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Simultaneous measurement of hormone release and secretagogue binding by individual pituitary cells

(luteinizing hormone/gonadotropin-releasing hormone/reverse hemolytic plaque assay/receptor autoradiography/pituitary gland)

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ABSTRACT **The quantitative relationship between receptor binding and hormone secretion at the single-cell level was investigated in the present study by combining a reverse hemolytic plaque assay for measurement of luteinizing hormone (LH) secretion from individual pituitary cells with an autoradiographic assay of ^{125}I-labeled gonadotropin-releasing hormone (GnRH) agonist binding to the same cells. In the plaque assay, LH secretion induces complement-mediated lysis of the LH-antibody-coated erythrocytes around the gonadotropes, resulting in areas of lysis (plaques). LH release from individual gonadotropes was quantified by comparing radioimmunoassayable LH release to hemolytic area in similarly treated cohort groups of cells; plaque area was linearly related to the amount of LH secreted. Receptor autoradiography was performed using ^{125}I-labeled GnRH-A (a superagonist analog of GnRH) both as the ligand and as the stimulant for LH release in the plaque assay; the developed silver grains appearing over cells in the center of plaques were measured microscopically. The grains appeared to represent specific and high-affinity receptors for GnRH because (*i*) no pituitary cells other than gonadotropes bound the labeled ligand and (*ii*) grain development was progressively inhibited by coincubation with increasing doses of unlabeled GnRH-A. Despite high correlations between mean grain number and mean plaque area in dose–response curves, the correlation coefficients for these parameters were low (range 0.02–0.38) in the individual cells comprising these groups. We conclude that GnRH receptor number for any individual gonadotrope is a weak determinant of the amount of LH it can secrete; nevertheless, full occupancy of all its GnRH receptors is required for any gonadotrope to reach its full LH-secretory capacity. Apparently the levels of other factors comprising the steps along the secretory pathway determine the secretory capacity of an individual cell.**

The quantitative relationship between receptor binding and hormone secretion has been difficult to establish at the single-cell level because of the presence of "spare receptors" on cells and because of the absence of methods permitting measurement of secretion by individual cells. In the present study, we combined a reverse hemolytic plaque assay for measurement of luteinizing hormone (LH) secretion from individual pituitary cells (1, 2) with an autoradiographic assay of ^{125}I-labeled gonadotropin-releasing hormone (GnRH) agonist binding to the same cells under conditions in which the spare receptors had been removed by trypsin treatment.

The hypothalamic decapeptide GnRH is the key neural regulator of the reproductive process of mammals (3, 4). It is synthesized by hypothalamic neurons and reaches the anterior lobe of the pituitary gland after secretion into the hypothalamohypophyseal portal circulation, the blood vessels comprising a direct link between the hypothalamus and pituitary gland. There, GnRH stimulates the release of LH from the gonadotropes (comprising about 5% of the anterior lobe cells) into the peripheral vasculature. LH induces ovulation in the female and testosterone secretion in the male. GnRH receptors having high affinity but low capacity are described as residing on the surface of gonadotropes (5, 6). Prior difficulties in investigating the relationship between receptor binding and hormone secretion are highlighted by the finding that only about 20% of the GnRH receptors need be occupied for maximal LH secretion (7) and that trypsin-dispersed pituitary cells show no binding to ^{125}I-labeled GnRH agonist in standard radioreceptor assays (7), although agonist-induced secretion can be evoked maximally from the same cells (1).

Here we report that with increasing doses of ^{125}I-labeled GnRH agonist, there is a corresponding increase in the average binding and hormone secretion when measured in groups of cells, as expected, but that these two measures vary independently of each other in the individual cells comprising these groups. Apparently, other components of the secretory pathway are the primary quantitative determinants of the amount of hormone secreted by individual cells.

MATERIALS AND METHODS

Pituitary cell cultures were prepared as described (8) from proestrous or diestrous-1 rats (Holtzmann, Madison, WI). The rats were maintained under daily lighting from 0600 to 1800. Pituitary glands were obtained after decapitation at 0700 and dispersed into single cells by use of trypsin as described by Hymer and Hatfield (9). The cells were suspended in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 0.1% bovine serum albumin (BSA) and antibiotics (100 units of penicillin G and 0.1 mg of streptomycin sulfate per ml).

Quantification of LH Release. Cohort cells were incubated either in Petri dishes for measurement of LH release as assessed by radioimmunoassay or in Cunningham slide chambers for measurement of hemolytic plaque area (2). Petri dishes coated with poly(L-lysine) were loaded with 3×10^5 freshly dispersed cells and were incubated in 2 ml of DMEM/0.1% BSA at 37°C in a humid 95% air/5% CO_2 atmosphere for various lengths of time with various concentrations of GnRH (Peninsula Laboratories, San Carlos, CA) (see ref. 10) as described in *Results*. The medium was assayed for LH content with the NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases) radioimmunoassay kit and the results were expressed in terms of the rat

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Abbreviations: LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; GnRH-A, the GnRH agonist Buserelin (des-Gly^{10}-[D-Ser(Bu^t)^6]GnRH ethylamide); ^{125}I-GnRH-A, ^{125}I-labeled GnRH-A; BSA, bovine serum albumin; NGF, nerve growth factor.

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LH standard rLH-RP-1 (biopotency = 0.03 × NIH-LH-S1). For measurement of plaque area, we mixed the dispersed pituitary cells (5×10^5 per ml) with an equal volume of an 18% suspension (vol/vol) of protein A-coupled ovine erythrocytes, coupled with chromium chloride as described previously (2), and infused a 30-μl aliquot containing 7.5×10^3 pituitary cells into a poly(L-lysine)-coated Cunningham slide chamber (composed of a glass coverslip spanning two strips of double-stick tape on the surface of a glass microscope slide; see ref. 2). Chambers were incubated for 45 min at 37°C in a humid 95% air/5% CO_2 atmosphere to permit attachment of the cells to the floor of the chamber as a monolayer. Chambers were then rinsed once with DMEM/0.1% BSA and filled with DMEM/0.1% BSA containing LH antiserum (final dilution 1:80) and GnRH if appropriate. Slides were incubated in the CO_2 chamber for 0.5–2.0 hr before the addition of complement (guinea pig serum; final dilution 1:20) to develop the plaques. After 30 min, the plaque reaction was terminated by infusion of 2% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4. The antiserum to ovine LH (oLH-AB 32-3) used in the plaque assay has been described (1); presorption of the antiserum with rat LH abolished the plaque reaction, whereas 100-fold greater concentrations of rat follicle-stimulating hormone and rat thyroid-stimulating hormone did not (1).

LH secretion by individual gonadotropes was derived from comparisons of plaque area and radioimmunoassayable LH release in similarly treated (time of incubation, dose of GnRH, and physiologic state) groups of cells. First, for each cohort pair, LH release from the 3×10^5 cells in Petri dishes was corrected to equal the numbers of pituitary cells (7.5×10^3) used in the plaque assay. In preliminary experiments, similar fractions (>90%) of the pituitary cells were shown to attach to the Petri dishes and Cunningham chambers. A regression coefficient for total radioimmunoassayble LH release vs. total plaque area was then calculated from the 14 paired treatment groups, which spanned an ≈50-fold range of secretory responses. The amount of LH secreted per gonadotrope (pg of LH RP-1) was calculated by multiplying the regression coefficient times the plaque area of individual gonadotropes.

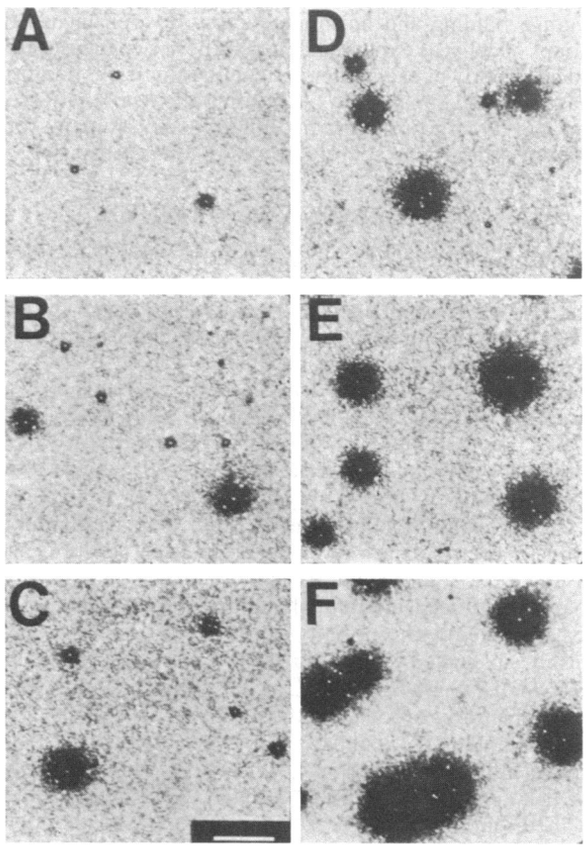

FIG. 1. Low-power, dark-field photomicrograph of LH plaques formed in response to treatment with various doses of GnRH-A (*A*, 0.5 pM; *B*, 1 pM; *C*, 2 pM; *D*, 5 pM; *E*, 10 pM; *F*, 50 pM). Incubation with the secretagogue lasted 2 hr. The speckled gray area is the ovine erythrocyte lawn, the black areas are the plaques, and the white dots in the centers of the plaques are gonadotropes. All of the secretory cells were derived from the pituitary gland of one proestrous rat. (Bar = 400 μm.)

Receptor Autoradiography. A GnRH superagonist analog [Buserelin (Hoechst); des-Gly^{10}-[D-Ser(Bu^t)^6]GnRH ethylamide, hereafter called GnRH-A] was radioiodinated using chloramine T and separated on a carboxymethylcellulose column using ammonium acetate at pH 4.5 as described (5, 11). Specific activity of the ^{125}I-labeled GnRH-A (^{125}I-GnRH-A) was estimated by comparing its bioactivity to that of unlabeled GnRH-A in the quantitative reverse hemolytic plaque assay for LH described above. Specific radioactivities of 1000–1200 μCi/μg (36.7–44.0 MBq/μg) were obtained.

^{125}I-GnRH-A was used as the secretagogue at concentrations of 0.1–100 pM in the plaque assay, which otherwise was performed as described above. Incubations lasted 2 hr. After plaque formation, we fixed the cells for 30 min by infusion of 2% glutaraldehyde (in 0.1 M phosphate buffer) into the Cunningham chamber. Coverslips were removed from the chamber and the cells were dehydrated with ethanol, air-dried, and dipped in Kodak NTB-2 emulsion (12). Cells were exposed for 2 weeks a −20°C and then developed in Kodak D-19 developer (5 min), rinsed in distilled water (30 sec), and fixed in Kodak fixer (5 min) (temperature = 15° ± 0.1°C) (12). After photographic processing the slides were stained lightly with toluidine blue, dehydrated, and mounted.

Areas of individual LH plaques were measured with a Bioquant video image-analysis system (2). In this system, the

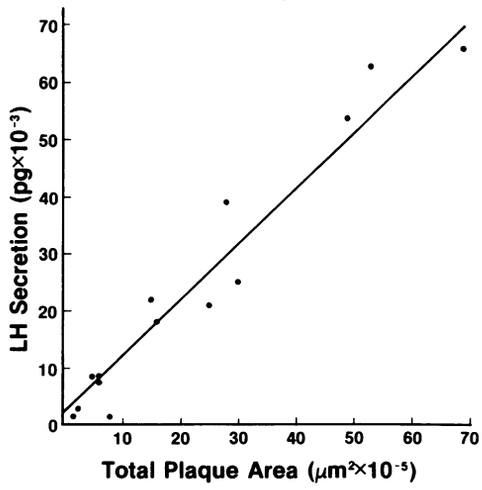

FIG. 2. Comparison of LH secretion from pituitary cells incubated either in Petri dishes for hormone measurement by radioimmunoassay (ordinate) or in Cunningham chambers for measurement of total plaque area (abscissa). Fourteen cohort groups of cells were treated similarly with GnRH to generate secretory responses covering a 50-fold range. Each point on the graph is the total plaque area formed by 150 gonadotropes (abscissa) or the mean content of triplicate cultures each radioimmunoassayed in duplicate (ordinate). Correlation coefficients in four such assays ranged from 0.93 to 0.98. LH concentrations are expressed in terms of the NIDDK standard rLH-RP-1 (see *Materials and Methods*). The statistical values for the data shown in the graph are as follows: $\bar{y} = 24 \times 10^3$ pg, $\bar{x} = 22 \times 10^5$ μm^2, $a = 2.5 \times 10^3$ pg, $b = 9.94 \times 10^{-3}$ pg/μm^2, and $r = 0.978$.

plaque is outlined by use of a digitizing pad. ^{125}I-GnRH-A binding to the same cells was measured with a Leitz MPV-Compact microphotometer; developed silver grains over each cell were measured by reflectance illumination with a ×63 oil-immersion objective. The error associated with reflectance measurements was low (mean coefficient of variation = 3.1%); this was determined by five repeated reflectance measurements on the same 14 cells, which spanned the range from lowest to highest grain numbers. Paired plaque areas and reflectance measurements were made for 50 plaque-forming cells per slide, with three slides per treatment group. All experiments were independently repeated at least three times, but the data presented are derived from a representative experiment in each case.

RESULTS

LH secretion from individual cells in culture was detected in our studies by means of a reverse hemolytic plaque assay that is based on complement-mediated lysis of LH antibody-coated erythrocytes coincubated with the pituitary cells (2, 8). LH secretion results in the lysis of indicator erythrocytes around the gonadotropes so that clear areas of lysis (plaques) surround them (Fig. 1). The presence of a plaque around a cell identifies it as a gonadotrope, and the area of a plaque is related to the amount of LH secreted.

Hormone release from individual cells was quantified by relating total radioimmunoassayable LH release from a group of cells incubated in Petri dishes to the total area of hemolysis caused by a similarly treated cohort group of cells undergoing the hemolytic plaque assay (Fig. 2). LH secretory activity in cohort groups of cells was manipulated over a 50-fold range, using various lengths of incubations combined with various doses of GnRH. Plaque area was linearly related to the amount of LH secreted (Fig. 2) (correlation coefficients of 0.93–0.98 were observed in four such assays). Thus, using the regression coefficient generated in such comparisons, we converted areas of individual plaques to quantity of LH secreted by single cells. As we reported previously (2), cells derived from a single pituitary gland treated with maximally effective doses of GnRH showed a 500- to 1000-fold difference between the cell secreting the smallest amount of LH and the one secreting the largest amount. For example, in the experiment of Fig. 2, this range was 3–2124 pg of the NIH-LH reference preparation. Correcting for the 1.5–3.0% abundance of LH in this preparation, we estimate the sensitivity of the assay (smallest detectable plaque) to be 1–5 amol. The linear relationship of plaque area and amount of LH secreted establishes that plaque area is a relative measure of LH secretion. Thus, plaque area can be used as a measure of LH release without the necessity of measuring LH release by radioimmunoassay in every experiment.

To examine the relationship between LH release and GnRH binding by single cells, we performed receptor autoradiography with ^{125}I-GnRH-A used both as the ligand and as the stimulant for LH release in the plaque assay. The developed silver grains appearing over cells in the center of plaques (Fig. 3) appeared to result from the presence on these trypsin-dispersed gonadotropes of specific and high-affinity receptors for GnRH. First, only cells forming plaques showed the presence of grains; i.e., no pituitary cells other than gonadotropes bound the labeled ligand. Second, the

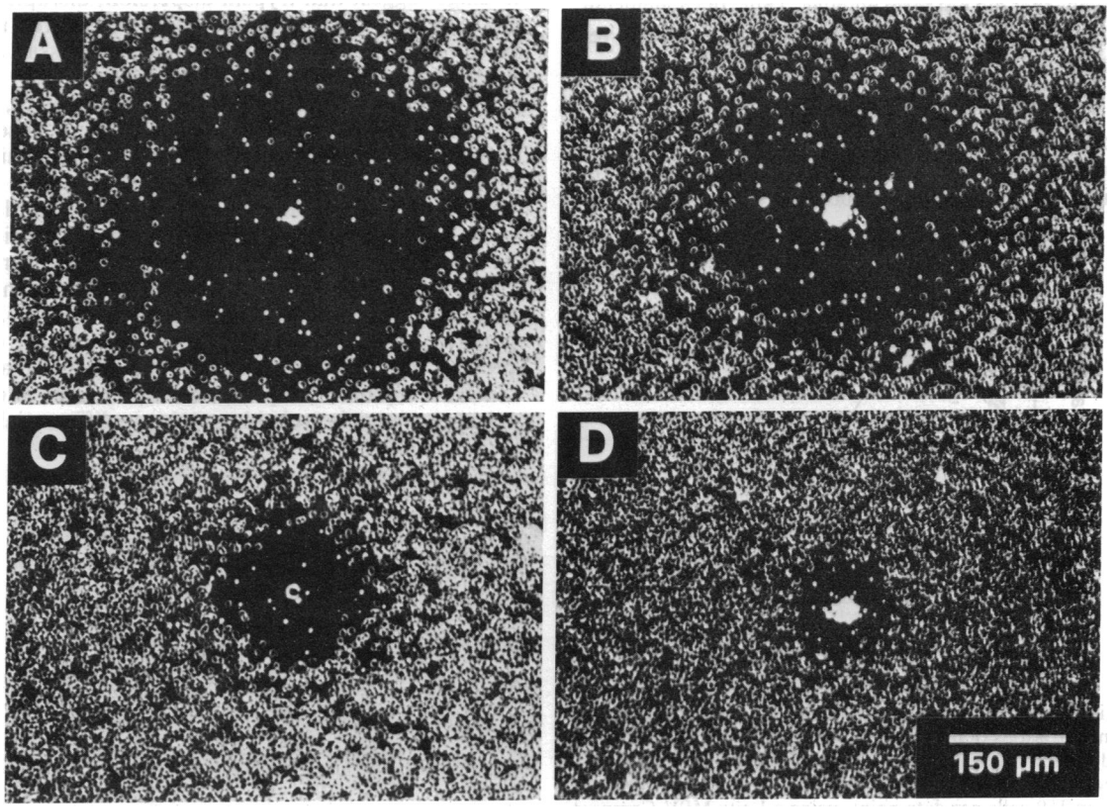

FIG. 3. Dark-field photomicrographs illustrating autoradiographic grain development over gonadotropes in the center of plaques. Silver grains appear as white dots in dark-field photomicrographs. Cells were obtained from proestrous rats. Background grains represent <3% of the total grains present over gonadotropes (see Fig. 4). All four cells were present in a single treatment group (100 pM ^{125}I-GnRH-A, a maximal dose, for 2 hr) and thus illustrate the low correlation between GnRH-A binding (grain number) and LH release (plaque area) exhibited by individual cells. *A* and *B* illustrate larger plaques with low (*A*) and high (*B*) grain numbers, whereas *C* and *D* are smaller plaques with low (*C*) and high (*D*) grain numbers. In seven different treatment groups (150 pairs of data in each group) the correlation coefficients ranged from 0.02 to 0.38.

number of grains observed was progressively inhibited by inclusion of increasing doses of unlabeled GnRH-A in the incubation medium (Fig. 4). Third, mean receptor binding (grain number) and mean LH secretion (plaque area) were highly correlated (r = 0.95) (Fig. 5). Finally, "spare receptors" on the gonadotropes appear to have been removed by trypsin, since the binding/LH secretion ratio remained constant over the full dose–response curve and maximal GnRH-A binding coincided with maximal LH secretion (Fig. 5).

Despite the high correlation between GnRH receptor binding and plaque area observed among group means (Fig. 5), the correlation coefficients for these two parameters fall to low levels when the individual cells comprising those groups are studied (overall correlation coefficient = 0.33; range of correlations within the seven individual groups = 0.02–0.38). This low correlation is illustrated in Fig. 3, where two larger plaques are seen to be associated with either low (*A*) or high (*B*) grain numbers, and two smaller plaques are also associated with low (*C*) or high (*D*) grain numbers.

Previous studies (1) of pituitary cells derived from proestrous rats demonstrated that all of the immunocytochemically identified gonadotropes were secretory (i.e., formed plaques), but at diestrus-1 only about 60% of them were; the remaining 40% contained LH as demonstrated by immunocytochemistry but still did not form plaques. To determine if such nonsecretory gonadotropes possess GnRH receptors, we performed receptor autoradiography on pituitary cells from diestrous-1 rats. As expected, during a 2-hr incubation period only about 56% as many plaques were formed in diestrous-1 cultures as in proestrous cultures, even though an equal number of pituitary cells was present in the Cunningham chambers (Fig. 6). Moreover, whereas all plaque-forming cells contained grains, none were found over cells that did not form plaques. Thus, the conclusion that nonsecretory gonadotropes at diestrus-1 lack GnRH receptors seems inescapable.

DISCUSSION

The heterogeneity in the amount of LH secreted by individual gonadotropes derived from the pituitary gland of a single rat is surprisingly large (500- to 1000-fold difference in the amount of LH secreted among gonadotropes). The determinants of this heterogeneity are unknown but do not appear to be artifacts of the experimental procedures used to disperse and culture the cells. Cells prepared as described in this paper show maximal secretory responses to GnRH that vary with physiologic state similar to those observed with undissociated fragments of pituitary glands (13–15). In addition, at maximal GnRH-A concentrations, 15-fold increases in LH secretion over baseline values are observed (Fig. 5). These findings suggest that trypsin dispersion, disruptions of cell–cell contacts, and the subsequent procedures involved in performing the reverse hemolytic plaque assay are not the determinants of individual cell heterogeneity.

In addition to this quantitative heterogeneity, qualitative heterogeneity among cells also has been observed; i.e., ≈40% of the gonadotropes derived from the pituitary glands of rats at the diestrus-1 stage of the estrous cycle were nonsecretory under conditions (maximally stimulatory doses of GnRH and prolonged incubation times) in which virtually all of the gonadotropes from proestrous rats were secretory (1). A 24-hr incubation with estradiol converted the nonsecretory gonadotropes into a secretory state (1). We have confirmed the existence of such nonsecretory cells in the present study and furthermore have shown that they lack GnRH receptors. The presence of such cells at diestrus but not at proestrus is probably not due to differential removal of

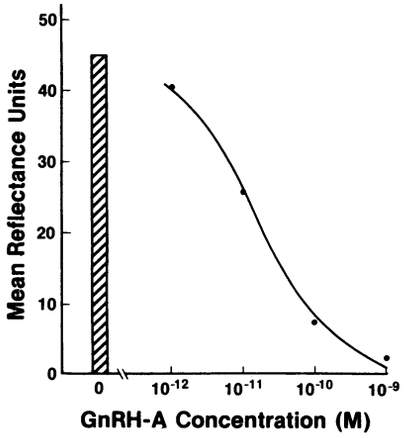

FIG. 4. Specificity of ^{125}I-GnRH-A binding to gonadotropes. Cells were incubated with ^{125}I-GnRH-A (50 pM, a maximal dose) and various concentrations of unlabeled GnRH-A for 2 hr and processed for autoradiography; pituitary cells were obtained from proestrous rats. Reflectance measurements (autoradiographic grain number) were made on 150 cells per data point, using a Leitz MPV-Compact microphotometer. GnRH at 10-fold higher concentrations exhibited a quantitatively similar inhibition curve. At maximally inhibitory concentrations of unlabeled GnRH-A, the number of grains remaining in LH plaques represented <3% of the grains present in the control group; when only unlabeled GnRH-A was used to stimulate LH plaque development, the number of grains present represented <1% of those present in the control group.

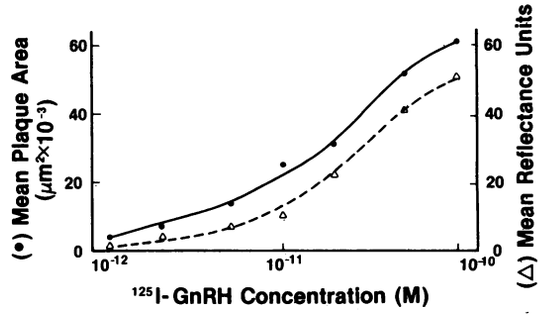

FIG. 5. Relationship between average GnRH receptor binding per cell (reflectance) and LH secretion per cell (plaque area) when ^{125}I-GnRH-A was used both as the receptor ligand and as the stimulant for LH release. The data shown were derived from seven doses of ^{125}I-GnRH-A; at each dose, reflectance and plaque area were measured on 150 cells. Incubations with the secretagogue lasted 2 hr, and cells were derived from the pituitary gland of a proestrous rat. The correlation coefficient for this assay was 0.95. Note that the binding/LH secretion ratio remained constant over the full dose–response range, including near-maximal doses of ^{125}I-GnRH-A.

FIG. 6. Comparison of total LH release and total GnRH receptor binding by groups of cells derived from the pituitary glands of proestrous and diestrous-1 rats. Incubation lasted 2 hr in the presence of a maximally stimulatory dose of ^{125}I-GnRH-A (100 pM). Only 55.6% as many cells formed plaques at diestrus-1 as at proestrus; a similar difference in the fraction of cells containing grains was also observed (see text).

receptors by trypsin at diestrus, since the relative secretory responsiveness of such cells is similar to that observed in nondissociated pituitary fragments (13–15). Thus, it seems likely that the increase in GnRH receptors observed in pituitary glands over the diestrus-1 to proestrus interval leading to the preovulatory LH surge (ref. 16 and Fig. 6) is due in part to induction of GnRH receptor formation on the nonsecretory gonadotropes by estrogens. Parenthetically, our observation of an increase in GnRH receptors over the diestrus-1 to proestrus interval (Fig. 6) confirms the findings of several other studies (16–18); this confirmation may be viewed as an additional validation of our method of GnRH receptor measurements.

In earlier studies on homogenates of undissociated rat pituitary glands with standard liquid radioreceptor assays, investigators have reported the existence of "spare" GnRH receptors; i.e., there was a 5-fold excess of receptors over the number required for full activation of the secretory response (7). However, GnRH receptors were undetectable in homogenates of trypsin-dispersed cells by the same assay (7), despite the fact that such cells retain maximal responsiveness to the secretagogue (1). Unpublished studies (J. D. Peck and J.D.N.) have confirmed the absence of GnRH receptors in trypsin-dispersed cells shown to be fully responsive to the LH-stimulatory actions of GnRH in a liquid radioreceptor assay (validated by confirming previous estimates of GnRH receptor number and affinity in undissociated rat pituitary glands). The results of the present report demonstrate that the autoradiographic approach permits the estimation of GnRH receptor number in trypsin-dispersed cells and thus apparently is more sensitive than standard liquid radioreceptor assays.

The data presented in Fig. 5, where the ratio of the LH secretory response and receptor number was constant over a full two-orders-of-magnitude dose/binding curve including near-maximal value, suggest that trypsin preferentially removed nonfunctional or "spare" receptors. In standard liquid radioreceptor assays on undissociated pituitary glands, the LH secretory response to GnRH reaches maximum when only 20% of the receptors are occupied (7). Our suggestion of a qualitative difference between functional and nonfunctional receptors is supported by the findings of Iwashita and Catt (19) that two GnRH receptor species of 60 and 53 kDa were detected by $NaDodSO_4$/polyacrylamide gel electrophoresis on photoaffinity-labeled homogenates of undissociated rat pituitary glands; only the 53-kDa species was detected after trypsin treatment. Preliminary studies (J. J. Mulchahey and J.D.N., unpublished) have confirmed the preferential loss of the 60-kDa GnRH receptor species after trypsin dispersion of cells and $NaDodSO_4$/polyacrylamide gel immunoblots with a GnRH receptor antibody (20). This differential sensitivity of the two species of GnRH receptors resembles findings about nerve growth factor (NGF) receptors on a pheochromocytoma cell line (PC12). Schechter and Bothwell (21) reported the existence of two classes of NGF receptors, which have similar equilibrium binding constants but which differ in dissociation constants by about 40-fold. Trypsin treatment of PC12 cells preferentially removed the rapidly dissociating class of NGF receptors. Furthermore, only the slowly dissociating class of NGF receptors was associated with the cytoskeleton (21) and only about 10% of the total cellular receptors were in this class (22). The relevance of these observations to ours with the GnRH receptor is not clear, but at minimum they provide a precedent for our conclusion about the existence of two classes of GnRH receptors having differential sensitivity to trypsin.

In view of the high correlations between mean grain number and mean plaque area in dose–response curves, our finding of a low correlation between receptor binding and LH secretion at the level of individual cells is surprising. Cells from diestrous and proestrous rats have low and high numbers of GnRH receptors matched by low and high rates of LH secretion (Fig. 6), and at seven different doses of ^{125}I-GnRH-A there is a high correlation between mean receptor binding per cell and mean LH release per cell (Fig. 5). Yet individual cells comprising these means exhibited low correlations between these parameters. We interpret these findings to mean that GnRH receptor number for an individual gonadotrope is a weak determinant of the amount of LH it can secrete; nevertheless, full occupancy of all its GnRH receptors (whether high or low in number) is required for the gonadotrope to reach its full LH secretory capacity (which may be high or low in amount). Clearly, other steps along the secretory pathway, such as hormone storage, are likely to be heterogeneous. Thus, it appears that the level of these or other factors, or perhaps a sum or product of the factors, comprising the chain of events linking receptor binding to LH secretion may determine the amount of hormone secreted by an individual cell.

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