Proc. Natl. Acad. Sci. USA
Vol. 77, No. 11, pp. 6692–6695, November 1980
Cell Biology

Receptor-mediated internalization of fluorescent gonadotropin-releasing hormone by pituitary gonadotropes

(fluorescence microscopy/hormone receptors/patching/endocytosis/receptor microaggregation)

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Communicated by James B. Wyngaarden, August 21, 1980

ABSTRACT **A bioactive, fluorescent derivative of gonadotropin-releasing hormone, <Glu-His-Trp-Ser-Tyr-D-Lys(N^{ϵ}-tetramethylrhodamine)-Leu-Arg-Pro-Gly-NH_2, was prepared. This peptide retained high-affinity binding (apparent dissociation constant, 3 nM) to the receptor for gonadotropin-releasing hormone and was utilized for microscopic visualization and localization of gonadotropin-releasing hormone receptors in cultured rat pituitary cells. The fluorescently labeled receptors were initially distributed uniformly on the cell surface and formed patches, which subsequently internalized (at 37°C) into endocytic vesicles. These processes were dependent on specific binding sites for the rhodamine-labeled peptide to gonadotrope cells. Cluster formation and internalization were markedly reduced in the absence of Ca^{2+}, which is required for gonadotropin secretion. It is possible that cluster formation, microaggregation, and internalization of gonadotropin-releasing hormone receptors may be important in eliciting biological effects or for the observed loss of tissue responsiveness after desensitization due to exposure to the homologous hormone.**

The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland is stimulated by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH; <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). The first step in stimulation is recognition of GnRH by specific binding sites (receptors) at the surface of the gonadotrope cells (1). Binding of ^{125}I-labeled metabolically stable GnRH analogs to pituitary plasma membranes (2, 3) has indicated the presence of a single class of high-affinity binding sites (dissociation constant K_d = 2–6 nM). Recently these analogs have been used to study the interaction of GnRH with its receptors in cultured rat pituitary cells.§

Certain studies have suggested that cyclic AMP may be involved in GnRH-stimulated gonadotropin release (4, 5). However, other studies have shown that LH release and cyclic AMP production can be uncoupled (6–9). Recently, a line of evidence has developed which suggests that calcium may play a central role in GnRH stimulation of gonadotropin release from cultured rat pituitary cells (10–13) and that the specific requirement for Ca^{2+} occurs at a postreceptor step (14). Nevertheless, the effect of GnRH on cellular receptor distribution is not known. Recently, image-intensified fluorescence microscopy has proven useful in visualizing the patterns and the mobility of receptors for epidermal growth factor, insulin, chemotactic peptide, enkephalin, and α_2-macroglobulin in intact cells (15–19). In the present studies, a bioactive, fluorescently labeled GnRH analog was used to visualize GnRH receptors in cultured rat pituitary cells. The receptors were initially distributed uniformly and subsequently formed surface clusters that became internalized. Cluster formation and internalization were substantially reduced in the absence of Ca^{2+}.

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MATERIALS AND METHODS

Pituitary Cells. Pituitaries were dispersed and cultured as described (10). Briefly, whole pituitaries were removed from female weanling rats (28 days) (Zivic–Miller) and collected in sterile medium 199 containing 0.3% bovine serum albumin (fraction V, Armour), 10 mM Hepes (Sigma) at pH 7.4, and gentamicin (Schering) at 20 μg/ml. Each pituitary was cut into six to eight pieces and allowed to settle in a 50-ml sterile conical centrifuge tube containing Hepes-buffered (10 mM) medium 199 (pH 7.4) with 0.3% bovine serum albumin (199/albumin). This medium was decanted and replaced twice with fresh 199/albumin to remove lysed cells and their contents released during mincing of the tissue.

A 10-ml portion of 0.25% collagenase (Worthington) and 0.10% hyaluronidase (Sigma) in 199/albumin was added to the decanted tissue fragments. The tube was then capped, placed on its side in a water bath (37°C), and shaken (100 cycles per min). Every 3 min the tube was removed and the contents were gently drawn repetitively into and out of a disposable sterile 10-ml pipette. After 15 min, the solution was filtered through organza cloth to remove residual tissue fragments. The filtrate, containing clumps of 5–10 cells, was brought to a final volume of 50 ml with 199/albumin.

After centrifugation at 225 × *g* for 10 min, the supernatant was discarded and the pellet was resuspended in 1–2 vol (ml per pituitary) of 199/albumin containing 10% horse serum (Microbiological Associates, Bethesda, MD), 2.5% fetal calf serum (Microbiological Associates), and gentamicin at 20 μg/ml. Five-milliliter samples of the resuspended cells were plated in 10-ml petri dishes containing coverslips and incubated at 37°C for 2 days. For fluorescence microscopy the cells were washed and incubated (37°C) in three changes of phosphate-buffered saline containing 0.3% bovine serum albumin and 1 mM $CaCl_2$ over a period of 1 hr.

Synthesis of Rhodamine-Labeled [D-Lys^6]GnRH. <Glu-His-Trp-Ser-Tyr-D-Lys(N^{ϵ}-tetramethylrhodamine)-Leu-Arg-Pro-Gly-NH_2 ([Rhod-D-Lys^6]GnRH) was prepared by reaction of [D-Lys^6]GnRH (0.5 mg) (Peninsula Laboratories, San Carlos, CA) with 2 equivalents of rhodamine (Rhod) isothiocyanate (tetramethyl form) (Research Organics, Cleveland, OH) in methanol in the presence of 1.2 equivalents of triethylamine. After standing at 24°C for 24 hr, the reaction mixture was evaporated to dryness under reduced pressure, dissolved

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; [Rhod-D-Lys^6]-GnRH, [N^{ϵ}-tetramethylrhodamine-D-Lys^6]GnRH; 199/albumin, medium 199/0.3% bovine serum albumin.

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§ Naor, Z, Clayton, R. N. & Catt, K. J. (1980) *62nd Annual Meeting of the Endocrine Society*, June 18–20, 1980, Washington, DC, p. 109.

in 0.2 ml of 1.0 M acetic acid, and applied to a Sephadex G-15 column (1.5 × 20 cm) that had previously been equilibrated and eluted with 1.0 M acetic acid. The [Rhod-D-Lys^6]GnRH peak (ϵ_{550} = 80,000 M^{-1} cm^{-1}), which eluted just behind the void volume, was collected and lyophilized. Thin-layer chromatography (silica gel, precoated plastic sheets, Brinkmann) revealed R_F values of 0.3 in BuOH/AcOH/H_2O (4:1:4, vol/vol) and 0.2 in chloroform/methanol (1:1, vol/vol).

Iodination, Pituitary Membrane Preparation, and Binding Assays. des-Gly^{10}-[D-Ser(*t*Bu)^6]GnRH ethylamide (Buserelin, Hoescht) was iodinated by using the chloramine-T method and separated on CM-cellulose as described for GnRH (20). Purified pituitary plasma membranes were prepared from adult female bovine pituitaries according to the modification of the method of Clayton *et al.* (2). Binding assays were conducted in polypropylene Microfuge tubes (Beckman) precoated with 1% bovine serum albumin. The labeled peptide (40,000 cpm, specific activity 100 μCi/μg; 1 Ci = 3.7 × 10^{10} becquerels) was incubated with 100–200 μg of protein of pituitary membranes in a total volume of 0.5 ml of 10 mM Tris·HCl, pH 7.4, containing 0.1% bovine serum albumin (120 min, 4°C). Separation of membrane-bound from free ligand was achieved by centrifugation at 12,800 × *g* for 5 min. Specific binding represents the bound radioactivity that can be displaced by incubation with 10 μM GnRH. Each value is the mean of triplicate incubations, which varied by less than 7%.

Image-Intensified Fluorescence Microscopy. The distribution and binding of the [Rhod-D-Lys^6]GnRH to cultured rat pituitary cells were visualized by using a Zeiss Photomicroscope III, equipped with epifluorescence optics, an oil-emersion Planapo 63/1.25 numerical aperture lens, and a Hamamatsu C1000 camera. This camera is especially sensitive and can detect very low levels of light. Video output was recorded on a Panasonic recorder (NV-8030) and displayed on a Hitachi TV monitor (VM-905AV) from which Polaroid photographs were taken.

RESULTS

[Rhod-D-Lys^6]GnRH was prepared by chemical modification of the ϵ-amino group in position 6 of [D-Lys^6]GnRH. [D-Lys^6]GnRH was selected as the starting material for derivatization with rhodamine because (*i*) substitution of the D amino acid in the sixth position of GnRH results in a more potent and metabolically stable derivative (21) and (*ii*) the ϵ-amino group of lysine serves as a spacer for substitution reactions and thus the GnRH sequence and its conformation are less likely to be disturbed. The analog appeared homogeneous on thin-layer chromatography and gave a negative test with ninhydrin reagent, indicating the absence of free amino groups. Spectrophotometric analysis of the derivative (Fig. 1) revealed spectral properties identical to those reported for coupled rhodamine, ϵ_{550} = 80,000 M^{-1} cm^{-1} and $\epsilon_{350} \approx$ 12,000 M^{-1} cm^{-1} (22). The inhibition of binding of ^{125}I-labeled Buserelin to pituitary membrane preparations by [Rhod-D-Lys^6]GnRH and GnRH is shown in Fig. 2. The apparent IC_{50} values (the concentration of unlabeled ligand at which the specific binding of labeled ligand is reduced by 50%) were found to be 3 nM and 40 nM for [Rhod-D-Lys^6]GnRH and GnRH, respectively. Both Buserelin and [Rhod-D-Lys^6]GnRH bind to the GnRH receptor with higher affinity than GnRH itself does. This may result from the increased peptidase resistance of these derivatives. In contrast, GnRH is susceptible to proteolysis and may accordingly have an apparently decreased affinity.

When cultured rat pituitary cells were incubated (120 min, 4°C) with 10 nM [Rhod-D-Lys^6]GnRH, in the presence of 1 mM Ca^{2+}, the fluorescent hormone was evenly distributed over the surface of the gonadotropes (Fig. 3*A*). The gonadotropes account for about 20% of the total pituitary cells in this preparation and are easily identifiable by morphological criteria. The uniform distribution observed is specific, because the fluorescence was much reduced in the presence of 10 μM [D-Ala^6]GnRH or 10 μM native GnRH (Fig. 3*B*). When the cells that had bound [Rhod-D-Lys^6]GnRH at 4°C were warmed on the microscope stage to 37°C, aggregation (Fig. 3*C*) and subsequent internalization of the fluorescent peptide by 20% of the pituitary cells could be observed (Fig. 3*D*). The internalized fluorescent hormone appeared in endocytic vesicles that displayed the saltatory motion characteristic of cytoplasmic organelles. When cytoplasmic streaming was observed by phase-contrast microscopy, the fluorescent vesicles moved with the same velocity and direction as the cytoplasmic organelles. Similar patterns were observed when cells were incubated directly with [Rhod-D-Lys^6]GnRH at 23°C or 37°C. Cluster formation was observed at 23°C after 30–40 min (similar to Fig. 3*C*), while some internalization was complete within 5–10 min at 37°C (similar to Fig. 3*D*). Internalization into endocytic vesicles was much more rapid at 37°C (10 min), and no further changes were observed during a further 60-min incubation. The nonspecific fluorescence, in the presence of 10 μM unlabeled hormone, reveals internalization by only about 1–2% of the total

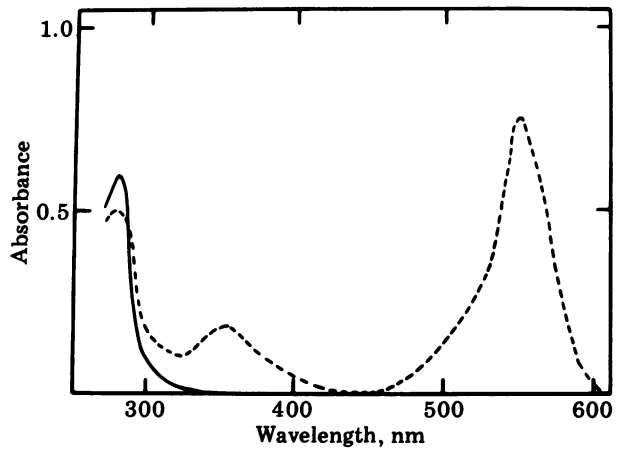

FIG. 1. Ultraviolet–visible absorption spectra of 7 μM [Rhod-D-Lys^6]GnRH (---) and 100 μM GnRH (—) in 0.1 M acetic acid.

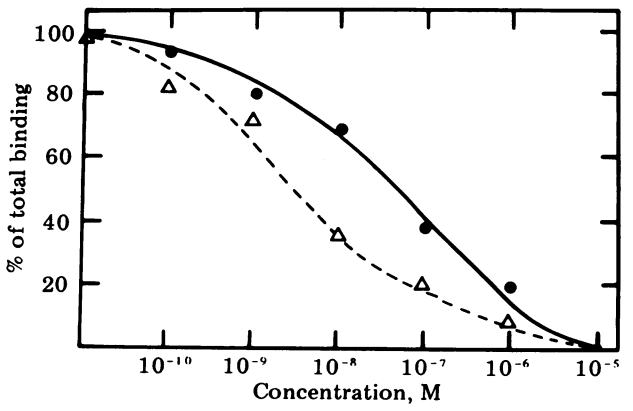

FIG. 2. Competition of binding of ^{125}I-labeled Buserelin to pituitary plasma membrane by GnRH (●—●) and [Rhod-D-Lys^6]GnRH (△---△). The radioactive Buserelin (40,000 cpm) was incubated with different concentrations of the tested compound for 120 min at 4°C in a final volume of 0.5 ml containing pituitary membrane (0.1–0.2 mg of protein per ml) and 0.1% bovine serum albumin, and the binding was measured. Values are means of triplicate incubations.

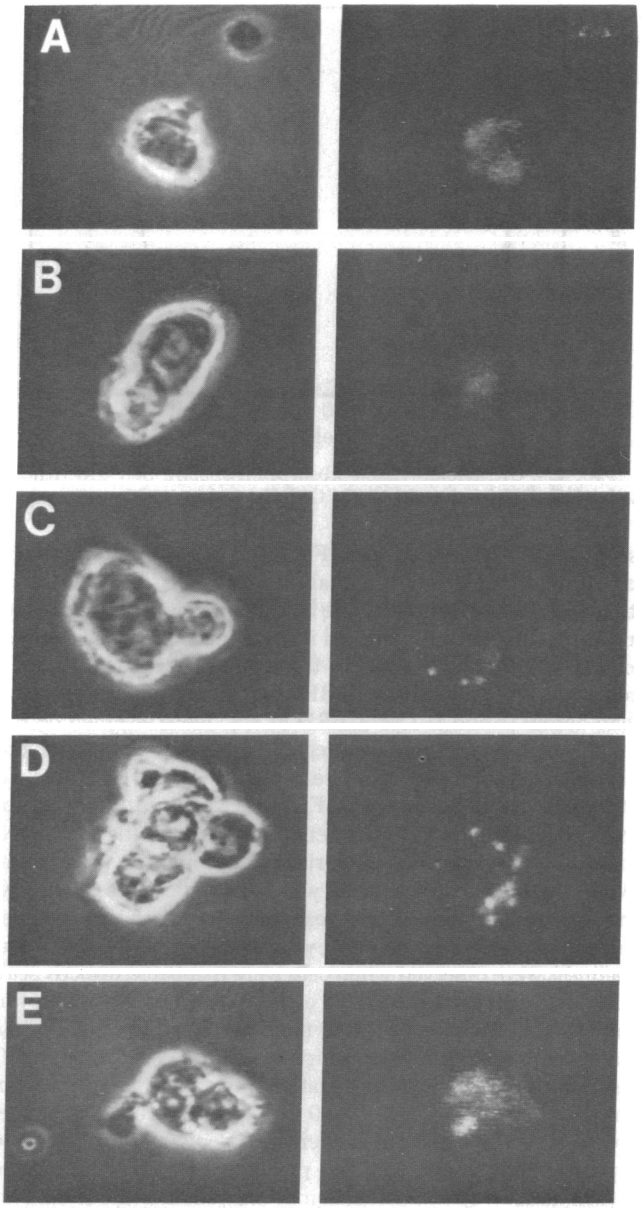

FIG. 3. Fluorescent visualization of [Rhod-D-Lys^6]GnRH binding to cultured rat pituitary cells. On the left, phase-contrast micrographs (×600); on the right, fluorescence micrographs of the same field. The cells were incubated for 120 min at 4°C in the presence of 1 mM Ca^{2+} with 10 nM rhodamine derivative (*A*); incubation in the presence of 10 μM [D-Ala^6]GnRH (*B*); warming the cells in *A* to 37°C, after 5 min (*C*) and after 10–15 min (*D*); incubation in the absence of 1 mM Ca^{2+} (*E*). Binding was terminated by rinsing the coverslips in 4°C buffer followed by immediate fixation in 1% formaldehyde (30 min at 4°C).

cells. This noncompetitive internalization may suggest nonspecific endocytosis by cells other than gonadotropes. Similar nonspecific endocytosis (≈1% of total cells) was observed when the cells were incubated with rhodamine alone.

Because extracellular Ca^{2+} (optimally, 1 mM) is an absolute and specific requirement for GnRH stimulation of LH release from pituitary cells, the distribution and behavior of GnRH receptors were examined in the absence of Ca^{2+}. Pituitary cells were preincubated (30 min) with 3 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid in phosphate-buffered saline containing 0.3% bovine serum albumin, followed by incubation with 10 nM [Rhod-D-Lys^6]GnRH, in the same buffer, at 23°C or 37°C for 60 min. Examination by fluorescence microscopy revealed that cluster formation and internalization are much reduced (<5%) compared to that observed in the presence of 1 mM Ca^{2+}, and most of the fluorescent hormone was uniformly distributed over the cell surface of the gonadotropes (Fig. 3*E*). The uniform distribution appeared to be brighter than that in the presence of 1 mM Ca^{2+}.

DISCUSSION

Cultured rat pituitary cells have been very useful in studying the mechanism of GnRH action. These cells respond to administration of GnRH by releasing LH. Because release is not markedly inhibited by cycloheximide, because GnRH does not stimulate incorporation of radioactive amino acids into LH acutely, and because LH release is accompanied by an equal depletion of cellular LH (refs. in ref. 13), it appears that GnRH does not substantially stimulate LH biosynthesis in these cultures when release occurs. Thus, the pituitary culture system offers a clear distinction between LH biosynthesis and release. Studies on the mechanism of action of GnRH have indicated that GnRH stimulation of LH release from these cells is mediated by a Ca^{2+}-dependent mechanism (10–13) and not via cyclic AMP (23).

The present study shows that in the presence of 1 mM Ca^{2+} the unoccupied GnRH receptors of the plasma membrane are diffusely and homogeneously distributed over the gonadotrope surface. GnRH binding to cells at 4°C does not alter receptor distribution. However, warming these cells to 37°C or GnRH binding at 37°C causes a rapid aggregation of the fluorescent peptide (and possibly its receptor) into intensely fluorescent patches on or near the cell surface. These aggregates are subsequently internalized into endocytic vesicles. The internalization is observed specifically with gonadotropes, which constitute 20% of the total pituitary cells under the conditions used. In addition, cluster formation and internalization are not seen with cultured cell lines (e.g., KB epidermoid cells, HF_3 human fibroblasts) that lack GnRH receptors. In the absence of Ca^{2+}, cluster formation and internalization are much reduced. Similar results have been observed with α_2-macroglobulin and epidermal growth factor (24), with which cluster formation and internalization did not occur in the absence of extracellular Ca^{2+}. It has been suggested (24) that a calcium-requiring transglutaminase enzyme may be involved in the formation of clusters. It is clear, however, that while Ca^{2+} may be required for optimal internalization, this does not preclude an additional intracellular locus of action of Ca^{2+} (12) for LH release in response to GnRH.

Whether clustering and internalization have any relevance to the biological release function of GnRH is presently unclear. Several recent studies suggest that receptor crosslinking or microaggregation, perhaps independent of internalization, may be important for hormonal action in some systems. Bivalent antibodies to the insulin receptors block insulin binding, bind to insulin receptors, and trigger many of the biological responses caused by insulin (25, 26). Monovalent Fab′ fragments are ineffective, but addition of anti-Fab′ fragments to crosslink the Fab′–receptor complexes restores the insulin-like activity (25). Furthermore, under certain conditions bivalent but not univalent antibodies directed against insulin (27) and EGF (28) can dramatically enhance the activity of very low concentrations of these hormones in fibroblasts. On the other hand, in certain other systems, such as low density lipoprotein and yolk protein (29), internalization of receptor-bound proteins serves as a selective transport system that directs specific ligands to specific subcellular organelles. In additon, nerve growth factor, which enters the cell at the tip of the axon, migrates in vesicles to the

cell body, where it apparently accumulates in the nucleus in an intact form (30, 31).

Sternberger *et al.* (32), using immunocytochemical staining with antisera to GnRH, showed that GnRH receptors are found in plasma membranes of rat pituitaries and are highly concentrated on the membranes of large secretory granules of gonadotropes. One hypothesis that they invoked to explain this phenomenon was that the receptors interact with GnRH on the membrane and then translocate to the granules. Evidence from fixed cell preparations labeled with a ferritin-GnRH conjugate also indicated that internalization of receptor-bound GnHR can occur (33). Because Ca^{2+} is required for both GnRH stimulation of LH release and internalization of hormone into endocytic vesicles (or secretory granules), and because the release (which starts within 15 min and reaches maximum at 180 min) occurs subsequent to internalization (10 min at 37°C), internalization of GnRH could play a role in LH release from cultured rat pituitary cells. Alternatively, internalization may simply be related to degradation of the hormone or receptors. The internalization of GnRH (and presumably its receptor) could explain the decrease in gonadotrope responsiveness to the hormone after chronic treatment with GnRH (34).

P.M.C. is the recipient of National Institutes of Health Research Career Development Award HD 00337 and National Institutes of Health Grant HD 13220.

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