Protein kinase G-mediated stimulation of basal Leydig cell steroidogenesis

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Andric SA, Janjic MM, Stojkov NJ, Kostic TS. Protein kinase G-mediated stimulation of basal Leydig cell steroidogenesis. Am J Physiol Endocrinol Metab 293: E1399–E1408, 2007.—The androgen-secreting Leydig cells produce cGMP, but the pathways responsible for generation and actions of this intracellular messenger have been incompletely characterized in these cells. Here, we show the presence of mRNA transcripts for the membrane-bound and soluble guanylyl cyclases (sGC), the cGMP-specific phosphodiesterase 5, and the cGMP-dependent protein kinase I (PKG I) and PKG II in purified rat Leydig cells from adult animals. Stimulation of both guanylyl cyclases and inhibition of phosphodiesterase 5 in vitro were accompanied by elevations in cGMP and androgen production, whereas inhibition of sGC and PKG led to a decrease in steroidogenesis. The stimulatory action of cGMP on steroidogenesis was preserved in cells with inhibited cAMP-dependent protein kinases. Experiments with exogenously added substrates revealed the dependence of cGMP-induced progesterone and androgen synthesis on cholesterol but not on 22-OH cholesterol, pregnenolone, progesterone, and Δ⁴-androstenedione.

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cGMP; steroidogenic acute regulatory protein

LEYDIG CELLS OF TESTES AND OTHER STEROIDOSUS TISSUES PRODUCE STEROIDS FROM A COMMON PRECURSOR, CHOLESTEROL. THIS IS A MULTIZYMATIC PROCESS, IN WHICH FREE CHOLESTEROL FROM INTRACELLULAR STORES IS TRANSFERRED TO THE OUTER AND THEN TO THE INNER MITOCHONDRIAL MEMBRANE. INSIDE MITOCHONDRIA, CHOLESTEROL IS CONVERTED TO PREGNENOLONE. THIS REACTION IS CATALYZED BY THE CYTOCHROME P-450 SIDE-CHAIN CLEAVAGE ENZYME, WHICH BELONGS TO THE CHOLESTEROL CLEAVAGE SYSTEM LOCATED ON THE MATRIX SIDE OF THE INNER MITOCHONDRIAL MEMBRANE. PREGNENOLONE IS FURTHER METABOLIZED TO PROGESTERONE BY MITOCHONDRIAL OR MICROSONAL 3β-HYDROXYSteroid dehydrogenases. IN SMOOTH ENDOPLASMIC RETICULUM OF LEYDIG CELLS, MATURATION OF PROGESTERONE TO ANDROSTENEDIONE IS CATALYZED BY 17α-HYDROXYLASE/C17–20 lyase (P450c17), WHEREAS FURTHER CONVERSION OF ANDROSTENEDIONE TO TESTOSTERONE DEPENDS ON 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY (35, 36, 43).

TRANSPORT OF CHOLESTEROL FROM INTRACELLULAR SOURCES INTO THE MITOCHONDRIA IS A RATE-LIMITING AND HORMONE-SENSITIVE STEP (35, 41, 42). LEYDIG CELLS PRODUCE ANDROGENS UNDER THE CONTROL OF LH OR ITS PLACENTAL CounterPART CHORIONIC GONADOTROPIN, AS WELL AS IN RESPONSE TO MULTIPLE INTRATESTICULAR FACTORS (13, 28, 38). LH/chorionic gonadotropin receptors belong to the Gs-coupled seven-transmembrane domain receptor family, whose activation leads to stimulation of adenyl cyclase (4). THE RESULTING ACCUMULATION IN cAMP INTRACELLULAR LEVELS AND THE CONCOMITANT ACTIVATION OF THE cAMP-DEPENDENT PROTEIN KINASE (PKA) LEADS TO PHOSPHORYLATION OF NUMEROUS PROTEINS, INCLUDING THE STEROIDOSUS ACUTE REGULATORY PROTEIN (StAR) (41, 46). StAR IS PREDOMINANTLY LOCALIZED IN STEROID-HORMONE-PRODUCING TISSUES AND CONSISTS OF A 37-kDa Precursor CONTAINING AN NH2-Terminal mitochondrial targeting sequence and several isolectric 30-kDa mature protein forms (14, 15, 44). STEROID PRODUCTION IN GONADAL AND ADRENAL CELLS REQUIRES BOTH DE NOVO SYNTHESIS AND PKA-DEPENDENT PHOSPHORYLATION OF StAR-37 PROTEIN (2). THE NEWLY SYNTHESIZED STAR IS FUNCTIONAL AND PLAYS A CRITICAL ROLE IN TRANSFER OF CHOLESTEROL FROM OUTER TO THE INNER MITOCHONDRIAL MEMBRANE, WHEREAS THE MITOCHONDRIAL IMPORT AND PROCESSING TO 30-kDa StAR PROTEIN TERMINATE THIS ACTION (3, 19, 25).

ALTHOUGH LEYDIG CELL STEROIDOSUS IS PREDOMINANTLY REGULATED BY cAMP/PKA, SEVERAL OTHER PATHWAYS COULD ALSO CONTRIBUTE TO THIS PROCESS (45), INCLUDING THE cGMP SIGNALING PATHWAY (21). cGMP IS GENERATED BY THE MEMBRANE-BOUND guanylyl cyclase (mGC) AND NITRIC OXIDE (NO)-DEPENDENT SOLUBLE guanylyl cyclase (sGC) AND ACTS AS AN INTRACELLULAR MESSENGER BY MODULATING THE FUNCTION OF EFFECTORS DIRECTLY OR THROUGH THE cGMP-DEPENDENT PROTEIN KINASE (PKG) (27). SPECIFICALLY, THERE IS EVIDENCE THAT Natriuretic peptides activate sGC IN THESE CELLS AND STIMULATE TESTOSTERONE PRODUCTION THROUGH BOTH Δ⁵ AND Δ⁴ PATHWAYS (21, 27, 29). IT HAS ALSO BEEN SHOWN THAT sGC IS OPERATIVE IN LEYDIG CELLS (10) AND THAT THE RESULTING INCREASE IN cGMP ALSO STIMULATES ANDROGENESIS (47). IN ZONA GLomerulosa cells, ACTIVATION OF PKG II BY cGMP REGULATES BASAL LEVELS OF ALDOSTERONE PRODUCTION AND PHOSPHORYLATION OF StAR PROTEIN (16).

AT THE PRESENT TIME, HOWEVER, THE MECHANISM BY WHICH cGMP CONTROLS TESTICULAR STEROIDOSUS AND THE ROLE OF StAR PROTEIN IN THIS PROCESS ARE INCOMpletely characterized. TO ADDRESS THESE QUESTIONS, IN THIS STUDY WE SYSTEMATICALLY ANALYZED THE EXPRESSION AND ROLE OF mGCs AND sGCs IN CONTROL OF STEROIDOSUS BY USING PURIFIED RAT LEYDIG CELLS. WE ALSO STUDIED CONTRIBUTION OF cGMP IN CONVERSION OF CHOLESTEROL AND SEVERAL OTHER PRECURSORS TO PROGESTERONE AND ANDROGENS. FINALLY, WE STUDIED THE RELATIONSHIP BETWEEN THE cGMP-SIGNALING PATHWAY AND StAR PROTEIN PHOSPHORYLATION. OUR RESULTS INDICATE THAT ACTIVATION OF mGC AND sGC LEADS TO STIMULATION OF STEROIDOSUS, WHICH IS MODULATED, AT LEAST IN part, BY PKG-DEPENDENT PHOSPHORYLATION OF StAR PROTEIN.

MATERIALS AND METHODS

MATERIALS. The RNAeasy kit for total RNA isolation was purchased from Quagen (Valencia, CA), and chemicals for RT-PCR analysis...
were obtained from Invitrogen (Gaithersburg, MD). The antisera for StAR protein were generous gifts from Douglas Stocco (Texas Tech University, Lubbock, TX) (8) and Dale B. Hales (Department of Physiology and Biophysics, University of Illinois, Chicago, IL) (18), whereas anti-testosterone-11-BSA serum 250 and anti-progesterone-11-BSA serum 337 were kindly supplied by Gordon D. Niswender (Colorado State University, Fort Collins, CO). The anti-mouse and anti-rabbit secondary antibodies linked to horseradish peroxidase were from Kirkegaard & Perry (Gaithersburg, MD). The monoclonal anti-phosphoserine detection kit, the phosphatase inhibitors cocktail set, KT5823, 4-(aminoethyl)-benzenesulfonyl fluoride hydro-chloride, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C natriuretic peptide (CNP), 4-cyano-3-methylisouquinoline, and N-[2-(p-bromo-cinnamyl)aminoethyl]-5-isouquinolinesulfonyl-famomide-2HCl (H98) were purchased from Calbiochem (La Jolla, CA). The cell-permeable cGMP analog 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP) was from RBI (Natick, MA). The protein A agarose resin slurry (1:1) was from Oncogene Research Products (San Ganon, West Orange, NJ). All other reagents were of analytic grade.

Leydig cell culture. Experiments were performed on purified Leydig cells obtained from normal 90-day-old male Wistar rats raised in the Animal Facility of Faculty of Sciences (University of Novi Sad). All experiments were approved by the Ethical Committee of the University of Novi Sad and were performed and conducted in accordance with Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council).

Isolation of interstitial cells was done according to Anakwe et al. (1), with some modification. Briefly, testes were quickly removed, decapsulated, placed in medium 199 containing 1.2 mg/ml collagenase, 1.5% BSA, 20 mM HEPES, sodium bicarbonate, and antibiotics (sensitivity 6 pg/tube; intra-assay coefficient of variation 6 – 8%). For measurement of nitrite levels in medium, sample aliquots were mixed with an equal volume of Griess reagent containing 0.5% sulfanilamide and 0.05% naphthyl-ethylenediamine in 2.5% phosphoric acid (all from Sigma). The mixture was incubated in dark for 10 min at room temperature, and the absorbance was measured at 546 nm (17).

Nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite. The amounts of cGMP secreted into the culture medium, comparable to what is produced within the cell (22), were measured by the cGMP EIA kit, which typically displays an IC50 of ~4 pmol/ml and a detection limit of <1 pmol/ml.

RNA isolation and RT-PCR. Total RNA from purified Leydig cells was isolated using RNAeasy kit (Qiagen), following a protocol recommended by the manufacturer, and its concentrations and purity were determined spectrophotometrically. To eliminate residual genomic DNA, RNA samples were treated with 4 IU DNase I. After DNase I treatment, 3 µg of total RNA from each sample were reverse transcribed into cDNA in a 20-µl reaction mixture containing oligo(dT)18 primer and Superscript III reverse transcriptase (Invitrogen) according to the supplier’s instructions. RNA samples were subjected to RT-PCR. An aliquot of 5 µl of the RT reaction was

Table 1. Sequences of primers used in RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mGC-A</td>
<td>X14773</td>
<td>5'-CGCAGAAACGGCCAGAGTTA-3'</td>
</tr>
<tr>
<td>mGC-B</td>
<td>M26896</td>
<td>5'-AGTCCCCTCCGAGCAGGATC-3'</td>
</tr>
<tr>
<td>mGC-C</td>
<td>M55636</td>
<td>5'-ATGAGAAGGTGACGCGGCT-3'</td>
</tr>
<tr>
<td>mGC-D</td>
<td>L37203</td>
<td>5'-CTGCTTGGCCGACCATAAGTCAA-3'</td>
</tr>
<tr>
<td>mGC-E</td>
<td>L36029</td>
<td>5'-CTGGGAGGTCTGCATGGCATT-3'</td>
</tr>
<tr>
<td>mGC-F</td>
<td>L36530</td>
<td>5'-CTGGAGGGATGTCGAGAAGAC-3'</td>
</tr>
<tr>
<td>mGC-G</td>
<td>U33847</td>
<td>5'-CACAGGAGCCTGGTGGAGA-3'</td>
</tr>
<tr>
<td>α1-sGC</td>
<td>U60835</td>
<td>5'-CCCAAGTGCTGCTTGAGATTCA-3'</td>
</tr>
<tr>
<td>α2-sGC</td>
<td>AF10963</td>
<td>5'-CCCAAGTGGCGAAGGAGAAGG-3'</td>
</tr>
<tr>
<td>β1-sGC</td>
<td>NM 012769</td>
<td>5'-GATCCGCAATTACGTCGCA-3'</td>
</tr>
<tr>
<td>β2-sGC</td>
<td>M57057</td>
<td>5'-TGAGGAGATGTCGACG-3'</td>
</tr>
<tr>
<td>PDE5</td>
<td>NM 153422</td>
<td>5'-TAATGCTCGAGTCAGGTCGGG-3'</td>
</tr>
<tr>
<td>PKGI</td>
<td>NM 174436</td>
<td>5'-TTGCGAGTCAGGTCGGG-3'</td>
</tr>
<tr>
<td>PKGII</td>
<td>NM 013012</td>
<td>5'-CAGCGAGGGATGAGAAGAAG-3'</td>
</tr>
</tbody>
</table>

Sequences were designed [phosphodiesterase 5 (PDE5) and cGMP-depen-
dent protein kinase II (PKG II)] or adopted from references (23, 40, 48). mgC, membrane-bound guanylyl cyclase; sGC, soluble guanylyl cyclase; F, forward; R, reverse.
amplified with PCR reagent system (Invitrogen) in a final volume of 50 μl containing 1.5 mM MgCl₂, 0.2 mM dNTP, 0.4 μM primers (Table 1), and 1.25 U of Taq DNA polymerase. To check DNA integrity, we used GAPDH and RS16. Reactions without RT served as negative controls. The PCR products were analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide.

**Immunoprecipitation and Western blot analysis.** Cells (3 × 10⁶/well) were washed twice with ice-cold PBS and lysed in 1-ml buffer containing 20 mM HEPES, 10 mM EDTA, 40 mM β-glycerophosphate, 1% tergitol, 2.5 mM MgCl₂, 1 mM DTT, 0.5 mM 4-(aminoethyl)-benzenesulfonyl fluoride hydrochloride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and a cocktail of phosphatase inhibitors [0.05 mM (−)-P-bromotetramisole oxalate, 10 μM cantharidin, and 10 nM micocystin LR; pH 7.5]. Concentration of proteins was estimated by the Bradford method using BSA as a standard (6). Equal amounts of cell lysates were mixed with the StAR protein antiserum and incubated overnight at 4°C with constant rotation. During additional overnight incubation at 4°C with constant rotation, immunoprecipitated complexes were recovered by 30 μl of protein A agarose resin slurry (1:1). Precipitated proteins were washed three times with 1 ml of lysis buffer. Final pellets were resuspended in the SDS-PAGE loading buffer, denatured for 5 min at 95°C, and loaded on SDS-PAGE 16% gels. Gels were analyzed by one-dimensional SDS-PAGE, using a discontinuous buffer system, and proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA), using a wet transfer, following the manufacturer’s recommendation. The immunodetection of the phosphorylated StAR was done with the use of monoclonal anti-phosphoserine antibodies 16B4 or 4A3, whereas StAR protein was detected with antisera to the StAR protein. The reactive bands were always determined with a Luminol-based kit (Pierce, Rockford, IL), and the reaction was detected by an enhanced chemiluminescence system.

![Image](https://via.placeholder.com/150)

**Fig. 1.** Expression and role of the membrane-bound guanylyl cyclase (mGC) and soluble guanylylcyclase (sGC) in steroidogenesis of Leydig cells in vitro. A and E: expression of mRNA transcripts for mGC (A) and α- and β-subunits of sGC (E) in purified Leydig cells. In this and Figs. 2 and 3, total RNA was isolated from primary culture of purified Leydig cells, treated with DNase, and subjected to RT-PCR using specific primers for each gene as described in MATERIALS AND METHODS and Table 1. Data shown are representative of 1 of 3 similar experiments. The quality of RNA was checked using primers for both GAPDH and RS16, and negative controls were performed in the absence of the enzyme in RT reaction (data not shown). B and C: stimulatory effects of atrial (ANP), brain (BNP), and C natriuretic peptides (CNP), the mGC agonists, on androgen (B) and cGMP (C) accumulation in culture medium. F and H: dose-dependent effects of 3,3′-(hydroxynitrosohydrazino)bis-1-propanamine (DPTA), a nitric oxide (NO) donor, on androgen (F) and cGMP (G) accumulation in culture medium. D and H: correlation between cGMP and androgen production. In this and Figs. 2–4, experiments were performed on primary culture of purified Leydig cells and plated in 96-well plates with density of 5 × 10⁴ cells per well in 0.2 ml of culture medium (DMEM-F12). Cells were placed in a CO₂ incubator to attach and recover after purification. After 3 h, incubation medium was discarded and cells were stimulated for 2 h at 34°C in CO₂ incubator, medium was collected, and cGMP and androgen levels were determined by ELISA and RIA, respectively, as described in MATERIAL AND METHODS. Experiments were done in cells with and without inhibited phosphodiesterases (PDEs) with 1 mM IBMX. Data points shown are means ± SE of 3 independent experiments, each performed in 4 or 6 replicates. *Significant difference between controls and treated cells, P < 0.05.
using X-ray film. The immunoreactive bands were analyzed as two-dimensional images with Image J (version 1.32; National Institutes of Health).

Calculations. The results shown are means ± SE of at least three independent experiments, each performed in sextuplicate or quadruplicate determinations. Asterisks indicate significant differences (P < 0.05) among means, estimated by Mann-Whitney unpaired nonparametric two-tailed test (for two-point data experiments) or one-way ANOVA followed by Student-Newman-Keuls multiple-range test (for group comparisons).

RESULTS
cGMP stimulates androgenesis in Leydig cells. We initially characterized the expression and role of guanylyl cyclases in Leydig cells in vitro. Our RT-PCR analysis showed the presence of mRNA transcripts for mGC-A, -B, -C, and -G in purified Leydig cells (Fig. 1A). To study the functional significance of these plasma membrane-associated enzymes on steroidogenesis, Leydig cells were treated with increasing concentrations of ANP, BNP, and CNP, the specific agonists for mGC-A, -B, and -C receptors, respectively. As expected, all three agonists increased cGMP levels in a concentration-dependent manner (Fig. 1C). This rise was accompanied by significantly increased basal androgen production (Fig. 1B). Among three agonists, CNP was the least effective in elevating cGMP and T/DHT levels. When combined together, the values for cGMP concentrations induced by mGC agonists linearly correlated with the corresponding androgen levels (Fig. 1D).

Leydig cells also expressed mRNA transcripts for α1-, β1-, and β2-subunits of sGC (Fig. 1E). The enzyme was functional, as documented by the ability of DPTA, a slowly releasable NO donor, to increase cGMP in a concentration-dependent manner in cultures without inhibition of phosphodiesterases (PDEs) (Fig. 1G). The accumulation of this cyclic nucleotide was severalfold higher in the presence of 1 mM IBMX (Fig. 1G). In contrast to cGMP production, DPTA induced stimulation of basal androgen production at low concentrations and inhibition at higher concentrations. Such a biphasic effect was observed in both experimental conditions, with and without addition of IBMX (Fig. 1F). Others observed similar effects of NO donors on androgen production (47) and found that NO molecules inhibit androgenesis independently of cGMP by binding to the heme molecule of cytochrome P-450 enzymes (11, 12, 37). To reduce the direct inhibitory effect of NO on hormone production, in further experiments cells were exposed to 10 μM DPTA, a concentration that induces maximal increases in T + DHT accumulation (Fig. 1F). In a stimulatory range of DPTA concentrations, a very strong correlation between cGMP and androgen production was observed (Fig. 1H).

The existence of a significant correlation between cGMP and androgen levels was consistent with a hypothesis that stimulation of androgen production resulted from increased cGMP...
production. To test this hypothesis, in a first experiment, Leydig cells were incubated for 15 min with NS2028, a specific sGC inhibitor, followed by 2-h incubation in NS2028-containing medium supplemented with 10 μM DPTA. Such a treatment led to a concentration-dependent decrease in cGMP accumulation in medium, independently of the status of PDE activity (Fig. 2B). Inhibition of cGMP production was accompanied by attenuated accumulation of androgen in the same samples (Fig. 2A).

In the second experiment, we increased cGMP levels by inhibition of phosphodiesterase 5 (PDE5) activity. As shown in Fig. 2C, Leydig cells in vitro expressed mRNA transcripts for this enzyme. To inhibit it, we treated cells for 15 min with increasing concentrations of T0156, followed by 2-h incubation in the presence of 1 and 10 μM DPTA. Consistent with the hypothesis, T0156 increased cGMP (Fig. 2E) and androgen (Fig. 2D) production, in a concentration-dependent manner, and DPTA was more effective in 10 μM than in 1 μM (data not shown).

The stimulatory action of cGMP appeared to be independent of the status of PKA activity. In cells treated with 1 μM H89, a specific PKA inhibitor, DPTA induced typical dose-dependent stimulation of androgen production (Fig. 3A). Similar effects were also observed in cells treated with 1 μM of another PKA inhibitor, 4-cyano-3-methylisouquinoline (data not shown). Furthermore, the addition of 8-Br-cGMP (0.1 mM), a cell-permeable cGMP analog, further increased T+DHT production even in cells stimulated with hCG (0.1 ng/well) and with inhibited PDEs (Fig. 3B).

Two closely related enzymes, PKG I and PKG II, usually mediate the effects of cGMP. Leydig cells expressed mRNA transcripts for both PKG I and PKG II (Fig. 3C). Consistent with the role of PKG in androgenesis, inhibition of this enzyme by KT5823 in cultures with blocked PDEs led to a dose-dependent inhibition of DPTA-induced androgen production (Fig. 3D). Similar effects were observed in cultures without inhibited PDEs (Fig. 3D). Thus PKG is responsible, at least in part, for the cGMP-dependent increase in testosterone production in Leydig cells.

cGMP stimulates conversion of cholesterol to progesterone and androgens. To investigate whether cGMP-PKG modulates activity of steroidogenic enzymes, we bathed primary cultures of purified Leydig cells in medium containing different substrates in the absence or presence of 10 μM DPTA. The progesterone production was stimulated when cholesterol, a steroid substrate carried to mitochondria by StAR, was present in incubation medium (Fig. 4A, left). In contrast, conversion of 22-OH cholesterol, a steroid substrate able to enter mitochondria without carrier, to progesterone was not affected by DPTA treatment (Fig. 4A, right). DPTA also increased androgen production when cholesterol but not 22-OH cholesterol was used as a substrate (Fig. 4B). Furthermore, DPTA did not stimulate conversion of pregnenolone to progesterone (Fig. 4C). The androgen production was also not affected when pregnenolone was present (Fig. 4D, left), suggesting that cGMP-PKG stimulated an earlier step in androgenesis, probably the transport of cholesterol to mitochondria. In the same experimental conditions, the P450c17-mediated conversion of progesterone to testosterone decreased, and Δ5-androstenedione transformation to testosterone mediated by 17β-hydroxysteroid dehydrogenase was not affected (Fig. 4D).
As stated before, the inhibitory effect of DPTA on progesterone transformation reflects inhibition of P450c17 activity by NO (11, 12, 37). Thus cGMP stimulated conversion of cholesterol to both progesterone and testosterone but did not modulate the activity of steroidogenic enzymes on downstream pathways, indicating that transport of cholesterol to mitochondria contributes to the cGMP effects on steroidogenesis.

**PKG-dependent phosphorylation of StAR protein.** Cholesterol is carried to mitochondria by the transport protein complex that includes StAR, and this is the rate-limiting step in steroidogenesis (41). Because PKA, PKC, and PKG II phosphorylate StAR (2, 16), it was reasonable to investigate whether this protein is also a substrate for PKGs in Leydig cells. To do this, we stimulated cells with 10 μM DPTA for variable times without blocking the PDE activities. At the end of incubation, medium was collected and used to measure nitrite, cGMP, progesterone, and androgen levels, whereas cells were scraped and subjected to immunoprecipitation with StAR anti-serum. Results of these investigations confirmed the time-dependent accumulation of nitrites (Fig. 5A), accompanied by increased cGMP levels (Fig. 5B), and the time-dependent increases in progesterone (Fig. 5C) and androgen (Fig. 5D) levels in culture medium. Stimulation of cGMP production by DPTA was also accompanied by significantly increased phosphorylation of the mature, 30-kDa StAR form, whereas phosphorylation levels of immature 37-kDa StAR precursor fluctuated during incubation with DPTA (Fig. 5E), giving the impression that posttranslational modification of StAR is dynamic and that de novo synthesis of StAR is ongoing. The increase in progesterone preceded elevation in androgen levels, and the rise in total StAR 30-kDa and 37-kDa was not time dependent, with the latter consistent with a complex pathway involved in co- and posttranslation modification of these proteins (2, 3, 14, 25).

In further experiments, Leydig cells were preincubated for 15 min with medium containing 1 μM NS2028 or solvent, and then 10 μM DPTA was applied. To provide a positive control for StAR protein phosphorylation, cells were also stimulated with supramaximal hCG concentration (100 ng/well). Two hours later, medium was collected for determination of messenger and hormone levels, and cells were scraped and used for immunoprecipitation-Western blot analysis. Results of these investigations confirmed that the DPTA-dependent increase in NO levels (Fig. 6A) was accompanied by elevation in cGMP (Fig. 6B), progesterone (Fig. 6C), and androgen (Fig. 6D) levels in medium. As expected, hCG stimulated steroidogenesis independently of the status of sGC activity.

DPTA treatment also increased StAR protein phosphorylation (Fig. 6E) and the level of total StAR proteins (Fig. 6F). Moreover, inhibition of sGC activity abolished all these effects of DPTA, a finding consistent with the idea that cGMP modulates steroid biosynthesis in Leydig cells most likely by increasing StAR phosphorylation. It is interesting that DPTA treatment increased phosphorylated and total StAR proteins to the same levels as observed in cells treated with supramaximal hCG concentrations (Fig. 6, E and F), but the level of testosterone production was higher in hCG-treated cells. The results
also confirmed the lack of effects of NS2028 on hCG-stimu-
lated StAR protein phosphorylation (Fig. 6, E and F).

Finally, we examined dependence of DPTA-induced StAR
protein phosphorylation on PKG activity. To do this, Leydig
cells were preincubated for 15 min with or without 10 μM
KT5823, followed by 2-h incubation in the presence of 10 μM
DPTA. The DPTA-induced NO release (Fig. 7A) and the
accompanied increase in cGMP production (Fig. 7B) were not

Fig. 5. Time course of NO-induced messenger/hormone production and modification of steroidogenic acute regulatory protein (StAR) in Leydig cells. 
A–D: effects of 10 μM DPTA on nitrite (A), cGMP (B), progesterone (C), and T + DHT (D) accumulation in culture medium. E and F: effects of DPTA treatment on phosphorylation of immature (37 kDa) and mature (30 kDa) StAR proteins (E) and their total intracellular levels (F). Experiments shown in this and Figs. 6 and 7 were done on purified Leydig cells plated in 100-mm Petri dishes (3 × 10⁶ cells·ml⁻¹·dish⁻¹) and in IBMX-free medium. At the end of 2-h incubation, medium was collected for measurement of NO, cGMP, progesterone, and androgens. Cells were washed three times with ice-cold 1 × PBS, lysed, and subjected to immunoprecipitation with anti-StAR antisera, followed by Western blots for either anti-phosphoserine (Ser-P; E) or StAR protein (F) levels. Representative blots are shown in E and F, and pooled data from scanning densitometry are shown, as bars below plots, on E (densitometry for anti-phosphoserine blot of 37- and 30-kDa StAR, respectively) or F (densitometry for StAR blot of 37- and 30-kDa StAR, respectively) and are means ± SE for 3 independent experiments. For other results, data points shown are means ± SE of 3 independent experiments, each performed 4 times. *P < 0.05 vs. control (0 min).

Fig. 6. Dependence of basal and DPTA-stimulated StAR protein modification and steroidogenesis on sGC activity in Leydig cells. 
A–D: effects of 1 μM NS2028 on 10 μM DPTA-induced increase in NO (A), cGMP (B), progesterone (C), and T + DHT (D) levels in culture medium. E and F: effects of NS2028 on DPTA-induced modification of StAR protein. Cells were incubated for 15 min in IBMX-free medium containing NS2028, followed by 2-h incubation in the presence of 10 μM DPTA. Cells treated with hCG (100 ng/well) were used as positive controls. *P < 0.05 vs. control (−DPTA, −NS2028, −hCG); †P < 0.05 vs. DPTA-treated (+DPTA, −NS2028, −hCG).
affected by inhibition of PKG, indicating that this enzyme does not modulate sGC activity in Leydig cells. In contrast, the DPTA-induced phosphorylation of StARs and increase in the levels of immunoreactive proteins (Fig. 7, E and F) were significantly affected by KT5823 treatment, suggesting a role of PKG in NO/cGMP-dependent modulation of this proteins. The physiological significance of PKG in this process was suggested in measurements of progesterone and androgen levels (Fig. 7, C and D). In the same experiments, we studied the possible involvement of PKA in DPTA-induced androgen synthesis and StAR phosphorylation; H89 was applied in concentrations that inhibit this enzyme. Results of these investigations showed that H89 did not affect DPTA-induced StAR protein phosphorylation and synthesis of progesterone and androgens (Fig. 7).

**DISCUSSION**

In this study, we systematically examined the role of cGMP signaling pathway in Leydig cell steroidogenesis and modification of StAR protein. Our investigations confirmed that both pathways for generation of cGMP, mGC and sGC, are expressed and operative in Leydig cells. The mRNA transcripts for mGC-A, mGC-B, and mGC-C were identified in purified Leydig cells, and application of agonists for these receptor enzymes stimulated cGMP and testosterone production in Leydig cells; ANP and BNP were always more effective than CNP. These findings are in line with observations in mouse, rat, and human Leydig cells presented by others in discussing the possible role of mGC agonists in autocrine and/or paracrine regulation of testicular function (21, 29–33). In addition, CNP seems to be a local regulator of ACTH-induced aldosterone secretion in bovine adrenal zona glomerulosa cells (20). We also detected the presence of transcripts for mGC-G, which is an orphan receptor (39). So far, mGC-G has been identified in the rat lung, small intestine, skeletal muscles, and kidney (27), as well as in mouse testicular tissue, but without the precise location within the testis (24).

RT-PCR analysis also showed the presence of mRNA transcripts for α1-, β1-, and β2-subunits of sGC. It is well established that many mammalian cells express α1β1-heterodimer and that α1β2-dimer is less active and probably has a regulatory role in cGMP production (22). At the present time, it is difficult to discuss the physiological relevance of β2-subunits in Leydig cells. Our experiments with DPTA, a slowly releasable NO donor, showed that sGC dimers are functional in these cells and generate cGMP in a time- and concentration-dependent manner. In vivo, the activity of this enzyme is controlled by NO synthases, which are expressed in human Leydig (10) and neighboring (29) cells.

NO not only stimulates sGC but also affects the function of either the cytochrome P-450 side-chain cleavage enzyme (11, 12) or P450c17 (37). The dual effects of NO in our experiments resulted in a biphasic modulation of androgen production, stimulatory at small concentrations and inhibitory at high concentrations. This observation is also in agreement with the literature (47). Three lines of evidence have indicated that the stimulatory effects of low-NO concentrations on androgen production are mediated by cGMP. First, a cell-permeable cGMP analog mimicked the action of NO donors on androgen production. Second, inhibition of sGC activity was associated with a drop in NO-dependent androgenesis. Third, inhibition of the cGMP-specific PDE5 stimulated testosterone production.

We also progressed in understanding the mechanism by which cGMP could stimulate steroidogenesis. As addressed in the introduction, the steroid hormones are all synthesized from a common substrate, cholesterol, by several successive enzymatic transformations in mitochondria and endoplasmic reticulum, and transport of cholesterol from intracellular sources into the mitochondria is the rate-limiting step (35, 41). Several PKA-phosphorylated proteins are important for steroidogene-
cis in mitochondria, and a small increase in cAMP is sufficient to induce a maximal rate of mitochondrial cholesterol transport and steroid synthesis (25). Among these proteins, StAR plays a critical role in steroidogenesis and the cAMP-PKA-dependent phosphorylation of this protein initiates the post- or cotranslation events responsible for increases in its biological activity (41, 42).

Our experiments support a role of StAR protein in cGMP-induced steroidogenesis, as activation of the NO/cGMP signaling pathway led to phosphorylation of this protein. The stimulatory action of cGMP was not mediated by PKA, as documented by the lack of effects of PKA inhibitors on cGMP-mediated facilitation of androgenesis. Here, we show that PKG is responsible for phosphorylated StAR protein. Previously, it has been shown that, in adrenal glomerulosa cells, PKG II-dependent stimulation of aldosterone production correlates with phosphorylation of StAR protein (16). We observed the presence of mRNA transcripts for both PKG I and II in Leydig cells. This observation could suggest that both PKG isoforms mediate the action of cGMP on steroidogenesis in Leydig cells, but additional experiments are required to clarify this issue. Type I kinase is a soluble protein, consisting of two splice forms, and mediates effects of natriuretic peptides and NO in cardiovascular cells. On the other hand, type II kinase is a membrane-associated enzyme. Both enzymes operate as dimers, although each monomer seems self-sufficient in its regulatory and catalytic properties (26).

We also show a parallelism in the actions of PKG on steroidogenesis and StAR protein modifications. In addition, NO-dependent cGMP accumulation stimulated the posttranslational modification of StAR, and this process was accompanied by an increase in progesterone and androgen production. These observations support the hypothesis that modification of StAR protein accounts for stimulatory effects of PKG on androgenesis. A more conclusive evidence for this hypothesis was obtained in experiments with variable substrates/precursors of testosterone and activation of sGC by DPTA. These experiments showed that NO-induced cGMP accumulation was accompanied by an increase in progesterone and androgen production but only when cholesterol was offered to cells as a substrate.

The stimulatory action of PKG on testosterone production could also be mediated through modulation of PDE activities. It is well known that PDE-catalyzed cyclic nucleotide degradation provides an important mechanism for regulating signaling cascades (9). The substantial evidence for the regulatory function of PDEs in Leydig cells has also been reported, including a small stimulatory effect of nonelective PDE inhibitors on testosterone release by primary Leydig cells (7, 29). Phosphorylation of PDEs by PKG and PKA also modulates the activity of these enzymes (5, 9). To address this issue, cells were bathed in medium containing 1 mM IBMX, an inhibitor that blocks the majority of PDEs, including PDE1 with an IC50 of 4 μM, as well as PDEs 2, 3, 4, 5, 6, 7, 10, and 11 with IC50 values of 2–100 μM (5). The ability of cGMP, as well as 8-BrcGMP, to further stimulate testosterone production in cells bathed in medium containing high concentration of PDEs inhibitor strongly argues against the hypothesis that PDE-gene-dependent phosphorylation of PDEs accounts for downregulation of their activities and elevation of cAMP levels and therefore testosterone production.

In conclusion, in this study, we have provided numerous data substantiating the hypothesis that, in addition to cAMP, cGMP also stimulates progesterone and androgen production in Leydig cells, suggesting the importance of both cAMP and cGMP in steroidogenesis in the Leydig cell, with both acting through the StAR protein but perhaps differing at the level of other enzymes in the production of androgens. Both mGC and StAR have the capacity to contribute to the regulation of steroidogenesis. We further showed for the first time a role of PKG in cGMP-dependent regulation of steroidogenesis in Leydig cells. The stimulatory action of cGMP-PKG on steroidogenesis was mediated at least in part by PKG-dependent phosphorylation and posttranslation modification of StAR protein. Further experiments are important to clarify the details of PKG actions on StAR protein.

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