THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 4, Issue of February 25, pp. 1915–1922, 1981
Printed in U.S.A.

Estradiol Receptor-mediated Regulation of Steroidogenesis in Gonadotropin-desensitized Leydig Cells*

(Received for publication, August 13, 1980, and in revised form, October 6, 1980)

Kaoru Nozu, Maria L. Dufau, and Kevin J. Catt

From the Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205

The steroidogenic lesion produced in Leydig cells by gonadotropin stimulation has been attributed to an inhibitory effect of estrogen on 17α-hydroxylase and 17,20-desmolase activity, with impaired conversion of progesterone to androgens. In animals treated with doses of human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH) that caused the decrease in desmolase activity, administration of the estrogen antagonist, Tamoxifen, prevented the development of the steroidogenic lesion. The changes in cytosol and nuclear estradiol receptors caused by treatment with hCG or GnRH were compared with testicular estradiol levels during hormone treatment. The 9 S cytosol estradiol receptor was of high affinity ($K_a \approx 2.8 \times 10^9$ M^{-1}) and low capacity (286 fmol/testis) and underwent nuclear translocation to a 5 S form after estradiol or gonadotropin treatment. Administration of hCG (2 μg) or GnRH (100 μg) caused reduction of cytosol estradiol receptors to 40% at 6 h, followed by a return to 70% at 18 h. The nuclear concentration of estradiol receptors reached a maximum of +200% (120 fmol/testis) at 6 h and fell to +35% at 18 h. Testicular estradiol content rose after 2 μg of hCG from 22 ± 3 to 68 ± 6 pg/testis at 6 h, and gradually returned to normal between 2 and 3 days. The steroidogenic lesion in desensitized Leydig cells, which was preceded by elevation of testicular estradiol and nuclear translocation of estradiol receptors, was prevented by Tamoxifen-induced depletion of cytosol estradiol receptors. These findings indicate that nuclear actions of testicular estradiol are responsible for the 17α-hydroxylase/17,20-desmolase lesion induced by hCG or GnRH. Such effects of endogenous estradiol formation on testosterone production may reflect a continuous modulating action of intratesticular estradiol upon androgen secretion by the Leydig cell.

The initial response of testicular Leydig cells to stimulation by luteinizing hormone or human chorionic gonadotropin is an increase in testosterone secretion (1). In both adult and immature rats, the early androgen response to LH[1] or hCG is soon followed by a refractory period, in which the testosterone responses to gonadotropin and exogenous cyclic AMP are markedly impaired for several days after hormone treatment (2–4). Such desensitization of the Leydig cell by gonadotropin has been shown to depend on several factors, including LH receptor loss and subsequent lesions in the steroid biosynthetic pathway (4, 5). These defects include a decrease in pregnenolone biosynthesis after treatment with high doses of hCG that cause marked LH receptor loss, and impaired conversion of progesterone to androstenedione after lower hCG doses that cause moderate loss of LH receptors (5). The latter defect in androgen formation is predominately due to decreased 17α-hydroxylase and 17,20-desmolase activities (5, 6), and has been attributed to an inhibitory effect of intratesticular estradiol formation upon testosterone biosynthesis (5). This proposal was based upon the known ability of exogenous estrogens to cause impairment of testosterone production by decreasing the activity of 17α-hydroxylase and 17,20-desmolase in the rat (7–9) and mouse (10) testis.

The possible participation of estrogen in the desensitizing effect of gonadotropins upon Leydig cell function is further suggested by the increased production of estradiol in LH-stimulated testes (11), and by the presence of estradiol receptors in the rodent testis (12–14). Recently, direct actions of estrogens on androgen secretion have been observed in hypophysectomized rats (7, 8), and the 17,20-desmolase lesion in hCG-treated animals has been shown to be prevented by treatment with the estrogen antagonist, Tamoxifen (11, 15). While these findings have emphasized the probability that endogenous estrogen is involved in the mechanism of hCG-induced testicular desensitization, there has been little direct evidence for the role of the estrogen receptors of the Leydig cell in the refractory process. To clarify the relationship between estrogen action and testicular steroidogenesis after gonadotropin treatment, we have characterized the cytosol and nuclear estrogen receptors of the Leydig cell, and their distribution after treatment with doses of hCG and GnRH that caused testicular desensitization and impaired androgen biosynthesis. These studies have demonstrated that Leydig cell estrogen-receptor complexes undergo nuclear translocation after gonadotropic stimulation, and that prevention of this process by estrogen antagonists results in abolition of the 17,20-desmolase and 17α-hydroxylase lesions in hCG-stimulated Leydig cells.

MATERIALS AND METHODS

Preparation and Incubation of Leydig Cells—Adult male rats (200 to 250 g) were obtained from Charles River Laboratories, Inc., Wilmington, MA, and treated with hCG or gonadotropin-releasing hormone to induce testicular desensitization. Usually, animals were given a single subcutaneous injection of partially purified hCG (Pregnyl, Organon, Inc., West Orange, NJ) of which 10 international units were equivalent to 1 μg of purified hCG, in 200 μl of phosphate-buffered saline, pH 7.4 (PBS). In some experiments, desensitization by endogenous LH was induced by subcutaneous injection of 100 μg of GnRH (Beckman Bioproducts, Silver Spring, MD) in PBS. For blockade of estrogen action, 2-μg doses of the antiestrogen Tamoxifen (*trans*-1(*p*-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: LH, luteinizing hormone; hCG, human chorionic gonadotropin; GnRH, gonadotropin-releasing hormone.

β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene; I. C. I. Organics, Inc., Stamford, CT) were given by intramuscular injection 1 h before, and 1 and 24 h after, the hCG injection. Control and hormone-treated animals were killed by decapitation at selected intervals and interstitial cells were prepared by collagenase digestion as described previously (16). In some experiments, Leydig cells were purified by centrifugation on 14 to 32% Metrizamide gradients (Accurate Chemical, Hicksville, NY) by a modification of the method described previously (17). The most highly active Leydig cells, in terms of LH receptor content and cAMP/testosterone responses to hCG stimulation, were present in bands located at densities of 1.085 and 1.105 g/ml, corresponding to refractive indices of 1.3622 and 1.3680. These components represent functionally similar components of the Leydig cells studied as the active interstitial cell fraction recovered in medium of refractive index 1.3655 (1.095 g/ml) in an earlier report (18), in which the values cited as density should be read as refractive index. A less active fraction of Leydig cells with abundant LH receptors but relatively low cAMP/testosterone responses to hCG was observed at 1.078 g/ml, and would have been excluded from the cell fraction analyzed in previous studies (5, 11, 17, 18). This lighter component contained numerous damaged cells with extensive vacuolization and mitochondrial changes, when examined by electron microscopy. In contrast, the active cells present in bands sedimenting at 1.085 and 1.105 g/ml showed the morphological features of normal Leydig cells, and were combined prior to quantitation in a Levy ultraplane counting chamber (Arthur Thomas, Phila., PA) after histochemical staining for Δ_4-3β hydroxysteroid dehydrogenase (18). The purified Leydig cells were incubated with purified hCG (10,000 international units/mg, prepared by Dr. R. E. Canfield, Columbia University, New York) or dibutyryl cyclic AMP (Sigma Chemical Co., St. Louis, MO). When pregnenolone formation was to be measured, inhibitors of 3 β-hydroxysteroid dehydrogenase (1 μM Cyanoketone, 17β-hydroxy-4,4,17-trimethyl-3-oxo-androst-5-ene-2α-carbonitrile, Upjohn Co., IL) and 17α-hydroxylase (10 μM spironolactone, 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androstene-17α-yl) propionic acid γ-lactone, G. D. Searle and Co., Chicago, IL) were added 20 min before the addition of stimuli, and control incubations were treated similarly (5).

Assays of Steroids—Testosterone production was measured by direct radioimmunoassay in the incubation media diluted 1:5 or 1:10 with PBS as described previously (18). Pregnenolone was measured by the method of DiPietro *et al.* (19) with a highly specific rabbit antiserum to the 11-hemisuccinate-albumin conjugate. Radioimmunoassay of 17α-hydroxyprogesterone was performed with an antiserum to the 3-carboxymethyloxime derivative. Because the antiserum to 17α-hydroxyprogesterone showed significant cross-reactivity with progesterone (14%), the two steroids were separated by thin layer chromatography when progesterone was present in the incubation medium. Radioimmunoassay of progesterone was performed according to the procedure described by De Villa *et al.* (20). Radioimmunoassays of androstenedione and dehydroepiandrosterone were performed with highly specific antisera as described previously (5). Radioimmunoassay of estradiol-17β was performed using a highly specific rabbit antiserum to 6-ketoestradiol conjugated to bovine serum albumin (Steraloids, Wilton, NH). Only minor cross-reaction was observed with the following phenolic steroids: estrone, 0.45%; estriol, 0.21%; 16-ketoestradiol, 2.2%; 16-epiestriol, 3.6%; estradiol-3-glucuronide, 0.23%; and estradiol-17-glucuronide, 0.09%. No cross-reaction was observed with any other steroid tested, including testosterone, androstenedione, progesterone, pregnenolone, 17α-hydroxyprogesterone, estradiol-3,17 disulfate, and estrone-3-glucuronide. For measurement of estradiol-17β in testis homogenates and Leydig cells, samples were extracted with ethyl acetate followed by partition between toluene and 1 N NaOH as described previously (21). Briefly, 3×10^8 Leydig cells or six testes were dispersed in 2 ml of PBS in a glass-Teflon homogenizer. The homogenates were transferred to 50-ml glass tubes and extracted with 30 ml of ethyl acetate after addition of tracer amounts of [^{3}H]estradiol-17β to account for recovery during the extraction procedure. The ethyl acetate layer was then transferred to a 50-ml glass tube and the contents were evaporated under a stream of N_2. The residue was dissolved in 5 ml of toluene, followed by addition of 15 ml of 1 N NaOH, and the mixture was vigorously shaken on a Vortex mixer for 1 min. After the alkaline partition step, the toluene layer containing the nonphenolic steroids was separated from the aqueous layer. The alkaline aqueous layer containing the phenolic steroids was then extracted with 30 ml of ether after adjusting the pH to 7.0 with 10 N HCl. The ether layer was decanted after freezing the aqueous layer with ethanol/dry ice, and evaporated under a stream of N_2. The dried samples were subsequently reconstituted in PBS, 0.1% bovine serum albumin, and analyzed for estradiol content by radioimmunoassay. The toluene phase was also evaporated and reconstituted for testosterone radioimmunoassay. The recoveries of testosterone and estradiol-17β were 95 and 60%, respectively. This method gave values similar to those obtained using LH-20 Sephadex column chromatography for separation prior to radioimmunoassay (22). The estradiol content of extracts prepared from purified Leydig cells was 0.88 ± 0.10 pg/10^6 cells, or 30 ± 3 pg/testis, calculated on the basis of the measured Leydig cell number of 34×10^6 cells/testis, and this accounted for the estradiol measured in extracts of testicular tissue. The number of Leydig cells per testis estimated by histochemical staining of the interstitial cell dispersion was in good agreement with the value obtained by morphological analysis of the testis (23).

Assay of LH Receptors in Dispersed Leydig Cells—Radioiodinated hCG tracer was prepared by the lactoperoxidase method and purified by chromatography on Sepharose-concanavalin A (Pharmacia Fine Chemicals, Inc.) as described previously (24). Leydig cells (1×10^6) were incubated for 3 h at 35°C with 2×10^5 dpm of ^{125}I-hCG (specific activity, 40 μCi/μg) with addition of 5 and 10 ng of hCG to ensure receptor saturation. Nonspecific binding was determined by incubation of cells with the radioactive hormone in the presence of 10 μg of unlabeled hCG. All binding capacities were calculated from six replicate estimations of specific ^{125}I-hCG binding at saturation, with corrections for specific activity and maximum bindability of the tracer preparation as previously described (1). The mean binding capacity was calculated for each of the experimental groups, and expressed as the number of LH receptor sites per Leydig cell.

Preparation of Cytosol and Nuclear Fractions for Determination of Estrogen Receptors—Purified Leydig cells (3.8×10^7), or decapsulated testes in groups of six, were dispersed in 2 volumes of 0.1 M Tris·HCl (pH 7.4) containing 0.5 mM disodium EDTA, 0.5 mM mercaptoethanol, and 0.25 M sucrose (TEM buffer/sucrose) using a Teflon-glass homogenizer. The homogenates were centrifuged at 800 × *g* for 10 min, and the pellet containing the nuclear fraction was washed once with 50 ml of TEM buffer/sucrose containing 5 mM $MgCl_2$ and 0.2% Triton X-100. After filtration through nylon mesh, this fraction was again centrifuged at 800 × *g* for 10 min. The pellet was washed once with 50 ml of TEM sucrose, 5 mM $MgCl_2$, and after centrifugation at 800 × *g*, was resuspended in 8 ml of the same buffer. The supernatant of the first centrifugation at 800 × *g* was centrifuged at 105,000 × *g* for 60 min to obtain the cytosol fraction.

Measurement of Cytosol and Nuclear Receptors—Aliquots (125 μl) of testicular cytosol were incubated with 20 nM [2,4,6,7-^{3}H]estradiol-17β (New England Nuclear, Boston, MA; specific activity, 94 Ci/mmol) and dissolved in 125 μl of TEM buffer for 1 h at 30°C in the presence or absence of a 100-fold excess of unlabeled estradiol. After incubation, 200 μl of a 50% suspension of hydroxyapatite in 20 mM phosphate-buffered saline, pH 7.4, was added; the samples were stood for 15 min at 4°C with agitation on a Vortex mixer every 5 min, and then centrifuged at 400 × *g* for 10 min. The hydroxyapatite pellets were washed three times with 3 ml of 20 mM phosphate-buffered saline, and estradiol-17β was extracted from the hydroxyapatite by shaking on a Vortex mixer with 1 ml of ethyl alcohol. The ethanol extracts were transferred to scintillation counting vials and mixed with 10 ml of Aquasol (New England Nuclear). In some experiments, the cytosol receptors were determined by density gradient centrifugation in 5 to 20% sucrose (25) or by gel filtration on Sephadex G-25 (26). After initial comparative studies, the hydroxyapatite method was used routinely for cytosol receptor assay, since it gave low nonspecific binding similar to that obtained by sucrose gradient centrifugation.

The degree of association of estradiol with cytosol receptors was maximal after incubation for 30 to 60 min at 30°C, and decreased at later times. During incubations at 35 and 40°C, the maximal binding was reduced when compared with incubations at 30 and 0°C, with a progressive decline after reaching maximal levels. These effects of receptor degradation were minimal at 0°C, when binding increased progressively over 3 h to reach the same maximum level attained after 1 h at 30°C. In the present studies, the more rapid method of equilibration at 30°C was employed for assay of cytosolic estradiol receptors.

Determination of nuclear receptors was performed by the exchange method of Anderson *et al.* (27). Briefly, nuclear suspensions (500 μl) were incubated with 500 μl of 10 nM [^{3}H]estradiol at 30°C for 60 min in the presence or absence of a 100-fold excess of unlabeled estradiol. After incubation, the nuclei were centrifuged at 800 × *g* for 10 min and washed twice with 2 ml of TEM sucrose buffer containing 5 mM $MgCl_2$. The nuclear pellet was extracted with 1 ml of TEM buffer, pH

8.5, containing 0.5 M KCl, at 4°C for 10 min with frequent agitation on a Vortex mixer. The resulting extracts were centrifuged at 3000 × *g* for 10 min. Aliquots (0.5 ml) of the supernatants were applied to columns of Sephadex G-25 (1.4 × 5.0 cm) and subsequently eluted with TEM buffer, pH 8.5, containing 0.5 M KCl. Eluted fractions of 1 ml were collected in glass counting vials (Packard, Inc., Downers Grove, IL), and after addition of 10 ml of Aquasol, the radioactivity was measured in a liquid scintillation β spectrometer. The Sephadex G-25 method (26) was selected because it consistently gave the lowest nonspecific binding in nuclear extracts.

Sucrose Gradient Centrifugation of Cytosol and Nuclear Receptors—Linear sucrose gradients (5 to 20%, w/w), in TEM buffer, pH 7.4, for cytosol and in TEM buffer, 0.5 M KCl, pH 8.5, for nuclear receptors, were prepared in centrifuge tubes for the Beckman SW 50.1 rotor. Samples were applied to the top of each gradient and centrifuged at 150,000 × *g* (cytosol) or 200,000 × *g* (nuclear) for 16 h at 4°C. Thirty fractions of 4 drops were collected from the bottom of each tube via a capillary into tubes (12 × 75 mm) and subsequently transferred to counting vials by addition of five 2-ml aliquots of Aquasol. Bovine serum albumin, γ-globulin, alcohol dehydrogenase, and hCG were used as standard proteins during density gradient fractionation.

RESULTS

Characteristics of Testicular Estradiol Receptors—The cytosol and nuclear estradiol receptors were first characterized in purified Leydig cells and testicular tissue in order to establish the experimental conditions for subsequent receptor studies during hormonal desensitization. Density gradient analysis of testicular cytosol showed a 9 S peak of specific [^{3}H]estradiol-receptor binding, and a nonspecific 4 S binding component that was not displaceable by 10^{-6} M unlabeled estradiol (Fig. 1, *left*). The nuclear extracts contained a 5 S peak of specific binding activity (Fig. 1, *right*) that varied reciprocally with the concentration of cytosolic receptor sites. When animals were treated with estradiol (1.5 μg, subcutaneously), the cytosolic receptors disappeared within 1 h, and there was a simultaneous increase in the 5 S nuclear receptors (data not shown), consistent with nuclear translocation of the 9 S cytosol receptors. After treatment with a single dose of hCG (10 μg, subcutaneously), the 9 S cytosolic and 5 S nuclear receptors showed changes similar to those observed above after estrogen administration (Fig. 1). Analysis of the same preparations by the Sephadex G-25 procedure revealed changes in specific binding to cytosol and nuclear receptors similar to those demonstrated by sedimentation analysis.

Equilibrium binding of [^{3}H]estradiol by the testis cytosol receptor during incubation at 30°C for 1 h was progressively inhibited by the presence of increasing concentrations (0.4 to 12.9 nM) of unlabeled estradiol. Testosterone concentrations up to 1 μM caused no inhibition of [^{3}H]estradiol binding to the cytosol receptor. Concentrations of Tamoxifen 10-fold higher than that of estradiol were required to produce half-maximal displacement of [^{3}H]estradiol. Scatchard plots of these binding data revealed the presence of a single class of binding sites with relatively high affinity for [^{3}H]estradiol. The association constant (K_a) determined by Scatchard analysis was 2.8×10^9 M^{-1}, almost identical with that obtained by direct analysis of the saturation curve. The binding capacity for estradiol was 286 fmol/testis, equivalent to about 5000 receptors/Leydig cell. Since the estradiol receptors were confined to the Leydig cells, subsequent analyses of these sites were performed with testicular extracts.

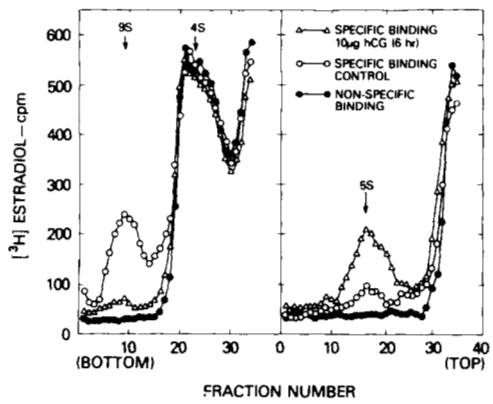

FIG. 1. **Sucrose gradient centrifugation profile of cytosol (*left*) and nuclear (*right*) estradiol receptors from purified Leydig cells.** The cytosol and nuclear sites were determined in Leydig cells from animals treated 6 h before with a single subcutaneous dose of hCG (10 μg).

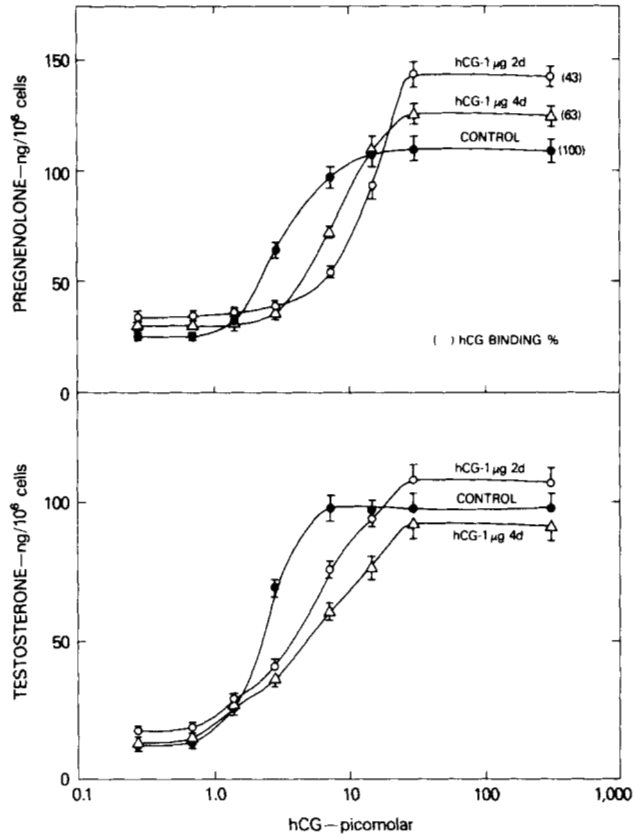

FIG. 2. **Dose-response curves for production of pregnenolone and testosterone by Leydig cells from normal and desensitized animals, prepared 2 and 4 days after subcutaneous injection of 1 μg of hCG.** Leydig cells were incubated with several concentrations (0.3 to 300 pM) of hCG in the presence of 0.1 mM (1-methyl 3-isobutyl xanthine) at 35°C for 3 h. Pregnenolone production in this and subsequent experiments was measured in the presence of 10^{-6} M cyanoketone and 10^{-5} M spironolactone.

Effects of hCG and GnRH on Androgen Biosynthesis—Administration of hCG by subcutaneous injection caused dose-dependent changes in the subsequent responses of Leydig cells to gonadotropin *in vitro*. After injection of 0.5 to 1 μg hCG, there was a 40 to 50% decrease in LH receptors and a 2-fold increase in the ED_{50} of hCG for testosterone stimulation at 2 and 4 days after treatment (Fig. 2, *lower panel*) with no change in maximum testosterone production. The ED_{50} of hCG for pregnenolone production was also increased when measured in the presence of enzyme inhibitors to prevent pregnenolone metabolism (Fig. 2, *upper panel*). Such increases in the ED_{50} of hCG correlated well with the number of LH receptors, which were reduced to 4460 and 6570 sites/cell, or to 43 and 63% of the control values, at 2 and 4 days,

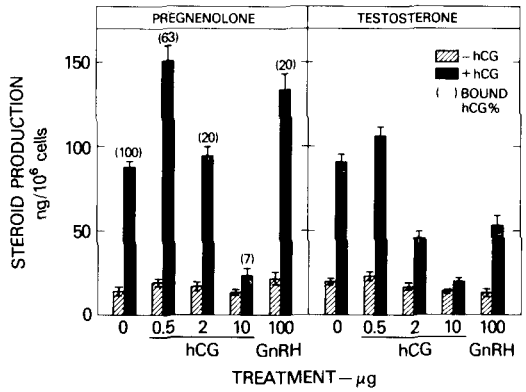

FIG. 3. **Production of pregnenolone (*left*) and testosterone (*right*) by Leydig cells isolated from testes of control, hCG-(0.5 to 10 μg), or GnRH (100 μg)-treated animals (2 days after subcutaneous injection) in response to *in vitro* stimulation with 100 ng/ml of hCG.** The *numbers in parentheses* represent the LH receptor content expressed as a percentage of the control values. Each *bar* represents the mean ± S.D. of four determinations.

respectively. These changes in LH receptors and Leydig cell sensitivity to hCG were accompanied by significant increases in maximal pregnenolone production. This positive effect of hCG presumably reflects the stimulating action of gonadotropin upon early steps of steroid biosynthesis (Fig. 2, *upper panel*; Fig. 3, *left*).

When the dose of hCG was increased to 2 μg, or when 100 μg of GnRH was given, the maximal testosterone response of Leydig cells to hCG *in vitro* was reduced to 50 and 60% of control values (Fig 3, *right*), respectively, whereas pregnenolone responses were either normal or increased (Fig. 3, *left*). This discrepancy between pregnenolone and testosterone production is caused by decreased activity of the 17α-hydroxylase and 17,20-desmolase steps of androgen biosynthesis, as described previously following intravenous administration of gonadotropin (5). Higher doses of hCG (up to 10 μg) caused more marked receptor depletion, and were followed by reduction of both pregnenolone and testosterone responses, indicating the presence of an additional lesion in the early biosynthetic pathway.

The "late" (17α-hydroxylase/desmolase) and "early" (pre-pregnenolone) biosynthetic lesions did not affect basal testosterone production, but caused marked changes in steroidogenic responses evoked by gonadotropic stimulation of Leydig cell function *in vitro*. The late lesion became apparent at 18 h (Fig. 4) as a marked reduction in maximum testosterone production during hCG stimulation, and lasted for up to 3 days after hCG treatment, or for 2 days after injection of GnRH (Fig. 5), with full recovery at 5 and 3 days after hCG and GnRH treatment, respectively (Fig. 5). The early steroidogenic lesion produced by high doses of hCG was also maximal at 2 to 3 days, but did not begin to recover until 5 days, and was completely reversed after 8 to 10 days. The time course of recovery of this early biosynthetic defect was generally similar to the return of LH receptors in the desensitized testis.

Steroid Production and Estrogen Receptors in Gonadotropin-desensitized Leydig Cells—Significant changes in the testicular content of testosterone, estradiol, and estrogen receptors, were observed in animals treated with hCG (0.5 to 10 μg) or GnRH (Fig. 6). There was a rapid, dose-related rise in the testosterone content of the testis, with 5-, 10-, 18-, and 9.5-fold increases after treatment with 0.5, 2, and 10 μg of hCG, and 100 μg of GnRH, respectively. These levels were maintained for about 6 h, and then declined to near-control values between 18 h and 2 days. After the highest dose of hCG (10 μg), a delayed secondary 7.6-fold increase in testosterone content occurred at 3 days (Fig. 6, *upper left*), and returned to control values at 5 days. These changes in testosterone content were followed by increases in estrogen content that were also dose-dependent, and reached maximal levels at 6 h (Fig. 6, *lower left*). However, estrogen levels declined more gradually, and in the animals treated with 2 and 10 μg of hCG or 100 μg of GnRH, testicular estrogen content returned to control values at 3 days. In the animals treated with 0.5 μg of hCG, there were transient and relatively small changes in estradiol formation. The gonadotropin-induced changes in testicular estradiol content were shown to occur in the Leydig cell by studies with purified Leydig cells from normal and hCG-treated animals (Fig. 7). Stimulation of control cells with hCG significantly increased estradiol production *in vitro* by about 70%. Also, Leydig cells from hCG-treated animals showed a marked increase in basal estradiol production that was no longer stimulated by hCG *in vitro* (Fig. 7).

Measurement of cytosolic estradiol receptors revealed no changes in the testes of animals treated with the lowest dose of hCG (0.5 μg). However, in animals treated with 2 or 10 μg of hCG or 100 μg of GnRH (Fig. 6, *lower right*), marked

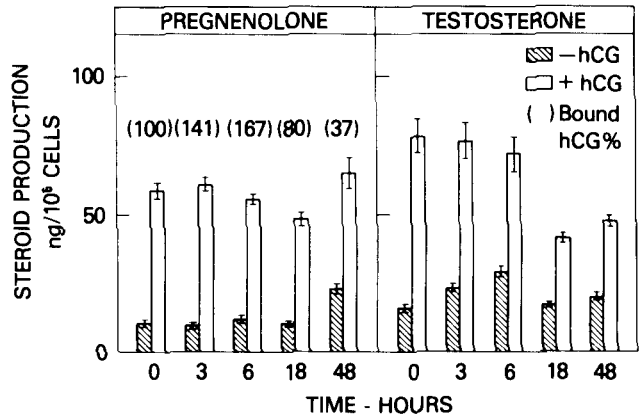

FIG. 4. **Time course of pregnenolone (*left*) and testosterone (*right*) production by Leydig cells isolated from testes of control or hCG (2 μg)-treated animals at intervals up to 48 h.** Each *value* represents the mean ± S.D. of three determinations. The *numbers in parentheses* indicate the LH receptor content expressed as percentage of control values.

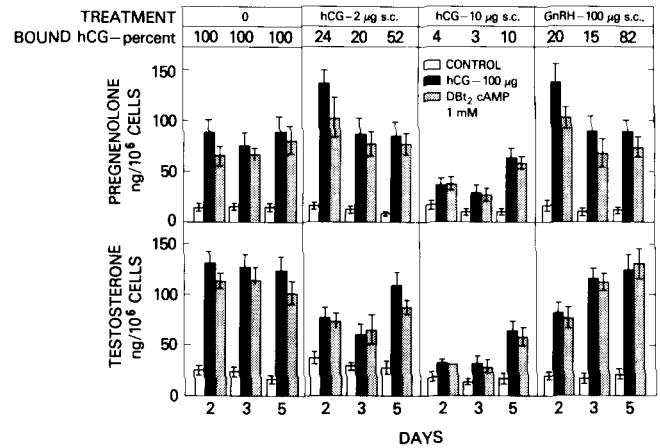

FIG. 5. **Time course of pregnenolone (*top*) and testosterone (*bottom*) production by Leydig cells isolated from testes of control, hCG-(2 to 10 μg), or GnRH (100 μg)-treated animals at 2, 3, and 5 days after the desensitizing injection.** Steroid responses were determined during incubation of dispersed cells with 100 ng/ml of hCG or 1 mM dibutyryl cyclic AMP (DBt_2 cAMP). Each *value* represents the mean ± S.D. of four determinations. s.c., subcutaneous.

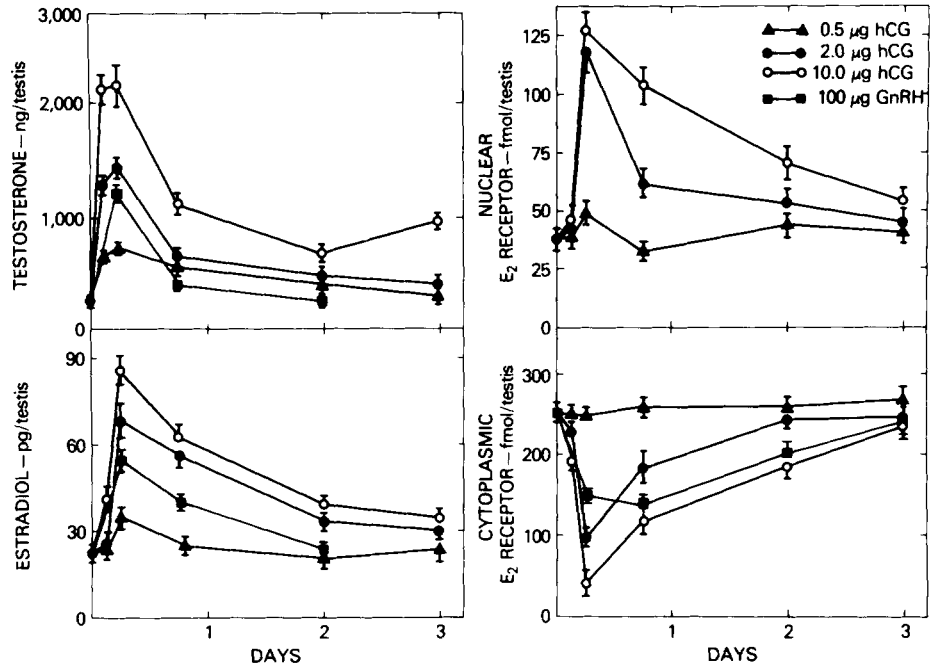

FIG. 6. **Testicular concentrations of estradiol and testosterone (*left*), and cytosol and nuclear estradiol receptors (*right*) following treatment of animals with hCG (0.5, 2, or 10 μg) and GnRH (100 μg).** Each *point* represents the mean ± S.D. of four determinations.

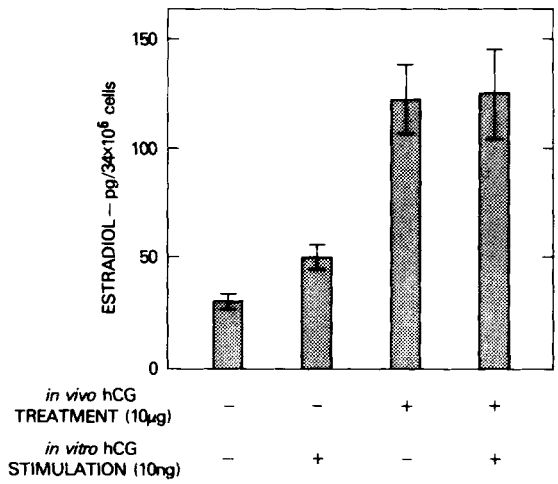

FIG. 7. **Estradiol response to gonadotropin in purified Leydig cells from normal rats and animals treated with 10 μg of hCG 6 h previously and incubated with or without 10 ng of hCG for 2 h.** The estradiol content of the cells was measured by radioimmunoassay as described in the test.

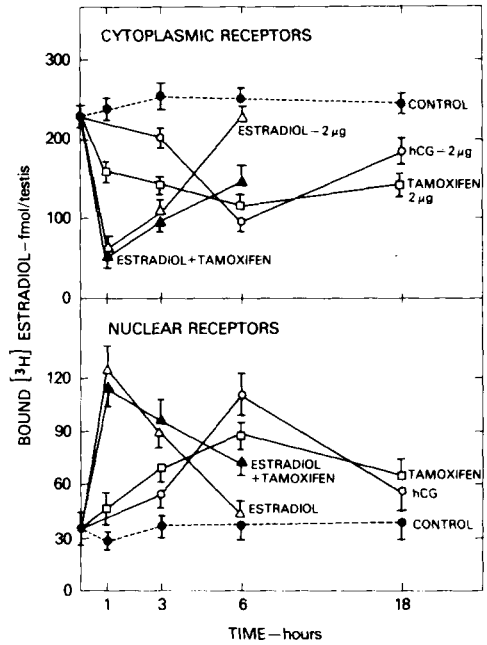

FIG. 8. **Effects of hCG or estradiol and/or Tamoxifen administration on the testicular content of cytosolic estrogen receptors (*top*), and on their translocation to nuclear sites (*bottom*).** Tamoxifen (2 μg) was injected intramuscularly 1 h before and 1 h after the subcutaneous injection of 2 μg of hCG or 2 μg of estradiol. Cytosol and nuclear estradiol receptors were determined by the hydroxyapatite procedure and Sephadex G-25 chromatography, respectively.

depletion of estradiol receptors occurred in parallel with the increase in the estrogen content of the testis, followed by gradual return to control values at 3 days. Nuclear translocation of estrogen cytosol receptors was maximal at the time of major depletion of cytosolic receptors, and showed a gradual return to near-control values at 3 days (Fig. 6, *upper right*). No nuclear translocation of cytosol receptors was observed in animals treated with the lowest hCG dose. The most marked changes in testicular estrogen content and receptors occurred in the animals in which steroidogenic lesions were observed, and the time of appearance of the steroid biosynthetic lesions followed the changes in estradiol receptors.

Effects of Estrogen Antagonist on Desensitized Leydig Cells—The effects of the antiestrogen Tamoxifen upon testicular estrogen receptors (Fig. 8) and steroidogenic lesions (Fig. 9) were analyzed in testicular tissue from hCG-treated rats. After treatment with 2 μg of Tamoxifen, an optimal dose for prevention of the late steroidogenic lesion, cytosol receptors were reduced at 1 h, became maximally depleted at 6 h, and showed only slight recovery at 18 h (Fig. 8). In contrast, treatment with hCG caused only minor changes in cytosol receptors at 3 h, followed by rapid depletion between 3 and 6 h (by 50%), and significant recovery of sites at 18 h. The changes in cytosol receptors caused by Tamoxifen were more gradual and prolonged than those caused by endogenous estradiol during hCG stimulation. This was also apparent when compared with the effects of treatment with exogenous estradiol (2 μg), which caused pronounced depletion of receptors (by 80%) within 1 h, followed by rapid recovery to the

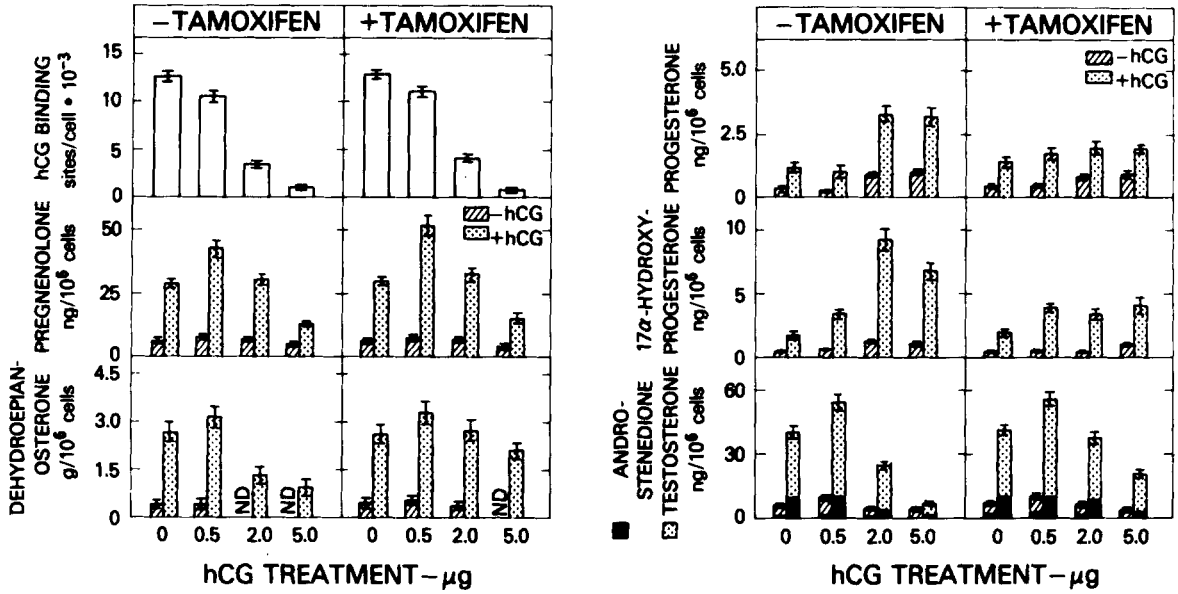

FIG. 9. **Effects of Tamoxifen treatment on the steroidogenic capacity of Leydig cells from animals treated with hCG.** Tamoxifen (2 μg) was administered by intramuscular injection 1 h before, 1 h after, and 24 h after a subcutaneous injection of 0.5, 2, or 5 μg of hCG. Leydig cells were prepared from testes of groups of eight animals killed 48 h after hCG injection. Cells were incubated in the absence or presence of 200 ng of hCG, a supramaximal steroidogenic dose. LH/hCG receptor content was determined in each of the experimental groups. Data are the mean ± S.D. of data from quadruplicate incubations.

control value at 6 h. The return of cytosol receptors was significantly delayed when Tamoxifen was administered together with estradiol. The depletion of cytosol estradiol receptors was accompanied by a reciprocal increase of nuclear receptors after treatment with estrogen or hCG (Fig. 8, *bottom*). The more prolonged changes in cytosol and nuclear receptors during hCG-induced increases of estradiol production are attributable to the long half-life of hCG and the consequently more sustained increase in testicular estradiol Fig. 6). Because of this prolonged effect of hCG, for complete prevention of the late steroid biosynthetic lesion, it was necessary to give a third dose of Tamoxifen 24 h after hCG treatment.

In parallel experiments, we investigated the effect of Tamoxifen on the steroid biosynthetic capacity of cells from animals treated with hCG (Fig. 9). Leydig cells from animals treated with 2 and 5 μg of hCG, with reduction of hCG receptors to 26 and 8%, showed marked reduction of testosterone, dehydroepiandrosterone, and androstenedione responses to hCG, with increase in 17α-hydroxyprogesterone and progesterone. These changes were typical of steroidogenic lesions of the 17α-hydroxylase and 17,20-desmolase steps of androgen biosynthesis. Pregnenolone production (measured in the presence of enzyme inhibitors) did not differ from control in the group treated with 2 μg of hCG, since the lesion was confined only to the distal enzymes. In the group treated with 5 μg of hCG, pregnenolone production was markedly reduced due to the additional steroidogenic lesion prior to the synthesis of pregnenolone. Simultaneous treatment with Tamoxifen prevented the decrease in 17,20-desmolase and 17α-hydroxylase activity in the animals treated with 2 μg of hCG. Also, the production of testosterone, androstenedione, and dehydroepiandrosterone by Leydig cells was similar to the control values, and accumulation of progesterone or 17α-hydroxyprogesterone did not occur. In the group treated with the higher dose of hCG, Tamoxifen again prevented the estrogen-dependent lesions in 17,20-desmolase and 17α-hydroxylase, as reflected by the increase in testosterone production (4-fold) and dehydroepiandrosterone (2-fold), and by the absence of accumulation of progesterone and 17α-hydroxyprogesterone. However, Tamoxifen did not prevent the early steroidogenic lesion, as shown by the sustained reduction in pregnenolone production (by 60%) and testosterone production (by 50%), despite reversal of the late (*i.e.* 17α-hydroxylase-17,20-desmolase) biosynthetic lesions by this compound.

DISCUSSION

The present studies have demonstrated that the estrogen receptors of the rat testis interstitium (12, 14) are present in the Leydig cell, and undergo nuclear translocation after occupancy by endogenous or exogenous estradiol. The cytosolic Leydig cell estrogen receptors possess high affinity and low capacity for estradiol, with K_a of 2.8×10^9 M^{-1} and binding capacity of 6.5 fmol/mg of protein. The affinity constant is similar to that reported in 26- and 28-day-old rats (12, 13), and is about one-tenth of that reported previously in the adult animals (14). The binding capacity in the adult animal was lower than the value of 14 to 16 fmol/mg of protein reported in 26- to 28-day old animals (12, 13), and that of 15 fmol/mg observed in 18-day-old rats.[2] The sedimentation coefficients of the cytosol and nuclear receptors were similar to those reported by others (28, 29). These receptors are specific for estradiol, and do not bind testosterone or other steroids. They are distinct from the androgen-binding protein that is localized in the seminiferous tubules and binds estradiol with low affinity (30, 31). The calculated number of cytosolic estradiol receptors per Leydig cell was about 5000; the nuclear receptors numbered about 750/cell in unstimulated testes, increasing to a maximum of 2100 sites/cell during stimulation with hCG or estradiol. About 60% of the nuclear receptors were extractable with 0.5 M KCl; the residual fraction with long term nuclear retention may correspond to the recently described tight binding category of nuclear receptors (32).

Stimulation of the testis by either exogenous or endogenous gonadotropin caused rapid increases in testosterone production that were closely followed by increases in estradiol content of the Leydig cell, with depletion of cytosol estradiol receptors and their concomitant translocation to the nucleus. A relationship between the late steroidogenic lesions produced

[2] K. Nozu, unpublished observations.

by hCG treatment and the effect of endogenous estrogen formation was suggested by the rapid increase in testicular estrogen content, and by the changes in estradiol receptors 12 h before the late enzymatic lesions in 17α-hydroxylase and 17,20-desmolase. The antiestrogen Tamoxifen was bound by estrogen receptors of the Leydig cell and caused nuclear translocation of the cytosol receptors. Therefore, the ability of Tamoxifen to prevent the late steroidogenic lesion in desensitized testes indicated that the nuclear actions of testicular estradiol are responsible for the 17α-hydroyxlase and 17,20-desmolase lesions induced by hCG and GnRH.

Nuclear translocation of the estradiol receptors was induced by administration of estrogen, hCG, or GnRH. The increase in nuclear estradiol receptors during hCG or GnRH treatment was dose-dependent and did not occur with low hCG doses (0.5 μg) that did not cause the steroidogenic lesions of 17α-hydroxylase and 17,20-desmolase. However, nuclear accumulation was always observed and was almost maximal with an intermediate dose of hCG (2 μg) that consistently caused the late steroid biosynthetic lesions. The highest hCG dose (10 μg), which also produced an early lesion prior to pregnenolone formation, caused only a minor additional increase in nuclear estradiol receptors.

Although only minute quantities of estradiol are produced in the Leydig cell, significant increases in endogenous estradiol production were observed during hCG stimulation *in vivo*. The fractional conversion of androgen to estrogen via the aromatase enzyme present in the Leydig cell (33, 34) is extremely small, forming only picograms of estrogen from a substrate that is produced in a 1000-fold greater amount. Thus, it is likely that this enzyme is rate-limiting for estrogen formation in the Leydig cell. However, such small increases in estrogen within the gonadotropin-stimulated target cell are sufficient to cause translocation of the estradiol receptor to the nucleus, with subsequent impairment of the enzymatic activities of 17α-hydroxylase and 17,20-desmolase. The inhibitory effect of gonadotropin-induced elevations of endogenous estradiol on the 17α-hydroxylase and 17,20-desmolase enzymes was completely prevented by Tamoxifen. This estrogen antagonist was used in minimal effective doses (11) to avoid changes in LH secretion (35) or direct effects on aromatase activity (36). The antagonist became bound to the cytosol estrogen receptor of the Leydig cell and caused nuclear translocation with prolonged retention of the complex. The prevention of the steroidogenic lesion in desensitized testes by Tamoxifen strongly suggests that the nuclear actions of testicular estradiol are responsible for the 17α-hydroxylase and 17,20-desmolase lesions induced by hCG and GnRH.

It is generally accepted that the estrogen·receptor complex undergoes translocation to nuclear sites in the target tissue and induces specific RNA and protein synthesis (37–39). This includes stimulation of the production of secreted proteins (40) and enzymes (41–43), and cell proliferation (44, 45). However, in the present study, endogenous estrogen inhibited the activities of the cytochrome P-450-dependent microsomal enzymes, 17α-hydroxylase and 17,20-desmolase. Recently, both endogenous and exogenous estradiol have been shown to reduce the testicular content of microsomal cytochrome P-450, and this decrease correlated with the impaired activity of the same enzymes in the Leydig cell (46). Although the mechanism of this unusual inhibitory action of estrogen is not clear, it could reflect the induction of proteins that are involved in the regulation of these enzyme systems. Such effects of estradiol on the enzymes of the androgen biosynthetic pathway may reflect a physiological modulating effect of intratesticular estrogen upon androgen secretion by the Leydig cells during regulation by pituitary gonadotropins.

Acknowledgments—We are grateful to Dr. C. Strott for providing the antiserum employed for pregnenolone assay, to Dr. G. Bialy and the Center for Population Research for providing the purified hCG employed in these studies, and to Ms. Christine Winters for performing radioimmunoassays of testicular estradiol-17β content.

REFERENCES

1. Dufau, M. L., and Catt, K. J. (1978) *Vitam. Horm.* **36,** 461–592
2. Hsueh, A. J. W., Dufau, M. L., and Catt, K. J. (1976) *Biochem. Biophys. Res. Commun.* **72,** 1145–1152
3. Hsueh, A. J. W., Dufau, M. L., and Catt, K. J. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 592–595
4. Tsuruhara, T., Dufau, M. L., Cigorraga, S., and Catt, K. J. (1977) *J. Biol. Chem.* **252,** 9002–9009
5. Cigorraga, S. B., Dufau, M. L., and Catt, K. J. (1978) *J. Biol. Chem.* **253,** 4297–4304
6. Chasalow, F., Marr, H., Haour, F., and Saez, J. M. (1979) *J. Biol. Chem.* **254,** 5613–5617
7. Kremers, P., Tixhon, C., and Gielen, J. (1977) *J. Steroid Biochem.* **8,** 873–875
8. Hsueh, A. J. W., Dufau, M. L., and Catt, K. J. (1978) *Endocrinology* **103,** 1096–1102
9. Melner, M. H., and Abney, T. O. (1980) *J. Steroid Biochem.* **13,** 203–210
10. Samuels, L. T., Uchikawa, T., Zain-ul-Abedin, M., and Huseby, R. A. (1969) *Endocrinology* **85,** 96–102
11. Cigorraga, S. B., Sorrell, S., Bator, J., Catt, K. J., and Dufau, M. L. (1980) *J. Clin. Invest.* **65,** 699–705
12. Brinkmann, A. O., Mulder, E., Lamers-Stalhofzen, G. J. M., Mechielsen, M. J., and van der Molen, H. J. (1972) *FEBS Lett.* **26,** 301–305
13. Kato, J., Onouchi, T., Okinaga, S., and Ito, N. (1974) *Endocrinology* **94,** 902–907
14. Abney, T. O. (1976) *Endocrinology* **99,** 555–566
15. Dufau, M. L., Hsueh, A. J., Cigorraga, S., Baukal, A. J., and Catt, K. J. (1978) *Int. J. Andrology,* Suppl 2, 193–239
16. Dufau, M. L., Mendelson, C., and Catt, K. J. (1974) *J. Clin. Endocrinol. Metab.* **39,** 610–613
17. Conn, P. M., Tsuruhara, T., Dufau, M. L., and Catt, K. J. (1977) *Endocrinology* **101,** 639–642
18. Dufau, M. L., Horner, K. A., Hayashi, K., Tsuruhara, T., Conn, P. M., and Catt, K. J. (1978) *J. Biol. Chem.* **253,** 3721–3729
19. DiPietro, D. L., Brown, R. D., and Strott, C. A. (1972) *J. Clin. Endocrinol. Metab.* **32,** 619–624
20. De Villa, G. O., Roberts, K., Wiest, W. G., Mikhail, G., and Flickinger, G. (1972) *J. Clin. Endocrinol. Metab.* **35,** 458–462
21. Dufau, M., Weinstein, R., and Kliman, B. (1969) *Clin. Chim. Acta.* **25,** 389–393
22. Karsch, F. J., Weick, R. F., Hotchkiss, J., Dierschke, D. J., and Knobil, E. (1973) *Endocrinology* **93,** 478–486
23. Mori, H., and Christensen, K. (1980) *J. Cell Biol.* **84,** 340–354
24. Dufau, M. L., Podesta, E. J., and Catt, K. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 1272–1275
25. Clark, J. H., Harding, J. W., and Upchurch, S. (1978) *J. Biol. Chem.* **253,** 7630–7634
26. Aten, R. F., Weinberger, M. J., and Eisenfeld, A. J. (1978) *Endocrinology* **102,** 433–442
27. Anderson, J., Clark, J. H., and Peck, E. J. (1972) *Biochem. J.* **126,** 561–567
28. Mulder, E., Brinckmann, A. O., Lamers-Stalhofzen, G. J. M., and van der Molen, H. J. (1973) *FEBS Lett.* **31,** 131–136
29. De Boer, W., De Vries, J., Mulder, E., and van der Molen, H. J. (1977) *Biochem. J.* **162,** 335–339
30. Vernon, R. G., Kopec, B., and Fritz, I. B. (1974) *Mol. Cell. Endocrinol.* **1,** 167–187
31. Sanborn, B. M., Steinberger, A., Tcholakian, R. K., and Steinberger, E. (1977) *Steroids* **29,** 493–502
32. Clark, J. H., and Peck, E. J. (1976) *Nature* **260,** 635–636
33. Canick, J. A., Makris, A., Gunsalus, G. L., and Ryan, K. J. (1978) *Endocrinology* **102A,** 138
34. Valladares, L. E., and Payne, A. H. (1979) *Endocrinology* **105,** 431–436
35. Bartke, A., Mason, M., Dalterio, S., and Bex, F. (1978) *J. Endocrinol.* **79,** 239–240
36. Watson, J., and Howson, J. W. H. (1977) *J. Reprod. Fertil.* **49,** 375–376
37. Walker, M. D., Negreanu, V., Gozes, I., and Kaye, A. M. (1979) *FEBS Lett.* **98,** 187–191

38. Huber, S., Ryffel, G. U., and Weber, R. (1979) *Nature* **278,** 65–67
39. Vician, L., Shupnik, M. A., and Gorski, J. (1979) *Endocrinology* **104,** 736–743
40. Chan, L., Means A. R. and O'Malley, B. W. (1978) *Vitam. Horm.* **36,** 259–295
41. Wu, C. (1979) *Biochem. Biophys. Res. Commun.* **89,** 769–776
42. Ghraf, R., Lax, E. R., and Schriefers, H. (1979) *J. Endocrinol.* **83,** 157–163
43. Lyttle, C. R., Garay, R. V., Desombre, E. R. (1979) *J. Steroid Biochem.* **10,** 359–363
44. Kirkland, J. L., La Pointe, L., Justin, E., and Stancel, G. M. (1979) *Biol. Reprod.* **21,** 269–272
45. Martin, L., and Finn, C. A. (1978) *J. Endocrinol.* **78,** 417–425
46. Nozu, K., and Matsuura, S. (1980) *Fed. Proc.* **39,** 2089