The antiprogestin RU486 dissociates LH and FSH secretion in male rats: evidence for direct action at the pituitary level

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Abstract

Administration of 4 mg of the antisteroid RU486 over 8 consecutive days to adult male rats dissociated in vivo and in vitro gonadotrophin secretion, increasing FSH and decreasing LH secretion. In subsequent experiments we evaluated the involvement of testicular or adrenal secretory products, as well as hypothalamic LHRH, in the effects of 4 consecutive days of RU486 treatment on the secretion of gonadotrophins. The first day of RU486 injection was designated day 1, subsequent days being numbered consecutively. Groups of rats injected with oil (0·2 ml) or RU486 (4 mg) were: (i) injected s.c. from day 1 to day 4 with the antiandrogen flutamide (10 mg/kg); (ii) bilateral orchidectomized (ORCH) on day 1; and (iii) bilateral adrenalectomized (ADX) on day 1. Controls were given flutamide vehicle or were sham operated. To ascertain whether the secretion of LHRH is involved in the effects of RU486 on gonadotrophin secretion, we measured the LHRH secretion into the pituitary stalk blood vessels at 1100 h on day 5 in oil- or RU486-treated rats. Additional oil- and RU486-treated rats were injected i.p. with 100 ng LHRH at 1000 h on day 5, or s.c. with 1 mg

LHRH antagonist (LHRH-ANT) at 1000 h on days 2 and 4. Controls were given saline. All animals were decapitated at 1100 h on day 5, trunk blood collected and serum stored frozen until FSH, LH and testosterone assays.

While ADX had no effect on FSH and LH secretion in either oil- or RU486-treated rats, the removal of androgen negative feedback with flutamide treatment or by ORCH substantially increased serum levels of FSH and LH in both oil- and RU486-treated rats, and thus annulled the effects of RU486. No differences in pituitary stalk plasma LHRH concentrations were found between oil- and RU486-treated rats. Injection of LHRH increased serum FSH and LH concentrations in oil-treated rats but only, and to a lesser extent, LH concentrations in RU486-treated rats. Treatment with LHRH-ANT decreased serum concentrations of FSH and LH in both oil- and RU486-treated rats. These results suggest that RU486 inhibited LHRH-stimulated LH secretion at the pituitary level, and that FSH secretion increased in response to a reduction in the negative feedback of androgen.

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Introduction

The antiprogestagen RU486 is a synthetic steroid with strong antiprogesterone and antiglucocorticoid activity (Philibert 1984, Baulieu 1989). It has been shown that administration of RU486 to female cyclic rats dissociates dioestrous secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Sánchez-Criado *et al.* 1990, 1992, 1993), reduces the magnitude of the preovulatory LH surge (Rao & Mahesh 1986, Sánchez-Criado *et al.* 1990) and abolishes the primary and secondary surge of FSH (Knox & Schwartz 1992, Knox *et al.* 1993, Sánchez-Criado *et al.* 1994). These effects of RU486 on FSH and LH secretion could be the result of the blockade of progesterone and/or glucocorticoid action (Lusting *et al.* 1988, Tébar *et al.* 1995). Also, results from recent experiments indicate that RU486 may affect FSH (Szabo *et al.* 1996, Ringstrom *et al.* 1997) and LH (Waring

& Turgeon 1992, Turgeon & Waring 1994, Levine 1997) secretion through the blockade of ligand-independent activation of progesterone receptor.

In a pilot experiment we found that in male rats which have pituitary progesterone receptors (Kato *et al.* 1985, Negro-Vilar *et al.* 1984) and negligible progesterone levels (Döhler & Wuttke 1975, Schwartz & Justo 1977), administration of RU486 increases FSH, while decreasing LH secretion. This effect is opposite to that found in females, in which RU486 decreases FSH and increases LH baseline secretion (Sánchez-Criado *et al.* 1990, 1992, 1993, 1994). Aiming at elucidating this intriguing effect of RU486 in male rats, in the present study we characterized the involvement of the hypothalamus and the steroid-secreting glands in the effects of RU486 on FSH and LH secretion in male rats. The results indicated that RU486, acting at the pituitary, decreased basal luteinizing hormone-releasing hormone (LHRH)-stimulated LH secretion.

Furthermore, the results suggest that FSH secretion increased in response to a partial reduction of negative androgen feedback.

Materials and Methods

Animals

Adult male Wistar rats weighing 250–300 g were used. The rats were housed (four per cage) under standard lighting (lights on from 0500 to 1900 h) and temperature (20–23 °C) conditions and had free access to rat chow and tap water. Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs, treatment and surgery

Antiprogestagen RU486 (mifepristone, 11β-(4-dimethyl-aminophenyl)-17β-hydroxy-17α-(1-propynyl)-estra-4,9-dien-3-one) was donated by Dr Sitruk-Ware (Exelgyn, Paris, France). This compound has a high affinity for progesterone and glucocorticoid receptors (Philibert 1984, Baulieu 1989). Injections consisted of 4 mg/0·2 ml oil (s.c.). Control injections consisted of oil.

Antiandrogen flutamide (4′-nitro-3′-trifluoro-methyl-isobutyranilide; Schering-Plough, Madrid, Spain) was dissolved in oil at a concentration of 20 mg/ml, and administered s.c. at 10 mg/kg per injection. This dose effectively blocks androgen action (Chandolia et al. 1991). Controls received oil.

The synthetic LHRH used (Peninsula 7201) was obtained from Peninsula Laboratories, Inc., Belmont, CA, USA. Immediately before use, the peptide was dissolved in saline to a concentration of 200 ng/ml. The animals were injected i.p. with 0·5 ml of this solution. Controls were given 0·5 ml saline.

The LHRH antagonist (LHRH-ANT) used was ORG.30276 (Ac-D-p-Cl-Phe-D-p-Cl-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH$_2$-CH$_3$-COOH) (Organon International BV, Oss, The Netherlands). Immediately before use, the peptide was dissolved in saline to a concentration of 5 mg/ml. Injections consisted of 0·2 ml of this solution. This dosage causes maximal suppression of LH serum levels (van den Dungen et al. 1989, Sánchez-Criado et al. 1993). Controls were injected with saline.

Bilateral orchidectomy (ORCH) was performed under light ether anaesthesia through the scrotal route between 0900 and 1100 h on day 1. Sham-ORCH consisted of exposing but not removing the testes.

Adrenalectomy (ADX) was performed under ether anaesthesia between 0900 and 1100 h on day 1. Adrenal glands were removed through a subcostal 1 cm incision on both sides. Sham-ADX consisted of exposing but not removing the adrenals.

Pituitary stalk blood collection

The pituitary stalk was exposed following the methods of Worthington (1966) and Sarkar et al. (1976) under midatrene (Glaxo Laboratories, Enghien, France). This anaesthetic consists of 9 mg alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione) and 3 mg alphadolone acetate (21-acetoxy-3α-hydroxy-5α-pregnane-11,20-dione) per ml. It was injected i.p. at a dosage of 0·5 ml/100 g body weight.

Pituitary incubation

Incubations were carried out as described previously (Rodriguez-Padilla et al. 1987). Briefly, the anterior pituitary was cut in half and the halves were incubated at 37 °C with constant shaking in an atmosphere of 95% CO$_2$–5% O$_2$. After 60 min of preincubation, FSH and LH concentrations were measured at 120 and 240 min of incubation.

Experiments

In the first experiment the effects of RU486 on FSH and LH secretion in vivo and in vitro were studied. Just after the collection of blood, rats were injected daily with RU486 or oil over 8 consecutive days. The first day of treatment was designated as day 1. Less than 0·5 ml blood was withdrawn at 1100 h by direct jugular venipuncture under light ether anaesthesia on days 1, 3, 5 and 7. At 1100 h on day 9 all animals were killed by decapitation, trunk blood was collected, and their pituitaries were rapidly collected for pituitary incubation. Serum was stored frozen until assayed for LH and FSH.

In the second experiment the possible involvement of testes or adrenal gland secretory products in the effects of RU486 on LH and FSH secretion were evaluated. Groups of rats injected with RU486 or oil were: (i) injected with flutamide from day 1 to day 4; (ii) ORCH; or (iii) ADX. Controls were sham-operated or injected with oil. All rats were decapitated at 1100 h on day 5. Trunk blood was collected and the serum was stored frozen until assayed for LH and FSH.

In the third experiment we explored the participation of the hypothalamic LHRH in the effects of RU486 on LH and FSH secretion. First, male rats received injections of oil or RU486 over 4 consecutive days and, on day 5, 144 ± 5·4 µl portal blood were collected for 30 min between 1000 and 1200 h. Secondly, oil- or RU486-injected rats were given LHRH at 1000 h on day 5 or LHRH-ANT at 1000 h on days 2 and 4. All animals were decapitated at 1100 h on day 5. Trunk blood was collected and serum stored frozen until assayed for gonadotrophins.

RIA of LHRH

Pituitary stalk plasma concentrations of LHRH were measured in duplicate in a direct RIA using the LHRH

antiserum HU60 supplied by Dr H F Urbanski (Neuroscience Division, Oregon Regional Primate Research Center, Beaverton, OR, USA). A complete characterization of this antiserum has been reported by Urbanski *et al.* (1990). Synthetic LHRH (Peninsula Laboratories) was used as the standard and labelled with ^{125}I by the chloramine-T method (Greenwood *et al.* 1963). The assay was routinely performed under disequilibrium conditions as reported by Sanchez-Criado *et al.* (1994). To avoid interference with other plasma proteins, bound and free LHRH were separated using a second antibody system (López *et al.* 1990). All samples were run in the same assay. Under these conditions, the sensitivity of the assay was 0·25 pg per tube with an intra-assay variability of 8%.

RIA of FSH and LH

The concentrations of FSH and LH were measured in duplicate in 25 µl samples by double-antibody RIA methods, using the RIA kits supplied by the National Institute of Diabetes, Digestive and Kidney Diseases (Baltimore, MD, USA) and following a microassay method described previously (Sánchez-Criado *et al.* 1993). Rat FSH-I-8 and LH-I-9 were labelled with ^{125}I using the chloramine-T method (Greenwood *et al.* 1963). Serum and medium FSH and LH concentrations were expressed as ng/ml and ng/hemihypophysis respectively of the reference preparations LH-rat-RP-3 and FSH-rat-RP-2. All samples were run in the same assay. The intra-assay coefficients of variation were 8 and 7% for LH and FSH respectively. The sensitivities of the assay were 20 and 7·5 pg/tube for FSH and LH respectively.

RIA of testosterone

Serum concentrations of testosterone were measured by the RIA method described previously (Rodriguez-Padilla *et al.* 1987), using antiserum supplied by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). All samples were run in the same assay. The intra-assay coefficient of variation was 6% and the sensitivity 5 pg/tube.

Data evaluation and statistical analysis

Results were expressed as means ± S.E.M. Data were analysed by one-way ANOVA using Duncan's multiple range test or Student's *t*-test when two means had to be compared. Results were considered significant at the $P < 0.05$ level.

Results

Effect of RU486 on FSH, LH and testosterone secretion in intact rats

Administration of RU486 over 8 consecutive days increased FSH serum concentrations throughout the ex-
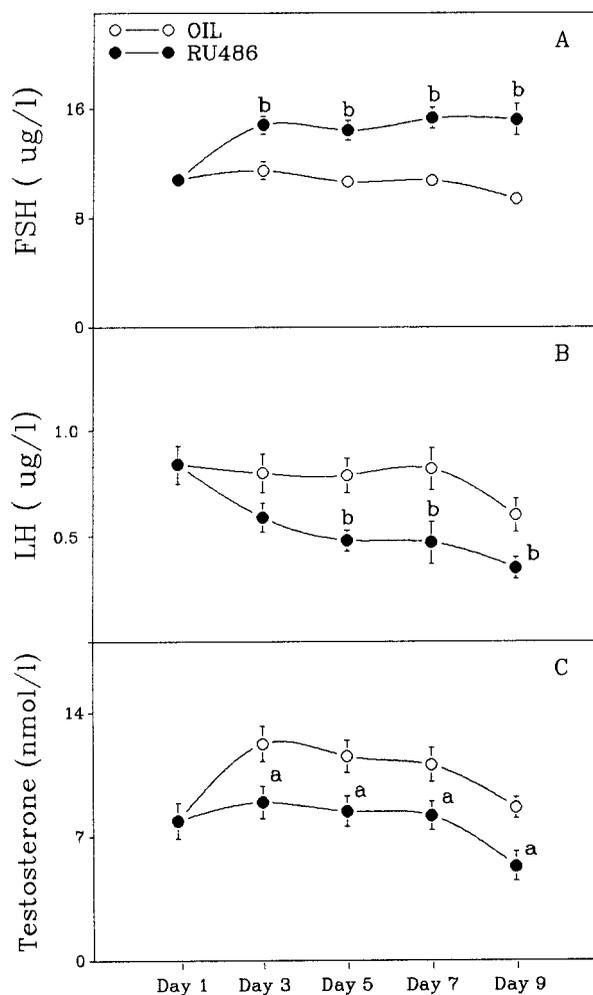
Figure 1 Serum concentrations of (A) FSH, (B) LH and (C) testosterone at 1100 h on days 1, 3, 5, 7 and 9 in adult male rats injected daily with 4 mg RU486 (s.c.) over 8 consecutive days starting on day 1. Control injections consisted of 0·2 ml oil. Values are means ± S.E.M. (12 rats per group). a=$P < 0.05$ and b=$P < 0.01$ vs oil-injected rats (Student's *t*-test).

periment (Fig. 1A). In contrast, LH (Fig. 1B) and testosterone (Fig. 1C) serum concentrations decreased in rats injected with RU486. Also, *in vitro* FSH secretion after *in vivo* administration of RU486 significantly increased for at least 4 h (Fig. 2A). Secretion of LH was significantly lower for the pituitaries of rats treated *in vivo* with RU486 at 120 and 240 min of incubation than for controls (Fig. 2B).

Effect of flutamide, ORCH or ADX on serum concentration of FSH and LH in rats injected with RU486

Treatment with RU486 over 4 consecutive days increased and decreased, respectively, the serum concentrations of FSH and LH in rats decapitated at 1100 h on day 5
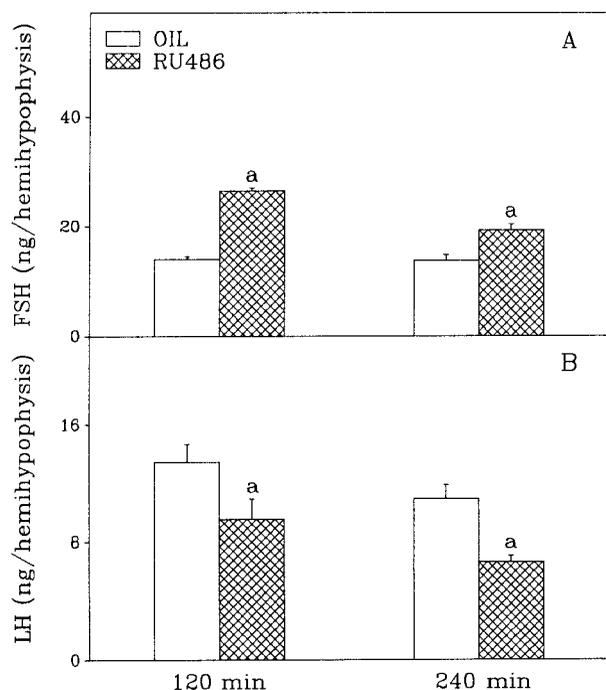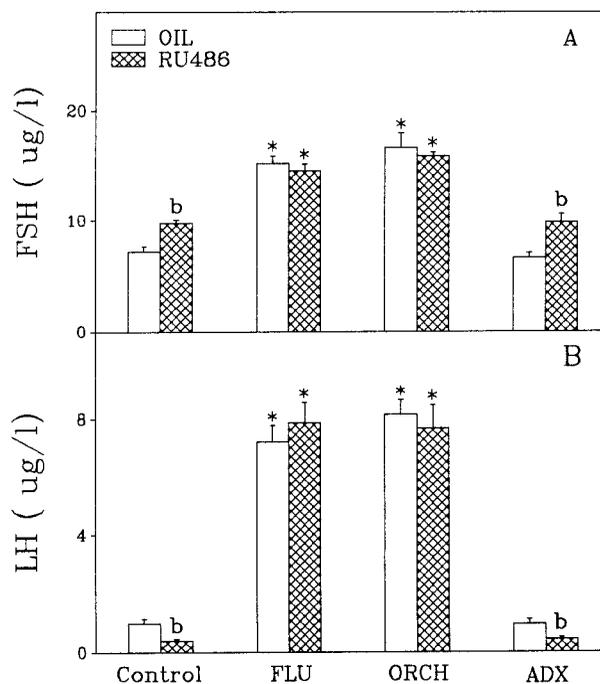
Figure 2 *In vitro* basal FSH (A) and LH (B) secretion after 120 and 240 min of incubation on day 9 from pituitaries of male rats injected *in vivo* with 4 mg RU486 (s.c.) over 8 consecutive days. Values are means ± S.E.M. a=*P*<0·01 vs oil-injected rats (Student's *t*-test).

Figure 3 Effects of antiandrogen flutamide (FLU), orchidectomy (ORCH) or adrenalectomy (ADX) on serum concentrations of (A) FSH and (B) LH at 1100 h on day 5 in male rats injected with 4 mg RU486/0·2 ml oil over 4 consecutive days starting on day 1. ORCH, ADX or sham-operations were performed on day 1. FLU treatment consisted of daily injections of 10 mg/kg over 4 consecutive days. Values are means ± S.E.M. (8–12 rats). b=*P*<0·01 vs oil-injected rats; *=*P*<0·01 vs the corresponding control group (flutamide-vehicle+sham-operated rats) (one-way ANOVA and Duncan's multiple range test).

(Fig. 3). While this effect of RU486 was also evident in ADX rats, it was absent in flutamide–treated rats and in ORCH rats, in which serum concentrations of both gonadotrophins substantially increased (Fig. 3).

LHRH secretion in RU486-treated rats: effect of LHRH and LHRH-ANT on FSH, LH and testosterone concentrations in rats injected with RU486

Plasma concentration of LHRH in the pituitary stalk on day 5 after 4 consecutive days of RU486 treatment (11·8 ± 0·9 ng/l, *n*=10) did not differ by the Student's *t*-test from that of oil-injected rats (12·1 ± 1·7 ng/l, *n*=10).

Injection of LHRH increased the serum concentrations of LH at 1100 h on day 5 in both oil- and RU486-treated rats. This effect of LHRH was greater in controls (Fig. 4B). The injection of LHRH increased FSH serum concentrations in oil-injected rats. No statistically significant effects of LHRH were noted in FSH serum concentrations in RU486-treated rats (Fig. 4A). LHRH-ANT treatment decreased serum concentrations of FSH and LH in both oil- and RU486-treated rats (Fig. 4A and B). Testosterone serum concentrations paralleled those of LH (Fig. 4C).

Discussion

Secretion of FSH and LH in male rats depends on the eliciting action of the hypothalamic decapeptide gonadotrophin–releasing hormone and on steroidal and non-steroidal inhibitory gonadal feedback (reviewed by Fink 1988). Administration of the antisteroid RU486 to adult male rats increased FSH secretion but decreased LH secretion. This dissociation of gonadotrophin secretion in male rats contrasts with the effect witnessed in females. In female rats blockade of progesterone actions by administration of RU486 decreases the magnitude of the pre-ovulatory LHRH surge, and increases LHRH secretion during the period of low secretion rate of gonadotrophin, without affecting LHRH pituitary responsiveness (Sánchez-Criado *et al.* 1994). Administration of RU486 to male rats decreased LH secretion without altering LHRH secretory rate (present results). Because of this, the effects of RU486 on FSH and LH secretion in males should be the result of either the blockade of the feedback effect of peripheral glands at the pituitary level and/or a change of pituitary responsiveness to LHRH.
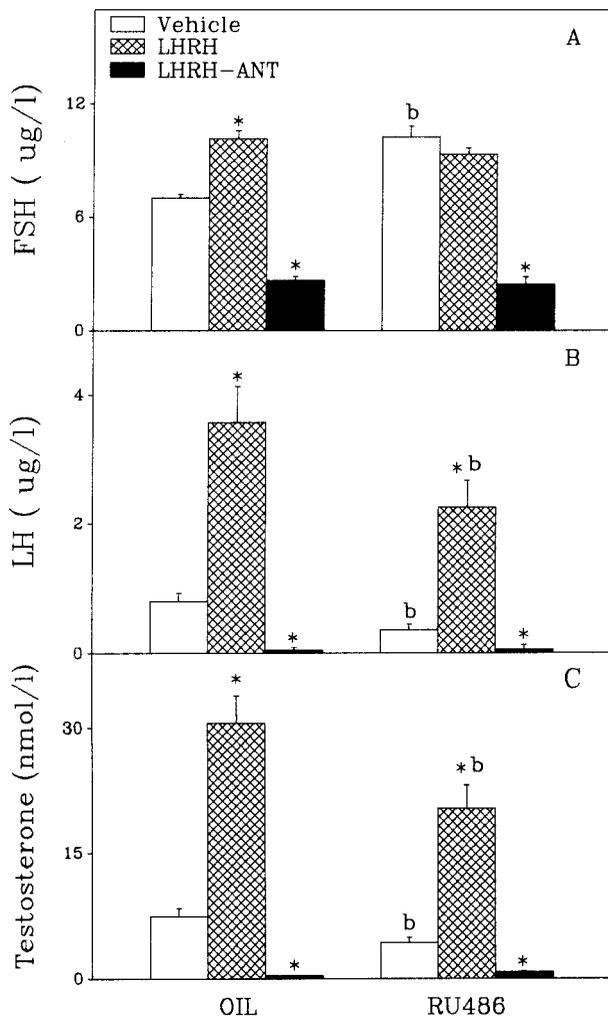
Figure 4 Effects of LHRH or LHRH antagonist (LHRH-ANT) on serum concentrations of (A) FSH, (B) LH and (C) testosterone at 1100 h on day 5 in oil- or RU486-treated rats. RU486 (4 mg) or oil (0·2 ml) was injected (s.c.) over 4 consecutive days. LHRH (100 ng/0·5 ml saline) was injected (i.p.) at 1000 h on day 5. LHRH-ANT (1 mg/0·2 ml saline) was injected (s.c.) at 1000 h on days 2 and 4. Values are means ± S.E.M. (10 rats). b=*P*<0·01 vs oil-treated rats; *=*P*<0·01 vs saline-injected rats (one-way ANOVA and Duncan's multiple range test).

Besides the antiprogestagenic activity, the synthetic steroid RU486 also has strong antiglucocorticoid and moderate antiandrogenic activity (Philibert 1984). In male rats adrenal secretory products play a modulatory role in the secretion of FSH and LH (Lorenzen *et al.* 1980). However, we do not consider that the effects of RU486 on FSH and LH secretion could be due to the blockade of glucocorticoid actions due to the following reasons: (i) glucocorticoids inhibit LHRH-stimulated gonadotrophin secretion in males (Ringstrom & Schwartz 1985); (ii) the antiglucocorticoid action of RU486 in the rat, as measured

by increased serum corticosterone concentrations, lasts only 24 h (Sánchez-Criado *et al.* 1997); and (iii) the present results have shown that the removal of circulating adrenal steroids by ADX did not modify serum levels of FSH and LH or interfere with the effects of RU486 on FSH and LH.

The finding that flutamide increased both FSH and LH secretion to the same extent as ORCH indicates, in agreement with the results of Decker *et al.* (1981) and Summerville & Schwartz (1981), that testosterone, where there is no marked elevation of serum FSH over LH, accounts for almost all of the FSH- and LH-suppressing activity of the testes. Thus, the increased serum FSH concentrations found in RU486-treated male rats was, probably, the consequence of a partial reduction in the negative feedback of androgen, although this interpretation is controversial (Rea *et al.* 1986, Paul *et al.* 1990). Also, the results show that LHRH injection of RU486-treated rats did not increase further FSH secretion and that treatment with LHRH-ANT reduced FSH secretion. This suggests that the reduction in the inhibitory feedback of testosterone and/or its metabolites at the pituitary allowed endogenous LHRH to stimulate the release of FSH but not of LH. Indeed, in agreement with previous data (Gay & Midgley 1969, Kotsuji *et al.* 1988), pituitary LH secretion appeared more sensitive to the inhibitory effects of androgen than of FSH since flutamide treatment or ORCH increased serum LH to values about 10-fold while FSH levels were only double those of control males. The exact mechanism by which RU486 stimulates FSH secretion in male rats is not known. However, because RU486 antagonizes the FSH-modulatory activity of activin in female rats (Szabo *et al.* 1998), and testosterone and LHRH affect FSH secretion through pituitary FSH-modulating proteins in males (Kirk *et al.* 1994, Besecke *et al.* 1996, Bilezikjian *et al.* 1996), direct or indirect effects of RU486 on FSH secretion are possible.

The above reasoning, however, does not explain the inhibitory effect of RU486 on LH secretion. Results showed that RU486 reduces basal and LHRH-stimulated LH secretion and that decreased testosterone levels cannot be involved since androgens produce an inhibitory effect on LH secretion (Drouin & Labrie 1976, Summerville & Schwartz 1981). Thus, the reduction in androgen negative feedback would be expected to enhance LH secretion. Additionally, the results showed that the effect of RU486 disappeared when the strong inhibitory influences of testosterone on LH were removed by ORCH or flutamide treatment (Luderer & Schwartz 1991, present results).

In female rats ovariectomized on the morning of pro-oestrus, treatment with RU486 was found to attenuate the LH surge (Tébar *et al.* 1996). This, and other findings (Levine 1997), have been explained by observing that, even in the absence of progesterone as a ligand, progesterone receptor in the gonadotroph can be trans-activated by LHRH through a progesterone-independent

activation of progesterone receptor (Waring & Turgeon 1992, Turgeon & Waring 1994). Similarly, in the male rat, the inhibitory effect of RU486 on LH secretion could be due, in the virtual absence of circulating progesterone (Döhler & Wuttke 1975, Schwartz & Justo 1977), to a progesterone receptor-mediated blockade of LHRH-stimulated LH secretion. Indeed, experiments in progress in our laboratory using oestrogen-primed anterior pituitary cell cultures from male rats indicate that progesterone receptor antagonist RU486 (2 nM) significantly reduces LHRH (10 nM)-augmented LH secretion. Also, although in most *in vivo* systems RU486 does not display any agonistic action (Baulieu 1991, Knox *et al.* 1996), the possibility that the effects of RU486 on FSH and LH secretion could be ascribed to a progesterone (Horwitz 1985, Meyer *et al.* 1990) or a glucocorticoid (Gruol & Altschmied 1993) agonistic action should not be discarded. Nevertheless, the finding that RU486 inhibits both endogenous and exogenous LHRH-stimulated LH secretion while enhancing endogenous LHRH-stimulated FSH secretion only, cannot be explained unless a differential regulation of gonadotrophin synthesis and secretion by LHRH, RU486 or androgen is considered. Accurate interpretation of the present results is not possible until more direct *in vitro* examination of the effects of RU486 on FSH and LH secretion in males is performed.

In summary, the results are consistent with the hypothesis that RU486 acting directly at the pituitary level decreases basal and LHRH-stimulated LH secretion. Also, despite the fact that the RU486-increased FSH secretion in male rats could be the result of an effect of RU486 at the pituitary through FSH-modulating proteins, the results suggest that increased FSH secretion may be the result of a partial reduction of the negative androgen feedback at the pituitary.

Acknowledgements

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