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Regulation of Progestin Biosynthetic Enzymes in Cultured Rat Granulosa Cells: Effects of Prolactin, β_2-Adrenergic Agonist, Human Chorionic Gonadotropin and Gonadotropin Releasing Hormone[1]

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ABSTRACT

Prolactin (Prl), β_2-adrenergic agents and human chorionic gonadotropin (hCG) are luteotropic in rats, whereas gonadotropin releasing hormone (GnRH) exerts direct inhibitory effects on ovarian steroidogenesis. The present study examined the modulation of the progestin biosynthetic pathway by the luteotropic agents, as well as the actions of GnRH. Rat granulosa cells were primed with follicle-stimulating hormone (FSH) to increase their responsiveness to the luteotropic agents. Subsequent treatment for 2 days with Prl, terbutaline (a β_2-adrenergic agonist) or hCG stimulated the production of progesterone, 20α-hydroxypregn-4-en-3-one (20α-OH-P), pregnenolone and the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD). In contrast, treatment with Prl or terbutaline, but not hCG, inhibited 20α-hydroxysteroid dehydrogenase (20α-HSD) activity by decreasing the apparent maximal velocity of the enzyme with no change in its K_m value. Concomitant treatment with GnRH inhibited progesterone, but increased 20α-OH-P production stimulated by Prl or terbutaline. These effects were associated with a stimulation of 20α-HSD activity, while neither 3β-HSD activity nor pregnenolone biosynthesis was decreased. In contrast, GnRH inhibited progesterone production in hCG-treated cells without affecting 20α-OH-P production. This was associated with an inhibitory effect of GnRH on pregnenolone biosynthesis with no effect upon 3β-HSD activity. Thus, Prl and the β_2-agonist stimulate progesterone production in granulosa cells by increasing pregnenolone production and 3β-HSD activity as well as by decreasing 20α-HSD activity, while hCG stimulates progesterone production by increasing pregnenolone production and 3β-HSD activity. The inhibitory effect of GnRH on Prl- or terbutaline-stimulated progesterone production appears to result from a preferential increase in 20α-HSD activity, while the GnRH inhibition of hCG-stimulated progesterone production appears to result from a preferential inhibition of pregnenolone production.

INTRODUCTION

Prolactin (Prl) and luteinizing hormone (LH)/human chorionic gonadotropin (hCG) are luteotropic in rodents (Smith, 1980; Rothchild, 1981). Adrenergic agents, specifically β_2-agonists, stimulate progesterone production by luteal tissues and catecholamines have been postulated to play a role in the maintenance of luteal functions (Condon and Black, 1976; Godkin et al., 1977; Jordan et al., 1978; Ratner et al., 1980; Adashi and Hsueh, 1981; Kliachko and Zor, 1981). Earlier studies have examined the regulation of ovarian steroidogenesis by Prl and LH/hCG utilizing in vivo models or following short-term incubations of dispersed corpora lutea cells or luteal tissue slices (Rubin et al., 1963; Armstrong et al., 1969, 1970; Hashimoto and Wiest, 1969; Loewit et al., 1970; Behrman et al., 1971; Rondell, 1974). Due to the inability to maintain long-term luteal cell steroidogenesis in vitro and the complications of in vivo experimentation, studies on the direct modulatory effect of various luteotropic and luteolytic agents on progestin biosynthetic enzymes were limited.

Employing granulosa cells cultured in serum-free media, we have recently demonstrated that follicle-stimulating hormone (FSH)

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treatment increases steroidogenic responsiveness of granulosa cells to LH, Prl and adrenergic agents (Erickson et al., 1979; Wang et al., 1979; Adashi and Hsueh, 1981; Jones and Hsueh, 1981a). These FSH-treated "granulosa-luteal" cells possess many functional characteristics similar to luteal cells and serve as a useful model for studying the actions of luteotropic and luteolytic agents in vitro. In the present study, we have used this culture system to examine the regulation of the progestin biosynthetic pathway by Prl, terbutaline (a β_2-adrenergic agonist) and hCG. In addition, the luteolytic actions of gonadotropin releasing hormone (GnRH) were examined in FSH-primed granulosa cells treated with various luteotropic hormones.

MATERIALS AND METHODS

Reagents and Hormones

Ovine FSH (NIH-FSH-S13; FSH activity 15 × NIH-FSH-S1 units/mg; LH activity 0.05 × NIH-LH-S1 units/mg; Prl activity <0.1% by weight) and ovine Prl (NIH-P-S13; Prl activity 30 IU/mg; LH and FSH activities <0.1% by weight) were the generous gifts of the National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Disease. Human chorionic gonadotropin (hCG CR-121; 13,450 IU/mg) was the gift of Dr. R. E. Canfield through the Center for Population Research of the National Institute of Child Health and Human Development. A highly purified preparation of ovine Prl (Prl-90C) with Prl activity comparable to NIH-P-S13 was prepared by the method of Li (1980) to eliminate LH and FSH contamination and was generously provided by Dr. H. Papkoff (University of California, San Francisco). Terbutaline sulfate [brethine sulfate; 5—(2—([1, 1—dimethylethyl] amino) 1—hydroxyethyl) 1,3— benzenediol] was provided by Mr. C. A. Brownley, Jr., of Ciba-Geigy Corp. (Summit, NJ). Gonadotropin releasing hormone (GnRH) was the generous gift of Dr. N. C. Ling (Salk Institute, La Jolla, CA).

McCoy's 5a medium (modified; without serum), penicillin-streptomycin solution, L-glutamine, and trypan blue stain were obtained from Grand Island Biological Co. (Santa Clara, CA). Progesterone, 20α-hydroxypregn-4-en-3-one (20α-OH-P), pregnenolone, and Δ^4-androstene-3,17-dione (androstenedione) were purchased from Sigma Chemical Co. (St. Louis, MO). Diethylstilbestrol was obtained from Steraloids, Inc. (Wilton, NH). 2α-Cyano, 4,4,17α-trimethyl-17β-hydroxy-androst-5-en-3-one (cyanoketone) was provided by J. Allen Campbell, The Upjohn Co. (Kalamazoo, MI).

7(n)—[^{3}H] Pregnenolone (19.3 Ci/mmol), 20α-[1,2-^{3}H] OH-progesterone (55.7 Ci/mmol), and [4-^{14}C] progesterone (51.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Labeled steroids were purified before use by thin-layer chromatography using the system, chloroform:ether (5:1, v/v). The precoated silica plates were obtained from MC/B Manufacturing Chemists, Inc. (Cincinnati, OH).

Granulosa Cell Cultures

Immature female Sprague-Dawley rats (21—23 days old) were hypophysectomized by Curtis Johnson Labs. (Chicago, IL) and delivered on the third post-operative day. Silastic capsules (10 mm) containing diethylstilbestrol were implanted at the time of surgery. Hypophysectomized animals were given a mixture of bread, milk, and tap water and physiological saline (0.9% NaCl solution) ad libitum.

Four to six days after surgery, granulosa cells were obtained from the hypophysectomized, diethylstilbestrol-treated rats as described previously (Hsueh et al., 1980). These cells were cultured in 35 × 10 mm Falcon tissue culture dishes (2 to 4 × 10^6 viable cells/dish) in 1 ml of McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 nM androstenedione. Cells were cultured at 37°C in a humidified 95% air, 5% CO_2 incubator. At the end of the incubation, culture media were collected and stored at −20°C until analyzed for progesterone and 20α-OH-P contents by radioimmunoassay.

To examine pregnenolone production, granulosa cells (approx. 2 × 10^5 viable cells/dish) were incubated for 2 days in culture medium with FSH (10 ng/ml) and 100 nM androstenedione. After the 2-day incubation, cells were washed twice with 2 ml of culture medium and reincubated for another 2 days with the appropriate hormones in the presence of cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) (Goldman et al., 1965). After this incubation, media were collected and stored at −20°C until analyzed for pregnenolone contents by radioimmunoassay. In an initial set of experiments, FSH-primed cells were incubated for 2 days in either culture medium alone (control) or with Prl (3 μg/ml) in the absence or presence of increasing concentrations of cyanoketone. The concentration of pregnenolone in the media was low (<0.7 ng/ml) in both control (Fig. 1A) and Prl-treated cultures (Fig. 1B) when the cells were incubated in the absence of cyanoketone. When increasing concentrations of cyanoketone were added, medium content of pregnenolone increased while the concentrations of progesterone and 20α-OH-P decreased in a dose-dependent manner. At 10^{-6} M cyanoketone, maximal concentrations of medium pregnenolone were observed in both control and Prl-treated cells; this concentration of cyanoketone is used throughout the present study.

Radioimmunoassay

Medium progesterone contents were measured using specific antiserum supplied by Dr. G. Abraham (Wang et al., 1979). Medium 20α-OH-P contents were measured using specific antiserum supplied by Ralph Schwall (University of California, San Diego). The 20α-OH-P antiserum #316 cross-reacts <0.1% with progesterone, C_{19}, C_{18}, and other C_{21} steroids. Medium pregnenolone contents were measured with specific antiserum supplied by Dr. George P. Chrousos (NICHD, Bethesda, MD); this antiserum cross-reacts <0.3% with progesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 20α-OH-P, estradiol and

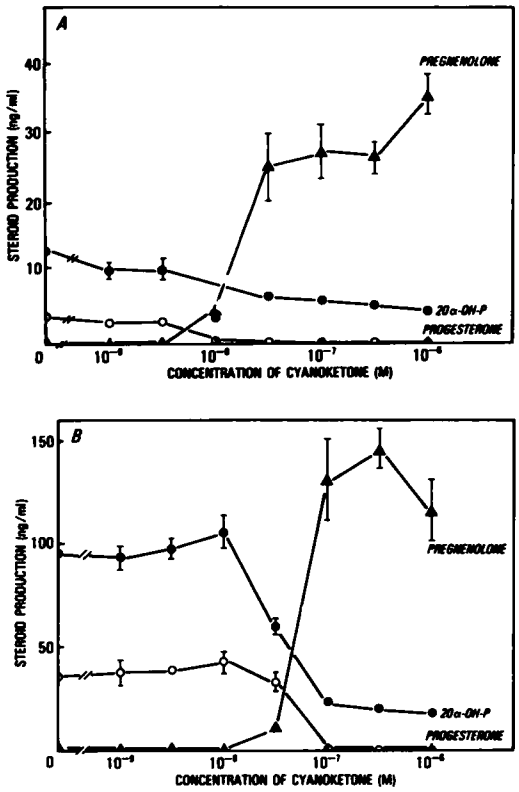

FIG. 1. Effect of increasing concentrations of cyanoketone on pregnenolone production by cultured granulosa cells. Granulosa cells (approx. 2 × 10^5 viable cells/dish) were incubated for 2 days in culture medium containing FSH (10 ng/ml). After 2 days, the cells were washed with 2 ml of medium and recultured for another 2 days with fresh medium alone (*A*) or Prl [NIH-P-S13; 3 μg/ml (*B*)] in the absence or presence of increasing concentrations of cyanoketone. Medium progesterone, 20α-OH-P, and pregnenolone contents were measured by radioimmunoassay. Data points represent mean ± SEM of four determinations. In several cases, the SEM is within the data point drawn in the figure.

testosterone. High concentrations (10^{-6} M) of diethylstilbestrol, GnRH, androstenedione, or cyanoketone in the culture medium did not interfere with the radioimmunoassays.

Assay of Steroidogenic Enzymes

The assay of 20α-HSD (EC 1.1.1.149) activity was based on a procedure developed by Eckstein et al. (1977) and modified by us (Jones and Hsueh, 1981b) in which 20α-HSD activity is measured as the rate of conversion of [^{3}H] 20α-OH-P to [^{3}H] progesterone. Unless indicated otherwise, a saturating concentration (50 μM) of 20α-OH-P was used in the enzyme assay.

The assay of 3β-HSD/$\Delta^{5,4}$-isomerase (EC 1.1.1.51/ EC 5.3.3.1) activity was based on a procedure developed by Murono and Payne (1979) in which 3β-HSD activity is measured as the rate of conversion of pregnenolone to progesterone. Our modification of this procedure has been described elsewhere (Jones and Hsueh, 1982). A saturating concentration (50 μM) of pregnenolone was used in the enzyme assay.

Statistical Analyses

Estimates of enzyme kinetic constants were calculated by a modified Lineweaver-Burk plot (Wilkinson, 1961) using a linear regression program. The estimates were adjusted and the standard errors were calculated using the statistical method of Wilkinson (1961). Other statistical analyses were performed using the Student's *t* test or, as indicated, analysis of variance (ANOVA). Comparisons with $P>0.01$ were not considered significant. The dose-response curves and ED_{50} values were derived by a four parameter logistic curve-fitting program (DeLean et al., 1978). Data points represent mean ± SEM of radioimmunoassay determinations from 4 separate cultures or enzyme activity determination from 3 separate assay tubes. For each figure, comparable results were obtained from at least 2 additional experiments.

RESULTS

Stimulation of Steroidogenesis by Prl, Terbutaline and hCG in FSH-Primed Granulosa Cells: Time Course of Action

We have shown that FSH treatment enhances the responsiveness of cultured rat granulosa cells to subsequent treatment with Prl (Wang et al., 1979; Jones and Hsueh, 1981a), β_2-adrenergic agents (Adashi and Hsueh, 1981; Jones and Hsueh, 1981a), and LH/hCG (Erickson et al., 1979) as indicated by increased progesterone production. To examine the time course of action of these tropic agents, granulosa cells were incubated for 2 days with FSH (10 ng/ml). The cultures were then washed and reincubated for another 2 days with fresh medium alone (control), or with Prl, terbutaline or hCG. Additionally, one set of cultures was incubated in the presence of 10^{-6} M cyanoketone to examine pregnenolone production. Throughout the 2-day incubation, sets of cultures were removed and media were analyzed for steroid content by radioimmunoassay. As shown in Fig. 2A, a low level of progesterone was detected in the media of control cultures, while Prl treatment stimulated progesterone production by approximately 10-fold at 48 h (9.4 ± 1.3-fold, N=4 experiments), compared with the controls. Treatment with hCG also resulted in an increase in medium progesterone during the first 24 h. This was followed by a slight decrease in progesterone concentration,

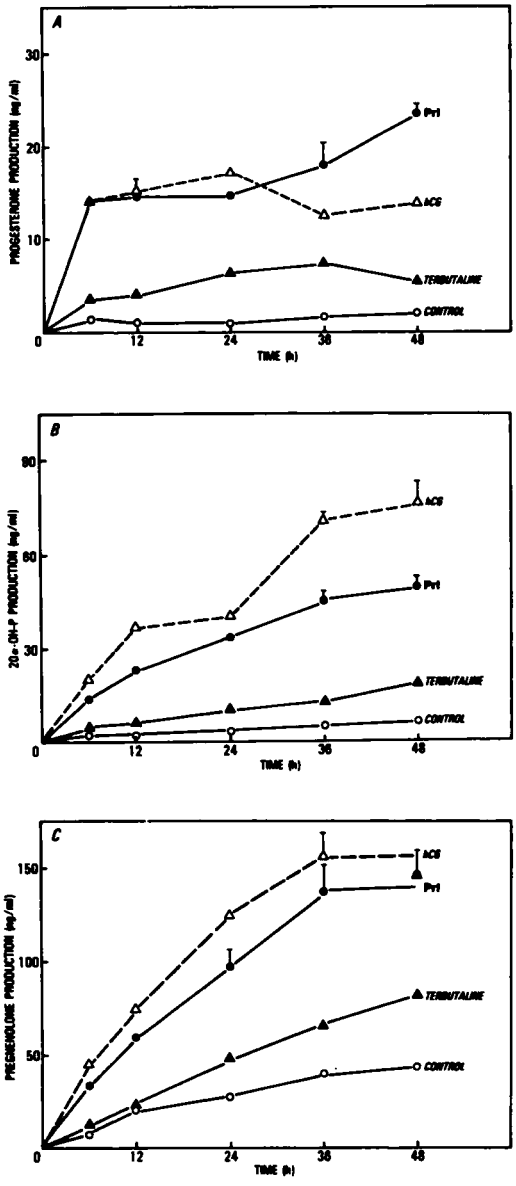

FIG. 2. Effects of treatment with Prl, terbutaline and hCG upon steroidogenesis by FSH-primed granulosa cells. Granulosa cells (approx. 2 X 10^5 viable cells/dish) were incubated for 2 days in culture medium with FSH (10 ng/ml). After incubation, the cells were washed and recultured for 2 days with fresh medium alone (*control*), with Prl (NIH-P-S13; 3 μg/ml), terbutaline (10^{-7} M), or hCG (30 ng/ml). Additionally, one set of cultures was incubated in the presence of 10^{-6} M cyanoketone to examine pregnenolone production. Throughout the 2-day incubation, sets of cultures were removed and medium progesterone (*A*), 20α-OH-P (*B*), and pregnenolone (*C*) contents were measured by radioimmunoassay. Data points represent mean ± SEM of four determinations. In several cases, the SEM is within the data point drawn in the figure.

representing an approximate 6-fold increase at 48 h (6.3 ± 0.8-fold; N=4 experiments). Similarly, terbutaline also increased medium progesterone during the 48 h of incubation (2.9 ± 0.3-fold; N=4 experiments).

In the same cultures, 20α-OH-P production was measured (Fig. 2B). Medium content of 20α-OH-P was low in the control cultures. Treatment with hCG, Prl or terbutaline for 48 h resulted in 17-fold (17.6 ± 1.8, N=4 experiments), 11-fold (9.0 ± 1.8, N=4), or 4-fold (3.5 ± 0.4; N=4) increases in 20α-OH-P production, respectively. The time courses of hCG and Prl actions were characterized by a plateau in 20α-OH-P production during the last 12 h of incubation, while 20α-OH-P accumulated in the media of terbutaline-treated cultures throughout the 48 h incubation.

As shown in Fig. 2C, medium content of pregnenolone increased in control cultures to 42.1 ± 2.3 ng/ml at 48 h. In cultures treated with either hCG or Prl, pregnenolone accumulated in the media during the first 36 h of incubation and plateaued during the last 12 h, resulting in a 3.4-fold increase in pregnenolone production at 48 h, as compared with the controls (hCG: 5.4 ± 1.0-fold and Prl: 4.2 ± 0.8-fold, N=7 experiments). Terbutaline treatment resulted in an increase in pregnenolone production throughout the 48 h incubation; at 48 h, medium pregnenolone content was approximately 2-fold greater than the controls (2.2 ± 0.2-fold; N=7 experiments).

Effect of Treatment with Prl, Terbutaline and hCG upon Pregnenolone Production by FSH-Primed Granulosa Cells: Dose-Response Studies

To examine the dose-dependency of the stimulation of pregnenolone production by Prl, terbutaline and hCG, FSH-primed granulosa cells were incubated for 2 days in medium containing 10^{-6} M cyanoketone (control), in the presence or absence of FSH (10 ng/ml), or increasing concentrations of Prl, hCG or terbutaline. FSH treatment stimulated pregnenolone production by approximately 2.6-fold while treatment with Prl resulted in a dose-dependent increase in pregnenolone production, reaching a maximal 4-fold increase with 10 μg/ml Prl (Fig. 3). The minimum effective dose was approximately 300 ng Prl/ml and the apparent ED_{50} value was 2 μg Prl/ml. Highly purified oPrl

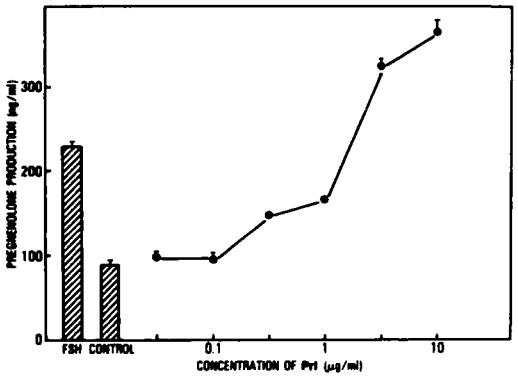

FIG. 3. Effect of treatment with Prl upon pregnenolone production by FSH-primed granulosa cells. Granulosa cells were primed with FSH as described in Fig. 2. The FSH-treated cells were washed and recultured for 2 days in fresh medium containing 10^{-6} M cyanoketone alone (*control*) or cyanoketone plus FSH (10 ng/ml) or increasing concentrations of Prl (NIH-P-S13). Medium pregnenolone contents were measured by radioimmunoassay. Data points represent mean ± SEM of four determinations. In several cases, the SEM is within the data point drawn in the figure.

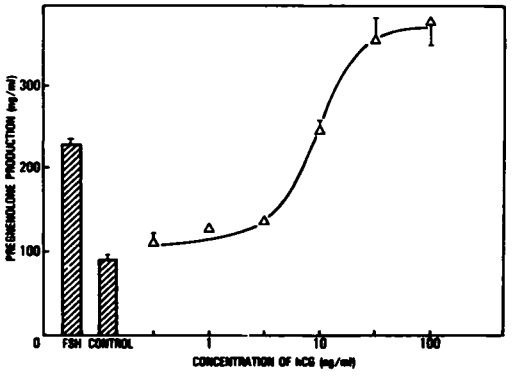

FIG. 4. Effect of treatment with hCG upon pregnenolone production by FSH-primed granulosa cells. Granulosa cells were primed with FSH as described in Fig. 2. The FSH-treated cells were washed and recultured for 2 days with fresh medium containing 10^{-6} M cyanoketone alone (*control*) or cyanoketone plus FSH (10 ng/ml) or increasing concentrations of hCG. Medium pregnenolone contents were measured by radioimmunoassay. Data points represent mean ± SEM of four determinations. In several cases, the SEM is within the data point drawn in the figure.

(Papkoff 90C) also stimulated pregnenolone production (control cells 50 ± 7 ng pregnenolone/ml; cells treated with 0.3 and 1.0 μg Prl/ml: 103 ± 10 and 170 ± 37 ng pregnenolone/ml, respectively; N=4 experiments).

Similarly, treatment with hCG also stimulated pregnenolone production in a dose-dependent manner, reaching a maximal (approx. 4-fold) increase at 30 ng hCG/ml (Fig. 4). The ED_{50} value was determined to be 9.63 ng hCG/ml.

As shown in Fig. 5, treatment with terbutaline also stimulated pregnenolone production in a dose-dependent manner, reaching a maximal (approx. 2.2-fold) increase at 10^{-7} M. The ED_{50} value was determined to 7.4×10^{-9} M.

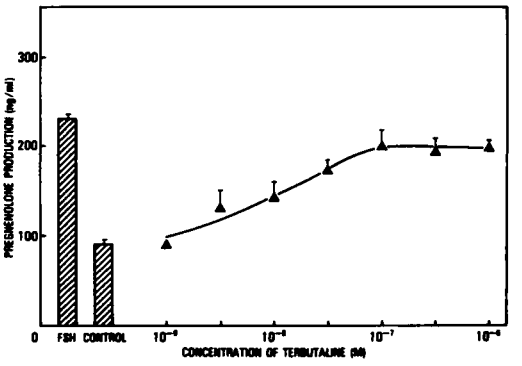

FIG. 5. Effect of treatment with terbutaline upon pregnenolone production by FSH-primed granulosa cells. Granulosa cells were primed with FSH as described in Fig. 2. The FSH-treated cells were washed and recultured for 2 days in fresh medium containing 10^{-6} M cyanoketone alone (*control*) or cyanoketone plus FSH (10 ng/ml), or increasing concentrations of terbutaline. Medium pregnenolone contents were measured by radioimmunoassay. Data points represent mean ± SEM of four determinations.

Effects of Treatment with FSH, Prl, Terbutaline and hCG upon 3β-HSD Activity of Cultured Granulosa Cells: Time Course of Action

To examine the hormonal modulation of 3β-HSD activity, granulosa cells were incubated in medium alone or with FSH. After 2 days of incubation, FSH-primed cultures were washed and reincubated in medium alone (control), or with Prl, terbutaline or hCG. At various intervals, 3β-HSD activities were determined as described in *Materials and Methods*. As shown in Fig. 6, FSH treatment increased enzyme activity in a time-dependent manner reaching 1.7- and 4.8-fold after 1 and 2 days of incubation. When FSH-primed cells were reincubated for 2 days in medium alone, 3β-HSD activity increased slightly (30% increase). When FSH-primed cells were treated with either Prl (NIH-P-S13) or hCG, 3β-HSD activity increased by

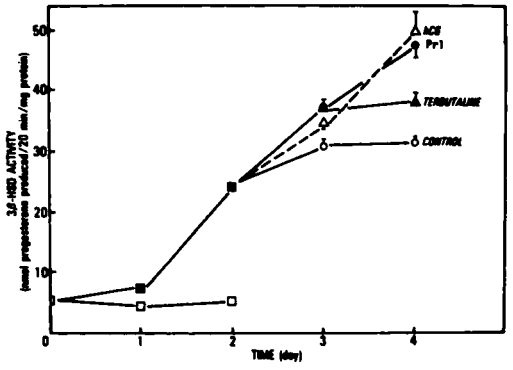

FIG. 6. Effects of treatment with FSH, Prl terbutaline and hCG upon 3β-HSD activity of cultured granulosa cells. Granulosa cells (approx. 4 × 10^5 viable cells/dish) were incubated in medium alone (*open squares*) or with FSH (10 ng/ml; *solid squares*). After 2 days of incubation, FSH-primed cultures were washed and reincubated in medium alone (*control*), or with Prl (NIH-P-S13; 3 μg/ml), terbutaline (10^{-7} M) or hCG (30 ng/ml). At various intervals, 3β-HSD activities were determined as described in *Materials and Methods*. Data points represent mean ± SEM of three determinations. In several cases, the SEM is within the data point drawn in the figure.

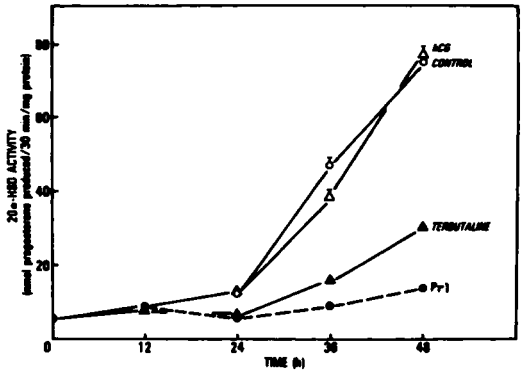

FIG. 7. Effects of treatment with Prl, terbutaline and hCG upon 20α-HSD activity of FSH-primed granulosa cells. Granulosa cells (approx. 4 × 10^5 viable cells/dish) were incubated for 2 days in culture medium containing FSH (10 ng/ml). After incubation, the FSH-treated cells were washed with 2 ml of medium and recultured for another 2 days with fresh medium alone (*control*), or with Prl (NIH-P-S13; 3 μg/ml), terbutaline (10^{-7} M), or hCG (30 ng/ml). At various intervals, 20α-HSD activities were determined as described in *Materials and Methods*. Data points represent mean ± SEM of three determinations. In several cases, the SEM is within the data point drawn in the figure.

approximately 2-fold during the 2-day incubation (hCG: 2.4 ± 0.6-fold; Prl: 2.2 ± 0.5-fold; N=3 experiments). Terbutaline treatment also increased enzyme activity by 1.6-fold during this time period (1.6 ± 0.2-fold; N=4 experiments). Furthermore, treatment with highly purified oPrl (Papkoff 90C; 1 μg/ml) also stimulated 3β-HSD activity (control cells: 30.0 ± 0.3 nmol progesterone produced/20 min per mg protein; cells treated with Prl: 43.5 ± 2.1 nmol progesterone produced/20 min per mg protein).

Effects of Treatment with Prl, Terbutaline and hCG upon 20α-HSD Activity of FSH-Primed Granulosa Cells: Time Course of Action

We have previously shown that FSH treatment for 2 days results in a slight (30–40%) increase in 20α-HSD activity (Jones and Hsueh, 1981a,b). To examine the time course of the modulation of 20α-HSD activity by Prl, hCG and terbutaline, granulosa cells were incubated for 2 days in medium containing FSH. After washing, the cultures were reincubated for another 2 days in medium alone (control), or with Prl, terbutaline or hCG. At various intervals, 20α-HSD activities were determined as described in *Materials and Methods*. As shown in Fig. 7, the enzyme activity of control cultures increased dramatically (12.5-fold) during the 2-day incubation; hCG treatment did not affect this increase. In marked contrast, treatment with Prl or terbutaline inhibited the increase in 20α-HSD activity by 81% or 59%, respectively.

Determination of V_{max} and K_m Values for 20α-HSD in FSH-Primed Cells Treated with Prl or Terbutaline

To examine the effect of treatment with Prl or terbutaline on the kinetic constants of 20α-HSD, granulosa cells were incubated for 2 days in medium containing FSH. After washing, the cultures were reincubated for 2 days in medium alone (control) or with Prl or terbutaline. Enzyme activities were determined in the presence of increasing concentrations of 20α-OH-P, as described in *Materials and Methods*. To determine the apparent enzyme kinetic constants (V_{max} and K_m), data were calculated by a modified Lineweaver-Burk plot (Wilkinson, 1961) as shown in Fig. 8A. A linear regression program was used to calculate the

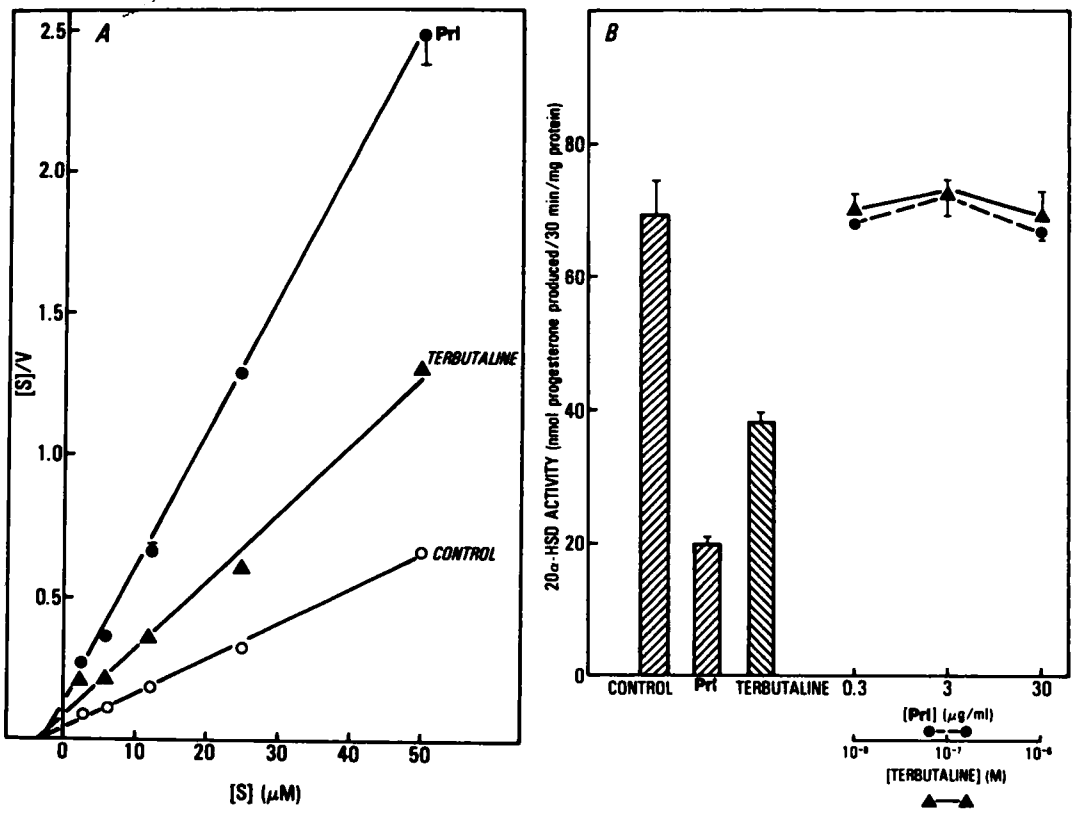

FIG. 8. Determination of V_{max} and K_m values for 20α-HSD in FSH-primed granulosa cells treated with Prl or terbutaline. Granulosa cells were primed with FSH as described in Fig. 7. The cells were washed and recultured for 2 days in culture medium alone (*control*), or Prl (NIH-P-S13; 3 μg/ml), or terbutaline (10^{-7} M). Aliquots of cell homogenate were incubated with increasing concentrations of 20α-OH-P and 20α-HSD activities were determined as described in *Materials and Methods*. *A)* The enzyme kinetic constants were estimated by a modified Lineweaver-Burk plot, using a linear regression program. The estimates were adjusted and statistical errors were calculated using the method of Wilkinson (1961). *B)* To examine a possible direct interference of the enzyme assay by Prl or terbutaline, cell homogenates were obtained from cultures incubated with medium alone (*control*) or with Prl (3 μg/ml) or terbutaline (10^{-7} M). During the enzyme assay, aliquots of cell homogenate from the control group were also incubated with increasing concentrations of Prl or terbutaline. Enzyme activities were determined as described in *Materials and Methods*. All data points represent mean ± SEM of three determinations. In several cases, the SEM is within the data point drawn in the figure.

slope (inverse of the apparent V_{max}) and X-intercept (negative value of the apparent K_m). The estimates were adjusted and statistical errors calculated (Wilkinson, 1961). Prl treatment resulted in a 74% decrease in the apparent maximal velocity from 83.7 ± 3.7 (control) to 21.7 ± 1.0 nmol progesterone produced/30 min per mg protein. Similarly, terbutaline treatment resulted in a 45% decrease in apparent V_{max} to 46.0 ± 4.0 nmol progesterone produced/30 min per mg protein. In contrast, the K_m value was not affected by treatment with the luteotropic agents (controls: 3.10 ± 0.53 μM; Prl-treated: 2.09 ± 0.47 μM; terbutaline-treated: 4.84 ± 1.27 μM). These data indicate that the inhibitory effect of Prl or terbutaline is due to a decrease in the concentration of the enzyme rather than changes in the affinity of the enzyme to its substrate.

The possibility that Prl or terbutaline may interfere with the enzyme assay was tested by incubating aliquots of cell homogenate, obtained from control cultures, with various concentrations of either Prl or terbutaline during the enzyme assay. As shown in Fig. 8B, treatment with Prl (3 μg/ml) or terbutaline (10^{-7} M) for 2 days inhibited 20α-HSD activity by 71% or 45%, respectively (*batched bars*). In contrast,

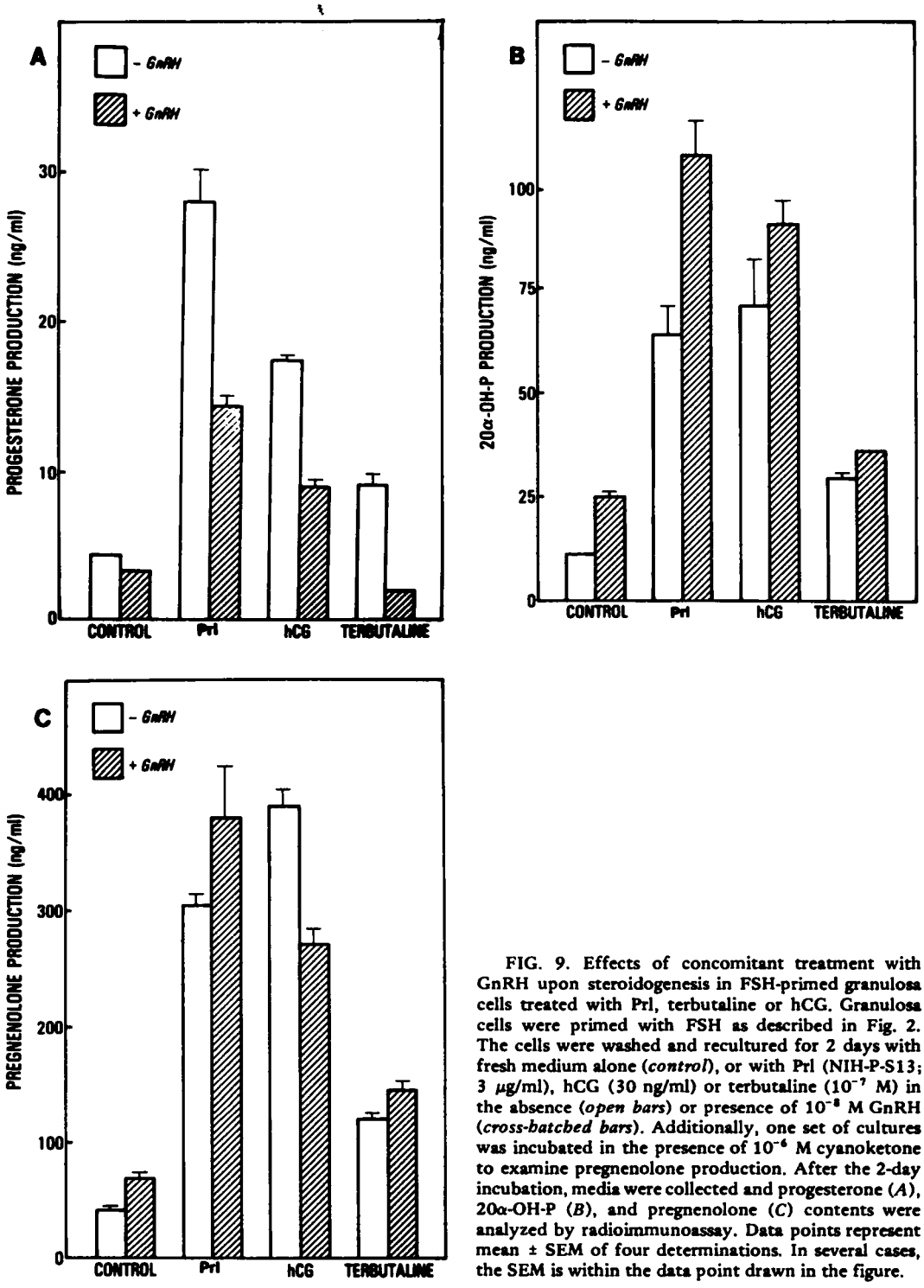

FIG. 9. Effects of concomitant treatment with GnRH upon steroidogenesis in FSH-primed granulosa cells treated with Prl, terbutaline or hCG. Granulosa cells were primed with FSH as described in Fig. 2. The cells were washed and recultured for 2 days with fresh medium alone (*control*), or with Prl (NIH-P-S13; 3 μg/ml), hCG (30 ng/ml) or terbutaline (10^{-7} M) in the absence (*open bars*) or presence of 10^{-8} M GnRH (*cross-batched bars*). Additionally, one set of cultures was incubated in the presence of 10^{-6} M cyanoketone to examine pregnenolone production. After the 2-day incubation, media were collected and progesterone (*A*), 20α-OH-P (*B*), and pregnenolone (*C*) contents were analyzed by radioimmunoassay. Data points represent mean ± SEM of four determinations. In several cases, the SEM is within the data point drawn in the figure.

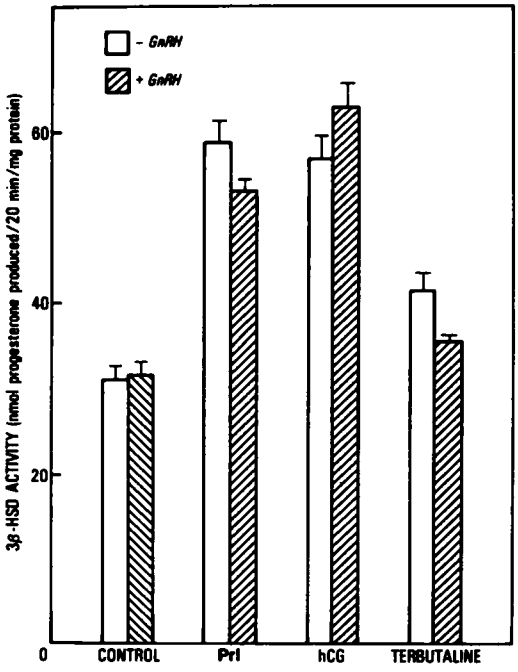

FIG. 10. Effects of concomitant treatment with GnRH upon 3β-HSD activity in granulosa cells treated with Prl, terbutaline or hCG. Granulosa cells were primed with FSH as described in Fig. 7. The cells were washed and recultured for 2 days in culture medium alone (*control*), or with Prl (NIH-P-S13; 3 μg/ml), hCG (30 ng/ml) or terbutaline (10^{-7}M) in the absence (*open bars*) or presence of 10^{-8} M GnRH (*cross-hatched bars*). Enzyme activities were determined as described in *Materials and Methods*. Data points represent mean ± SEM of three determinations.

the presence of either Prl or terbutaline during the enzyme assay did not affect enzyme activity (ANOVA; P=0.28).

Effects of Concomitant Treatment with GnRH upon Steroidogenesis by Granulosa Cells Treated with Prl, hCG or Terbutaline

To examine the modulatory effects of GnRH on steroidogenesis, granulosa cells were primed for 2 days with FSH. After washing, the cultures were reincubated for 2 days in medium alone (control) or with Prl, hCG or terbutaline in the absence or presence of 10^{-8} M GnRH. Additionally, one set of cultures was incubated in the presence of 10^{-6} M cyanoketone and various hormones to examine pregnenolone production. After the 2-day incubation, steroid content in the medium was analyzed by radioimmunoassay. Treatment with GnRH alone did not significantly affect progesterone production as compared with the untreated controls (Fig. 9A). In contrast, concomitant treatment with GnRH decreased progesterone production stimulated by Prl, hCG or terbutaline by 48% (54 ± 4; N=3 experiments), 48% (51 ± 4; N=3) and 78% (60 ± 11; N=3), respectively.

In the same cultures, medium concentrations of 20α-OH-P were measured (Fig. 9B). Treatment with GnRH alone resulted in a 2.2-fold (2.6 ± 0.2; N=3 experiments) increase in 20α-OH-P production, compared with the untreated controls. Similarly, concomitant treatment with GnRH enhanced Prl- or terbutaline-stimulated 20α-OH-P production by 1.7-fold (1.65 ± 0.09; N=3 experiments) and 1.2-fold (1.49 ± 0.14; N=3 experiments), respectively. In contrast, GnRH did not significantly affect hCG-stimulated 20α-OH-P production.

As shown in Fig. 9C, treatment with GnRH alone resulted in a 1.6-fold (1.55 ± 0.18; N=4 experiments) increase in pregnenolone production, compared with the untreated controls. Concomitant treatment with GnRH did not affect pregnenolone production stimulated by Prl but caused a slight (22 ± 4%; N=4 experiments) increase in terbutaline-stimulated pregnenolone production. In marked contrast, concomitant treatment with GnRH decreased hCG-stimulated pregnenolone production by 30% (40 ± 8%; N=4 experiments).

Effects of Concomitant Treatment with GnRH upon the Activities of 3β-HSD and 20α-HSD in Granulosa Cells Treated with Prl, hCG or Terbutaline

To examine possible modulatory effects of GnRH on 3β-HSD and 20α-HSD activities, granulosa cells were incubated for 2 days in medium containing FSH. After washing, the cells were reincubated for 2 days in medium alone (control), or with Prl, hCG or terbutaline in the absence or presence of 10^{-8} M GnRH. After 2 days, enzyme activities were determined as described in *Materials and Methods*. As shown in Fig. 10, treatment with GnRH alone or in combination with any of the luteotropic agents did not significantly affect 3β-HSD activity. Furthermore, GnRH treatment did not significantly affect the activity of 20α-HSD in control cultures (Fig. 11). In contrast, concomitant treatment with GnRH increased 20α-HSD activities of Prl-, hCG- and terbutaline-treated cultures by 3.6-fold (5.2 ±

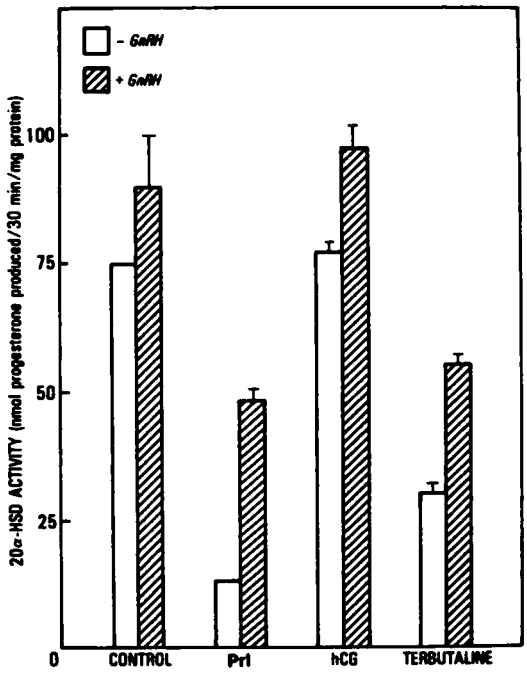

FIG. 11. Effects of concomitant treatment with GnRH upon 20α-HSD activity in granulosa cells treated with Prl, terbutaline or hCG. Granulosa cells were primed with FSH as described in Fig. 7. The cells were washed and recultured for 2 days in culture medium alone (*control*) or with Prl (NIH-P-S13; 3 μg/ml), hCG (30 ng/ml) or terbutaline (10^{-7} M) in the absence (*open bars*) or presence of 10^{-8} M GnRH (*cross-hatched bars*). Enzyme activities were determined as described in *Materials and Methods*. Data points represent mean ± SEM of three determinations. In several cases, the SEM is within the data point drawn in the figure.

0.2; N=6), 1.3-fold (1.8 ± 0.2; N=6), and 1.8-fold (2.5 ± 0.1; N=6), respectively.

DISCUSSION

The results of this study demonstrate that: *1*) treatment with Prl, hCG or terbutaline increases pregnenolone production in a dose- and time-dependent manner; *2*) treatment with Prl, hCG or terbutaline increases 3β-HSD activity of FSH-primed granulosa cells; *3*) treatment with either Prl or terbutaline inhibits the increase of 20α-HSD activity in FSH-primed granulosa cells, while treatment with hCG has no effect; *4*) the inhibition of 20α-HSD activity by Prl or terbutaline is characterized by a decrease in the apparent maximal velocity of the enzyme with no effect on its K_m value; and *5*) the inhibition of Prl- or terbutaline-stimulated progesterone production by GnRH is associated with increases in both 20α-OH-P production and activities of 20α-HSD enzyme with no inhibition of pregnenolone production, while the GnRH inhibition of hCG-stimulated progesterone production is associated with decreased pregnenolone production with no effect on 20α-OH-P production.

Prl, hCG and β_2-adrenergic agents stimulate progesterone production by rat luteal cells. The present culture of FSH-primed "granulosa-luteal" cells provided a model for the study of the mechanisms of action of the luteotropic agents in vitro. In the present study, Prl was shown to increase pregnenolone production in a dose- and time-dependent manner. Serum Prl concentrations have been shown to increase to 3.66 mIU/ml during proestrus in rats (Smith et al., 1975). Since 0.1 μg Prl/ml (3.0 mIU/ml) did not increase pregnenolone production (Fig. 3), it is unlikely that Prl-stimulated pregnenolone production contributes significantly to progesterone production during the rat estrous cycle. However, it is possible that the Prl stimulation of pregnenolone production may be important during early pregnancy when serum Prl concentrations increase to approximately 11 mIU/ml (Beattie et al., 1977). Earlier studies have indicated that Prl increases cholesterol synthesis in rat corpora lutea (Armstrong et al., 1969, 1970; Behrman et al., 1971), which may lead to increased pregnenolone production. In the cultured granulosa cells, Prl also increased 3β-HSD activity which is consistent with histochemical evidence showing that Prl increases the 3β-HSD activity of rat granulosa cells (Madej, 1980).

In vivo studies suggested that the major direct effect of Prl upon luteal steroidogenesis is due to the inhibition of the metabolism of progesterone to 20α-OH-P (Armstrong et al., 1969; Hashimoto and Wiest, 1969; Lamprecht et al., 1969; Raj and Moudgal, 1970). The results of the present study demonstrate that Prl treatment in vitro decreases the apparent maximal velocity without affecting the K_m value of 20α-HSD, suggesting that Prl decreases the cellular concentration of the enzyme. This concept is strengthened by the observation that Prl treatment inhibited the time-dependent increase in 20α-HSD activity in FSH-primed cells (Fig. 7). Thus, in the FSH-primed rat granulosa cells, Prl increases progesterone production by increasing pregnenolone produc-

tion and 3β-HSD activity and by decreasing the metabolism of progesterone to 20α-OH-P.

Like Prl, the β_2-adrenergic agonist decreases 20α-HSD activity and increases pregnenolone production. The present study demonstrates, for the first time, that treatment with a β_2-adrenergic agonist increases pregnenolone production and 3β-HSD activity in rat granulosa cells. Although there are no extensive studies on the regulation of progestin biosynthesis by catecholamines in vivo, Burden and Lawrence (1977) have observed that ovarian denervation or chemical sympathectomy decreases 3β-HSD activity of rat corpora lutea, suggesting that catecholamines may influence 3β-HSD activity in vivo. In an earlier study, we demonstrated that β-adrenergic agonists inhibit 20α-HSD activity of FSH-primed granulosa cells (Jones and Hsueh, 1981a). The present results suggest that β-adrenergic agonists inhibit 20α-HSD activity by decreasing the cellular concentration of the enzyme without affecting its K_m value. Time course studies of 20α-HSD activity also indicates that the increase of 20α-HSD activity in FSH-primed cells is inhibited by terbutaline treatment (Fig. 7). Thus, the results suggest that β-adrenergic agents increase progesterone production by stimulating pregnenolone production and 3β-HSD activity, as well as by decreasing the metabolism of progesterone to 20α-OH-P. This mechanism not only explains the β-adrenergic agonist stimulation of progesterone production by cultured granulosa cells (Adashi and Hsueh, 1981; Kliachkno and Zor, 1981; Fig. 2), but may also serve to explain the catecholamine stimulation of progesterone production by corpora lutea (Condon and Black, 1976; Godkin et al., 1977; Jordan et al., 1978; Ratner et al., 1980).

Treatment with hCG stimulated pregnenolone production and 3β-HSD activity. Similarly, the mechanism by which LH or hCG stimulates progesterone production in vivo appears to involve the stimulation of pregnenolone production, possibly by facilitating the intramitochondrial movement of cholesterol to the site of side-chain cleavage enzymes, and the stimulation of 3β-HSD activity (Rubin et al., 1963; Loewit et al., 1970; Rondell, 1974; Boyd et al., 1975; Suzuki and Tamaoki, 1979; Madej, 1980; Henderson et al., 1981). Treatment with hCG did not inhibit 20α-HSD activity (Figs. 2 and 7). In the hCG-treated group, medium progesterone decreased during the last 24 h of incubation while 20α-OH-P increased; this correlates with the spontaneous rise in 20α-HSD activity observed during the same time period. A possible regulatory role of LH/hCG in vivo on ovarian 20α-HSD activity is unclear (Hashimoto and Wiest, 1969; Loewit et al., 1970; Hickman-Smith and Kuhn, 1976; Loewit and Zambelis, 1979; Suzuki and Tamaoki, 1979). However, our data are consistent with the finding by Eckstein et al. (1977) who observed that the level of ovarian 20α-HSD activity in proestrous rats was maximal before the first LH surge; increases in serum LH did not affect 20α-HSD activity. Thus, in the FSH-primed rat granulosa cells, hCG (LH) increases progesterone production by increasing pregnenolone production and the conversion of pregnenolone to progesterone without affecting 20α-HSD activity.

The relationship between 20α-HSD activity and 20α-OH-P production deserves further comment. Treatment with hCG does not affect 20α-HSD activity, while 20α-OH-P production is actually increased. In contrast, Prl and terbutaline inhibit 20α-HSD activity, but stimulate 20α-OH-P production. In the granulosa cells, the level of 20α-OH-P depends on the activity of 20α-HSD as well as the availability of the enzyme substrate progesterone. Although hCG does not affect 20α-HSD activity during the 2-day incubation, the levels of enzyme activity in the control cells increase by an order of magnitude. Thus, the high basal level of 20α-HSD activity, coupled with the hCG-stimulated progesterone production, leads to the increase in 20α-OH-P production. While Prl and terbutaline inhibit 20α-HSD activity, enzyme activity is not completely inhibited. As a result, the stimulation of progesterone biosynthesis by Prl and terbutaline leads to a net increase in 20α-OH-P production.

Earlier studies have demonstrated that GnRH treatment inhibits progesterone production stimulated by Prl or LH in FSH-primed granulosa cells (Hsueh and Jones, 1981). Treatment with GnRH agonists also inhibits progesterone production induced by LH, hCG or epinephrine in rat luteal cells (Hsueh and Jones, 1981). However, the mechanisms of the inhibitory effect of GnRH on progesterone production induced by these luteotropic agents were not examined. Our studies on the modulatory effects of GnRH on progestin biosynthesis stimulated by Prl, hCG and terbutaline are summarized in Fig. 12. These results indicate

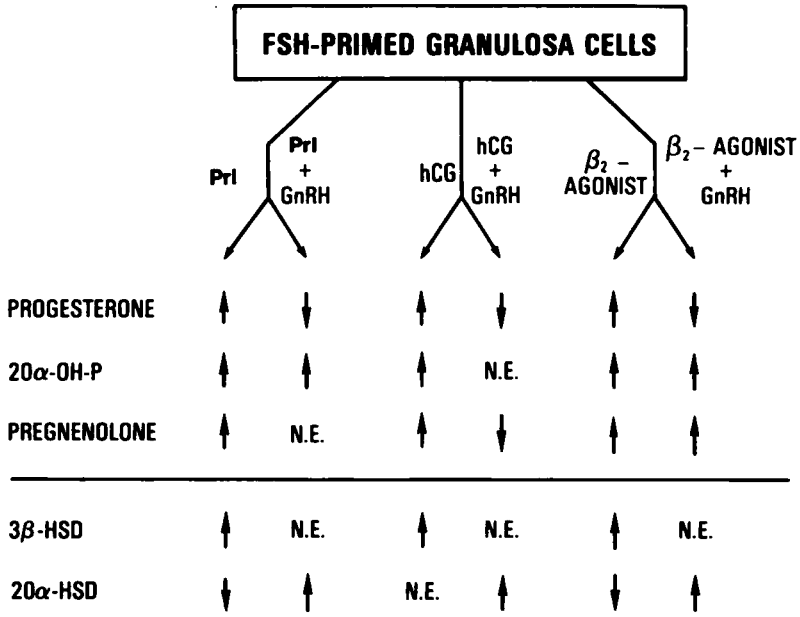

FIG. 12. Modulation of ovarian progestin production and steroidogenic enzymes by GnRH in FSH-primed granulosa cells treated with various luteotropic agents. *N.E.*=No effect.

that the GnRH inhibition of progesterone production stimulated by Prl or a β-adrenergic agonist is due primarily to an increase in 20α-HSD activity, resulting in an increase in the metabolism of progesterone to 20α-OH-P. In contrast, the GnRH inhibition of hCG-stimulated progesterone production appears to be the result of an inhibition of pregnenolone production with no effect on 20α-OH-P production. Although 20α-OH-P appears to be the major metabolite of progesterone in rat granulosa cells (Nimrod, 1981), one cannot rule out the possibility that GnRH treatment stimulates the formation of additional degradative pathways.

In this report, the regulation of the progestin biosynthetic pathway was examined in FSH-primed granulosa cells which acquire many functional characteristics similar to those of the luteal cells. Although these granulosa-luteal cells may, in some subtle ways, differ from luteal cells in vivo because the latter are derived from both granulosa and thecal cells, the present culture system provides a useful model system for the study of the functions of luteinized granulosa cells. Future studies on in vitro regulation of steroidogenic enzymes by these diverse luteotropic and luteolytic agents should provide new information regarding the mechanism of action of these hormones.

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