Photoaffinity-labeling and fluorescence-distribution studies of gonadotropin-releasing hormone receptors in ovarian granulosa cells

(photostimulation/patching/internalization)

Eli Hazum and Abraham Nimrod

Department of Hormone Research, The Weizmann Institute of Science, Rehovot, 76100, Israel

Communicated by Ephraim Katchalski-Katzir, November 23, 1981

ABSTRACT Photoaffinity labeling of rat ovarian granulosa cells and membrane preparations with a bioactive photoaffinity derivative of gonadotropin-releasing hormone resulted in identification of two specific components with apparent molecular weights of 60,000 and 54,000. Fluorescent visualization of gonadotropin-releasing hormone receptors in these cells, by using a bioactive rhodamine derivative of the hormone, indicated that the fluorescently labeled receptors were initially distributed uniformly on the cell surface and then formed patches that subsequently internalized (at 37°C) into endocytic vesicles. These processes were dependent on specific binding sites for the rhodamine-labeled peptide on the granulosa cells. These studies may provide an experimental basis for understanding the molecular events involved in the action of the hormone in the ovary.

Gonadotropin-releasing hormone (GnRH) is believed to mediate the hypothalamic control of pituitary gonadotropin secretion. However, recent studies have indicated an extrapituitary action of GnRH and its agonists (1-9). It has been shown that the hormone and its agonists inhibit a variety of female reproductive functions, including ovarian steroidogenesis, ovulation, ovum transport, and implantation (1-9). These data suggest that the hormone has a direct ovarian site that can play a role in its antifertility effect. Indeed, the presence of steroidal high-affinity binding sites for GnRH has recently been shown in culture cells as well as in ovarian membrane preparations (10-15). These receptors exhibited binding characteristics similar to those of GnRH receptors in the anterior pituitary. In view of these observations, GnRH receptors of rat granulosa membranes have been identified directly by using a photoaffinity-labeling technique. In addition, GnRH receptors in rat granulosa cells have been visualized by using image-intensified fluorescence microscopy.

MATERIALS AND METHODS

Granulosa Cells and Membrane Preparation. Granulosa cells were collected from immature (26-day-old) rats 48 hr after subcutaneous administration of 15 units of pregnant mare serum gonadotropin (Gestyl, Organon). Cells were expressed from the ovarian follicles into phosphate-buffered saline/0.1% bovine serum albumin (for binding studies) or into McCoy’s 5A tissue culture medium (for culture and fluorescence microscopy). Granulosa cell cultures were carried out as described (16), except that serum-coated glass coverslips were introduced into the culture dishes to serve as substrate for cell adhesion. After 24 hr of culture, the coverslips were washed and incubated (37°C, 1 hr) in three changes of phosphate-buffered saline/0.2% bovine serum albumin/1 mM CaCl₂.

Membranes were prepared by homogenizing freshly collected granulosa cells with a Dounce homogenizer at 4°C in assay buffer (10 mM Tris-HCl, pH 7.4/0.1% bovine serum albumin/1 mM dithiothreitol) and centrifuged for 10 min at 1000 g. The supernatant was then centrifuged for 20 min at 20,000 g. The pellet was suspended in assay buffer, centrifuged at 20,000 g for 20 min, and resuspended in assay buffer.

Iodination and Binding Assay. [35S]-Serine-bovine, [35S]-Gly, [35S]-ethyylamide]-GnRH (buserelin, provided by J. Sandow, Hoechst, Frankfurt) and [35S]-azidobenzoyl-D-[35S]-Lys6]-GnRH ([N6-azidobenzoyl]-D-Lys6]-GnRH) were iodinated by the lactoperoxidase method as described (17-19). The specific activity of the labeled peptides was 1500 μCi/μg (1 Ci = 3.7 × 10¹² becquerels).

Binding assays were carried out as described (17, 18). Briefly, the labeled peptide (40,000 cpm) was incubated with 50-100 μg of granulosa membrane protein in (total volume) 0.5 ml of assay buffer for 90 min at 4°C. The homogenate was filtered at 10 μl of ice-cold incubation buffer, the filters were then assayed in a gamma counter. Specific binding represents the bound radioactivity that can be displayed by simultaneous or prior addition of 1 μM unlabeled buserelin. Each value is the mean of duplicate incubations, which varied by <7%.

Synthesis of Rhodamine-Labeled and Azidobenzoyl-Labeled [35S]-GnRH. [35S]-tetramethylrhodamine-D-[35S]-Lys6]-GnRH ([Rhod-D-Lys6]-GnRH) and [35S]-azidobenzoyl-D-[35S]-Lys6]-GnRH ([AB-D-Lys6]-GnRH) were prepared as described (17, 20). Briefly, [35S]-GnRH was treated with 2 molar equivalents of rhodamine isothiocyanate (tetramethyl form) or 2 equivalents of (4-azidobenzoyl)-N-hydroxy succinimide in methanol in the presence of 1.2 equivalents of triethylamine. The two analogs appeared homogeneous on thin-layer chromatography and showed spectral properties identical to those reported for coupled azidobenzoaryl and rhodamine derivatives (17, 20).

Photolysis and NaDODSO₄/Polyacrylamide Gel Electrophoresis. Granulosa cell membranes (0.9 mg) were incubated with [35S]-labeled [AB-D-Lys5]-GnRH (1,000,000 cpm) in the presence or absence of 1 μM buserelin in 1.5 ml of assay buffer at 4°C in the dark. After 90 min, the membranes were photolysed (7 min at 4°C, optimal conditions) with a mercury lamp at a distance of 10 cm and washed twice with assay buffer by centrifugation; then, the pellet was boiled in 1% NaDODSO₄/10 mM dithiothreitol. Aliquots were prepared and run on elec-


**RESULTS**

Apparent IC\textsubscript{50} values for inhibition of binding of \textsuperscript{125}I-labeled buserelin to granulosa membrane preparations by GnRH, buserelin, [AB-D-Lys\textsuperscript{6}]GnRH, and [Rhod-D-Lys\textsuperscript{6}]GnRH are given in Table 1. These values are similar to the corresponding IC\textsubscript{50} values in pituitary membrane preparations (17, 18, 20) and consistent with previous reports (10–15) that both receptors exhibit similar binding affinities for GnRH and its analogs.

Photoactivation of \textsuperscript{125}I-labeled [AB-D-Lys\textsuperscript{6}]GnRH after incubation (90 min at 4°C) with granulosa membranes (Fig. 1) or granulosa cells (not shown) resulted in identification of two specific bands, with apparent molecular sizes of 60,000 and 54,000 daltons. The intensities of these specific bands decreased in a dose–response manner in the presence of various concentrations (1, 10, and 100 nM) of buserelin, [d-p-Glu\textsubscript{1},d-Phe\textsubscript{2},d-Trp\textsubscript{3}]\textsuperscript{6}GnRH (a GnRH antagonist), or GnRH (not shown). Identical results were obtained if the membranes were irradiated without washing or were washed (after binding) and then photolyzed. As shown in Fig. 1, some of the radioactivity did not penetrate to the gel under reducing conditions (the amount of radioactivity was varied in different experiments), which suggests formation of receptor aggregates.

Incubation of cultured rat granulosa cells (120 min at 4°C) with 10 nM [Rhod-D-Lys\textsuperscript{6}]GnRH resulted in uniform distribution of the fluorescent hormone over the surface of the cells (Fig. 2A). The uniform distribution observed represents specific binding since the fluorescence was much reduced in the presence of 10 \textmu M [d-Ala\textsubscript{6}]GnRH or native GnRH (Fig. 2B). If the cells that had bound [Rhod-D-Lys\textsuperscript{6}]GnRH at 4°C were then warmed to 37°C, aggregation (Fig. 2C) and subsequent internalization (Fig. 2D) of the fluorescent peptide by granulosa cells could be observed. 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\textsuperscript{6}]GnRH at 23°C or 37°C. Cluster formation was observed at 23°C after 30–40 min (similar to Fig. 2C), while internalization into endocytic vesicles was observed after 30 min at 37°C. No further changes were seen during a further 60-min period of incubation at 37°C.

**DISCUSSION**

GnRH receptors in rat anterior pituitary and rat ovarian granulosa cells exhibit similar binding affinities for GnRH and its analogs (10–15). These data suggest the presence of similar binding sites for GnRH in these two locations. Our previous study (19) has indicated that photofinity labeling of GnRH receptors of plasma membrane preparations from pituitary glands of the rat results in identification of a single specific band that has an apparent \( M \text{r} \) of 60,000. The present study suggests that some differences exist between the GnRH binding sites of the pituitary and of ovarian granulosa cells, as the latter have an additional specific component of \( M \text{r} \), 54,000. This additional band is probably not a degradation product of the receptor since all studies were carried out at 4°C and this specific band was consistently present in all experiments. The \( M \text{r} \), 54,000 component may be related to the different functional effects of the hormone at the two locations (pituitary and ovary), while the common \( M \text{r} \), 60,000 band may be related to the similarity in binding and recognition properties toward the hormone. Because of the specialized portal blood system, it is likely that the endogenous GnRH secreted by the hypothalamus does not exert a significant effect on the gonad. Nevertheless, a substance from ovarian follicular fluid that has GnRH-like activity, but is immunologically distinct from GnRH, has been identified recently (23).
FIG. 2. Fluorescent visualization of [Rhod-D-Lys$^\text{a}$]GnRH binding to cultured rat granulosa cells. (Left) Phase-contrast micrographs. (x600.) (Right) Fluorescence micrographs of the same fields. Cells were incubated for 120 min at 4°C with 10 nM rhodamine derivative without (A) and with (B) 10 μM [D-Ala$^6$]GnRH. Cells in A were warmed to 37°C for 20 min (C) and for 30-40 min (D). Binding was terminated by rinsing the coverslips in buffer at 4°C followed by immediate fixation in 1% formaldehyde (30 min at 4°C).
The present study also shows that unoccupied GnRH receptors of the plasma membrane are diffusely and homogeneously distributed over the granulosa cell surface and that GnRH binding to cells at 4°C does not alter receptor distribution. Warming these cells to 37°C or binding of GnRH at 37°C causes 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. This pattern of receptor-mediated internalization of GnRH by granulosa cells is similar to the pattern observed in pituitary cells (20).

Recent studies have indicated that receptor crosslinking at the cell surface is by itself sufficient to trigger the subsequent biological events of hormone action in some systems (for review, see ref. 24). Although ligand internalization and degradation may occur, it is not a prerequisite for ligand function. On the other hand, in some other systems, internalization and degradation of hormone–receptor complexes is important for biological activity (24). In the pituitary, it has been recently shown that neither formation of large-scale clusters nor internalization of GnRH receptors are required for luteinizing hormone release (25, 26). Whether crosslinking, clustering, or internalization of GnRH receptors have any relevance to the biological function of GnRH in the ovary is currently not known.

We thank Dr. J. Schlessinger for providing the image-intensified fluorescence microscope. This work was supported by the Ford Foundation and the Rockefeller Foundation, New York.