Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 753-759

DEGRADATION OF LUTEINIZING HORMONE - RELEASING HORMONE AND ANALOGS BY ADENOHYPOPHYSEAL PEPTIDASES

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Received March 9, 1981

SUMMARY

The degradation of Luteinizing Hormone - Releasing Hormone (LH-RH) and several analogs by the adenohypophyseal pyroglutamate aminopeptidase, "LH-RH degrading endopeptidase" and post-proline-cleaving enzyme has been investigated using a fluorescamine-based assay. The rates of hydrolysis of these peptides have been compared, and the influence of amino acid substitutions or deletions in LH-RH on the enzymatic reactions is discussed. Superactive agonists modified in position 6 and 10 are degraded more slowly, but there is no strict colinearity between the resistance to degradation and the prolonged and enhanced biological activity of these analogs.

INTRODUCTION

After the elucidation of the structure of LH-RH [1] (<Glu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$) (1,2) many analogs have been synthesized and tested for biological activity. Analogs containing D-amino acids in position 2 and 3 were found to act as antagonists, whereas analogs modified in position 6 and 10 have prolonged and enhanced LH/FSH releasing activity [for a review see (3)] . It has been suggested that the superactivity of

[1] LH-RH, Luteinizing Hormone - Releasing Hormone; Cbz-, benzyloxycarbonyl-; tBu, tertiary butyl; Me, methyl; EA, ethylamide; <Glu, pyroglutamic acid

0006-291X/81/100753-07$01.00/0

these agonists is principally due to a decreased rate of degradation (4-8). The use of crude tissue extracts and the small number of agonists tested, however, did not allow any detailed statements about this correlation. Since the degradation of LH-RH by adenohypophyseal extracts is mainly due to the pyroglutamate aminopeptidase, the "LH-RH degrading endopeptidase" and the post-proline-cleaving enzyme (9), we have investigated the degradation of LH-RH and several analogs by these enzymes.

MATERIALS AND METHODS

Materials: Fluorescamine was a product from Hoffmann-La Roche, Basel, Switzerland. LH-RH (2-10) nonapeptide amide and LH-RH (6-10) pentapeptide amide were purchased from UCB, Belgium; glycinamide and ethylamine from Fluka, West Germany. LH-RH, LH-RH (1-9) ethylamide, [D-Ala^6]LH-RH and [D-Phe^2,D-Ala^6]LH-RH were obtained from Bachem AG, Bubendorf, Switzerland. All other analogs were synthesized by conventional methods of peptide synthesis.

Determination of the bioactivities: The relative biological activities of the LH-RH agonists were determined as described (10).

Enzyme purification: The pyroglutamate aminopeptidase, the "LH-RH degrading endopeptidase" and the post-proline-cleaving enzyme were purified as described (11,12).

Degradation of LH-RH and analogs: All incubations were performed at 37°C.

The pyroglutamate aminopeptidase and the "LH-RH degrading endopeptidase" were incubated with 20-500 µM LH-RH or analog in 40 µl of 100 mM potassium phosphate buffer pH 7.4, 2 mM dithioerythritol, 2 mM EDTA and 50 mM potassium phosphate buffer pH 8.0, 1 mM dithioerythritol, respectively. After 0, 10, 20 and 30 min, aliquots of 5 µl were withdrawn from the reaction mixture and diluted with 200 µl of 100 mM sodium phosphate buffer pH 8.0. The samples were then rapidly mixed with 75 µl of a 0.03 % solution of fluorescamine in dry dioxane. The fluorescence of the samples was determined in a Perkin-Elmer fluorescence spectrophotometer MPF 44 (excitation at 390 nm, emission at 475 nm). For each experiment, a standard curve was constructed using various amounts of LH-RH (2-10) nonapeptide amide and LH-RH (6-10) pentapeptide amide, respectively, which were diluted in phosphate buffer and reacted with fluorescamine as described for the samples.

To determine the degradation of LH-RH and glycinamide containing analogs by the post-proline-cleaving enzyme, the enzyme was incubated with the substrates (1-50 µM) in 800 µl of 100 mM sodium phosphate buffer pH 7.4, 1 mM dithioerythritol. At given time intervals, aliquots of 100 µl were withdrawn from the reaction mixture and diluted with 100 µl of 100 mM sodium phosphate buffer pH 7.4. After mixing the samples with the fluorescamine solution, the fluorescence was determined as described above. The standard curve was constructed using various amounts of glycinamide.

The ethylamide containing analogs (10-400 µM) were incubated with the post-proline-cleaving enzyme in 80 µl of the buffer described above. Aliquots of 10 µl were withdrawn at the given time intervals and diluted in 90 µl of 100 mM sodium phosphate buffer pH 7.4. After addition of 30 µl of 500 mM potassium borate buffer pH 9.0 (yielding a final pH of 8.6), the samples were

mixed with the fluorescamine solution. Before determining the fluorescence, the pH of the samples was adjusted to 7.4 by addition of 70 µl of 50 mM phosphoric acid. The standard curve was constructed using various amounts of ethylamine.

The K_M and v_{max} values were determined on the basis of Lineweaver Burk plots. The relative rates of hydrolysis (v_{rel}) were calculated in the following way: Since the physiological concentration of LH-RH is far below the K_M values of the three peptidases, the Michaelis-Menten equation can be simplified to $v = v_{max} \times S / K_M$. The relative rate of hydrolysis of an analog then equals

$$v_{rel} = \frac{K_M(\text{LH-RH}) \times v_{max}(\text{analog})}{v_{max}(\text{LH-RH}) \times K_M(\text{analog})} \times 100\ \%$$

Inhibition of [^{3}H]LH-RH degradation by LH-RH and analogs: The test for the degradation of [^{3}H]LH-RH has been described previously (9). To determine the concentrations of the peptides leading to 50 % inhibition of the rate of degradation of [^{3}H]LH-RH by the individual enzymes (IC_{50}), various amounts of LH-RH and analogs (0.01 - 1 mM) were included in the incubation mixture. Since the reaction exhibits first order kinetics, the rate constants were calculated from the equation

$$k_1 = \frac{1}{t} \ln \frac{100}{100 - H}$$

where t is the time in minutes and H is the percent hydrolysis at time t.

Protein determination: Protein was determined according to (13).

RESULTS AND DISCUSSION

Due to the N-terminal pyroglutamic acid residue and the lack of lysine residues, LH-RH does not react with fluorescamine, a commonly used reagent for the sensitive assay of amino acids, peptides and other primary amines. Cleavage of any peptide bond (with the exception of the Arg^8-Pro^9 bond), however, leads to the formation of fragments containing a free amino group. Therefore the degradation of LH-RH and analogs can easily be monitored by determining the amount of generated fragments using fluorescamine. For several analogs which are not hydrolyzed by the pyroglutamate aminopeptidase and the "LH-RH degrading endopeptidase", we have determined their potency in inhibiting the degradation of [^{3}H]LH-RH by these two enzymes.

The pyroglutamate aminopeptidase (table 1) cleaves LH-RH at the $<Glu^1$-His^2 peptide bond (9). Modifications of LH-RH at position 6 only slightly influence the degradation by this enzyme, whereas LH-RH (1-9) ethylamide is degraded

Table 1. Degradation of LH-RH and analogs by the pyroglutamate aminopeptidase

Substance	Bioactivity (relative to LH-RH)	K_M (μM)	v_{max} (pmol/min×μg of protein)	IC_{50} (μM)	v_{rel} (%)
LH-RH	1	76	62	47	100
[des-His^2]LH-RH	(inhibitory)	(neglegible rate)		26	0
[D-Phe^2]LH-RH	(inhibitory)	280	4	n.d.	2
[D-Phe^2,D-$Trp^{3,6}$]LH-RH	(inhibitory)	312	12	n.d.	5
[D-<Glu^1,D-Phe^2,D-$Trp^{3,6}$]LH-RH	(inhibitory)	(neglegible rate)		210	0
Cbz-[D-$Ser(tBu)^6$]LH-RH (2-9) EA	0	(no hydrolysis)		>1000	0
[D-$Ser(tBu)^6$]LH-RH	70	62	64	n.d.	126
LH-RH (1-9) EA	5	109	41	n.d.	48
[D-$Ser(tBu)^6$]LH-RH (1-9) EA	150	72	45	n.d.	76

For details see under "Methods". n.d., not determined.

at a significantly lower rate than LH-RH. This effect may be brought about by steric interactions between the C- and N-terminus, which seem to be in close proximity in the active conformation of LH-RH (14). Analogs containing a D-Phe^2 residue are degraded very slowly. The D-<Glu^1 derivative is hydrolyzed at a neglegible rate, but it inhibits the degradation of [^{3}H]LH-RH, albeit much less than LH-RH itself. Cbz-[D-$Ser(tBu)^6$]LH-RH (2-9) ethylamide is not degraded by the pyroglutamate aminopeptidase and does not inhibit the degradation of [^{3}H] LH-RH. These results are in agreement with the known specificity of this exopeptidase for <Glu-X structures. The fact, however, that [des-His^2]LH-RH is degraded extremely slowly, shows that more detailed studies about the substrate specificity of this enzyme are necessary.

The "LH-RH degrading endopeptidase" (table 2) cleaves LH-RH preferentially at the Tyr^5-Gly^6 peptide bond and subsequently the resulting N-terminal fragment at the His^2-Trp^3 bond (12). In agreement with this we find that analogs containing D-amino acids in position 6 are not cleaved by this enzyme, but they are as effective as LH-RH in inhibiting the degradation of [^{3}H]LH-RH. Modifications at positions remote from the scissile peptide bond also influence the

Table 2. Degradation of LH-RH and analogs by the "LH-RH degrading endopeptidase"

Substance	Bioactivity (relative to LH-RH)	K_M (μM)	v_{max} (pmol/ min×μg of protein)	IC_{50} (μM)	v_{rel} (%)
LH-RH	1	200	1270	190	100
[des-His^2]LH-RH	(inhibitory)	250	3174	n.d.	200
[D-Phe^2]LH-RH	(inhibitory)	175	608	n.d.	55
[D-Phe^2, D-$Trp^{3,6}$]LH-RH	(inhibitory)	(no hydrolysis)		70	0
[D-Phe^2,D-Ala^6]LH-RH	(inhibitory)	(no hydrolysis)		190	0
[D-Ala^6]LH-RH	8	(no hydrolysis)		245	0
[D-$Ser(tBu)^6$]LH-RH	70	(no hydrolysis)		205	0
LH-RH (1-9) EA	5	71	794	n.d.	176
[$Ser(tBu)^7$]LH-RH (1-9) EA	20	100	866	n.d.	136
[D-$Ser(tBu)^6$]LH-RH (1-9) EA	150	(no hydrolysis)		180	0

For details see under "Methods". n.d., not determined.

degradation by this endopeptidase. It is interesting to note that analogs containing additional aromatic amino acid residues are more effective in inhibiting the degradation of [^{3}H]LH-RH. This agrees with preliminary studies (unpublished data), which indicate that enzyme - ligand binding is strongly influenced by hydrophobic interaction forces.

The post-proline-cleaving enzyme (table 3) hydrolyzes the Pro^9-Gly^{10} peptide bond of LH-RH (11). The K_M (2.3 μM) is extremely low for a proteolytic enzyme. This may explain the fact that the degradation of [^{3}H]LH-RH by tissue extracts is mainly due to this enzyme (15). The LH-RH (1-9) ethylamides are degraded very slowly. Modifications in positions remote from the scissile peptide bond also influence the degradation. As in the case of the "LH-RH degrading endopeptidase", these effects may not only be due to changes in the substrate conformation, but also to differences in the interaction of amino acid residues of the substrates with the substrate-binding region of these two endopeptidases. It should be noticed that, based on these effects, amino acid substitutions or deletions rendering analogs more resistant to degradation

Table 3. Degradation of LH-RH and analogs by the post-proline-cleaving enzyme

Substance	Bioactivity (relative to LH-RH)	K_M (μM)	V_{max} (pmol/ min×μg of protein)	v_{rel} (%)
LH-RH	1	2.3	2126	100
[D-Phe^2,D-Trp^{3,6}]LH-RH	(inhibitory)	0.7	1485	229
[D-Phe^2,D-Trp^{3,6},(N^{im}-Me)Leu^7]LH-RH	(inhibitory)	1.6	1143	77
[D-Phe^2,D-Ala^6]LH-RH	(inhibitory)	0.6	868	157
[D-Ala^6]LH-RH	8	6.1	1371	24
[D-Ser(tBu)^6]LH-RH	70	1.4	618	48
LH-RH (1-9) EA	5	30	1577	6
[Ser(tBu)^7]LH-RH (1-9) EA	20	34	457	2
[D-Ser(tBu)^6]LH-RH (1-9) EA	150	10	1201	13

For details see under "Methods".

by one enzyme may lead to an increased rate of hydrolysis by another enzyme (compare v_{rel} of [D-Phe^2,D-Trp^{3,6}]LH-RH and LH-RH (1-9) ethylamide for the individual enzymes).

It is interesting to note that LH-RH agonists which are more resistant to degradation by the "LH-RH degrading endopeptidase" and the post-proline-cleaving enzyme have a prolonged and enhanced LH/FSH releasing activity. Since modifications at position 2 yield antagonists, the pyroglutamate aminopeptidase cannot be considered in this context. Additional modifications of these antagonists at position 6 renders them also more effective (16). Therefore it is attractive to speculate that the "LH-RH degrading endopeptidase" and the post-proline-cleaving enzyme might be involved in the degradation of LH-RH in vivo.

Although the superactive agonists are more resistant to degradation, there is no strict colinearity between the biological activity and resistance to degradation. This indicates that resistance to degradation is not the only factor in rendering these analogs superactive, but that increased receptor binding or stimulation (16-19) must be considered also. Studies about the receptor binding activity of LH-RH and analogs, however, are hampered by

ligand degradation. The results presented in this paper may help to overcome these difficulties and to design superactive analogs for further studies.

ACKNOWLEDGEMENTS

We thank Drs. R. Geiger and W. König, HOECHST AG, Frankfurt for kindly providing several of the peptides tested here. We thank Dr. H. Kleinkauf for continual interest and the Deutsche Forschungsgemeinschaft for financial support.

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