PRODUCTION AND CHARACTERIZATION OF AN ANTISERUM TO SYNTHETIC GONADOTROPIN-RELEASING HORMONE

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SUMMARY

The synthetic decapeptide "luteinizing hormone-releasing hormone" (LH-RH) was rendered antigenic by reaction of its histidine or tyrosine residues (7 : 3 approx.) with p-diazonium phenylacetic acid and coupling of the azo-derivatives formed to bovine serum albumin (BSA). Immunization of rabbits yielded antisera that bound ^{125}I-labeled LH-RH (approx. 50 pg) at dilutions up to 1:200,000 and showed no cross-reaction with unrelated hypothalamic and pituitary hormones, extracts from rat cerebral cortex, and with small fragments of LH-RH. Cross-reaction was minimal (0.2%) with the free acid analogue of LH-RH, and moderate with des-pGlu LH-RH (20%), des-pGlu-His-LH-RH (2.4%) and with LH-RH analogues in which a single residue (No. 4-6 or No. 8) was exchanged by an amino-acid of similar character (1.2-12%). Biologically active hypothalamic extract and LH-RH produced parallel ^{125}I-LH-RH-binding inhibition curves, providing immunochemical support for the identity of the native releasing hormone with synthetic LH-RH.

Recently the structure of a molecule isolated from porcine (1) and ovine (2) hypothalamus has been elucidated, which is endowed with both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) releasing activity. This molecule, a decapeptide, was synthetized and named gonadotropin-releasing or LH-releasing hormone (LH-RH), inferring that it plays a part in the physiological control of pituitary secretion. This paper describes the synthesis of an antigenic protein-conjugate of this oligopeptide and the specificity of a potent antiserum generated by this antigen in rabbits. Such an antiserum should be useful for establishing the physiological function of LH-RH, for studying its cellular localization and for the development of a radioimmunoassay procedure for this hormone.

MATERIALS AND METHODS

Preparation of antigenic conjugate of LH-RH. p-Diazonium phenylacetic acid was attached to synthetic LH-RH (a generous gift of Dr. N. Yanaihara) and the resulting azo-derivative was coupled to bovine serum albumin (BSA) (Fig. 1). p-Aminophenyl acetic acid (10 μmol) in 25 μl of cold 2N-HCl was diazotized by addition of sodium nitrite (10 μmol) in cold water (25 μl; 4°C). After standing for 8 min at 4°C, 75 μmol

$NaHCO_3$ dissolved in 150 μl ice-cold water (pH 8.5) was added. This reaction mixture was immediately combined with a solution of LH-RH (11.8 mg, i.e. approx. 10 μmol) in 90 μl of 60% aq. N,N'-dimethylformamide containing 20 μmol $NaHCO_3$. The colorless solution turned orange-brown within a few min. The reaction was allowed to proceed for 12 h at 4°C. The mixture was then acidified to pH 2 with 2N-HCl and extracted several times with 2 vol. ether. The ether washes were discarded, and the aqueous phase adjusted with 0.5M-$NaHCO_3$ to a neutral pH. Analysis of this solution by TLC in the solvent systems (i) acetic acid:n-butanol:ethyl acetate:water (1:1:1:1, v/v) and (ii) ethanol:water (7:3, v/v) revealed the presence of a single brownish component moving faster than LH-RH in both systems.

The product was attached to BSA (Crystalline grade, Miles Labs., Kankakee, Ill.), using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl at pH 5.0 (3).

Immunization. Five mature rabbits (3 females and 2 males) were given two sets of multiple intradermal injections of the LH-RH--BSA conjugate in complete Freund's adjuvant, 10 days apart, using on each occasion a total of 0.25 mg of the antigen supplemented with 1.6 units of a vaccine against Hemophilus pertussis (Pertussis Vaccine Fluid, U.S.P., Eli Lilly & Co., Indianapolis). The rabbits were bled at two-week intervals and the sera were examined for the presence of antibodies.

Iodination of LH-RH. LH-RH was labeled with ^{125}I using the chloramine T method (4): LH-RH (2 μg) in 50 μl of 0.5M sodium phosphate buffer (pH 6.9) was allowed to react with 2 mCi of Na ^{125}I (IMS.30, 1.7 x 10^4 Ci/gm, 100 mCi/ml; Amersham, England) in the presence of 50-100 μg of chloramine T (5-10 mg/ml) for 30 seconds at 4°C. The reaction was stopped by the addition of 150 μg sodium metabisulfite (5 mg/ml). The labeled hormone was separated from free iodide by application to a Sephadex G-10 column (0.5 x 7.0 cm) and elution with 0.01 M phosphate-buffered saline (PBS), pH 6.9. Specific activities of 300-360 μCi/μg were achieved.

Radioimmunoassay. Each assay tube received a known amount of unlabeled LH-RH, or of a heterologous peptide, or an aliquot of a tissue extract, in a total volume of 0.5 ml PBS (pH 6.9); "blank" tubes received only the buffer. A solution of ^{125}I-labeled LH-RH (about 50-100 pg in 0.1 ml) was added, followed by 0.2 ml of antiserum appropriately diluted with 0.05 M - EDTA in PBS containing 1% of normal rabbit serum. (The dilution of the antiserum was chosen so as to yield 40-50% binding of the labeled hapten in the absence of unlabeled peptide). This mixture was incubated for 4 h at 37°C with shaking. An adequate amount of goat antiserum to rabbit gamma-globulin, made up to 0.2 ml with PBS, was then added and the incubation continued for 15 h at 4°C. Cold PBS (1.5 ml)

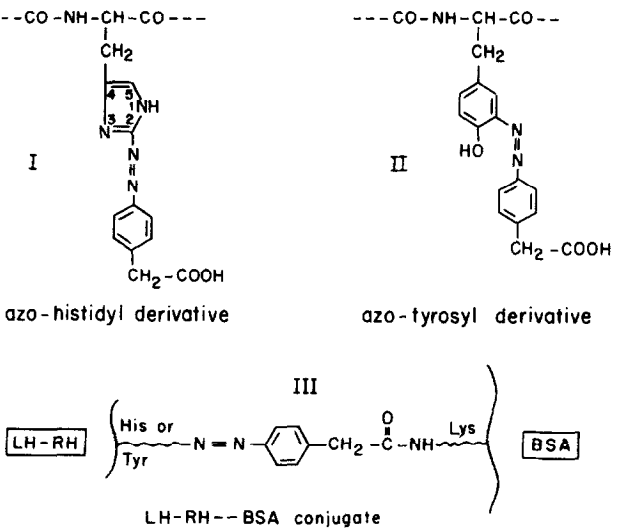

Fig. 1. Mode of coupling of LH-RH to BSA (schematic).

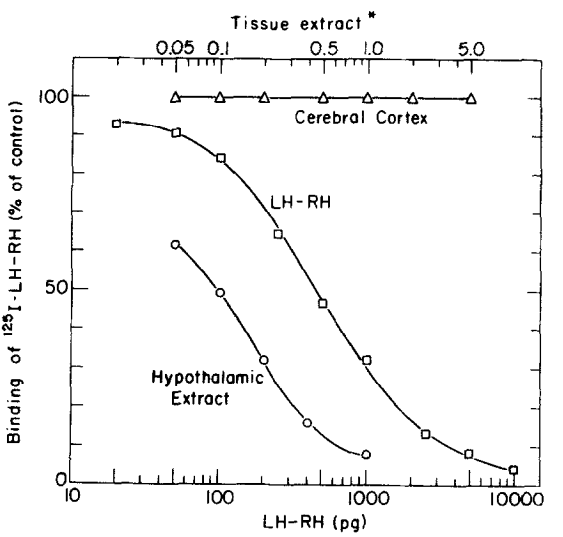

Fig. 2. Inhibition of binding of ^{125}I-LH-RH to antiserum by (i) unlabeled LH-RH, (ii) rat hypothalamic extract (NIAMD-Rat HE-RP-1) and (iii) extract of rat cortex (control). Anti LH-RH--BSA serum (No. 5) used at 1:20,000 dilution.
(*) Units indicate number of hypothalami or weight of cerebral cortex (in multiples of 30 mg) from which the extract assayed was derived.

was added, and the tubes were centrifuged at 1,200 x g for 30 min. The supernatants were discarded by aspiration and the radioactivity in the precipitates measured in a Packard Auto-Gamma Spectrometer.

Table 1. Inhibition of the binding of ^{125}I-LH-RH to antiserum by homologous hapten and by heterologous peptides. Serum to LH-RH--BSA (No. 4) used at 1:200,000 dilution.

No.	Compound	Relative Activity (%)
1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LH-RH)	100
2	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-COOH	0.2
3	pGlu-His-Trp-Thr-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	12
4	pGlu-His-Trp-Ser-Phe-Gly-Leu-Arg-Pro-Gly-NH_2	7.0
5	pGlu-His-Trp-Ser-Tyr-Ile-Leu-Arg-Pro-Gly-NH_2	6.4
6	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Lys-Pro-Gly-NH_2	1.2
7	H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	20
8	H-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	2.4
9	H-Leu-Arg-Pro-Gly-NH_2	1.8
10	pGlu-His-Trp	<0.05
11	H-Trp-Ser-Tyr-Gly-Leu-Arg-NH_2	
12	H-Trp-Ser-Tyr-Gly-OH	
13	pGlu-His-Trp-Ser-methyl ester	
14	pGlu-His	
15	Ser-Tyr	
16	TRH (pGlu-His-Pro-NH_2)	
17	MIF (Pro-Leu-Gly-NH_2)	
18	Vasopressin	
19	Oxytocin	
20	Bradykinin	
21	Eledoisin	
22	Luteinizing hormone (NIAMD-Rat LH-RP-1)	
23	Follicle stimulating hormone (NIAMD-Rat FSH-RP-1)	
24	Prolactin (NIAMD-Rat prolactin-RP-1)	

Compounds No. 7, 9, 11, 12 were gifts from Dr. N. Yanaihara, No. 3, 4 and 5 from Dr. A.V. Schally, No. 2, 8, 10, 14 and 15 from Dr. W.F. White and No. 6 and 13 from Dr. R. Geiger. Compound 16 was purchased from Calbiochem A.G., and 18-21 from Sigma Chemical Co. MIF was synthetized in our laboratory.

Tissue extracts. Rat hypothalamic extract was the reference preparation NIAMD-Rat HE-RP-1. Cerebral cortex of mature male rats was extracted with acetic acid in methanol (5).

RESULTS

Structure of antigen. Diazonium salts can react either with the histidine or the tyrosine residue of LH-RH (Fig. 1, I and II). Spectral analysis showed that at pH 6.3 the azo-LH-RH derivative absorbed maximally at 345 nm; at pH 12 the maximum shifted to 395 nm, with a shoulder at 425-450 nm. This bathochromic shift with increasing basicity is characteristic of azo-histidyl derivatives (6, 7). However, a slight increase in absorbance at 500 nm at the alkaline pH suggested a contribution of azo-tyrosyl linkages

which absorb at this wavelength (6, 7). These inferences were borne out by amino-acid analysis (8) of the azo-LH-RH derivative, which showed that modification of histidine residues and of tyrosine residues occurred in the ratio of 7:3.

Spectral analysis of the LH-RH--BSA conjugate at 340 nm indicated that on the average about 27 residues of LH-RH were attached to each molecule of the carrier protein.

Formation of antibodies to LH-RH. Antibodies binding ^{125}I-labeled LH-RH appeared in the serum of all five rabbits one month after the first immunizing injection. Four months later the serum of one male rabbit (No. 4) bound 50% of the labeled hapten (50-100 pg) at 1:200,000 final dilution; the corresponding titres of the sera from the other rabbits ranged from 1:4,000 to 1:100,000. Palpation of the testes of the two male rabbits immunized revealed extreme gonadal atrophy (diameter reduced to $<1/5$).

Specificity of the antisera to LH-RH. The extent to which the antiserum to LH-RH was able to bind heterologous peptides is shown in Table 1.

Presence of material immunochemically related to LH-RH in hypothalamic extract. A rat hypothalamic extract with LH-releasing activity (NIAMD-Rat HE-RP-1) inhibited the binding of ^{125}I-LH-RH by the antiserum to LH-RH--BSA. The inhibition curve was parallel to that given by unlabeled synthetic LH-RH (Fig. 2). An extract of rat cerebral cortex did not inhibit the binding of LH-RH.

DISCUSSION

The specificity of antibodies to a hapten protein conjugate is often influenced by the site through which the hapten is coupled to its carrier (9). A number of approaches have recently been used to render LH-RH antigenic:adsorption to Al_2O_3 (10); condensation to BSA with the carbodiimide reagent by an unspecified mechanism (11); coupling to BSA of the free carboxyl analogue of LH-RH by the mixed anhydride reaction (12) or of LH-RH itself using bis-diazotized benzidine (12). In the latter instance, coupling through the tyrosine and/or histidine residues of LH-RH seems likely, but since a symmetrical bifunctional reagent is used in a one-step reaction, dimerization and oligomerization of LH-RH cannot be ruled out. All these methods yielded antisera of low binding capacity: the titres reported ranged from 1:120 to 1:1,200. The antisera produced in the present experiments had titres of up to 1:200,000. An asymmetric bifunctional reagent was used in a two-step coupling procedure. Spectral and amino acid analysis indicated that this method, under the conditions chosen (pH 8.5), resulted in preferential formation of an azo-histidyl derivative and to a minor extent (30%) of an azo-tyrosyl derivative of LH-RH.

The antisera elicited by this antigen showed no cross-reaction (<0.05%) with four unrelated hypothalamic oligopeptide hormones (Table 1, 16-19) and with the nonapeptide bradykinin, the hendecapeptide eledoisin, the pituitary gonadotropins, or with small fragments of LH-RH (Table 1, 10-15). Moderate cross-reactions were observed with the des-pGlu nonapeptide (20%) and des-pGlu, His octapeptide (2.4%) derivatives of LH-RH . The antiserum described by Barker et al. (11) gave 100% cross-reaction with this octapeptide. However, it is not known whether these fragments occur in nature. Analogues in which single amino-acid residues No. 4-6 or No. 8 were exchanged by different amino-acids of similar chemical character (Table 1) gave only minor cross-reaction (1.2-12%). Interestingly, the LH-RH free acid (Table 1, No. 2) showed little competition (0.2%) with intact LH-RH for binding sites, suggesting that unmasking of the carboxyl group causes a conformational change in the decapeptide molecule, possibly by interaction with its basic residues. This lack of cross-reaction may be of practical importance , since deamidation of the C-terminal amino acid was shown to be an early step in the metabolism of TSH-RH (13). An antiserum generated with LH-RH-COOH failed to distinguish between this analogue and intact LH-RH (12), whereas that produced by use of the diazo-benzidine method (12) showed no such cross-reaction (0.6%).

The des-pGlu LH-RH derivative, which gave 20% cross-reaction with our antiserum, is devoid of LH-releasing activity (potency <0.01% of LH-RH; ref. 14). On the other hand, [Phe^5]-LH-RH shows only 7% cross-reaction with our antiserum, but has 44% the LH-releasing activity of LH-RH (15). Antibody and receptor often recognize different aspects of the hapten molecule (9).

The parallel ^{125}I-LH-RH-binding inhibition curves produced by unlabeled LH-RH and an extract of rat hypothalamus active in the LH-releasing assay indicate immunochemical similarity between synthetic LH-RH and the native releasing hormone.

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