Effects of a depot formulation of the GnRH agonist leuprolrelin on the ultrastructure of male rat pituitary gonadotropes *

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Summary. To clarify the acute and chronic effects of GnRH agonists on pituitary gonadotropes, changes in the ultrastructure of male rat gonadotropes were examined immunocytochemically and morphometrically after the administration of a one-month depot formulation of the GnRH agonist, leuprolrelin. Immediately after the depot administration, the relative amounts of secretory granules drastically decreased in gonadotropes concomitantly with a marked increase in the plasma LH level. After the acute hyperstimulated phase, secretory granules in gonadotropes were gradually restored although the newly synthesized granules were less densely immunolabeled for LHβ; their relative amounts and sizes were still significantly smaller than the controls after depot treatment for 28 days. Eighty-four days after the leuprolrelin depot administration, however, the ultrastructural characteristics of pituitary gonadotropes appeared to recover as observed in controls: there were no significant differences in the relative amounts, sizes, and labeling densities for LHβ of secretory granules, and the amounts of chromogranin A (CgA) and secretogranin II (SgII) were restored in secretory granules to control levels. When the rats were repeatedly treated with the leuprolrelin depot at intervals of 4 weeks, the expression and intracellular storage levels of gonadotropins remained highly suppressed, judging from the labeling density for LHβ. These findings suggest that the depot formulation of the GnRH agonist could suppress both the biosynthesis and release of gonadotropins for a month by synergistically depleting the intracellular storage of secretory granules at the onset of the treatment and by inducing the subsequent desensitization of the GnRH receptor signaling.

Introduction

Mammalian reproduction is regulated by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH), synthesized in hypothalamic neurons, is released into the hypothalamo-hypophysial portal system and then stimulates pituitary gonadotropes to synthesize and secrete gonadotropins by binding to the corresponding GnRH receptor(s) expressed on these cells (McArdle et al., 2002; Pawson and McNeilly, 2005; Cheng and Leung, 2005). Gonadotropins released from pituitary gonadotropes in turn regulate the gametogenesis and biosynthesis of sex-steroids in gonads of both sexes.

Since GnRH plays a pivotal role in the regulation of the pituitary-gonadal axis, a variety of GnRH analogues, both agonists and antagonists, have been developed by substituting its amino acid residues (Conn and
Crowley, 1994; Millar et al., 2004). Some of these GnRH analogues have been applied for the treatment of sex-steroid-dependent diseases such as endometriosis, uterine leiomyoma, prepubertal maturation, and metastatic prostate cancer (Barbieri, 1992). Of note, the depot formulation of GnRH agonists is used clinically for medical castration to block the proliferation of prostate cancer (Hellerstedt and Pienta, 2002). To date, however, there has been only limited information on the ultrastructural changes in pituitary gonadotropes under the chronic influence of GnRH agonists although these cells are regarded as a main target of GnRH agonists.

Pituitary gonadotropes synthesize and secrete two distinct peptide hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Ultrastructurally, gonadotropes possess two discernible subsets of secretory granules, each of which exclusively contains two distinct granin proteins besides gonadotropins: one containing chromogranin A (CgA) and other containing secretogranin II (SgII) (Watanabe et al., 1991). Granins are widely distributed in a variety of peptide-producing endocrine tissues, such as the adrenal medulla, endocrine pancreas, and pituitary gland, in which they are specifically localized to secretory granules (Wiedemann and Huttner, 1989; Winkler and Fischer-Colbrie, 1992). Our previous studies have also described the changes in the expression and localization of these two granins in gonadotropes under various experimental conditions (Watanabe et al., 1993, 1998a, b; Jeziorkowski et al., 1997). Besides the physiological roles of granins in gonadotropes, the examination of the expression and the intracellular localization of these two granins may be a useful tool to evaluate the status of gonadotropes under experimental conditions (Crawford and McNeilly, 2002; Crawford et al., 2002; McNeilly et al., 2003).

The present study aimed to clarify the acute and chronic effects of GnRH agonists on pituitary gonadotropes by examining the morphological changes of rat gonadotropes after administration of a depot formulation of a representative GnRH agonist, leuprolrel. For this purpose, we analyzed the intracellular localizations of gonadotropins and granins within gonadotropes, using immunocytochemical and morphometrical methods. The results enable a discussion of the influence of the GnRH agonist on gonadotropes in both acute and chronic phases, which will provide a cellular basis for the clinical application of the GnRH agonist depot formulation.

Materials and Methods

Antisera

Gonadotropes were identified with a polyclonal antiserum (rabbit origin) against the ovine LHβ-subunit (oLH β; kindly provided by Dr. Matozaki, Gunma University). Rabbit polyclonal antisera against C-terminal fragments of rat chromogranin A (CgA-C) and secretogranin II (SgII-C) were previously characterized by immunoblotting and adequate immunocytochemical preadsorption tests (Sakai et al., 2003).

An Alexa Fluor 488-labeled goat anti-rabbit IgG antibody was purchased from Molecular Probes (Eugene, OR, USA). Colloidal gold particles conjugated to secondary antibodies were purchased from British Biocell International (Cardiff, UK).

Animals and experimental procedures

One hundred and twenty adult male Wistar rats (body weight: ca. 200 g) were divided into eight experimental groups (15 rats each) and kept in plastic cages placed in a well-ventilated room (temperature 23 ± 1°C; relative humidity 55–65%) with ad libitum access to food and water. These animals were maintained and used in accordance with the Guide for Care and Use of Laboratory Animals of ILAR (National Research Council of USA 1996) under the permission of the experimental animal welfare committee of Asahikawa Medical College (permission number: #03009).

At 8 weeks of age, rats of seven groups (Groups B–H) were subcutaneously injected with 3 mg/kg of a GnRH agonist depot (leuprolrel; leuprolide acetate; Takeda Pharmaceutical Co., Osaka) for one-month depot suspension (3.75 mg/ml). These rats were maintained for a further 1 day (group B), 4 days (group C), 7 days (group D), 14 days (group E), 28 days (group F), or 84 days (group G), and were then used for experiments as described below. The rats of group H repeatedly received the same dosage of the leuprolrel depot at intervals of 28 days and were then used for experiments 84 days after the first administration. The rats of group A received only a suspension vehicle and were used directly for experiments as controls.

Tissue and blood plasma preparation for biochemical analyses

After the rats were anesthetized with pentobarbital (25 mg/kg; i.p.), blood was collected into tubes containing heparin (100 units per tube) by puncturing the inferior
vena cava (n = 5 per each experimental group). The blood samples were then centrifuged at 4°C for 10 min, and the plasma of each sample was divided into aliquots and stored at −20°C until assay.

For immunoblotting, pituitaries were excised from the same animals immediately after blood sampling. Tissue samples were then quickly frozen in liquid nitrogen and stored at −70°C until used. Testes were also excised from each rat, and their wet weights were measured for evaluating the effects of the GnRH agonist depot.

**Measurement of plasma LH concentration**

Plasma concentrations of LH were measured using a commercially available enzyme-immunoassay kit (rat luteinizing hormone (rLH) EIA assay system; code: RPA 2562; Amersham Biosciences Corp., Piscataway, NJ), according to the manufacturer’s instructions. The amount of LH in the sample was read from a standard curve and expressed in ng of the standard preparation of rat LH provided by the supplier.

**Immunofluorescence microscopy**

Five rats from each experimental group were anesthetized with pentobarbital (25 mg/kg; i.p.), then perfused with physiological saline, followed by a solution of 4% paraformaldehyde (PA) in a 0.1 M phosphate buffer (PB, pH 7.4) containing 4% sucrose. After fixation by perfusion, pituitaries were immersed in the same fixative for 2 h at 4°C. After washing thoroughly with 0.1 M PB containing 7.5% sucrose, the tissue samples were dehydrated in graded ethanol and embedded in epoxy resin (Epon 812). Serial sections of 0.5 μm thickness were then cut with an ultramicrotome and mounted on microscopic slides.

After removal of the resin by sodium methoxide (Grube and Kusumoto, 1986), the semi-thin sections were incubated with 2% normal goat serum (30 min, 20°C) and then with antisera against LHβ (12 h, 4°C). For the second antibody reaction, the sections were incubated for 1 h at 20°C with Alexa Fluor 488-labeled goat anti-rabbit IgG. Coverslips were then mounted in 90% glycerol (vol/vol in PBS) containing 0.1% p-phenylenediamine dihydrochloride (Sigma Chemical Co, USA). Antibodies were diluted in 0.01 M PB/0.15 M saline (pH 7.4) containing 0.05% Tween 20, and the sections were thoroughly washed between each step in 0.01 M PB/0.5 M saline (pH 7.4) containing 0.1% Tween 20. Stained sections were observed with a fluorescence microscope (Olympus, Tokyo).

**Immunoelectron microscopy**

Tissue preparation for immunoelectron microscopy was performed as described in our previous study (Sakai et al., 2005). Briefly, five rats from each experimental group were anesthetized and perfused with physiological saline, followed by a solution of 0.05% glutaraldehyde (GA)/4% PA in 0.1 M PB (pH 7.4) containing 4% sucrose. After fixation by perfusion, pituitaries were immersed in the same fixative (2 h, 4°C), cut with a vibratome, and then post-fixed with 0.2% OsO4 in 0.1M PB containing 7.5% sucrose for 1 h at 4°C. After post-fixation, the tissue sections were dehydrated in 70% ethanol containing 1% phosphotungstic acid (Wako Pure Chemicals, Osaka; 3 times for 20 min, 4°C). These fixed and dehydrated tissue sections were then infiltrated into pure LR White resin monomer (London Resin Co., Hampshire, UK; three times for 20 min, 4°C) and polymerized at the bottom of gelatin capsules filled with fresh LR White resin monomer for 24 h at 60°C.

Immunogold labeling of pituitary tissues embedded in LR White resin was performed as previously described (Sakai et al., 2003, 2005). Briefly, ultra-thin sections from the LR white-embedded tissues were directly incubated with 5% non-immune goat serum for blocking (20 min, 20°C), and subsequently incubated with the primary antisera for 12 h at 4°C as follows: goat anti-LHβ (diluted 1:4000), anti-CgA (1:500), and anti-SgII (1:1000). For double immunostaining, the two-face technique by Bendayan (1982) was applied: one face of the ultrathin sections was immunostained with the anti-CgA or anti-SgII antisera, and subsequently the other face was immunostained with the anti-LHβ antiserum. Intracellular localization of granins and LHβ were distinguished by labeling with different sizes of colloidal gold particles (size of particles: 5, 10, and 15 nm in diameter) conjugated to goat anti-rabbit IgG. Between each step, the sections on grids were washed three times in 0.02 M Tris-HCl buffered 0.5 M saline, pH 8.2, containing 0.1% BSA.

Following the immunoreactions, the sections were contrasted with saturated aqueous solutions of uranyl acetate and lead citrate and examined with an electron microscope (JEM-1010, JEOL, Tokyo).

**Morphometrical analysis**

After immunostaining with the anti-LHβ antiserum, the cytoplasmic areas of pituitary gonadotropes were randomly photographed at a magnification of x12,000 (n = 24 photographs per experimental group). The profile areas of the cytoplasm and secretory granules of the gonadotropes in each photograph were individually determined.
Fig. 1. Changes in the immunoreactivity for LHβ in the anterior pituitary of male rats receiving a one-month leuprolrelin depot. Figures A to H correspond to the experimental groups A to H, respectively. All of the semi-thin sections were simultaneously immunostained and photographed under identical conditions. Bar = 20 μm.
Fig. 2. Effects of the leuprolilin depot on the ultrastructure and immunoreactivity for LHβ in pituitary gonadotropes. The sections were simultaneously immunolabeled with an identical dilution (1:4000) of the antiserum against LHβ (size of immunogold particles: 15nm). Figures A to H correspond to the experimental groups A to H, respectively. Bar = 500 nm
measured with NIH Image software (written by W. Rasband, National Institutes of Health, USA), and the volume densities of secretory granules to cytoplasm were calculated by dividing the sum of profile areas of secretory granules with the total profile area of the corresponding cytoplasm. Diameters of each secretory granule observed in the photographed fields were simultaneously measured with the software and mean diameters of secretory granules of each experimental group were calculated.

To evaluate relative labeling densities of immunogold particles on secretory granules, the number of immunogold particles on secretory granules was counted, and then their numbers were divided by the corresponding total profile area of secretory granules in the observed fields.

These morphometrical analyses were also performed for the ultra-thin sections immunostained for CgA or SgII.

**Immunoblot analysis**

The pituitaries obtained from each rat (n = 5 per experimental group) were individually homogenized with 1 ml of 0.01 M Hepes/NaOH (pH 7.4), containing 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, 10 μg/ml leupeptin (Peptide Institute Inc., Osaka), 10 μg/ml pepstatin (Peptide Institute Inc.), and 0.4 mM p-amidino-phenyl methanesulfonyl fluoride (Wako Pure Chemicals). Homogenized samples from each rat were individually stored on ice for 1 hr and centrifuged at 10,000 g for 20 min at 4°C to remove debris.

These tissue extracts from each rat were loaded onto 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes according to the method described by Towbin et al. (1979). The membranes were soaked in PBS containing 5% BSA (Sigma Chemical Co.) to block nonspecific binding and then incubated with antisera against rat granins (dilutions of antisera; 1:2000). Immunodetection was carried out with a chemiluminescent ECL kit (Amersham Biosciences Corp.,) according to the manufacturer's protocol. The relative amounts of these granins were evaluated by densitometry. The electrophoresis and immunoblot detection were independently repeated three times to confirm the results.

**Statistical analysis**

The results were statistically analyzed with KaleidaGraph software (version 3.6; Synergy Software, PA, USA) by one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test when a significant interaction (p<0.05) was calculated.

| Table 1. Plasma concentrations of immunoreactive (ir-) LH and wet weights of a testis during the treatment with the leuprolelin depot. |
|---|---|---|---|
| Group | Days after the depot administration | Plasma ir-LH (ng/ml) | Testis wet weight (g) |
| A | no treatment | 5.57 ± 0.21 | 0.91 ± 0.17 |
| B | 1 d | 20.39 ± 3.60*** | 1.10 ± 0.01 |
| C | 4 d | 6.73 ± 0.83 | 0.75 ± 0.03 |
| D | 7 d | 3.18 ± 0.21 | 0.61 ± 0.02 |
| E | 14 d | 3.30 ± 0.40 | 0.55 ± 0.01* |
| F | 28 d | 3.24 ± 0.20 | 0.41 ± 0.05** |
| G | 84 d (single dosage) | 3.06 ± 0.15 | 1.02 ± 0.07 |
| H | 84 d (repeated dosage) | 3.17 ± 0.17 | 0.41 ± 0.05** |

Legend: Both plasma ir-LH levels and wet weights of the testes significantly fluctuated during the leuprolelin depot treatment (Mean ± SEM; p<0.001, ANOVA). Values that are significantly different from the controls (group A) are indicated with asterisks (*p < 0.05, **p < 0.01 and ***p < 0.001, Tukey's HSD post-hoc test)
Results

Plasma LH levels and testis weights

To assess the effects of the one-month depot formulation of leuprolin on the release of gonadotropins, the circulating LH levels and weights of testes excised from rats were measured (Table I).

The levels of immunoreactive LH (ir-LH) in the blood plasma measured by an EIA kit were markedly increased after 1 day of the treatment (group B, p<0.001 versus non-treated control rats, group A), indicating that leuprolin rapidly acts as a strong agonist for GnRH receptor (Okada et al., 1991; Plosker and Brogden, 1994). The plasma ir-LH levels promptly decreased with 7 days of the treatment (group D) and remained below the basal level after treatment for 28 days (groups E-F). The wet weight of testes decreased gradually after the depot administration and significantly fell by about 45.2% on day 28 of treatment (p<0.01), suggesting that the effects of gonadotropins on the target organ were suppressed for 4 weeks by the leuprolin depot administrated.

After the period described above, the weights of testes recovered to the control levels when the rats did not receive any additional depot, and no significant difference in the weights was found between group A (control) and group G (84 days after a single dosage of the one-month leuprolin depot). In contrast, the weights of testes from rats that repeatedly received the same dosage of the depot every 4 weeks (group H) remained significantly lower compared with those of the control rats (p<0.01). However, no significant elevation in the plasma ir-LH levels of the rats of group G, as compared with group H, was detected with the EIA kit used in the present study. Although the precise cause of the discrepancy between the bioactivity and the ir-LH level could not be determined, at the least, the secretion of bioactive gonadotropins in the animals of group G appeared to have recovered, judging from the changes in testis weights. In contrast, the secretion of bioactive gonadotropins of rats in group H, which repeatedly received the leuprolin one-month depot, was continuously suppressed for 12 weeks from the first administration.

Immunofluorescence microscopy

Concomitant with the surge in the plasma ir-LH level at the onset of the leuprolin depot treatment, the immunoreactivity for LHβ in the anterior pituitary was remarkably reduced (Fig. 1B), indicating that a large amount of stored gonadotropins was released in response to leuprolin. In these hyperstimulated gonadotropes, a

Fig. 3. Changes in the volume densities (A), mean diameters (B), and relative labeling densities for LH β (C) of secretory granules in gonadotropes (Mean ± SEM). There were significant fluctuations in morphometrical parameters during the treatment with the leuprolin depot (ANOVA, p < 0.05), and the results of the subsequent post-hoc test (Tukey’s HSD test) are described in the Results.
faint immunoreactivity for LHβ was observed but was restricted to the periphery of the cells (Fig. 1B). Even after this acute stimulated state, the immunoreactivity for LHβ was not restored in gonadotropes by 28 days of the treatment, suggesting that the sustained desensitization of the GnRH receptor caused by the leuprolrelin depot results in the suppression of gonadotropin biosynthesis — as previously reported (Murase et al., 2005). The immunoreactivity for LHβ remained faint by 84 days of the treatment, when the leuprolrelin depot was repeatedly administrated every 4 weeks (Fig. 1H). In contrast, the immunoreactivity for LHβ was restored within gonadotropes by 84 days of the treatment when the rats did not receive any additional depot (Fig. 1G).

**Ultrastructural changes in gonadotropes**

At the electron microscopic level, most secretory granules within gonadotropes were translocated to the periphery of gonadotropes immediately after the depot administration (Fig. 2B). Such peripheral localization of secretory granules was continuously observed after
Fig. 5. Intracellular localizations of SgII in gonadotropes during the leuprolrelin depot treatment; control (A and E), at days 28 (B) and 84 (C and F) of the treatment with a single dosage of the depot, and at day 84 (D and G) of the treatment with the repeated dosage of the depot (group H). The localizations of SgII and LHβ in A–D were respectively visualized with immunogold particles of 15 and 5 nm in diameter, while those of CgA and SgII in E–G were respectively visualized with immunogold particles of 15 and 10 nm. Intermediate granules, as seen in Figure 4, are indicated with arrowheads. Bars = 100 nm.
treatment for 7 days (Fig. 2C, D). After this period, secretory granules were also gradually restored in the central area of gonadotropes (Fig. 2E, F) although the sizes of the granules were apparently smaller than those of controls. When the rats did not receive any additional depot, the density, size, and distribution of secretory granules seemed to recover after treatment for 84 days to the level seen in the control rats (Fig. 2G). Although the individual sizes of granules appeared smaller than those of the controls, the relative amount of secretory granules appeared to be partially restored after treatment for 84 days, even when the rats repeatedly received the depot every 4 weeks (Fig. 2H).

The ultrastructural changes described above were confirmed by morphometrical analyses (Fig. 3). Cytoplasmic volume density (Vv) of secretory granules within gonadotropes significantly decreased immediately after the leuprolelin depot administration and reached the lowest level after treatment for 7 days (Fig. 3A). Then the Vv of secretory granules gradually increased to the control level by 84 days of the treatment, when the rats received a single dose of the depot (group G). The Vv of secretory granules of group H, where the rats repeatedly received the depot, was also significantly increased from the lowest level seen at the onset of the depot treatment (p<0.05, compared with groups C and D), but it was still lower compared with the controls (not statistically significant).

The changes in the mean diameter of secretory granules were similar to those in the Vv described above; the diameter of secretory granules decreased immediately after the depot administration, reached the lowest level after treatment for 7 days, and then gradually increased to the control level (Fig. 3B).

The changes in the relative labeling density for LHβ in secretory granules, however, showed patterns distinct from those in the Vv and diameter; the relative labeling density for LHβ did not decrease immediately in response to the leuprolelin depot but fell with a delay of a few days (Fig. 3C). Then the labeling density for LHβ remained significantly lower than the controls after treatment for 28 days (p<0.05). After this period, the relative labeling density for the LHβ of group G was restored to the control level, whereas that of group H remained significantly lower than the controls (p<0.001), suggesting that the relative amounts of gonadotropins within secretory granules were not restored under the influence of leuprolelin.

**Intracellular localization of CgA and Sg II**

As we previously reported (Watanabe et al., 1991, 1998a; Jeziorowski et al., 1997), two representative granins, CgA and SgII, are localized separately to large- and small-sized secretory granules in male rat gonadotropes, respectively (Fig. 4A, 5A, E). As expected from the observations described above, secretory granules immunolabeled for CgA and SgII were drastically decreased in gonadotropes at the onset of the treatment with the leuprolelin depot (data not shown).

After the acute treatment phase with the depot, immunogold particles indicative of CgA and SgII could be observed in gonadotropes although the relative amounts and sizes of the CgA-containing granules appeared much lower compared with the controls (Fig. 4B). Based on the morphometrical analyses, the Vv (Fig. 6A) and diameter (Fig. 6C) of CgA-positive secretory granules were significantly lower at day 28 of the treatment than those of the controls (p<0.001, both of Vv and diameter).

Although the relative amounts of SgII-containing secretory granules decreased in response to leuprolelin, the individual sizes of SgII-containing secretory granules were considerably constant (Fig. 5B); no significant difference in the diameter of SgII-positive secretory granules was found between the gonadotropes at day 28 of treatment and the controls (Fig. 6D), while the Vv of SgII-containing granules was significantly lower than that of the controls at the same time point (p<0.001, Fig. 6B).

After a single depot administration, secretory granules containing CgA and/or SgII were restored within the gonadotropes after 84 days, as seen in the controls (Fig. 4C, 5C); no significant differences in the Vvs of CgA- (Fig. 6A) and SgII- (Fig. 6B) positive secretory granules were found between groups A and G. The individual sizes of SgII-positive secretory granules appeared to be unchanged, while the sizes of CgA-positive granules were significantly increased after 84 days of treatment and were similar to the controls. Some of the CgA-positive large-sized granules contained a core-like structure (Fig. 4C; arrowheads) that was immunolabeled with anti-SgII (Fig. 5C, E; arrowheads), suggesting that they are the intermediate type of secretory granules we previously reported in male gonadotropes stimulated by castration or a single administration of GnRHI (Watanabe et al., 1993). Since these large-sized intermediate granules might be counted as "SgII-positive" ones, the mean diameter of the SgII-positive secretory granules of group G could be overestimated (Fig. 6D). Besides these occasionally observed, intermediate granules most secretory granules within the gonadotropes contained either CgA or SgII (Fig. 5F).

In contrast to group G, the Vvs of CgA- and SgII-positive granules of group H remained significantly smaller compared with the controls (p<0.001, Fig. 6A, B). The diameter of CgA-positive granules in group H
was significantly smaller compared with the controls (p<0.001), whereas that of SgII-positive ones did not significantly differ from those of both controls and rats at day 28 of the treatment (Fig. 6C, D).

Relative amounts of CgA and SgII in the pituitary gland

The changes in the relative amounts of both CgA and SgII evaluated by immunoblotting seemed to be well correlated with the morphological findings described above; the relative amounts of both CgA and SgII promptly decreased to the lowest levels within a week in response to leuprolelin administration and were then gradually restored in the pituitary (Fig. 7). At day 84 of treatment, the relative amounts of CgA and SgII in the pituitary extracts of rats which received only a single dose of the depot (group G) were fully restored to the control levels, whereas those of rats which repeatedly received the depot every 4 weeks (group H) were lower than the control levels.
Fig. 7. Changes in the relative amounts of CgA and SgII in the pituitary extracts. Representative blots (A), which were immunostained with anti-CgA (upper panel) and anti-SgII (lower panel) antisera, and the changes in the relative densities evaluated by densitometry (CgA [B]; SgII [C]), are shown. Equal amounts of proteins (10 μg lane) extracted from the pituitary glands were applied, and SDS-PAGE and immunoblotting analyses were independently repeated three times. The relative densities of each blot are expressed by assuming the average of the control rats (group A) as 100% (Mean ± SEM, n = 3).
Discussion

Since many diseases that originate from genital organs are possibly aggravated by gonadal androgens or estrogens, various ways to block the action of sex steroids have been considered as putative treatments for such diseases (Barbieri, 1992). In the urological field, the proliferation of prostate cancer cells could be suppressed in most cases if the action of intrinsic androgens were completely blocked or depleted (Hellerstedt and Pienta, 2002). Although surgical castration is the most direct way to deplete intrinsic gonadal androgens, GnRH analogues have been also used as an alternative "medical castration" to avoid surgical risks.

Among various analogues, GnRH agonists, not antagonists, were first used clinically — although they stimulate the secretion of pituitary gonadotropins at the onset of the treatment. Paradoxically to their own action, the sustained exposure of GnRH agonists to the pituitary gonadotropins causes desensitization of the surface GnRH receptors, resulting in an effective blocking of the intracellular signaling pathways for the biosynthesis and secretion of gonadotropins (Conn and Crowley, 1994; Millar et al., 2004).

Although the precise mechanisms underlying the desensitization of the GnRH receptors should be further investigated, depot formulations that continuously release GnRH agonists for months have been developed for clinical use (Okada et al., 1991; Goldspiel and Kohler, 1991). Biochemical analyses with these GnRH agonist depots have demonstrated that the prolonged desensitization of the GnRH receptor induced by the depot causes suppression of both the expression (Murase et al., 2005) and the release (Rajfer et al., 1984; Okada et al., 1991; Murase et al., 2005) of gonadotropins although the ultrastructural changes of functionally altered gonadotropes have not been described to date.

Our present study with a GnRH agonist depot demonstrated that the volume density of secretory granules immunolabeled for LHβ drastically decreased after the depot administration (14.0% of the control level at day 4 of treatment) and that this occurred concomitantly with a marked increase in the plasma ir-LH levels. These findings are consistent with the changes in the plasma concentration (Okada et al., 1991; Murase et al., 2005) and the pituitary storage (Murase et al., 2005) levels of gonadotropins previously reported. After the acute stimulated phase described above, secretory granules in gonadotropes were gradually restored although their relative amounts were still significantly lower than the controls after 28 days of the depot treatment.

These gradually restored granules were less densely immunolabeled with the anti-LHβ antiserum (55.0 and 44.2% of the controls at days 7 and 28, respectively), suggesting that the newly synthesized secretory granules are not sufficiently filled with gonadotropins. Although we could not immunocytochemically examine the effect of leuprolrelin on the pituitary storage of FSH in the present study, a biochemical study clearly demonstrated that the administration of the depot formulation of leuprolrelin results in a significant decrease of FSH concentration in the pituitary gland (Sudo et al., 1990). These observations indicate that the agonists can deplete the intracellular storage of secretory granules filled with gonadotropins at the very onset of treatment and that the agonists can desensitize the GnRH receptor(s) by prolonged exposure, resulting in a suppressed biosynthesis of gonadotropin subunits. By the combination of these acute and chronic effects, smaller amounts of agonists effectively suppress the release of gonadotropins for longer periods compared with antagonists (Conn and Crowley, 1994).

Individual evaluation of the mean sizes of CgA-positive and SgII-positive secretory granules revealed that the size of the CgA-positive ones was more affected by the GnRH agonist treatment: the mean diameter of CgA-positive ones at day 28 of the treatment was 69.7% of the controls, while that of SgII-positive ones was 100.2% of the controls. These findings also indicate that the number of SgII positive secretory granules was altered in response to the GnRH agonist depot treatment, whereas their individual sizes were considerably constant without regard to the treatment. In contrast, both the number and individual sizes of CgA-positive ones were largely affected by the treatment — although the cause of this difference between CgA and SgII is unclear.

After the depot had completely released effective amounts of the GnRH agonist, the ultrastructure of pituitary gonadotropes almost returned to that of controls and no significant differences in the morphometrical parameters — except for the mean diameter of SgII-positive granules — were found between groups A and G in the present study. A similar reversibility in the ultrastructure of gonadotropes has also been reported in the canine pituitary after cessation of a chronic administration of a GnRH agonist (Dubé et al., 1987). In addition to these ultrastructural properties, the storage levels of two representative granins, CgA and SgII, were fully restored in the pituitary gonadotropes 84 days after receiving a single dosage of the leuprolrelin depot. Although the intermediate type of secretory granules was occasionally observed, these findings suggest that the effects of the depot formulation of the GnRH agonist, leuprolrelin, are practically reversible after a single dosage.
In summary, our present study demonstrates an ultrastructural basis for the acute and chronic effects of a GnRH agonist depot on the pituitary gonadotrope. In addition, the reversibility of the effects of the GnRH agonist on gonadotropes was morphologically demonstrated. Although we have mainly described the changes in secretory granules within pituitary gonadotropes in the present study, the ultrastructure and biochemical properties of other cell organelles, especially the Golgi apparatus and rER upstream of the secretory granules, are possibly affected by the administration of the depot formulation of leuprolin. Since the GnRH analogues drastically influence the hypothalamic-pituitary-gonadal axis, secondary effects of leuprolin on the rest of the pituitary endocrine cells should be also analyzed. Further studies by combining biochemical and morphological analyses on the actions of GnRH analogues would provide better pharmacological regimens for the treatment of sex-steroid-dependent diseases.

Acknowledgments
We are grateful to Takeda Pharmaceutical Co. for a generous supply of the depot formulation of leuprolin.

References


