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Down-regulation of pituitary receptors for luteinizing hormone-releasing hormone (LH-RH) in rats by LH-RH antagonist Cetrorelix

(suppression of pituitary–gonadal axis/binding characteristics/*in vitro* desaturation)

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ABSTRACT **Antagonists of luteinizing hormone-releasing hormone (LH-RH), unlike the LH-RH agonists, suppress gonadotropins and sex steroid secretion immediately after administration, without initial stimulatory effects. [Ac-D-Nal(2)^1,D-Ph(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH (SB-75; Cetrorelix) is a modern, potent antagonistic analog of LH-RH. In this study, the binding characteristics of receptors for LH-RH in membrane fractions from rat anterior pituitaries were investigated after a single injection of Cetrorelix at a dose of 100 μg per rat. To determine whether the treatment with Cetrorelix can affect the concentration of measurable LH-RH binding sites, we applied an *in vitro* method to desaturate LH-RH receptors by chaotropic agents such as manganous chloride ($MnCl_2$) and ammonium thiocyanate (NH_4SCN). Our results show that the percentages of occupied LH-RH receptors at 1, 3, and 6 h after administration of Cetrorelix were approximately 28%, 14%, and 10%, respectively, of total receptors. At later time intervals, we could not detect occupied LH-RH binding sites. Ligand competition assays, following *in vitro* desaturation, demonstrated that rat pituitary LH-RH receptors were significantly ($P < 0.01$) down-regulated for at least 72 h after administration of Cetrorelix. The lowest receptor concentration was found 3–6 h after Cetrorelix treatment and a recovery in receptor number began within ≈24 h. The down-regulation of LH-RH binding sites induced by Cetrorelix was accompanied by serum LH and testosterone suppression. Higher LH-RH receptor concentrations coincided with elevated serum hormone levels at later time intervals. Our results indicate that administration of LH-RH antagonist Cetrorelix produces a marked down-regulation of pituitary receptors for LH-RH and not merely an occupancy of binding sites.**

More than 20 years have passed since the isolation, determination of structure, and synthesis of the hypothalamic hormone controlling the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (1). In the past 2 decades, >3000 agonists and antagonists of this decapeptide, called luteinizing hormone-releasing hormone (LH-RH) have been synthesized in view of their expected therapeutic applications (2–4). While repeated chronic administration of LH-RH agonists is required to induce a suppression of LH and FSH and reduction in the levels of sex steroids, similar effects can be obtained after a single injection of LH-RH antagonists (2–7). During the past few years, several highly potent antagonistic analogs of LH-RH containing neutral amino acids D-citrulline or D-homocitrulline at positions 6 and free of edematogenic and anaphylactoid reactions were synthesized by our group and tested *in vitro* and *in vivo* (3, 4, 8, 9). Among these analogs [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH (SB-75; Cetrorelix) proved to be the most powerful (9).

It is well established that chronic administration of LH-RH agonists reduces the number of pituitary receptors for LH-RH, a phenomenon that has been called down-regulation, and induces desensitization of the pituitary gonadotrophs (2–4, 10, 11). Although the principal mechanism of action of LH-RH antagonists was thought to be the competitive blockade of LH-RH receptors, recent data indicate that prolonged treatment with potent LH-RH antagonist Cetrorelix also down-regulates pituitary LH-RH receptors in rats (12–15).

The purpose of this study was to investigate the binding characteristics of receptors for LH-RH in membrane fractions from rat anterior pituitaries after a single injection of Cetrorelix. The pattern of changes in number and affinity of receptors for LH-RH was defined after administration of Cetrorelix followed by *in vitro* desaturation of LH-RH antagonist from its receptors by chaotropic agents, such as manganous chloride ($MnCl_2$) and ammonium thiocyanate (NH_4SCN) (16, 17). The use of such techniques allows estimation of total LH-RH receptor concentration and not only of measurable free receptors. The timing of receptor events after administration of Cetrorelix was compared with the sequel of inhibitory effects on gonadotropin and sex steroid secretion in male rats.

MATERIALS AND METHODS

Chemicals. LH-RH antagonist [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LH-RH (Cetrorelix), originally synthesized in our laboratory by solid-phase methods (9), was made by Asta Medica (Frankfurt on Main, Germany) as Cetrorelix acetate (D-20761). LH-RH agonist [D-Trp^6]LH-RH was synthesized by solid-phase methods and supplied by Debiopharm SA (Lausanne, Switzerland). Buserelin ([D-Ser(Bu^t)^6, Pro^9-NH-Et]LH-RH) was a gift from Hoechst Pharmaceuticals. Other chemicals, unless otherwise mentioned, were obtained from Sigma.

Iodination. Iodinated derivatives of [D-Trp^6]LH-RH were prepared by the chloramine-T method as described (18) with some modifications. Briefly, radioiodination of 5 μg of peptide in 80 μl of 0.5 M phosphate buffer (pH 7.5) was carried out with ≈2 mCi of $Na^{125}I$ (1 Ci = 37 GBq) (Amersham, Arlington Heights, IL) for 35 sec at room temperature. The reaction was initiated with 10 μg of chloramine T in 10 μl of 0.05 M phosphate buffer and was terminated by adding 50 μg of

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Abbreviations: LH, luteinizing hormone; LH-RH, LH releasing hormone; FSH, follicle-stimulating hormone.

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L-cysteine (in 10 μl of 0.01 M HCl). The radiolabeled hormone was purified by reversed-phase HPLC on a Vydac C-18 column, using 0.1% aqueous trifluoroacetic acid (TFA) as solvent A, and 0.1% TFA in 70% aqueous acetonitrile as solvent B. Elution was carried out by a linear gradient of 35–55% solvent B in 20 min. The effluent was monitored by UV absorption at 280 nm and a flow-through radioactivity detector constructed from a ratemeter (SML-2; Technical Associates, Canoga Park, CA) in our laboratory. One-milliliter fractions were collected in borosilicate glass tubes containing bacitracin (30 μg/ml) and bovine serum albumin (10 mg/ml) in 1 ml of 0.25 M phosphate buffer (pH 7.5). The fraction corresponding to the monoiodinated compound and identified by its elution position, radioactivity, and UV peak, was stored at −70°C for *in vitro* studies. This tracer could be stored for as long as 4 weeks at −70°C and the stability during storage was checked by HPLC. Specific activity, as calculated by isotope recovery, ranged between 1800 and 2000 Ci/mmol.

Animals and Experimental Protocol. Young adult male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing 250–300 g were used. The animals were allowed standard rat diet and tap water ad libitum and were maintained under controlled conditions in a 12-h light/12-h dark schedule at 24°C ± 2°C. Groups of 10–15 rats received one s.c. injection of Cetrorelix acetate at a dose of 100 μg per rat, dissolved in distilled water containing 5% (wt/vol) mannitol. Control animals received vehicle injections only. Rats were anesthetized with pentobarbital (50 mg per kg of body weight) and blood samples of 500 μl were taken from the jugular vein before (time 0) and 1, 3, 6, 24, 48, and 72 h after administration of Cetrorelix for measurement of hormone levels. Serum was separated by centrifugation, and the supernatant was diluted 1:1 with 0.1 M phosphate buffer containing 0.5% sodium azide. Immediately after blood samples were collected, rats were sacrificed by decapitation. Anterior pituitaries were removed, cleaned, and frozen on dry ice and then stored at −70°C until analyses of LH-RH receptors.

Membrane Preparation and Receptor Binding Assays. Pituitary membrane fractions were prepared as described (19). Receptor binding of LH-RH was performed as reported (20) using a sensitive *in vitro* ligand competition assay based on binding of radiolabeled [D-Trp^6]LH-RH to rat anterior pituitary membrane homogenates. Briefly, membrane homogenates containing 40–80 μg of protein were incubated in triplicate with 60,000–75,000 cpm (≈0.15 nM) [^{125}I][D-Trp^6]LH-RH as radioligand and with increasing concentrations (10^{-12}–10^{-6} M) of nonradioactive peptides in a total vol of 150 μl of binding buffer. At the end of the incubations, 125-μl aliquots of suspension were transferred onto the top of 1 ml of ice-cold binding buffer containing 1.5% (wt/vol) bovine serum albumin in siliconized polypropylene microcentrifuge tubes (Sigma). The tubes were centrifuged at 12,000 × *g* for 3 min at 4°C (Beckman J2-21M). Supernatants were aspirated, and the bottoms of the tubes containing the pellet were cut off and assayed in a γ counter (Micromedic Systems, Huntsville, AL). Protein concentration was determined by the method of Bradford (21) using a Bio-Rad protein assay kit (Bio-Rad).

Desaturation by Chaotropic Agents. A certain proportion of the pituitary LH-RH receptors may be occupied by the administered Cetrorelix. Although rat pituitaries were homogenized and fractionated and the final crude membrane fractions were washed twice by resuspending them in ice-cold homogenization buffer, a certain portion of these receptors may remain occupied after preparation of membranes. These LH-RH receptors would be undetectable unless the bound Cetrorelix is removed. The *in vitro* desaturation of LH-RH antagonist Cetrorelix from their receptors was performed using chaotropic agents such as $MgCl_2$, $MnCl_2$, NH_4SCN and CF_3COONa (17). Briefly, 150-μl aliquots of crude membrane homogenates were incubated with different concentrations of dissociating agents, ranging from 0.1 to 3 M in a final vol of 500 μl on ice. After 5 min of incubation, 3 ml of homogenization buffer was added and the tubes were centrifuged at 4000 × *g* for 15 min at 4°C. The supernatant was aspirated, and the pellet was resuspended in binding buffer and used immediately for radioreceptor assay. This procedure generally resulted in a loss of 20–35% in total protein content of the membrane fraction. In some studies, receptor binding assay was performed before the desaturation procedure and the specific binding was calculated. Investigating the same samples, the dissociation was carried out using chaotropic agents and the final binding parameters were determined again. Some binding studies were also performed with pituitary membrane fractions of control (untreated) rats, which had been preincubated *in vitro* with 200 pM Cetrorelix, a quantity sufficient to occupy all available binding sites. After treatment of these membranes with chaotropic agents, rebinding was performed, always utilizing the same original tubes and membrane fractions and adding only fresh [^{125}I][D-Trp^6]LH-RH and unlabeled peptide as competitor. All studies were performed at 4°C.

Hormone Determination. LH was determined by RIA with materials provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (rat LH-RP-3/AFP-7187B/, rat LH-I-9/AFP-10250C/, and anti-rat LH-S-II/AFP-C697071/). Serum testosterone levels were determined by the Coat-a-Count RIA kit from Diagnostic Products (Los Angeles).

Mathematical Analysis of the Binding Data. Specific ligand binding capacities and affinities were calculated by the Ligand-PC computerized curve-fitting program of Munson and Rodbard (22) as modified by McPherson (23). To determine the types of receptor binding, dissociation constants (K_d values), and the maximal binding capacity of receptors (B_{max}), LH-RH binding data were also analyzed by the Scatchard method (24). Statistical significance was assessed by Duncan's new multiple range test (25).

RESULTS

Characteristics of [^{125}I][D-Trp^6]LH-RH Binding in Untreated Rat Pituitaries. Analyses of the typical displacement of radiolabeled [D-Trp^6]LH-RH by the same unlabeled peptide revealed that the one-site model provided the best fit, indicating the presence of one class of high-affinity LH-RH receptors in membranes of rat pituitaries. The computerized nonlinear curve fitting and the Scatchard plot analyses of the binding data demonstrated that the high-affinity binding sites had a mean (±SE) K_d of 7.46 ± 0.89 nM with a mean maximal binding capacity (±SE) (B_{max}) of 1197.1 ± 30.5 fmol per mg of membrane protein (Fig. 1). The pituitary membranes used were from control untreated rats. Binding of [^{125}I][D-Trp^6]LH-RH was specific, reversible, and linearly related to protein concentration of rat pituitary membrane (data not shown). The specificity was demonstrated by the observation that buserelin and Cetrorelix completely displaced [^{125}I][D-Trp^6]LH-RH, but none of the structurally or functionally unrelated peptides tested (such as epidermal growth factor, somatostatin 14, human growth hormone-releasing hormone, and gastrin-releasing peptide) could inhibit the binding of [^{125}I][D-Trp^6]LH-RH at concentrations as high as 1 μM.

Measurement of Total and Free LH-RH Receptors Using *in Vitro* Desaturation. To determine whether the treatment with Cetrorelix can affect the concentration of measurable LH-RH binding sites, we applied an *in vitro* method to desaturate LH-RH receptors by chaotropic agents. Using this technique, we could estimate the total number of LH-RH receptors. Thus, not only the measurable free receptors, but all the occupied and unoccupied binding sites that remain after treatment with LH-RH antagonist were detected. Of all the chaotropic agents

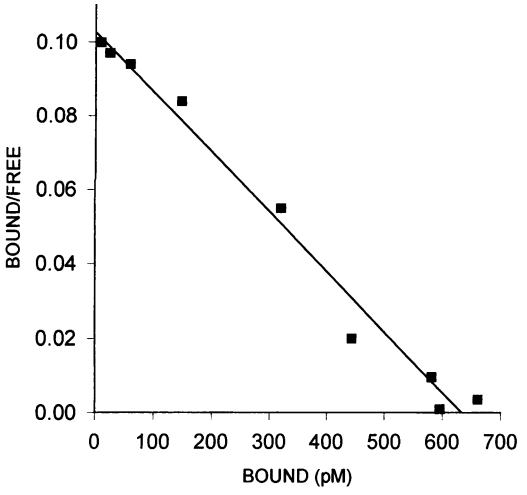

FIG. 1. Representative example of Scatchard plots of [^{125}I][D-Trp^6]LH-RH binding to the membrane fraction isolated from untreated rat pituitaries. Specific binding was determined as described. Each point represents mean of triplicate determinations.

tested, 0.2 M $MnCl_2$ and 0.1 M NH_4SCN were the most effective for both desaturation and rebinding. The use of 0.1 M $MgCl_2$ was nearly equally as effective as these agents for dissociation, but it was not suitable for rebinding. In our experiments, CF_3COONa was the least effective for desaturation studies. Generally, 0.5 M or higher concentrations of all agents were not suitable, since they cause damage to LH-RH receptors and, as a result, the binding sites were not able to rebind [^{125}I][D-Trp^6]LH-RH. In our experiments, we found that the percentage of occupied LH-RH binding sites 1, 3, and 6 h after administration of Cetrorelix was approximately 28%, 14%, and 10%, respectively, of total receptors. At later time intervals, we could not detect occupied LH-RH binding sites, indicating that the concentrations of measurable free receptors were identical with total LH-RH receptor levels. To check the validity of our results, pituitary membrane fractions of untreated rats were preincubated with 200 pM SB-75 *in vitro*. Treatment of these membranes with $MnCl_2$ or NH_4SCN was effective in removing bound Cetrorelix. This allowed a rebinding of [^{125}I][D-Trp^6]LH-RH to the receptors, which was similar in magnitude to that in membranes not exposed to a saturating concentration of Cetrorelix during the first incubation (Table 1).

Characteristics of LH-RH Binding in Rat Pituitaries After Cetrorelix Treatment. The evaluation of LH-RH receptors, following *in vitro* desaturation with chaotropic agents, showed that a single injection of Cetrorelix in a dose of 100 μg per rat, produced a radical decrease in the number of total LH-RH binding sites in a time-dependent manner, as compared to that in the untreated control group (Table 2). The lowest B_{max} values were found 3–6 h after Cetrorelix treatment, but the LH-RH receptors were still detectable. The concentration of receptors of LH-RH was down-regulated significantly ($P < 0.01$) for at least 72 h (Table 2). The time course of Cetrorelix-induced decrease in LH-RH receptor levels showed that a recovery in receptor number began within ≈24 h and continued for the period of the study. The concentration of receptors increased markedly at 48 h and the levels at 72 h were even higher. However, the number of receptors at 72 h was still significantly lower than those in controls. K_d values were not altered significantly, except that 6 h after the treatment there was a slight increase in affinity. Our results clearly demonstrate the down-regulation of LH-RH receptors and not merely an occupancy of LH-RH binding sites by LH-RH antagonist Cetrorelix.

Table 1. Effect of presaturation and dissociation with chaotropic agents on rebinding of [^{125}I][D-Trp^6]LH-RH to rat pituitary membrane receptors

Treatment	K_d, nM	B_{max}, fmol per mg of protein
Control (untreated)	7.46 ± 0.89	1197.1 ± 30.5
Cetrorelix (200 pM)	ND	ND
Cetrorelix (200 pM) then 0.2 M $MnCl_2$	6.13 ± 1.71	1043.3 ± 98.5
Cetrorelix (200 pM) then 0.1 M NH_4SCN	8.07 ± 1.42	1008.9 ± 123.6

Results are means ± SEM. Receptor binding studies, dissociation by chaotropes, and rebinding were performed as described. Preincubation with unlabeled Cetrorelix was performed at 4°C for 90 min. ND, not detectable.

Serum LH and Testosterone Levels. The fall in LH and sex steroid levels after administration of LH-RH antagonists was previously demonstrated by us and other investigators (5, 7, 8, 14, 15). However, we thought that it would be important to examine the timing of the fall in LH and testosterone levels following Cetrorelix administration under the conditions of our experiment aimed at receptors. Serum LH and testosterone levels in rats injected with Cetrorelix are shown in Table 3. Serum LH levels were significantly suppressed by Cetrorelix for 72 h, as compared to the control group (0.93 ± 0.18 ng/ml). The maximal suppression was seen 3 and 6 h after injection, with nadir values of 0.20 ± 0.04 ng/ml and 0.20 ± 0.06, respectively. Forty-eight hours after Cetrorelix administration, LH concentrations increased slightly and reached ≈50% of the control value. Serum testosterone levels were also significantly ($P < 0.01$) suppressed after administration of Cetrorelix. Twenty-four hours after injection of 100 μg of Cetrorelix, serum testosterone fell below the detection limit of the RIA (0.20 ng/ml). Testosterone levels then increased to 2.55 ± 0.32 ng/ml at 48 h and remained in that range for at least another 24 h. However, 72 h after Cetrorelix administration, serum LH and testosterone were still significantly below normal levels. The patterns of the decline and the recovery of the pituitary receptors for LH-RH and serum LH levels are shown in Fig. 2.

DISCUSSION

LH-RH antagonists were originally designed and synthesized for contraception (3, 4). However, a variety of experimental work and, more recently, clinical studies indicate that LH-RH

Table 2. Characteristics of total LH-RH receptors on anterior pituitary membranes in control rats and in animals injected with LH-RH antagonist Cetrorelix (100 μg per rat; s.c.) evaluated before (time 0) and at several time periods after peptide administration and after *in vitro* desaturation

Time, h	K_d, nM	B_{max}, fmol per mg of protein
0	7.46 ± 0.89	1197.1 ± 30.5
1	6.28 ± 1.45	619.4 ± 32.9†
3	5.06 ± 0.71	193.5 ± 2.9†
6	3.89 ± 0.50*	203.2 ± 18.9†
24	4.99 ± 0.62	310.3 ± 36.5†
48	5.61 ± 1.30	567.1 ± 125.7†
72	6.26 ± 0.01	837.7 ± 41.5*

Results are means ± SEM. Binding characteristics were determined from 12-point ligand competition experiments in triplicate tubes using [^{125}I][D-Trp^6]LH-RH as radioligand in two or three independent experiments. There were 10–15 rats per group. Results were obtained after *in vitro* desaturation of Cetrorelix from its receptors by chaotropic agents ($MnCl_2$, NH_4SCN). Control (time 0) values for K_d and B_{max} were obtained before treatment with Cetrorelix.
*$P < 0.05$.
†$P < 0.01$.

Table 3. Serum levels of LH and testosterone in control rats and in animals injected with LH-RH antagonist Cetrorelix (100 μg per rat; s.c.) evaluated before (time 0) and at several time periods after Cetrorelix administration

Time, h	LH, ng/ml	Testosterone, ng/ml
0	0.93 ± 0.18	5.69 ± 0.74
1	0.27 ± 0.06†	4.11 ± 0.21†
3	0.20 ± 0.04†	0.77 ± 0.15†
6	0.20 ± 0.06†	0.26 ± 0.05†
24	0.26 ± 0.09†	ND
48	0.49 ± 0.03*	2.55 ± 0.32†
72	0.46 ± 0.07*	2.34 ± 0.17†

Results are means ± SEM. Control (time 0) values were obtained before the treatment with Cetrorelix. ND, not detectable.
*$P < 0.05$.
†$P < 0.01$.

antagonists can be used for the treatment of sex steroid-dependent or -sensitive tumors and other conditions in which inhibition of steroid secretion is desirable (3, 4, 6, 8, 26–29). The use of antagonistic analogs of LH-RH would avoid transient stimulation of the release of gonadotropins and sex steroids, which occurs initially in response to LH-RH agonists, thus preventing the temporary clinical flare-up of the disease (4, 27). Among these antagonists, Cetrorelix has been shown to be one of the most powerful (5, 9). LH-RH antagonists act on the same receptor sites as LH-RH and produce an immediate inhibition of the release of LH, FSH, and sex steroids (3, 4, 6, 7, 9, 14, 15, 26, 29). The mode of action of LH-RH antagonists varies in some aspects from that of the agonists inasmuch as down-regulation of receptors is not required for an inhibitory effect, which is immediate, due to occupancy of receptors (12, 30). Nevertheless, recent evidence from our laboratory showed that prolonged treatment with modern LH-RH antagonist Cetrorelix causes a marked down-regulation of pituitary LH-RH receptors in rats (12, 14, 15). Those results are in agreement with findings made in other laboratories (13). This down-regulation of pituitary LH-RH receptors is reversible and a complete recovery occurs 2–5 months after cessation of treatment (14, 15). Previous studies which did not show that this down-regulation occurs, were done with early, weak antagonists and/or injected in inadequate doses (31). Clinical studies in normal subjects and patients with benign prostate hyperplasia (BPH) and prostate cancer show protracted suppression of LH and testosterone levels by Cetrorelix and

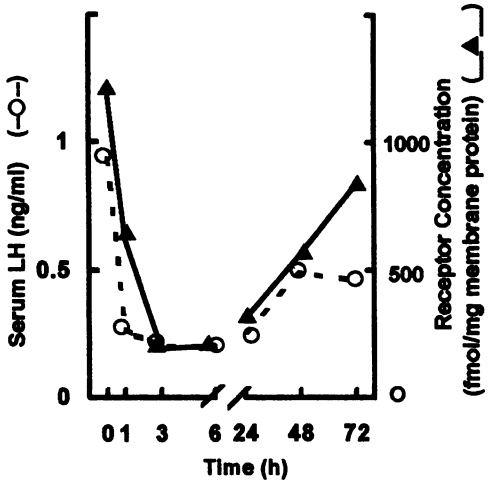

FIG. 2. Time course of serum LH Levels (○) and pituitary LH-RH receptor concentrations (▲) in rats, evaluated before (time 0) and at several time periods after administration of Cetrorelix. SEMs were omitted for the sake of clarity in both curves.

suggest that the receptor down-regulation could also occur in men (4, 27–29).

In the present study, we were able to demonstrate a clear down-regulation of rat pituitary LH-RH receptors after a single administration of Cetrorelix in a dose of 100 μg/rat. Using an *in vitro* method to desaturate Cetrorelix from its receptors by chaotropic agents we could show a major, time-dependent decrease in the concentration of receptors and not merely an occupancy of LH-RH binding sites by LH-RH antagonist Cetrorelix. Utilizing of such techniques allows the estimation of total LH-RH receptor concentration and not only of measurable free receptors. Both the occupied and unoccupied LH-RH binding sites that remain after treatment with the antagonist can be detected after desaturation. Our results showed that the percentage of rat pituitary LH-RH receptors reversibly occupied by Cetrorelix is 28% after 1 h, decreasing to 10% after 6 h. At later time intervals, the receptors occupied by Cetrorelix cannot be detected.

The mechanism by which chaotropic agents act to dissociate LH-RH antagonist from its specific binding sites is not clear at present. Several lines of evidence indicate that chaotropic ions act by decreasing water structure (32). Since hydrophobic interactions are a major factor stabilizing biological membranes, chaotropic ions tend to destabilize cell membranes and enzyme complexes as a result of their interaction with the water molecule (16). It is also possible that these agents lead to diminution of protein–protein interactions and a reversible denaturation of the membrane protein receptors.

This study also revealed that the down-regulation of pituitary LH-RH receptors induced by Cetrorelix in the first 24 h after administration is accompanied by the fall in serum LH and testosterone levels. The reduction in LH levels clearly paralleled a decrease in receptor numbers (Fig. 2). At later time periods a recovery occurs in receptor concentrations and serum hormone levels and both rise markedly. However, normal levels of receptors and serum LH and testosterone are not achieved for more than 72 h. Our results indicate that administration of potent LH-RH antagonists like Cetrorelix to rats produces a marked down-regulation of receptors for LH-RH in pituitary and not merely an occupancy of LH-RH binding sites. A similar down-regulation may occur also in men (4, 27, 28). The down-regulation of pituitary LH-RH receptors by LH-RH antagonists in other species and the internalization of bound antagonist–receptor complex need further investigation. In conclusion, the treatment with LH-RH antagonist, similarly to that with the LH-RH agonists, appears to lead to down-regulation of pituitary LH-RH receptors, contrary to previous views (33). These phenomena might have clinical relevance for planning therapy with LH-RH antagonists.

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