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Involvement of Protein Kinase C and Cyclic Adenosine 3′,5′-Monophosphate-Dependent Kinase in Steroidogenic Acute Regulatory Protein Expression and Steroid Biosynthesis in Leydig Cells[1]

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ABSTRACT

This study investigated the roles of the protein kinase C (PKC) and protein kinase A (PKA) pathways in regulating constitutive steroidogenesis and steroidogenic acute regulatory (STAR; herein designated by its common name, StAR) protein in R2C Leydig tumor cells. Inhibition of PKC and phospholipase C resulted in significant decreases in steroid production, phosphorylation of cAMP-responsive element binding (CREB) protein, and *Star* gene transcription under basal conditions in R2C cells. These observations were corroborated in MA-10 and mLTC-1 Leydig tumor cell lines, in which activation of PKC by phorbol-12-myristate-13-acetate (PMA, 10 nM) increased CREB phosphorylation and total StAR (tot-StAR) protein expression. However, induction of StAR protein by PMA did not result in the expected concomitant increase in steroids because PKC failed to phosphorylate StAR, the biologically active form of the protein. However, in conjunction with PMA, minor increases in PKA activity using submaximal doses of $(Bu)_2$cAMP (0.05–0.1 mM; a concentration range insufficient for induction of StAR), were able to stimulate dramatic increases in both phospho-StAR (P-StAR) and steroid production. Human chorionic gonadotropin stimulation also resulted in a further enhancement in P-StAR and progesterone production when added to PMA-treated MA-10 cells. Similar results for tot-StAR and P-StAR expression were observed in primary cultures of immature rat Leydig cells treated with PMA and submaximal doses of $(Bu)_2$cAMP. In summary, the present study demonstrates that basal activities of both PKC and PKA play important roles in the constitutive steroidogenic characteristics of R2C cells. This study also demonstrates for the first time a role for PMA-induced PKC in StAR protein regulation and the requirement for submaximal doses of cAMP to produce steroids in Leydig cells.

cAMP, CREB, kinases, Leydig cells, PKA, PKC, PMA, progesterone, signal transduction, StAR, steroid biosynthesis, testosterone

INTRODUCTION

The rate-limiting enzymatic reaction in steroid biosynthesis (i.e., the conversion of free cholesterol to pregnenolone) is catalyzed by the cytochrome P450 side chain cleavage enzyme (CYP11A1; herein called $P450_{scc}$) [1–3]. However, the physiologically relevant rate-limiting step is the transfer of free cholesterol from the outer mitochondrial membrane to the inner membrane where the $P450_{scc}$ resides [4]. This transfer is mediated in part by the steroidogenic acute regulatory (StAR) protein. Steroidogenesis in tissues such as the adrenal, ovary, and testis cannot occur without ongoing StAR synthesis [5, 6]. Inhibition of protein synthesis by cycloheximide blocks both hormone-induced StAR synthesis and steroid production in steroidogenic cells [5, 6].

R2C rat Leydig tumor cells constitutively synthesize steroids. This constitutive steroidogenesis has been studied in several laboratories and compared with that in MA-10 cells, a mouse Leydig tumor cell line [4, 7–10]. MA-10 cells produce steroids and StAR protein when stimulated by trophic hormones, while R2C cells do not require external stimulation and exhibit inherently high basal levels of steroids and StAR protein. Earlier, we investigated the manner by which R2C cells are equipped to constitutively produce steroids at such high levels. We found that the scavenger receptor-type B class I (SCARBI or SRBI), hormone-sensitive lipase (LIPE or HSL), and StAR, all of which are used in the trafficking of cholesterol for steroidogenesis, are constitutively expressed in R2C cells at levels much higher than those observed in MA-10 cells under basal conditions [11]. We further demonstrated that the levels of the peripheral benzodiazepine receptor (BZRP; herein called PBR), a protein also involved in the transfer of free cholesterol into the inner mitochondrial membrane [10], were, surprisingly, much higher in MA-10 cells than that in R2C cells [12]. Because it has been shown that PBR is an essential component of cholesterol transfer in steroidogenic cells [13], perhaps it is the proposed relationship between StAR protein and PBR [14] that is altered in R2C cells and thus responsible for constitutive steroidogenesis.

Given these observations, we sought to determine the mechanism for the constitutively high basal levels of proteins supporting steroid production in R2C cells and, as a target, we chose to focus on the StAR protein in the present study. Because a recent investigation from our laboratory demonstrated that the absence of the dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1 (NR0B1 or DAX1) transcription factor in R2C cells is only partially responsible for the inherently high steroidogenic output of the cells [15], we assumed that other factors are also implicated.

Leydig cells are specialized interstitial cells in the testis that produce the testosterone required for spermatogenesis

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[16, 17]. The signal that initiates steroidogenesis in Leydig cells is the binding of LH to the LH receptor (LHCGR) [18, 19] and the activation of numerous downstream pathways that in turn regulate steroid biosynthesis [20–22]. There are many lines of evidence indicating that LH can activate both the protein kinase A (PKA) and protein kinase C (PKC) signaling pathways. Using preovulatory follicles from immature rats, Morris and Richards showed that the PKA inhibitor H89, and the PKC inhibitor calphostin-C, were able to inhibit LH-induced progesterone synthesis in granulosa cells [23]. Furthermore, they found that following activation of the LH receptor, phospholipase C (PLC) is able to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) liberating diacylglycerol (DAG), which can fully activate PKC, and inositol 1,4,5-triphosphate (IP_3), which can trigger the release of calcium from the endoplasmic reticulum. The authors suggested that granulosa cells respond to an LH surge through both the PKA and PKC pathways. An investigation using porcine endometrial cells [24] also demonstrated that stimulation with LH increased cAMP and IP_3 concentrations. Oxytocin was even more effective at increasing the concentrations of cAMP. In addition, turnover of IP_3 was accelerated by both LH and oxytocin. Unlike PKA, whose role in steroidogenesis and StAR expression has been studied extensively, the involvement of PKC in the pathways controlling StAR and steroid biosynthesis has only recently been implicated. The precise mechanism through which the PKC pathway is involved in steroid production and *Star* gene regulation has not been demonstrated in the Leydig cell.

When R2C cells, which have a 2-fold higher PKA activity under basal conditions than MA-10 cells, were treated with H89 to inactivate PKA, constitutive synthesis of *Star* mRNA and steroids was significantly inhibited, indicating that a basal level of PKA activity is crucial for the cells' constitutive steroidogenic phenotype [11]. Therefore, we hypothesize that in the basal state, R2C cells have activated signaling pathways that render the R2C cells constitutively steroidogenic. To test this hypothesis, we attempted to determine the role of the PKC and PKA pathways in constitutive StAR and steroid production. Using the R2C, MA-10, and mLTC-1 Leydig tumor cell lines as well as primary cultures of immature rat Leydig cells, we activated PKC and PKA with phorbol-12-myristate-13-acetate (PMA; a synthetic analog of DAG) and $(Bu)_2$cAMP, respectively, to determine the role of each pathway in the regulation of StAR and steroid biosynthesis.

MATERIALS AND METHODS

Reagents

Dibutyryl cAMP ($(Bu)_2$cAMP), U73122, 22(R)-hydroxycholesterol, H89, PMA, hCG, and Waymouth MB 752/1 medium were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS), gentamicin, and horse serum were purchased from Invitrogen Life Technologies (Carlsbad, CA). GF-109203X (Bisindolylmaleimide I) and Gö6983 were purchased from Calbiochem (San Diego, CA).

Cell Culture and Animals

R2C cells were grown in Waymouth MB 752/1 medium supplemented with 15% horse serum and 2.5% (v/v) FBS in the presence of 0.4% (v/v) gentamicin. MA-10 cells were grown in Waymouth MB 752/1 medium supplemented with 15% (v/v) heat-inactivated horse serum in the presence of 0.4% (v/v) gentamicin. Dr. Pulak R. Manna (Texas Tech University Health Sciences Center, Lubbock, TX) kindly provided the mLTC-1 cells. Primary cultures of immature rat Leydig cells were isolated as previously described [25]. Experimental protocols using animals were approved by the Institutional Animal Care and Use Committee at Morehouse School of Medicine-Atlanta University Center, where these studies were conducted. The cell lines and primary Leydig cell cultures were incubated in 5% CO_2 at 37°C.

Western Blot Analysis

Cells were cultured in 6-well plates in triplicate, incubated with different inhibitors for 15 min, and followed with stimulating or nonstimulating treatments. Total cell lysates were prepared in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM PMSF, 2 μg/ml of aprotinin, and 1% [v/v] Nonidet P-40) and assayed for protein content after sonication [26]. Equal amounts of protein were analyzed by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with specific antibodies recognizing total StAR (tot-StAR), cytochrome P450 17α-hydroxylase/C17-20 lyase (CYP17A1; herein called CYP17; kind gifts from Dr. Walter L. Miller, University of California, San Francisco, CA) [27, 28], phospho-StAR (P-StAR; generated against a peptide conjugated to keyhole limpet hemocyanin corresponding to amino acids 190–199 of mouse StAR with Ser194 phosphorylated; obtained from Bethyl Laboratories, Montgomery, TX), phospho-Ser/Thr PKA substrate (Cell Signaling Technology Inc., Beverly, MA), phospho-cAMP responsive element binding (P-CREB; Cell Signaling Technology) or total-CREB (tot-CREB; Cell Signaling Technology). A second rabbit anti-porcine CYP17 antibody was obtained from Dr. Dale B. Hales (University of Illinois at Chicago, Chicago, IL) [29]. After incubation with secondary antibodies (anti-mouse-horseradish peroxidase [HRP]-conjugated or anti-rabbit-HRP conjugated), antibody binding was determined using enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA) and exposure to x-ray film (Marsh Bio Products, Inc., Rochester, NY). The integrated optical density of the protein bands was quantified using the BioImage Visage 2000 (BioImage Corp., Ann Arbor, MI).

Northern Blot Analysis

Northern blot analysis for *Star* was performed as previously described [11, 30]. Briefly, total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA (10 μg) was loaded on a 1% agarose gel containing 5.2% (v/v) formaldehyde. Northern blot analysis of *Star* mRNAs was performed using the nonradioactive, North2South Hybridization kit (Pierce, Rockford, IL). Blots were hybridized with biotin-labeled mouse *Star* cDNA probes and exposed to x-ray film (Marsh Bio Products) for different lengths of time. After stripping the membrane, the biotin-labeled *18S* (*Rn18s*) cDNA probes were hybridized and analyzed for use as a loading control.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA as described [11]. Briefly, 2 μg of total RNA was used for the RT reaction using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 37°C for 1.5 h. PCR reactions were performed using 2 μl of RT reaction products with the addition of the PCR master mix containing dNTP, *Taq* DNA polymerase (Promega), [α-^{32}P] dCTP (2 μCi of 3000 Ci/mmol), and specific oligonucleotide primers for *Star* (forward, 5′-TTCAAGCTGTGTGCTGGAAGCTCCTA-3′ [16–41 base pairs]; reverse, 5′-TTAACACTGGGCCTCAGAGGCAGGGC-3′ [830–855 base pairs]). The primers that served as a reaction control were for ribosomal protein L19 mRNA (*Rpl19*) (forward, 5′-GAAATCGCCA-ATGCCAACTC-3′; reverse, 5′-TCTTAGACCTGCGAGCCTCA-3′). The expected sizes of *Star* and *Rpl19* PCR products were 840 and 400 base pairs, respectively. Optimum conditions made during the exponential phase of the amplification procedure were established to assure measurements.

Dual Luciferase Reporter Assay

Various segments of the mouse *Star* promoter (−966, −254, −151, and −110) were previously cloned into the pGL3-Basic vector (Promega), thereby controlling expression of a reporter gene encoding firefly luciferase. MA-10 cells were plated in 12-well plates (1×10^5 cells/well) and transfected on the next day with 0.4 μg of individual constructs using Effectene reagent (Qiagen, Valencia, CA) following previously optimized conditions in accordance with the manufacturer's instructions. To correct for variations in transfection efficiency, 10 ng of pRL-SV40 encoding *Renilla* luciferase was cotransfected as a normalization control. On the second day of transfection, the medium was changed. On the third day,

the cells were thoroughly washed and treated with various stimuli for 6 h in serum-free Waymouth media. To determine the level of luciferase activity, cells were washed and lysed with 100 μl of passive lysis buffer provided with the Dual-Luciferase Reporter assay system (Promega) using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) following the manufacturer's instructions. Firefly luciferase activity was measured by addition of 50 μl of Luciferase Assay Reagent II to 10 μl of cell lysates. Stop & Glo Reagent (50 μl) was added to quench firefly luciferase activity and measure *Renilla* luciferase activity. Normalized relative luciferase units or activity was calculated as the ratio of firefly luciferase:*Renilla* luciferase activity obtained from the same cell lysates.

Radioimmunoassay

Steroid production was assessed with radioimmunoassay. Cells were cultured in duplicate, thoroughly washed, and treated in serum-free culture media for each treatment. The medium was recovered and progesterone or testosterone concentration was measured as described earlier [31]. In most cases, steroid levels were normalized using total cellular protein or cell number and expressed as picograms per micrograms (pg/μg) of protein or nanograms per cell number.

Statistics

Each experiment was repeated at least three times using triplicate samples within each experiment. The data are expressed as the mean ± SEM. Statistical analyses were performed using the Student *t*-test (two-sample assuming unequal variances), and *P* values less than 0.05 were accepted as significantly different [15].

RESULTS

Constitutive StAR and Steroid Production in R2C Cells Requires PLC and PKC Activity

In an effort to determine whether constitutive steroidogenesis in R2C cells is the result of increased activity of the PKC pathway, the possibility that PLC, the enzyme that produces the DAG activator of PKC, was constitutively activated in R2C cells was examined. U73122, a specific inhibitor of PLC, inhibited R2C cell steroid production by 50% (Fig. 1A). To determine whether this observation was the result of nonspecific toxicity of U73122, cells were incubated with 22(R)-hydroxycholesterol (22R-OHC) in the presence of U73122. The hydrophilic 22R-OHC can readily diffuse unassisted across the mitochondrial membranes and reach the $P450_{scc}$ enzyme system where it is converted to pregnenolone and then to progesterone by 3β-hydroxysteroid dehydrogenase (HSD3B). U73122 had no effect on progesterone synthesis when 22R-OHC was used as a substrate; therefore, its inhibitory effects were located upstream of $P450_{scc}$. In parallel with the observed decrease in steroid production, RT-PCR was used to demonstrate a concomitant 40% reduction in *Star* mRNA levels in R2C cells treated with U73122 (Fig. 1B), while the levels of *Rpl19* were not changed.

To determine whether PKC is also constitutively active in the R2C cell line, cells were treated with two PKC inhibitors, GF-109203X (GFX) and Gö6983. Following 6 h of treatment with either inhibitor, the high basal levels of steroid normally observed in R2C cells were reduced by 70% (Fig. 2A). Furthermore, whereas R2C cells cultured in the presence or absence of either PMA (10 nM) or $(Bu)_2$cAMP (1 mM) for 6 h showed little change in steroid output, in the presence of either inhibitor, steroid levels decreased by 70% in PMA-treated cells and by 40%–45% in $(Bu)_2$cAMP-treated cells. Using the same treatments followed by RT-PCR, transcriptional levels of *Star* in R2C cells decreased significantly following PKC inhibition even in the presence of PMA or $(Bu)_2$cAMP (Fig. 2B).

To understand the mechanism through which PKC affects *Star* gene expression, we examined the expression patterns and phosphorylation states of CREB using the same treatments described above. Results from these experiments revealed that R2C cells had constitutively increased levels of P-CREB under basal conditions as shown in Figure 2C, and stimulation of the PKA pathway resulted in a slight increase in P-CREB levels. The levels of P-CREB in basal and PMA-stimulated R2C cells were significantly decreased by the PKC inhibitors (70%–80% reduction). The same PKC inhibitors were not as effective in reducing the levels of $(Bu)_2$cAMP-induced P-CREB (45% reduction). When stained for tot-CREB, no changes were observed following any of the treatments. The basal level of P-CREB in R2C cells was assessed following H89 treatment and was found to be significantly decreased (data not shown). These results indicate that constitutive steroid and StAR produc-

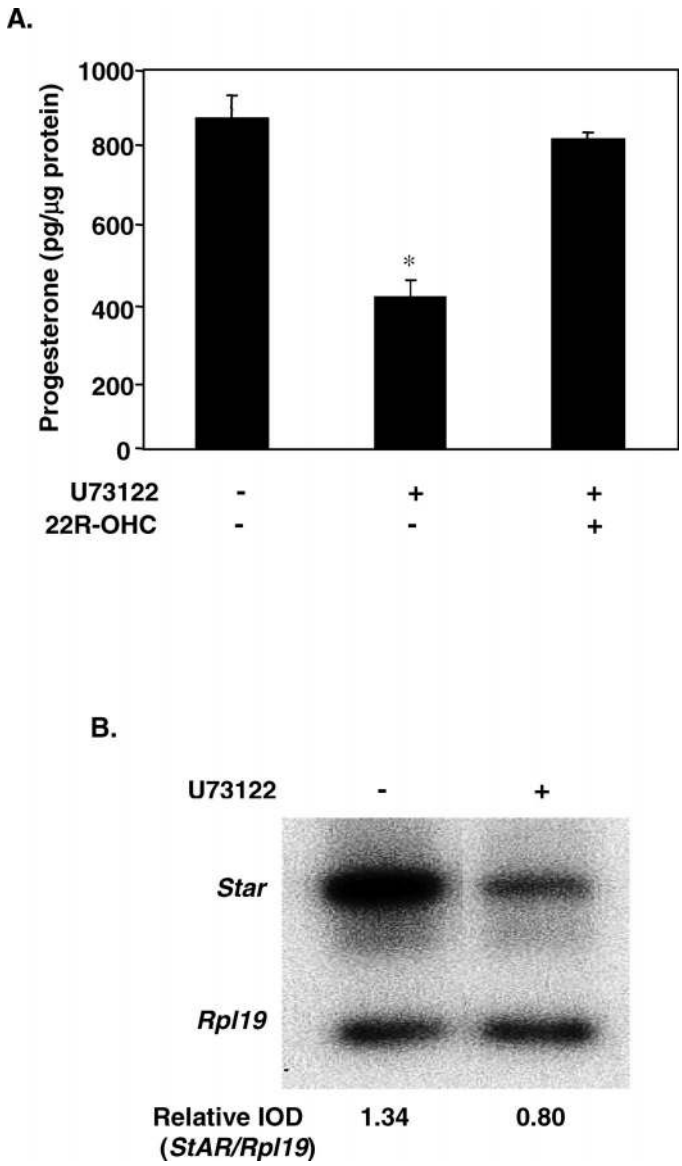

FIG. 1. Involvement of constitutive activation of PLC in steroid and StAR synthesis in R2C cells. **A**) Serum-free Waymouth media was added and treated with vehicle (dimethylsulfoxide, controls) and the PLC inhibitor (U73122, 100 μM) without or with 22(R)-hydroxycholesterol (25 μM, a positive control), for 6 h and tested for progesterone production. Statistical analysis was performed as described in *Materials and Methods* (*$P < 0.05$). Small bars in the graph indicate SEM. **B**) RT-PCR analysis for *Star* and *Rpl19* (reaction control).

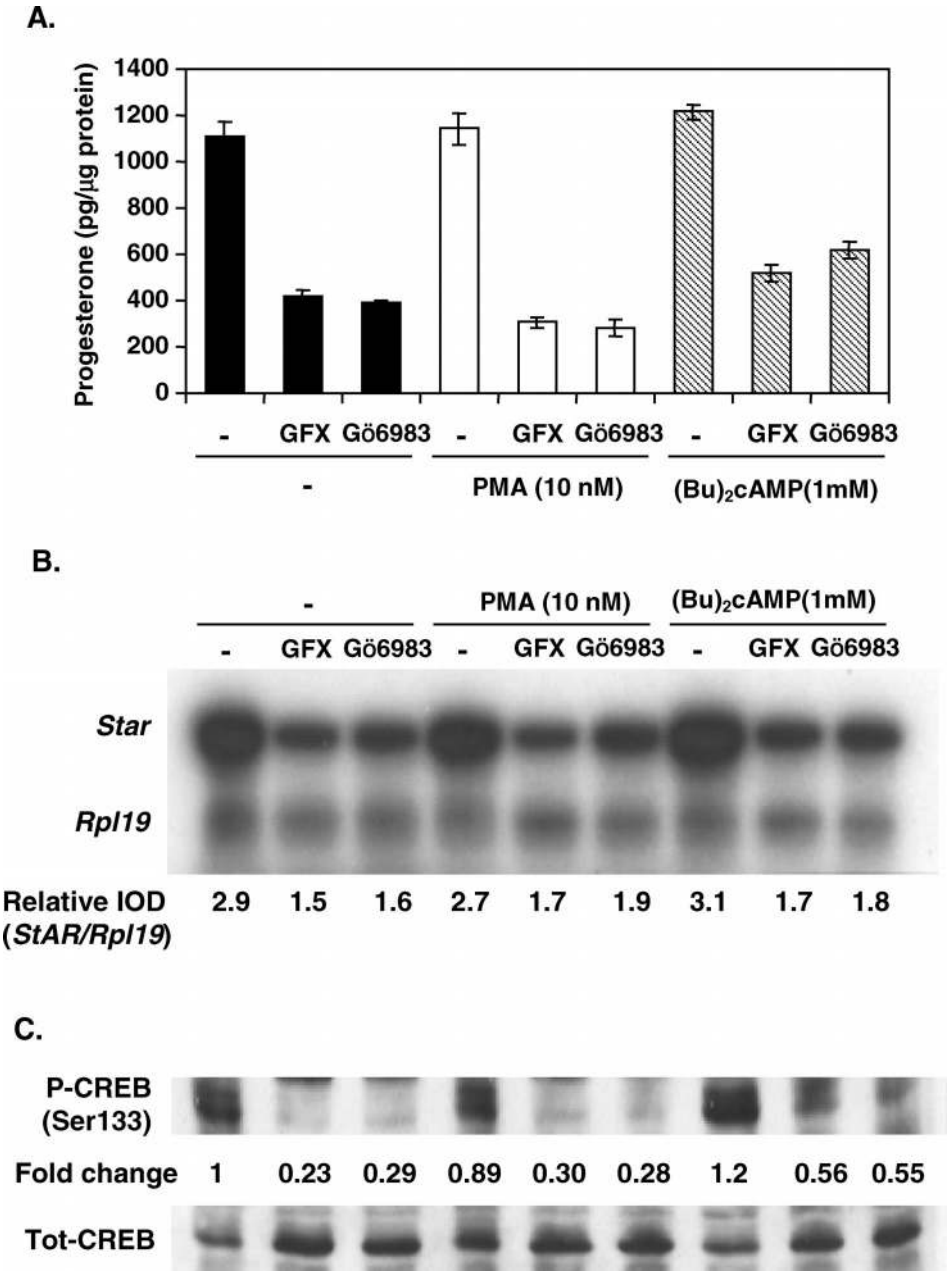

FIG. 2. Involvement of constitutive PKC activation in steroid and StAR expression in R2C cells. R2C cells were treated with 25 μM of either GFX or Gö6983 for 15 min followed by 10 nM PMA or 1 mM $(Bu)_2$cAMP for 6 h. The media were analyzed for steroid production (**A**), and the cells were used for RT-PCR (**B**) to determine the transcriptional levels of *Star* and *Rpl19* (loading control) genes and Western blot analyses (**C**) to observe the levels of phosphorylated CREB (P-CREB) and total CREB (tot-CREB). Small bars in the graph indicate SEM. Figures are representative of three independent experiments with similar results.

tion in R2C cells are, in part, due to the constitutive phosphorylation of CREB that occurs as a result of the intrinsically activated PLC/PKC or PKA pathways (or both).

PKC Stimulates StAR Expression but Not Steroid Production in MA-10 Cells

To further explore the role of the PKC pathway in steroidogenesis, MA-10 cells were stimulated with $(Bu)_2$cAMP or PMA. MA-10 cells expressed extremely low basal levels of StAR and P-CREB, but stimulation with either compound strongly increased the levels of these proteins (Fig. 3A, top and middle panels). Concomitant with these changes, steroid production increased only in cells stimulated with $(Bu)_2$cAMP (Fig. 3B). When PKA or PKC activity was blocked with H89 or GFX, respectively, the levels of StAR and P-CREB were strongly attenuated as was $(Bu)_2$cAMP-stimulated progesterone synthesis.

PMA Stimulation Requires Submaximal Doses of $(Bu)_2$cAMP to Synthesize Steroids in MA-10 Cells

To better understand the roles of PKA and PKC, the levels of StAR protein and steroid synthesis induced with different doses of PMA were compared with those obtained with different doses of $(Bu)_2$cAMP (Fig. 4A) in MA-10 cells. Concentrations of $(Bu)_2$cAMP below 0.1 mM did not appreciably affect StAR protein and steroid synthesis. Treatment with 10 and 50 nM of PMA stimulated StAR protein production to levels comparable to those observed with 0.5 and 1.0 mM $(Bu)_2$cAMP, respectively, but again, without significantly affecting steroid production. Thus, stimulation of PKC activity using PMA can induce expression of StAR protein, but this induction is insufficient to drive steroid biosynthesis.

Because of these results, MA-10 cells were manipulated in an attempt to mimic the PKC and PKA activities [11]

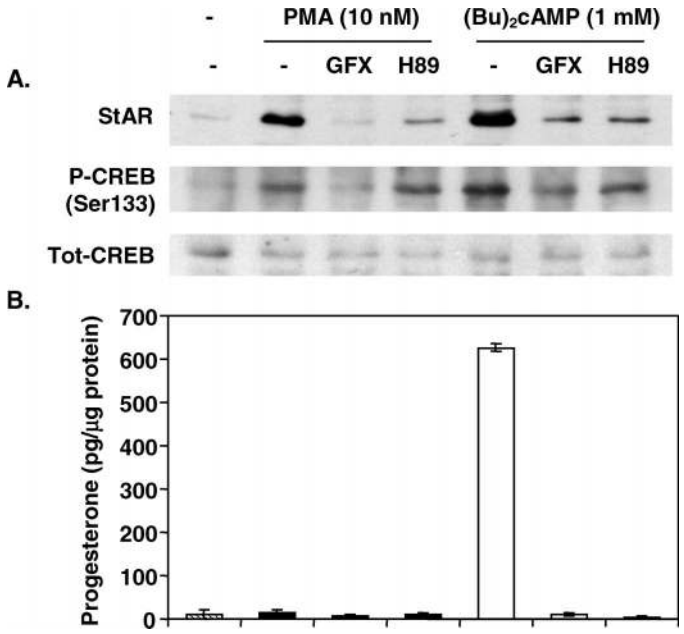

FIG. 3. PMA induces StAR but not steroid production in MA-10 cells. Cells were incubated in serum-free Waymouth media, treated with the PKC- and PKA-specific inhibitors GFX (25 μM) and H89 (25 μM), respectively, for 15 min, followed by PMA (10 nM) or $(Bu)_2$cAMP (1 mM) stimulation for 6 h. To determine the levels of P-CREB, cells were stimulated for 1 h. Equal amounts of proteins were analyzed for StAR, P-CREB, and tot-CREB by Western blot analysis (**A**). Medium was analyzed for progesterone production (**B**). Small bars in the graph indicate SEM.

found in R2C cells. MA-10 cells were stimulated with 10 nM PMA in the presence of increasing but low doses of $(Bu)_2$cAMP (0.005–0.1 mM, which by themselves did not affect StAR protein and steroid synthesis appreciably). As shown in Figure 4B, compared to the controls, a significant increase in StAR protein occurred following PMA stimulation with little change in steroid biosynthesis. In the presence of 0.005 and 0.01 mM $(Bu)_2$cAMP, PMA-treated MA-10 cells showed no further induction of StAR protein and a small and gradual increase in steroid production. However, in the presence of 0.05 and 0.1 mM $(Bu)_2$cAMP (still submaximal concentrations), StAR protein levels were further increased compared with that of PMA-only treatment, and dramatic increases in steroids were observed in the presence of PMA. Under these conditions, steroid levels were comparable to those measured after stimulation with 0.5 mM $(Bu)_2$cAMP only. These results indicate that the production of steroids in response to PMA requires a minimal PKA activity, which, by itself is insufficient to elicit steroid synthesis or StAR expression.

PMA Stimulates Star Gene Transcription

To determine whether PMA affected the abundance of *Star* mRNA, experiments on *Star* promoter activity and Northern blot analyses were performed. Different mouse *Star* promoter-luciferase constructs were transfected into MA-10 cells and promoter activity was assessed using a dual luciferase reporter assay. PMA alone slightly increased *Star* promoter activity compared with that of control groups (Fig. 5A). The activity of the *Star* promoter was also stimulated when both 10 nM PMA and 0.05 mM $(Bu)_2$cAMP were used, but the activities were less than those of 1 mM $(Bu)_2$cAMP. It was also noted that PMA-responsive elements were likely present within the −151/−110 region of

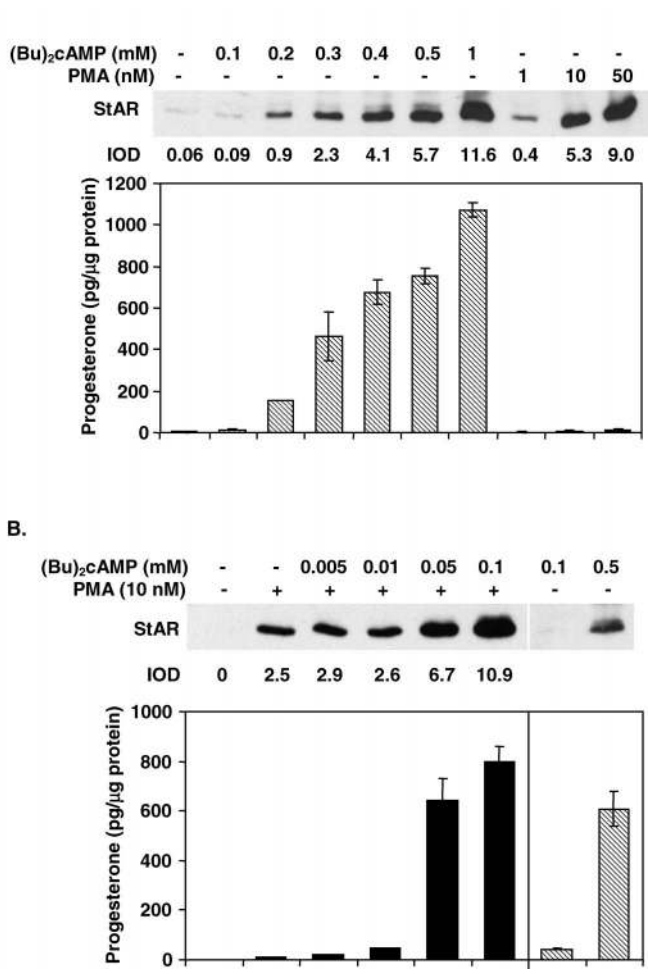

FIG. 4. Comparison of StAR expression and steroid production in MA-10 cells stimulated with $(Bu)_2$cAMP, PMA, or costimulated with both. **A**) Cells were stimulated with different concentrations of PMA or $(Bu)_2$cAMP for 6 h and analyzed for StAR protein expression and progesterone production. **B**) Cells were costimulated with both PMA (10 nM) and increasing doses of $(Bu)_2$cAMP for 6 h to determine the levels of StAR and steroids. Small bars in the graph indicate SEM.

the *Star* promoter. Also, the activity of the −966/−1 promoter construct was always lower than those of the −254/−1 or −151/−1 constructs, a pattern previously observed [32, 33]. This unexplained observation could indicate the presence of an inhibitory element between −966 and −254 in the *Star* promoter. The result of Figure 5A paralleled those obtained with Northern blot analysis (Fig. 5B). While PMA alone increased *Star* mRNA, 0.05 mM $(Bu)_2$cAMP alone was not able to induce *Star* mRNA expression. Simultaneous treatment with PMA plus 0.05 mM $(Bu)_2$cAMP increased *Star* mRNA levels beyond those observed with PMA alone or with 1 mM $(Bu)_2$cAMP. Even 1 mM $(Bu)_2$cAMP-induced *Star* mRNA levels were further increased by costimulation with PMA.

In the Presence of PMA, a Submaximal Dose of $(Bu)_2$cAMP Is Required for the Phosphorylation of StAR in MA-10 Cells

It was consistently observed that while PMA can induce StAR expression, it requires the addition of $(Bu)_2$cAMP to induce a steroidogenic response. Because our previous data suggested that PMA potentiates both *Star* gene transcription and translation, we hypothesized that the lack of steroido-

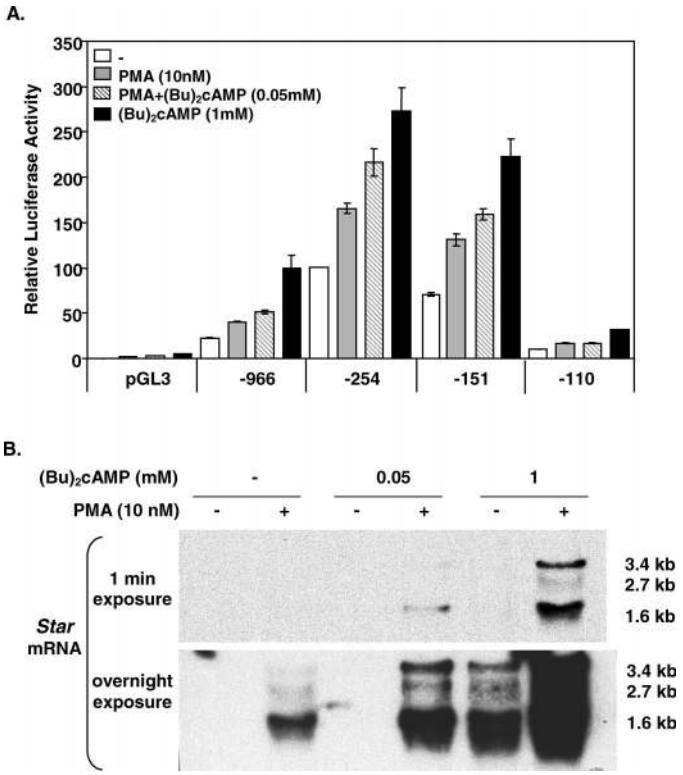

FIG. 5. Transcriptional regulation of the *Star* gene by PMA in MA-10 cells. **A**) Different lengths of the mouse *Star* promoters (−966, −254, −151, and −110) containing a luciferase reporter gene transfected into MA-10 cells and treated with 10 nM PMA, PMA + 0.05 mM $(Bu)_2$cAMP, and 1 mM $(Bu)_2$cAMP for 6 h. The promoter activity was determined as described in *Materials and Methods*. Small bars in the graph indicate SEM. **B**) Northern blot analysis for *Star* mRNA was performed on RNA isolated from MA-10 cells treated with 0.05 mM $(Bu)_2$cAMP and 1 mM $(Bu)_2$cAMP in the absence and presence of PMA (10 nM) for 6 h. Upper and lower panels show different exposure times of the same blot to examine the *StAR* transcripts.

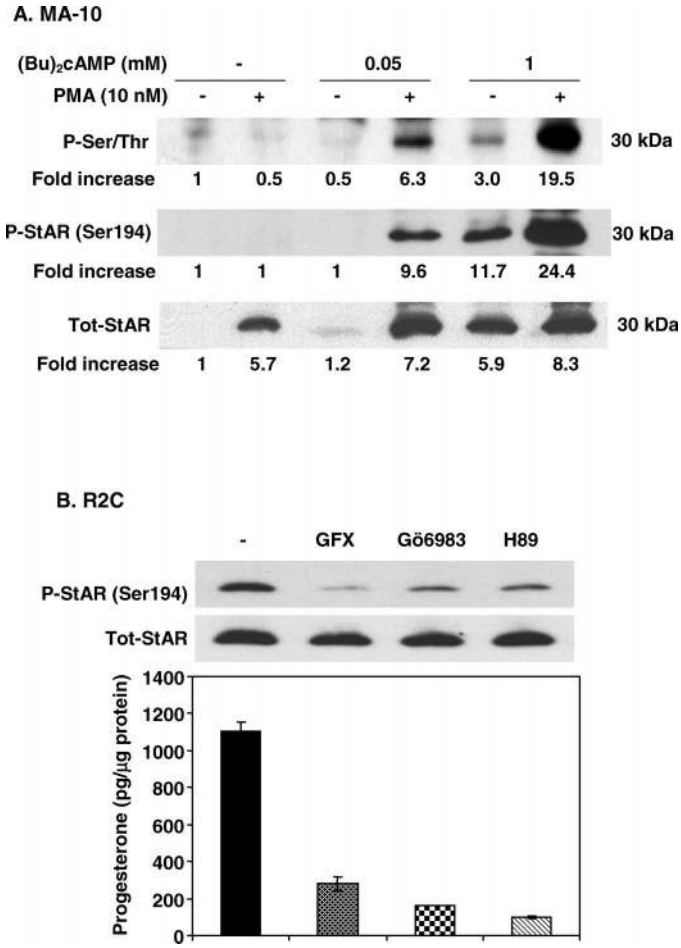

FIG. 6. Role of PKC and PKA (a submaximal dose of $(Bu)_2$cAMP) in MA-10 and R2C cells. **A**) MA-10 cells were stimulated using 0, 0.05, and 1 mM $(Bu)_2$cAMP in the presence or absence of 10 nM PMA for 6 h. Western blot analyses were performed using a specific antibody to detect tot-StAR and two separate antibodies to detect P-StAR. The top panel shows the 30-kDa protein bands detected by a phospho-Ser/Thr (p-Ser/Thr)-specific PKA substrate antibody purchased from Cell Signaling. The middle panel represents the phosphorylated StAR using a phospho-Ser194-specific antibody (p-Ser194) of StAR protein. The bottom panel shows the tot-StAR using an antibody to tot-StAR. **B**) R2C cells were treated with GFX (25 μM) and Gö6983 (25 μM) to inhibit the PKC pathway and H89 (25 μM) to inhibit the PKA pathway and followed by 6 h of incubation. The protein levels of P-StAR and tot-StAR were analyzed by Western blot, and the production of progesterone was tested by radioimmunoassay. Small bars in the graph indicate SEM.

genesis in PMA-treated MA-10 cells is due to its inability to activate StAR activity at a posttranslational level. More specifically, in the presence of PMA, a submaximal dose of $(Bu)_2$cAMP was required to induce PKA activity required for the phosphorylation and full activation of the StAR protein. To discriminate between tot-StAR and P-StAR, two different antibodies were used to recognize P-StAR protein; one recognizes the phospho-Ser/Thr PKA substrate and the other detects phosphorylated Ser194 on StAR. Stimulation of MA-10 cells with PMA alone results in abundant synthesis of nonphosphorylated tot-StAR (Fig. 6A). Importantly, the submaximal dose of $(Bu)_2$cAMP (0.05 mM), when used alone, was unable to induce either tot-StAR or P-StAR. In contrast, treatment of MA-10 cells with both PMA and submaximal $(Bu)_2$cAMP generated high levels of tot-StAR protein, and the protein was phosphorylated. As expected, cells stimulated with 1 mM of $(Bu)_2$cAMP showed high levels of tot-StAR and P-StAR, and the levels could be further increased when cells were stimulated simultaneously with PMA. The results were similar using both antibodies to recognize P-StAR.

Constitutive Activation of PKC and PKA Pathways Are Responsible for the R2C Cells' Steroidogenic Phenotypes

R2C cells were treated with inhibitors of the PKC (GFX and Gö6983) and PKA (H89) pathways to determine whether both pathways are critical for constitutive steroid production and whether P-StAR and tot-StAR are significantly altered by these inhibitors. As demonstrated in Figure 6B, P-StAR was constitutively present under basal conditions in R2C cells. Inhibition of both pathways resulted in significant decreases in the levels of P-StAR as well as corresponding decreases in steroid production. Tot-StAR levels were slightly decreased in R2C cells in response to the inhibitors, indicating that StAR protein was not yet significantly degraded within 6 h of treatment. These studies once again demonstrated the importance of the phosphorylated form of StAR in steroid synthesis.

PMA Requires a Submaximal Dose of $(Bu)_2$cAMP to Induce Steroid Production in mLTC-1 Cells

The combined effects of PMA and $(Bu)_2$cAMP on steroid production were also tested in another cell line. The

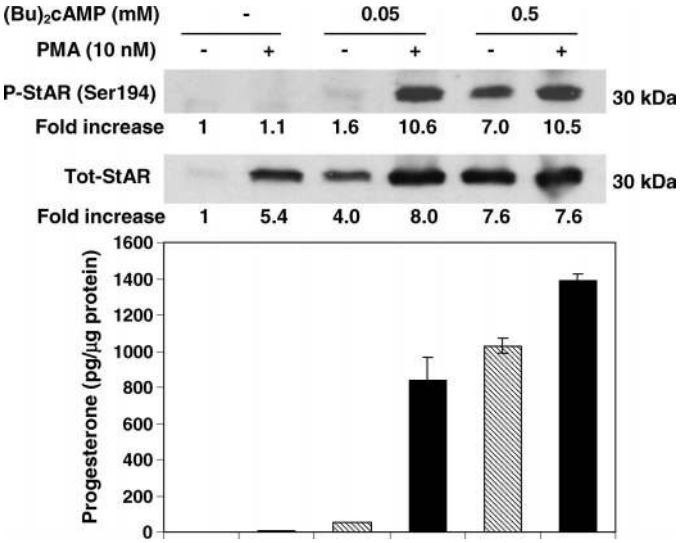

FIG. 7. Regulation of StAR phosphorylation and steroid production in mLTC-1 cells using PMA and a low dose of $(Bu)_2$cAMP. Cells were stimulated similar to those treated in Figure 6. Western blot analysis was performed using cell lysates to determine the levels of tot-StAR and phosphorylated-Ser194 on StAR (P-StAR) using each specific antibody. The media were recovered and analyzed for progesterone production. Small bars in the graph indicate SEM. The data are representative of three separate experiments with similar results.

mLTC-1, mouse Leydig tumor cell line, was treated with or without PMA in the presence or absence of either 0.05 or 0.5 mM $(Bu)_2$cAMP. As shown in Figure 7, while PMA alone induced the expression of unphosphorylated StAR, no steroid production was observed. Treatment of mLTC-1 cells with 0.05 mM $(Bu)_2$cAMP alone was able to induce a slight increase in unphosphorylated StAR expression but only slight increases in P-StAR and steroid production. However, PMA plus 0.05 mM $(Bu)_2$cAMP resulted in high levels of P-StAR and steroids. Using the same experimental design the levels of P-CREB were also determined by Western blot analysis and showed that P-CREB was also increased by PMA stimulation (data not shown). In summary, both the PKA and PKC pathways appear to act in concert to regulate StAR protein expression and steroidogenesis in Leydig tumor cell lines. A general schematic summarizing how PMA and a submaximal dose of $(Bu)_2$cAMP appear to work together in MA-10 cells is depicted in Figure 8.

PMA and hCG Synergistically Increase Progesterone Production in MA-10 Cells

Additional studies attempted to determine whether the PMA-induced PKC pathway has a synergistic effect on StAR expression and steroid production in the presence of trophic hormones. As observed earlier, PMA stimulated strong expression of unphosphorylated StAR in MA-10 cells without a comparable increase in steroid production (Fig. 9). Stimulation with hCG induced both total and P-StAR production within 6 h and resulted in a corresponding increase in progesterone synthesis. Addition of hCG in combination with PMA resulted in increases in tot-StAR and P-StAR levels (2.4-fold and 4-fold, respectively), and an increase in progesterone of 2.6-fold over treatment with hCG only. This result demonstrates that PMA significantly enhances trophic hormone-stimulated StAR and steroid production.

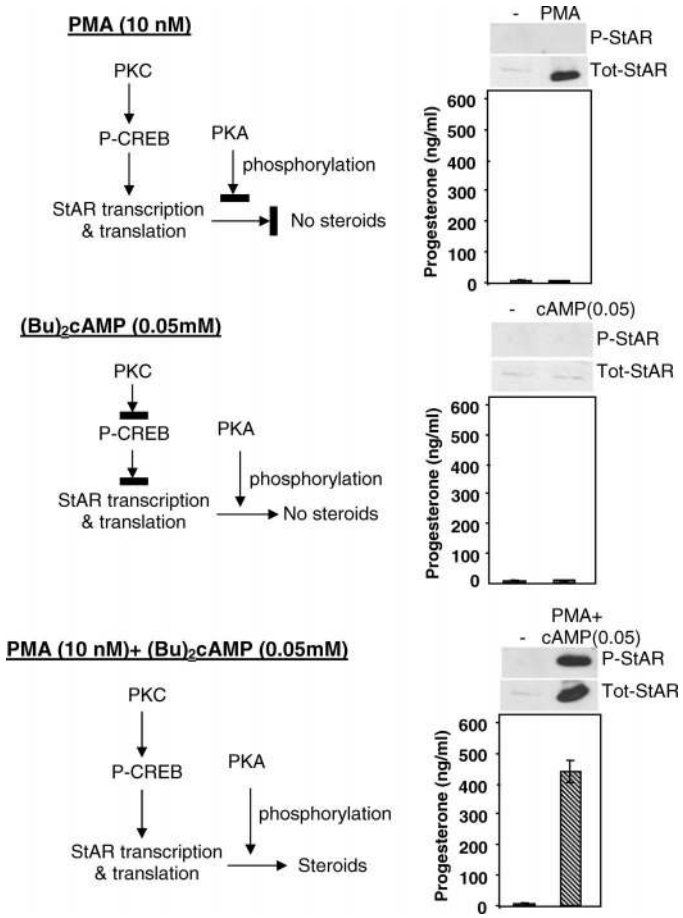

FIG. 8. Diagram of the interaction of PMA-induced PKC pathways with and without a submaximal stimulation of the PKA pathway by $(Bu)_2$cAMP in the regulation of StAR expression and steroidogenesis. Arrows with black bars indicate pathways that are not activated; arrows without black bars indicate activated pathways. Panels on the right correspond to the levels of P-StAR, tot-StAR, and progesterone obtained in each experimental paradigm using MA-10 cells.

Effects of PMA on Steroidogenic Enzymes in Isolated Immature Rat Leydig Cells

In Leydig tumor cells, 10 nM PMA was sufficient to synthesize *Star* mRNA and StAR protein, but only in the presence of a low dose of $(Bu)_2$cAMP or hCG was StAR phosphorylated and steroids synthesized. To investigate this phenomenon in primary cultures, Leydig cells were freshly isolated from 10-day-old rats. The cells were treated with PMA and two different doses of $(Bu)_2$cAMP in a manner identical to the treatment of MA-10 and mLTC-1 cells with the exception that the incubation times were both 6 and 24 h. Primary cultures had very low levels of P-StAR and tot-StAR under basal conditions at both 6 (Fig. 10A) and 24 h (Fig. 10B). In primary cultures of rat Leydig cells, PMA was able to induce tot-StAR but not P-StAR, consequently resulting in no changes in steroid levels (Fig. 11). This situation was similar at both 6 (Fig. 11A) and 24 h (Fig. 11B). In contrast, addition of 0.05 mM $(Bu)_2$cAMP to PMA-treated cells was able to induce the phosphorylation of StAR (Fig. 10), as observed in the Leydig tumor cell lines, and a corresponding increase in progesterone at both 6 and 24 h (Fig. 11, A and B). As anticipated, high doses of $(Bu)_2$cAMP resulted in dramatic increases in both tot-StAR and P-StAR, which corresponded to increases in progesterone and testosterone. In these experiments we ob-

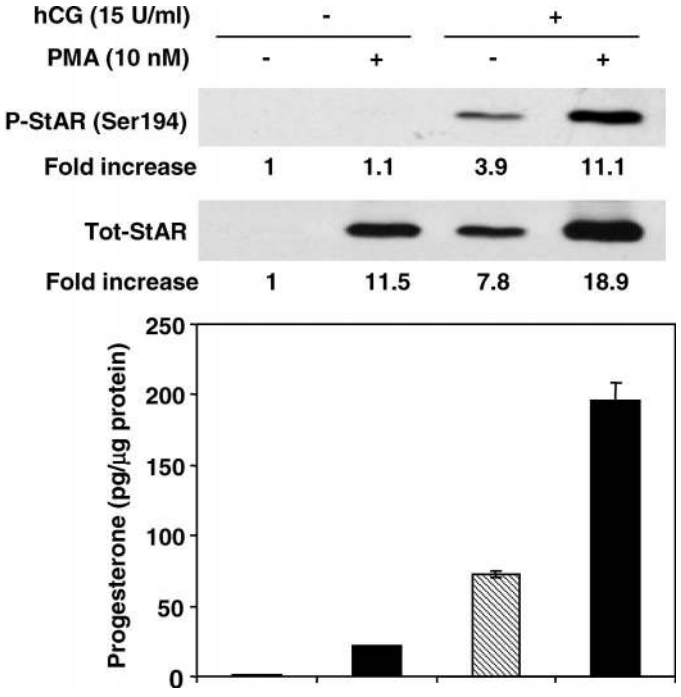

FIG. 9. PMA synergistically increases hCG-induced P-StAR and progesterone production in MA-10 cells. Cells were treated with or without hCG in the absence or presence of 10 nM PMA for 6 h. Proteins were analyzed for tot-StAR and P-StAR by Western blot analysis, and the media were recovered and analyzed for progesterone synthesis. Small bars in the graph indicate SEM. The data are representative of two separate experiments with identical results.

served that costimulation of primary Leydig cells with 0.5 mM $(Bu)_2$cAMP and PMA resulted in high levels of progesterone but significantly decreased levels of testosterone. Similar patterns were also observed in response to LH- or hCG-and-PMA-treated cells and are consistent with earlier observations that PMA can inhibit CYP17 enzyme activity in rat and mouse Leydig cells [34, 35]. In an attempt to determine whether PMA inhibited the expression of CYP17, Western blot analyses were performed. When the levels of CYP17 were normalized to actin, there was no significant difference in its expression at either 6 or 24 h (Fig. 12) in the absence or presence of PMA. These results

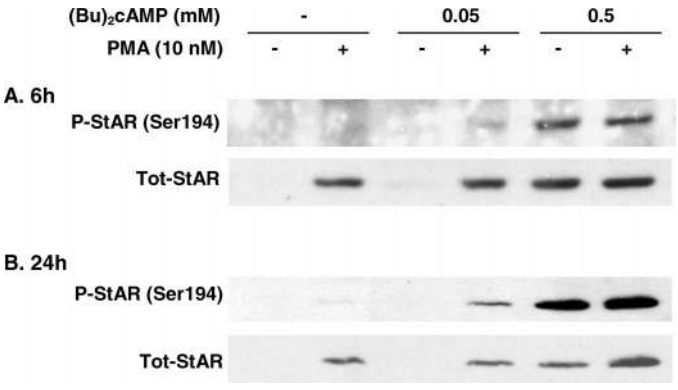

FIG. 10. Differential effects of PMA on StAR expression in freshly isolated rat Leydig cells. Immature rat Leydig cells were treated with low (0.05 mM) and high (0.5 mM) doses of $(Bu)_2$cAMP, respectively, in the absence and presence of 10 nM PMA for 6 (**A**) and 24 h (**B**). Total cell lysates were analyzed for P-StAR and tot-StAR by Western blotting. The data are representative of four independent experiments with similar results.

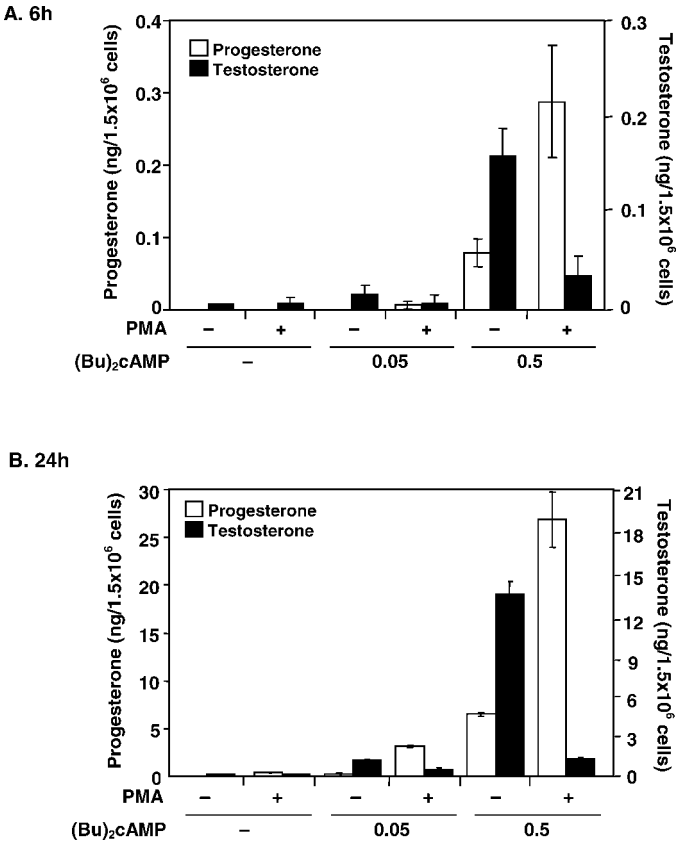

FIG. 11. Effects of PMA on steroidogenesis in freshly isolated rat Leydig cells. Immature rat Leydig cells were treated with low (0.05 mM) and high (0.5 mM) doses of $(Bu)_2$cAMP, respectively, in the absence and presence of 10 nM PMA for 6 (**A**) and 24 h (**B**). Medium was analyzed for progesterone and testosterone by radioimmunoassay. Small bars in the graph indicate SEM. The data are representative of four independent experiments with similar patterns.

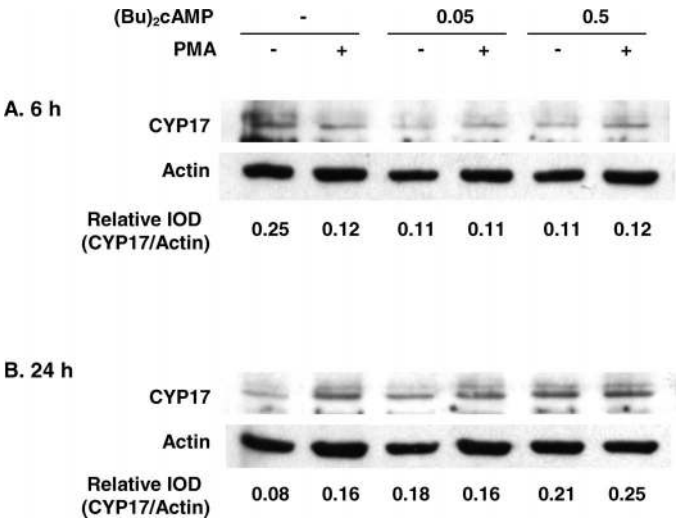

FIG. 12. Effects of PMA on CYP17 expression in freshly isolated rat Leydig cells. Immature rat Leydig cells were treated with low (0.05 mM) and high (0.5 mM) doses of $(Bu)_2$cAMP, respectively, in the absence and presence of 10 nM PMA for 6 (**A**) and 24 h (**B**). Total cell lysates were analyzed for CYP17 by Western blotting. Levels of actin were also determined as a loading control. Relative integrated optical density of the ratio using CYP17:actin was represented on the bottom of each blot. The figures are representative of two different experiments using two different antibodies for CYP17 (anti-human CYP17 and anti-porcine CYP17) with identical results.

demonstrated that PMA inhibited testosterone production by affecting the activity of CYP17 and not its expression. The manner in which PMA inhibits CYP17 activity remains unknown. Most importantly, however, these studies show that, as seen in Leydig tumor cells, PMA stimulation of primary cultures of Leydig cells can induce unphosphorylated StAR protein expression, but a low dose of cAMP is required to phosphorylate and activate StAR and stimulate steroid synthesis.

DISCUSSION

In the present study, we continued investigations on the factors that render R2C cells constitutively steroidogenic. The importance of the PKA pathway was suggested when R2C cells were treated with H89, an inhibitor of PKA, and decreases in both *Star* mRNA levels and steroid production were observed [11]. Given the complex phenotype exhibited by R2C cells described in earlier studies [4, 7–12, 15], we perceived that in addition to PKA, other regulatory mechanisms were likely involved in steroidogenesis. In this study the functional impact of the PKC pathway in R2C cells was examined by testing both PKC itself and PLC, the enzyme that generates the PKC activator, DAG. Inhibition of either PLC or PKC in R2C cells resulted in significant decreases in *Star* mRNA and steroid production without any adverse effects on the activity of the $P450_{scc}$ enzyme. The basal levels of P-CREB in R2C cells were, interestingly, also decreased by PKC inhibition, indicating that phosphorylation of CREB may result from activation of both the PKA and the PKC pathways. Studies showed that PMA, like $(Bu)_2$cAMP, was able to increase P-CREB levels in MA-10 cells and suggested that upon activation, both the PKA and PKC pathways converged at CREB phosphorylation. The mouse *Star* promoter has been well characterized and shown to contain three separate cAMP-responsive element (CRE) half sites [36]. The regulation of those CRE half sites by the ATF/CREB/CREM family of transcription factors has been thoroughly studied [36–39]. The phosphorylation of CREB by PKA and PKC pathways was first reported in 1991 by Sakurai et al. [40], who used an in vitro system to test the specific phosphorylation sites of recombinant human CREB-1. Their results illustrated that PKA phosphorylated Ser62, and PKC phosphorylated Ser340 and Ser367 in CREB-1. Later, Xie and Rothstein showed that PKC phosphorylates CREB on Ser133 in B cells [41]. Recently, it was demonstrated that phosphorylation of CREB at Ser133 in PC12 cells was increased within 15 min in response to forskolin, PMA, epidermal growth factor, and nerve growth factor [42]. Consistent with these observations, we conclude that R2C and MA-10 cells express CREB that is phosphorylated on Ser133 by PKA (stimulated by $(Bu)_2$cAMP) and PKC (stimulated by PMA), because the antibody used in the present study was specific for phospho-Ser133 in CREB.

PMA itself, although capable of increasing StAR protein levels, was not able to stimulate steroid production in MA-10 cells, confirming earlier observations that described PMA as a weak stimulator of steroidogenesis in rat Leydig cells and Y-1 adrenocortical cells [43, 44]. Similar results from other studies also suggested that the inability of PMA to induce a comparable increase in steroid production to that of StAR protein was due to a requirement for a PKA-dependent posttranslational modification [45, 46]. In accordance with these observations we demonstrated that PMA treatment alone could not increase steroid production significantly, because the PMA-induced StAR protein was not activated by phosphorylation.

Phosphorylation of the StAR protein requires the action of the PKA pathway as demonstrated in an earlier observation that showed that PMA failed to generate phosphoprotein species of StAR that are present in hCG-, $(Bu)_2$cAMP-, or forskolin-stimulated MA-10 cells [47]. Studies evaluating the role of phosphorylation in StAR activity demonstrated that the phosphorylation of StAR at Ser194 was functionally more important than the phosphorylation at other potential PKA sites in the StAR protein [48]. A 50% decrease in StAR activity occurred when Ser194 was mutated to alanine. Loss of this site in heterologous cell systems reduced StAR activity by 70%–80% [49, 50]. In agreement with these observations, results of the present study demonstrated that the PMA-stimulated PKC pathway increased StAR protein levels, but the protein was not phosphorylated, and consequently, was incapable of mediating steroid synthesis. However, when PMA-treated MA-10 and mLTC-1 cells were exposed to submaximal doses of $(Bu)_2$cAMP, high levels of steroid production were obtained, presumably through PKA-mediated phosphorylation of the PMA-induced StAR protein. As seen in Figure 6B, under basal conditions, R2C cells contain high levels of P-StAR that were dramatically decreased following inhibition of either the PKC or PKA pathways. These results indicate that R2C cells can produce P-StAR basally because of the presence of constitutive levels of PLC/PKC and PKA activities not observed in unstimulated MA-10 cells. It was also noted that the sensitivity of MA-10 and mLTC-1 cells to submaximal doses of $(Bu)_2$cAMP was quite different. Mouse LTC-1 cells were more sensitive to low doses of $(Bu)_2$cAMP than were MA-10 cells (Fig. 7). The reasons for this difference are not presently clear.

A role for other factors in sensitizing Leydig cells to low levels of PKA stimulation has been previously described. For example, stimulation of Leydig cells with LH, hCG, or cAMP was linked to increased chloride ion conductance [51–53]. Removal of extracellular chloride ions significantly enhanced 0.1 mM $(Bu)_2$cAMP-stimulated StAR and steroid production in MA-10 cells [54]. The authors suggested that when PKA is submaximally activated, cAMP-dependent regulation of steroidogenesis is likely to be influenced by the synthesis and specific phosphorylation of proteins, perhaps one of them being StAR. Wang et al. [55] reported that submaximal doses of $(Bu)_2$cAMP were involved in steroid production and StAR expression following the production of arachidonic acid metabolites via one of its metabolizing pathways. In MA-10 cells, the addition of arachidonic acid alone had no effect on StAR protein levels and steroid production, whereas both parameters were significantly increased when submaximal doses of $(Bu)_2$cAMP were added simultaneously with arachidonic acid. The hypothesis that minimal levels of PKA are critical for steroidogenesis was also addressed by Wang et al. in another study [56]. MA-10 cells treated with a cyclooxygenase 2-specific inhibitor displayed no change in StAR protein or steroid synthesis, but significant increases in these two parameters were observed when a submaximal dose of $(Bu)_2$cAMP was added in conjunction with the inhibitor. The precise mechanism involved in this observation is not yet fully understood. However, the present study clearly demonstrates the absolute requirement for a low level of PKA activity for the phosphorylation of StAR protein in PMA-induced steroidogenesis, as illustrated in the diagram in Figure 8.

The involvement of the PKC pathway in Leydig cell steroidogenesis has been documented, and the mechanisms that regulate steroidogenesis and StAR expression via PKC action appear to be highly dependent on the cells and their tissue types, as well as the external stimuli influencing these cells. Several studies have shown that the PKC pathway is involved in the activation of steroid synthesis [31, 34, 57], while others have observed negative relationships [31, 34, 35, 58–60] or no effect [61] in various cell types. It appears that the concentration of PMA is one critical factor in controlling trophic hormone effects on steroid production. An earlier study showed that 100 ng/ml (162 nM) PMA treatment had an inhibitory effect on hCG-stimulated progesterone production in MA-10 cells. This observation was consistent with cAMP content, because 162 nM PMA had an inhibitory effect on the accumulation of cAMP induced by hCG [31]. However, in the presence of hCG, the 10 nM PMA used in the present study stimulated both P-StAR and progesterone production in MA-10 cells, indicating that in the presence of trophic hormone, low concentrations of PMA are stimulatory, whereas higher concentrations are inhibitory.

In the present study, stimulation of freshly isolated rat Leydig cells with 10 nM PMA and low doses of cAMP resulted in a decrease in testosterone levels (Fig. 10), whereas P-StAR levels were increased. However, levels of progesterone were greatly increased in these cells. These observations indicated that PMA had differential effects on the steroidogenic enzymes in the pathways that produce progesterone and testosterone. In an early study, Welsh et al. [34] treated immature rat testicular cells with 10 ng/ml (16.2 nM) PMA in the presence of hCG and showed that 17α-hydroxyprogesterone, androstenedione, and testosterone production were inhibited by 80%–90%, while progesterone was increased in these cells. Because testosterone production was also inhibited by PMA following the exogenous addition of progesterone and 17α-hydroxyprogesterone under both basal and stimulated conditions, the authors suggested that PMA inhibited CYP17 activity. A similar report demonstrated that PMA inhibited cAMP-induced *Cyp17* mRNA expression, the activity of CYP17, and testosterone production in mouse Leydig cells [35, 62]. Also, 10 nM PMA was shown to be stimulatory for expression of *Hsd3b* mRNA but inhibitory for expression of *Cyp17* mRNA in human H295R adrenocortical cells [62]. Thus, in our study, the decrease in testosterone and the concomitant increase in progesterone are undoubtedly due to an inhibition of CYP17 activity by PMA. This may mirror what occurs in ovarian theca cells after the LH surge in that they switch from producing androgens to progestagens even though stimulatory levels of LH remain in the ovary [63, 64]. When we measured the expression levels of CYP17 protein in immature rat Leydig cells, decreases in CYP17 protein were not observed (Fig. 12). Thus, as seen in previous studies [35, 62], we concluded that PMA results in an inhibition of CYP17 enzyme activity, but not its expression. The mechanism of this inhibition remains unknown.

The PMA receptor, PKC, was identified more than two decades ago [65–67]. Nikula and Huhtaniemi studied the effects of PKC activation by PMA on cAMP and testosterone levels using Percoll-purified rat Leydig cells [68]. Activation of PKC with PMA or OAG (1-oleoyl-2-acetyl-sn-glycerol) alone did not affect levels of intracellular cAMP; however, when used in conjunction with other treatments, each was shown to have profound effects on cAMP levels. They were inhibitory on hCG-stimulated cAMP production, but stimulatory with cholera toxin and forskolin. The authors concluded that PKC activation by LH might affect the coupling between the LH receptor and guanine nucleotide binding protein, alpha inhibiting (GNAI) or guanine nucleotide binding protein, alpha stimulating (GNAS). Another study supporting this idea resulted from the infection of Sf9 cells with LH receptor constructs. The data demonstrated the activation of adenylyl cyclase and PLC through interaction with GNAS and GNAI2, respectively, and resulted in increases in cAMP and IP_3 [69]. This result suggests that LH/hCG binding to the LH receptor leads to the activation of the PKA and PKC pathways through the independent targeting or interaction of guanine nucleotide binding protein subfamilies. Moreover, independent stimulation of both pathways may allow for significant levels of testosterone synthesis without the need for high levels of LH. This could be useful, for instance, in maintaining high basal steroid production in the testes between LH pulses.

In summary, the present study indicates that constitutive stimulation of the PLC/PKC pathway in the presence of low basal PKA activity is primarily responsible for the high level of basal steroidogenesis observed in R2C cells and that activation of the PKC pathway potentiates steroidogenesis in the presence of hCG or cAMP analogs in Leydig cells. Collectively, the data demonstrate that while activation of PKC by PMA can induce transcription and translation of *Star,* low doses of cAMP are required to phosphorylate and activate StAR protein before it can mediate cholesterol transfer and result in steroid synthesis. The increase in expression of StAR but lack of steroid synthesis following stimulation by PMA represents a novel observation in Leydig cells. The subsequent increase in steroids by submaximal levels of cAMP in PMA-treated cells demonstrates the absolute requirement for minimal PKA activity and phosphorylation of StAR in the regulation of steroidogenesis.

ACKNOWLEDGMENTS

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