J. steroid Biochem. Vol. 23, No. 6A, pp. 1053–1058, 1985
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0022-4731/85 $3.00 + 0.00
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# THE ANTIPROGESTERONE STEROID RU-486 DOES NOT IMPAIR GONADOTROPIN-STIMULATED LUTEAL ADENYLYL CYCLASE ACTIVITY OR GONADOTROPIN RELEASE BY PITUITARY CELLS

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(*Received* 28 *December* 1984)

**Summary**—Administration of the antiprogesterone synthetic steroid RU-486 (17$\beta$-hydroxy-11$\beta$-[4-dimethylaminophenyl-l]-17$\alpha$-[prop-l-ynyl]-estra-4,9-dien-3-one) in human and non-human primates induces menstruation and is promising as a new approach to fertility control. To explore the sites of action of RU-486, we investigated in this study the effects of RU-486 upon gonadotropin-stimulable adenylyl cyclase in membrane preparations obtained from human corpus luteum and upon LH and FSH release by a dispersed rat anterior pituitary cell culture. In the presence of a wide range of concentrations ($10^{-10}$ to $10^{-6}$ M), RU-486 failed to alter basal or hCG-stimulated adenylyl cyclase activities under conditions allowing either maximal or submaximal hormonal activation. Additionally, enzyme stimulation by GMP-P(NH)P (100 $\mu$M), NaF (10 mM) or forskolin (100 $\mu$M) was not affected by a high concentration ($10^{-6}$ M) of RU-486. These data indicate that RU-486 does not affect gonadotropin receptor binding nor does it interfere with cAMP generation. It is unlikely, therefore, that the compound may modulate human luteal function through changes in plasma membrane lipid mobility or modifications of reactions occurring in plasma membranes as suggested for other steroids in several membrane systems. The present observations are compatible with previously published *in vivo* studies suggesting that RU-486 activity does not involve a direct antigonadotropic effect at the primate corpus luteum level.

We also found that RU-486 ($10^{-12}$ to $10^{-7}$ M) did not alter the basal release of gonadotropins by the pituitary cells, nor did the compound impair the response of these cells to maximally or submaximally effective concentrations of LHRH. Thus, these data suggest that the anti-reproductive actions of RU-486 involve no direct effect upon pituitary function. Taken together, these findings support the concept that RU-486 exerts its effects on the luteal phase exclusively by a local action upon the endometrium.

## INTRODUCTION

The antiprogesterone activity of the new steroid analog, RU-486 (17$\beta$-hydroxy-11$\beta$-[4-dimethylaminophenyl-1]-17$\alpha$-[prop-1-ynyl]-estra-4,9-dien-3-one), has been demonstrated in several species, including the human female [1, 2]. This property appears to be consistent with its binding characteristics as a progesterone receptor antagonist [1]. Recent studies in the human and in non-human primates indicate that RU-486 is effective for interrupting early pregnancy [2, 3], as well as for induction of menses when the compound is administered during the luteal phase of the menstrual cycle [2, 4]. These observations suggest that RU-486 may open a new and promising approach for human fertility control. The sites of action of RU-486, however, remain to be elucidated. Previously, we have observed that administration of RU-486 during the luteal phase of regularly cycling Rhesus monkey induces early uterine bleeding without affecting the daily serum concentrations of LH, FSH, estradiol and progesterone [4]. These data indicate that the compound most likely exerts its effects by acting directly at the endometrial level. A similar conclusion has been drawn by Healy *et al.*[5], using castrated, estrogen- and progesterone-treated Cynomolgus monkeys. Others, however, have reported that the induction of menses in normally cycling women receiving RU-486 during the luteal phase is accompanied by a decrease in serum levels of LH, FSH, estradiol and progesterone during treatment [2]. This raises the questions of whether the induction of menstruation may also involve a direct inhibiting effect of RU-486 upon pituitary and/or luteal function.

Although the actions of steroid antagonists through the corresponding receptors in cytoplasm and nucleus of target cells are well recognized, there are considerable data illustrating effects of steroids upon membrane receptors, plasma membrane fluidity and membrane bound enzymes including adenylyl cyclase activity [6–9]. These observations led us to examine whether RU-486 may exert an anti-

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gonadotropin activity at the ovarian levels by altering basal and gonadotropin-stimulable adenylyl cyclase using a cell-free system prepared from human corpus lutea. In addition, we also investigated the ability of RU-486 to interfere directly with basal LH and FSH release as well as with LHRH response of dispersed rat anterior pituitary cell cultures.

## EXPERIMENTAL

### *Materials*

[$\alpha$-$^{32}$P]ATP (20–25 Ci/mmol) was purchased from ICN (Irvine, CA); [$^{3}$H]cAMP (20–30 Ci/mmol) from Amersham (Arlington Heights, IL); creatine phosphate, myokinase, creatine phosphokinase, ATP (Na-salt; catalog no. A-2383), cAMP, EDTA and Tris from Sigma Chemical Co. (St Louis, MO); GMP-P(NH)P (abbreviation used: GMP-P(NH)P, guanyl 5′-yl imidodiphosphate) from Boehringer-Mannheim (Mannheim, Federal Republic of Germany); trypsin and Dulbecco's Modified Eagle medium (DMEM) from Grand Island Biological Co. (Grand Island, NY); NaF from Fisher Scientific Co. (Waltham, MA); and forskolin from Calbiochem (La Jolla, CA). LHRH (Abbot lot 19-193 AL, LHRH-NIH-NICH-72-2722) was purchased from Abbot Laboratories (North Chicago, IL) and a highly purified hCG (14,000 IU/mg) from Radioassay Systems Laboratories, Inc. (Carson, CA). RU-486 was obtained from Dr Edouard Sakiz of Roussel Uclaf, Paris, France. All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

### *Collection of corpora lutea*

Corpora lutea were obtained from the ovaries of regularly cycling women, aged 23–36 years, undergoing exploratory laparotomies at the medical Center Hospital, San Antonio, Texas, for a variety of benign gynecological conditions. The study was approved by the Institutional Review Board. None of the women were pregnant or hormonally medicated, and the ages of the corpora lutea were between 6 and 12 days, as assessed by histology and cycle dates. Immediately after removal, the luteal tissue was placed in iced Krebs–Ringer bicarbonate buffer prepared with one half the recommended amount of $CaCl_2$ and transported to the laboratory.

### *Assay of human luteal adenylyl cyclase*

Washed membrane particles were prepared from human corpora lutea as described previously [10]. Adenylyl cyclase activity was determined by the method of Birnbaumer *et al.*[11]. The optimal conditions for the assay of the enzyme from human luteal membranes have been described elsewhere [10]. Aliquots (10 $\mu$l) of membrane particle preparation (about 10 $\mu$g protein) were assayed in triplicate for adenylyl cyclase activity in a final volume of 50 $\mu$l containing 2.0 mM [$\alpha$-$^{32}$P]ATP (50 cpm/pmol), 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM [$^{3}$H]cAMP (10,000 cpm), nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine kinase (111 U/ml), 0.1 mg/ml myokinase (1950 U/mg) and 25 mM Tris–HCl buffer, pH 7.5. Incubations were performed at 32°C for 10 min and terminated by the addition of 0.1 ml of a solution containing 40 mM ATP, 10 mM cAMP and 1% sodium dodecyl sulfate. The [$^{32}$P]cAMP formed and [$^{3}$H]cAMP present as recovery maker were isolated by double chromatography over Dowex 50 and alumina, and quantified by liquid scintillation counting [10]. In each experiment, a batch of membrane particles from a single corpus luteum was used. In order to test tissue variability, the experiments were repeated with at least one batch of membranes from a different corpus luteum. All experiments showed a similar pattern of results. Because of variations in absolute values among experiments, results from different experiments were not averaged. Thus, the figures and tables present data obtained in single representative experiments.

### *Dispersed rat anterior pituitary cell culture*

Sixty-day old female Holtzman rats were used as donors for anterior pituitary cell cultures. The animals were utilized at random stages of the estrous cycle. The preparative system consisted of cells dispersed by trypsinization and grown for 3 days in monolayer as has been detailed in previous publications [12, 13]. The cultures were preincubated for 2 h in the absence (control groups) or presence of RU-486 ($10^{-12}$ to $10^{-7}$ M) and the media were removed and subjected to radioimmunoassay to determine LH and FSH. The cells were immediately covered with Dulbecco's Modified Eagle Medium and incubated with or without a fixed concentration of LHRH and in the absence (control groups) or presence of increasing concentrations of RU-486 to make a final vol of 1 ml. Following a 2-h incubation, the culture media were assayed for LH and FSH release by radioimmunoassay.

### *Radioimmunoassay (RIA)*

A double antibody RIA, using NIAMDD standards and antibodies, was used to estimate levels of LH and FSH as described elsewhere [12]. Samples were incubated with the first antibody for 24 h, followed by the addition of a second antibody and a further 24-h incubation. The intra- and interassay coefficients of variation did not exceed 10 and 15%, respectively.

### *Other procedures*

To minimize contamination with "guanyl nucleotide-like" material, the components of the nucleoside triphosphate-regenerating system were subjected to purification. To accomplish this, creatine phosphokinase and myokinase were passed over Sephadex G-25 and creatine phosphate was mixed with

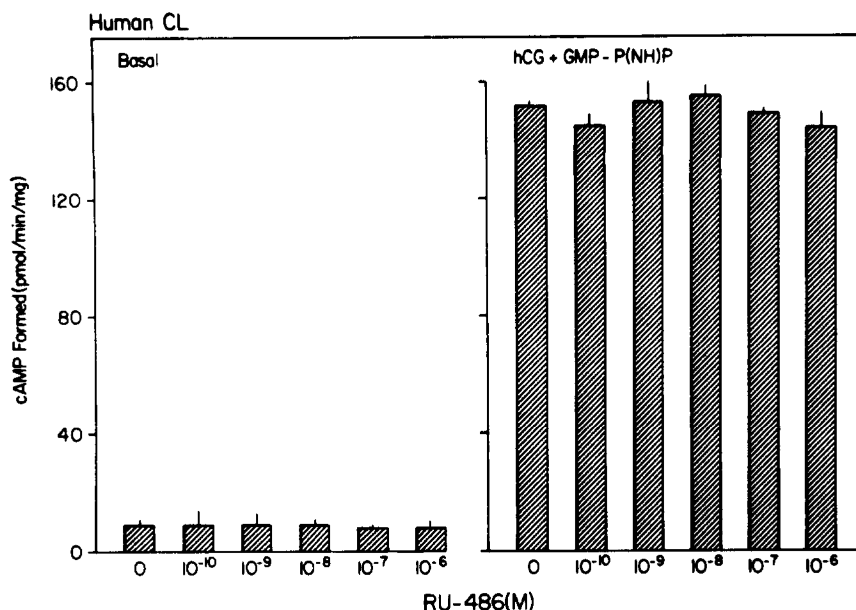


Fig. 1. Effect of RU-486 on basal and maximal hCG responsiveness of human luteal adenylyl cyclase. Enzyme activities were assayed at varying concentrations of RU-486 under basal conditions (left panel) and in the presence of a saturating concentration of hCG (10 $\mu$g/ml) plus a maximally effective concentration of GMP-P(NH)P (100 $\mu$M) (right panel).

activated charcoal (Norit A) at 0–4°C according to the procedures described by Iyengar *et al.*[14]. Forskolin and RU-486 were added as a dilution of a stock solution dissolved in absolute ethanol. Final concentrations of ethanol were 0.5 and 2% in adenylyl cyclase assays using forskolin and RU-486, respectively, and 0.1% in the cell cultures. An equivalent final concentration of ethanol was present in the corresponding control groups. Proteins were measured by the method of Lowry *et al.*[15] using bovine serum albumin as standard. Student's $t$-test or analysis of variance, followed by Duncan's multiple range test [16] were utilized for statistical evaluations. The results are expressed as the mean ± SEM.

## RESULTS

*Effects upon human luteal adenylyl cyclase activity*

The effects of increasing concentrations of RU-486 upon maximal gonadotropin stimulation are shown in Fig. 1. Maximal gonadotropin responsiveness was obtained using a maximally effective concentration of hCG in the presence of a saturating concentration of the hydrolysis-resistant GTP analog, GMP-P(NH)P [10]. Under such conditions, the hCG-stimulated activity was not affected by RU-486, nor did the compound alone alter the activity of the enzyme in the absence of any stimulator (Fig. 1). This absence of any observable effect by RU-486 could not be attributed to the condition which allowed maximal hormone responsiveness. Thus, a similar pattern of results was obtained when RU-486 was added in the presence of hormone alone (Fig. 2); it became apparent that, although hCG stimulation was significantly reduced in the absence of added guanyl nucleotide, the response was not altered by RU-486. Additional experiments also demonstrated that the compound did not affect the stimulation of human luteal adenylyl cyclase by different types of non-hormonal activators such as guanyl nucleotide, NaF and the diterpene forskolin (Fig. 3).

*Effects upon dispersed rat anterior pituitary cell cultures*

We next studied the effects of RU-486 upon basal LH and FSH release by dispersed pituitary cell cultures. As shown in Fig. 4, the compound did not significantly alter the spontaneous release of either LH (upper panel) or FSH (lower panel). Similarly,

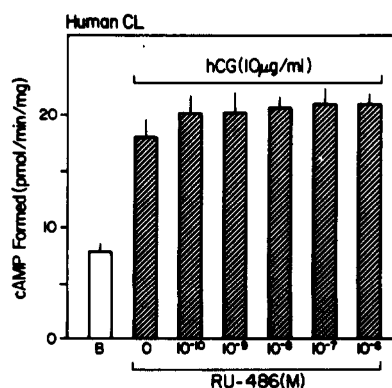


Fig. 2. Effect of RU-486 on hCG stimulation of human luteal adenylyl cyclase in the absence of added guanyl nucleotide. Enzyme activities were assayed at varying concentrations of RU-486 in the presence of hCG alone (10 $\mu$g/ml). B, basal activity.

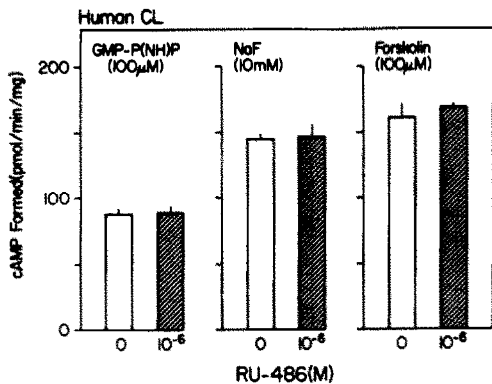


Fig. 3. Effect of RU-486 on adenylyl cyclase activity from human corpus luteum membranes in response to GMP-P(NH)P, NaF and forskolin. Adenylyl cyclase activities were determined in the absence or presence of $10^{-6}$ M RU-486. When present, the concentration of both GMP-P(NH)P and forskolin was 100 $\mu$M, and that of NaF was 10 mM.

the response of cell cultures to LHRH was not significantly affected by RU-486. This failure of RU-486 to affect the LHRH-stimulated release of LH and FSH was evident not only when maximally effective concentrations of LHRH were employed, e.g. 5 ng/culture or higher concentrations [13] (data not shown), but also under conditions resulting in submaximal response, as observed in the presence of 1 ng LHRH/culture (Fig. 5).

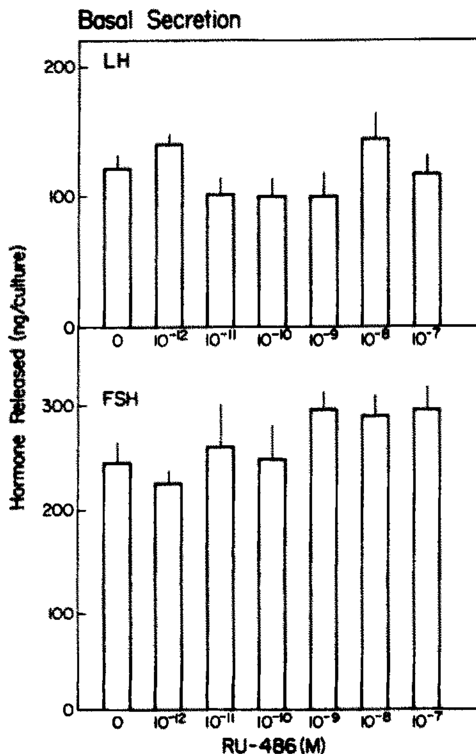


Fig. 4. Effect of RU-486 on the spontaneous release of LH (upper panel) and FSH (lower panel) by dispersed rat anterior pituitary cells. The cells were trypsin-dispersed and grown for 3 days in monolayer before utilization. After 2 h incubation in the absence or presence of varying concentrations of RU-486, the media were removed and subjected to RIA to determine LH and FSH. Each data point represents the mean ± SEM of three individual cultures.

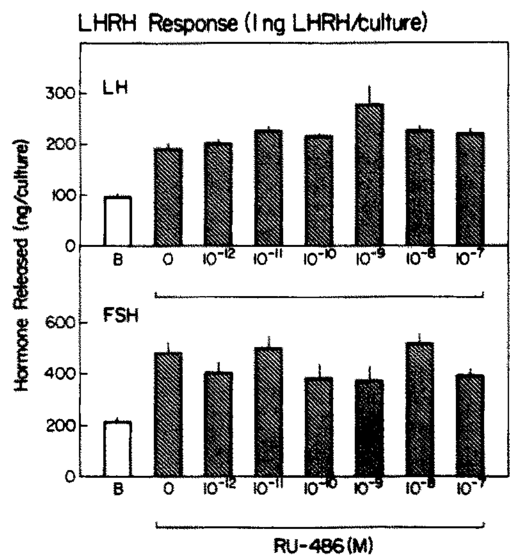


Fig. 5. Effect of RU-486 on the response of dispersed rat anterior pituitary cell cultures to LHRH. The cultures were preincubated for 2 h in the absence (control groups) or presence of RU-486 ($10^{-12}$ to $10^{-7}$ M) and the media were removed as described in the text. Cells were then incubated with 1 ng LHRH/culture in the absence (control groups) and presence of varying concentrations of RU-486. After 2 h, the media were removed and the LH (upper panel) and FSH (lower panel) levels were determined by RIA. B, basal hormone release. Each data point represents the mean ± SEM of three individual cultures.

## DISCUSSION

Conflicting data among groups of investigators concerning the effects of RU-486 upon the hormonal cycle have led to the conjecture that this agent does not act exclusively at the endometrial level [2, 4, 5, 17]. In the present study, we have demonstrated that RU-486 does not directly alter responsiveness of gonadotropin-sensitive adenylyl cyclase in membranes obtained from human corpora lutea. Failure to alter hCG-responsive adenylyl cyclase would imply that RU-486 does not affect gonadotropin receptor binding or cAMP synthesis and/or degradation in this membrane system. It is unlikely, therefore, that the compound may modulate human luteal function through changes in plasma membrane lipid mobility or modifications of reactions occurring in plasma membranes as suggested for other steroids in several membrane systems [6–9]. Although it cannot be excluded that RU-486 can exert its effects at some steps beyond gonadotropin-stimulated cAMP production in the human luteal cell, the results described here are compatible with previously published *in vivo* studies suggesting that the effects of RU-486 do not involve a direct antigonadotropin activity at the level of the primate corpus luteum. Accordingly, we have ob-

served that RU-486 administration during the luteal phase in primates can induce early onset of menses, despite the simultaneous administration of doses of hCG that mimic the circulating hCG levels of early pregnancy in Rhesus monkeys [4]. Also, these results are consistent with data showing that induction of early onset of uterine bleeding by RU-486 administration does not affect serum progesterone concentration in regularly cycling Rhesus monkeys [4], and with studies in castrated Cynomolgus monkeys indicating that RU-486 can act irrespective of any actions of this agent upon progesterone secretion by the primate corpus luteum [5]. Thus, these observations conflict with the hypothesis [18] that RU-486 may shorten the life span of the human corpus luteum when administered in normal menstrual cycles, and provide further support to the concept that RU-486 has no direct luteal site of action in primates.

We have also demonstrated that RU-486 does not directly inhibit LH and FSH release by pituitary cell cultures, nor does the compound impair the response of these cells to LHRH. These data would suggest the RU-486 activity involves no direct effect at the pituitary level. However, it is interesting to consider that the pituitary cell culture used in this study was prepared from female rats utilized at random stages of the estrous cycle. With this consideration, we cannot completely preclude the likelihood that the antiprogesterone activity of RU-486 may alter gonadotropin secretion by the pituitary when progesterone is required to establish the normal gonadotropic surge in the menstrual cycle [19]. In addition, the effects of RU-486 upon gonadotropin release by cultured pituitary cells were studied using short-term exposure to the steroid. Taking into account that RU-486 may act through progesterone receptor and that progesterone receptor translocation and subsequent nuclear processing may occur 30–60 min after *in vitro* progesterone treatment [20], one might expect that the period of exposure used in our study would be sufficient to allow steroid action. We feel, however, that a definitive conclusion concerning the effects of RU-486 upon gonadotropin secretion by altered pituitary cells awaits long-term exposure to the steroid. Although more extended analyses are warranted, it should be noted that the present data are consistent with reports indicating that induction of menstruation by RU-486 is not accompanied by changes in daily serum LH and FSH levels in regularly cycling monkeys [4] or in castrated monkeys receiving exogenous estradiol and progesterone treatment [5].

Taken together, these findings suggest that induction of early menstruation by RU-486 is unlikely to be secondary to a direct inhibitory action of RU-486 upon pituitary or luteal function. In the absence of such sites of action, these data argue that the induction of uterine bleeding and pregnancy termination with RU-486 are due to a primary local effect of the compound upon the endometrium.

*Acknowledgements*—We are grateful to Dr Edouard Sakiz of Roussel Uclaf, Paris, France for the kind supply of RU-486. We wish to thank Ms Rowena Bray for excellent technical assistance and Ms Kim Morren and Ms Gretta Small for their help in preparing the manuscript.

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