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FINAL REPORT:

Title: Direct and indirect actions of a gonadotropin-releasing hormone agonist on ovarian cells in vivo and in vitro.

USAID Award or Contract Number: DAN-5053-G-SS-8028-00

Period of Total Award: 08-31-1988 to 09-30-1990

Professional Personnel:

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Publications: (copies enclosed)

Smith CJ, Greer TB, Banks TW, Sridaran R. (1989). The response of large and small luteal cells from the pregnant rat to substrates and secretagogues. Biology of Reproduction 41:1123-1132.

Sridaran R, Krishna A, Srivastava RK. (1991). Effects of long-term treatment of a gonadotropin-releasing hormone agonist on ovarian function in the rat. Chapter 47 in Signalling mechanisms and gene expression in the ovary. G. Gibori, Ed., Springer-verlag Inc., New York: 390-394.

The objective of this proposal was to elucidate the physiological significance of gonadotropin-releasing hormone (GnRH) as a regulatory molecule in the ovary.

Specific aim: To examine in detail the short-term and long-term treatment of GnRH agonist (GnRH-Ag) on the synthesis of gonadotropins and their receptors; and also the consequences of these changes on ovarian follicular development and steroidogenesis.

Study: Young adult female rats (160-175 gm) of Sprague-Dawley (CD strain, cobb variety) were obtained from Charles River Laboratories (Wilmington, MA). Rats were exposed to a daily 14L:10D photoperiod (lights on from 0600 to 2000 h) at 23-25 C. The estrous cyclicity of rats were monitored by taking vaginal smears daily. Only the rats that exhibited at least 2 regular estrous cycles were chosen for the experiment. Purina rat chow and tap water were available ad libitum. Each rat was implanted sc on the dorsal surface with a minipump (2002; Alza Corp., CA) containing a GnRH-Ag (WY-40972, a gift from Wyeth-Ayerst Laboratories) starting on the day of estrus. Each minipump released continuously 0.2, 1 or 5 µg/day of GnRH-Ag for either a period of 7 days (short-term treatment) or a period of 28 days (long-term treatment). Sham surgery was performed in controls, but they received no treatment. Rats were weighed individually, every week, for the duration of the experiment. At the end of the experiment, rats were dissected while they were under ether anesthesia. After obtaining the total ovarian weight, the ovaries were processed for light microscopy. Ovaries were sectioned serially 6-8 µ and were stained with hematoxylin and eosin. Follicles were counted in every tenth section at 100x magnification. Then, rats were exsanguinated by bleeding via the abdominal aorta. The blood was allowed to clot at the room temperature, centrifuged at 4 C and serum stored frozen for the measurement of steroid and peptide hormones as described by us previously. Neuropeptide Y (NPY) levels were also measured in the hypothalami obtained from these rats. Differences between two groups were analyzed by Student's t-test. A p value less than 0.05 was considered significant.

The results obtained from long-term treatment of GnRH-Ag has been published as a Chapter in a book (please see the enclosed copy). Briefly, the continuous administration of GnRH-Ag at the dose of 1 or 5 µg/day suppressed the number of small and big antral and atretic follicles. The

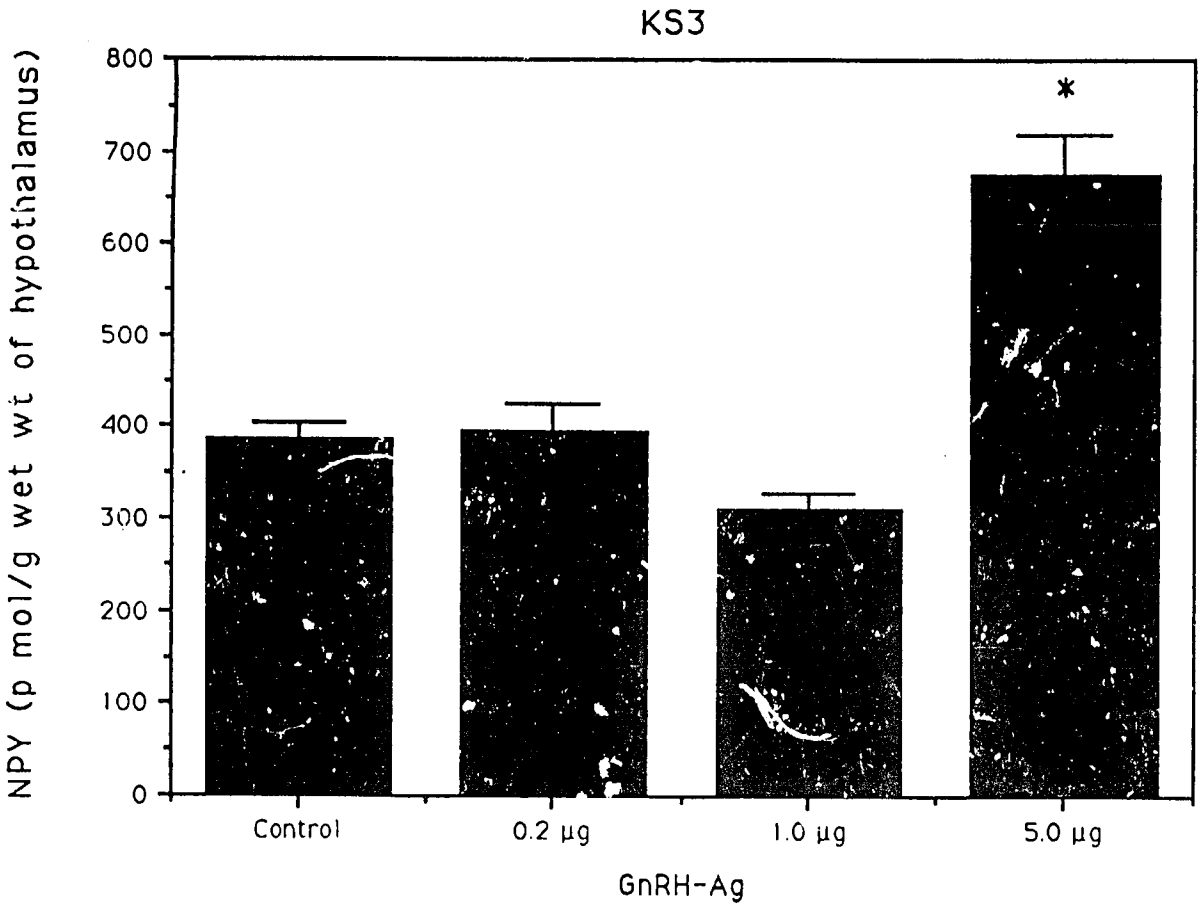
recruitment of new big follicles was totally absent and small follicles was greatly reduced. This is indicative of the absence of a secondary FSH surge in these rats. The results also suggest that GnRH-Ag arrests the growth of antral and atretic follicles of both sizes, as reflected in the secretion of various steroid hormones. The reduction in the steroid hormonal milieu and associated changes in the follicular development has exhibited an interruption in the estrous cyclicity of these rats and thus an inhibition of ovulation. The dose-dependent weight gain noticed in these rats is an interesting observation. In our hands, NPY levels in the hypothlami obtained from these rats (Figure enclosed) indicate that GnRH-Ag at the dose of 5 µg/day elevated hypothalamic NPY levels. NPY has been shown to play an important role in the neural regulation of feeding behavior. Therefore, the weight gain triggered by the administration of GnRH-Ag in these rats may play a role in the stimulation of feeding in these rats which may be associated with the secretion of NPY, endogenous opioid peptides and/or catecholamines. The data obtained from short-term treated rats are given in a table (see enclosure).

Specific Aim: Pattern of LH- and FSH-induced cAMP and steroid production (in vitro) by follicles (preantral, early and late antral) isolated from ovaries of rats treated (in vivo) with various GnRH-Ag dosage regimen.

Study: The study had been carried out as described in tne proposal. Steroid and cAMP levels have been measured in most of the samples. When these measurements in the remaining samples are completed the data will be analyzed.

Specific Aim: To investigate the effect of GnRH on oocyte maturation.

Study: The duration of the award period and funds were insufficient to conduct this study primarily because we needed to study NPY levels in the hypothalami of earlier study that was not in the original proposal.


KS3
NPY (p mol/g wet wt of hypothalamus)
800
700
600
500
400
300
200
100
0
*
Control
0.2 µg
1.0 µg
5.0 µg
GnRH-Ag

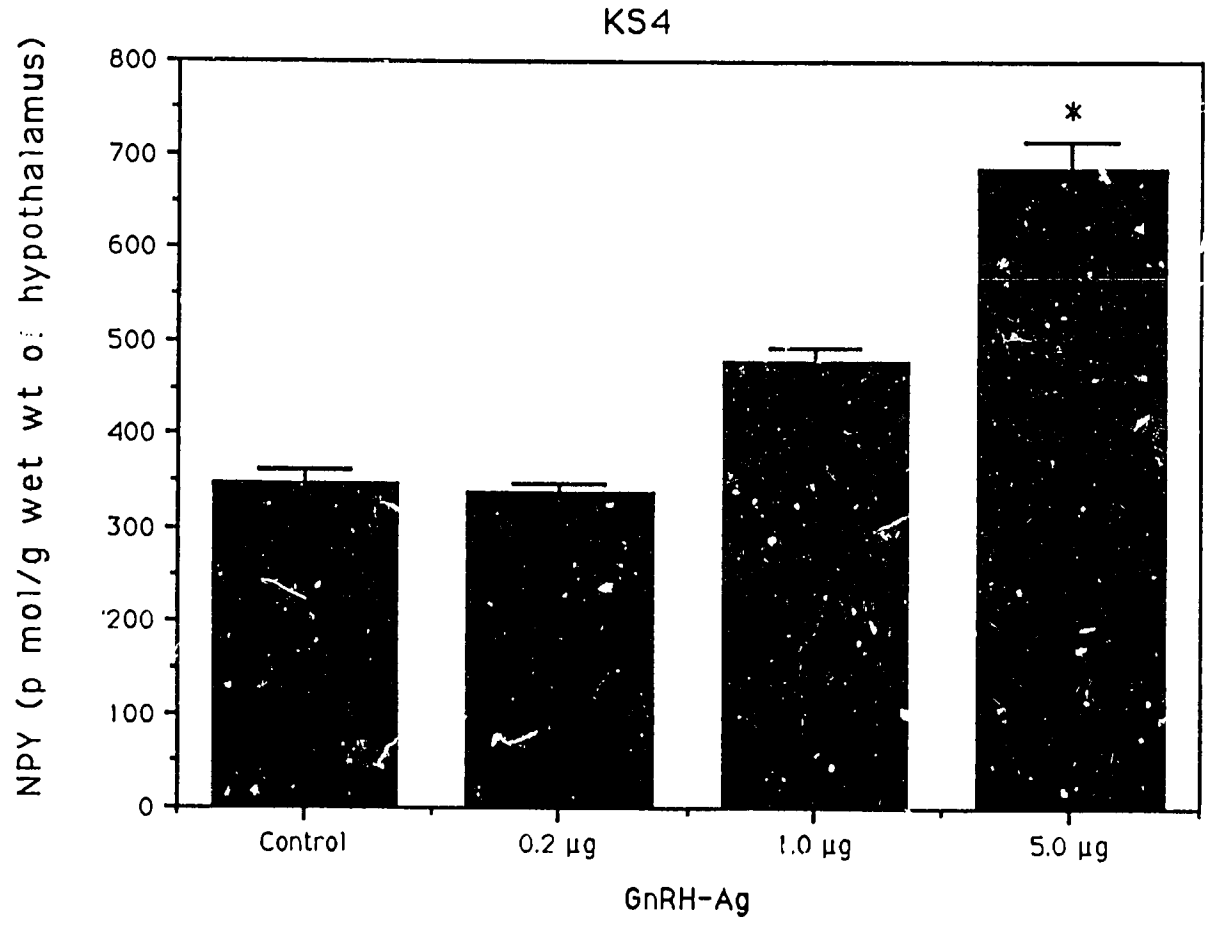

KS4
NPY (p mol/g wet wt of hypothalamus)
800
700
600
500
400
300
200
100
0
*
Control
0.2 µg
1.0 µg
5.0 µg
GnRH-Ag

TABLE 1. EFFECT OF SHORT-TERM (7 DAYS) TREATMENT OF GnRH AGONIST

Treatment	Body Weight Gain (gm)	Ovarian Weight (mg)	Uterine Weight (mg)
Control	9.4 ± 1.3*	137.1 ± 5.5	493.0 ± 24.9
0.2 µg/day	14.0 ± 2.2	102.3 ± 11.0	555.0 ± 62.0
1.0 µg/day	23.4 ± 2.2	90.3 ± 4.8	287.2 ± 7.8
5.0 µg/day	24.3 ± 2.6	101.0 ± 4.8	314.5 ± 15.6

*Values are expressed as the Mean ± SE

EFFECTS OF LONG-TERM TREATMENT OF A GONADOTROPIN-RELEASING HORMONE AGONIST ON OVARIAN FUNCTION IN THE RAT

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ABSTRACT

To examine in detail the long-term treatment (28 days) of a gonadotropin-releasing hormone agonist (GnRH-Ag; Wyeth-40972) on ovarian function, rats were treated continuously with 0.2, 1 or 5 μg/day of GnRH-Ag using an osmotic minipump (Alza). Each rat was implanted sc on the dorsal surface with a minipump starting on the day of estrus. Only the rats that exhibited at least 2 regular estrous cycles were chosen for the experiment. Sham surgery was performed in controls, but they received no treatment. The ovarian histology revealed the inhibitory effect of GnRH-Ag on the follicular development. The number of big antral follicles was 4.1±1.5 in 0.2 μg/day of GnRH-Ag group as compared to 8.2±1.5 in controls. No big antral follicles were present in the remaining treated groups. The two high doses of GnRH-Ag suppressed the number of small antral follicles and also big and small atretic follicles when compared to controls ($p<0.001$). GnRH-Ag at the dose of 0.2 μg/day was without any effect on these follicles. The ovarian weight was decreased by the treatment at all doses. The peripheral levels of progesterone in rats treated with 0, 0.2, 1 or 5 μg of GnRH-Ag were 32±6, 17±3, 18±2, and 18±4 ng/ml, respectively. The peripheral levels of testosterone and estradiol showed similar suppression by various doses of GnRH-Ag except the testosterone levels at 518±77 in 0.2 μg treated group were not different from controls at 531±76 pg/ml. The body weight gain of rats treated with 0.2, 1 or 5 μg of GnRH-Ag was 63±4, 88±8 and 97±5 when compared to 54±6 gm in controls. These data suggest that GnRH-Ag arrests the growth of antral and atretic follicles of both sizes which is reflected in the secretion of the various steroid hormones. The dose dependent weight gain in these rats is an interesting observation and the mechanism by which this is triggered is under investigation.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) analogs are associated with the inhibition of a variety of female reproductive functions such as ovarian steroidogenesis, ovulation, ovarian transport,

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Chapter 47: In Signalling mechanisms and gene expression in the Ovary. G. Gibori, Ed., Springer-Verlag Inc. New York: 390-394, 1991.

5

ovarian implantation, pregnancy, and uterine growth (1). GnRH and its analogs are also shown to delay puberty, induce premature meiotic maturation of ova within preantral and preovulatory follicles and disrupt the normal ovarian cycle (1). Although the GnRH agonists are known to halt follicular development (2,3), no studies quantified changes in follicle numbers and their size distribution following long-term treatment.

MATERIALS AND METHODS

Young adult female rats (160-175 g) of Sprague-Dawley (CD strain, Cobb variety) were obtained from Charles River Laboratories (Wilmington, MA). Rats were exposed to a daily 14L:10D photoperiod (lights on from 0600 to 2000 h) at 23-25°C. The estrous cyclicity of rats were monitored by taking vaginal smears daily. Only the rats that exhibited at least 2 regular estrous cycles were chosen for the experiment. Purina rat chow (Ralston-Purina Co., St. Louis, MO) was available ad libitum.

Each rat was implanted sc on the dorsal surface with a minipump (2002; Alza Corp., CA) containing a GnRH-agonist (GnRH-Ag; WY-40972, a gift from Wyeth-Ayerst Laboratories) starting on the day of estrus. Each minipump released continuously 0.2, 1, or 5 μg/day of GnRH-Ag for a period of 14 days. Therefore, on day 15, each minipump was replaced with a new pump containing identical drug treatment, with the rat under brief ether anesthesia. Sham surgery was performed in controls, but they received no treatment. Rats were weighed individually, every week, for the duration of the experiment. On day 29, the rats were dissected while they were under ether anesthesia. After obtaining the total ovarian weight, the ovaries were processed for light microscopy. Ovaries were sectioned serially 6-8 μ and were stained with hematoxylin and eosin. Follicles were counted in every tenth section at 100x magnification according to the classification reported (4). At the end of the experiment, rats were exsanguinated by bleeding via the abdominal aorta. The blood was allowed to clot at the room temperature, centrifused at 4°C and serum stored frozen at -20°C for the measurement of progesterone, testosterone and estradiol by radioimmunoassay as described by us previously (5).

Differences between two groups were analyzed by Student's t-test. A p value of less than 0.05 was considered significant.

RESULTS

Effect of GnRH-Ag Treatment on Ovarian Follicles (Table 1).

The ovarian histology revealed the inhibitory effect of GnRH-Ag on the follicular development. The number of big antral follicles in 0.2 μg/day of the GnRH-Ag group was decreased when compared to controls. No big antral follicles were present in the

remaining treated groups. The two high doses of GnRH-Ag suppressed the number of small antral follicles and also big and small atretic follicles when compared to controls ($p<0.001$). However, GnRH-Ag at the dose of 0.2 μg/day was without any effect on these follicles.

TABLE 1. Effect of GnRH Agonist Treatment on Ovarian Follicles[a]

Treatment	Big Antral Follicle	Small Antral Follicle	Big Atretic Follicle	Small Atretic Follicle
Control	8.2±1.5*	14.3±2.3	8.5±2.0	10.0±1.8
0.2 μg/day[b]	4.1±1.5	10.7±1.3	7.5±1.1	13.0±1.9
1.0 μg/day	0.0	2.0±1.2	0.0	4.8±1.9
5.0 μg/day	0.0	1.6±0.6	0.0	2.3±0.9

*Values are expressed as the Mean±SE
[a]Number of follicles counted in one ovary from GnRH agonist-treated rats
[b]Dose of GnRH-Ag

Effect of GnRH-Ag Treatment on Serum Levels of Steroids (Table 2).

TABLE 2. Effect of GnRH Agonist Treatment on Serum Levels of Steroids

Treatment	Progesterone (ng/ml)	Testosterone (pg/ml)	Estradiol (pg/ml)
Control	32±6*	531±76	53±19
0.2 μg/day[a]	17±3	518±77	20±9
1.0 μg/day	18±2	229±15	16±8
5.0 μg/day	18±4	197±22	11±3

*Values are expressed as the Mean±SE
[a]Dose of GnRH-Ag

The peripheral levels of serum progesterone in rats treated with all doses of GnRH-Ag were suppressed by the treatment. Whereas, the two high doses of GnRH-Ag suppressed the serum testosterone and estradiol levels, GnRH-Ag at the dose of 0.2 was ineffective.

The two high doses of GnRH-Ag increased the body weight of these rats ($p<0.05$), as compared to control. The treatment at 0.2

μg/day had not shown such effect on the body weight gain, although an increasing trend was noticed. The ovarian weight was decreased by the treatment at all doses.

Effect of GnRH-Ag Treatment on Body and Ovarian Weights (Figure 3).

TABLE 3. Effect of GnRH Agonist Treatment on Body and Ovarian Weights

Treatment	Body Weight Gain (gm)	Ovarian Weight (mg)
Control	53.9±6.6*	137.1±5.5
0.2 μg/day[a]	62.8±3.8	46.1±5.7
1.0 μg/day	88.2±7.7	78.7±2.8
5.0 μg/day	96.5±4.6	54.0±3.1

*Values are expressed as the Mean±SE
[a]Dose of GnRH-Ag

DISCUSSION

The continuous administration of GnRH-Ag at the dose of 1.0 or 5.0 μg/day suppressed the number of small and big antral follicles following long-term treatment. The treatment also suppressed the number of big and small atretic follicles. The recruitment of new big follicles, was totally absent and new small follicles was greatly reduced. This is indicative of the absence of a secondary FSH surge in these rats (6). The minimum dose of GnRH-Ag appears to be 1.0 μg/day to be inhibitory on the follicular development by the continuous mode of administration. The results also suggest that GnRH-Ag arrests the growth of antral and atretic follicles of both sizes, as reflected in the secretion of various steroid hormones. The reduction in the serum steroid hormonal milieu and associated changes in this follicular development has exhibited an interruption in the estrous cyclicity of these rats and thus an inhibition of ovulation. These inhibitory effects of GnRH-Ag could be due to its inhibition of the pre-ovulatory proestrus gonadotropin-surge (7) and/or to its direct effect on the ovary for rat ovaries are known to have receptors for GnRH (8) and the GnRH-agonists are known to halt follicular development in hypophysectomized rats (2).

The dose dependent weight gain noticed in these rats is an interesting observation, and the mechanism by which this is triggered is presently under investigation in this laboratory. Neuropeptide Y (NPY), another neuropeptide like GnRH, is abundant

in the rat brain and has been shown to play an important role in the neural regulation of feeding behavior (9). It has been speculated that NPY releases endogenous opioid peptide or catecholamines in critical brain areas, which may in turn activate feeding in the rats. Therefore, the weight gain triggered by the administration of GnRH-Ag in these rats may play a role in the stimulation of feeding in these rats which may be associated with the secretion of NPY, endogenous opioid peptides and/or catecholamines.

ACKNOWLEDGEMENTS

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KEY WORDS

Gonadotropin-releasing hormone agonist, follicular development, sex steroids, body weight gain, estrous cyclicity

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The Response of Large and Small Luteal Cells from the Pregnant Rat to Substrates and Secretagogues[1]

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ABSTRACT

Large (>22 μm) and small (12 – 21 μm) luteal cells from Day 8 pregnant rats were separated by elutriation after enzyme dissociation. Aliquots of cells were incubated for 4 h at 37°C in Medium 199 alone (control) or with medium containing dibutyryl cyclic adenosine 3′, 5′-monophosphate (cAMP) at 0.5 mM or 5 mM; rat luteinizing hormone (LH) at doses of 1, 10, 100, or 1000 ng/ml; 10 μg/ml 25-OH-cholesterol; or 10 ng/ml testosterone. Production of progesterone, testosterone, and estradiol was measured by radioimmunoassay. Both cell types showed a similar increase in estradiol synthesis when stimulated with LH (1 μg/ml) or dibutyryl cAMP (5 mM); however, large luteal cells aromatized exogenous testosterone, whereas small luteal cells did not. Large luteal cells produced increased amounts of progesterone at lower doses of dibutyryl cAMP (0.5 mM) and LH (10 ng/ml), compared to small cells, which required 5 mM dibutyryl cAMP or 1 μg/ml LH for minimal stimulation. Dibutyryl cAMP (5 mM) also resulted in an increase of testosterone release from small luteal cells. Progesterone synthesis in both cell types was enhanced by 25-OH-cholesterol. These results suggest that the two cell types differ functionally with respect to steroidogenesis during pregnancy, and that the large luteal cells appear to be the primary site of progesterone and estradiol production at this stage of pregnancy.

INTRODUCTION

Two different types of steroidogenic cells in the corpus luteum have been described in several mammalian species including various ruminants (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982), pigs (Lemon and Loir, 1977), rabbits (Hoyer et al., 1986), humans (Chegini et al., 1988), and, recently, rats (Nelson and Khan, 1987). Many of these studies have reported that the two cell types differ functionally as well as structurally. The majority of such studies, however, have focused on ruminants.

The two steroidogenic cell types within the cow corpus luteum are the small and large luteal cells. Small luteal cells possess a variably shaped nucleus with dense heterochromatin lining the periphery of the nucleus; large luteal cells have a spherical nucleus with dispersed chromatin (Hansel and Dowd, 1986). Both large and small luteal cells contain steroidogenic organelles (Hansel and Dowd, 1986). In rats, small luteal cells span a size range of 12 – 21 μm, and large cells are >22 μm (Nelson and Khan, 1987).

Previous studies have partially elucidated the steroidogenic processes in rat corpus luteum. During early pregnancy, the corpus luteum of the rat produces testosterone in response to luteinizing hormone (LH) (Sridaran et al., 1981). Testosterone is then aromatized within the corpus luteum to estradiol (Elbaum and Keyes, 1976), and estradiol further enhances progesterone synthesis (Takayama and Greenwald, 1973; Gibori et al., 1977) by increasing the availability of cholesterol, a substrate for progesterone synthesis (Khan et al., 1984, 1985). This results in the high titers of progesterone necessary to maintain pregnancy (Niswender et al., 1985). However, it is not yet clear which steroidogenic processes occur in which cell type.

To further clarify how steroidogenesis occurs in the rat corpus luteum, we used Day 8 pregnant rats to examine the response of separated large and small luteal cells to substrates and secretagogues. The substrates chosen included 25-OH-cholesterol and testosterone; the secretagogues were LH and dibutyryl cyclic adenosine 3′, 5′-monophosphate (cAMP).

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10

MATERIALS AND METHODS

Chemicals

Medium 199, Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}, trypan blue, collagenase type IV, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA) (endotoxin-tested), testosterone, $NaHCO_3$, and dibutyryl cAMP were purchased from Sigma Chemical Co. (St. Louis, MO). DNase I and dispase (neutral protease) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). 25-OH-cholesterol was purchased from Steraloids (Wilton, NH); rLH (NIDDK-rLH-7) was supplied from the National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD. Our source of diethyl ether was Mallinckrodt (Paris, KY), and Diagnostic Products Corp. (Los Angeles, CA) was the source for the estradiol-17β radioimmunoassay (RIA) kit. The following reagents were purchased from Ted Pella, Inc. (Tustin, CA): paraformaldehyde, uranyl acetate, and lead citrate. Glutaraldehyde, cacodylate, OsO_4, and propylene oxide were purchased from Electron Microscopy Sciences (Fort Washington, PA), and Polybed 812 was from Polysciences (Warrington, PA). Ethanol, 200-proof, was obtained from Pharmco (Philadelphia, PA). ^{3}H-Progesterone was purchased from NEN Research Products (Boston, MA), and the progesterone antiserum (GDN-337) was provided by Dr. G. D. Niswender. ^{125}I-Testosterone and testosterone antiserum (catalog #172) were obtained from Radioassay Systems Laboratories, Inc. (Carson, CA).

Animals

Timed-pregnant Sprague-Dawley rats were obtained from Holtzman Co. (Madison, WI) and housed at 23 – 25°C with a 14L:10D photoperiod (lights on from 0600 to 2000 h). Purina rat chow (Ralston-Purina Co, St. Louis, MO) and water were available ad libitum.

Tissue Collection

On Day 8 of pregnancy (with Day 1 as day of insemination), the ovaries were dissected while the rats were under ether anesthesia. The corpora lutea were removed, cleaned of any adhering follicles with fine-tipped forceps, and placed in Medium 199, which contained 2.2 g/l $NaHCO_3$ and had been gassed with 95% O_2:5% CO_2. For each experiment, 200-300 corpora lutea were used. After all of the corpora lutea were dissected, the incubation medium was changed to HBSS with 0.1% BSA, but without Ca^{2+} and Mg^{2+}, to which collagenase (50 U/ml), dispase (2.4 U/ml), and deoxyribonuclease (Dnase; 200 U/ml) were added.

Cell Separation

The methodology employed for enzyme incubation and tissue dissociation followed the basic procedures by Nelson and Khan (1987). The enzyme solution consisted of 50 U/ml collagenase, 2.4 U/ml dispase, 200 U/ml DNase, and 0.1% BSA in HBSS without Ca^{2+} and Mg^{2+}. Four incubations lasting 30 min each were carried out in a shaking water bath at 37°C. The tissue was then incubated in 0.02% EDTA:0.1% BSA for 15 min. After centrifugation at 200 × *g* for 5 min, the EDTA solution was replaced with HBSS without Ca^{2+} and Mg^{2+}.

The tissue was dissociated by repeatedly resuspending the tissue/cells: the tissue and clumped cells were allowed to settle and the suspended cells were gently transferred to a new test tube. After dissociation, the cells were separated by centrifugal elutriation using the Beckman JE-6B elutriation system and rotor and a standard chamber. The parameters for elutriation were the same as used by Fitz et al. (1982). The nonsteroidogenic cells, including red blood cells and fibroblasts, were collected in a separate fraction and discarded. Subsequent collections from the elutriator resulted in a separation of small and large luteal cells. Cells of each type were counted with a hemocytometer and viability was calculated after incubation in 0.4% trypan blue. The size of each cell was compared to the calibrated lines within the hemocytometer to determine cell type. Cell type was also verified by electron-microscopic examination of cells from each collected fraction from 4 experiments. The purity of each fraction was assessed and averaged 97 ± 3.4% for small luteal cells and 83 ± 7.3% for large luteal cells. The percent viability averaged 86 ± 11% for small luteal cells and 67 ± 14% for large luteal cells. Results were expressed as 10^5 viable cells.

Incubation

Aliquots of approximately 2 – 7 × 10^5 viable cells were incubated as cell suspensions in a shaking water bath for 4 h at 37°C in Medium 199 containing 2.2 g/l $NaHCO_3$ gassed with 95% O_2:5% CO_2, with or without substrates or secretagogues. The two substrates used were testosterone (10 ng/ml) and 25-OH-cholesterol (10

μg/ml). The doses were based upon previous tissue culture experiments in our laboratory and upon reports from other laboratories (Toaff et al., 1982; Silavin et al., 1984). The two secretagogues included dibutyryl cAMP and rLH. The medium did not contain BSA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or serum, and was maintained at a pH of 7.4.

RIA

The medium and cells were assayed for synthesis of progesterone (Gibori et al., 1977) and testosterone (Sridaran et al., 1981) by RIA as previously described. Sensitivity was 10 pg/tube for the testosterone assays and 0.05 ng/tube for the progesterone assays. Estradiol-17β was measured by a double-antibody RIA kit after extraction with anhydrous diethyl ether. The extraction procedure has been previously described (Korenman et al., 1974). Interassay coefficient of variation was 9.8%, and the intraassay coefficient of variation was 2.7%. Accuracy of the assay was assessed by correlating potencies of the standards to potencies of extracted samples to which estradiol standards were added. The product-moment correlation coefficient at 0.982 was significant ($p<0.01$). Sensitivity of the estradiol assays was 5 pg/ml of reconstituted extract.

Electron Microscopy

The elutriated cells were fixed overnight in a 2.5% glutaraldehyde:2% paraformaldehyde solution buffered with 0.1 M cacodylate at pH 7.4. The cells were post-fixed in 2% OsO_4 in cacodylate. After washing, they were stained en bloc in saturated aqueous uranyl acetate, dehydrated with ethanol, and infiltrated with propylene oxide followed by Polybed 812. To infiltrate with Polybed 812, cells were suspended in the Polybed 812:propylene oxide solutions then centrifuged between each solution change. They were embedded in Polybed 812.

The cells were sectioned on a Porter-Blum MT2B ultramicrotome and placed on 200-mesh copper grids for electron microscopy. Sections were stained with uranyl acetate followed by lead citrate, and examined in a JEOL JEM 1200 EX transmission electron microscope. Thick sections were placed on glass slides and stained with toluidine blue.

Statistics

There were 5 – 7 replicates/treatment in each experiment, and 2 – 5 independent experiments were performed for each substrate or secretagogue treatment. Data from independent experiments were pooled whenever possible. However, in some treatments, the absolute concentrations of hormones differed considerably among cell preparations so that it was not feasible to pool all data. In those instances, the results for each experiment are presented in a table. The hormone levels were divided by the number of cells used in each incubation so as to express the data as hormone concentration/10^5 cells. This computation decreased the values of the final data. In addition, whenever hormone potencies fell below the sensitivity of that assay, a zero value was assigned for data analysis. Because the data expressed here represent means, it may appear that some fall below the level of sensitivity when actually the mean value is low due to some "zero" (undetectable) values.

The data were analyzed by one-way analysis of variance followed by the Tukey test when differences were significant. Where appropriate, Student's *t*-test was used to compare differences between two groups. All steroid hormone data are expressed as the mean (± standard deviation) concentration of hormone/ml/10^5 viable cells.

RESULTS

Figures 1 and 2 are micrographs of large luteal cells, after elutriation. Figure 1 is a light micrograph of

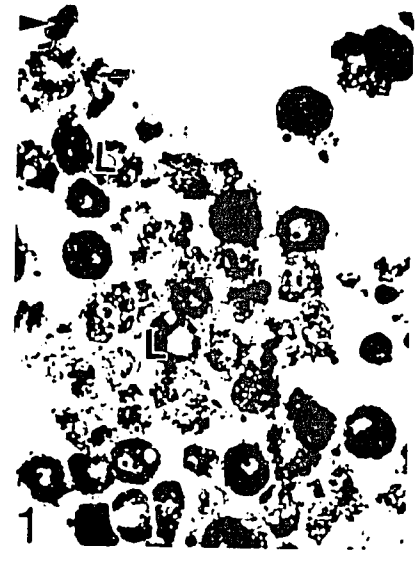

FIG. 1. Light micrograph of large luteal cells (*L*) after elutriation. *Arrow* points to contamination by smaller cells. Much of the debris is an artifact of preparation for resin sections, due to repeated centrifugations of the cells in the viscous Polybed 812 (×240).

12

FIG. 2. Light micrograph of live large luteal cells *(L)* after elutriation showing absence of debris (×240).

epoxy-embedded cells, whereas Figure 2 shows live large luteal cells as they appear in the cell suspension. The large luteal cells used in this study were greater than 22 μm in diameter and possessed a large, centrally located, spherical nucleus (Fig. 1). Contamination of large cells by smaller cells averaged 17 ± 7.3%. Typically, clumps of smaller cells could be found occasionally in the large luteal cell fraction (Fig. 1, arrow). The debris seen in Figure 1 is an artifact of epoxy infiltration and it was not present in the fraction of live large luteal cells after elutriation (Fig. 2). The cytoplasm of large luteal cells contained abundant smooth endoplasmic reticulum, mitochondria with tubular cristae, and lipid droplets (Fig. 3).

Elutriated small luteal cells had a size range of 12 – 21 μm, and are shown in Figures 4 and 5. The small luteal cell fraction was rarely contaminated by large luteal cells (average contamination was 3 ± 3.4%), but did contain approximately 6% small, nonsteroidogenic cells, as quantified by ultrastructural appearance (Fig. 4, arrow). Figure 5 shows live small luteal cells after elutriation to demonstrate that the debris shown in Figure 4 is an artifact of epoxy infiltration. Small luteal cells also appeared steroidogenic (Fig. 6). Their nuclei were more variable in location and shape, and possessed dense heterochromatin along the periphery of the nucleus (Fig. 6).

Progesterone production in large luteal cells increased in a dose-related manner to LH, with a minimal response to 10 ng/ml LH (Fig. 7). Dibutyryl cAMP also stimulated progesterone synthesis in large luteal cells at doses of 0.5 and 5 mM, with a greater response to the higher dose (5 mM) (Fig. 8). Incubation with the substrate 25-OH-cholesterol (10 μg/ml) enhanced progesterone production in large luteal cells (Fig. 9), whereas treatment with testosterone (10 ng/ml) had no effect (3.4 ± 2.4, controls; 3.2 ± 3.6 ng/ml/10^5 cells, testosterone-treated cells; data pooled from 3 experiments).

Small luteal cells synthesized increased levels of progesterone in response to only the highest doses of LH (1 μg/ml) (Fig. 7) and dibutyryl cAMP (5 mM) used (Fig. 8). Exposure to 25-OH-cholesterol also augmented progesterone production in small luteal cells (Fig. 9), whereas treatment with testosterone (10 ng/ml) showed no effect (1.1 ± 0.3, controls; 1.7 ± 0.7 ng/ml/10^5 cells, testosterone-treated cells; data pooled from 3 experiments).

Estradiol production increased in both small and large luteal cells incubated with LH at 1000 ng/ml (Table 1), and the lower doses of LH had no effect on estradiol levels. Estradiol synthesis in large luteal cells also increased after incubation with 0.5 mM dibutyryl cAMP in 2 of 4 experiments (Table 2). However, when these data were pooled, no differences were found between controls and cells incubated with 0.5 mM dibutyryl cAMP. Estradiol synthesis did not increase when small luteal cells were incubated with 0.5 mM dibutyryl cAMP (Table 2), whereas both cell types synthesized increased levels of estradiol in response to the higher dose of dibutyryl cAMP (5 mM; Table 2).

Incubation with substrates stimulated estradiol production in only the large luteal cells, as evidenced by an increase when cells were incubated with testosterone, whereas no response was seen in small luteal cells (Fig. 10). Additionally, when cells were incubated with 25-OH-cholesterol, estradiol production increased in the large luteal cells in 2 of 3 experiments (Table 3). When these data were pooled, the increase was still significant at $p<0.05$. The 25-OH-cholesterol was ineffective in small luteal cells (Table 3).

Testosterone levels increased in the media containing large luteal cells treated with 1000 ng/ml LH, but the lower doses of LH had no effect on testosterone levels (Table 1). Nevertheless, only small luteal cells responded to dibutyryl cAMP (5 mM) with increased testosterone concentrations in the media (Fig. 11). The substrate 25-OH-cholesterol did not alter testosterone levels in either small or large luteal cells (1.7 ± 1.2, small cells control; 5.7 ± 6.4, cholesterol-treated small cells; 3.7 ± 3.6, large cells control; 14.0 ± 13.1 pg/ml/10^5 cells, cholesterol-treated large luteal cells).

TABLE 1. Estradiol and testosterone production (pg/ml/10^5 cells) by small and large luteal cells after incubation with LH.[ab]

Steroid/cells	Doses of LH (ng/ml)				
	0	1	10	100	1000
Estradiol production:					
Small cells	2.0 ± 2.7	2.7 ± 1.2	1.4 ± 1.3	3.1 ± 0.6	8.3 ± 6.9*
Large cells	2.8 ± 1.3	5.2 ± 0.3	5.4 ± 3.5	4.1 ± 0.8	22.3 ± 13.9*
Testosterone production:					
Small cells	1.1 ± 1.2	0.0 ± 0.0[c]	1.4 ± 1.5	0.0 ± 0.0[c]	2.1 ± 2.5
Large cells	3.6 ± 3.5	0.0 ± 0.0[c]	5.2 ± 3.9	5.1 ± 4.6	8.4 ± 3.8*

[a]Results are expressed as the mean ± SD.

[b]The hormone levels were divided by the quantity of cells used in the incubation, which decreased the values of the final data.

[c]All replicates, within this mean had hormone values below the sensitivity level of the assay.

*Significantly different from controls ($p<0.05$).

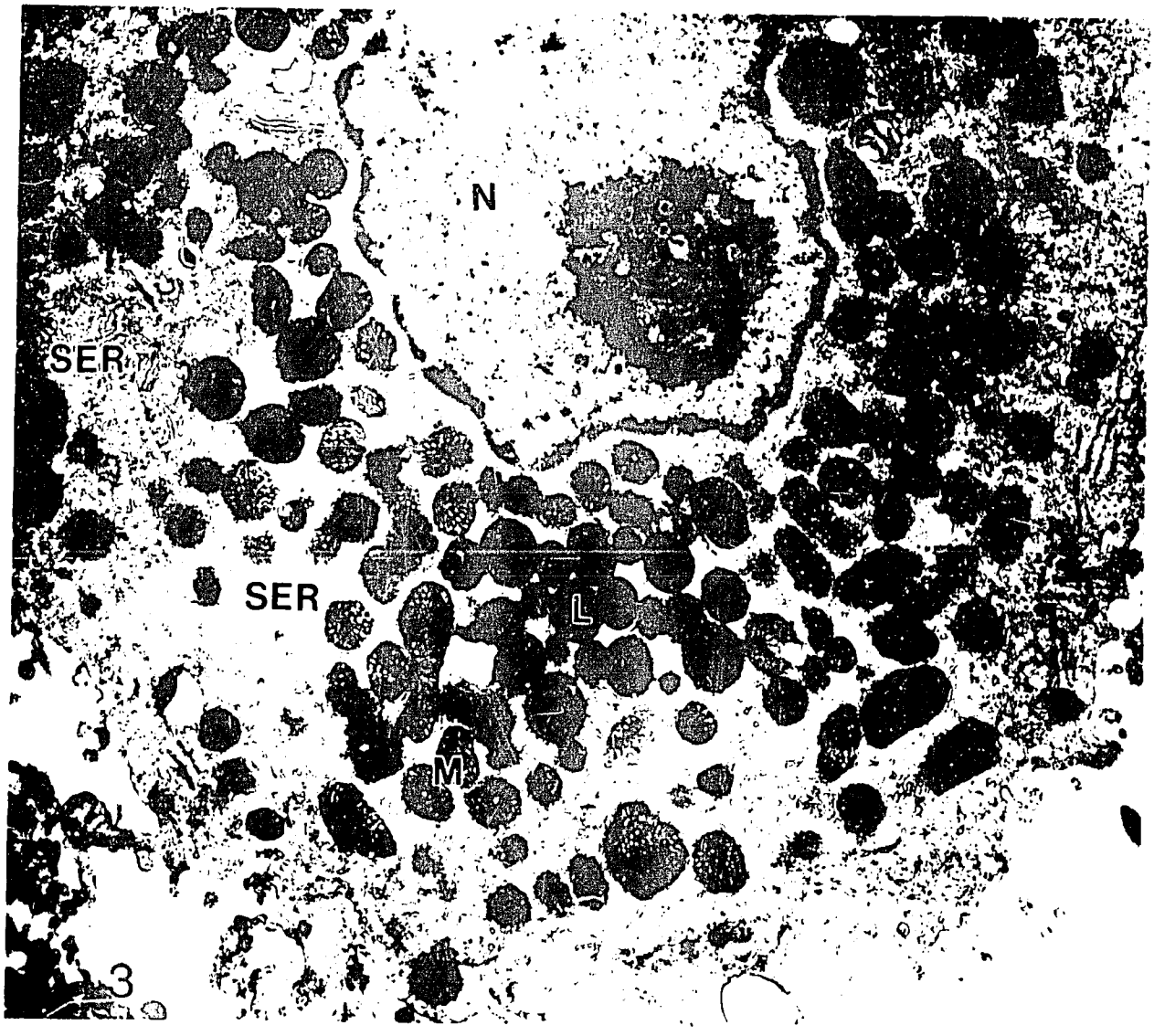

FIG. 3. Electron micrograph of large luteal cell after elutriation. The spherical nucleus (*N*) is centrally located. Steroidogenic organelles are numerous. *SER*, smooth endoplasmic reticulum; *M*, mitochondria with tubular cristae; *L*, lipid droplet (×8000).

14

FIG. 4. Light micrograph of small luteal cells *(S)* after elutriation. *Arrow* points to contamination by smaller cells that are probably nonsteroidogenic. Much of the debris is an artifact of preparation for resin sections, due to repeated centrifugations of the cells in the viscous Polybed 812 (×240).

FIG. 5. Light micrograph of live small luteal cells *(S)* after elutriation (×240).

DISCUSSION

In pregnant rats, large luteal cells appear to be the major source of progesterone from the corpus luteum for several reasons. Basal levels of progesterone are higher in media containing large luteal cells versus small luteal cells. Large luteal cells produce progesterone in a dose-related manner to rat LH, and progesterone synthesis in large cells is stimulated by a 100-fold lower dose of LH than in small luteal cells. This is physiologically relevant since on Day 8 of pregnancy, steroidogenesis is dependent upon LH (Sridaran et al., 1981). Also, large luteal cells synthesize increased progesterone in response to a 10-fold lower dose of dibutyryl cAMP than do small cells. The response of large luteal cells to dibutyryl cAMP corroborates data obtained in our laboratory from undissociated tissue. Luteal tissue incubated with 0.5 mM dibutyryl cAMP shows a 2-fold increase in progesterone synthesis (unpublished observation), suggesting that progesterone production by luteal tissue in response to cAMP may be mediated by the large luteal cells, since these cells also show a 2-fold increase in progesterone synthesis when incubated with 0.5 mM dibutyryl cAMP.

Large luteal cells from Day 3 pregnant rats possess more LH receptors than do small luteal cells, but both cell types at this stage of pregnancy release progesterone in a similar manner to 1 μg/ml LH (Nelson et al., 1987). In addition, Kumai et al. (1986) reported that less dense rat luteal cells, ranging in size from 25 – 40 μm, produced more progesterone in response to human chorionic gonadotropin than more dense cells ranging in size from 15 – 40 μm. In the present study on Day 8 pregnant rats, progesterone production in small luteal cells was stimulated by LH, but this stimulation was minimal compared to that seen in large luteal cells. The different response of small luteal cells to LH at different stages of pregnancy may reflect functional changes occurring in the corpus luteum as pregnancy progresses, or differences in incubation times or other methodology.

Unstimulated large luteal cells produce more progesterone than small luteal cells not only in rats, but in

TABLE 2. Estradiol production (pg/ml/10^5 cells) by small and large luteal cells after incubation with dibutyryl cAMP.[a,b]

	Doses of dibutyryl cAMP		
Cells	0	0.5 mM	5 mM
Experiment 1			
Large cells	1.6 ± 0.3	3.8 ± 1.4*	
Small cells	2.0 ± 0.5	2.4 ± 1.9	
Experiment 2			
Large cells	1.0 ± 1.0	0.8 ± 0.5	2.8 ± 1.2*
Small cells	0.0 ± 0.0[c]		1.0 ± 1.0*
Experiment 3			
Large cells	0.4 ± 0.3	1.9 ± 0.4*	
Small cells	0.6 ± 0.2		3.5 ± 0.8*
Experiment 4			
Small cells	0.3 ± 0.6		5.4 ± 0.8*
Experiment 5			
Large cells	0.1 ± 0.1	0.4 ± 0.2	13.3 ± 6.4*
Small cells	0.0 ± 0.0[c]	0.2 ± 0.1	4.0 ± 2.1*

[a]Results are expressed as the mean ± SD.

[b]The hormone levels were divided by the quantity of cells used in the incubation, which decreased the values of the final data.

[c]All replicates within this mean had estradiol values below the sensitivity level of the assay.

*Significantly different from controls ($p<0.05$).

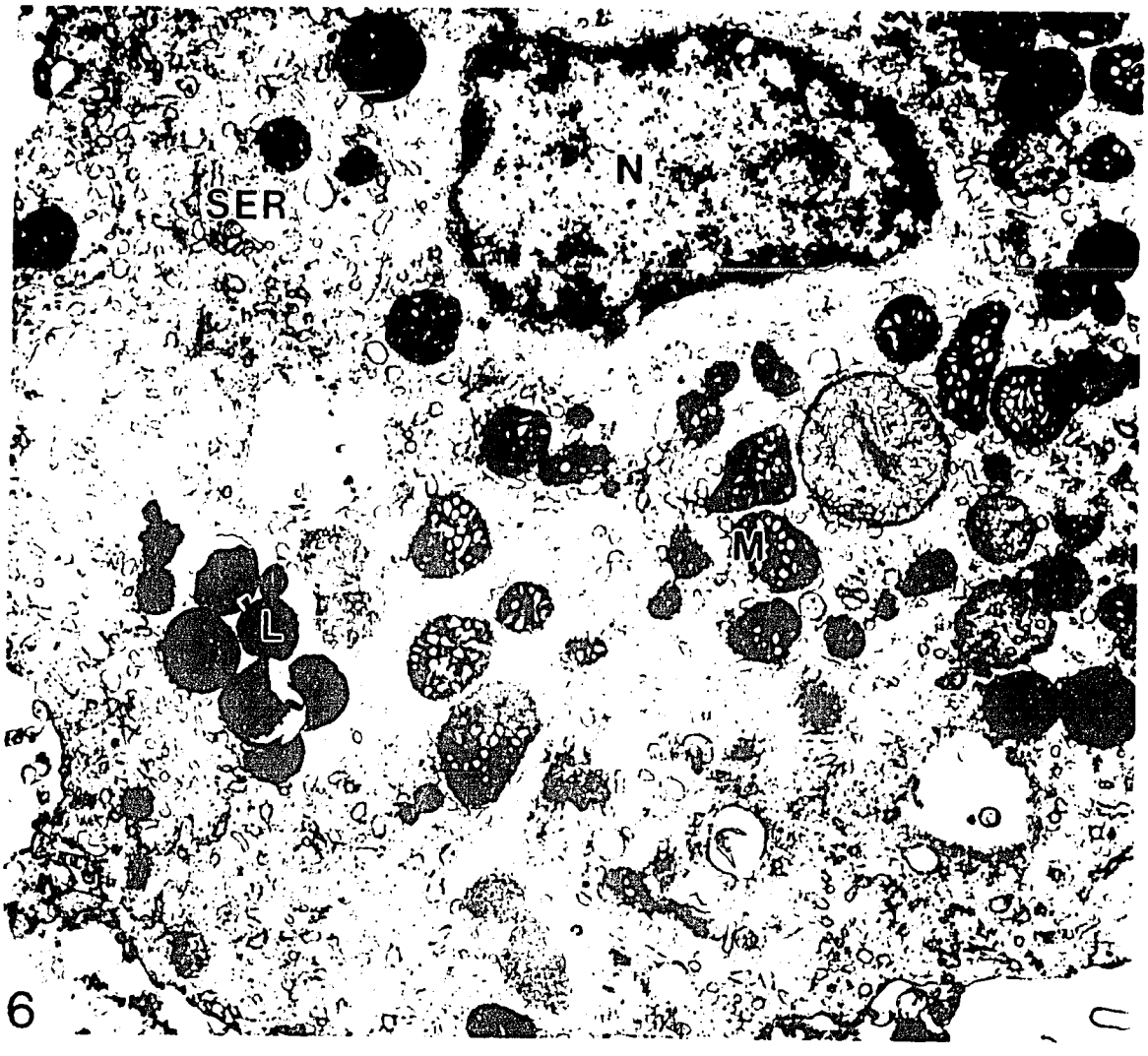

FIG. 6. Electron micrograph of small luteal cell after elutriation. The cytoplasm, although occupying less area than in large luteal cells, also contains structures indicative of steroidogenesis. *SER*, smooth endoplasmic reticulum; *M*, mitochondria with tubular cristae; *L*, lipid droplet; *N*, nucleus (×13,500).

pigs (Lemon and Loir, 1977), cows (Ursely and Leymarie, 1979), and sheep (Fitz et al., 1982). However, the steroidogenic response of large luteal cells to LH differs. Large luteal cells in cows (Ursely and Leymarie, 1979), sheep (Fitz et al., 1982), and rabbits (Hoyer et al., 1986) could not be stimulated by LH to produce increased levels of progesterone. Instead, the small luteal cells of these species showed stimulated progesterone synthesis after incubation with LH (Ursely and Leymarie, 1979; Fitz et al., 1982; Hoyer et al.,

TABLE 3. Estradiol production (pg/ml/10^5 cells) in separated luteal cells after incubation with 10 μg/ml 25-OH-cholesterol.[ab]

	Large luteal cells		Small luteal cells	
	Control	Cholesterol treatment	Control	Cholesterol treatment
Experiment 1	1.6 ± 0.3	2.3 ± 1.0	2.0 ± 0.5	3.5 ± 2.0
Experiment 2	0.8 ± 0.1	1.7 ± 0.3*	0.2 ± 0.1	0.4 ± 0.2
Experiment 3	1.0 ± 0.5	8.1 ± 4.0*	0.5 ± 0.4	1.1 ± 0.4

[a]Results are expressed as the mean ± SD.

[b]The hormone levels were divided by the quantity of cells used in the incubation, which decreased the values of the final data.

*Significantly different from controls ($p<0.01$).

1986). In spite of that, Niswender et al. (1985) have concluded that, in the sheep corpus luteum, when the number of each cell type and relative ability of each to synthesize progesterone are calculated, large luteal cells produce most of the progesterone. On Day 8 of pregnancy in rats, there are 46.1% large luteal cells and 53.9% small luteal cells in the corpus luteum (non steroidogenic cells excluded; Smith et al., unpublished results). Because the ratio of large to small cells in the tissue is close to 1:1 and large luteal cells demonstrate a greater ability to synthesize progesterone, the major source of progesterone in the rat corpus luteum also appears to be the large luteal cells.

Large and small luteal cells in rat corpora lutea show an enhancement of progesterone production after exposure to 25-OH-cholesterol. This observation corroborates earlier work suggesting that cholesterol availability, stimulated by estradiol, is a critical factor in synthesizing the high levels of progesterone needed to maintain pregnancy in rats (Gibori et al., 1984; Khan et al., 1984, 1985). In this study, estradiol production in both cell types was stimulated by LH and dibutyryl cAMP. Yet, although estradiol levels were similar in LH-treated and dibutyryl cAMP-treated cell types, progesterone production resulting from these treatments was much more enhanced in large luteal cells. Since small luteal cells responded similarly to large cells in production of progesterone upon incubation with 25-OH-cholesterol, it implies that large luteal cells may contain more cholesterol or have a greater ability to synthesize cholesterol or to free cholesterol from the esterified form in response to estradiol.

Also, Medium 199 contains cholesterol; thus, the possibility exists that this cholesterol could have elevated basal steroid production. However, compared to 25-OH-cholesterol, cholesterol is not very soluble in aqueous solutions and is metabolized less rapidly to pregnenolone (Lambeth et al., 1982; Toaff et al., 1982; Quinn et al., 1985). The increase in progesterone production upon incubation with 25-OH-cholesterol, compared to controls, indicated that the presence of cholesterol in Medium 199 did not stimulate steroidogenesis to maximal or near maximal levels.

Both cell types from Day 8 pregnant rats were capable of estradiol production without the addition of sub-

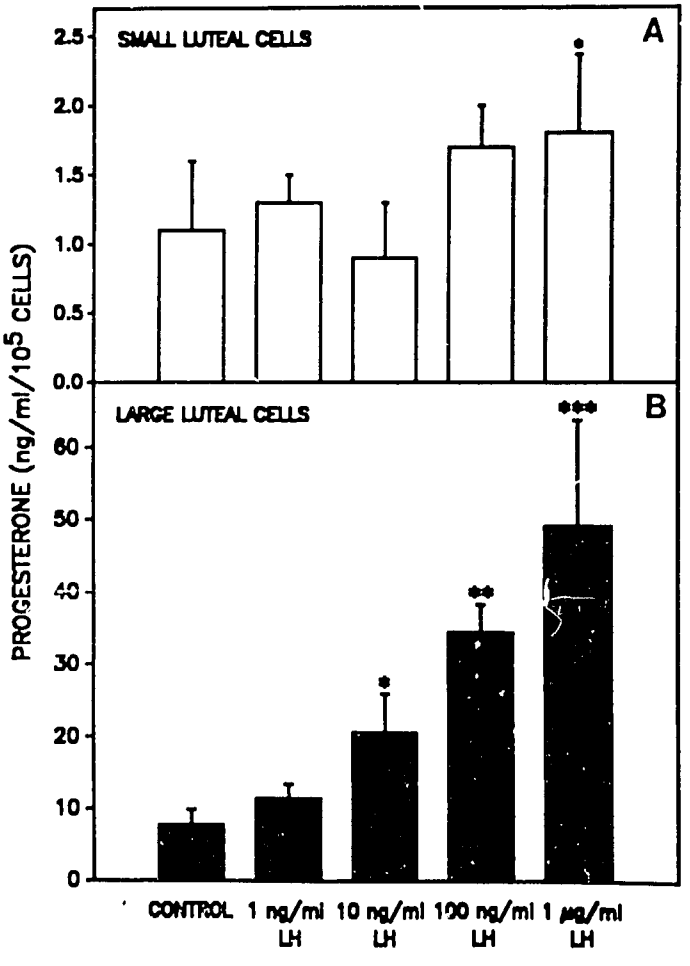

FIG. 7. Mean (± SD) progesterone levels in medium containing luteal cells of pregnant rats after incubation with increasing doses of LH. *(A)* Small luteal cells produced increased amounts of progesterone only after incubation with the highest dose of LH (1 µg/ml). *(B)* With the exception of the LH dose of 1 ng/ml, progesterone production by large luteal cells increased significantly with each higher dose of LH, as compared to the preceding dose (denoted by *asterisks*).

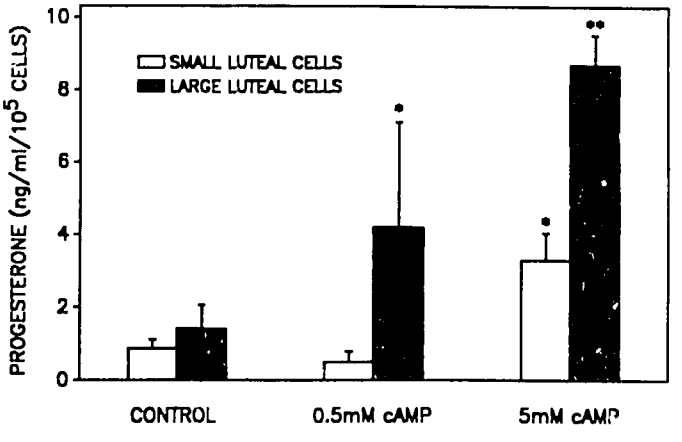

FIG. 8. Mean (± SD) progesterone levels in medium after incubation with dibutyryl cAMP. Data from 4 independent experiments were pooled. A single *asterisk* denotes a significant increase in hormone production as compared to the control; two *asterisks* indicate a significant increase as compared to the preceding dose.

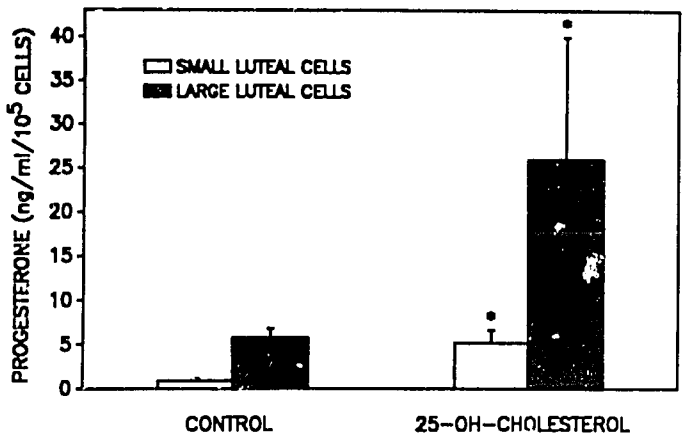

FIG. 9. Mean (± SD) progesterone levels in medium after incubation with 10 μg/ml 25-OH-cholesterol. Data from 2 independent experiments were pooled (*asterisk*, $p<0.05$ compared to respective control).

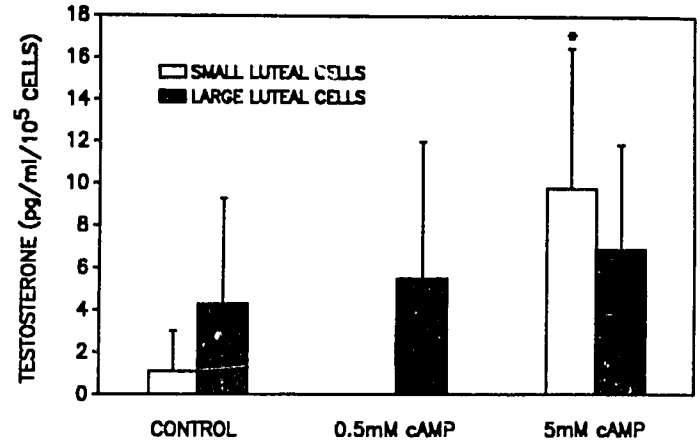

FIG. 11. Mean (± SD) testosterone levels in medium after incubation with dibutyryl cAMP. Data from 4 independent experiments were pooled. Variations in absolute hormone concentrations differed between experiments resulting in high standard errors when data were pooled (*asterisk*, $p<0.05$ compared to respective control). Testosterone levels in medium containing small luteal cells incubated with 0.5 mM dibutyryl cAMP were undetectable.

strates. In this study. LH and dibutyryl cAMP stimulated estradiol synthesis in both cell types; hence, it follows that both cell types were also producing androgens. Yet, aromatization of exogenous testosterone occurred only in large luteal cells, suggesting that aromatase activity is low in small luteal cells until they are treated with LH or dibutyryl cAMP. Small luteal cells also produced increased quantities of testosterone when incubated with cAMP but not with LH. This difference could be explained by the relative time needed to activate aromatase activity and synthesize additional substrate (testosterone). Testosterone levels may not be elevated after LH incubation due to conversion to estradiol without sufficient replacement. Small luteal cells from Day 3 pregnant rats also do not aromatize exogenous testosterone, whereas large luteal cells exhibit high aromatase activity at this stage of pregnancy (Gibori et al., 1988).

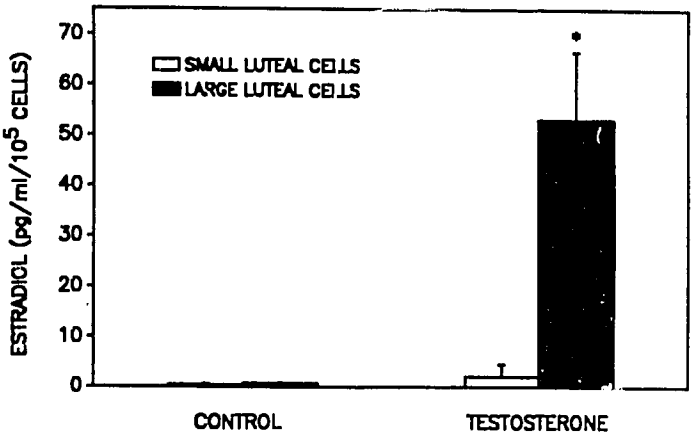

FIG. 10. Mean (± SD) estradiol levels in medium after incubation with 10 ng/ml testosterone. Data from 2 independent experiments were pooled (*asterisk*, $p<0.05$ compared to respective control).

In conclusion, both cell types from Day 8 corpora lutea have the capability to produce testosterone, estradiol, and progesterone. However, large luteal cells appear to be the major source of progesterone and estradiol and can synthesize these hormones in the absence of small luteal cells. Since estradiol enhances progesterone production (Gibori et al., 1977; Gibori and Keyes, 1978), the new information presented here indicates that estradiol may have an autocrine role within the large luteal cells. We are currently examining that question in more detail. Also, further investigation needs to be directed toward the cellular source of androgens at this stage of pregnancy.

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