

## Corpus Luteum Function and Adenylyl Cyclase Stimulability in the Rat after an Estradiol Benzoate-Induced Ovulatory Surge of Luteinizing Hormone: Role of Prolactin\*

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**ABSTRACT.** Estradiol benzoate (E; 20 µg, sc) was given on diestrus day 1 to 4-day cycling rats to advance the ovulatory LH surge by 1 day. Estrogen treatment is known to result in the maintenance of two apparently functional sets of corpora lutea (CL). Previously, our laboratory has demonstrated that E treatment results in the maintenance of LH-stimulable adenylyl cyclase activity in the 4-day-old CL on the day of expected proestrus. We investigated whether the effect of E was mediated by PRL by using an erythrocytine analog CB-154 to inhibit PRL release. We measured LH- and isoproterenol (Iso)-stimulable adenylyl cyclase activity and progesterone levels in 4- and 1-day-old CL and progesterone levels in serum. Iso was used as a control hormone to monitor receptor-mediated regulation of adenylyl cyclase activity. Progesterone was measured by RIA to examine whether LH-stimulable adenylyl cyclase activity correlated with CL function.

We found that E treatment resulted in increased LH-stimulable adenylyl cyclase activity and elevated luteal progesterone

in the 4-day-old CL when compared to control CL; serum progesterone was also elevated after E treatment. CB-154 plus E treatment prevented the elevation of tissue and serum progesterone and prevented the increase in LH stimulability of adenylyl cyclase activity in 4-day-old CL. PRL plus CB-154 plus E reversed the effect of CB-154 plus E and resulted in increased LH stimulability of CL cyclase and in elevated tissue and serum progesterone. LH-stimulable adenylyl cyclase activity and tissue progesterone content were low in all 1-day-old CL. Interestingly, Iso-stimulable adenylyl cyclase activity was high (8- to 10-fold) in all 4-day-old CL.

We confirm that E treatment 1) advanced the LH surge and ovulation by 1 day, 2) resulted in pseudopregnancy, and 3) maintained LH-stimulable adenylyl cyclase activity after a desensitizing LH surge. We conclude that PRL is part of the means by which estrogen acts to maintain LH-stimulable adenylyl cyclase activity and functionality in 4-day-old CL. (*Endocrinology* 106: 382, 1980)

**I**N THE rat under physiological conditions, luteal regression takes place at the end of pregnancy, at the end of pseudopregnancy, and between diestrus day 2 and proestrus during the estrous cycle. The triggers and/or hormones responsible for regression in each instance are poorly understood. Current thinking ascribes roles to PRL, prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), LH, estrogen, and uterine factors acting in as yet undefined manners. Luteal regression can also be triggered experimentally. Thus, injection of PGF<sub>2α</sub>, estrogen, LH, or hCG to normal pseudopregnant rats or of PRL to rats bearing corpora lutea (CL) that had not been exposed to PRL for at least 96 h (1) will lead to luteal regression. In recent years, our laboratory has been characterizing the luteal events that follow the administration of high luteolytic doses of hCG or LH. We determined that in pregnant and pseudopreg-

nant rats, treatment with hCG results in desensitization of luteal LH-stimulable adenylyl cyclase within 6–12 h and in luteolysis characterized by a drop in CL weight (2). This sequence of responses is not restricted to the rat, since it also occurs in the rabbit (2–4), and may be the mechanism by which the luteal phase of the menstrual cycle of primates and humans is shortened after LHRH (Labrie, F., Ovarian Workshop, 1978) (5). The connection between luteal cyclase desensitization and steroidogenic shutdown is not clear, but it appears that the two events have a positive correlation.

There are instances in which high levels of LH do not seem to cause luteolysis. One of them arises in rats that are subjected to estrogen treatment on diestrus day 1. This treatment, as shown by Krey and Everett (6) and Ying and Greep (7), results in a 24-h advancement of the ovulatory surge of LH, advancement of ovulation, formation of a new set of CL without apparent loss of the old set of CL, and initiation of a period of pseudopregnancy. In an earlier report (8), we showed that the LH-stimulable adenylyl cyclase activity in CL of the cycle on the day of proestrus is low and responds poorly to LH

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(1.2- to 1.3-fold), and that an estrogen treatment that advances ovulation results in 4-day-old CL that on the day of expected proestrus have a more active adenyl cyclase system (responding 3-fold to LH). This degree of responsiveness is comparable to that seen in CL of rats on their fourth day of pseudopregnancy (2). To gain further insight into the interaction of LH with CL and into possible regulatory aspects of this interaction, we investigated whether the old CL, found to maintain a LH-stimulable adenyl cyclase and reported to survive structurally, also retained functionality, *i.e.* secrete progesterone. Furthermore, we tested whether the cyclase-protecting and possible function-protecting action(s) of estrogen are dependent on PRL. We found that CL in estrogen-treated rats are fully protected against the desensitizing and luteolytic actions of LH, and that this protection is indeed dependent on PRL.

### Materials and Methods

#### *Animals, treatments, preparation of sera and homogenates, and determination of ovulation*

Female rats were obtained at 60 days of age from Charles River (CD, outbred) and housed in air-conditioned quarters with lights on from 0500–1900 h. Food and water were available *ad libitum*. Vaginal smears were taken 7 days a week by saline lavage at 1000–1100 h. Rats were chosen for the experiment after they had shown at least two consecutive 4-day cycles. Six groups of rats were subjected to the treatment schedules outlined in Fig. 1. All injections were given *sc*. Oil injections of 0.1 ml sesame seed oil (Fisher Scientific Co., Waltham, MA) with or without 20  $\mu$ g estradiol benzoate (E; Sigmal Chemical Co., St. Louis, MO) were given at 1230 h on diestrus day 1. Oil injections of 0.2 ml sesame seed oil with or without 1.0 mg CB-154 (2-bromo- $\alpha$ -ergocryptine; Sandoz Pharmaceuticals (Hanover, NJ) were given at 12-h intervals starting at 1000 h on diestrus day 1. Saline injections of 0.2 ml with or without 200  $\mu$ g PRL (NIH-PRL-S11, Hormone Distribution Program, NIAMDD, NIH) were also given at 12-h intervals starting at 1000 h on diestrus day 1. All rats were sacrificed by decapitation at 0900 h on the day of expected proestrus. Blood was collected from the trunk, allowed to clot at room temperature, and left overnight at 4–10 C. Serum was separated the next morning by centrifugation and stored at –20 C until assayed for progesterone content (within the next 6 months). Occurrence of ovulation(s) on the night before was determined by examination of ovaries for ovulation points and positive identification of freshly ovulated ova within the ovarian bursa and upper oviduct. To this end, both ovaries enclosed in their respective bursas, the oviducts, and approximately 2 cm of uterine horn were removed immediately after sacrifice and placed into ice-cold Krebs-Ringer bicarbonate buffer prepared with half of the recommended amount of  $\text{CaCl}_2$  (9). After trimming away on an ice-cold petri dish the uterine horns and any adhering tissues, the ovary still enclosed in its bursa and joined to its oviduct was placed in the cavity of a concaved microscope slide filled with approximately 100  $\mu$ l Krebs-Ringer bicarbonate buffer. The bursa was then slit open, the ovary was removed, and the

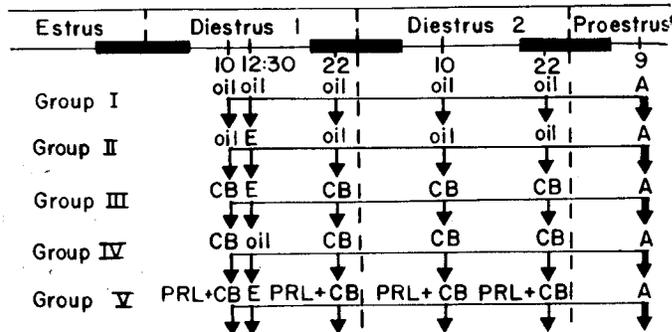


FIG. 1. Treatment schedule for the induction of advanced ovulation and for testing the effects of estrogen CB-154 and PRL on adenyl cyclase activity and serum and luteal progesterone levels. Sesame oil (0.2 ml) with or without CB-154 (1 mg) was injected *sc* beginning at 1000 h on diestrus day 1 and continued every 12 h through 2200 h on diestrus day 2 into 4-day-old cycling rats maintained on a 14-h light-10-h dark schedule. PRL (200  $\mu$ g in 0.2 ml saline) was injected *sc* beginning at 1000 h on diestrus day 1 and continued every 12 h through 2200 h on diestrus day 2 in groups III, V, and VI. A single injection of sesame oil (0.1 ml) with or without 20  $\mu$ g E was administered *sc* at 1230 h on diestrus day 1 to all groups. At 0900 h, all animals were decapitated, blood was collected, and serum was prepared. The ovaries were removed into ice-cold Krebs-Ringer bicarbonate buffer; 4-day old CL and, when present, 1-day-old CL were dissected. Separate homogenates of each type of CL were prepared and assayed immediately for adenyl cyclase activity in the presence and absence of LH (10  $\mu$ g/ml) and Iso ( $10^{-4}$  M). Within 6 months, serum and luteal progesterone levels were determined in samples that had been kept at –20 C. See text for further details.

solution, bursa, and oviduct were examined for the presence of ova using a Zeiss binocular microscope at  $\times 100$  magnification (Carl Zeiss, Inc., New York, NY). From the intact ovaries, the CL of the cycle and, when appropriate, newly formed CL were dissected within 1–2 h under a dissecting microscope using Graefe forceps (Roboz Surgical Instrument Co., Washington, D.C.) and were placed until homogenization into iced Krebs-Ringer bicarbonate buffer. Newly formed CL could be distinguished from the CL of the cycle by their more vascular appearance, smaller size, and the presence of a distinct fresh ovulation point. Dissected CL were counted, blotted dry, weighed, homogenized as described earlier (8), and assayed either immediately for adenyl cyclase activities or later after storage at –20 C for up to 6 months for progesterone content. One type of CL (original from the cycle or newly formed 1 day old) from one rat yielded enough homogenate to assay for basal, LH-stimulated, and isoproterenol (Iso)-stimulated adenyl cyclase activities in duplicate, to determine the protein in duplicate, and to carry out two assays for progesterone with triplicate determinations.

#### *RIA of progesterone*

**Reagents and solutions.** Antiserum GDN 337, kindly supplied by Dr. Gordon D. Niswender and raised in sheep against a bovine serum albumin conjugate of 11-hydroxyprogesterone, was diluted in 50 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.1% gelatin (PBS-gel) and used at a final dilution of 1:3,000. Solutions of progesterone were made in absolute ethanol and diluted in water to determine their con-

centrations using spectrophotometry at 240 nm [ $E_M$  (absorption maximum) of 17,000] or diluted 1:100 in PBS-gel to yield a stock. Progesterone solutions to construct standard curves were made by serial dilution of the stock solution into PBS-gel. Charcoal (Norit A, Sigma Chemical Co.) was washed and dried before use. The washing procedure, carried out to effect separation of fines and nonsedimenting charcoal, consisted of suspending approximately 100 g charcoal in 1 liter  $H_2O$ , stirring for 5 min, and allowing the mixture to settle for 45 min. After this time, both floating charcoal and nonsedimented charcoal were removed by suction. This procedure was repeated twice more. The resulting washed charcoal was then heated in an oven at 100 C until dry and stored until used. Dextran-coated charcoal, used to separate free from bound progesterone, was prepared by suspending washed charcoal (3.12%) and dextran T-70 (0.62%; Pharmacia Fine Chemicals, Piscataway, NJ) in PBS-gel, mixing well by stirring for 1 h, and storing overnight in the cold (4–10 C). [ $1,2,6,7-^3H$ ]Progesterone (100 Ci/mmol; supplied in benzene; New England Nuclear Corp., Boston, MA) was evaporated under a stream of nitrogen and diluted to the desired concentration in cold PBS-gel.

**RIA.** Incubations were carried out at 8–12 C overnight in a final volume of 300  $\mu$ l PBS-gel containing approximately 12,000 cpm [ $1,2,6,7-^3H$ ]progesterone, 7–3,650 pg unlabeled progesterone as standards or unknowns, progesterone antibody GDN 337 at a final dilution of 1:3,000, and between 50–300 cpm labeled progesterone carried over as recovery marker (see below). Unknowns were routinely added in 10- to 200- $\mu$ l aliquots, tracer in 50- $\mu$ l aliquots, and antibody in 40- $\mu$ l aliquots. Separation of free from bound progesterone was achieved by the addition of 1.0 ml ice-cold dextran-coated charcoal suspension, followed by a 15- to 20-min incubation in iced water and centrifugation in the cold. Bound progesterone was determined in 1.0-ml aliquots of the supernatant by liquid scintillation counting. We found the progesterone antibody to be quite temperature sensitive, and care was taken to keep the working dilution of the GDN 337 antibody ice cold during distribution into the incubation tubes. In addition, 10-point standard curves (in duplicate) were run after every 100 assay tubes to check for constancy of displacement throughout. Standard curves were constructed using a logit-log transformation of the percent bound *vs.* percent unlabeled progesterone data (10). Only assays with standard curves having logit-log transformations with correlation coefficients greater than 0.98 were used. Unknowns giving displacements of percent bound of more than 80% or less than 20% were re-assayed at lower and higher dilutions, respectively. Coefficients of variation for interassay variability (3 serum pools; 11–14 assays) and intraassay variability (10–15 determinations/assay; 3 assays) were  $13.7 \pm 3.5\%$  and  $11.0 \pm 2.09\%$  (mean  $\pm$  SD), respectively (11). As recently shown by Gibori *et al.* (12) and verified by us, antibody GDN 337 is very specific for progesterone, the major cross-reactant being corticosterone, which interferes at a level of 2.5%, but it is poorly extracted by petroleum ether. Since tissue (CL) as well as serum were assayed for progesterone, a preliminary study was done to insure that only progesterone was being measured. To this end, samples (10  $\mu$ l tissue homogenate or 50  $\mu$ l serum) were extracted twice with 3.0 ml petroleum ether (analyzed by J. T. Baker Co., Phillips-

burg, NJ; boiling point, 35–60 C), evaporated at 37 C under a stream of air, and then either directly resuspended in 500  $\mu$ l PBS-gel and assayed for progesterone content without further treatment or first subjected to chromatography over Sephadex LH-20 and then resuspended in PBS-gel and assayed for progesterone content. In two such experiments it was found that after correction for recovery (see below), the estimates of progesterone content in either tissue homogenates or sera obtained using chromatographed samples did not differ significantly from those obtained using samples not chromatographed over LH-20. All progesterone values reported below were therefore obtained on samples that were extracted with petroleum ether, evaporated, resuspended in PBS-gel, and assayed without further purification steps. Aliquots (40  $\mu$ l) of liver homogenates prepared in the same way as CL homogenates were used as tissue blanks; liver homogenates, when assayed for progesterone content, gave values that were indistinguishable from blank values. Forty microliters contained 4 times as much protein as was used for the assay of CL.

**Chromatography of progesterone.** The evaporated petroleum ether extracts were taken up in 200  $\mu$ l solvent and applied to columns (1 cm in diameter) containing 800 mg Sephadex LH-20. The columns were developed with isooctane-benzene-methanol (90:5:5) as described by Carr *et al.* (13). Progesterone-containing fractions were collected, evaporated under a stream of air, and resuspended in 500  $\mu$ l PBS-gel, as described above.

**Recovery estimates.** To monitor recovery, [ $^3H$ ]progesterone (~3000 cpm; 100 Ci/mmol) in 10  $\mu$ l PBS-gel was added to each aliquot (serum or CL homogenate) to be extracted with petroleum ether, and 75–100  $\mu$ l of the final suspension in PBS-gel were counted for  $^3H$  content in a liquid scintillation counter. Recoveries with and without chromatography were routinely 85–95% and 60–75%, respectively.

#### Adenylyl cyclase assays

Ten-microliter aliquots of CL homogenates were assayed at 32.5 C in a final volume of 50  $\mu$ l containing 3.0 mM [ $\alpha-^{32}P$ ]ATP ( $5-20 \times 10^6$  cpm), 5.0 mM  $MgCl_2$ , 1.0 mM [ $^3H$ ]cAMP (~10,000 cpm), 1.0 mM EDTA, an ATP-regenerating system (consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 0.02 mg/ml myokinase), 25 mM Tris-HCl (pH 7.0), and, when present, 10  $\mu$ g/ml LH (NIH-LH-S19, Hormone Distribution Program, NIAMDD, NIH) or  $10^{-4}$  M (–)-Iso (a gift from Dr. F. C. Nachod, Sterling Winthrop Research Institute, New Castle on Tyne, United Kingdom).

Proteins were assayed by the method of Lowry *et al.* (14) using bovine serum albumin as standard.

#### Other materials

[ $\alpha-^{32}P$ ]ATP used in adenylyl cyclase assays was synthesized and supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies in Reproductive Biology, Baylor College of Medicine. All other materials and sources were recently described (15).

#### Statistics

Statistical significance of differences between groups was calculated using Student's *t* test.

## Results

In initial experiments we confirmed and expanded the earlier findings by Everett (16), Krey and Everett (6) and Ying and Greep (7) as well as our own that estrogen treatment of 4-day cycling rats on diestrus day 1 results in advancement of ovulation, pseudopregnancy characterized by two sets of CL on the day of expected proestrus, and maintenance of an LH-stimulable adenylyl cyclase in the original set of CL of the cycle (Table 1). New 1-day-old CL, in accordance with earlier findings with normal 1-day-old CL present in ovaries of cycling rats on the day of estrus, showed little or no LH-stimulable activity. As shown in Table 1, 4-day-old CL of both control proestrus rats and estrogen-treated rats showed good Iso-stimulable adenylyl cyclase activity.

Absolute adenylyl cyclase activities found in this study were lower than those reported earlier by an average

TABLE 1. Effect of treating rats on diestrus day 1 with estrogen on ovulation, luteal weight, and luteal adenylyl cyclase activities as seen on the day of proestrus

Measurements	Treatment on diestrus day 1		
	Control: 4-day-old CL <sup>a</sup>	E <sup>b</sup>	
		4-day-old original CL	1-day-old new CL
Ovulation			
Ova	0/4 <sup>c</sup>	6/8	
Ovulation points	0/4	8/8	
Tissue wt (mg/CL)	1.32 ± 0.06	1.62 ± 0.05	
Adenylyl cyclase activities			
Absolute activities (pmol cAMP/mg protein · min) <sup>d</sup>			
Basal	6.3 ± 0.3	6.4 ± 0.7	6.0 ± 1.2
LH	9.6 ± 0.6 <sup>e</sup>	17.6 ± 2.7 <sup>f</sup>	5.5 ± 1.0 <sup>g</sup>
Iso	46.5 ± 5.0	42.6 ± 2.1 <sup>h</sup>	27.5 ± 3.9 <sup>i</sup>
NaF	31.5 ± 2.9	46.1 ± 3.2	43.1 ± 3.0
Relative stimulation of activity (-fold over basal)			
LH	1.5 ± 0.1 <sup>j</sup>	2.8 ± 0.4 <sup>k</sup>	1.0 ± 0.1 <sup>l</sup>
Iso	7.4 ± 0.7	7.2 ± 0.9 <sup>m</sup>	5.3 ± 0.9 <sup>n</sup>
NaF	5.2 ± 0.5	7.7 ± 1.0	8.6 ± 1.3

<sup>a</sup> Animals were treated as described for groups I in Fig. 1 and sacrificed at 0900 h on the day of (expected) proestrus.

<sup>b</sup> Animals were treated with 20 µg E at 1230 h on diestrus day 1, as described for group II in Fig. 1, and sacrificed at 0900 h on the day of (expected) proestrus.

<sup>c</sup> The numerator indicates the number of animals in which ova or ovulation points were found. The denominator indicates the total number of animals examined.

<sup>d</sup> Values represent the mean ± SEM of the number of animals examined for ovulation.

<sup>e-n</sup> *e* vs. *f*, *j* vs. *k*, and *m* vs. *n*, *P* < 0.05; *j* vs. *l*, *P* < 0.02; *f* vs. *g*, *h* vs. *i*, and *k* vs. *l*, *P* < 0.005.

factor of about 2.0 (17). The reasons for this are not clear. However, in spite of this, the relative stimulations due to LH obtained earlier and those presented here are very similar. Thus, in our earlier studies, relative stimulation of adenylyl cyclase by LH in CL of control animals on proestrus was 1.34-fold and that in CL from estrogen-treated animals on the same day was 2.84-fold; measurement of the same parameters in our present studies gave values of relative stimulations of 1.5-fold and 2.8-fold, respectively (Table 1). In none of the studies reported below did basal adenylyl cyclase activities vary significantly between treatment groups. Relative stimulations (activities in the presence of hormones/activity in the absence of added hormone) will be presented.

Ovulatory levels of LH (or hCG) cause desensitization of rat adenylyl cyclase to LH under most circumstances, including those existing on the afternoon of proestrus; on days 5, 7, and 9 of pseudopregnancy; and on days 5, 9, 7, and 15 of pregnancy (2, 7, 17, 18). However, results shown in Table 1 indicate that proper hormonal manipulation of luteal tissue, such as that resulting from treatment of rats with estrogen on diestrus day 1 and leading by 1200 h of diestrus day 2 to an adenylyl cyclase that responds to LH 2.91-fold over basal (7), will give rise to a situation where desensitization does not occur after exposure to an ovulatory dose of LH. An increase in serum LH on the afternoon of diestrus day 2 is not the only hormonal change that follows treatment with E. One additional change that occurs under these circumstances is an increase in the levels of serum PRL (19). Since under physiological conditions, PRL is known to rescue the CL of the cycle and participate in its transformation into and maintenance as a CL of pseudopregnancy (20, 21), and since estrogen treatment of the type given in the studies reported here leads to a pseudopregnant state, we investigated whether maintenance of an LH-stimulated CL adenylyl cyclase on the day of expected proestrus depends on PRL. To this end we treated cycling rats with both estrogen (to advance the ovulatory surge of LH) and an ergocryptine derivative, CB-154, known to block pituitary PRL release (22). The treatment schedules for the experimental group (group III) as well as for various control groups (groups IV-VI), which include treating rats with CB-154 alone and providing replacement therapy with PRL, are presented schematically in Fig. 1. The effects of these treatments on advancement of ovulation and on hormonal stimulability of adenylyl cyclase in 4-day-old CL on the day of (expected) proestrus are shown in Fig. 2. Figures 3 and 4 show the effects of three treatments on luteal progesterone content and on serum progesterone levels, respectively. From a statistical point of view, it was found that LH stimulability of adenylyl cyclase in 4-day-old CL of rats treated with E did not differ (*P* > 0.1) from that of rats treated with E plus CB-

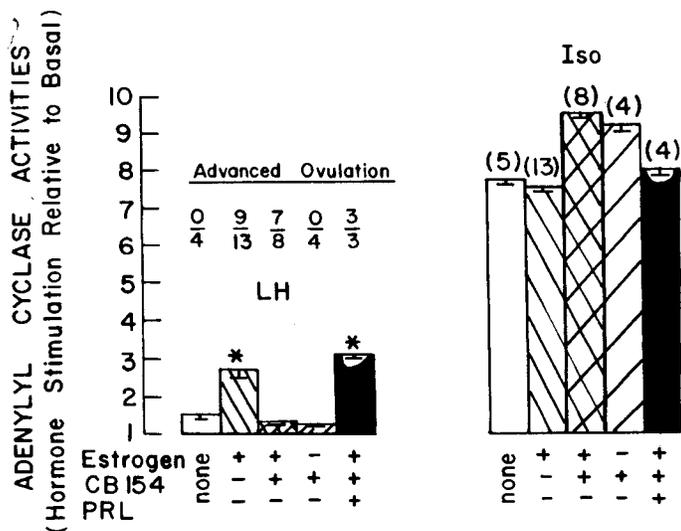


FIG. 2. The effect of an estrogen-induced ovulatory LH surge in the presence or absence of PRL on adenylyl cyclase activity in 4-day-old rat CL. A 1-day advancement of the ovulatory surge of LH due to E (20 µg) treatment was verified by finding ova in the ovarian bursa and upper oviduct on proestrus. The ratios shown under *Advanced Ovulation* represent the number of animals in which ova were found over the number of animals that were examined. The degree of stimulation of adenylyl cyclase in homogenates of 4-day-old CL in the presence of LH (10 µg/ml) or Iso (10<sup>-4</sup> M) relative to basal activity is shown. The number of animals in each group for all cyclase determinations is given in parentheses over the appropriate treatment group where Iso was the stimulating hormone. The asterisk indicates that the mean degree of hormonal stimulation was significantly greater ( $P < 0.01$ ) in that treatment group when compared to the mean of degree of stimulation observed in the homogenates of CL from group I (oil treatment only).

154 plus PRL but was significantly elevated over that of rats either not treated ( $P < 0.05$ ) or treated with E plus CB-154 ( $P < 0.0005$ ) or with CB-154 alone ( $P < 0.001$ ). Finally, no significant difference was seen between LH stimulability in CL of control rats and that in either E plus CB-154- or CB-154-treated rats ( $P > 0.05$ ). Statistically similar results were found when progesterone content in 4-day-old CL or serum progesterone were the dependent variables. Thus, from the data presented in Fig. 2, it can be concluded that maintenance of luteal LH-stimulable adenylyl cyclase activity on the day of (expected) proestrus requires pituitary PRL and that the combined estrogen plus PRL treatment resulted in the maintenance of an LH-stimulable cyclase even though an ovulatory surge of LH had occurred (ova were present in three out of three such treated rats). Thus, an LH-stimulable adenylyl cyclase could not be found in CL on the day of expected proestrus if estrogen treatment was combined with the administration of 1 mg CB-154/12 h, a dose that was shown by Smith *et al.* (20) to inhibit rat pituitary PRL secretion, but did not inhibit the LH surge (rats treated with CB-154 plus estrogen ovulated, as shown in Fig. 2). LH-stimulable adenylyl cyclase was again found in CL of expected proestrus if estrogen plus

CB-154-treated rats were given replacement therapy with 200 µg PRL/12 h (Fig. 2).

None of the treatments affected the catecholamine-mediated stimulability of luteal adenylyl cyclase (Fig. 2). In fact, all luteal tissues tested showed Iso-stimulable activity regardless of variations seen in LH-stimulable activity. Stimulability by NaF was unaltered by any of the treatments (not shown).

We investigated the effect of the treatments outlined in Fig. 1 on progesterone content in 4- and 1-day-old CL. Results from this experiment are shown in Fig. 3. All luteal tissues contained appreciable levels of progesterone, indicating synthetic capacity. Early elevation of serum PRL due to estrogen treatment (estrogen plus oil and estrogen plus CB-154 plus PRL, but not estrogen plus CB-154) resulted in 4-day-old CL that contained significantly ( $P < 0.01$ ) more progesterone than either the 1-day-old CL induced by the same treatment or the 4-day-old control CL.

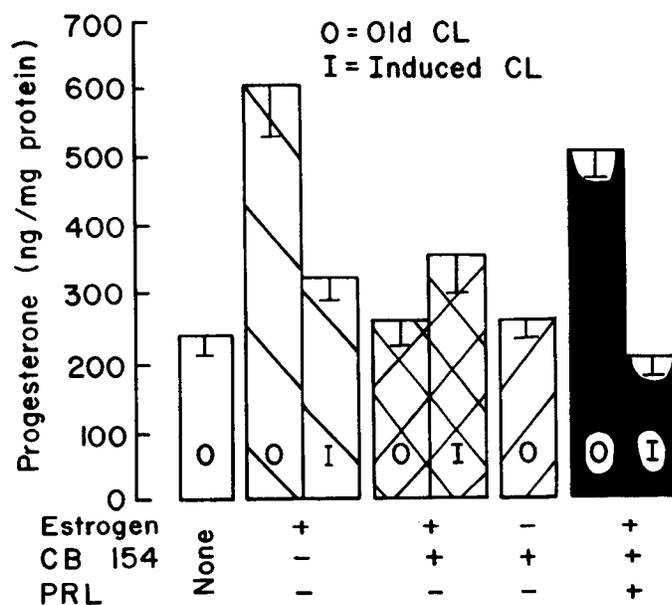


FIG. 3. The effect of estrogen (20 µg) and PRL alone or in combination on the progesterone content of 4-day-old rat CL (O) and newly induced 1-day-old CL (I), when present. CB-154 (1 mg/12 h) was given to prevent the increase in endogenous PRL seen after estrogen administration. Progesterone was measured by RIA. Liver was used as a control tissue. The numbers of animals in each group was: control (O), n = 6; estrogen (O), n = 9 or (I), n = 9; estrogen plus CB-154 (O), n = 6 or (I), n = 5; CB-154 (O), n = 5; estrogen plus CB-154 plus PRL (O), n = 7 or (I), n = 6; and PRL (O), n = 5. The mean values  $\pm$  SEM are represented. Relevant statistics: Progesterone in old CL of estrogen-treated rats *vs.* progesterone in old CL of control, estrogen plus CB-154-treated, and CB-154-treated groups *vs.* progesterone in new CL,  $P < 0.01$ ; same for progesterone in old CL of estrogen plus CB-154 plus PRL-treated rats when compared to any values except that found in old CL of estrogen only-treated rats. Progesterone in old CL of estrogen-treated rats was not significantly different ( $P > 0.1$ ) from progesterone of old CL in estrogen plus CB-154 plus PRL-treated rats. For further details, see Fig. 1 and the text.

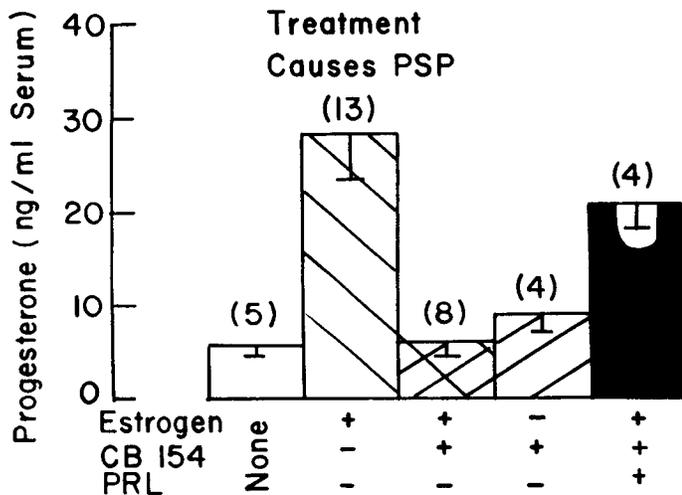


FIG. 4. The effect of an estrogen-induced ovulatory LH surge in the presence or absence of PRL on serum progesterone levels in the rat on the day of expected proestrus. E (20  $\mu$ g) was used to advance the LH surge by 1 day and to induce pseudopregnancy. CB-154 (1 mg/12 h) was given to block the estrogen-induced increase in serum PRL. PRL (200  $\mu$ g/12 h) replacement was used to determine if CB-154 treatment was reversible. Progesterone levels were determined by RIA. The numbers in parentheses represent the numbers of animals in each group. The mean values  $\pm$  SEM are represented. Relevant statistics: Progesterone in serum of rats treated with estrogen or with estrogen plus CB-154 plus PRL vs. serum progesterone in control rats, rats treated with estrogen, CB-154, or estrogen plus CB-154,  $P < 0.005$ . Progesterone in serum of estrogen-treated rats vs. that in estrogen plus CB-154 plus PRL-treated rats, NS ( $P > 0.1$ ). For further details, see Fig. 1 and the text.

The effects of the various treatments described in Fig. 1 on serum progesterone levels on the morning of (expected) proestrus are shown in Fig. 4. Serum progesterone levels were elevated to those expected on day 4 of pseudopregnancy (23). Blockade of pituitary PRL secretion resulted in low serum progesterone levels even though ovulation was advanced. Replacement therapy with PRL resulted in actively secreting CL, as seen by the elevated serum progesterone levels (Fig. 4). In those treatment groups where serum progesterone was elevated, luteal progesterone levels in the 4-day-old CL were also elevated.

The induction of pseudopregnancy by estrogen treatment was verified in a separate group of rats. After estrogen treatment on diestrus day 1, daily vaginal smears were taken. A predominately leucocytic smear was interpreted as indicating pseudopregnancy. In four of the five rats followed, the smears remained leucocytic for 12–16 days.

### Discussion

Two aspects of CL function and its regulation, reported in this article, merit comment. First, the ability of the CL to produce progesterone and, hence, remain functional is directly correlated with the maintenance of an

LH-stimulable cyclase system. Secondly, desensitization of luteal adenylyl cyclase and luteolysis, as induced by LH, do not always occur.

To determine if LH-stimulable adenylyl cyclase activity and CL function correlate, it is necessary to examine both serum and tissue levels of progesterone. The data show that LH-stimulable cyclase activity in the 4-day-old CL was elevated by the same treatments (estrogen in the presence of PRL) that resulted in elevated serum progesterone. These treatments (as well as the administration of estrogen plus CB-154) also induced new CL which did not have LH-stimulable cyclase activity. Thus, elevated serum progesterone could potentially come solely or partially from the new induced CL rather than from the 4-day-old CL exposed to the ovulatory LH surge. The induced CL are thought not to be major contributors to serum progesterone levels for several reasons. Low levels of serum progesterone are observed on estrus or on day 1 of pregnancy or pseudopregnancy (21, 23); thus, newly induced CL are thought to secrete very little progesterone. In this study, all newly induced CL contained approximately the same amount of progesterone regardless of treatment; however, the progesterone levels in the 4-day-old CL varied between treatment groups, being higher after exposure to both estrogen and PRL than in control CL. Increased tissue progesterone content in the 4-day-old CL directly correlated with elevated serum progesterone. Therefore, the 4-day-old CL that were exposed to estrogen and PRL and that maintained an LH-stimulable cyclase system after an ovulatory LH surge appear to remain functional.

One should note that progesterone production correlated with LH-stimulable cyclase activity only. Recent studies using CL from rats (24), cows (25, 26), and sheep (27) have demonstrated that progesterone production can be stimulated by catecholamines such as epinephrine. This study showed that the CL on proestrus in control animals is highly responsive to catecholamines (8-fold stimulation); in fact, the CL adenylyl cyclase was more responsive to catecholamines than to LH (1.3-fold stimulation). If LH stimulability of the adenylyl cyclase was low, serum progesterone was low even though catecholamine responsiveness remained high. These results cause one to wonder whether catecholamines affect progesterone production under physiological circumstances. Clearly, further experiments are required to elucidate the role of catecholamines in ovarian function.

The maintenance of a catecholamine-responsive cyclase system in CL from control rats on proestrus suggests that adenylyl cyclase and its ability to couple to hormone receptors has not been lost. The lack of response to LH may be due to a selective loss of LH receptors or to an uncoupling of these receptors from the cyclase system by proestrus. In recent years, the induc-

tion of receptors by hormones has been demonstrated. Richards and Williams have shown that PRL can induce LH receptors in granulosa cells in FSH- and estrogen-primed immature rats (28) and can maintain LH receptors in pregnant rats (29). Our findings are consistent with a PRL induction of LH receptors, since in the absence of PRL, LH stimulatory and progesterone secretion were low even though LH and estrogen were present.

It is now known that the proestrus surge of LH is accompanied by a PRL surge, both surges being induced by estrogen (30), yet this PRL surge comes too late to rescue the CL which undergo final regression as the new ones develop. The administration of E on diestrus day 1 advances both the PRL surge by 48 h and the LH surge by 24 h (31) and leads to the rescue of cycle CL and the establishment of a pseudopregnancy characterized by diurnal surges of PRL (20). The fact that after this type of treatment old CL are not only rescued but maintain activity and LH responsiveness in spite of being exposed to an ovulatory (and potentially desensitizing and luteolytic) surge of LH suggests two possibilities: 1) at 3 days of age, CL have not yet developed a luteolytic susceptibility and desensitizing response to LH, and 2) there is a pattern of hormonal manipulation (including PRL and estrogen) that, if presented to the CL at the right time, leads to inhibition of the luteolytic effect of LH and to protection against the desensitizing action of LH. If protection occurs, then estrogen alone is not enough, as seen in estrogen plus CB-154-treated rats. Also, PRL alone does not appear to be sufficient, since 5- and 7-day-old CL of pseudopregnancy supported by PRL (21, 32) are readily desensitized after hCG treatment (17). The 9-day-old CL of pregnancy, thought to depend on both PRL and estrogen (32, 33), also responds to hCG by desensitizing its adenylyl cyclase, the putative protection is also not due to the mere presence of both of these hormones. Thus, if conditioning of CL to become resistant to the deleterious effects of an ovulatory dose of LH occurs, it must depend either on an additional hormone or on the time, sequence, and dose pattern to which the CL is subjected after a single 20- $\mu$ g E administration at 1200 h on diestrus day 1.

Alternatively, the possibility has to be considered that the 3-day-old CL, rescued by estrogen plus PRL, has not yet developed its sensitivity to the luteolytic and desensitizing actions of LH. Experiments performed by Spies and coworkers (34) suggest that the response of the rabbit CL of early pseudopregnancy to ovulatory doses of LH is indeed altered. Thus, these authors showed that such CL were insensitive to the luteolytic actions of LH on day 2 but were sensitive on day 4. A similar situation may hold in estrogen-treated rat CL. If the 3-day-old rat CL are indeed insensitive, the situation would be analo-

gous to that seen with PGF<sub>2 $\alpha$</sub> . The luteolytic effect of PGF<sub>2 $\alpha$</sub>  cannot be seen in rat CL on day 3 of pseudopregnancy, but it can be seen by day 7 (35). Data at hand do not as yet enable us to distinguish between protection and insensitivity.

In contrast to the estrogen-treated rats, PRL levels are not elevated before the day of proestrus in the control rats (20) and must have remained low in CB-154 plus estrogen-treated rats, since the effect of CB-154 could be overcome by PRL administration (Figs. 1-3). Without preexposure to PRL, some irreversible changes in the LH responsiveness of the CL occurs and progesterone production can no longer be enhanced or maintained. Regression ensues. Data from our laboratory (36) suggest that one change that can occur involves a lack of ability of PRL to maintain an LH-responsive adenylyl cyclase if treatment with PRL has not been initiated before 1200 h on diestrus day 2. Further studies are required to define more precisely the means by which estrogen and PRL together alter CL function and adenylyl cyclase stimulatory.

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