antisense riboprobe, respectively.

Testes were dissected from wild-type and PDE8A KO mice and were rapidly frozen in Tissue-Tek O.C.T. compound (Sakura, Torrence, CA) on dry ice. Sections (20 μm) were cut in a cryostat, mounted on a Superfrost plus microslide (VWR Scientific, West Chester, PA), and air dried. The sections were

fixed in 4% paraformaldehyde for 15 min at room temperature, treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, and dehydrated through a graded ethanol series (30, 60, 80, 95, and 100%). The sections were then incubated with hybridization buffer [50% deionized formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 100 mM DTT, 1 mM EDTA, 1 mg/ml yeast tRNA, 250 μg/ml salmon sperm DNA, and 10 mM Tris·HCl (pH 8.0)] in a humid chamber at 60°C for 4 h. After rinsing in 2× SSC (standard saline citrate; 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.2), the sections were dehydrated through a graded ethanol series. Hybridization was performed in buffer containing ^{35}S-labeled antisense or sense probe (40 pg/34,000–38,000 cpm per μl) under plastic coverslips in a humid chamber at 60°C overnight. The coverslips were gently removed, and the sections were washed with 2× SSC containing 10 mM DTT for 30 min twice at 60°C. The sections were then incubated for 30 min at 37°C with 20 μg/ml RNaseA in 0.5 M NaCl, 10 mM Tris·HCl (pH 7.5), and 1 mM EDTA, then washed in 50% formamide, 2× SSC containing 10 mM DTT for 30 min at 60°C, in 1× SSC containing 10 mM DTT for 30 min at 60°C, and further in 0.1× SSC containing 10 mM DTT for 30 min at 60°C. Finally, the sections were dehydrated in ethanol (70, 95, and 100%), air dried, and exposed on BioMax XAR film (Eastman Kodak, Rochester, NY) for 9 h. To see the cell distribution of PDE8A mRNA hybridization, the slides were coated with Autoradiography NTB emulsion (Eastman Kodak) and exposed for 1 week at 4°C. The slides were developed, fixed, and counterstained with hematoxyline and mounted in Canada Balsam (Sigma-Aldrich).

PDE8A Immunoprecipitation and Assay. Mouse sperm were isolated from the cauda epididymis by a swim-out method (38) and homogenized in PBS containing 1% Nonidet (Roche Applied Science, Indianapolis, IN), 1 mM EGTA, 20 mM DTT, 0.2 mM PMSF, 1 mM *para*-amino benzamidine, and Sigma protease inhibitor mixture (Sigma-Aldrich). The homogenate was centrifuged for 5 min at 16,000 × *g* in a microcentrifuge and 200-μl aliquots of the supernatant, equivalent to 10^6 sperm, were incubated overnight with 20 μl of a 1:1 slurry of protein G-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in the presence or absence of PDE8A antibody (1:100, 121-AP; Fabgennix, Frisco, TX). The next day, the immunoprecipitate was washed three times with PBS and then assayed for PDE activity in the presence of 10 nM [^{3}H]cAMP substrate, 40 mM Mops (pH 7.5), 15 mM Mg-acetate, 2 mM EGTA, and 0.2 mg/ml BSA as in ref. 39.

Immunohistotochemistry. Freshly frozen mouse testes were embedded in Tissue-Tek O.C.T. compound and then sectioned on a cryostat at 20 μm per slice. Tissue sections or isolated Leydig cells were dried and fixed in 4% (wt/vol) paraformaldehyde/PBS (pH 7.4) at room temperature for 10 min and washed three times with PBS. The slides were preincubated with blocking buffer (5% donkey serum, 1 mg/ml BSA, and 0.1% Triton X-100 in PBS) for 1 h at room temperature, incubated with an anti-PDE8A antibody (200 μl, 1:100, C-15; Santa Cruz Biotechnology) in PBS containing 1% donkey serum, 1 mg/ml BSA, and 0.1% Triton X-100 overnight at 4°C, washed in PBS containing 0.05% Tween 20 three times for 20 min, incubated with donkey anti-goat Alexa546 (200 μl, 1:500; Invitrogen) in PBS containing 1 mg/ml BSA and 0.1% Triton X-100 for 2 h at room temperature and washed. The slides were further incubated with blocking buffer containing 5% goat serum for 1 h at room temperature, incubated with cytochrome P450 side chain cleavage enzyme (CYP11A) antibody (200 μl, 1:250; Chemicon International Inc., Temecula, CA) overnight at 4°C, washed in PBS containing 0.05% Tween 20 three times for 20 min, and incubated with goat anti-rabbit Alexa488 (1:500; Invitrogen) as above. The sections were finally counterstained with TO-PRO-3 (1:1000; Invitrogen) in PBS for 5 min, washed, and mounted in SlowFade Gold antifade reagent (Invitrogen). To test the PDE8A antibody specificity, the antibody solution was preincubated with 2.5 μg/ml antigen peptide (Santa Cruz Biotechnology) for 2 h at room temperature before staining. Some sections were incubated without the primary antibodies to determine background due to the secondary antibodies. The immunostaining signals were visualized with a confocal microscope (Leica SL; Leica Microsystems Inc., Bannockburn, IL). β-galactosidase activity, expressed with a nuclear localization signal by the LacZ gene in the targeting cassette, was assessed by X-gal staining. The sections were first incubated with anti-CYP11A antibody followed by anti-rabbit alkaline phosphatase conjugate (1:500; Invitrogen) and NBT/BCIP (Roche) for color development. X-gal staining was then performed as described (40) in the X-gal mixture [1 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% Nonidet P-40, 5 mM EGTA, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, and 1 mg/ml X-gal in PBS, pH 7.4] at 37°C overnight. The sections were washed and mounted with glycerol/Tris-buffered saline.

Leydig Cell Purification. Leydig cells were isolated as described in ref. 41. Briefly, testes from adult mice were decapsulated and dispersed in a shaking water bath at 34°C for 10 min in 10 ml of Medium 199 (M-199) with 0.25 mg/ml collagenase D, 35 mU/ml Dispase II, and 6 μg/ml DNase I. To terminate tissue dispersion, 40 ml of 1% BSA in M-199 media containing 15 mM Hepes, 4 mM sodium bicarbonate, and 25 μg/ml soybean trypsin inhibitor was added to dilute the original suspension. Tubes were then capped and inverted several times. Seminiferous tubules were allowed to settle by gravity, and the supernatant containing the interstitial cells was collected. The procedure was repeated two times to further harvest interstitial cells. The cells were pelleted in 50-ml tubes by centrifugation at 800 × *g* for 20 min at 4°C and then fractionated by using a continuous Percoll (GE Healthcare, Piscataway, NJ) gradient (55% Percoll in HBSS buffered with 15 mM Hepes and 25 μg/ml soybean trypsin inhibitor) in a total volume of 35 ml. The gradients were formed *in situ* by centrifugation in a JA-20 fixed angle rotor (Beckman Coulter, Fullerton, CA) at 23,700 × *g* for 30 min at 4°C. A tube containing density marker beads (GE Healthcare) was used as a reference to identify the different density layers. Leydig cells were recovered starting at a density of 1.07 g/ml to the bottom of the gradient. Five volumes of HBSS were added to dilute the Percoll, and the cells were pelleted at 200 × *g* for 10 min at 4°C. The final pellet obtained from two testes, enriched in Leydig cells, was resuspended in 4 ml of DMEM/F-12 supplemented with 1% BSA and used immediately for the experiments.

3β-Hydoxysteroid Dehydrogenase Assay. Leydig cells were incubated in 96 wells at 37°C for 2 h as described (42) in the presence of PBS containing 0.1% BSA, 1.5 mM NAD^+, 0.25 mM nitroblue tetrazolium, and 0.2 mM 5β-androstane-3β-ol,17-one with the addition of 0.5% Triton X-100, and the absorbance was read at 570 nm.

Measurement of Testosterone Production. Leydig cells were resuspended as above and 100-μl aliquots were dispensed in 96-well plates. Cells were stimulated in 150-μl final volume with the indicated concentrations of recombinant LH for 3 h in a 5% CO_2 incubator at 37°C. Recombinant human LH was obtained from the National Hormone & Peptide Program (A. F. Parlow, Torrance, CA). Testosterone released into the media of duplicate cell samples for each LH concentration was measured by using an immunoenzymatic assay kit from Neogen (Lexington, KY).

All data are expressed as the mean ± SD. The EC_{50} values for LH action on testosterone production were calculated by nonlinear fitting of the LH concentration-dependence curves obtained for each Leydig cells preparation by using a software package (GraphPad Prism 4.0; GraphPad Software, San Diego, CA). Statistical analysis was performed by Student's *t* test. Differences were regarded as significant at $P < 0.05$.

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1. Beavo JA, Brunton LL (2002) *Nat Rev Mol Cell Biol* 3:710–718.
2. Schwartz JH (2001) *Proc Natl Acad Sci USA* 98:13482–13484.
3. Soderling SH, Beavo JA (2000) *Curr Opin Cell Biol* 12:174–179.
4. Conti M (2002) *Biol Reprod* 67:1653–1661.
5. Zaccolo M, Di Benedetto G, Lissandron V, Mancuso L, Terrin A, Zamparo I (2006) *Biochem Soc Trans* 34:495–497.
6. Mehats C, Andersen CB, Filopanti M, Jin SL, Conti M (2002) *Trends Endocrinol Metab* 13:29–35.
7. MacFarland RT, Zelus BD, Beavo JA (1991) *J Biol Chem* 266:136–142.
8. Nikolaev VO, Gambaryan S, Engelhardt S, Walter U, Lohse MJ (2005) *J Biol Chem* 280:1716–1719.
9. Saez JM (1994) *Endocr Rev* 15:574–626.
10. Stocco DM, Wang X, Jo Y, Manna PR (2005) *Mol Endocrinol* 19:2647–2659.
11. Catt KJ, Dufau ML (1973) *Nat New Biol* 244:219–221.
12. Mendelson C, Dufau M, Catt K (1975) *J Biol Chem* 250:8818–8823.
13. Soderling SH, Bayuga SJ, Beavo JA (1998) *Proc Natl Acad Sci USA* 95:8991–8996.
14. Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB (1998) *Biochem Biophys Res Commun* 246:570–577.
15. Vasta V (2007) in *Phosphodiesterases in Health and Disease*, eds Beavo J, Francis S, Houslay M (CRC, Boca Raton, FL), in press.
16. Glavas NA, Ostenson C, Schaefer JB, Vasta V, Beavo JA (2001) *Proc Natl Acad Sci USA* 98:6319–6324.
17. Baxendale RW, Fraser LR (2005) *Mol Reprod Dev* 71:495–508.
18. Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) *Science* 288:1822–1825.
19. Jeon YH, Heo YS, Kim CM, Hyun YL, Lee TG, Ro S, Cho JM (2005) *Cell Mol Life Sci* 62:1198–1220.
20. Varnerin JP, Chung CC, Patel SB, Scapin G, Parmee ER, Morin NR, MacNeil DJ, Cully DF, Van der Ploeg LH, Tota MR (2004) *Protein Expr Purif* 35:225–236.
21. Anawalt BD, Merriam GR (2001) *Endocrinol Metab Clin North Am* 30:647–669.
22. Yialamas MA, Hayes FJ (2003) *Best Pract Res Clin Endocrinol Metab* 17:223–236.
23. Morley JE, Haren MT, Kim MJ, Kevorkian R, Perry HM, III (2005) *J Endocrinol Invest* 28, 76–80.
24. Matsumoto AM (2002) *J Gerontol A Biol Sci Med Sci* 57:M76–M99.
25. Ochsenkuhn R, De Kretser DM (2003) *Int J Androl* 26:195–201.
26. Makhsida N, Shah J, Yan G, Fisch H, Shabsigh R (2005) *J Urol* 174:827–834.
27. Ishak KG, Zimmerman HJ (1987) *Semin Liver Dis* 7:230–236.
28. Sheckter CB, Matsumoto AM, Bremner WJ (1989) *J Clin Endocrinol Metab* 68:397–401.
29. Liu S, Veilleux A, Zhang L, Young A, Kwok E, Laliberte F, Chung C, Tota MR, Dube D, Friesen RW, Huang Z (2005) *J Pharmacol Exp Ther* 314:846–854.
30. Card GL, England BP, Suzuki Y, Fong D, Powell B, Lee B, Luu C, Tabrizizad M, Gillette S, Ibrahim PN, *et al.* (2004) *Structure (London)* 12:2233–2247.
31. Swinnen JV, D'Souza B, Conti M, Ascoli M (1991) *J Biol Chem* 266:14383–14389.
32. Walton M, Anderson RA (2005) *Curr Drug Targets Immune Endocr Metabol Disord* 5:249–257.
33. Amory JK (2005) *Treat Endocrinol* 4:333–341.
34. Huggins CB (1969) *J Urol* 102:119–120.
35. Taylor BL, Zhulin IB (1999) *Microbiol Mol Biol Rev* 63:479–506.
36. Vreede J, van der Horst MA, Hellingwerf KJ, Crielaard W, van Aalten DM (2003) *J Biol Chem* 278:18434–18439.
37. Amezcua CA, Harper SM, Rutter J, Gardner KH (2002) *Structure (London)* 10:1349–1361.
38. Westenbroek RE, Babcock DF (1999) *Dev Biol* 207:457–469.
39. Thompson WJ, Appleman MM (1971) *Biochemistry* 10:311–316.
40. Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T, Bonventre JV (2005) *J Clin Invest* 115:1743–1755.
41. Dong Q, Salva A, Sottas CM, Niu E, Holmes M, Hardy MP (2004) *J Androl* 25:973–981.
42. Payne AH, Downing JR, Wong KL (1980) *Endocrinology* 106:1424–1429.