0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 1
Printed in U.S.A.

Mechanism of the Inhibitory Action of RU486 on the Secondary Follicle-Stimulating Hormone Surge*

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ABSTRACT

Recent evidence utilizing RU486 has implicated progesterone (P) and glucocorticoids, in addition to a drop in serum inhibin, in the development of the secondary FSH surge on the morning of estrus. To assess the role of these steroids, we treated proestrous female rats with the antiprogestin/antiglucocorticoid RU486 (6 mg/kg sc) at 1230 h, and with dexamethasone (dex; 8.4 or 16.2 mg/kg sc), or with the steroid biosynthesis inhibitor aminoglutethimide (AG; 150 mg/kg ip) at 1030 h, alone or in combination with RU486. The effects of these treatments on uterine ballooning and intraluminal fluid content (an index of P action), ovulation, and serum levels of P, corticosterone (B), FSH, LH, and inhibin-α at 1830 h proestrus and 0900 h estrus were examined. In accord with previous work from our laboratory, RU486 caused uterine intraluminal fluid retention on the morning of estrus and significantly suppressed the preovulatory surges of both FSH and LH, and the secondary surge of FSH without affecting the fall in inhibin-α. Treatment with dex alone raised serum FSH at both 1830 h proestrus and 0900 h estrus, coincident with suppression of serum inhibin-α. When administered in combination with RU486, dex partially reversed the increased uterine intraluminal fluid retention at 0900 h estrus, but did not modify the inhibitory effect of RU486 on the primary gonadotropin surges or the secondary surge of FSH. AG alone significantly suppressed serum P, B, and gonadotropins (LH to a greater extent than FSH) at 1830 h proestrus and blocked ovulation and uterine intraluminal fluid release at 0900 h estrus; it did not, however, suppress the secondary FSH surge or prevent the fall in serum inhibin-α. When administered 2 h before RU486, AG did not prevent the RU486-induced inhibition of the primary gonadotropin surges or the secondary FSH surge. We conclude from these results that development of the secondary FSH surge does not require P or glucocorticoid action and that RU486 suppression of the secondary FSH surge does not involve blockade of binding of these steroids to their receptors. Our data are compatible with ligand-independent activation of the P receptor, susceptible to blockade by RU486, as the mechanism underlying the enhanced secretion of FSH from the gonadotrope on the morning of estrus. (*Endocrinology* **137:** 85–89, 1996)

THE PITUITARY gonadotropins LH and FSH, which regulate steroidogenesis and gametogenesis, respectively, are secreted by the same gonadotropes (1). Although secretion of both LH and FSH is stimulated by GnRH and suppressed by gonadal steroids, regulation of these hormones diverges at certain stages of the rat estrous cycle. Specifically, on late proestrus and early estrus, when serum LH declines to basal levels, FSH secretion remains high. This sustained elevation of serum FSH, known as the secondary surge of FSH, is responsible for recruitment of the next cohort of follicles (2).

The gonadal protein inhibin is a key regulator of FSH secretion and FSHβ messenger RNA (mRNA) synthesis, but does not affect LH secretion (1, 3–5). Measurement in serum of the α subunit of inhibin revealed that serum FSH is inversely related to serum inhibin in the rat as well as in other species (3, 6, 7). Passive immunization with an antiserum to the α subunit of inhibin on diestrus leads to a prompt, sustained rise in serum FSH in female rats (6); iv administration of antiinhibin-α on proestrus raises serum FSH above the secondary FSH surge on the morning of estrus (8, 9). The preovulatory surges of LH and FSH on the afternoon of proestrus in the rat decrease mRNA for the inhibin subunits in the ovary (10) and also reduce the α subunit of inhibin in serum late on proestrus (7). This fall in inhibin has been considered until recently the principal factor underlying the development of the secondary FSH surge. Our demonstration that the progesterone/glucocorticoid receptor antagonist RU486 administered on proestrus attenuates the rise in FSH on the morning of estrus, despite a fall in serum inhibin-α (9, 11) and that RU486 prevents the action of an antiserum to inhibin-α administered on proestrus from raising serum FSH on estrus (8, 9) raised the possibility that progesterone (P) or corticosterone (B), in addition to a drop in inhibin, are factors responsible for the development of the secondary FSH surge. Alternatively, the cellular mechanism(s) mediating the action of reduced serum inhibin may be coupled to activation of the P receptor (PR) or the glucocorticoid receptor (GR) in a ligand-independent fashion (12).

RU486 can block the action of both P and glucocorticoids (13), although through different mechanisms with respect to receptor binding, receptor stabilization, and DNA binding and transcriptional enhancement by its complexes (14). The present study was undertaken to determine whether attenuation of the secondary FSH surge by RU486 treatment is due to blockade of the action of P or B or both steroids. Dexamethasone (dex) was administered early at proestrus to saturate the GR before RU486 treatment. Aminoglutethimide (AG), an inhibitor of the side-chain cleavage enzyme (15, 16)

Received August 28, 1995.
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* This work was supported in part by NIH Grants PO1 HD-21921, P30 HD-28048, and RO1 HD-07504 (to N.B.S.), and T32 HD-07068 (to K.L.K.).

and aromatase (17) in both the ovary and the adrenal, was used to reduce serum levels of both P and B. Although suppression of the actions of either B or both B and P failed to block the secondary FSH surge, the inhibitory action of RU486 on this surge persisted, suggesting that RU486 exerts its effects on serum FSH through a mechanism independent of blockade of P or glucocorticoid action.

Materials and Methods

Animals

Cycling female Sprague-Dawley rats (Charles River, Portage, MI) were housed three or four per cage in facilities approved by the American Association for the Accreditation of Laboratory Animal Care, and maintained in controlled temperature (20 C) and lighting conditions (14-h light, 10-h dark; lights on at 0500 h), with standard rat chow and tap water provided *ad libitum*. Estrous cyclicity was monitored by daily vaginal smears; only rats that had exhibited at least two consecutive 4-day estrous cycles were included in the study. Rats were killed by decapitation at 1830 h proestrus or at 0900 h estrus; trunk blood was collected for hormone measurements; serum was separated and stored at -20 C until RIA. Laparotomy was performed to assess the presence of uterine ballooning and ovulation (the number of ova was not counted). Accumulation of uterine intraluminal fluid, an index of the E_2/P status of the animal (18), was determined by excising the uterus and weighing it before and after extrusion of the fluid through an incision in each uterine horn. The experimental protocol was approved by the Northwestern University Institutional Animal Care and Use Committee.

Drug treatments

Three separate studies were performed to elucidate the role of glucocorticoids and P in RU486 action on serum FSH. In Exp 1 and 3, RU486 was dissolved in sesame oil with slight warming at a concentration of 6 mg/ml; 6 mg/kg was administered sc at 1230 h on proestrus; 1 ml/kg oil sc served as the control. In Experiment I, RU486 treatment was preceded by sc injection of a 1.5- or 3-fold molar excess of dexamethasone in oil (DexI or DexII, 8.2 or 16.4 mg/kg) at 1030 h on proestrus. In Exp 2 and 3, aminoglutethimide (AG), 75 mg/ml propylene glycol (PG) was dissolved by slight warming; 150 mg/kg was administered ip at 1030 h proestrus, PG served as the vehicle control. Dex, AG, and PG were purchased from Sigma (St. Louis, MO); RU486 was kindly provided by Roussel-UCLAF (Romainville, France).

Hormone assays

Serum FSH, LH, P, B, and inhibin-α were assayed in duplicate by double antibody RIA as described previously (9, 19). FSH and LH concentrations are expressed in terms of the NIDDK rat FSH-RP-2 and LH-RP-3 standards. To preclude interassay variability, all samples from an experiment were analyzed in the same assay.

Data analysis

All results are the mean ± SE of groups of four to six rats. Significance of treatment effects was assessed by analysis of variance, using the CRISP statistical software package (CRUNCH Software, San Francisco, CA). A P value of < 0.05 was considered significant.

Results

Exp 1: effects of pretreatment with dexamethasone on RU486 effects on serum gonadotropins, steroids, inhibin-α, and uterine ballooning and ovulation

The effects of RU486 administered at 1230 h proestrus on uterine intraluminal fluid content, serum FSH, LH, and inhibin-α are shown in Fig. 1. RU486 blocked the action of P, as indicated by uterine ballooning and intraluminal fluid retention on the morning of estrus (a). This effect of RU486 was highly significant ($P < 0.001$) and was significantly attenuated by treatment with either dose of dex ($P = 0.04$). Serum P on either proestrus or estrus was not significantly affected by treatment with either RU486, dex, or the combination of both drugs (b); serum B, on the other hand, was suppressed to undetectable levels by either dose of dex (c), confirming the efficacy of the treatment. RU486 markedly suppressed serum FSH at both 1830 h proestrus and 0900 h estrus in both control and dex-treated rats (d; $P < 0.001$). Dex treatment alone significantly raised serum FSH on both proestrus ($P = 0.01$) and estrus ($P < 0.001$). At 1830 h

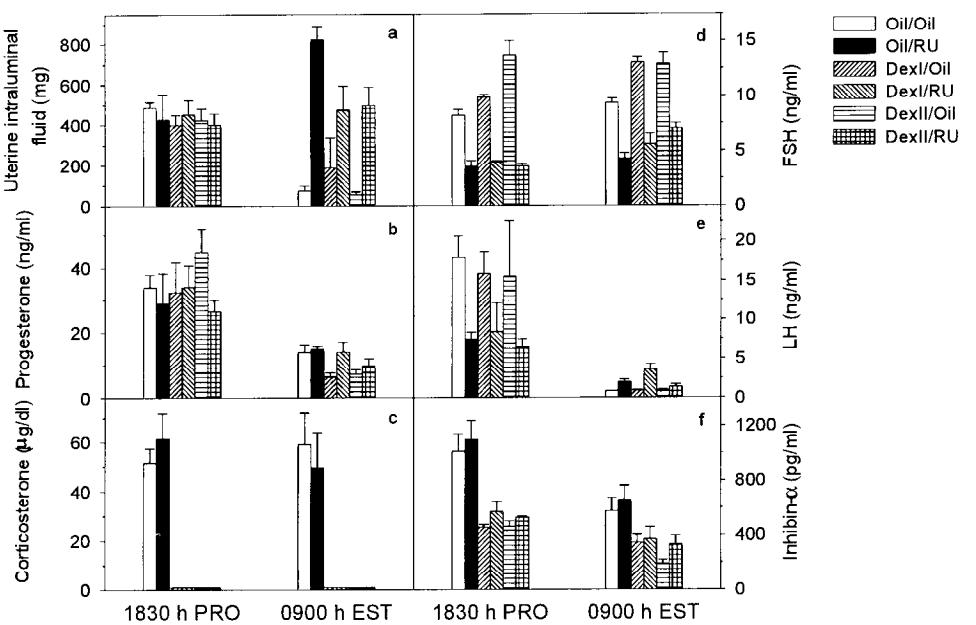

FIG. 1. Effects of RU486 (6 mg/kg sc) injected at 1230 h proestrus, preceded by dexamethasone (8.2 or 16.4 mg/kg sc) at 1030 h proestrus, on uterine intraluminal fluid content (a), and serum FSH (b), LH (c), and inhibin-α (d) at 1830 h proestrus and at 0900 h estrus. Each *bar* is the mean and the *error bars* the SE of four to five rats. Significant effects of treatment are described in *Results*.

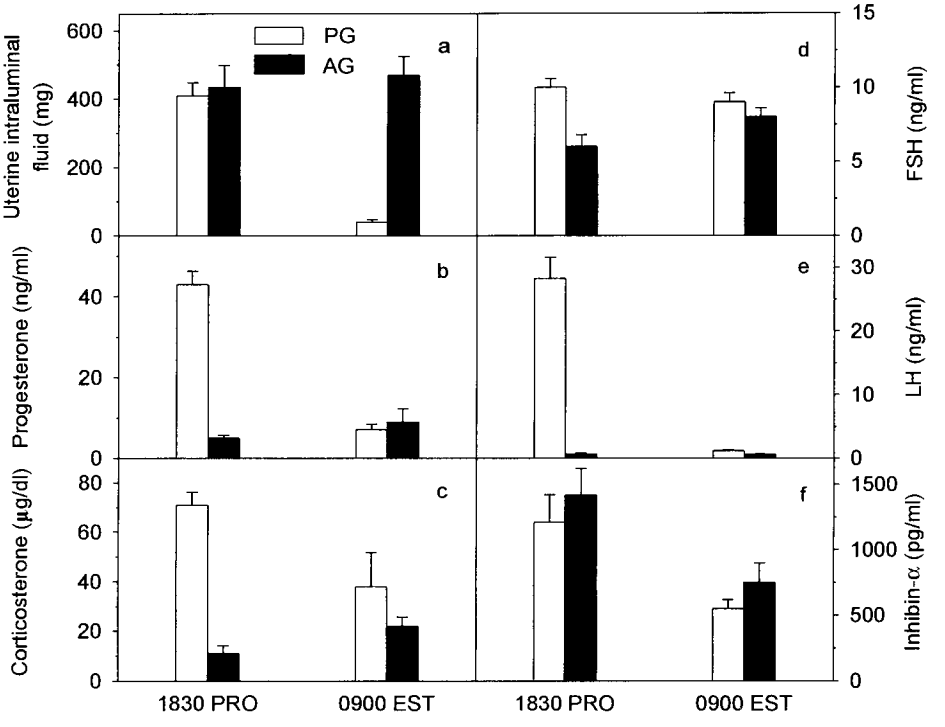

FIG. 2. Effects of aminoglutethimide (AG; 150 mg/kg ip), injected at 1030 h proestrus, on uterine intraluminal fluid content (a), and serum P (b), corticosterone (c), FSH (d), LH (e), and inhibin-α (f) at 1830 h proestrus and at 0900 h estrus. Each *bar* is the mean and the *error bars* the SE of six rats. Significant effects of treatment are described in *Results*.

proestrus, RU486 administration significantly suppressed serum LH, irrespective of dex treatment (e; $P = 0.006$). In contrast, RU486 modestly, but significantly raised the low serum LH present at 0900 h estrus ($P < 0.001$); this rise was partially reversed by dex ($P = 0.006$). Serum inhibin-α (f) was not affected by RU486 administration at either time examined but was significantly suppressed by dex at both 1830 h proestrus and 0900 h estrus ($P < 0.001$). Overall, serum inhibin-α was significantly lower at 0900 h estrus than at 1830 h proestrus ($P < 0.001$). Under all conditions where dex exerted a significant effect, the two doses of dex tested were indistinguishable, *i.e.* both doses appeared to be maximally effective. The effect of RU486 treatment on ovulation was variable and was not affected by prior treatment with either dose of dex. Ovulation was detected in 5/5 control and 1/4 RU486-treated animals; after dex treatment, ovulation was present in 7/10 control and 8/10 RU486-treated rats.

Exp 2: effects of treatment with AG on serum gonadotropins, steroids and inhibin-α, uterine ballooning and ovulation

Figure 2 depicts the effects of AG administered at 1030 h proestrus on uterine intraluminal fluid content, serum P, B, FSH, LH, and inhibin-α at 1830 h proestrus and 0900 h estrus AG treatment dramatically suppressed serum P (b) and B (c) at 1830 h proestrus ($P < 0.001$ for both steroids), but had no effect on the lower steroid levels present on the morning of estrus. By blocking the secretion of P, AG treatment prevented the release of uterine intraluminal fluid and prolonged uterine ballooning on the morning of estrus (a; $P < 0.001$), as well as blocking ovulation in all animals (0/5 AG-treated *vs.* 6/6 control). AG reduced the primary surge of FSH from 9.9 ± 0.6 to 5.6 ± 0.8 ng/ml at 1830 h proestrus ($P = 0.002$) but did not significantly affect the secondary surge of FSH at 0900 h estrus (d), coincident with a significant drop of serum inhibin-α (f; $P < 0.001$). AG treatment markedly suppressed the preovulatory surge of LH (1.2 ± 0.1 *vs.* 28.3 ± 3.3 ng/ml, $P < 0.001$) at 1830 h proestrus but did not affect the normally low serum LH at 0900 h estrus (e).

Exp 3: effect of treatment with AG on RU486-induced suppression of the secondary FSH surge

Treatment of rats with RU486 at 1230 h proestrus, with AG at 1030 h proestrus or with both drugs resulted in prolonged uterine ballooning and significantly increased intraluminal fluid content at 0900 h estrus (a; $P < 0.001$ and $= 0.02$ for RU486 and AG respectively); there was no significant interaction between the two treatments. Uterine ballooning normally present at 1830 h proestrus was not significantly affected by either agent. Ovulation was detected in 5/5 control animals and 5/6 animals treated with RU486; ovulation was blocked in all animals receiving AG alone (0/5) or AG followed by RU486 (0/7). Serum P and B were unaffected by RU486, but were significantly suppressed by AG treatment at 1830 h proestrus (b and c; $P < 0.001$ for both steroids). A profound suppression of serum FSH by RU486 was evident at both 1830 h proestrus and 0900 h estrus (d; $P < 0.001$ at both times); this suppression of both the primary and secondary FSH surge was not affected by prior administration of AG, which alone significantly lowered serum FSH at 1830 h proestrus ($P = 0.01$), but not at 0900 h estrus. Serum LH was dramatically suppressed by both treatments, separately or in combination, on proestrus evening (Fig. 3e; $P = 0.002$ and < 0.001 for RU486 and AG effects, respectively). As in previous experiments, serum LH concentration increased slightly, but significantly, in RU486-treated animals on the morning of estrus ($P = 0.003$) but was not affected by AG alone or in combination with RU486 (e). In all treatment

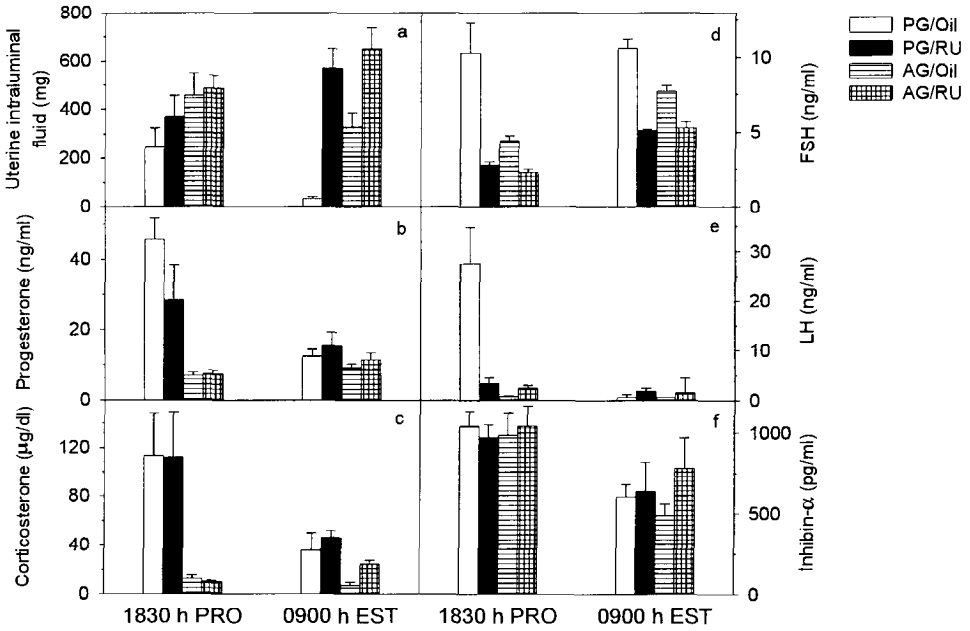

FIG. 3. Effects of RU486 (6 mg/kg) injected at 1230 h proestrus, preceded by AG (150 mg/kg ip) at 1030 proestrus on uterine intraluminal fluid content (a), and serum P (b), corticosterone (c), FSH (d), LH (e), and inhibin-α (f) at 1830 h proestrus and at 0900 h estrus. Each *bar* is the mean and the *error bars* the SE of five to six rats. Significant effects of treatment are described in *Results*.

groups, serum inhibin-α was significantly lower at 0900 h estrus than at 1830 h of the preceding evening of proestrus (f; $P < 0.001$ for time effect). None of the drug treatments affected significantly serum inhibin-α levels at either time of the study, nor was there a significant interaction between time and any of the drug treatments.

Discussion

The secondary surge of FSH which occurs, without an accompanying rise in serum LH, early in the morning of estrus, and plays a critical role in recruiting follicles for the next estrous cycle, is likely the result of an interplay among the ovarian factors that specifically regulate FSH secretion (5, 20) and FSHβ mRNA levels (21), inhibin, activin, and follistatin. Of these FSH-regulating proteins, the secretion of only inhibin has been characterized in detail in relation to periovulatory changes in circulating FSH (3, 7). Although participation of activin and its binding protein, follistatin, remains to be demonstrated, reduced circulating inhibin and diminished expression of its subunits in the ovarian follicles on proestrus evening is a well-established cause of the secondary, GnRH-independent rise of serum FSH on estrus morning (10).

A fall in inhibin, however, does not invariably lead to a rise in serum FSH. Recent work from our laboratory and others indicates that treatment with the antiprogestin/antiglucocorticoid RU486 on proestrus can block the effect of either a normal (11) or antiinhibin-α-induced (8, 9) fall in serum inhibin on serum FSH on the morning of estrus. These findings suggested that in addition to the fall in serum inhibin, P and glucocorticoids, whose actions are blocked by RU486, are required to induce the secondary surge of FSH on estrus; the objective of the present study was to test the above hypothesis.

The results of the present work clearly rule out an obligatory role of either steroid's action in eliciting the secondary FSH surge. Administration of maximally effective doses of dex 2 h before RU486 treatment on proestrus, did not prevent suppression of the secondary FSH surge by RU486 on the morning of estrus. Moreover, marked suppression of serum B on the evening of proestrus by AG administered on proestrus morning, failed to prevent development of the secondary FSH surge or its suppression by RU486 on estrus morning, lending additional support to the view that RU486 action does not involve blockade of glucocorticoid binding to the GR. This conclusion is consistent with the earlier findings of Campbell *et al.* (22), who reported that adrenalectomy performed at 0800 h of proestrus did not prevent the rise in serum FSH at 0830 h estrus but is contrary to that put forth recently by Tébar *et al.* (23). The latter authors demonstrated that adrenalectomy at 1100–1200 h of proestrus suppressed and administration of B at 1500 and 1700 h of proestrus partially restored the elevated serum FSH at 0200 h estrus, and interpreted this as evidence of a key role of glucocorticoids in induction of the secondary FSH surge. There is no ready explanation of the discrepancy between the conclusions of Tébar *et al.* (23) and those of the present study; they are presumably due to differences in experimental design, such as the length of time intervening between treatment and blood collection for hormone assay.

The present data similarly rule out a requirement for P action on late proestrus in eliciting the secondary FSH surge. Rats given AG on the morning of proestrus exhibited marked suppression of serum P at 1830 h proestrus, coincident with partial suppression of the primary FSH surge and complete elimination of the preovulatory LH surge and ovulation the next morning. Nevertheless, the secondary FSH surge and the fall in serum inhibin-α persisted, and RU486 still suppressed the secondary FSH surge under these conditions. The finding that a significant fall in serum inhibin-α had occurred despite the complete absence of the preovulatory LH surge was surprising and unexpected in the face of the prevailing view that this surge was required to suppress

inhibin synthesis late on proestrus (10). Evidently, the preovulatory surge of FSH, which can substitute for LH, and which was only partially blocked by AG treatment, was sufficient to reduce inhibin synthesis to a degree required to elicit the normal rise in serum FSH.

Although both RU486 and AG completely abolished P action, as indicated by the failure of uterine intraluminal fluid to be released on the morning of estrus, there were striking differences between the effects of the two treatments. First, whereas AG completely blocked the preovulatory LH surge at 1830 h proestrus and ovulation on the morning of estrus, RU486 suppressed only partially the preovulatory LH surge, and the residual surge was sufficient to allow ovulation to occur in most animals. A second and notable difference in the outcome of the two treatments was the persistence of the secondary FSH surge after AG, compared with its profound suppression by RU486, implying significant differences in the two drugs' mode of action.

Although the present findings argue against a blockade of P or B binding to their respective receptors as the mechanism whereby RU486 suppresses the secondary FSH surge, they do not rule out blockade by RU486 of ligand-independent activation of the PR. Members of the steroid hormone superfamily are known to be activated, in addition to their authentic ligands, by agents that promote intracellular protein phosphorylation, *e.g.* cAMP (12). An example of such ligand-independent activation of the PR, blocked by RU486 and occurring in the absence of P has been proposed by Turgeon and Waring (24) as the mechanism underlying GnRH self-priming in gonadotropes *in vitro*; a process in which the PR is activated through a cAMP-mediated pathway. In addition, Mani and co-workers reported activation of the hypothalamic PR that governs sexual behavior by agonists of the dopamine D1 receptor in the absence of P (25), another process blocked by antiprogestins, including RU486. It is worth noting that in the present study, RU486 attenuated the preovulatory surges of both gonadotropins in the virtual absence of P in AG-treated animals, suggesting suppression of ligand-independent activation of the gonadotrope-associated PR, rather than blockade of the binding of the authentic ligand P to its receptor.

In conclusion, the present work provides evidence that the ability of RU486 to suppress the secondary FSH surge involves a mechanism independent of blockade of P or B binding to their receptors and suggests a role for ligand-independent activation of the PR in inducing the secondary FSH surge.

Acknowledgments

We are grateful to NIDDK for the reagents for the FSH and LH RIAs and to Roussel-UCLAF (Romainville, France) for the generous gift of RU486 used in this study. Brigitte Mann and Stephanie Kluge provided excellent technical assistance.

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