

Effects of Withdrawal of Exogenous Estradiol from Pseudopregnant Rabbits: Transient Nature of Loss of Luteal Function and Reversal of Estradiol-Induced Suppression of Luteinizing Hormone-Responsive Adenylyl Cyclase*

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ABSTRACT. We have reported in recent studies that exogenous estradiol (E_2) suppresses luteal LH-responsive adenylyl cyclase activity in pseudopregnant rabbits. The purpose of the present study was to determine whether this suppression is reversible. High or low level E_2 -filled Silastic capsules or empty capsules were sc implanted in day 5 pseudopregnant rabbits. On day 8 of pseudopregnancy, the high level E_2 implants were either sham replaced, replaced with low level E_2 implants, or replaced with empty capsules. The low level E_2 implants and empty capsules were sham replaced. Animals from each of the five resulting groups were killed on days 9–12 of pseudopregnancy (1, 2, 3, and 4 days postimplant manipulation). As previously reported using an intermittent injection protocol, exogenous E_2 had little effect upon serum progesterone concentrations. Both high and low level E_2 implants suppressed the luteal LH-responsive adenylyl cyclase, but the suppression due to the low level E_2

implants was not as great as that for the high level E_2 implants. Within 24 h of switching from high to low level E_2 implants, LH-responsive adenylyl cyclase activity increased from the level found for animals with high level E_2 throughout to that found for animals with low level E_2 throughout. Total withdrawal of exogenous E_2 resulted in a precipitous fall in serum progesterone concentrations, as predicted by previous studies. However, within 4 days of withdrawal, both serum progesterone and luteal LH-responsive adenylyl cyclase activity had returned to control values. E_2 implants also suppressed serum LH concentrations and follicular LH-responsive adenylyl cyclase activities. Both of these effects were reversed within 24–48 h after implant withdrawal. We conclude, therefore, that effects of exogenous E_2 are reversible and that the previously reported E_2 -induced dependency upon exogenous E_2 is related to the experimental protocol used. (*Endocrinology* 109: 2129, 1981)

IT HAS been established that estradiol (E_2) is the primary luteotropin in rabbits (1–7). However, at least two other hormones present under normal physiological conditions can exert effects on corpora lutea (CL). Our laboratory has shown that both LH and isoproterenol can stimulate luteal adenylyl cyclase (8–11). In addition, LH can desensitize adenylyl cyclase activity (9, 10) and can cause functional luteolysis in spite of a continuous supply of E_2 (10).

We have recently reported that high levels of exogenous E_2 cause a suppression of luteal LH-responsive adenylyl cyclase (10, 11), and there are several reports which suggest that treatment of pseudopregnant rabbits with high levels of exogenous E_2 can cause a dependence upon exogenous E_2 (11–13), since the loss of progesterone secretion occurs after the cessation of E_2 treatment. Although the exact cause of the loss of luteal function

after E_2 withdrawal is not currently known, a likely explanation is that the exogenous E_2 suppresses gonadotropin secretion and, therefore, follicular development. Upon exogenous E_2 withdrawal, the follicles are not capable of E_2 secretion, and luteal regression is the result. It is possible, however, that an impaired LH-responsive adenylyl cyclase could contribute to the loss of luteal function, since in the past, we have found a good positive correlation between serum progesterone and luteal LH-responsive adenylyl cyclase activity. In a previous study (11), we attempted to determine whether the suppression of the LH-responsive adenylyl cyclase by exogenous E_2 is permanent or reversible. Unfortunately, the data were inconclusive. For this reason, a new experimental protocol has been established, the results of which are reported herein.

Materials and Methods

Materials

E_2 -filled Silastic capsules (id, 3.35 mm; od, 4.65 mm; 12 mm in length) were prepared according to the method of Holt *et al.*

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(12) and are referred to as high level implants. Other E₂-filled Silastic capsules (id, 1.57 mm; od, 2.41 mm) were prepared according to the method of Legan *et al.* (14), except that the total length of the capsule was 9 mm, with a 3-mm fill length. These capsules are referred to as low level implants. hCG was a gift from Dr. John B. Jewell (Ayerst Laboratories, Rouses Point, NY) and was dissolved in 0.15 M saline to yield a concentration of 250 IU/ml. LH (NIH-LH-S19, obtained from the NIAMDD) was kept as a stock solution (1 mg/ml) in 0.15 M NaCl. (-)Isoproterenol was a gift from Dr. F. C. Nachod (Sterling Winthrop Research Institute, Rensselaer, NY) and kept as a 10⁻²-M stock solution in 10⁻³ M HCl. NaF (Fisher Scientific Co., Waltham, MA) was kept as a 1-M stock solution. Prostaglandin E₁ (PGE₁; kept as a 1 mg/ml stock solution in 10 mM Tris-base) was a gift from Dr. John E. Pike (Upjohn Co., Kalamazoo, MI), as were the steroid-11-hemisuccinate-tyrosine-methyl-esters (steroid-11-TME). For adenylyl cyclase assays, stock solutions of LH, isoproterenol, NaF, and PGE₁ were diluted with water to 50 µg/ml, 500 µM, 50 mM, and 50 µg/ml, respectively; 10 µl of each solution were used in the assays. Creatine phosphate and creatine kinase were obtained from Calbiochem (La Jolla, CA); myokinase, ATP (Tris-salt), cAMP, EDTA, and Tris were purchased from Sigma Chemical Co. (St. Louis, MO); [α -³²P]ATP (20–50 Ci/mmol), synthesized according to the procedure of Walseth and Johnson (15) and purified according to the method of Birnbaumer *et al.* (16), was supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine (Houston, TX). [³H]cAMP (10–15 Ci/mmol) was obtained from Amersham/Searle (Arlington Heights, IL). All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

Animals

Virgin New Zealand White rabbits (3–4 kg) were housed in individual cages in air-conditioned quarters and were fed *ad libitum* Purina rabbit chow for at least 15 days before the initiation of experiments. Pseudopregnancy was induced by the iv injection of 100 IU hCG (0.4 ml). The day after hCG injection was designated day 1 of pseudopregnancy. This mode of induction of pseudopregnancy causes no appreciable difference in luteal adenylyl cyclase activities during the time span studied here compared to levels seen in CL from pregnant rabbits (8, 9).

Treatments and preparation of sera and CL homogenates

On the morning of day 5 of pseudopregnancy, rabbits were anesthetized with Nembutal and sc implanted with high level E₂ capsules, low level E₂ capsules, or empty capsules. On the morning of day 8 of pseudopregnancy, rabbits with high level E₂ capsules had the capsules sham replaced (high:high group), replaced with low level capsules (high:low group), or replaced with empty capsules (high:none group). Rabbits with low level implants had the implants sham replaced (low:low group), as did rabbits with empty capsules (none group). All implant and sham replacements were performed without anesthetic. Rabbits

from each group were killed 24, 48, 72, and 96 h after implant or sham replacement (days 9, 10, 11, and 12 of pseudopregnancy, respectively). The rabbits were killed by cervical dislocation, blood was collected by cardiac puncture, and the ovaries were removed and placed in ice-cold Krebs-Ringer bicarbonate buffer prepared with half the recommended amount of CaCl₂ (17). The blood was allowed to clot at room temperature for about 10 min and was placed in a refrigerator for 4 h, after which it was spun in a refrigerated centrifuge for 10 min to obtain serum.

The largest antral follicles found were dissected free of the ovaries and placed in ice-cold Krebs-Ringer bicarbonate buffer in a small watch glass. The follicles were opened to release the follicular fluid, but care was taken not to lose excessive amounts of granulosa cells. The follicles and loose granulosa cells were transferred to a Dounce homogenizer and homogenized in 500 µl ice-cold 27% (wt/wt) sucrose in 10 mM Tris-HCl and 1 mM EDTA (STE buffer), pH 7.5, with 10 strokes of a tight pestle. The follicular homogenates were filtered through a silk screen, as described previously (8). In each case, the Dounce homogenizer was washed with 150 µl STE buffer, and the wash was also filtered. The washes were combined with the first filtrates, and the resulting homogenates were used for the adenylyl cyclase assays. The CL were dissected free of the ovaries, cleaned of adhering intestinal tissue, and kept in ice-cold Krebs-Ringer bicarbonate buffer until further processing (30 min to 1 h). Before homogenization, CL were blotted and weighed. Homogenization was performed in 10 vol ice-cold 27% (wt/wt) STE buffer, as previously described (8); the homogenates were diluted with 10 vol ice-cold buffer and filtered through a silk screen. Homogenates were analyzed for adenylyl cyclase activity within 30 min, for protein by the method of Lowry *et al.* (18) after storage at -20 C for 1 or 2 days, and for E₂ content within 9 months, during which they were stored at -20 C.

Adenylyl cyclase assays

Adenylyl cyclase activity in 20-µl aliquots of homogenates were determined, as described earlier (8), at 32.5 C in 50 µl medium containing 3.0 mM [α -³²P]ATP (~5 × 10⁶ cpm for luteal homogenates and ~15 × 10⁶ cpm for follicular homogenates), 5.0 mM MgCl₂, 1 mM EDTA, 1 mM [³H]cAMP (~10,000 cpm), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl. When present, the LH concentration was 10 µg/ml, the isoproterenol concentration was 100 µM, the NaF concentration was 10 mM, and the PGE₁ concentration was 10 µg/ml. The final pH of the incubation (10 min) was 7.0. The [³²P]cAMP formed was isolated by the method of Salomon *et al.* (19), as modified by Bockaert *et al.* (20). Results are expressed as picomoles of cAMP formed per min/mg protein. Protein was determined by the method of Lowry *et al.* (18) using bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago, IL) as standard.

Progesterone assays

To determine procedural losses, [1,2-³H]progesterone (New England Nuclear Corp.; ~1.5 × 10³ cpm; SA, 50 Ci/mmol) was added to all serum samples. The samples (400 µl) were then

extracted with 10 vol petroleum ether. The dried extracts were reconstituted with 1.2 ml 0.1% gelatin-0.01 M phosphate-buffered saline, pH 7.4 (gel-PBS), 0.2 ml of which was counted in a liquid scintillation counter to determine recovery.

The labeled hormone for this assay was [¹²⁵I]iodoprogesterone-11-hemisuccinate-tyrosine-methyl-ester ([¹²⁵I]P₄-11-TME), which was prepared as follows. P₄-11-TME (Upjohn; 400 pmol in 2 μl) was reacted with 0.7 mCi (320 pmol in ~2 μl) of Na¹²⁵I (Isotex, Houston, TX) using 7 nmol chloramine-T (Eastman Kodak, Rochester, NY; 2 μl) as oxidant. The reaction was stopped after 2 min by the addition of 21 nmol (5 μl) sodium metabisulfite. The reaction mixture was extracted with petroleum ether, dried under N₂, and reconstituted by dissolving the residue in 75 μl methanol and then adding 40 μl H₂O (65% methanol). The reconstituted iodination product was run over a Waters 0.4 × 30 cm μBondapak C₁₈ high pressure liquid chromatography (HPLC) column using 65% methanol (1 ml/min) as the developing solvent. Inorganic ¹²⁵I ran with the solvent front. Two major organic iodine-containing peaks were obtained (Fig. 1A). The first, eluting at approximately 17 min, was used for the assays. Increasing the amount of ¹²⁵I used for the iodination increased the peak 2 to peak 1 ratio of ¹²⁵I twice as much as the peak 2 to peak 1 ratio of the absorbance at 254 nm (A₂₅₄). In the procedure described below, the counts per min nonspecifically bound were about twice as high for peak 2 as for peak 1. Therefore, we believe that peak 1 is monoiodo-P₄-11-TME, whereas peak 2 is diiodo-P₄-11-TME.

We would have preferred using the peak 2 iodination product (presumably diiodo), since half as much unknown would be required for displacement of the labeled steroid. Unfortunately, the presumed diiodo-P₄-11-TME bound with higher affinity to the antibody than did the presumed monoiodo-P₄-11-TME, such that at antibody dilutions yielding equal fractions of added

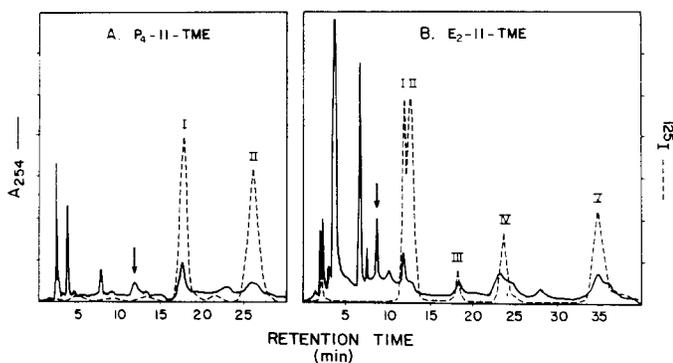


FIG. 1. Separation of steroid-11-TME iodination products using HPLC. P₄-11-TME (A) and E₂-11-TME (B) were iodinated as described in *Materials and Methods*. The separations were accomplished using a Waters μBondapak C₁₈ column through which 65% methanol was run at 1 ml/min, creating a back-pressure of approximately 1400 psi. The arrows indicate the positions of A₂₅₄ peaks for uniodinated P₄-11-TME (A) and uniodinated E₂-11-TME (B). A₂₅₄, Absorbance of effluent at 254 nm, as measured by a Waters model 440 absorbance detector. ¹²⁵I, Tracing of radioactivity as measured by juxtaposing a Nuclear-Chicago (Des Plaines, IL) model 2650 Geiger counter to the effluent tubing. The units for both A₂₅₄ and ¹²⁵I are arbitrary.

iodinated steroid bound (~25°C; 1:60,000 for peak 1 and 1:120,000 for peak 2), 50% inhibition was achieved with approximately 240 pg progesterone/tube using presumed monoiodo-P₄-11-TME, whereas about 330 pg progesterone/tube were required when presumed diiodo-P₄-11-TME was used as the labeled ligand. Retention times of the steroid-11-TME and iodination products gradually decreased with increasing age of the column. To some extent, this can be compensated for by gradually decreasing the methanol concentration in the developing solvent. The HPLC chromatographs in Fig. 1 were obtained using a new column.

The samples (all sera from a particular day of postimplant manipulation) and standards (Calbiochem) were pipetted in duplicate into 12 × 75-mm assay tubes and brought to 500 μl with gel-PBS. Two hundred microliters of antiserum GDN 337 (supplied by Dr. Gordon D. Niswender) at a dilution of 1:60,000 in 1:100 NRS (1 part normal rabbit serum plus 99 parts gel-PBS; final assay dilution, 1:240,000) and 100 μl [¹²⁵I]P₄-11-TME (~20,000 cpm) were added simultaneously to each tube at room temperature. In addition, three tubes received 100 μl labeled progesterone plus 1.7 ml gel-PBS (total count tubes), and three tubes with 500 μl gel-PBS received 200 μl 1:100 NRS and 100 μl labeled hormone (nonspecific binding tubes). All tubes were transferred to a cold room, where they were incubated overnight. The following day, 1 ml 0.5% charcoal-0.05% dextran was added in the cold room to each tube (except for the three total count tubes). After a 15-min incubation in the cold, the tubes were spun for 15 min in a refrigerated centrifuge, and the supernatants were poured into clean 12 × 75-mm tubes and counted for 1 min each in a Searle 1197 γ-counter (Searle Analytical, Des Plaines, IL). Assay results were analyzed using a computer program based on the assay statistics described by Midgley, Jr., *et al.* (21) and Duddleson *et al.* (22). Cross-reactivities of various other steroids with the GDN 337 antiserum using this assay procedure are not significantly different than those described by Gibori *et al.* (23) using a tritiated progesterone as labeled hormone, except for 5α-pregnan-3,20-dione (67.7%) and pregnenolone (15.0%).

Using these procedures, nonspecific binding was 1.6 ± 0.1%, and the zero competition tubes gave 25 ± 1% binding. The 50% inhibition point was about 240 ± 26 pg/tube. The assay sensitivity (2 SD of one set of triplicate zero competition tubes near the beginning of the assay and a second set of triplicates near the end of the assay) was 7 ± 3 pg/tube, and the slope of the standard curve was -1.76 ± 0.04. These values are the mean ± SEM of nine assays. To control for interassay variation, a standard serum was run in each assay. The coefficient of variation for this serum was 9.4%.

E₂ assays

E₂ was assayed in chromatographed benzene extracts of serum samples and luteal homogenates using the antibody and basic assay developed by England *et al.* (24). Each assay contained a sample from one animal per group. Briefly, [2,4,5,7,16,17-(N)-³H]E₂ (New England Nuclear Corp.; 1.5 × 10³ cpm in 20 μl; SA, 130 Ci/mmol) was added to each serum (1 ml) and tissue (150 μl homogenate plus 350 μl 0.1% gel-PBS) sample. Benzene (9 ml for serum; 4.5 ml for homogenates) was added to

each sample for extraction. The extracts were dried under N_2 , reconstituted with 400 μ l benzene-methanol (9:1), and applied to Sephadex LH-20 columns (2.6-ml column bed) equilibrated with the same solvent. After chromatography on Sephadex LH-20 columns using the same solvent, the samples were dried and reconstituted with 0.9 ml gel-PBS, 200 μ l of which were used for determination of recovery. The set-up of the assay was similar to that of the progesterone assay with the following exceptions. GDN 930 was the antiserum used and was added as a 1:200,000 dilution (in 1:100 NRS; final assay dilution, 1:800,000) at room temperature. After a 1-h incubation at room temperature, the samples were transferred to a cold room and incubated for an additional 0.5 h; approximately 12,000 cpm [^{125}I]E₂-11-hemisuccinate-tyrosine-methyl-ester ([^{125}I]E₂-11-TME) were added to each tube, and the samples were incubated for a further 1.5 h in the cold room. At this point, the charcoal-dextran was added. The tubes were then processed as described above for progesterone assays.

The [^{125}I]E₂-11-TME was prepared by reaction of 500 pmol E₂-11-TME (Upjohn; 5 μ l) with 0.22 mCi (100 pmol in ~0.5 μ l) of Na ^{125}I (Isotex) using 8 nmol chloramine-T (Eastman; 5 μ l) as oxidant. The reaction was stopped after 30 sec using 52.5 (2.5 μ l) nmol sodium metabisulfite. The reaction mixture was extracted with benzene, dried under N_2 , and reconstituted by dissolving the residue in 75 μ l methanol and then adding 40 μ l water. The reconstituted extract was chromatographed over a HPLC μ Bondapak C₁₈ column, as described above for progesterone derivatives, using 65% methanol in water as the developing solvent. Inorganic iodide eluted with the solvent front. Five major organic ^{125}I peaks were obtained (Fig. 1B). Of these, the first three were suitable for use in the RIAs. The first two peaks, eluting at approximately 11-14 min, had similar properties and were pooled for use. The third peak, although suitable for use, yielded a high level of nonspecific binding (~4.3% vs. ~1.2% for peaks 1 and 2). The fourth peak yielded moderate nonspecific binding (2.6%), but poor specific binding (~8.8% vs. ~28.0% for peaks 1, 2, and 3 at the same antibody dilution). The fifth peak did not specifically bind to the antibody.

Using these procedures, nonspecific binding was $2.9 \pm 0.1\%$ and the zero competition tubes gave $29.5 \pm 4.8\%$ binding. The 50% inhibition point was 8.1 ± 1.5 pg/tube. The assay sensitivity was 198 ± 80 fg/tube, and the slope of the standard curve was -2.16 ± 0.11 . These values are the mean \pm SEM of 28 assays. The coefficient of variation for a standard serum run in these assays was 12.1%.

LH assays

Serum LH was assayed according to the basic procedures for double antibody RIA described by Niswender *et al.* (25). All samples were assayed in a single assay. The primary antibody was AFP-8-1-28 used at a final dilution in the assay of 1:4,000,000. Rabbit LH used for both iodination and standard was AFP-559-B. We are indebted to Dr. A. F. Parlow (NIAMDD Pituitary Hormone Distribution Program) for supplying both the rabbit LH and the antirabbit LH serum. Briefly, sample (300 μ l) or standard assay tubes were brought to 500 μ l using 0.1% gel-PBS. Then, 200 μ l of a 1:1,000,000 dilution of AFP-8-1-

28 antibody in 1:100 NGpS [1 part normal guinea pig serum (Colorado Serum Lab) plus 99 parts 0.05 M EDTA-PBS] were added, and the tubes were allowed to incubate at 4 C for 24 h. One hundred microliters of [^{125}I]iodoLH (~50,000 cpm) were added, and the tubes were allowed to incubate for 48 h at 4 C. Then 200 μ l goat antiguinea pig γ -globulin (G4-1P4, Antibodies, Inc., Davis, CA; 1:12 in 0.1% gel-PBS) were added to the tubes, which were incubated for an additional 24 h at 4 C. The tubes were then centrifuged for 15 min at 4 C, the supernatants were poured off, and the pellets were counted in a γ -counter.

Using these procedures, nonspecific binding was $1.5 \pm 0.1\%$, and the zero competition tubes gave $37.9 \pm 0.6\%$ binding. The 50% inhibition point was 3.0 ± 0.1 ng/tube. The assay sensitivity was 92.9 ± 31.7 pg/tube, and the slope of the standard curve was -2.25 ± 0.08 . These values are the mean \pm SEM of four assays. The coefficient of variation for a standard serum run in these assays was 10.2%.

Statistics

Multiple comparisons were made using analysis of variance, and differences were considered significant when $P < 0.05$.

Results

As described in *Materials and Methods*, we explored the effects of E₂ implants and implant withdrawal over a period that lasted from 1-4 days. We determined the serum E₂ levels 1, 2, 3, and 4 days after implant withdrawal, sham withdrawal, and replacement with low level E₂ implants in animals that had initially received high or low level E₂ implants or empty implants. In this way we obtained what are referred to as the high:high, high:low, high:none, low:low, and none groups. We found that the levels of serum E₂ varied with the size of implant present on the day of sacrifice, and that the number of days after implant manipulation made no difference. Therefore, the data shown in Fig. 2 are the serum E₂ concentrations resulting from each of the five different treatments regardless of the day of sacrifice, *i.e.* the mean \pm SEM of all 4 days combined. It can be seen that the high level implants yielded serum E₂ concentrations approximately 4.1 times the control level, and the low level implants produced serum E₂ concentrations approximately 2.2 times the control level.

Table 1 contains the data from adenylyl cyclase assays of luteal homogenates. Exogenous E₂ appeared to have no effect upon basal adenylyl cyclase activity. In agreement with previous studies from this laboratory (10, 11), high levels of exogenous E₂ suppressed LH-responsive adenylyl cyclase activity by about 50%. The low level E₂ implants also suppressed LH-responsive adenylyl cyclase, but only by about 63% as much as the high level implants. Within 2 days after implant replacement, the high:low group had LH-responsive adenylyl cyclase activities comparable to those of the low:low group, and within 4 days,

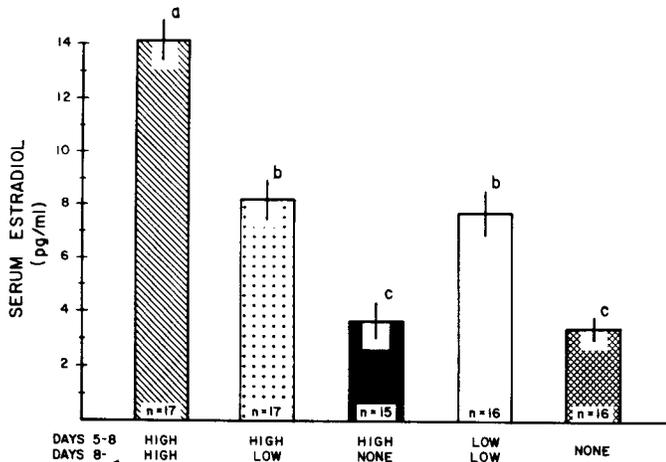


FIG. 2. Serum E₂ concentrations resulting from E₂ implant treatments. The treatments described in the text are indicated under each bar. Since there were no statistical differences within each treatment group from day to day over days 9–12, each bar represents the mean \pm SEM of data from all rabbits for the designated treatment regardless of the day of sacrifice. (There were several rabbits from which insufficient quantities of sera were obtained for assay.) Groups with different letters above the bars are significantly different at $P < 0.05$.

the high:none group had LH-responsive adenylyl cyclase activities which were not different from the control value. Isoproterenol-responsive adenylyl cyclase activities on days 9 and 10 followed a pattern similar to those found with LH, except that the suppression due to exogenous E₂ was not as great (24–36% decrease). On days 11 and 12, exogenous E₂ had no apparent effect upon isoproterenol-responsive adenylyl cyclase activity. On days 9 and 10, E₂ treatment had no effects on PGE₁-responsive adenylyl cyclase, but on days 11 and 12, a slight suppression of adenylyl cyclase activity was noted. E₂ treatments had no effects on fluoride stimulation of adenylyl cyclase in our study.

Figure 3 is a representation of the stimulation of luteal adenylyl cyclase by LH relative to basal (*i.e.* fold stimulation). We have found in the past that this is a useful means of presenting data, especially in light of the fact that the coefficients of variation for the different groups are usually substantially lower when the data are presented in this way. Whereas the absolute values of LH-responsive adenylyl cyclase (in picomoles per min/mg) of the high:high group were not different from those of the low:low group on 2 of the 4 days, the low:low group values were about 1.5 times greater than the high:high group values on each of the days ($P < 0.0005$, $P < 0.005$, $P < 0.025$, and $P < 0.0005$ on days 9–12, respectively) when expressed as stimulation relative to basal. In addition, switching from high to low level E₂ implants resulted in a return of LH-stimulated adenylyl cyclase activity relative to basal to levels found with low level implants within 1 day of implant replacement. Four days were

required for the fold stimulation by LH to return to control levels after high level implants were replaced with empty implants (the high:none group on day 12 is not statistically different from the pooled mean of the none groups for days 9–12).

Figure 4 represents the serum progesterone concentrations for each of the treatment groups. On day 9, the high level implant caused a slight (35%) but significant ($P < 0.05$) increase in serum progesterone over the control value. This effect of exogenous E₂ was not seen at any of the other times studied, and in general, exogenous E₂ had no effect upon serum progesterone concentrations, as has been reported previously (10–13). In addition, switching from high level to low level implants had no effect on serum progesterone concentrations. Replacing high level implants with empty capsules resulted in a precipitous fall in serum progesterone within 24 h, as reported by others (12, 13). However, unlike these other reports, from that point on the serum progesterone concentrations progressively increased. Although at 2 days after E₂ withdrawal, the mean serum progesterone levels of the withdrawal group was less than that of the control group, the difference was not significant. The serum concentrations of progesterone were nearly identical in the two groups 3 and 4 days after the withdrawal of E₂.

Figure 5 contains the day 9 follicular adenylyl cyclase data along with the day 9 luteal adenylyl cyclase data for comparison. Decreasing the serum concentration of E₂ by implant manipulation had an effect on basal follicular adenylyl cyclase similar to that found in the CL, *i.e.* basal adenylyl cyclase activity was slightly ($P < 0.005$) lower than the control level. The presence of exogenous E₂ in itself had no effect on the basal cyclase activity in follicles, but did suppress LH-responsive adenylyl cyclase activity by about 25% ($P < 0.025$), though not to the same extent as in CL. Two and 3 days after implant manipulation, exogenous E₂ was continuing to suppress LH-responsive adenylyl cyclase activities. When exogenous E₂ treatments were discontinued, follicular adenylyl cyclase activities returned to control values (data not shown).

The serum LH data shown in Fig. 6 indicate that the E₂ implants suppressed serum LH concentrations. Despite the fact that the data in Fig. 6 are from a single bleeding 24 h after implant manipulation, it is apparent that LH secretion was returning to control levels in the group which had the high level implants removed.

Discussion

We have previously reported that high levels of exogenous E₂ suppress luteal LH-responsive adenylyl cyclase activity and that reversal of this suppression was neither rapid nor complete (11). Although the data presented

TABLE 1. Adenylyl cyclase activities in homogenates of CL of rabbits subjected to various E₂ treatments

Treatment schedule ^a	n	Addition to adenylyl cyclase assays ^b				
		None	LH	ISO	NaF	PGE ₁
Day 5 8 9						
↓ ↓ ↓ A						
High high	9	9.4 ± 0.6 ^{c,d}	26.1 ± 1.3 ^{c,e}	25.4 ± 1.7 ^{c,d}	55.9 ± 7.9	25.7 ± 2.8
High low	9	8.5 ± 0.5 ^c	31.7 ± 2.6 ^c	27.5 ± 2.5 ^{c,d}	56.1 ± 3.5	24.5 ± 2.3
High none	8	9.2 ± 0.5 ^c	21.7 ± 1.8 ^e	23.2 ± 1.7 ^c	57.9 ± 6.9	25.1 ± 1.9
Low low	9	9.3 ± 0.3 ^{c,d}	36.9 ± 1.7 ^d	29.2 ± 1.6 ^d	60.2 ± 4.0	26.1 ± 2.3
None none	8	10.6 ± 0.7 ^d	54.9 ± 4.5 ^f	39.8 ± 4.1 ^e	67.2 ± 5.6	30.3 ± 3.2
Day 5 8 10						
↓ ↓ ↓ A						
High high	4	10.8 ± 0.9	26.3 ± 1.8 ^c	26.1 ± 1.5 ^c	61.4 ± 7.1	27.0 ± 0.6
High low	4	9.8 ± 0.6	36.7 ± 4.7 ^d	28.8 ± 1.8 ^c	68.6 ± 10.2	24.5 ± 1.8
High none	4	10.4 ± 1.7	25.0 ± 3.3 ^c	26.5 ± 1.1 ^c	62.7 ± 6.7	25.7 ± 4.1
Low low	4	8.9 ± 0.5	33.0 ± 4.7 ^{c,d}	26.2 ± 2.1 ^c	61.1 ± 4.6	21.2 ± 0.9
None none	4	10.4 ± 1.2	47.0 ± 3.4 ^d	34.4 ± 3.1 ^d	74.4 ± 9.9	26.6 ± 2.6
Day 5 8 11						
↓ ↓ ↓ A						
High high	4	9.5 ± 0.6	25.2 ± 1.4 ^c	28.2 ± 1.8	66.4 ± 10.5	21.7 ± 1.3 ^c
High low	4	9.8 ± 0.7	33.6 ± 2.0 ^{d,e}	34.2 ± 2.3	67.7 ± 5.5	26.7 ± 1.4 ^{d,f}
High none	4	8.4 ± 0.9	30.5 ± 2.7 ^{c,d}	26.7 ± 2.6	64.9 ± 5.9	22.1 ± 2.0 ^{c,d}
Low low	4	8.8 ± 0.5	31.1 ± 2.0 ^{c,d}	30.7 ± 4.1	59.1 ± 6.7	25.7 ± 1.2 ^{d,e,f}
None none	4	8.6 ± 0.8	40.2 ± 5.2 ^e	31.5 ± 4.8	66.3 ± 4.3	27.2 ± 2.0 ^f
Day 5 8 12						
↓ ↓ ↓ A						
High high	5	9.0 ± 0.7	18.4 ± 2.7 ^c	23.4 ± 3.0 ^c	60.8 ± 7.3	19.6 ± 2.9 ^c
High low	5	8.4 ± 0.6	28.6 ± 1.7 ^d	25.2 ± 3.2 ^{c,d}	63.9 ± 5.6	20.0 ± 2.0 ^c
High none	4	9.2 ± 0.6	34.4 ± 1.7 ^e	30.6 ± 1.0 ^d	73.8 ± 5.1	23.4 ± 2.6 ^{c,d}
Low low	5	8.1 ± 0.7	29.1 ± 2.1 ^{d,e}	26.3 ± 1.7 ^{c,d}	62.9 ± 5.4	23.3 ± 1.0 ^{c,d}
None none	4	7.8 ± 0.5	30.0 ± 2.1 ^{d,e}	24.2 ± 2.9 ^{c,d}	69.4 ± 7.1	27.7 ± 5.5 ^d

^a A, Day of autopsy.^b Values are the mean ± SEM in picomoles per min/mg homogenate protein. Groups without a common superscript have activities that differ at $P < 0.05$.

here appear to contradict that conclusion, there are two probable explanations for the seemingly contradictory results. First, in the previous report, injections of E₂ produced transiently much higher serum E₂ values than those produced by Silastic implants. Second, since the dose of E₂ was switched late in pseudopregnancy (day 11), it could not be adequately determined if the suppression of adenylyl cyclase activity could be reversed before the onset of normal luteal regression.

These data also appear to contradict the finding that withdrawal of high levels of exogenous E₂ causes luteal regression in pseudopregnant rabbits (11-13). However, this difference can also be explained. Day *et al.* (11) used 15 µg/12 h E₂, administered by injection, which, as mentioned above, would cause transiently much higher serum concentrations of E₂ than that released by the high level implants used here. Holt *et al.* (12) and Bender *et al.* (13) inserted high level E₂ implants on day 1 of pseudopregnancy and thereby suppressed follicular development to

the point that implant withdrawal caused a fall of serum E₂ to nondetectable levels. In the present study, the high level implant was inserted on day 5, and large antral follicles containing LH-stimulable adenylyl cyclase activity were observed in the presence of high estrogen concentrations. Furthermore, the fact that serum E₂ concentrations were at control values 24 h after withdrawal of the E₂ implant indicates that the follicles were producing E₂ normally at that time. Therefore, this experiment could be likened to that of Bender *et al.* (13), in which the E₂ implants were replaced 24 h after their withdrawal. They found a sharp fall in serum progesterone concentrations after withdrawal, followed by a somewhat more gradual increase back to control levels after the implant was replaced. In our study, we had a very similar finding, except that the replacement of E₂ was endogenous rather than exogenous.

In previous studies, this laboratory found a positive correlation between the functional activity of the CL and

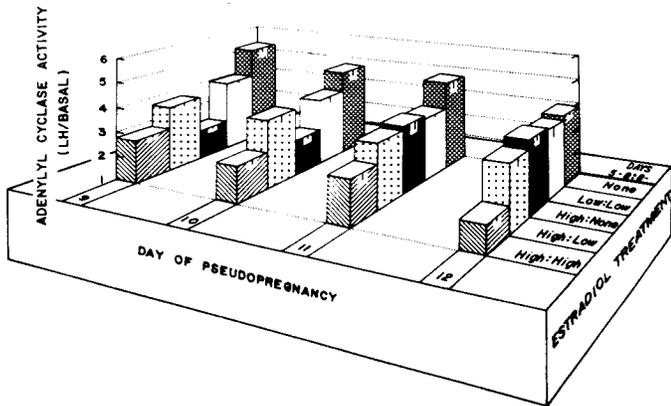


FIG. 3. Luteal LH-responsive adenylyl cyclase activities resulting from the various E₂ treatments. Each bar represents the mean and SEM of the fold stimulation of adenylyl cyclase by LH (LH-stimulated activity/basal activity; left-most axis) on each day of pseudopregnancy from days 9-12 (front-most axis) for each E₂ treatment (right-most axis). Days 9-12 of pseudopregnancy correspond to days 1-4 postimplant manipulation. Significant differences ($P < 0.05$) and number of rabbits (n) were as follows: day 9, None (n = 8) > low:low (n = 9) = high:low (n = 9) > high:high (n = 9) > high:none (n = 8); day 10, none (n = 4) > low:low (n = 4) = high:low (n = 4) > high:none (n = 4) = high:high (n = 4); day 11, none (n = 4) > low:low (n = 4) = high:low (n = 4) = high:none (n = 4) > high:high (n = 4); day 12, none (n = 4) = low:low (n = 5) = high:low (n = 5) = high:none (n = 4) > high:high (n = 5).

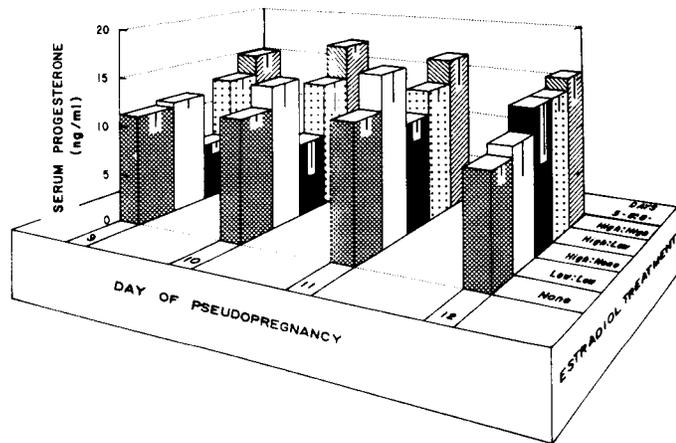


FIG. 4. Serum progesterone concentrations resulting from the various E₂ treatments. Each bar represents the mean and SEM of the serum progesterone concentration (left-most axis) on each day of pseudopregnancy from days 9-12 (front-most axis) for each E₂ treatment (right-most axis). Note that the order of E₂ treatments is the reverse of that in Fig. 3. Days 9-12 of pseudopregnancy correspond to days 1-4 postimplant manipulation. The number of rabbits in each group on each day is given in Fig. 3. The only significant differences ($P < 0.05$) between treatment groups occurred on day 9 (1 day postimplant manipulation) and were as follows: high:high > none and high:none < all other groups.

luteal LH-stimulated adenylyl cyclase activity (8-10). The exception to this was the lack of change in serum progesterone concomitant with the suppression of luteal LH-stimulated adenylyl cyclase activity by exogenous E₂

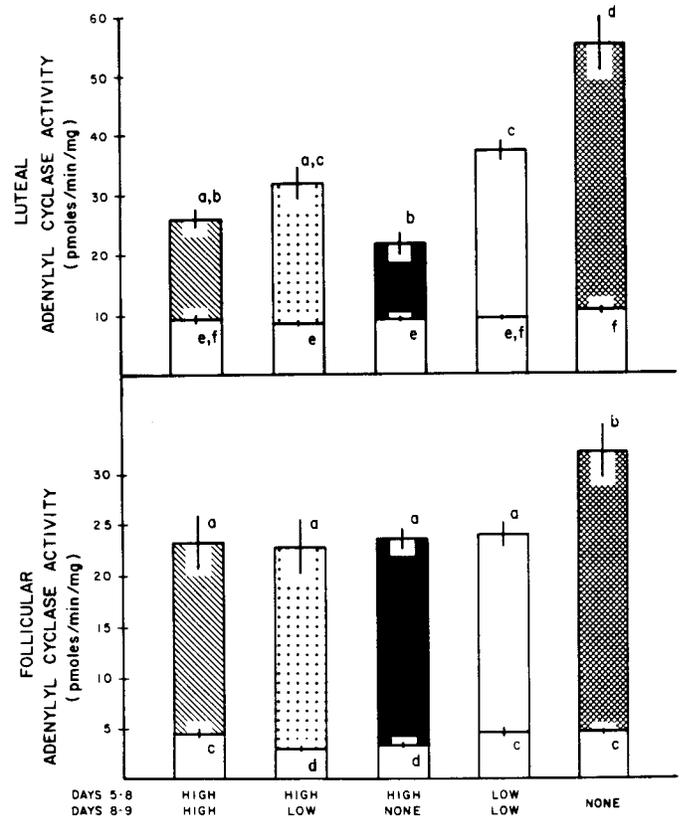


FIG. 5. Day 9 luteal (upper panel) and follicular (lower panel) adenylyl cyclase activities resulting from the various E₂ treatments. The smaller white bars are the means \pm SEM of the basal adenylyl cyclase activities, and the larger bars are the means \pm SEM of the adenylyl cyclase activities in response to LH. In the upper panel, n = 9, 9, 8, 9, and 8 from left to right; in the lower panel, n = 4 for all groups. Within each panel, bars with different letters represent significantly different ($P < 0.05$) group means. Note that the degree of suppression of LH-responsive adenylyl cyclase activity by E₂ is considerably greater in luteal tissue than in follicular tissue.

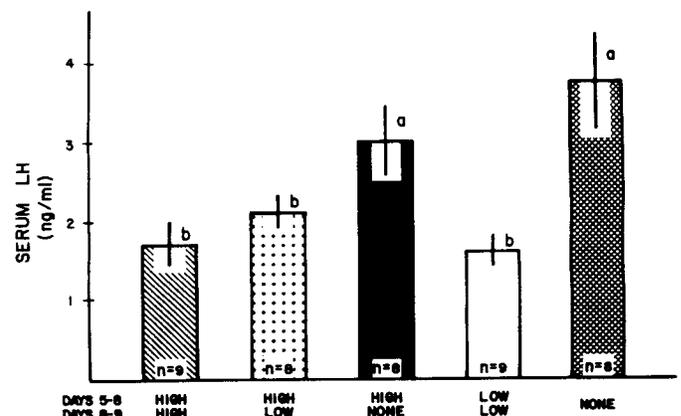


FIG. 6. Day 9 serum LH concentrations resulting from the various E₂ treatments. Each bar represents the mean \pm SEM of the indicated number of rabbits. Groups with different letters above the bars are significantly different at $P < 0.05$.

(Ref. 11 and the present study). However, despite the fact that LH-responsive adenylyl cyclase activity is attenuated by the presence of exogenous E_2 , it does not continue declining after the initial fall to a level which, although lower than normal, is not associated with non-functional CL. It is possible that the attenuation of luteal LH-responsive adenylyl cyclase activity is offset by the exogenous E_2 , and serum progesterone remains constant as a result. Since 1) LH is present in pseudopregnant rabbit serum, 2) CL have a LH-responsive adenylyl cyclase, and 3) LH-responsive adenylyl cyclase activity, under normal physiological conditions, is a good indicator of functional activity, it has been our contention that LH plays a role in luteal function. Bill, Jr. (26), recently reported that pseudopregnancy is normal in hypophysectomized E_2 -treated rabbits. Therefore, we are inclined to modify our hypothesis to state that LH may play a role in luteal function, but is not necessary. A functional hormone-responsive adenylyl cyclase may be of more importance than a specific LH-responsive adenylyl cyclase. Data from the current study and previous hCG/LH desensitization studies (10, 27, 28) would indicate that the modes of stimulation of luteal adenylyl cyclase by LH and catecholamine are similar, though not identical. Until it can be demonstrated that luteal function is normal in the absence of both LH and catecholamines, we will retain the hypothesis that a hormone-responsive adenylyl cyclase may be of importance in luteal function.

We can only speculate as to the cause of suppression of luteal LH-responsive adenylyl cyclase by E_2 . One possibility is that the exogenous E_2 suppresses the secretion of LH and possibly other gonadotropins, which could result in altered luteal LH receptor content and, hence, a decrease in responsiveness to LH [gonadotropins induce follicular LH receptors in the rat (29)]. This, however, would not explain the effect on the catecholamine-responsive adenylyl cyclase activity. Therefore, a second hypothesis is that the exogenous E_2 , either directly or indirectly, causes an alteration in the mechanism which couples hormone receptors to adenylyl cyclase. Although hormone receptors are highly specific, the coupling unit need not be. These hypotheses are currently under investigation in our laboratory. The partial protection against the hCG/LH-induced fall in serum progesterone afforded by E_2 which has been reported by our laboratory (10, 30) could also be explained by changes in LH receptor or coupling of the receptor to the adenylyl cyclase. For example, if there are decreased LH receptor numbers or altered coupling mechanisms, the luteolytic dose of hCG/LH would probably be less effective.

In summary, the suppression of LH-responsive adenylyl cyclase by E_2 is reversible and does not appear to change progesterone secretion. Although the withdrawal of E_2 produced a decline in progesterone, progesterone

secretion was restored to normal, presumably by estrogen of follicular origin. These findings indicate, therefore, that these implants do not establish a dependency of the CL on supraphysiological concentrations of E_2 ; instead, the CL are dependent upon a relatively uninterrupted supply of E_2 . Finally, we have proposed that the same mechanisms which cause a decrease in the ability of LH to stimulate luteal adenylyl cyclase in the presence of supraphysiological serum E_2 concentrations may also explain the partial protection by E_2 of the CL against hCG/LH-induced desensitization and luteal regression.

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