SYNTHESIS AND BIOLOGICAL ACTIVITY OF ANTAGONISTS OF LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH)

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Three analogs of LH-RH were synthesized by the solid phase method and were tested for agonist and antagonist activity in vitro. Des-His^2-[3-(2-naphthyl)-Ala^3]-LH-RH(I) and des-His^2-[3-(2-naphthyl)-Ala^3,D-Ala^6]-LH-RH(II) have no agonist activity and inhibit the release of LH and FSH by LH-RH. Des-His^2-[δ-N-Isopropylorn^8]-LH-RH has slight agonist activity and also significant antagonist activity at the doses tested.

INTRODUCTION

The isolation and structure elucidation of luteinizing hormone-releasing hormone (LH-RH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (1,2), has led to the synthesis and testing of many analogs for agonist and antagonist activity. The search for antagonists of peptide hormones is still an empirical exercise. Our approach here has been to combine the known inhibitory effect of omitting the histidine residue in position 2 (3,4) with our previous experience of producing partial agonists by more-or-less isosteric replacements in positions 3 and 8 (5). Thus we report the synthesis and in vitro activity of des-His^2-[3-(2-naphthyl)-Ala^3]-LH-RH(I), des-His^2-[3-(2-naphthyl)-Ala^3, D-Ala^6]-LH-RH(II) and des-His^2-[δ-N-isopropylorn^8]-LH-RH(III).

EXPERIMENTAL

The three analogs were synthesized by the solid phase method (6) on 1% crosslinked chloromethyl polystyrene containing 0.75 meq Cl/g, supplied by Lab Systems Inc., San Mateo, Ca. Reaction of the resin with Boc Gly triethylammonium salt gave 0.37 mmole Gly/g resin. The syntheses were carried out in a glass apparatus with stirring by a stream of nitrogen. α-N-Boc amino acids were used throughout; the side chain of Arg was protected as the N^G-tosyl derivative and those of Tyr and Ser as the benzyl ether.

Coupling reactions were carried out with dicyclohexyl carbodiimide and the Boc groups were removed by successive treatments with N HCl in HOAc for 25 min and 25% trifluoroacetic acid in $MeCl_2$ for 15 min. The decapeptide amides obtained by ammonolysis of the protected peptide resin were reduced by Na in liquid NH_3 (7) and purified by desalting on Sephadex G-15, eluting with 50% HOAc, gel filtration on Sephadex G-15, eluting with 0.2 N HOAc and finally by partition chromatography on Sephadex G-25 (fine) with 1-BuOH, HOAc, H_2O (4:1:5). Based on Boc Gly resin, the yields of pure peptides I-III were 24.7%, 32.9% and 28.1%, respectively. Analytical data on I, II and III are presented in Table I.

BIOLOGICAL METHODS

The determination of hormonal activities was performed *in vitro* using pituitaries of 20 day old female Sprague-Dawley rats (Charles River Laboratory). Two pituitaries were incubated at 37° in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10 ml Teflon beakers in a Dubnoff shaker. The pituitaries were incubated for a total of 6 hrs. Medium was removed each hour for radioimmunoassay (RIA) of LH and FSH and then fresh medium was added. After two preincubation periods (P_1, P_2), a LH-RH analog was added to the

Table I: Analytical data of LH-RH analogs

Analog I: $[\alpha]_D^{23}$ - 48.17° (c, 0.69, 1M HOAc); TLC[a] R_f^1 0.27, R_f^2 0.57, R_f^3 0.64; TLE[b] E_{Glu} 0.19; Amino acid anal.[c] Glu 0.99, 3-(2-Naphthyl)-Ala 1.0, Ser 0.84, Tyr 0.99, Gly 1.99, Leu 0.93, Arg 0.99, Pro 1.0.

Anal. calcd. for $C_{51}, H_{69}N_{13}O_{12} \cdot CH_3COOH \cdot 3\ H_2O$:
C, 54.59; H, 6.78; N, 15.52.

Found: C, 54.57; H, 6.73; N, 15.69.

Analog II: $[\alpha]_D^{23}$ - 44.4° (c, 0.49, 1M HOAc); TLC[a] R_f^1 0.29, R_f^2 0.60, R_f^3 0.64; TLE[b] E_{Glu} 0.21; Amino acid anal.[c] Glu 1.0, 3-(2-Naphthyl)-Ala 0.87, Ser 0.78, Tyr 0.93, Ala 1.0, Leu 0.97, Arg 1.0, Pro 0.99, Gly 0.99.

Anal. calcd. for $C_{52}H_{71}N_{13}O_{12} \cdot CH_3COOH \cdot 4\ H_2O$:
C, 53.94; H, 6.95; N, 15.14.

Found: C, 53.86; H, 7.07; N, 14.90.

Analog III: $[\alpha]_D^{23}$ - 47.46° (C, 0.48, 1M HOAc; TLC[a] R_f^1 0.23, R_f^2 0.57, R_f^3 0.59; TLE[b] E_{Glu} 0.26; Amino acid anal.[c] Glu 1.03, Trp 1.1, Ser 0.88, Tyr 1.01, Gly 2.01, Leu 1.0, δ-N-Isopropylorn 0.96, Pro 0.99.

Anal. calcd. for $C_{51}H_{72}N_{12}O_{12} \cdot 2\ H_2O$:
C, 56.65; H, 7.09; N, 15.54.

Found: C, 56.63; H, 6.92; N, 15.25

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a) R_f^1, R_f^2, and R_f^3 values refer to the solvent systems 1-BuOH-HOAc-H_2O (4:1:5), 1-BuOH-HOAc-H_2O-EtOAc (1:1:1:1) and 1-BuOH-HOAc-H_2O-Pyr (30:6:24:20), respectively. Silica gel plates were used for tlc.

b) E_{Glu} is the relative mobility on TLE with respect to Glu (= 1.0) at 400 V for 2 hrs and in pH 1.9 buffer on silica gel plate.

c) Analyses were carried out by AAA Laboratories, Seattle, Washington 98125.

incubation medium during the last 4 hourly incubation periods (I_3, I_4, I_5, I_6) and LH-RH was added at I_5 and I_6. The studies were performed in triplicate. All results represent the Δmμg/ml mean LH and FSH release of 6 values ± SEM. The Δ values were calculated by subtracting the amount of hormonal release at I_3, I_4, I_5 and I_6 from the respective hormonal release at P_2. The agonist activity was determined from the hormonal release at I_3 and I_4, and the antagonist activity from the hormonal release at I_5 and I_6. The RIA reagents for FSH were distributed by NIAMDD, NIH. Dr. G. Niswender supplied the anti-ovine LH serum No. 15 for the rat LH-assay, and Dr. L. E. Reichert supplied an ovine LH preparation for labeling and the LH rat reference preparation. The values for these assays are calculated in terms of millimicrograms of the following standards: LH-LER-1240-2 (0.60 NIH-LH-SI units/mg) and FSH (2.1 NIH-FSH-SI units/mg).

TABLE II

DES-HIS^2-[3-(2-NAPHTHYL)-L-ALA^3]-LH-RH IN VITRO AGONIST ACTIVITY

#	Dose Peptide I_3, I_4	LH			FSH		
	m g/ml medium	m g/ml medium	SEM	p value vs 2	m g/ml medium	SEM	p value vs 2
1	-	5	± 13	ns	- 1211	± 463	ns
2	-	14	± 28	-	338	± 671	-
3	100	2	± 35	ns	832	±1212	ns
4	1,000	0	± 35	ns	1281	± 536	ns
5	10,000	12	± 12	ns	2291	±1266	ns
6	100,000	- 18	± 4	ns	- 60	± 726	ns

RESULTS AND DISCUSSION

The data in Tables II-V show that the two analogs containing a naphthylalanine residue have no LH-RH agonist activity *in vitro*, and that both analogs inhibit the action of added LH-RH. The better antagonist of the two, des-His^2-[3-(2-naphthyl)-L-Ala^3, D-Ala^6]-LH-RH, completely blocks the release of both LH and FSH at 1 µg/ml medium, *i.e.*, at a concentration about 3,000 times that of LH-RH. The other, des-His^2-[3-(2-naphthyl)-L-Ala^3]-LH-RH, also inhibits completely the release of LH at 1 µg/ml but shows inhibition of FSH release only at 10 and 100 µg/ml.

It is well known that a D-residue in position 6 heightens both the agonist and antagonist response of LH-RH analogs, probably by stabilizing a conformation that binds to the receptor well (3,4) and/or by providing resistance to proteolytic breakdown of the peptide.

TABLE III

DES-HIS^2-[3-(2-NAPHTHYL)-L-ALA^3]-LH-RH *IN VITRO* ANTAGONIST ACTIVITY

#	Dose Peptide I_3,I_4,I_5,I_6 mµg/ml medium	Dose LH-RH I_5,I_6	LH Δmµg/ml medium	LH SEM	LH p value vs 2	FSH Δmµg/ml medium	FSH SEM	FSH p value vs 2
1	Saline	-	1	±24	<.001	-1014	± 501	<.001
2	-	0.3	134	± 9	-	5342	±1150	-
3	100	0.3	201	±37	ns	7319	± 905	ns
4	1,000	0.3	24	±21	<.001	6178	±1079	ns
5	10,000	0.3	14	± 7	<.001	2901	± 379	∿.05
6	100,000	0.3	-31	±11	<.001	84	± 323	<.001

TABLE IV

DES-HIS^2-[3-(2-NAPHTHYL)-L-ALA^3]-[D-ALA^6]-LH-RH IN VITRO AGONIST ACTIVITY

#	Dose Peptide I_3,I_4	LH			FSH		
	mμg/ml medium	Δmμg/ml medium	SEM	p value vs 1	Δmμg/ml medium	SEM	p value vs 1
1	-	-16	±21	-	- 501	± 325	-
2	100	46	±34	ns	- 957	± 925	ns
3	1,000	4	±17	ns	- 522	±1265	ns
4	10,000	13	±21	ns	380	± 268	ns
5	100,000	- 1	±10	ns	-1323	± 178	.05
6							

TABLE V

DES-HIS^2-[3-(2-NAPHTHYL)-L-ALA^3]-[D-ALA^6]-LH-RH IN VITRO ANTAGONIST ACTIVITY

#	Dose Peptide I_3,I_4,I_5,I_6	LH-RH I_5,I_6	LH			FSH		
	mμg/ml medium		Δmμg/ml medium	SEM	p value vs 1	Δmμg/ml medium	SEM	p value vs 1
1	-	.3	207	±18	-	3079	± 253	-
2	100	.3	180	±27	ns	3929	±1493	ns
3	1,000	.3	20	±17	.001	-1649	±1122	.01
4	10,000	.3	4	±15	.001	- 634	± 147	.001
5	100,000	.3	4	±12	.001	-2400	± 325	.001
6								

TABLE VI

DES-HIS^2-[δ-N-ISOPROPYL-ORN^8]-LH-RH IN VITRO AGONIST ACTIVITY

#	Dose Peptide I_3,I_4 mμg/ml medium	LH Δmμg/ml medium	SEM	p value vs 2	FSH Δmμg/ml medium	SEM	p value vs 2
1	-	5	± 8	ns	596	±177	ns
2	-	-1	± 6	-	840	±192	-
3	100	14	±10	ns	284	±247	ns
4	1,000	20	± 7	.05	1860	±416	.05
5	10,000	25	± 8	.05	1733	±361	.05
6	100,000	16	±13	ns	674	±238	ns

TABLE VII

DES-HIS^2-[δ-N-ISOPROPYL ORN^8]-LH-RH IN VITRO ANTAGONIST ACTIVITY

#	Dose Peptide I_3,I_4,I_5,I_6 mμg/ml medium	LH-RH I_5,I_6 mμg/ml medium	LH Δmμg/ml medium	SEM	p value vs 2	FSH Δmμg/ml medium	SEM	p value vs 2
1	-	-	25	±14	.01	374	±158	.001
2	-	.3	111	±22	-	7360	±745	-
3	100	.3	103	± 9	ns	6245	±312	ns
4	1,000	.3	125	±10	ns	6468	±461	ns
5	10,000	.3	67	±13	ns	4135	±438	.01
6	100,000	.3	54	±15	.05	1698	±385	.001

Analog III (Tables VI and VII) has slight agonist activity at 1 μg and 10 μg but it is not dose-related. It inhibits LH and FSH release significantly at 10 μg/ml but not at 1 or 0.1 μg.

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