

TOPOGRAPHICAL LOCALIZATION OF THE RECEPTORS FOR LUTEINIZING HORMONE-RELEASING HORMONE ON THE SURFACE OF DISSOCIATED PITUITARY CELLS

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ABSTRACT

A derivative of the hypothalamic peptide luteinizing hormone-releasing hormone (LHRH) has been coupled to ferritin and the conjugate purified by gel chromatography. In its ability to stimulate the secretion of luteinizing hormone from pituitary cells *in vitro*, the conjugate has the same potency and specificity as the native peptide. When dissociated pituitary cells maintained in short-term culture are lightly fixed with formaldehyde and then incubated with the conjugate, examination in the electron microscope shows an even distribution of ferritin particles over the free cell surface of the gonadotrophin cells. This binding appears to be specific for the LHRH receptor since it is prevented by a 10-fold excess of native peptide. In addition to the gonadotrophin cells, some somatotrophin and thyrotrophin cells bind conjugate on their free surfaces under similar conditions.

If living cells are incubated with the conjugate for 15 min, the bound conjugate becomes aggregated and then concentrated in one localized area of the cell surface. In this area, which lies immediately above the juxtannuclear Golgi complex, the plasma membrane is frequently invaginated in a manner which suggests that the bound, aggregated conjugate is internalized by endocytosis.

KEY WORDS pituitary · LHRH · hormone receptor · cell surface · endocytosis

Studies on the interaction between peptide and polypeptide hormones and their cell surface receptors have indicated the quantitative nature of the interaction in several systems (4, 5, 13). The topographical distribution of hormone receptors has, however, been less well studied, and little is known of the spatial relationships and redistributive events which may occur as a consequence of a hormone binding to its surface receptor. Studies of the effects of multivalent ligands on the redistribution of lymphocyte plasma membrane components serve to illustrate the plasticity of cell surface pro-

teins (19, 23), while an increasing number of reports suggest that the availability and the location of hormone receptors may be subject to a rapid and profound change primarily as a result of the receptor population binding hormone (4, 9, 11). Approaches employing hormone probes which are both biologically active and identifiable with a microscope are available (17), and these allow this question to be examined directly. In the work reported here, we have developed an analogue of the hypothalamic peptide hormone luteinizing hormone-releasing hormone (LHRH) as a labeled probe. This hormone is a specific secretagogue for gonadotrophin secretion in the anterior pituitary (21), and we have modified it for use in conjunc-

tion with dissociated pituitary cells to localize its specific receptor sites on the cell surface. We have established that the LHRH receptors are predominantly, and probably exclusively, located on the pituitary cell surface and demonstrated that, before their interaction with the peptide, these receptors are evenly distributed over the surfaces of the gonadotrophin cells. We have also found that when conjugate is applied to living cells, a redistribution of the binding sites occurs so that the even distribution observed in fixed cells is replaced by an aggregation and preferential concentration at one pole of the cell. At this pole the concentrated conjugate is apparently lost both by sloughing into the surrounding medium and by endocytosis into the cell.

MATERIALS AND METHODS

Poly-L-lysine (type II mol wt 1,000–4,000) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, U. K. Culture media were obtained from Gibco Bio-cult Ltd., Paisley, U. K.; ferritin from Miles Laboratories, Slough, U. K.; Biogel A-5M and P2 from Bio-Rad Laboratories, St. Albans, Herts, U. K.; and hemocyanin (keyhole limpet) from Calbiochem Ltd., Hereford, U. K. ^{125}I (carrier-free 100 mCi/ml) was obtained from the Radiochemical Centre, Amersham, U. K. All other reagents were of the highest analytical grade obtainable.

The competitive inhibitor des histidine² (des-his²) D-alanine⁶ (D-ala⁶) LHRH proethylamide was kindly provided by Dr. Roger Guillemin (Salk Institute, La Jolla, Calif.).

Methods

PREPARATION OF *p*-GLUTAMIC ACID-HISTIDINE-TRYPTOPHAN-SERINE-TYROSINE-D-HS-LYSINE-ARGININE ETHYLAMIDE: The peptide used in the present work was prepared by step-wise elongation from L-proline ethylamide to give the pentapeptide Z-tyrosine(Bu^t)-D-lysine(Boc)-leucine-arginine-proethylamide.¹ The benzyloxycarbonyl group was removed by hydrogenolysis and the dipeptide azide Z-tryptophan-serine N₃ added to give a heptapeptide. After hydrogenation the dipeptide *p*-glutamic acid-histidine N₃ was added in a similar manner and the protected nonapeptide purified by chromatography upon Sephadex LH-20. The two side chain protecting groups were removed from tyrosine and lysine by treatment with trifluoroacetic acid-water (9:1). Purification by partition chromatography on Sephadex G-25 with an *n*-butanol-

¹ Abbreviations used in this paper: Boc, t-butyloxycarbonyl; Bu^t, t-butyl; HS, hemisuccinate; and Z, benzyloxycarbonyl.

acetic acid-pyridine water system (5:1:1:5) was followed by gel chromatography on Sephadex G-25 in 1 M acetic acid. The product was homogeneous as judged by paper electrophoresis at pH 2.1, 6.5, or 8.9, by thin-layer chromatography on silica plates in *n*-butanol-acetic acid-water (4:1:5) and by amino-acid analysis.

Preparation of the hemisuccinate of the nonapeptide was carried out in anhydrous pyridine with a threefold excess of 1:4 ^{14}C succinic anhydride at 4°C. Formation of the hemisuccinate derivative of the epsilon amino group of the lysine residue was followed by paper electrophoresis at pH 6.5 until only the less basic radioactive product was observed. This was isolated by gel chromatography upon Sephadex G-25 in 1 M acetic acid.

COUPLING THE LHRH PEPTIDE TO FERRITIN: Ferritin (6 × crystallized, cadmium-free) was further purified by recrystallization according to Breese and Hsu (3). Conjugation of the peptide (0.16 μM) to the purified ferritin (0.014 μM) was carried out using 10-fold excess 1-ethyl-3-(3-dimethyl-amino-propyl)carbodiimide in 1 ml of H₂O at 4°C for 3–4 days. After conjugation, the reaction mixture was applied to 30 × 2.5 cm column of Biogel A-5M in 0.4 M phosphate buffer pH 7.2. The proportion of ferritin to peptide was indicated by the spectrophotometric estimation (A310) of ferritin and radioimmunoassay of the peptide (detailed below). In the total conjugate there was a 10% incorporation of peptide. In the pooled fractions used in the experiments described, the ratio of peptide to ferritin was 1:1. For use with cells, the conjugate was diluted initially with double-strength culture medium and thereafter accordingly in single-strength medium.

CELL DISSOCIATION: Pituitaries from male, 200 g Sprague-Dawley rats were removed and dissociated by the method of Hymer et al. (16). The cell suspension produced was freed from fine cell debris by centrifugation through 4% bovine serum albumin, resuspended in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal calf serum and plated out in aliquots of 4 × 10⁶ cells in 2.5-cm diameter petri dishes coated with poly-L-lysine (14). The cells were incubated for 48 h at 37°C for them to become optimally responsive to LHRH stimulation (14) and were then rinsed three times in DMEM containing 0.1% gelatin (DMEM/gel) before being fixed or treated with conjugate as described below. For bioassay, 48-h-incubated cells were similarly rinsed in DMEM/gel before being incubated for 1 h within 1 ml DMEM/gel (control) with additions as indicated in the appropriate legend. Dishes were used in triplicate and after the 1-h incubation the media were connected, centrifuged for 15 min at 3,000 g and then freeze-stored at -20°C until assay.

RADIOIMMUNOASSAY: LH was assayed by the N.I.A.M.D.D. homologous double antibody radioimmunoassay (22). N.I.A.M.D.D. rat LH-RP-1 was used as standard and goat antirabbit Fab₁ as precipitating antibody. The assay had a sensitivity of 2.65 ± 0.34 (SD) ng N.I.A.M.D.D. rat LH RP-1.

D-Lysine⁶ (D-lys⁶) LHRH analogues were radioimmunoassayed by using a protocol similar to that employed for LH and employing an antiserum raised in rabbit against a D-lys⁶ LHRH hemisuccinate-hemocyanin conjugate prepared in the same manner as the D-lys⁶ LHRH-ferritin conjugate. Iodinated antigen was prepared by the method of Greenwood et al. (10) modified so that the chloramine-T oxidation step was extended to 15 s. Free ¹²⁵I was removed by gel chromatography on Biogel P₂. The assay has a sensitivity of 1 ng/ml. Displacement of D-[¹²⁵I]lys⁶ LHRH by D-lys⁶ LHRH and the D-lys⁶ LHRH-ferritin conjugate produced identical curves, and it was therefore concluded that the free and conjugated peptides were immunologically similar.

INCUBATION WITH CONJUGATE AND FIXATION FOR ELECTRON MICROSCOPY: Several kinds of fixation protocol were employed. In procedures in which living cells were incubated with conjugate, the incubation was followed by five 1-min rinses in DMEM/gel at 5°C and then fixation in Karnovsky fluid (18) diluted 1:1 with DMEM/gel. Alternatively, cells prefixed for 2 min in 0.1% formaldehyde in 0.1 M cacodylate buffer at pH 7.2 were used. These prefixed cells were then rinsed 3 × 2 min in PBS containing 1 μg/ml gelatin and 0.25 μg/ml borohydride to remove and inactivate unreacted aldehyde (20). Thereafter, they were incubated with conjugate, rinsed in DMEM/gel at 5°C, and fixed in Karnovsky fluid diluted with DMEM/gel as for living cells.

Fixed cells were all finally scraped from the dish surface with a polythene policeman and processed as described by Hopkins and Farquhar (15). In order to increase the contrast of the ferritin, ultrathin sections were stained on the grid according to the method of Ainsworth and Karnovsky (1).

RESULTS

Fig. 1 shows the profile obtained when the D-lys⁶ hemisuccinate conjugated to ferritin as described in Materials and Methods is chromatographed on Biogel A5M in 0.4 M phosphate buffer. Aliquots taken for bioassay and immunoassay indicate that bioactive and immunoactive peptide is present in the two peaks containing ferritin and in the peak corresponding to free D-lys⁶ LHRH hemisuccinate used as standard. Rechromatographing of fractions 75–80 which were subsequently used for binding studies showed no biological activity or immunoreactivity in the position expected for free peptide (Fig. 1 B).

Microscope examination of material from the first peak containing ferritin (fractions 35–40) by negative staining showed varying degrees of gross aggregation in the ferritin. In fractions 75–80, most of the ferritin micelles were clearly separated

and monomeric. The dose response curve derived using the ferritin conjugate from fractions 75–80 shows that, in terms of its ability to induce LH release from dissociated cells, the conjugate has a biological activity equal to that of D-lys⁶ LHRH and to that of the native peptide (Fig. 2). The half-maximal dose levels and the threshold dose levels are the same for peptides and conjugate at 2.5×10^{-8} M and 10^{-7} M, respectively. To explore the possibility that the observed biological activity of the conjugate was due to free peptide dissociated from the conjugate during the bioassay incubation, 1 ml of conjugate was incubated for 6 h with 8×10^6 cells and then collected and rechromatographed on Biogel A-5M. Radioimmunoassay for D-lys⁶ LHRH in the eluent showed that most of the peptide immunoreactivity remained associated with the conjugate.

The specificity of the conjugate indicated by its ability to displace radiolabeled peptide from the dissociated cells will be the subject of a later publication. As shown in Fig. 3, however, the specificity of the ferritin-peptide conjugate for the LHRH receptor can be demonstrated by the influence of the des-His² D-ala⁶ proethylamide LHRH analogue on the LH releasing activity of the conjugate. This competitive inhibitor of LHRH (6.25) has the same effect, at the same dose level, on conjugate as it does on the native peptide.

Topographical Localization

Initially, dissociated cells incubated for 48 h postdissociation were used without prefixation. The cells were rinsed free of serum with three changes of DMEM/gel and then incubated with conjugate for 15 min at 20°C. The conjugate was used at a concentration equivalent to 10^{-7} M LHRH (as determined by its immunoreactivity) and was included in DMEM/gel. At the end of this incubation, the cells were given five 1-min rinses with DMEM/gel at 5°C in order to remove free, unbound conjugate and then fixed in dilute Karnovsky fluid for 15 min and processed for electron microscopy as described in Materials and Methods.

In preparations derived by using this protocol, the conjugate associated with the cells occurs as coarse aggregates and is usually preferentially localized in one area of the cell surface (Figs. 4 and 5). The aggregates are associated not only with gonadotrophin cells but also with other secretory cell types, most notably somatotrophin and thyro-

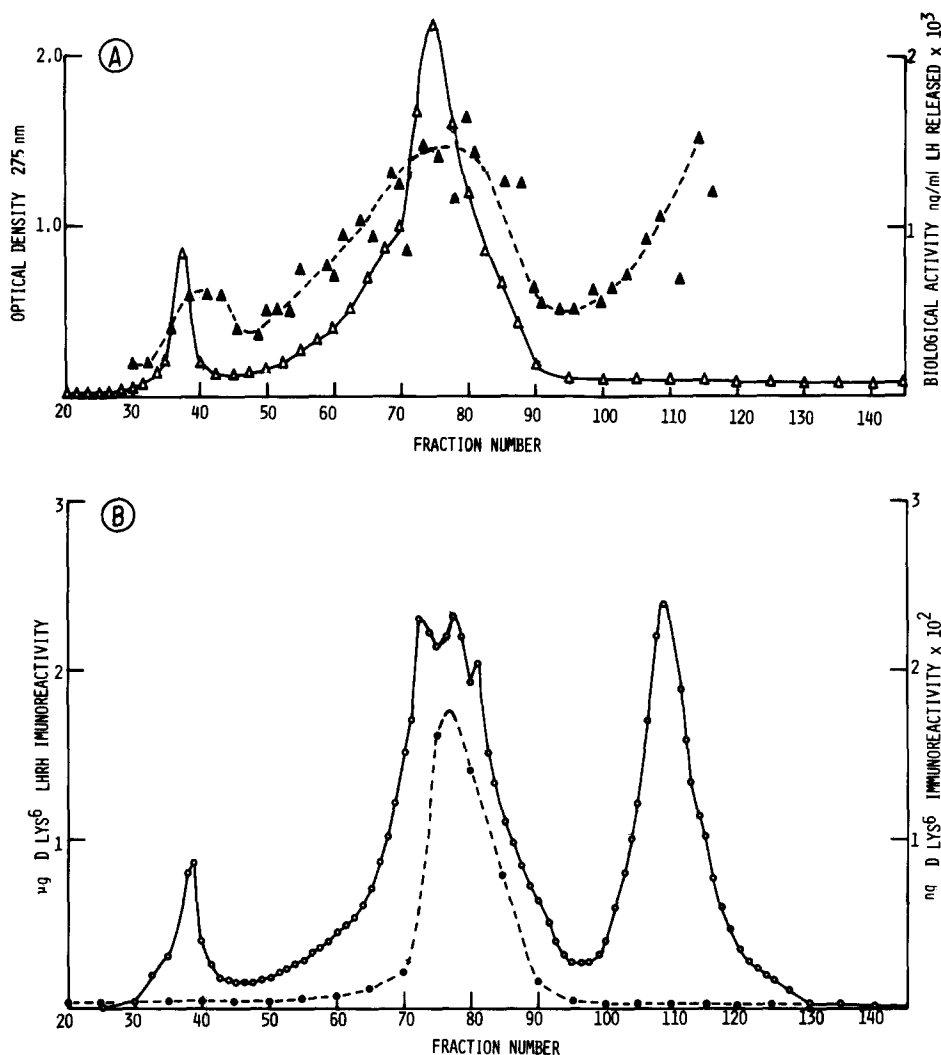


FIGURE 1 (A) OD profile (Δ - Δ) obtained by chromatographing ferritin-peptide conjugate on a 30×2.5 -cm column of Biogel A-5M in 0.4 M phosphate buffer. As indicated (\blacktriangle) each fraction was bioassayed for LH-releasing activity. Activity was present in the two main ferritin peaks and in the position of free peptide (in fractions 100-115+). (B) Profile obtained when fractions of the eluent shown in Fig. 1 A were tested for immunoreactivity using antiserum raised against a D-lys⁶ LHRH HS-hemocyanin conjugate. The distribution of the peptide as shown by immunoreactivity (\circ - \circ) is the same as that shown by bioassay. Also shown is the immunoreactive profile (\bullet - \bullet) obtained when an aliquot of pooled fractions 75-80 is rechromatographed. In this profile, there is no immunoreactivity in fractions 100-120, indicating that the conjugate in fractions 75-80 contains negligible free peptide.

trophin cells.² Occasionally, the accumulation of aggregates is very dense, and the surface layers

appear to be about to slough away. Free clumps of aggregate are occasionally seen in these preparations.

² In Figs. 4-11, identification of the various cell types is based primarily on the size of the secretory granules. The basis of this identification has been documented in detail elsewhere (8, 15).

In the area in which aggregated conjugate becomes concentrated, the plasma membrane is frequently invaginated. These invaginations always include aggregated conjugate, and below them in

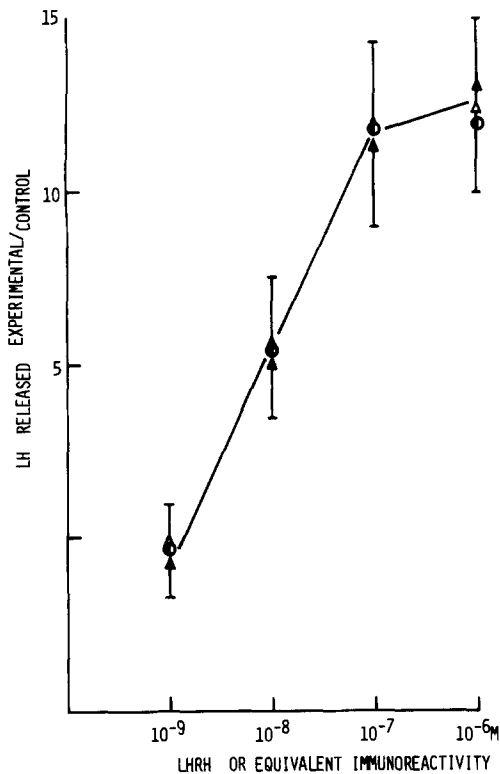


FIGURE 2 Dose response curve showing the LH-releasing activity of the LHRH native peptide (○—○), the D-lys⁶ (●—●) and D-lys⁶-HS analogues (△—△) and the D-lys⁶ HS-ferritin conjugate (▲—▲). All of these peptides and peptide derivatives have the same half-maximal (2×10^{-8} M) and maximal response ($\sim 10^{-7}$ M). The dissociated cells used in this bioassay were incubated for 48 h before being rinsed free of serum. Dishes in triplicate were then incubated for 1 h with either the peptide or peptide conjugates. Basal LH release 50 ± 6.4 ng/ 10^6 cells. Variation bars show the total SEM of all of the samples at any given peptide concentration.

the juxtanuclear Golgi area lysosome-like structures packed with conjugate are often present (Fig. 5). In these preparations, intracellular deposits of ferritin aggregates were also observed in the nonsecretory follicular cells (8). These aggregates were always contained within lysosome-like bodies and no binding of individual ferritin micelles to the surfaces of these cells was apparent.

In preparations preincubated for 30 min with 10^{-6} M LHRH and in which the conjugate incubation also included 10^{-6} LHRH, coarse aggregates are absent although particulate ferritin does occur both within the intercellular spaces between aggregated cells and on the free cell surface where

there are minor irregularities such as microvilli (Figs. 6 and 7).

From these observations it was concluded that the coarse aggregation of the ferritin conjugates is probably caused by a redistribution of conjugate after it has bound to the cell surface. Preliminary observations in which the incubation with conjugate was carried out at 5°C showed little evidence of aggregation. As an alternative approach and in order to examine the possibility that a redistribution of the LHRH receptors is induced by the binding of the ferritin conjugate, we lightly prefixed the cells with formaldehyde before adding the conjugate. Thus, cells attached to dishes and incubated for 48 h were rinsed three times with serum-free medium and fixed for 2 min in 0.1% formaldehyde in cacodylate buffer (pH 7.4). To remove and inactivate unreacted aldehyde, the cells were then rinsed 3×2 min in PBS containing $1 \mu\text{g/ml}$ gelatin and $0.25 \mu\text{g/ml}$ sodium borohydride (20). Thereafter, the cells were treated as before, the control pretreatment and incubation with 10^{-6} M LHRH being carried out on the prefixed cells.

The distribution of ferritin-LHRH conjugate on prefixed cells is illustrated in Figs. 8-11. The ferritin probe is distributed over the entire free surface of all gonadotrophin cells. Binding is also observed on the free surfaces of some somatotrophin and thyrotrophin cells. In these preparations there is little evidence of conjugate aggregation, and, where groups of two to three ferritin micelles occur on the cell surface, it is likely that they are derived from the minority population of aggregates seen in negatively stained preparations of the conjugate. To estimate the number of binding sites, we have scored groups of two or more ferritin micelles as one and counted the number bound per micromolar of free surface in a random selection of gonadotrophin cells. By assuming that a gonadotrophin cell is a sphere with an average diameter (D) of $13 \mu\text{m}$ (± 1.4) and an average section thickness of $0.7 \mu\text{m}$, we have taken the area of a sphere as $2\pi D^2$ and estimated the number of sites over the entire cell surface accordingly. The number of micelles bound/square micrometer of membrane in gonadotrophin cells is 32 ± 12 , giving an overall estimate of $\sim 30 \times 10^4$ per cell. In thyrotrophin and somatotrophin cells, the numbers are extremely variable, and we have observed cells with populations of micelles as dense as 3,000 and 2,800 per μm^2 , respectively. Binding of conjugate to follicular cells was not observed in

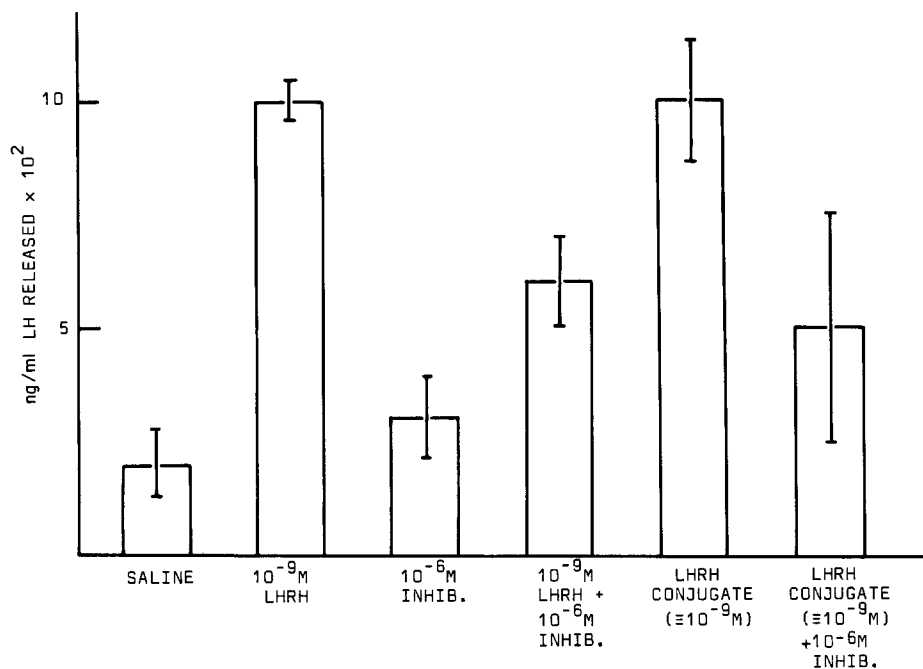


FIGURE 3 Effect of des-his² D-ala⁶ LHRH proethylamide on the ability of the native LHRH peptide and the D-lys⁶ LHRH-ferritin conjugate to release LH. Dissociated cells were plated out at 4×10^6 per dish, incubated for 48 h, rinsed free of serum and incubated in triplicate for 1 h as indicated. Variation bars indicate \pm SEM.

prefixed preparations. In control preparations of prefixed cells preincubated with 10^{-6} M LHRH, the distribution of ferritin conjugate is in all respects the same as that observed with unfixed cells that have similarly been preincubated with native peptide (Figs. 6 and 7).

To examine the possibility that the distribution of cell surface components may be altered in cells plated out on plasma-coated dishes, cells initially aliquoted onto uncoated dishes were also examined. In these preparations after a 48-h incubation fewer cells survive the initial rinsing procedure and, because there is considerable reaggregation, the amount of free surface available for examination is much reduced. Nevertheless, in cells from these preparations prefixed with 0.1% formaldehyde, the bound conjugate has an even distribution similar to that shown in Figs. 8 and 9.

DISCUSSION: The ability of the D-lys⁶ analogue of LHRH to retain its biological activity when it is conjugated to a macromolecular component via the epsilon amino group of the D-lys⁶ residue was initially shown by the studies of Amos et al. (2). These workers found that when conjugated to polyglutamic acid (average mol wt,

100,000) this peptide possessed significant LH-releasing activity in vivo. Our studies with D-lys⁶ LHRH proethylamide conjugated to ferritin are in agreement with this finding, although for a reason that as yet remains unclear we were unable to conjugate the peptide to the ferritin directly and an extended linkage provided by the succinylation of the amino group was therefore used. The potency of this ferritin conjugate together with the shape of its dose-response curve and relative degree of inhibition induced by the competitive inhibitor des-His² D-ala⁶ LHRH proethylamide (6, 25) all serve to demonstrate that the D-lys⁶ LHRH conjugate is interacting with the pituitary cells in a manner similar to that of the native peptide.

The difference in molecular size between the conjugate and the peptide allows the conjugate to be readily separated from the unconjugated peptide by gel chromatography, and it is thus most unlikely that any of the biological activity demonstrable with the fractionated conjugate can be attributed to contaminating peptide. In this context, it is worth noting that the potency of the conjugate and the nature of its interaction with the gonadotrophin cells as shown by bioassay provides strong

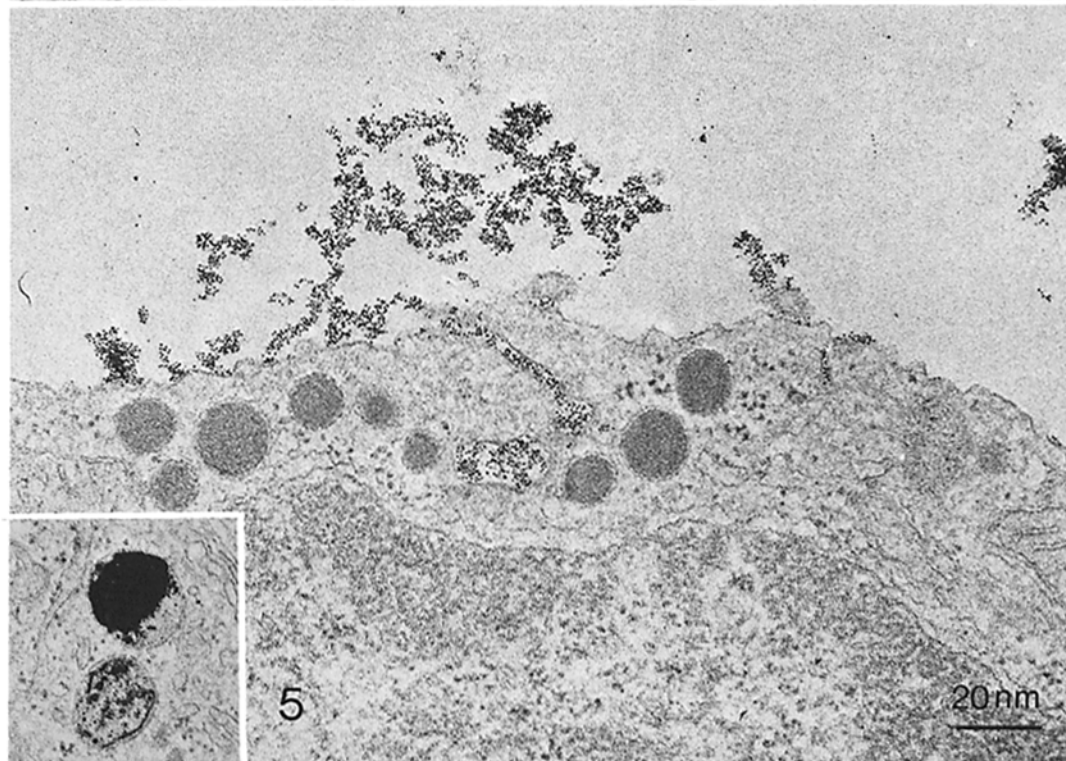
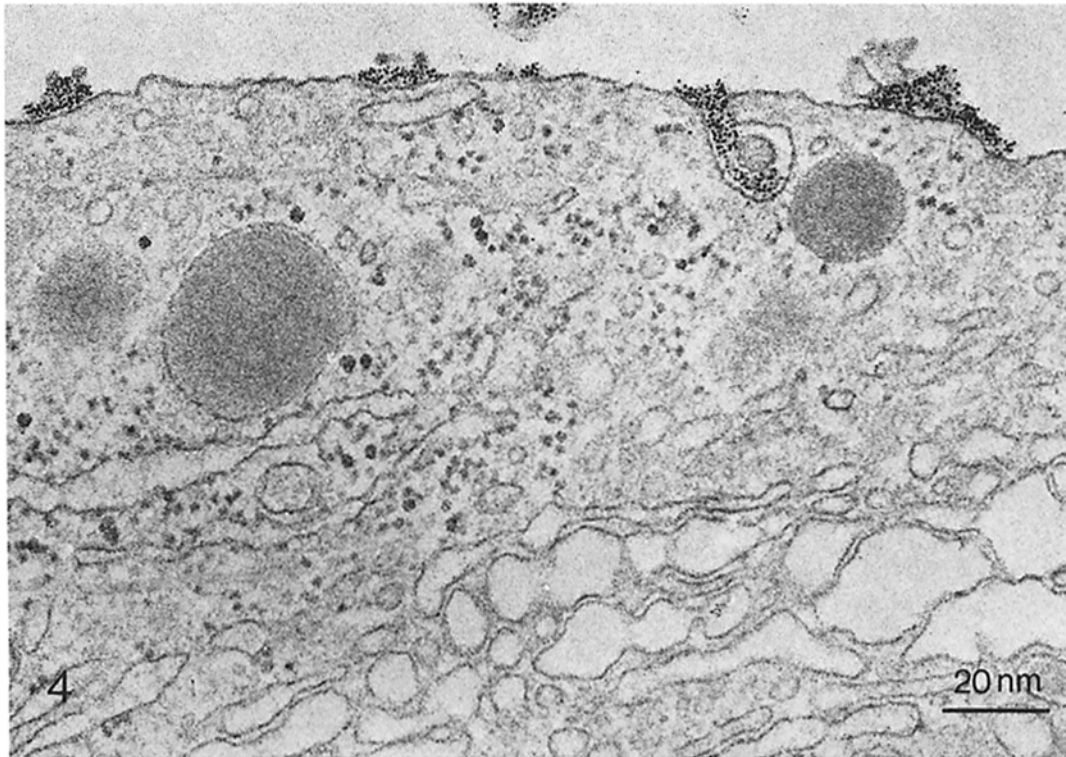
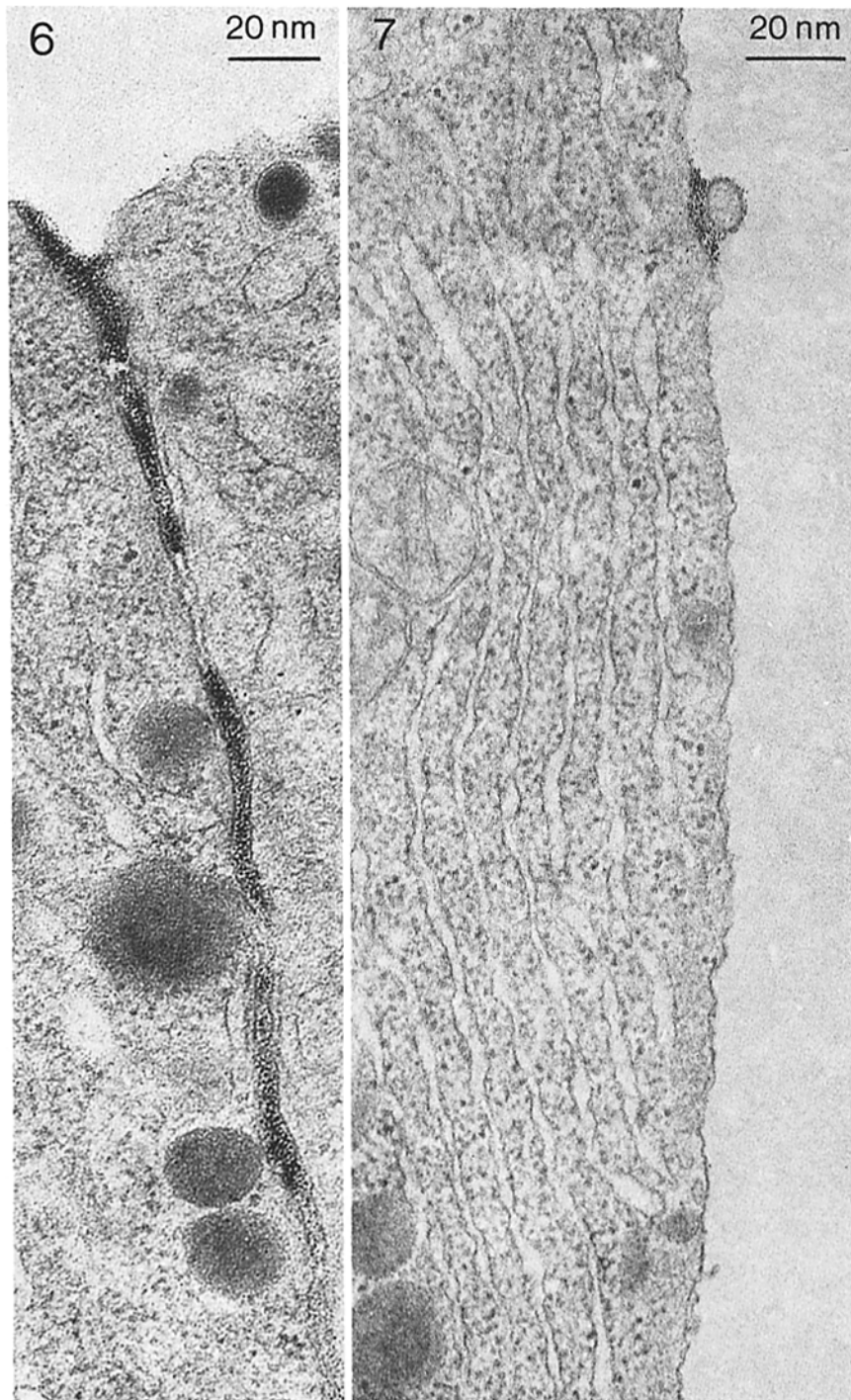
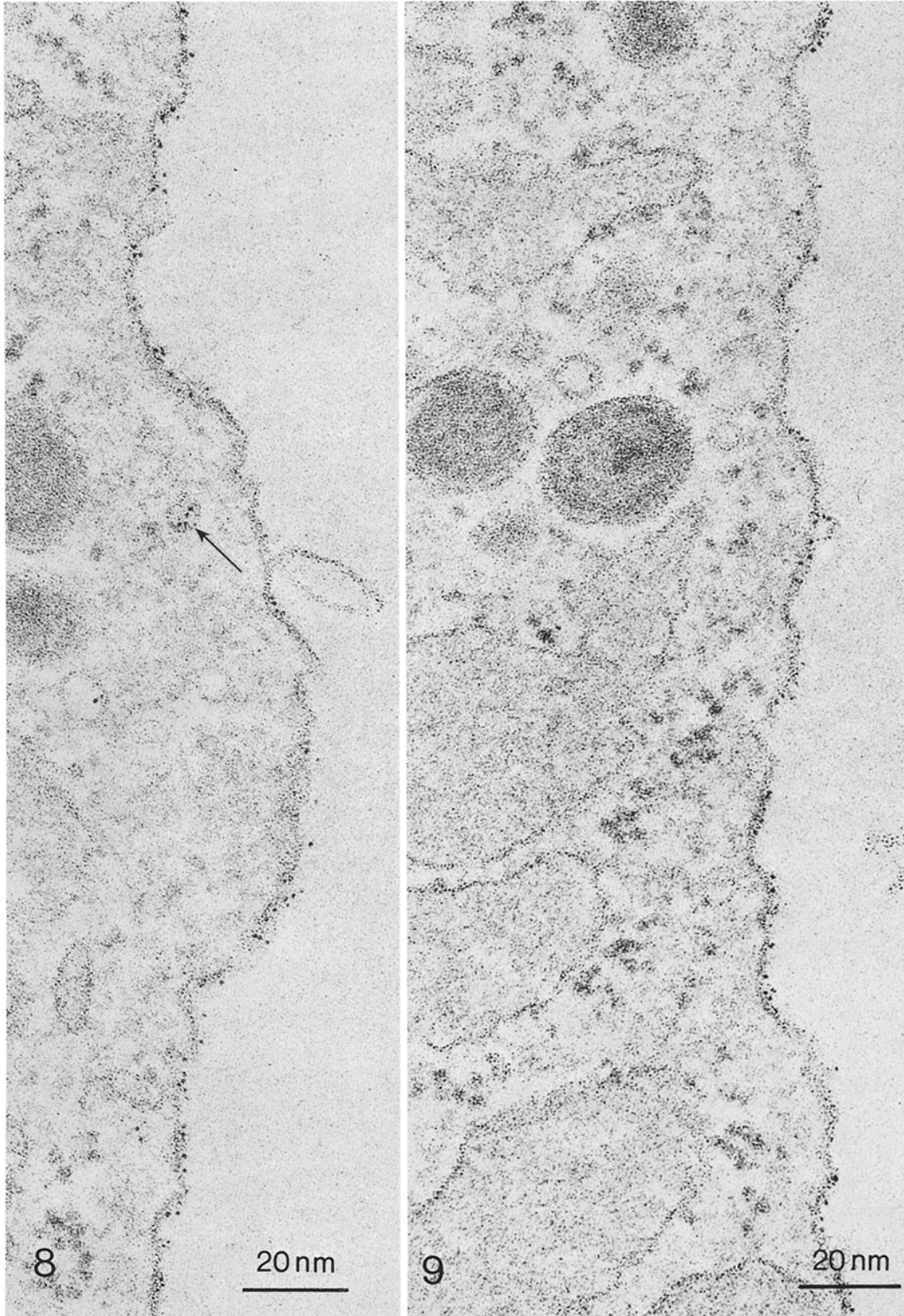


FIGURE 4 Periphery of a gonadotrophin cell incubated unfixed for 15 min with peptide-ferritin conjugate. The conjugate is aggregated into small focal concentrations. Invagination of the plasma membrane occurs immediately above the Golgi area. $\times 70,000$.

FIGURE 5 Periphery of a thyrotrophin cell incubated unfixed for 15 min with peptide-ferritin conjugate. The aggregated conjugate forms a thick, fragmentary cap in this area while the plasma membrane forms deep invaginations containing conjugate. $\times 55,000$. The *inset* shows lysosome-like structures containing conjugate; these were often observed in the vicinity of the Golgi complex in these preparations. $\times 23,500$.



FIGURES 6-7 Preparation incubated unfixd (15 min) with peptide-ferritin conjugate in the presence of 10^{-6} M LHRH after a 30-min preincubation with 10^{-6} M LHRH. Conjugate is confined to intercellular areas within aggregates (Fig. 6) and to surface irregularities such as folds and microvilli (Fig. 7). In these control preparations, ferritin particles are rarely found on the free surface. Fig. 6, $\times 60,000$. Fig. 7, $\times 65,000$.



FIGURES 8-9 Periphery of gonadotrophin cells prefixed in 0.1% formaldehyde before being incubated with the peptide-ferritin conjugate. The ferritin micelles are evenly distributed along the surface of the plasma membrane. The arrow indicates a vesicle-like structure containing ferritin; these profiles are uncommon; and since the cells are prefixed, they presumably represent indentations of the plasma membrane confluent with the extracellular space. Both electron micrographs, $\times 80,000$.

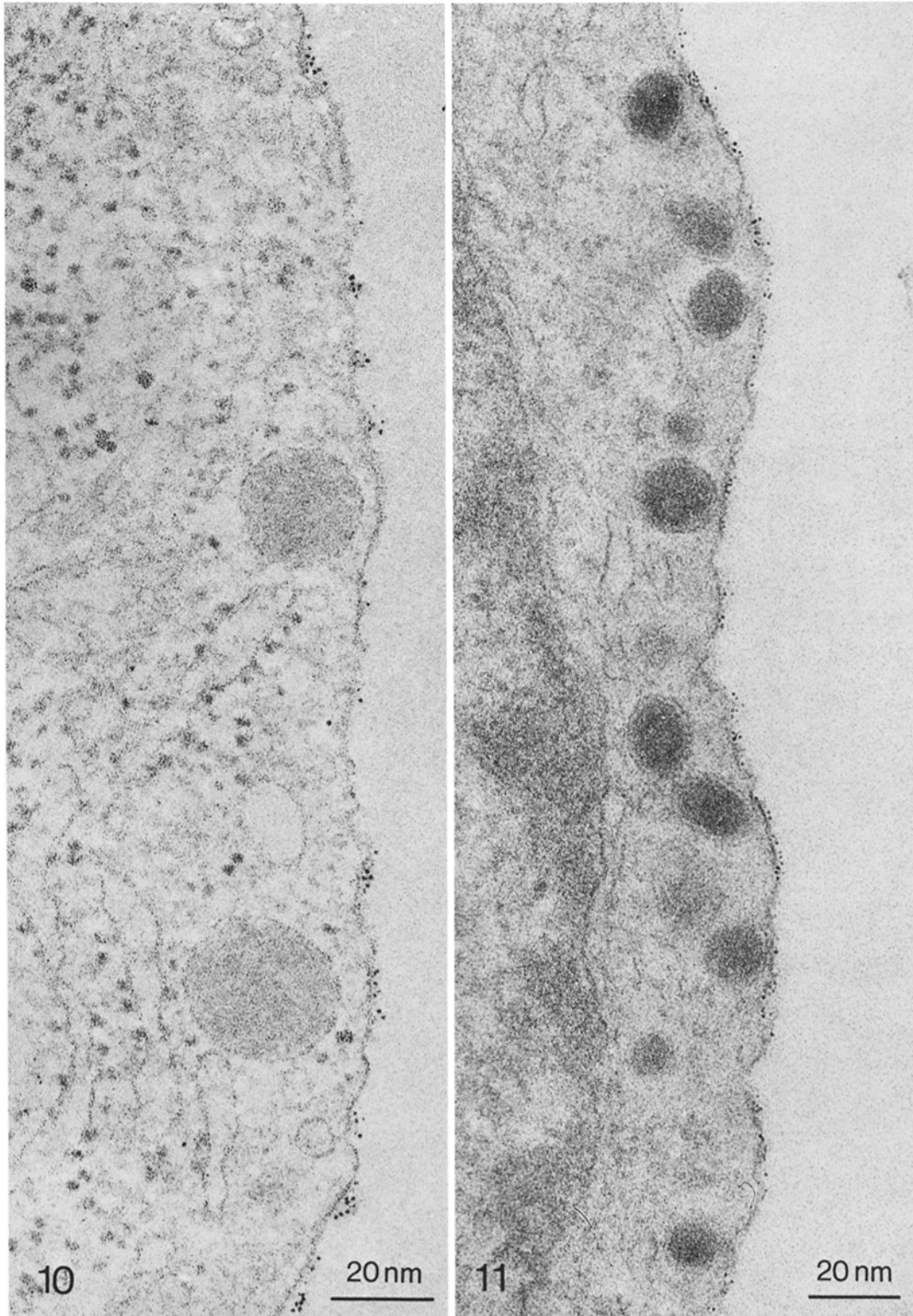


FIGURE 10 Periphery of a somatotrophin cell prefixed before being incubated with the peptide-ferritin conjugate. Small clusters of ferritin micelles (probably reflecting some aggregation in this conjugate preparation) are distributed at intervals along the cell surface. $\times 85,000$.

FIGURE 11 Periphery of a thyrotrophin cell prefixed before being incubated with the conjugate, and demonstrating the densest population of binding sites observed in this cell type. $\times 70,000$.

evidence for the exclusive location of LHRH receptors on the extracellular surface of the plasma membrane at a site which is directly accessible to this large (500,000 mol wt) macromolecular probe. The suggestion that an additional primary site of LHRH action may have an intracellular location (24) is thus not supported by our data. In purifying the conjugate by gel chromatography, it has not been possible to remove, entirely, free ferritin from the peptide-ferritin conjugate. However, the absence of conjugate on the free surface of prefixed cells incubated in the presence of a 10-fold excess of free peptide serves to demonstrate that in this system the nonspecific binding by both free ferritin and ferritin conjugate is negligible.

From the foregoing considerations, we conclude that the binding of ferritin conjugate observed on the free cell surfaces of prefixed preparations directly demonstrates the distribution of LHRH receptor sites on these cells. With the use of the conjugate with prefixed cells, it is clear that these receptor sites have a rather even distribution over the gonadotrophin cell surface. The effect of prefixation with aldehyde on the binding of LHRH has not been quantitatively evaluated, and thus for the time being any precise assessment of the number of receptors on each gonadotrophin cell must be tentative. Unfortunately, until a pure or enriched preparation of gonadotrophin cells is available a meaningful estimate of the number of receptor sites per gonadotrophin cell cannot be made by the usual averaging methods of quantitative binding assay. It is of interest to note, however, that the number of receptors on each gonadotrophin cell estimated in the present study is of the same order of magnitude as that reported for other peptide and polypeptide hormone receptors in other experimental systems (13).

The functional significance of the conjugate binding to secretory cells other than gonadotrophin cells is unclear. There is evidence from studies on thyrotrophin-releasing-hormone (TRH) that hypothalamic peptides may indeed have more than one type of target cell in the anterior pituitary, but for TRH there is also good physiological evidence that the peptide regulates the secretion of more than one kind of secretory cell (12). LHRH has not been similarly implicated in the regulation of either somatotrophin or thyrotrophin secretion. The binding of LHRH to cells other than gonadotrophin cells may, of course, be qualitatively different, and it may be that in this loca-

tion the peptide has a developmental influence rather than any direct, acute effect on secretory product release.

The binding of conjugate to the nonsecretory follicular cells observed in the postfixed preparations probably reflects the function of these cells as active phagocytes (8), and it is unlikely to be related to a specific LHRH-cell surface receptor interaction. This view is supported by the observation that in prefixed preparations the follicular cells did not bind conjugate on their free surface.

There is accumulating evidence to suggest that a prolonged hormone receptor interaction induces a redistribution and an eventual reduction in the receptor population specific for the hormone employed. Direct quantitative evidence in favor of this interpretation has been obtained in several systems (4, 9, 11), but for LHRH in particular the evidence remains circumstantial since it has thus far only been shown that a short-term exposure is sufficient to cause dissociated anterior pituitary cells to become refractory to further stimulation by the peptide (14).

The only direct information presently available on the spatial distribution of peptide hormone receptors comes from the recent study on the binding of α -melanocyte-stimulating hormone (MSH) to mouse melanoma cells (7, 26, 27). These studies indicate that the MSH receptor sites are initially distributed at one pole of the cell over the Golgi area and, although prefixation was not employed, since binding was carried out at low temperature, it seems unlikely that any redistribution of receptor site had occurred. In gonadotrophin cells, an aggregation and polar concentration of LHRH receptors clearly can occur, and it is perhaps worth noting that this redistribution takes place without any experimental interference with the subplasma-membrane organization of microtubules and microfilaments. Nevertheless, it remains to be established that these redistributive processes represent a normal consequence of LHRH binding to its receptors since a ligand bearing more than one binding site would also be expected to cross-link and aggregate receptor sites in a manner analogous to that observed in other cells treated with multivalent ligands (19, 23). With the peptide-ferritin probe used in the present study, one cannot exclude the possibility that a proportion of ferritin micelles in the conjugate bear more than one peptide. Studies intent on examining this aspect, using preparations pre-

treated with native peptide but in which the cells are prefixed before the addition of the conjugate, are in progress.

Studies with fluorescein-labeled or radio-labeled hormone probes are unlikely to allow any cross linkage between receptors. However, in the studies with fluorescein-labeled α -MSH (7) and, more recently, in those in which the binding of ^{125}I -epidermal growth factor to human fibroblasts has been followed (4), the evidence nevertheless suggests that receptors bearing hormone become internalized within hours of the binding taking place. The functional significance of this process is unclear, but it has been suggested that internalization of the receptor-hormone complex may serve to introduce the complex into the intracellular location at which its primary influence is directed. The profiles observed in the present study also indicate that receptor-bound LHRH can be internalized and transported into lysosome-like structures in the Golgi area. However, should this process of internalization in the gonadotrophin cell prove to be the normal consequence of LHRH binding, any consideration of its functional significance will have to be related to the knowledge that the most significant early consequence (within 1 min) of LHRH stimulation is LH release by exocytosis (14), a plasma membrane-related event which probably occurs over the entire cell surface.

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