LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH) ACTIVITY OF SOME SYNTHETIC POLYPEPTIDES. I. FRAGMENTS SHORTER THAN DECAPEPTIDE.

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SUMMARY. The luteinizing-releasing hormone (LH-RH) activity of 11 synthetic polypeptides, some of which were speculatively reported as having LH-RH activity, was assayed in vivo and in vitro against pure natural and synthetic LH-RH. In vivo tests showed that (pyro)Glu-Tyr-Arg-Trp-NH_2 had only 1 part in 7800 of the activity of the LH-RH decapeptide. (Pyro) Glu-Val-Ser-NH_2, (pyro)Glu-Ser-Val-NH_2 and (pyro)Glu-Gln-Ala-NH_2, were inactive in vivo in doses as high as 5 - 20 μg/rat. Synthetic (pyro)Glu-His-Pro-amide (thyrotropin-releasing hormone) and a synthetic decapeptide proposed as growth-hormone-releasing hormone showed no LH-RH activity in doses up to 100 μg. N-terminal tripeptide and tetrapeptide fragments of LH-RH as well as the C-terminal octapeptide of LH-RH were also inactive. The C-terminal nonapeptide had an extremely low LH-RH activity (about 1 part in 50,000). The structure-activity relationship of LH-RH has been briefly discussed.

The isolation and the determination of the structure of porcine luteinizing hormone-releasing hormone (LH-RH) in our laboratory (1,2,3,4) paved the way for its synthesis by our group (3,5) and subsequently by others (6,7,8,9,10,11,12,13). While all these syntheses were based on the decapeptide sequence established in our laboratory (3,4), it was also speculatively announced that tripeptides (pyro)Glu-Val-Ser-NH_2 and (pyro)Glu-Ser-Val-NH_2, implied to exist in hypothalamic extracts of sheep (14), as well as (pyro)Glu-Gln-Ala-NH_2 (15), have LH-RH activity. Similarly, Chang et al. (16) reported the synthesis of a tetrapeptide (pyro)Glu-Tyr-Arg-Trp-NH_2 and showed that it released LH in rats, but did not compare its activity with the LH-RH decapeptide. The synthesis of various analogs of LH-RH will be important in order to establish the structure-activity relationship for this hormone and for attempting to create a synthetic inhibitor of LH-release (17). This paper reports the results of quantitative assays of these and other synthetic polypeptides against natural and synthetic LH-R

MATERIALS AND METHODS

The natural LH-RH (AVS-77-33 #215-269) used in these experiments as a standard, was a pure material of porcine origin, isolated as described before (2), and homogeneous electrophoretically and chromatographically. Previously, we have shown that the LH-RH activity and FSH-releasing hormone (FSH-RH) activity of at least 5 synthetic preparations of LH-RH (5,6,7,11,12) made by classical or solid phase methods were quantitatively identical with those of the natural material (17,18). Other synthetic preparations of LH-RH (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 were used in these experiments. These were as follows: 1) A product from the solid phase synthesis of the LH-RH decapeptide (lot AVS-77-55 #215-271) conducted in our laboratory as described previously (5) was purified by preparative ion-exchange chromatography on carboxymethylcellulose followed by 900 transfers by counter-current distribution in 1-butanol : acetic acid : water = 4 : 1 : 5 (2) rather than by other methods (5,18). 2 and 3) Products of the syntheses of LH-RH by classical methods made by Geiger et al. at Farbwerke Hoechst (6) (lot R-4') and Immer et al. (13) at Ayerst and Co. (lot No. A.Y. - 24031 - 2). Other synthetic polypeptides were obtained or prepared as follows:

1. (pyro)Glu-Ser-Val-NH_2 (lot No. 571 - 1876), (pyro)Glu-Val-Ser-NH_2 (lot No. 571 - 1717), (pyro)Glu-His-Trp-NH_2, and (pyro)Glu-Tyr-Arg-Trp-NH_2 were synthesized in the laboratory of one of us (R.G.) by classical methods and repurified.

2. Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Gln-Ala, corresponding in structure to the proposed growth hormone-releasing hormone (GH-RH) was synthesized by Veber et al. (19) and supplied by Dr. R. Hirschmann, Merck, Sharp and Dohme Research Lab.

3. (Pyro)Glu-His-Pro-NH_2 (thyrotropin-releasing hormone, TRH) and (pyro)Glu-His-Trp-NH_2 were synthesized by solid phase methods at Abbott Laboratories, North Chicago, Illinois and kindly supplied by Dr. W.F. White of Abbott Laboratories.

4. (Pyro)Glu-Gln-Ala-NH_2 and C-terminal octapeptide H-Trp-Ser-Tyr-Gly-Leu-Arg-

Pro-Gly-NH_2 (des-(pyro)Glu-des-His-LH-RH) and nonapeptide H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (des-(pyro)Glu-LH-RH) fragments of LH-RH were made in the laboratory of one of us (N.Y.) by classical methods and repurified.

5. (Pyro)Glu-His-Trp-OH (lot No. AY - 24000 - 243 - I) was made by classical methods at Ayerst Laboratories and supplied by Dr. M. Gotz.

All the samples of synthetic LH-RH and other polypeptides were homogeneous electrophoretically and chromatographically. Their synthesis and purification will be reported elsewhere.

Their LH-RH activity were determined in vivo by the stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone (1,2,20), followed by radioimmunoassay for rat LH (21) using the anti-ovine LH-serum No. 15 provided by Dr. G. Niswender. The results are expressed in terms of NIH-LH-S-17. The responses to synthetic LH-RH and to synthetic polypeptides were usually examined at 2 or 3 dose levels. Serum LH levels after injection of samples were compared with those obtained after administration of saline and natural LH-RH (1,2,18). The increase in serum LH levels over those given by saline was used as the index of LH-RH activity. Each sample was measured at least in triplicate. In vitro measurement of LH and FSH-releasing activity was carried out as described previously (18). The significance of differences between groups was determined by Duncan's new multiple range test (22). Factorial analyses, calculations of potency and the limits of error were made as reported before (18).

RESULTS

Serum LH levels found 20 min after the intravenous administration of saline, natural LH-RH, three batches of synthetic LH-RH decapeptide, and various synthetic polypeptides are shown in Table I. The potencies of all synthetic peptides, as compared with that of natural LH-RH, are listed in Table II. It can be seen that all three synthetic samples of (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 raised serum LH to levels similar to those obtained after

Table I

SERUM LH LEVELS IN OVARIECTOMIZED RATS PRETREATED WITH ESTROGEN AND PROGESTERONE AFTER ADMINISTRATION OF NATURAL AND SYNTHETIC LH-RH AND VARIOUS POLYPEPTIDES

Sample	Dose ng/rat	Plasma LH Level ng/ml ± S.E.	P vs Saline
Saline	---	7.2 ± 0.6	----
Natural LH-RH	0.5	18.5 ± 1.1	0.01
	2.5	48.3 ± 0.8	0.01
Synthetic LH-RH, AVS-77-55	0.5	19.2 ± 6.0	0.05
	2.5	47.3 ± 10.3	0.05
Saline	---	6.4 ± 0.6	----
Natural LH-RH	0.5	14.5 ± 1.4	0.05
	2.5	37.9 ± 2.5	0.01
Synthetic LH-RH, Hoechst R-4'	0.5	11.3 ± 0.7	0.05
	2.5	48.3 ± 6.7	0.01
Saline	---	8.4 ± 1.8	----
Natural LH-RH	0.5	16.8 ± 3.0	0.05
	2.5	48.6 ± 3.0	0.01
Synthetic LH-RH, Ayerst AY 24 031 2	0.5	19.5 ± 3.7	0.05
	2.5	51.4 ± 4.2	0.01
Saline	---	8.0 ± 1.2	----
Natural LH-RH	0.5	13.6 ± 0.4	0.05
	2.5	70.0 ± 9.4	0.01
(pyro)Glu-Tyr-Arg-Trp-NH_2	1000	6.0 ± 0.1	NS
	2500	9.8 ± 1.5	NS
	5000	14.0 ± 0.9	0.05
	12500	47.8 ± 2.8	0.01
Saline	---	8.2 ± 1.5	----
Natural LH-RH	0.5	16.4 ± 1.8	0.05
	2.5	42.3 ± 4.3	0.01
(pyro)Glu-Ser-Val-NH_2	1000	8.5 ± 0.5	NS
	5000	5.8 ± 0.8	NS

Sample (Cont.)	Dose ng/rat	Plasma LH Level ng/ml ± S.E.	P vs Saline
Saline	----	6.9 ± 0.7	----
Natural LH-RH	0.5	9.7 ± 0.3	0.05
	2.5	41.3 ± 3.5	0.01
(pyro)Glu-Val-Ser-NH_2	50	7.4 ± 1.6	NS
	500	6.1 ± 1.2	NS
	5000	7.4 ± 0.7	NS
Saline	----	5.6 ± 0.8	----
Natural LH-RH	0.4	12.7 ± 0.4	0.01
	4.0	61.8 ± 17.8	0.05
(pyro)Glu-Gln-Ala-NH_2	2000	5.2 ± 0.2	NS
	20000	6.5 ± 1.4	NS
Saline	----	9.7 ± 0.7	----
Natural LH-RH	5	87.6 ± 14.2	0.01
(pyro)Glu-His-Pro-NH_2	1000	11.0 ± 0.8	NS
	100,000	11.2 ± 0.4	NS
Synthetic GH-RH	10,000	10.3 ± 1.2	NS
Saline	----	5.5 ± 0.2	----
Natural LH-RH	0.5	10.7 ± 1.7	0.05
	2.5	39.5 ± 1.9	0.01
Des-(pyro)Glu-des-His-LH-RH	1000	7.3 ± 1.4	NS
	5000	4.1 ± 0.8	NS
Saline	----	5.3 ± 0.9	----
Natural LH-RH	0.5	15.4 ± 3.4	0.05
	2.5	31.4 ± 1.4	0.01
Des(pyro)Glu-LH-RH	4000	7.8 ± 0.1	NS
	20,000	14.5 ± 0.6	0.01
Saline	----	7.6 ± 1.0	----
Natural LH-RH	0.5	19.5 ± 4.5	0.05
	2.5	47.2 ± 8.4	0.01
(pyro)Glu-His-Trp-OH	25,000	7.5 ± 0.9	NS
	125,000	6.1 ± 0.2	NS

Sample	Dose ng/rat	Plasma LH Level ng/ml ± S.E.	P vs Saline
Saline	----	7.0 ± 1.2	----
Natural LH-RH	0.5	13.6 ± 1.3	0.05
	2.5	37.9 ± 8.3	0.01
(pyro)Glu-His-Trp-NH_2	5000	8.1 ± 1.4	NS
	25,000	7.5 ± 0.7	NS
Saline	----	7.7 ± 0.7	----
Natural LH-RH	0.5	13.8 ± 0.9	0.05
	2.5	45.0 ± 9.7	0.01
(pyro)Glu-His-Trp-Ser-NH_2	5000	6.8 ± 1.0	NS
	25,000	8.4 ± 0.2	NS

administration of natural LH-RH. The potencies of all three batches of synthetic LH-RH were the same or very near that of natural LH-RH. As reported before (9), (pyro)Glu-Tyr-Arg-Trp-NH_2 raised serum LH levels. However, the doses required to obtain this effect were very large and consequently its potency is only 0.00013 (0.013 percent or 1 part in 7800) of that of LH-RH decapeptide which was accepted as 1.00 or 100 percent. All the other synthetic polypeptides which included (pyro)Glu-Val-Ser-NH_2, (pyro)Glu-Ser-Val-NH_2, (pyro)Glu-Gln-Ala-NH_2, GH-RH, TRH did not stimulate LH release in vivo at doses as high as 5-100 µg/rat. The N-terminal tripeptide and tetrapeptide fragments of LH-RH and C-terminal octapeptide of LH-RH (des-(pyro)Glu-des-His-LH-RH) were similarly inactive in doses of 5-125 µg. Since natural LH-RH and synthetic LH-RH are active in this test in doses as low as 0.25 nanog (1,2), the LH-RH activity of these polypeptides, if indeed any at all, must be 20,000 - 400,000 times lower than that of the LH-RH decapeptide. C-terminal nonapeptide of LH-RH (Des-(pyro)Glu-LH-RH) had only about 1 part in 50,000 of the activity of natural LH-RH. In addition, (pyro)Glu-Ser-Val-NH_2, (pyro)Glu-Val-Ser-NH_2 and (pyro)Glu-Gln-Ala-NH_2, in doses as large as 4-32 µg/ml did not stimulate the release of FSH in vitro from isolated rat pituitaries. LH-RH decapeptide is active in this test at doses of 0.5 ng/ml. (Pyro)Glu-Ser-Val-NH_2 in doses of

Table II

POTENCY ESTIMATES OF SYNTHETIC POLYPEPTIDES AGAINST PURE NATURAL LH-RH

Peptide	% LH-RH Activity with 95% confidence limits
Natural LH-RH	assumed 100%
Synthetic LH-RH (AVS-77-55)	103 (68 - 172%)
Synthetic LH-RH (Hoechst) lot R-4'	120 (77 - 178%)
Synthetic LH-RH (Ayerst) lot 2	115 (77 - 170%)
(pyro)Glu-Tyr-Arg-Trp-NH_2	0.013 (0.008 -0.019%)
(pyro)Glu-Ser-Val-NH_2	<0.004%
(pyro)Glu-Val-Ser-NH_2	inactive (<0.01%)
(pyro)Glu-Gln-Ala-NH_2	inactive (<0.001%)
(pyro)Glu-His-Pro-NH_2 (TRH)	inactive (<0.0005%)
GH-RH synthetic	inactive
Des-(pyro)Glu-LH-RH	<0.002%
Des-(pyro)Glu-Des-His-LH-RH	inactive
(pyro)Glu-His-Trp-OH	inactive (<0.0004%)
(pyro)Glu-His-Trp-NH_2	inactive (<0.001%)
(pyro)Glu-His-Trp-Ser-NH_2	inactive (<0.001%)

4-32 μg/ml occasionally induced a marginal stimulation of LH release in vitro without showing a dose-response relationship. Its LH-RH activity in vitro, if any, was less than 1 part in 60,000 of that of LH-RH decapeptide.

DISCUSSION

The results reported in this paper demonstrate that when synthetic polypeptides, which were announced (14,15) or reported (16) to possess significant LH-RH activity, were subjected

to quantitative assays and rigorous comparison with natural and synthetic LH-RH according to standard bioassay techniques, their LH-RH activity was found to be negligible or absent. Thus, (pyro)Glu-Tyr-Arg-Trp-amide (16), which does not correspond to any amino acid sequence of LH-RH but which contains four of the amino acids of LH-RH, has some LH-RH activity but this is only 0.013 percent or 1 part in 7800 of activity of LH-RH decapeptide. This activity was provisionally explained by the relationship of the side chains of tyrosine and arginine in the configuration of the hormone (16). However, the extremely low degree of LH-RH activity of this synthetic tetrapeptide and other analogs of LH-RH, shorter than 10 amino acids, raises again an important point, namely what degree of biological activity is required before a polypeptide hormone can be said to possess it. The low activity of this tetrapeptide renders unlikely its practical clinical or even veterinary application, because of high dosages that would have to be employed to elicit LH release. Moreover, although tetrapeptides are cheaper to synthesize than decapeptides, the relative cost of synthesis per unit of LH-RH activity is much higher for the tetrapeptide than for LH-RH decapeptide.

Tripeptides (pyro)Glu-Val-Ser-NH_2 and (pyro)Glu-Ser-Val-NH_2 which were said to possibly exist in sheep hypothalamic extracts and announced as having LH-RH activity (14) were inactive *in vivo* at doses of 5 μg. Similarly, synthetic (pyro)Glu-Gln-Ala-NH_2, which was said to release LH and FSH *in vivo* and *in vitro* (15) was without effect *in vivo* in doses as high as 20 μg. This dose is 80,000 times greater than the minimal active dose of LH-RH decapeptide *in vivo*. Similarly, none of these three peptides released FSH *in vitro* in doses as high as 32,000 ng/ml. McCann and Fawcett (private communication) are in complete agreement with us as to the lack of LH-RH activity *in vivo* of (pyro)Glu-Val-Ser-NH_2, (pyro)Glu-Ser-Val-NH_2 and (pyro)Glu-Gln-Ala-NH_2. A synthetic decapeptide which stimulates the release of growth hormone (19) and (pyro)Glu-His-Pro-NH_2 (TRH) were also inactive *in vivo* in doses up to 100 μg. Consequently, they do not possess LH-RH activity at doses up to 200,000 times greater than the minimal active dose of LH-RH decapeptide.

The lack of LH-RH activity or low LH-RH activity of other polypeptides including the N-terminal tripeptide and tetrapeptide fragment of LH-RH as well as C-terminal octapeptide and nonapeptide fragments of LH-RH, combined with information derived from previous studies on the effects of various enzymes and reagents on the biological potency activity of LH-RH (23) suggest the following tentative conclusions as to the structure activity relationship of LH-RH: 1) The amino acid sequence necessary for eliciting a stimulation of LH release is quite specific, as in the case of other biological effects exerted by different polypeptides; 2) It is unlikely that highly active fragments can be obtained from LH-RH as in the case of gastrin tetrapeptide amide fragment (24) , and 3) The complete amino acid sequence of LH-RH may be necessary for maximal activity, i.e. for optimal binding to the receptors and a strong functional effect.

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