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THREE LUTEINIZING HORMONE-RELEASING HORMONE LIKE SUBSTANCES IN A TELEOST FISH BRAIN: NONE IDENTICAL WITH THE MAMMALIAN LH-RH DECAPEPTIDE[1]

Faith H. Barnett, Joel Sohn, Seymour Reichlin and Ivor M.D. Jackson

Division of Endocrinology, Department of Medicine,
New England Medical Center Hospital, Tufts University
School of Medicine, Boston, Massachusetts 02111

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Acetic acid extracts of codfish brain contain three different immunoreactive (IR) luteinizing hormone-releasing hormone (LH-RH) like materials which differ from the mammalian LH-RH decapeptide by their behavior on chromatographic separation (molecular sieving, ion exchange and high performance liquid chromatography), and their reaction with two well characterized antisera directed against different regions of the LH-RH molecule. One of the IR LH-RH like substances is a larger molecular weight compound (1800 to 4,000 daltons), which may be a precursor of LH-RH extended at the N-terminus. The other two are approximately the same size as mammalian LH-RH and their nature is best explained by the occurrence of single amino acid substitutions at the C-terminal region of the 'authentic' decapeptide.

INTRODUCTION

In higher vertebrates the hypothalamus regulates the anterior pituitary gland through hypothalamic releasing hormones which are secreted into the pituitary portal vessel system for transport to the adenohypophysis (1). While it is generally accepted that the teleost adenohypophysis is also regulated by the hypothalamus this appears mediated in most instances by direct innervation or by paracrine (cell to cell) effects since interdigitation of hypothalamus with pituitary occurs in osteichthyes (2,3). The mammalian luteinizing hormone-releasing hormone (LH-RH) decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) first isolated from porcine and ovine hypothalami is biologically active in teleosts (4,5) and has been identified by radioimmunoassay (RIA) (6,7) and immunohistochemistry (8-10) in the hypothalamus and brain tissue of various species of bony fish. In recent studies

[1] All correspondence and reprint requests should be sent to Dr. I.M.D. Jackson at the above address.

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King and Millar (11) have reported on heterogeneity of LH-RH in vertebrate species and have shown that immunoreactive LH-RH in teleost fish brain is not identical to synthetic LH-RH as shown by lack of parallel displacement in LH-RH immunoassay curves, and by behavior in different chromatographic systems.

In this study we have examined the nature of LH-RH present in codfish brains using two well characterized antisera as molecular probes directed against different regions of the decapeptide. By means of molecular sieving, ion exchange chromatography and high performance liquid chromatography (HPLC) we have identified two LH-RH molecules which seem to differ from one another and from synthetic LH-RH by a single amino acid substitution, as well as a third immunoreactive material which appears to be a "big" LH-RH.

MATERIALS AND METHODS

Characterization of antisera to LH-RH

Antibody IJ-29 was raised in a rabbit following immunization of LH-RH conjugated to bovine thyroglobulin by the bis diazotized benzidine method (12). This antiserum does not recognize the deamidated free acid form of LH-RH and has been used to study LH-RH distribution by RIA, immunohistochemical localization and physiologic importance in a number of vertebrate species (13-15). Antibody R-42 provided by Dr. T. Nett, has been extensively utilized by many investigators to study LH-RH (16,17). The structural requirements of each of these antibodies have been fully determined (17). R-42, a conformational antibody, requires an intact N-terminus and C-terminus, but will react against a decapeptide with selective amino acid substitutions. In contrast, IJ-29 is a sequential antibody which reacts against the C-terminal tetrapeptide amide Leu^7-Arg^8-Pro^9-$GlyNH_2{}^{10}$; any changes at these loci will reduce or abolish immunoreactivity while changes at the 1-6 site are without effect. From a comparison of the antigenic requirements of these two antisera (Fig 1), it is apparent that a decapeptide with an amino acid substitution only at the Arg^8 or (less likely) Leu^7 locus would produce a substance recognized by R-42 but not IJ-29. Additionally, any N-terminal extension of LH-RH which would necessitate opening of the pyroglutamyl ring could be identified only by IJ-29 but not R-42.

Radio-Immuno Assays

The RIA for LH-RH follows the procedures of Nett and Adams (16) with minor modifications. The sample (or standard) in 0.2 ml phospho buffered saline (PBS) containing 0.1% gelatin, 0.1 ml ^{125}I-LH-RH (labeled by lactoperoxidase and purified on Sephadex G-25) and antibody (1:200,000 for R-42, 1:96,000 for IJ-29) are incubated for 24 hrs. Bound hormone is separated from free using dextran coated charcoal. The current sensitivity (least amount which can be distinguished from zero by greater than 2SD) is 1 pg for R-42 and 2 pg for IJ-29. All RIA determinations are calculated on a Hewlett-Packard 9830 A computer using a program derived by Rodbard (18) for this purpose. It should be noted that the determinations of the LH-RH like materials (LMs) in codfish brain are expressed in terms of synthetic LH-RH equivalents. The absolute levels of the peptide(s) may be higher.

Tissue Extraction

The procedures were modified from that previously utilized for the extraction of hypophysiotropic factors from porcine hypothalami (19). Preliminary studies were undertaken on goldfish (Carassius auratus) which were anesthetized on ice prior

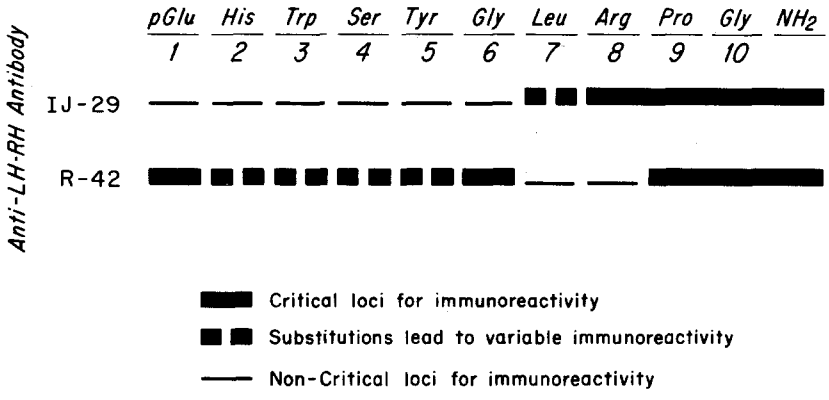

Fig 1. Characterization of the antigenic specificity of the LH-RH antisera utilized in this study. Adapted from the findings of Copeland et al (17).

to rapid removal of their brains for extraction and then characterization of the LH-RH-like material (LM). Following recognition of the lack of identity of goldfish LH-RH like substance(s) with synthetic LH-RH, subsequent studies were undertaken with codfish (Gadus morhua morhua) so enabling larger quantities of teleost brain to be processed. The extraction procedures undertaken were essentially similar for both species. After being caught, the codfish were kept on ice and the brains removed uaually 5 - 72 hrs after death. Immediately following removal the brain tissue was placed in 10 volumes of 1N acetic acid and frozen at -20C prior to transport to the laboratory in dry ice. The material was just allowed to thaw, homogenized in a Polytron (Brinkman) and boiled for 15 min. The homogenate was centrifuged, the precipate re-extracted with 1N acetic acid and both supernatants pooled and lyophilized. The dried extracts were reconstituted in 0.1N acetic acid, re-extracted with acetone (volumes 2:1) and then defatted with petroleum ether. The organic phase was discarded, the acetone removed by air flow and the residual phase lyophilized. This freeze dried material was reconstituted in 1N acetic acid for column chromatography.

Chromatography

The codfish brain extract was chromatographed on a Sephadex G-25 (Pharmacia) column (1 x 40 cm) in 1N acetic acid and the fractions assayed for LH-RH using IJ-29 and R-42 antisera. The only immunoreactive codfish LH-RH material recognized by IJ-29 eluted just after the void volume on Sephadex G-25. The molecular weight of this material was evaluated in the less polar Biogel (Biorad) systems (P-2, P-4, P-6) using 0.01 M ammonium acetate pH 4.7 as eluant. To exclude the possibility that this material was LH-RH aggregated or bound to a carrier protein, whole brain extract was incubated in dissociation conditions of 6M guanidine-HCl with 5% mercaptoethanol in 0.01 M acetic acid (4C, 5 hr) prior to Sephadex G-25 chromatography. The immunoreactive LH-RH-LM identified by R-42 eluting from Sephadex G-25 in a single peak was lyophilized and then re-constituted in 0.002 M ammonium acetate for cation exchange chromatography on a carboxy methylcellulose (CMC) (Sigma) column similar to that described by King and Millar (11). An 18 x 1 cm column was equilibrated at room temperature (22 ± 1C), the sample added and after the first 20 ml had been collected the solvent changed to 0.06 M ammonium acetate. The major peak of LH-RH-LM on CMC was further processed on HPLC utilizing a reversed phase C_{18} μ-Bondapak column 3.9 mm x 30 cm, solvent ethanol 25% in 0.01 M ammonium phosphate, pH 4.6, flow rate 1.5 ml per minute and pressure 1500 psi.

RESULTS and DISCUSSION

When assayed by IJ-29 a relatively small quantity of immunoreactive (IR) LH-RH eluted shortly after the void volume but no other material reacting with this anti-

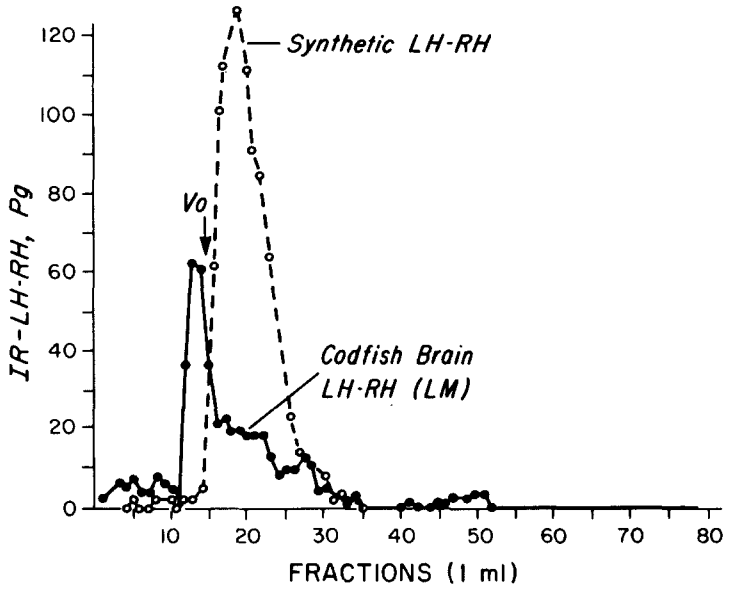

Fig 2. Chromatography of an acetic acid extract of codfish brain on a Biogel P2 column (exclusion limits 1800 daltons) utilizing 0.01M ammonium acetate as eluant. Void volume was determined using blue dextran dissolved in eluant buffer. The fractions were assayed using anti-LH-RH antibody LJ-29 which requires an intact C-terminus for recognition but is unaffected by changes at the N-terminal end of the molecule. A single LH-RH-LM peak was identified which came out in the void volume, whereas synthetic LH-RH (molecular weight 1182) eluted after the void volume.

body was found. Treatment with guanidine HCl did not alter the early elution pattern, indicating that it is neither an LH-RH aggregate, nor bound to a larger carrier protein. Since this material was excluded from Biogel P2 (Fig 2) but not Biogel P4 the molecular weight of this immunoreactive LH-RH-LM is estimated to lie between 1800 and 4,000 daltons - higher than that of synthetic LH-RH (1182 daltons). In view of its reactivity with LJ-29 this substance must have an intact C-terminus, while its size indicates that it is not a fragment. We propose that this particular LH-RH-LM is a precursor of the "authentic" LH-RH decapeptide with an extension at the N-terminus, comparable to the N-terminal extended somatostatin-28 described by Pradayrol et al (20) in porcine intestinal extract. We estimate 'big' LH-RH-LM constitutes less than 5% of the total LH-RH-LM present in teleost brain (see below). Whether this substance is processed further in teleost brain is unknown. It should be noted that Millar et al (21) have described the presence of higher molecular weight immunoreactive species of LH-RH in ovine brain and speculated that they were possible precursors of the decapeptide.

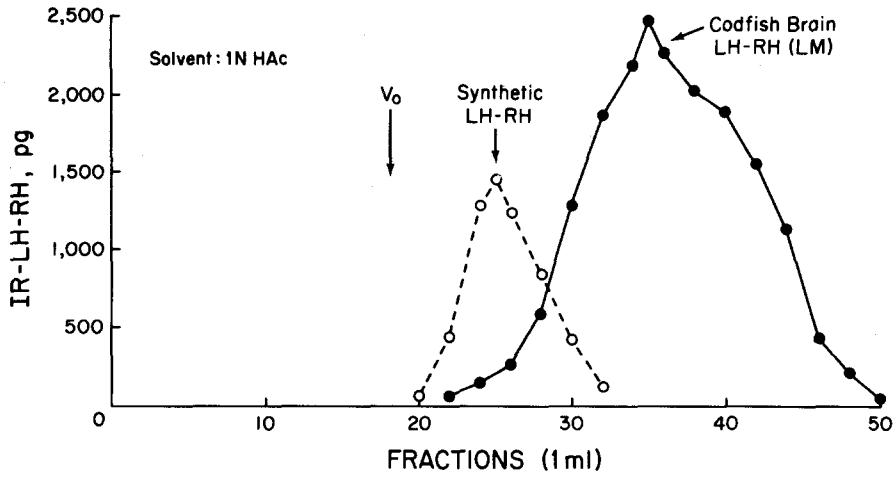

Fig 3. Sephadex G-25 chromatography of codfish brain extract in 1N acetic acid (HAc) compared with synthetic LH-RH. The immuno-reactive (IR) LH-RH-like material (LM) present in codfish brain eluted later than synthetic LH-RH in this system. The antibody used in the RIA was R-42 (see text and Fig 1 for details of antigenic specificity). Not shown in this figure is the higher molecular weight species of IR-LH-RH-LM which elutes at the Vo and is recognized by antibody IJ-29 but not R-42.

In contrast to the findings with IJ-29, immunoassays utilizing the R-42 antibody revealed large quantities of LH-RH-LM which eluted just beyond the position of synthetic LH-RH on Sephadex G-25 (Fig 3). This Sephadex G-25 peak of LH-RH-LM was readily separable from synthetic LH-RH on ion exchange chromatography since it eluted in 2 peaks (Fig 4) prior to that of the LH-RH decapeptide, which is strongly retained on CMC. Peak I, the earlier minor peak, amounted to 14% of the LH-RH-LM immunoreactivity while Peak II, the major peak which has an intermediate affinity for CMC compared with Peak I and synthetic LH-RH constituted 86% of the LH-RH-LM immunoreactivity assayed by R-42. When Peak I was lyophilized and reconstituted in 1N acetic acid and re-chromatographed on Sephadex G-25 it eluted with the single fish brain LH-RH-LM peak as assayed by R-42. However, when this specific Sephadex G-25 peak was re-run on CMC it eluted solely as Peak I indication that it is a distinct molecular species of LH-RH-LM.

When Peak II on CMC - the predominant form of LH-RH-LM in codfish brain - was subjected to HPLC, codfish LH-RH exhibited an enhanced retention time as compared to synthetic LH-RH (Fig 5) - emphasizing the separate identity of this substance from the mammalian LH-RH. Our studies in the goldfish were qualitatively identical

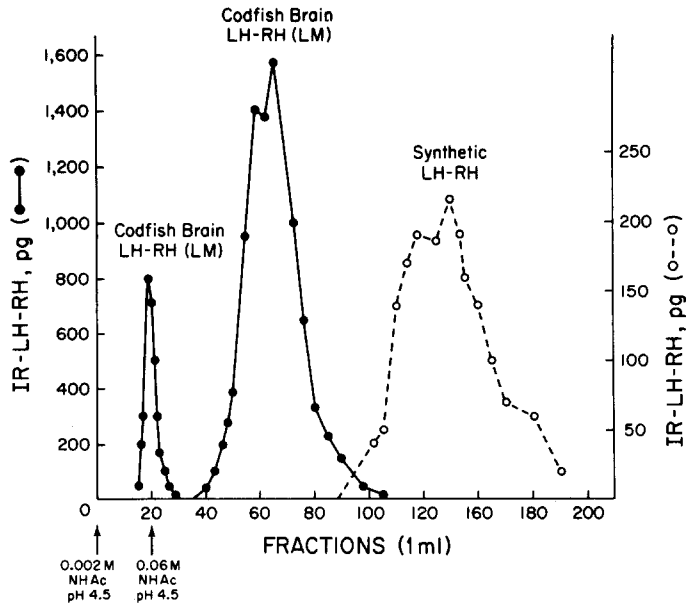

Fig 4. Elution profiles of codfish brain LH-RH-LM following Sephadex G-25 chromatography on CM cellulose cation exchange chromatography. The fractions were eluted with 0.002 M ammonium acetate (NH Ac) for the initial 20 ml and 0.06 M NH Ac. Note synthetic LH-RH is retarded compared with LH-RH-LM which elutes in two peaks -- a minor one at 20 ml and a major from 40 to 100 ml. IR-LH-RH was determined using antibody R-42. The peak 1 was not reactive with LJ-29 antibody and when lyophilized and rechromatographed on Sephadex G-25, it eluted identical to the single codfish brain LH-RH-LM (R-42) as shown on Figure 3.

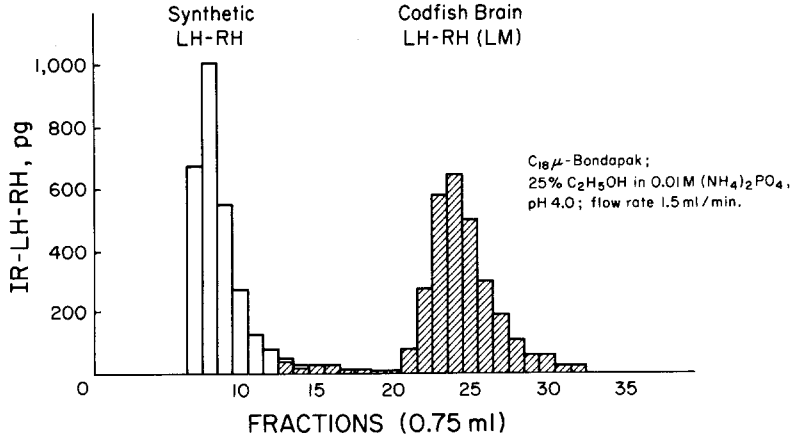

Fig 5. High performance liquid chromatography (HPLC) of the CMC peak 2 (see Fig 4) codfish brain LH-RH-LM on a C_{18} μ-Bondapak reverse phase column 3.9 mm x 30 cm using isocratic separation. Codfish brain LH-RH-LM and synthetic LH-RH were injected separately onto the column and the fractions were assayed for LH-RH by RIA using R-42 antibody. The codfish brain LH-RH-LM exhibits an enhanced retention time compared with synthetic LH-RH and the two substances can be readily separated from each other in this system.

with that obtained in the codfish suggesting that the presence of such LH-RH-LMs in the central nervous system (CNS) is likely a characteristic of the teleostei order.

The findings reported here thus provide evidence of three LH-RH like substances in fish brain and extend the report by King and Millar (11) of a single LH-RH-LM in teleost brain distinct from the mammalian decapeptide. Since the mammalian LH-RH is related, but not identical to any of the LH-RH-LMs we have been able to detect in the codfish brain, it is unlikely to be a significant physiologic teleost LH-RH; this provides a possible explanation for the need to administer higher doses of synthetic LH-RH per body weight to stimulate the pituitary-gonadal axis in bony fish than mammals (4,22).

The specificity of the antibodies utilized in this study allows us to propose definite hypotheses regarding the structure of the various LH-RH-LMs identified in the codfish brain. A substance recognized by R-42 but not LJ-29 could have an amino acid substitution in the LH-RH decapeptide at one of two sites - Arg^8 or Leu^7 with the antigenic determinants of the antisera favoring a point change at the Arg^8 locus (Fig 1). The elution profiles of codfish brain LH-RH on CMC provide evidence of two distinct LH-RH related peptides with different amino acid substitutions (probably at the same locus) in the C-terminal region of the mammalian decapeptide. The behavior of both these substances on cation exchange chromatography indicates that they are less positively charged than synthetic LH-RH while the retention on HPLC of the predominant codfish brain LH-RH-LM indicates a more hydrophobic material than synthetic LH-RH. These lead to the speculation that the major teleost brain LH-RH-LM has an amino acid substitution at the 8-position in the decapeptide-less positively charged and more hydrophobic than Arg. Confirmation of this view and determination of the nature of the amino acid point change requires amino acid analysis, which in itself may allow derivation of the codfish LH-RH-LM structure, as well as sequencing. Such studies are currently underway in our laboratory.

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