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PITUITARY RECEPTOR BINDING ACTIVITY OF ACTIVE, INACTIVE, SUPERACTIVE AND INHIBITORY ANALOGS OF GONADOTROPIN-RELEASING HORMONE

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SUMMARY

We have previously described the binding of biologically active ^{125}I gonadotropin-releasing hormone to the 10,800 x g membrane fraction prepared from 7-day castrate adult female rat anterior pituitary glands. Specific binding with two equilibrium association constants (10^9 liters per mole and 10^5 liters per mole) was found and an equilibrium competitive binding radioreceptor assay established. In order to further characterize the gonadotropin-releasing hormone receptor, 20 synthetic analogs with known bioactivity were tested in the radioreceptor assay. In vivo biological activity correlated with high affinity receptor binding but not with low affinity binding. Inhibitory analogs with no in vivo biological activity and weak antagonistic properties did not bind, while in vivo active or superactive analogs bound to high affinity receptors. These findings suggest that the high affinity gonadotropin-releasing hormone receptor binds only biologically active gonadotropin-releasing hormone like peptides and that this binding may be the initial step in gonadotropin-releasing hormone actions at the pituitary level.

By analogy with other peptide hormones, the initial step in hypothalamic decapeptide GnRH[1] mediation of FSH and LH release from the pituitary is binding to a membrane receptor protein (1). The direct study of membrane binding of GnRH was initially unsuccessful or gave variable results because of the unavailability of a high specific activity radioactively-labeled GnRH which retained full biological activity. Our laboratory has previously reported the preparation of high specific activity ^{125}I GnRH with full biological activity suitable for direct studies of membrane binding (3). This was possible since monoiodination occurred at the tyrosine in the number 5 position which fortunately is not critical for receptor binding. Using this biologically active radiolabel, we have

[1] GnRH = gonadotropin-releasing hormone; FSH = follical stimulating hormone; LH = luteinizing hormone; AVP = arginine vasopressin; TRH = thyrotropin releasing hormone; SRIF = somatostatin; ACTH = adrenocorticophic hormone; KA = equilibrium association constant determined by Scatchard analysis (9).

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established a GnRH radioreceptor assay and found both high affinity (K_A=$10^9$1/mole) and low affinity (K_A=$10^5$1/mole) specific binding of GnRH using the 10,800 x g membrane fraction of rat anterior pituitary glands. In addition, low affinity (K_A=$10^5$1/mole) binding of GnRH was noted in membrane fractions of a number of tissues including liver, spleen, kidney and lung (4,5). Modulation of high affinity pituitary binding in castrate female rats following estrogen treatment in vivo or in vitro was noted, while low affinity binding was not affected by this treatment (6). Steps beyond GnRH binding are poorly described at present and controversy remains as to whether GnRH actions are mediated by adenyl cyclase or another second messenger (7).

In order to further characterize the GnRH receptor, the binding of twenty active, inactive, superactive, and inhibitory analogs of GnRH was studied in the radioreceptor assay and correlated with available data on the biological activity of these analogs.

MATERIALS AND METHODS

Materials: Synthetic GnRH was obtained from Bachem, Inc., Torrance, Calif. Twenty analogs of GnRH with known bioactivity synthesized by Dr. David H. Coy, Tulane University School of Medicine, New Orleans, Louisiana were obtained for study through the cooperation of the Contraceptive Development Branch, National Institutes of Child Health and Human Development, NIH, Bethesda, Maryland. Biological gonadotropin-releasing activity was determined by Dr. Coy using an immature male rat bioassay system previously described (2).

Iodination: Synthetic GnRH was iodinated as previously described (3) and separated from unreacted GnRH by chromatography on CM-25 Sephadex (Pharmacia Inc., N.J.). The major peak yielded monoiodinated tyrosine following pronase digestion and migrated as a single peak when chromatographed on QAE Sephadex (Pharmacia Inc., N.J.) at pH 9.2.

Animals and Tissue Preparation: 150 gm female Wistar rats (Hilltop Laboratories) castrated seven days previously were decapitated. Their pituitary glands were harvested and placed in Medium 199 (Gibco Inc., Berkeley, CA) at 4° C within

one minute. The glands were homogenized gently with a Dounce homogenizer on ice and centrifuged at 900 x g for 5 minutes. The supernatant was then centrifuged at 10,800 x g for 10 min. The pellet was resuspended in assay buffer (.01 M Tris HCl, 1 mM $MgCl_2$, 1mM Dithiothreitol, pH 7.8) and centrifuged at 10,800 x g for 15 min. This pellet was resuspended in assay buffer at a concentration of 700 µg/ml protein as determined by the Lowry method (8). This required approximately one anterior pituitary per assay tube.

Radioreceptor Assay Protocol: 12 x 75 mm polypropylene test tubes (Falcon Plastics) were pre-soaked in 1% bovine serum albumin (Miles-Pentex, Ill.) for 24 hr. Each assay tube contained 200 µl assay buffer, 100 µl of various concentrations of synthetic GnRH or GnRH analogs, 100 µl ^{125}I GnRH (ca. 4,000 cpm) and 400 µl of the 10,800 x g membrane pellet prepared as described above. This mixture was incubated at 4° C for 30 min. The assay tubes were then centrifuged at 15,000 x g for 10 min to separate bound and free hormone. Both the counts bound in the pellet and the supernatant were counted. At each concentration of GnRH, GnRH analog, or zero-added peptide triplicate binding determinations were made. Analogs with similar structures or biological activities were stratified through different assays. Binding affinity for each analog was determined by Scatchard analysis (9).

RESULTS

While low affinity ($K_A=10^5$ l/mole) competitive binding in the GnRH radioreceptor assay was noted for all the analogs tested (see table I), high affinity binding ($K_A=10^9$ l/mole) was only noted for active or superactive analogs (fig. 1A and 1B). Inactive analogs did not compete for binding to high affinity receptors as shown in figure 1C. These analogs did competitively displace GnRH in the microgram range corresponding to low affinity binding.

At the amino terminus of the decapeptide, modifications in the number one position decreased or abolished biological activity and high affinity binding proportionately. The basic histidine residue in the 2 position

Table 1. Binding Activity and Biological Activity of GnRH Analogs

Analog	Bioactivity (relative to GnRH)	Receptor Binding Activity 10^9 l/mole	10^5 l/mole
GnRH	100%	100%	100%
Hyp^{1}GnRH	0.001%	0%	100%
AcSar^{1}GnRH	72%	50%	100%
Dphe^{2}GnRH	inhibitor	0%	100%
des His^{2}Dleu^{6}GnRH	inhibitor	0%	100%
5F-Trp^{3}GnRH	5%	0%	100%
Asn^{4}GnRH	inactive	0%	100%
Me^{5}Phe^{3}GnRH	70%	0%	100%
D Me^{5}Phe^{6}GnRH	1400%	100%	100%
D leu^{6}desGly^{10}GnRHEA	2000-3000%	100%	100%
D phe^{6}desGly^{10}GnRHEA	2000-3000%	100%	100%
des Gly^{10}GnRHEA	500%	100%	100%
D leu^{6}GnRH	600%	100%	100%
D leu^{6}NMeleu^{7}GnRH	600%	100%	100%
D leu^{6}D arg^8 GnRH	1%	0%	100%
Hyp^{9}GnRH	5%	0%	100%
Dphe^{2}phe^{3}Dphe^{6}GnRH	inhibitor	100%	100%
Dtrp^{2}phe^{3}Dphe^{6}GnRH	inhibitor	0%	100%
Dphe^{2}Dtrp^{3}Dphe^{6}GnRH	inhibitor	0%	100%
Dphe^{2}pro^{3}Dphe^{6}GnRH	inhibitor	0%	100%
Dphe^{2}Dpro^{3}Dphe^{6}GnRH	inhibitor	0%	100%

In each case displacement at both high and low affinity was assessed. Each result is based on two separate radioreceptor assays with triplicate determinations at each concentration of GnRH or analog added. Results listed as 100% were not significantly different from the binding characteristics of GnRH itself. All those listed as 0% showed no significant displacement of counts up to 1000 pg/ml. added GnRH analog. Only AcSar^{1}GnRH (Fig. 1A) showed intermediate binding (ca. 50%), significantly different from GnRH.

was also essential for binding and activity. The modification of serine to asparagine in the number 4 position abolished biological activity and high affinity binding. This modification was basically a residue substitution of an amino group for a hydroxyl group. Modifications in the central region

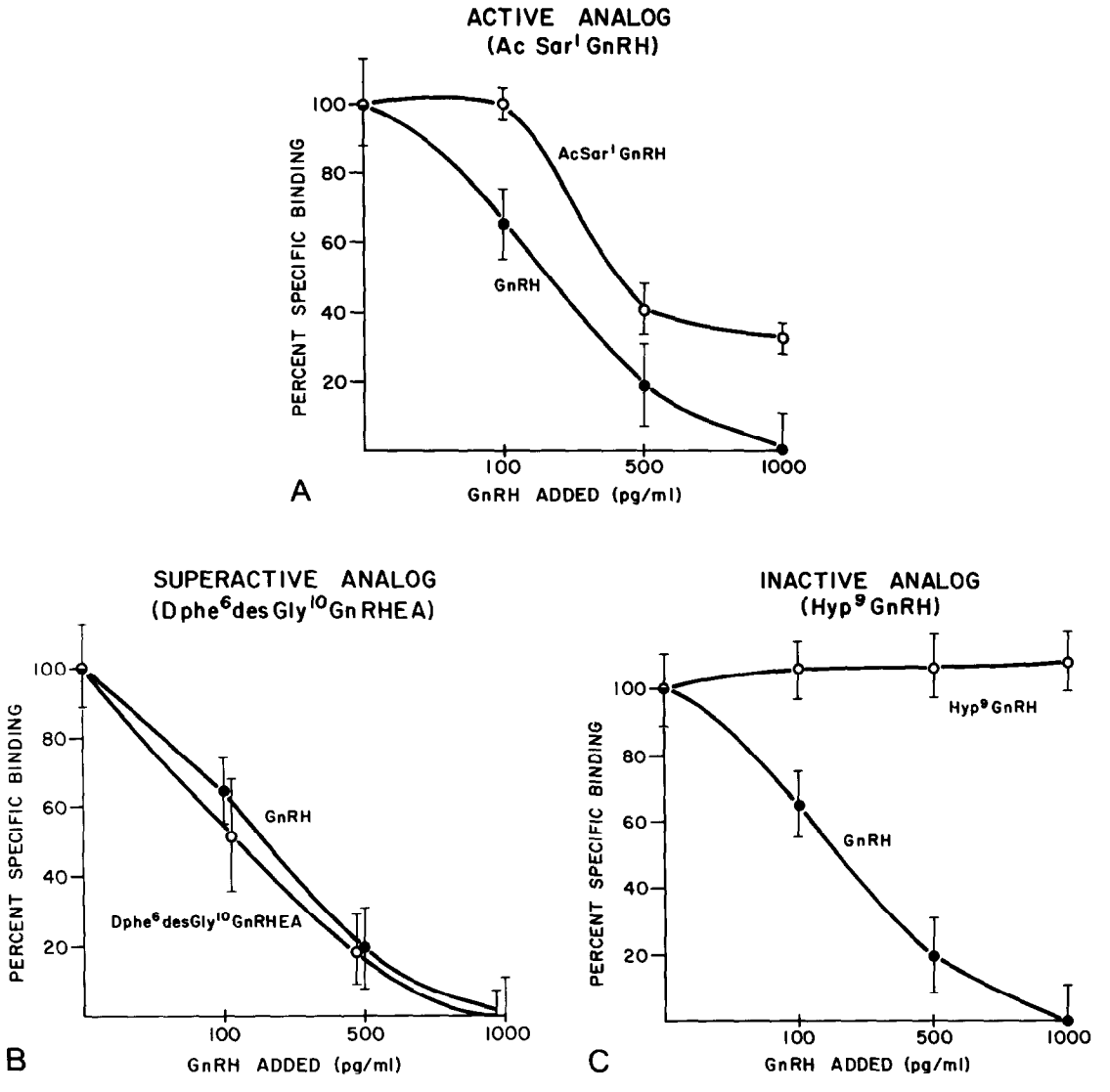

Fig. 1 High affinity binding of GnRH and analogs A) Active analog B) Superactive analog C) Inactive analog. Percent specific binding on the ordinate normalized between 0 added GnRH or analog and 1000 pg/ml. peptide concentration added shown on the abscissa. GnRH is shown for comparison in each case.

of the peptide (positions 5, 6 and 7) did not decrease binding or activity. Arginine in the 8 position and proline in the 9 position were critical for binding and activity. At the carboxy terminus the ethylamide substitution in the ten position increased bioactivity but resulted in equivalent competition

for high affinity binding (see fig. 1B). Analogs designed to antagonize GnRH but with no intrinsic biological activity did not bind to the high affinity receptor.

DISCUSSION

Biological activity correlated well with high affinity binding by the anterior pituitary membrane fraction. These findings suggest that the site discriminates among chemically similar peptide analogs based on their ability to fit the binding site stereochemically. This discrimination was highly selective distinguishing L-arginine from D-arginine and serine from asparagine. In addition, the biological activity of those analogs which fit well suggests that initial binding by the pituitary is necessary for GnRH actions on the gonadotroph cells.

Low affinity binding did not correlate with biological activity. Low affinity binding in the pituitary occurring together with high affinity binding has been observed by other investigators in the ovine (10) and bovine (11) pituitary. We have noted low affinity binding alone in a number of non-pituitary tissues including liver, kidney, spleen and lung, and postulated that this binding may have a role in the metabolic clearance or degradation of GnRH. Degradative enzymes derived from the hypothalamus (12) have recently been shown to act on GnRH analogs, and the low binding affinity is similar to that found for a number of enzyme systems. A simple high-capacity low affinity reservoir for GnRH would explain the low circulating levels of GnRH and its multiphasic clearance curve. Alternatively, this binding may represent a receptor directed at a closely related peptide which is cross-reacting with GnRH. However, AVP, TRH, SRIF, ACTH, prolactin, LH and FSH do not react with either low or high affinity in this system. A previous study of GnRH binding by Pedroza et al. (13) indicated that both a superactive and inhibitory GnRH analog displaced ^{125}I GnRH more effectively in a similar crude membrane preparation from rat anterior pituitary than did GnRH itself. Independent observations by that group have since yielded results similar in essence to those reported here (14).

In summary, new evidence is presented here that high affinity GnRH binding represents GnRH receptor activity which may, in turn, mediate the biological actions of GnRH on the pituitary gland. The low affinity binding observed is less specific, but is specific to GnRH-related analogs only. It may best be considered a GnRH acceptor site without a known physiological role at present. Further study of the steps beyond receptor binding will be necessary to elucidate the mechanisms involved in receptor-mediated transduction of GnRH activity.

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