SYNTHESIS, BIOLOGICAL ACTIVITY AND RESISTANCE TO ENZYMIC DEGRADATION OF LUTEINIZING HORMONE–RELEASING HORMONE ANALOGUES MODIFIED AT POSITION 7

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1. Introduction

Luteinizing hormone–releasing hormone (LHRH) can be rapidly degraded by peptidases present in the hypothalamus and the anterior pituitary of the rat [1–4]. A specific endopeptidase has been recognized which initially cleaves the decapeptide pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 at the Gly^6–Leu^7 bond, yielding the corresponding hexa- and tetrapeptide fragments [1–3]. Since LHRH-degrading activity is present both at the site of synthesis and release of LHRH (the hypothalamus) and its site of action (the pituitary), it is possible that these peptidases play a physiological role in controlling the level of the peptide in the body.

Investigation of the pattern of biodegradation of the neurohormone may guide the design and the synthesis of stable, long-acting LHRH analogues with possible, valuable clinical applications. We have demonstrated a correlation between the increased biological potencies of some LHRH analogues (substituted at position 6 by D-amino acids) and their resistance to enzymic degradation [5]. Here we have synthesized 3 new LHRH analogues modified at position 7 by hydrophobic amino acids: [Phe^7]-LHRH, [Cha^7]-LHRH and [$cLeu^7$]-LHRH, and examined their biological activity and their stability to enzymic attack.

Abbreviations: Amino acid derivatives and peptides follow the IUPAC–IUB Commission of Biochemical Nomenclature and Symbols as outlined in Eur. J. Biochem. (1972) 27, 201–207 and J. Biol. Chem. (1975) 250, 3215–3216; Cha, cyclohexyl-L-alanine; cLeu, cycloleucine; DMF, *N,N'*-dimethylformamide; DCC, *N,N'*-dicyclohexylcarbodiimide; Dnp, 2,4-dinitrophenyl

2. Materials and methods

Cyclohexyl-L-alanine was a generous gift of Miles-Yeda, Rehovot. 1-Aminocyclopentane-1-carboxylic acid (cycloleucine) was purchased from Sigma Chemical Co., St Louis, MO.

2.1. *Synthesis of [Phe^7]-LHRH, [Cha^7]-LHRH and [$cLeu^7$]-LHRH*

Peptides were synthesized using the standard solid-phase procedure [6,7]. A chloromethylated copolymer of styrene and 1% divinylbenzene (200–400 mesh, 0.74 mmol Cl/g, Bio-Rad Labs., Richmond, CA) was substituted to a level of 0.53 mmol Gly/g. Amino acids were then successively coupled, as their N^{α}-*t*-butyloxycarbonyl derivatives to the glycine–resin by activation with DCC [6,7]. Sidechain functions were protected as follows: histidine with N^{im}-Dnp; serine with *O*-benzyl; tyrosine with *O*-benzyl; arginine with N^{G}-nitro. Cleavage of the protected peptides from the resin was achieved by reaction with a saturated solution of ammonia in methanol (2 days at 4°C). Under these conditions, the N^{im}-Dnp protecting group of histidine was also removed. Treatment of the cleaved products with liquid HF containing 10% anisole (1 h at 0°C) [8] resulted in removal of all other protecting groups.

The crude peptides were purified by preparative high-voltage paper electrophoresis (Whatman no. 3 paper, 60 min at 60 V/cm) in pyridine–acetate buffer (pH 3.5); the overall yield of synthesis being 48%, 45% and 40% for [Phe^7]-LHRH, [Cha^7]-LHRH and [$cLeu^7$]-LHRH, respectively.

2.2. *Biological assay*

The peptide analogues were tested for their LH-

releasing activity [9]. Pituitaries from 12-day-old female rats were incubated in 1 ml Krebs–Ringer bicarbonate containing 1 mg glucose/ml (KRBG, pH 7.4), for 2 h at 37°C under an atmosphere of 95% O_2:5% CO_2. This medium was then decanted and substituted by 1 ml KRBG containing LHRH or an LHRH analogue (1×10^{-9}–5×10^{-7} M), and incubation was continued for 90 min. Aliquots of the medium were then analysed for LH by radioimmunoassay, using the kit kindly supplied by the National Institute of Arthritis and Metabolism and Digestive Diseases (NIAMDD), Rat Pituitary Hormone Program.

2.3. *Enzyme preparation*

Anterior pituitaries (~9 mg each) from 5 rats were detached immediately after decapitation and immersed in a Thomas homogenizing vessel containing 1 ml phosphate-buffered saline (PBS, pH 6.9). The tissue was homogenized (10 strokes) using a teflon pestle, and then centrifuged for 10 min at 17 000 × *g*. The resulting supernatant was adjusted by addition of PBS to 30 mg wet wt tissue/ml.

2.4. *Degradation reaction*

Degradation was performed essentially as in [1]. Briefly, LHRH or its analogue (500 ng) was incubated in 1 ml final vol. PBS with enzyme preparation equivalent to 3, 1.5 and 0.75 mg tissue. The reaction was allowed to proceed for 20 min at 37°C and was terminated by placing the tubes in boiling water for 3 min. The tubes were centrifuged for 10 min at 3000 × *g* and the residual LHRH in the supernatant was determined by radioimmunoassay [10].

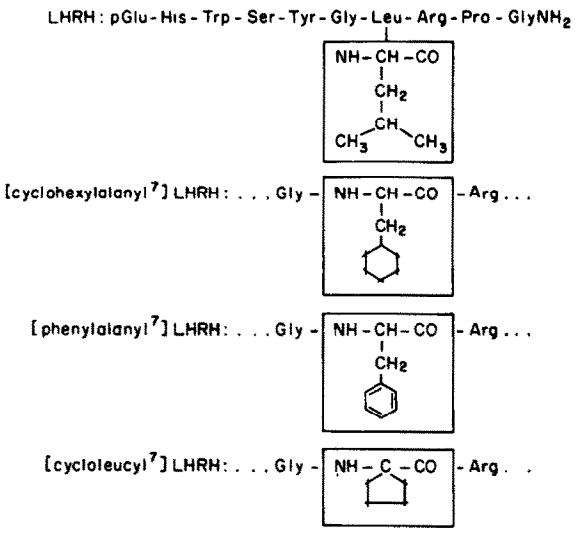

Fig.1. LHRH analogues modified at position 7.

Table 1
Amino acid analyses and electrophoretic mobilities of LHRH and its analogues

		LHRH[c]	[Phe⁷]-LHRH	[Cha⁷]-LHRH	[cLeu⁷]-LHRH
Electrophoretic mobility (R_{LHRH})[a]		1.00	1.00	0.96	1.06
Amino acid analyses[b]	Glu	1.02	0.95	0.96	0.98
	His	1.01	0.94	0.96	1.03
	Ser	0.98	0.96	0.94	0.99
	Tyr	1.01	0.95	0.95	0.98
	Gly	2.00	2.08	2.07	1.97
	Arg	1.00	0.97	0.99	1.01
	Pro	0.98	1.00	1.11	1.06
	Leu	1.09	–	–	–
	Phe	–	1.05	–	–
	Cha	–	–	0.98	–
	cLeu	–	–	–	0.97

[a] Electrophoresis was performed on Whatman no. 3 paper for 1 h at 60 V/cm in pyridine–acetate buffer (pH 3.5)

[b] Amino acid analyses were performed on a Spinco-Beckman model 120C amino acid analyzer. The peptides assayed were hydrolysed with 6 N HCl containing 4% phenol in evacuated sealed tubes at 110°C for 20 h. Tryptophan was not determined

[c] LHRH (kindly donated by Ayerst Research Labs., St Laurent, Canada) was used as a standard

3. Results and discussion

Three analogues of LHRH modified at position 7 were synthesized by the solid-phase method: [Phe^7]-LHRH, [Cha^7]-LHRH and [cLeu^7]-LHRH (fig.1). The 3 peptides were homogenous as determined by thin-layer chromatography, using 3 independent solvent systems: (1-butanol:acetic acid:water, 4:1:1, by vol.); (1-butanol:acetic acid:pyridine:water, 15:3:10:12, by vol.); (chloroform:2% acetic acid:methanol, 12.4:9, by vol.); and by high-voltage paper electrophoresis (table 1). Amino acid analyses showed ratios close to the theoretical (table 1).

The LH-releasing activities of [Phe^7]-LHRH, [Cha^7]-LHRH and [cLeu^7]-LHRH as tested by the in vitro biological assay (see section 2) at several dose levels were found to be 50%, 66% and 51% of that of LHRH, respectively.

In the standard pituitary extract degradation assay (see section 2), 64% of LHRH was decomposed. The relative degradation of the analogues, expressed as a percentage of LHRH cleavage under identical conditions, are as follows: [cLeu^7]-LHRH, <3%; [Phe^7]-LHRH, 18% and [Cha^7]-LHRH, 65%.

The relative stability of [cLeu^7] and [Phe^7] LHRH analogues, and to a much lesser extent that of [Cha^7]-LHRH, again indicates that indeed the Gly^6–Leu^7 bond is the preferred site of enzymic attack. However, in contrast to analogues modified at position 6, the resistance to enzymic degradation was not accompanied by an increase in biological potency. It is possible that the steric hindrance introduced into the LHRH molecule by the cyclic moieties at position 7 (Phe, Cha, cLeu) restricts the peptide's free rotation, preventing it from assuming an 'active' conformation and thus interfering with the binding to the pituitary receptor.

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