In Vitro Effects of Interactions of Follicle-Stimulating Hormone, Luteinizing Hormone, and Prolactin on Progesterone Synthesis by Rat Luteal Cells during Pregnancy (43910)

WEI YUAN* AND GILBERT S. GREENWALD†,[1]

Department of Animal Science, University of Missouri, Columbia, Missouri 65211 and Department of Physiology,† Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160*

Abstract. **The *in vitro* ability of ovine (o) follicle-stimulating hormone (FSH), (o)luteinizing hormone (LH), (o)prolactin (PRL), and recombinant human FSH (rhFSH) to stimulate progesterone (P_4) synthesis by rat corpora lutea on Day 4 of pregnancy was investigated. Dispersed luteal cells (large + small cells) were incubated in the presence of the gonadotropins (1–100 ng) alone or in various combinations (10 ng each) for 4 or 24 hr. Given alone, all the ovine preparations stimulated P_4 in a dose-dependent manner with even 1 ng of each hormone significantly enhancing P_4 production. Significantly, rhFSH—which is devoid of LH contamination—at 10 and 100 ng also stimulated P_4 production, thus clearly establishing for the first time that FSH is a luteotropic hormone in the rat. The combination of oFSH + LH + PRL (10 ng each) significantly stimulated P_4 synthesis to a greater extent than the combination of any two hormones or individual hormones at both 4 hr or an additional 24 hr of incubation ($P < 0.05$). This verified *in vitro* a previously established *in vivo* luteotropic complex. One hundred nanamolars of phorbol 12-myristate 13-acetate (PMA) did not affect basal P_4 secretion but inhibited cAMP, oFSH, and oLH stimulation of P_4. Thus, the luteotropic effects of FSH, LH, and activators of protein kinase A are antagonized by the protein kinase C pathway.** [P.S.E.B.M. 1995, Vol 209]

Recent *in vitro* studies have shown that follicle-stimulating hormone (FSH) acts as a luteotropic hormone in the pregnant hamster (1, 2). Moreover, on Day 4 of pregnancy combined incubation with FSH, luteinizing hormone (LH), and prolactin (PRL) produces the optimal synthesis of progesterone (1, 2). This agrees with *in vivo* experiments in which hamsters hypophysectomized on Day 4 of pregnancy (day of nidation) were injected with various combinations of gonadotropins; pregnancy was maintained by a minimal luteotropic complex of PRL and FSH (3). Small doses of LH synergize with the minimal luteotropic complex, whereas large amounts of LH lead to luteolysis (4).

The hamster corpus luteum (CL) has received little attention compared with the extensive study in the rat (5), and, therefore, in order establish the significance of these findings in other laboratory rodents, we directed our attention to the rat. We were especially interested in testing the *in vitro* effects of gonadotropins on rat luteal cells because rats hypophysectomized on Day 6 of pregnancy maintain pregnancy when injected daily with prolactin and FSH (6). Similarly, rats hypophysectomized on Day 6 of gestation require daily administration of highly purified FSH, LH, and PRL for optimal maintenance of viable fe-

[1] To whom requests for reprints should be addressed at Department of Physiology, University of Kansas Medical Center, Kansas City, KS 66160-7401.

Received November 1, 1994. [P.S.E.B.M. 1995, Vol 209]
Accepted March 13, 1995.

0037-9727/95/2094-0376$10.50/0
Copyright © 1995 by the Society for Experimental Biology and Medicine

tuses (7). The problem in interpreting the *in vivo* results is that distinguishing between the effects of FSH on follicular development and its direct actions on the CL is not feasible; hence, the *in vitro* approach was essential.

The current investigation differs in two respects from previous *in vitro* studies of the rat CL: the *in vitro* effects of ovine (o) FSH or recombinant human FSH (rhFSH) on progesterone synthesis are explored for the first time; and the additive effects of the gonadotropins as luteotropins are also evaluated. An additional objective is to determine the second messenger systems through which FSH and LH act to stimulate luteal progesterone synthesis, to compare with our previous results with the hamster (2).

Materials and Methods

Reagents. Dispase (Grade II, Ca^{++} and Mg^{++}-free) and Nutridoma NS (containing low-density lipoprotein, transferrin, BSA, vitamins, amino acids, and insulin) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Forskolin, phorbol 12-myristate 13-acetate (PMA), dibutyryl cAMP (db-cAMP), dimethyl sulfoxide (DMSO), aprotinin, collagenase (Type V), bovine calf serum (BCS), deoxyribonuclease I (DNase I, Type IV), penicillin, streptomycin and pregnant mare serum gonadotropin (PMSG) were purchased from Sigma Chemical Co. (St. Louis, MO). Hanks' balanced salt solution (HBSS, without Ca^{++} and Mg^{++}) was obtained from JRH Biosciences (Lenexa, KS). Human chorionic gonadotropin (hCG) was from Schein (Phoenix, AZ). ITS (a combination of insulin, transferrin, selenium, and BSA) was purchased from Becton Dickinson Labware (Bedford, MA). McCoy's 5A medium was obtained from Life Technologies Inc. (Grand Island, NY) and Ham's Nutrient Mixture F-12 from JRH Biosciences (Lenexa, KS). Ovine FSH (oFSH-19), oLH (oLH-25), and oPRL (oPRL-19) were kindly provided by National Hormone and Pituitary Program (Rockville, MD) and rhFSH was generously provided by Ares Advanced Technology (Randolph, MA).

Luteal Cell Dispersion. The immature PMSG-hCG treated rat was selected as the model in view of its widespread use as a close approximation of the hormonal changes encountered during normal gestation (8). Sprague-Dawley immature female rats on Day 25 (average weight 70–80 g) were purchased from Sasco Laboratories (Omaha, NE) and injected sc with 5 IU PMSG at 09:00 hr and 2 days later given 5 IU hCG at 09:00 hr and caged with fertile males at 17:00 hr on the same day. The following morning, when 28 days old, the rats were checked for the presence of a vaginal plug or sperm in the vaginal smear which was considered Day 1 of pregnancy. On Day 4 of pregnancy at 09:00 hr the rats were decapitated, ovaries removed and immediately placed in ice cold HBSS. Day 4 was selected for comparison with our previous experiments with the hamster. Intact corpora lutea were dissected from the ovaries under a dissecting microscope. Any follicles adherent to the CL were carefully removed. The methods used for cell isolation were described previously (1). Corpora lutea were usually pooled from two rats for each enzymatic dissociation. The CL were placed in the enzyme solution consisting of 2.4 U/ml Dispase, 0.1 mg DNase I, and 50 IU collagenase/ml. Dissociation was carried out with sequential incubations (4×30 min) at 37°C in a shaking water bath. The CL were mechanically and gently agitated several times during each incubation. Following each 30 min incubation, the enzyme solution was removed from the CL fragments. Aprotinin (1 mg/ml), a protease inhibitor, was added to the enzyme solution containing luteal cells which was centrifuged (100*g*) for 10 min. The supernatant was discarded following centrifugation and fresh incubation medium (1:1 McCoys and Ham's F-12 media with 1% ITS and 100 IU penicillin/ml and 100 mg streptomycin/ml) was added to the pellet of luteal cells. The washing procedure was repeated three times until the final incubation media, containing cells, looked clear. Total large and small luteal cells were counted with a hemocytometer. Cell viability was checked following isolation using 0.4% trypan blue.

Cell Incubation and Hormonal Treatment. Following isolation, cell numbers were adjusted to 100,000 luteal cells (large + small cells) per ml and plated in 24-well plates (Costar, Cambridge, MA) at 1.0 ml/well with 10% BCS. The plates were placed overnight (16 to 20 hr) in an incubator with 95% air, 5% CO_2, 100% humidity at 37°C. After overnight incubation, the media in the plates were discarded, and 1 ml fresh incubation media containing 5% Nutridoma NS plus various hormones were added and the plates incubated for 4 or 24 hr. After overnight incubation with BCS, the cells were then cultured with Nutridoma NS because it is the best medium to sustain optimal synthesis of progesterone (1). The medium in each well was then collected for progesterone determination by RIA.

The dose response effects of oFSH, oLH oPRL, and rhFSH (0, 1, 10, 100 ng) on progesterone synthesis on Day 4 of pregnancy was first tested. After overnight incubation fresh media plus hormones were added to each well and the cells incubated for a total of 24 hr. The media were then collected for progesterone determination.

In subsequent experiments, 10 ng of each hormone was the selected dose. After overnight incubation following isolation, fresh media with 5% Nutridoma NS and hormones were added, and the plates were incubated for 4 hr. The media were then col-

lected and replaced with fresh media and hormones for an additional 24 hr of incubation.

To test the effect of activation of protein kinase C (PKC) on progesterone synthesis, 100 nM PMA, 10 μM db-cAMP, 1 mM forskolin (PMA and forskolin were dissolved in DMSO), oFSH, and oLH, or a combination of PMA and these reagents were added to the plates and cells were incubated for 4 hr. The doses of the reagents were based on our results with the hamster (2). When the luteal cells were removed from the culture plates, cell viability was checked again. Cell viability was 84%–87% following enzymatic dissociation, 76%–79% and 72%–75% respectively after 4 or 24 hr of incubation.

The media were collected at the end of incubation and stored at −20°C. Progesterone was determined by RIA. For each hormone or combination tested, duplicate samples of luteal cells were run in each incubation and there were three replicates for each treatment.

Progesterone Determination. The concentration of progesterone in the incubation media was determined by RIA without extraction using progesterone antiserum developed in rabbits (9). Labeled [1,2,6,7-^{3}H] progesterone was purchased from Dupont (Boston, MA). The mean sensitivity of the assay at 95% binding was 5 pg/tube. Progesterone concentration was expressed as ng/100,000 cells.

Statistical Analysis. The data were analyzed by a randomized complete block design. The ANOVA analysis was carried out with the SuperANOVA program of Abacus Concepts, Inc. (Berkeley, CA) using a Macintosh Classic II computer followed by Duncan's New Multiple Range test. Treatment effects were evaluated at each incubation time. Differences at a probability of $P < 0.05$ were considered statistically significant.

Results

On Day 4 of pregnancy, the rat CL consisted of 15.4% ± 0.92% large luteal cells (>20 μm) and 84.6% ± 0.91% small luteal cells (<20 μm); the number of large luteal cells was about four times greater than in the hamster CL on Day 4 (1).

Ovine FSH, oLH, and oPRL enhanced progesterone synthesis by rat luteal cells on Day 4 of pregnancy in a dose-dependent manner, and even 1 ng of each hormone significantly stimulated progesterone (Fig. 1). Although 1 ng rhFSH was ineffective, 10 and 100 ng significantly enhanced progesterone secretion after 24 hr of incubation (Fig. 2) comparable to the effects of 10 and 100 ng of oFSH after 4 or 24 hr of incubation. Progesterone synthesis was significantly stimulated by FSH, LH, or PRL, but no additive effects were observed when any two of the hormones were combined (Fig. 3). However, a striking increase in progesterone synthesis occurred when all three hormones were added to the incubation medium ($P < 0.05$). This was observed at both 4 and 24 hr (Fig. 3). Similar results were obtained with CL of pseudopregnancy on Day 4 (data not shown).

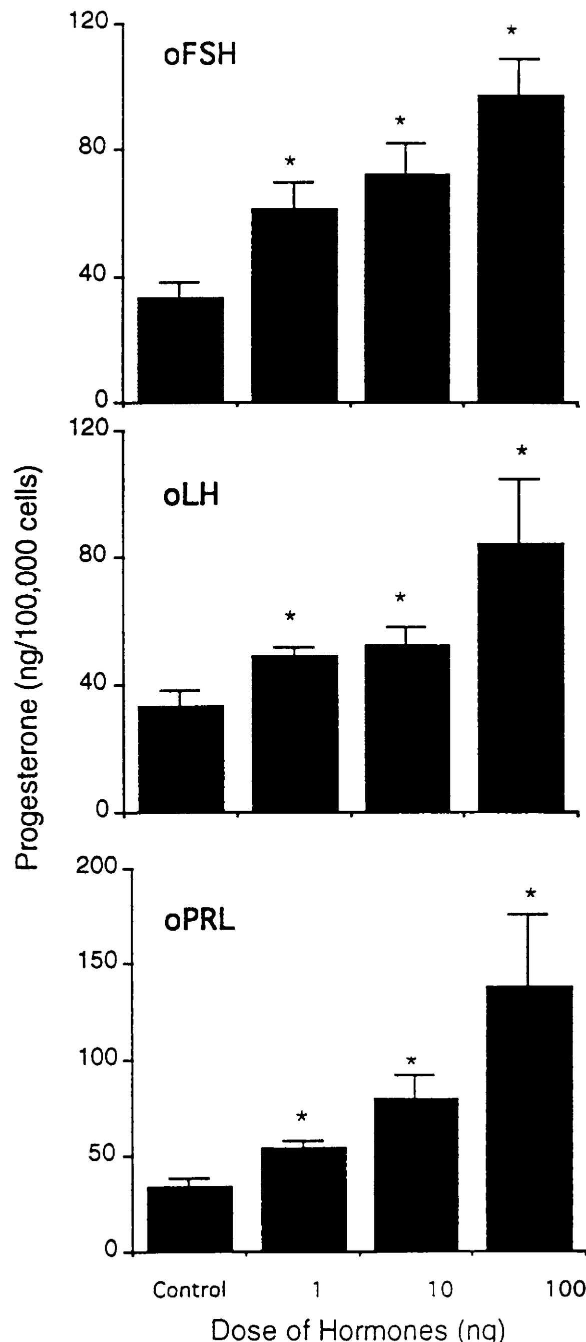

Figure 1. The dose-response effects of oFSH, oLH, and oPRL on progesterone production by rat luteal cells on Day 4 of pregnancy; incubation period: 24 hr. The values with stars differ significantly from the basal control ($P < 0.05$). Note that even 1 ng of each hormone significantly increased progesterone production. In all figures, the values represent the Mean ± SEM of duplicate determinations from three separate experiments.

The purpose of the final experiment was to determine through which second messengers FSH or LH increased progesterone synthesis. After 4 hr of incubation, progesterone synthesis was increased not only

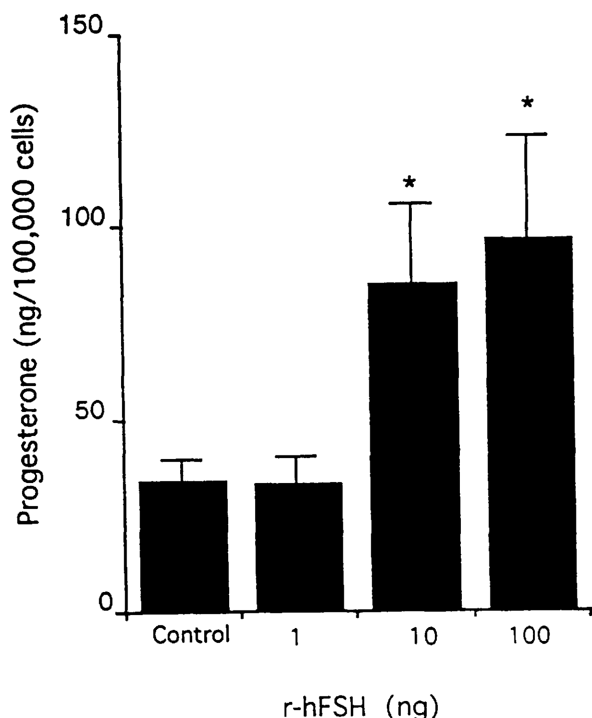

Figure 2. The dose response effects of rhFSH on progesterone synthesis by rat luteal cells on Day 4 of pregnancy. Incubation period: 24 hr. The values with stars differ significantly from control ($P < 0.05$).

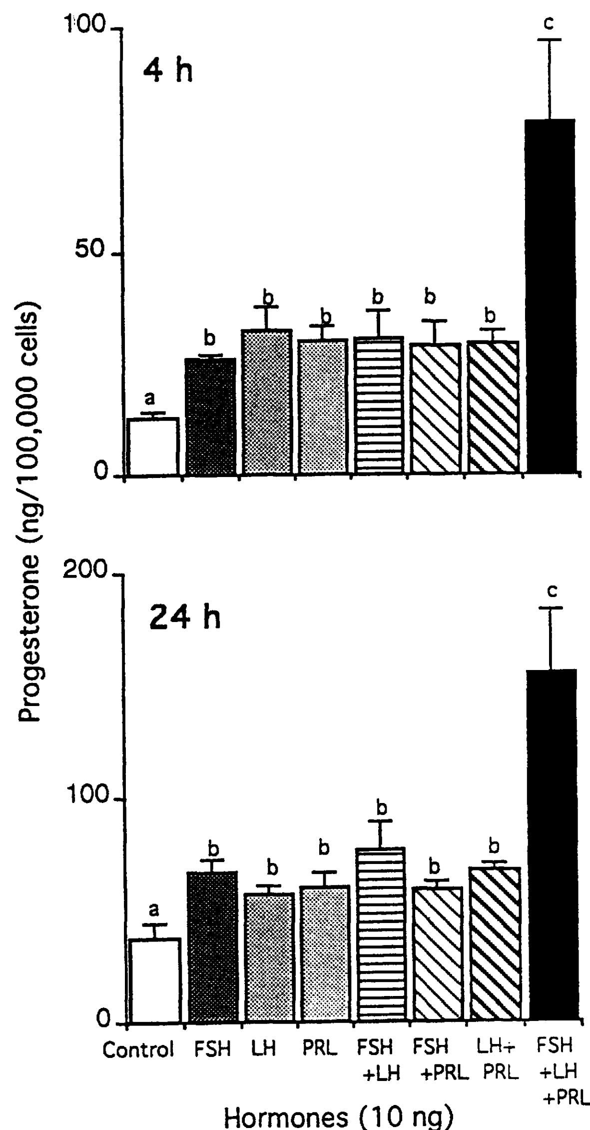

Figure 3. Effects of 10 ng oFSH, oLH, and oPRL or various combinations of these hormones on progesterone synthesis by rat luteal cells on Day 4 of pregnancy. Incubation period: 4 hr or an additional 24 hr. The values without a common letter are significantly different ($P < 0.05$). Note: oFSH + LH + PRL stimulated progesterone more than any other treatment ($P < 0.05$).

by 10 ng of ovine FSH or LH, but also by 10 μM cAMP or 1 μM forskolin (Fig. 4). The protein kinase C activator, PMA, by itself did not affect basal progesterone levels (Fig. 4) but significantly lowered progesterone production for all groups except for treatment with forskolin (Fig. 4).

Discussion

Although the *in vivo* effects of FSH and PRL as a minimal luteotropic complex in the rat were established 25 years ago (6), the present study, for the first time provides direct *in vitro* evidence that FSH enhances progesterone synthesis by rat luteal cells during pregnancy (Fig. 1). Since the rat luteal cells are treated with FSH *in vitro,* this minimizes the possible direct influence of follicular estradiol on CL *in vivo* (6, 7). The results with rhFSH, lacking LH contamination, especially corroborate the luteotropic effects of FSH in the rat (Fig. 2). Thus, the rat joins the hamster in demonstrating a direct *in vitro* role of FSH as a luteotropic hormone. It is noteworthy that highly purified human FSH is as effective as human LH in stimulating the *in vitro* production of progesterone in long-term cultured luteinized human granulosa cells collected from hyperstimulated cycles (10). Similarly, FSH stimulates *in vitro* progesterone production by porcine luteal cells at the early luteal phase (11), and FSH receptors are present in the bovine CL (12). Therefore, FSH likely plays a more universal role as a luteotropic hormone than previously recognized.

On Day 4 of pregnancy in the rat, after luteal dissociation there are about four times as many large cells as in the hamster. Our results agree very well with Nelson *et al.* (13), who reported 15.4% large luteal cells on Day 3 of rat pregnancy. This species difference in the number of large luteal cells may account for the considerably higher basal progesterone production (Fig. 1) than in the hamster (1). This agrees with results of an earlier study which showed on Day 4 of pregnancy a 5-fold greater serum level and *in vitro* production of progesterone by the rat compared with the hamster (14).

Prolactin and LH both stimulated progesterone

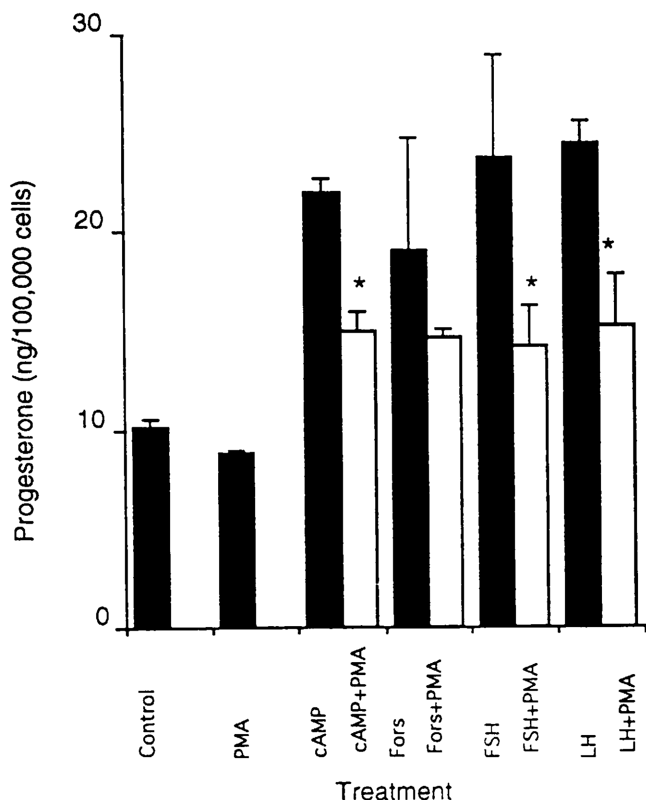

Figure 4. Effects of 100 n*M* phorbol 12-myristate 13-acetate (PMA) on 10 μ*M* db-cAMP-, 1 μ*M* forskolin-, 10 ng oFSH- or oLH-stimulated progesterone secretion by rat luteal cells on Day 4 of pregnancy after 4 hr incubation. The values with stars differ from db-cAMP-, oFSH-, and oLH-stimulated progesterone, respectively ($P < 0.05$).

synthesis by the dispersed rat luteal cells (Fig. 1 and 3) confirming previous results (15–17). The dose-response pattern for LH-stimulated progesterone production (Fig. 1) was similar to the profile obtained by culturing large luteal cells for 3 or 4 hr from rat CL dissociated on Day 3 or 8 of pregnancy (13, 15). In our experiments, the cells were cultured for 4 or 24 hr with 10 ng ovine LH (Fig. 3). The selected dose was based on our previous experiments with dissociated hamster luteal cells (1). A recent study used mixed rat luteal cells stimulated by 250 ng/ml of bovine LH (18). This resulted in 3 hr in a 4-fold increase in progesterone production compared with basal levels, whereas in our experiments with 10 ng LH there was only a 2-fold increase in progesterone (Fig. 3) It is possible that the use of a higher dose of oLH (e.g., 100 ng as in Fig. 1) might have elicited a greater response, but we considered 10 ng a more physiological *in vitro* treatment based on serum levels of 7 ng LH on Day 4 of pregnancy (19). Moreover, 100 ng FSH has considerable LH contamination which would have complicated the interpretation of the results.

It was interesting that on Day 4 of pregnancy, the combination of PRL, FSH, and LH produced the maximal secretion of progesterone—at both 4 and 24 hr of incubation (Fig. 3). This agrees with the additive effects of the hormones in dissociated hamster luteal cells (1). In the case of the hamster, PRL does not act via cAMP (2), but the situation may be quite different in the rat since the literature reports that PRL, acting *in vitro,* does increase cAMP (16, 17).

The mated immature rat was used as the model of pregnancy based on the similar hormonal profile to adult pregnant rats (8, 14, 18, 19). Logistically, it was more efficient than maintaining a large colony of adult rats which would have to be followed for several cycles before they could be utilized. Therefore, the possibility cannot be excluded that the use of pregnant rats might yield different results, (e.g., the response to FSH), although we consider this unlikely.

Luteal cells from hamsters and rats differ in several respects in response to the gonadotropins. In the hamster, combining any two hormones stimulates progesterone production, greater than any single hormone (1). However, this is not the case in the rat (Fig. 3). Since the rat CL contains a higher ratio of large luteal cells which contain more LH or PRL receptor binding sites (13), it is possible that either hormone alone has already maximally stimulated progesterone synthesis so that interaction of two hormones does not occur.

Another species difference is that in the rat, 10 ng rhFSH increases progesterone production as much as 10 ng oFSH (Fig. 1 and 2). In hamster luteal cells, however, progesterone production by 10 ng rhFSH is less than oFSH. As little as 1 ng prolactin stimulates progesterone synthesis by rat luteal cells whereas PRL by itself does not increase progesterone secretion by hamster luteal cells until Day 12 of pregnancy (1). It therefore seems likely that rat luteal cells are much more sensitive to the minimal luteotropic complex of FSH and PRL, especially to PRL than the hamster. This may be attributable to a higher ratio of large luteal cells containing more PRL receptor binding sites (13).

Phorbol 12-myristate 13-acetate at 100 n*M,* the same dose used in the hamster (2), did not affect progesterone synthesis in rat luteal cells on Day 4 of pregnancy but inhibited cAMP-, FSH- and LH-stimulated progesterone secretion (Fig. 4), similar to the results obtained with hamster luteal cells (2). Thus, activation of PKC blocks the *in vitro* luteotropic effects of FSH and LH during pregnancy in both species. The phorbol ester, PMA, mimics some of the luteolytic effects of $PGF_{2\alpha}$ by inhibiting progesterone synthesis and cAMP accumulation by isolated rat luteal cells (20). Small and large luteal cells of the ovine CL are similarly affected by PMA (21). The new information added by the present study is that the luteotropic activity of FSH is also inhibited by PMA.

We thank the National Hormone and Pituitary Program, Rockville, MD for kindly providing us with oLH, oPRL, and oFSH. We

also would like to thank Ares Advanced Technology and Ares Serono Group for rhFSH. We are grateful to Ms. Darlene Limback for her excellent assistance. The research was supported by a grant from the National Institutes of Health (HD-00596).

1. Yuan W, Greenwald GS. Luteotropic effects of FSH: I. FSH has in vitro luteotropic and synergistic effects with LH and prolactin on progesterone production by hamster luteal cells during pregnancy. Biol Reprod **51**:43–49, 1994.
2. Yuan W, Greenwald, GS. Luteotropic effects of FSH: II. FSH, LH and prolactin effects on second messenger systems in the corpus luteum of the pregnant hamster. Biol Reprod **51**:472–479, 1994.
3. Greenwald GS. Luteotropic complex of the hamster. Endocrinology **80**:118–130, 1967.
4. Greenwald GS. Further evidence for a luteotropic complex in the hamster: Progesterone determination of plasma and corpora lutea. Endocrinology **92**:2235–2242, 1973.
5. Gibori G. The corpus luteum of pregnancy. In: Adashi EY, Leung PCK, Eds. The Ovary. New York: Raven Press, pp261–317, 1993.
6. Greenwald GS, Johnson DC. Gonadotropic requirements for the maintenance of pregnancy in the hypophysectomized rat. Endocrinology **83**:1052–1064, 1968.
7. Ahmad NW, Lyons R, Papkoff H. Maintenance of gestation in hypophysectomized rats with highly purified pituitary hormones. Anat Rec **164**:291–304, 1969.
8. Nuti KMB, Sridharan BN, Meyer RK. Reproductive biology of PMSG-primed immature female rats. Biol Reprod **13**:38–44, 1975.
9. Surve AH, Barco I, Brinckerhoff JH, Kirsh SJ. Plasma levels of progesterone in pseudopregnant rabbits actively immunized with a progesterone protein conjugate. Biol Reprod **15**:343–349, 1976.
10. Sjögren A, Hillensjö T, Hamberger L. The regulatory role of FSH in steroid formation by luteinized human granulosa cells in culture. Human Reprod **2**:469–473, 1987.
11. Gregoraszczuk EL. Effect of various doses of FSH upon luteal cell function in tissue culture. Endocrinol Exp **23**:195–203, 1989.
12. Manns JG, Niswender GD, Braden T. FSH receptor in the bovine corpus luteum. Theriogenology **22**:321–328, 1984.
13. Nelson SE, McLean MP, Jayatilak PG, Gibori G. Isolation, characterization, and culture of cell subpopulations forming the pregnant rat corpus luteum. Endocrinology **130**:954–966, 1992.
14. Wada Y, Greenwald GS. Comparison of in-vitro production of progestagens by the corpora lutea of early pregnancy of the rat and hamster. J Reprod Fertil **71**:149–154, 1984.
15. Smith CJ, Greer TB, Banks TN, Sridaran R. The response of large and small luteal cells from the pregnant rat to substrates and secretogogues. Biol Reprod **41**:1123–1132, 1989.
16. Basuray R, Jaffe RC, Gibori G. Role of decidual luteotropin and prolactin in the control of luteal cell receptors for estradiol. Biol Reprod **28**:551–556, 1983.
17. Gitay-Goren H, Lindenbaum ES, Kraiem Z. Effects of prolactin on steroidogenesis and cAMP accumulation in rat luteal cell culture. Mol Cell Endocrinol **65**:195–201, 1989.
18. Tekpetey FR, Armstrong DT. Catecholestrogen modulation of steroid production by rat luteal cells: Mechanism of action. Mol Cell Endocr **101**:49–57, 1994.
19. Taya K, Greenwald GS. In vivo and in vitro ovarian steroidogenesis in the pregnant rat. Biol Reprod **25**:638–691, 1981.
20. Baum SM, Rosberg S. A phorbol ester, phorbol 12-myristate 13-acetate and a calcium ionophore, A 23187, can mimic the luteolytic effect of prostaglandin $F_{2\alpha}$ in isolated rat luteal cells. Endocrinology **120**:1019–1026, 1987.
21. Wiltbank MS, Diskin MG, Niswender GD. Differential actions of second messenger systems in the corpus luteum. J Reprod Fertil **43**(Suppl):65–75, 1991.