STUDIES ON INHIBITION OF THE LUTEINIZING HORMONE-RELEASING HORMONE BY AN IRREVERSIBLE INHIBITOR AT THE RECEPTOR SITE

by

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[Leu^2, Leu^3, D-Ala^6]-LHRH is an analog of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2(LHRH) and inhibits the release of LH and FSH induced by LHRH. This analog and inhibitor has been modified with the objective of developing an active-site-directed irreversible inhibitor. The modification consisted of replacing < Glu^1 with Chl^1 which is the moiety of chlorambucil (a nitrogen mustard). The Chl analog inhibited the release of LH and FSH by LHRH after addition prior to LHRH and after three changes of the incubation medium; in contrast, [Leu^2, Leu^3, D-Ala^6]-LHRH and [des-His^2]-LHRH only inhibit release when added together with LHRH. The Chl analog released LH and FSH but not TSH or GH, indicating that its agonist and antagonist activities could be specific at the receptor site for LHRH.

INTRODUCTION

Baker reviewed the design of active-site-directed irreversible inhibitors (1). Such compounds may be considered to act by first forming an analog-receptor complex, which then by neighboring group reactions within this complex, may result in the formation of a covalent linkage between the chemically reactive group on the analog and an appropriate group on or in the proximity of the receptor.

The nitrogen mustard, p-[N,N-bis(2-chloroethyl)amino]phenylbutyric acid (chlorambucil, Chl), contains a chemically reactive group that has the ability to form a covalent bond with nucleophilic sites. This Chl residue has been incorporated by Stewart and coworkers into numerous peptide sequences, including bradykinin (2), and the luteinizing hormone-releasing hormone (LHRH or < Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)(3). Paiva and coworkers have described the incorporation of this residue into sequences of angiotensin I (4) and angiotensin II (5).

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Humphries et al. (6) have recently reported that a decapeptide, [Leu^2,Leu^3]-LHRH, inhibited the LHRH-induced release of LH and FSH, in vitro, and did not release LH or FSH. Wan et al. (7) found that [Leu^2,Leu^3,D-Ala^6]-LHRH and [Val^2, Leu^3, D-Ala^6]-LHRH completely inhibited the LHRH-induced release of LH and FSH at one-tenth the inhibitory dosage of [Leu^2, Leu^3]-LHRH. Folkers et al. reported (8) that the latter analog, however, did not inhibit the in vitro release of FSH by partially purified FSHRH.

With this background, the analog, [Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH has been prepared for studies on irreversible inhibition. This analog differs from our inhibitor, [Leu^2, Leu^3, D-Ala^6]-LHRH, only in position 1 in which the Chl residue has replaced the <Glu residue.

EXPERIMENTAL

[Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH was synthesized by the solid-phase method with the benzhydrylamine resin on a Beckman 990 Peptide Synthesizer, essentially as described (6). The side chain functionalities of Arg, Tyr, and Ser, were protected by tosyl, 2,6-dichlorobenzyl, and benzyl, respectively. Chlorambucil was used unprotected. The final, protected resin, derived from 2 g Boc-Gly-benzhydrylamine resin (0.98 mequiv. Gly), was treated with anhydrous (CoF_3)liquid HF containing 10% anisole for 1 hr at 0°. The HF was removed in vacuo, and the peptide-resin mixture was washed with ether to remove anisole. The peptide was removed by extraction with $CHCl_3$ and then with 10% acetic acid. The $CHCl_3$ was evaporated, and the residue was combined with the acetic acid extract, and the mixture was lyophilized to yield about 480 mg of peptide.

The analog was purified by partition chromatography on Sephadex G25 with 1-BuOH, acetic acid, water (4:1:5). The main peak (355 mg) gave the ratios: Leu, 3x1.03; Ser, 0.76; Tyr, 1.04; Ala, 1.03; Arg, 1.02; Pro, 0.99; Gly, 1.07. The analog contained 5.56% chlorine (theory 5.56%); was homogeneous and had the following R_f values in the tlc systems (chloro-tolidine positive and ninhydrin negative): 0.93 in EtOAc, pyridine, AcOH, H_2O (5:5:1:3); 0.62 in $CHCl_3$, MeOH, conc, NH_4OH (60:45:20); 0.64 in 1-BuOH, pyridine, AcOH, H_2O (30:20:6:24); 0.73 in 2-propanol, 1 N AcOH (2:1); 0.69 in $CHCl_3$:MeOH:AcOH:H_2O (65:30:4:1); $[\alpha]_D$ -43.74 (c=1.07,MeOH).

BIOLOGICAL METHODS

Pituitaries (2 pituitaries/beaker) of 20-day old female rats (Sprague-Dawley) were incubated in a Dubnoff shaker (90 cycles/min) at 36° in lactated Ringers solution (Travenol Laboratories) for 6 hr (P_1, P_2, I_3, I_4, I_5, I_6) in 10-ml Teflon beakers. The medium was changed hourly. Synthetic LHRH was dissolved in distilled water and [Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH was dissolved in propylene glycol (PG). The LHRH (0.3 mμg/30 μl) was always added during incubation periods I_5 and I_6 while the Chl-analog (25 μl PG/dose) was added to I_3

TABLE I. AGONIST ACTIVITY, IN VITRO, OF [Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH

Dose of Analog mμg/ml medium	LH			FSH		
	Δ mμg/ml medium	SEM	p value vs 1	Δ mμg/ml medium	SEM	p value vs 1
-*	48	± 21	-	2052	± 395	-
100	102	± 19	ns	4608	± 1162	ns
1,000	320	± 58	0.001	7539	± 985	<0.001
10,000	486	± 58	<0.001	8102	± 1381	<0.01
100,000	>652	-	<0.001	8424	± 428	<0.001

Δ = mean (6) of P_2 minus I_3 and I_4

* = 25 μl PG

and I_4 to determine the LHRH agonist activity and to only P_1, P_1 and P_2, or P_1, P_2 and I_3, for determination of the LHRH antagonist activity. For control tests, 25 μl PG alone was added at the latter times.

Rat FSH, TSH, and GH were measured by using the reagents and RIA methods supplied by the NIAMDD-NIH pituitary program. 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 labelling and the rat preparation for reference. The values for these assays are calculated in terms of mμg of the following standards: LH-LER-1240-2 (0.60 NIH-SI units/mg), FSH (2.1 x NIH-FSH-SI units/mg), TSH (0.22 USP bovine units/mg) and GH (0.6 IU/mg).

RESULTS AND DISCUSSION

The data in Table I show that [Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH has definite LH and FSH releasing activity, in vitro. Such results are surprising since [Leu^2, Leu^3, D-Ala^6]-LHRH had no LH or FSH releasing activity at 100 μg/ml in this same in vitro system (7). This agonist activity could indicate that the Chl-analog can reach the LH and FSH cell receptor sites, or that the agonist activity is due to some other reaction.

The specificity of the LH and the FSH agonist activity, in vitro, was evaluated by measuring the release of GH and TSH as well as of LH and FSH, when the Chl-analog was added to the incubation medium. The LH and FSH release was found to increase, but the GH and TSH release did not increase. These results indicate that the LH and FSH agonist activity of the Chl-analog appears to be specific by an effect at the receptor sites of the gonadotrophs rather than by a non-specific effect.

Since this analog has agonist activity, the analog and LHRH were not concomitantly added to the medium for evaluation of LHRH antagonist activity. Var-

TABLE II. EFFECT OF [Chl[1], Leu[2], Leu[3], D-Ala[6]]-LHRH ON THE RELEASE, IN VITRO, OF LH AND FSH INDUCED BY LHRH

Additions to Medium				LH		FSH	
Analog μg/ml P_1	P_2	I_3	LHRH mμg/ml I_5 and I_6	Δ mμg/ml medium ± SEM	p value vs 2	Δ mμg/ml medium ± SEM	p value vs 2
-*			-	7 ± 8	<0.001	285 ± 154	<0.001
-*			0.3	243 ± 55	-	4784 ± 778	-
10			0.3	68 ± 17	0.01	2509 ± 324	0.02
25			0.3	40 ± 33	<0.01	341 ± 359	<0.001
50			0.3	-18 ± 19	0.001	-271 ± 154	0.001
				8 ± 4	<0.001	300 ± 163	
-*			0.3	307 ± 46	-	7219 ± 1296	
1			0.3	279 ± 80	ns	5490 ± 319	ns
3			0.3	120 ± 37	0.01	2891 ± 232	0.01
10			0.3	38 ± 17	<0.001	352 ± 109	<0.001
30			0.3	95 ± 29	<0.01	536 ± 242	<0.001
50			0.3	28 ± 27	<0.001	325 ± 201	<0.001
-*	-*		0.3	276 ± 37	-	6200 ± 968	
1	1		0.3	178 ± 26	<0.05**	2853 ± 173	<0.01
3	3		0.3	93 ± 17	0.001**	895 ± 185	<0.001
10	10		0.3	30 ± 23	<0.001**	295 ± 158	<0.001
30	30		0.3	-102 ± 31	<0.001**	1327 ± 199	<0.001
1	1	1	0.3	105 ± 50	0.02**	1032 ± 126	<0.001

Δ = mean (6) or I_4 minus I_5 and I_6

* = 25 μl PG

** = vs 8

ious dosages of the Chl-analog were added during P_1, P_1 and P_2, or P_1, P_2 and I_3 incubation periods, and LHRH was added to I_5 and I_6. During this 6 hr incubation period the medium was changed hourly.

When the Chl-analog was added only 1,2 or 3 times during the first 3 hourly incubation periods, the degree and duration of the LH and FSH agonist activity depended on the analog dose level. For instance, levels of 1, 3, 10, 30 and 50 μl/ml medium of the Chl-analog raised the LH and FSH levels at P_1. By I_4 both the LH and FSH levels had returned to basal levels.

The antagonist activity of the Chl-analog was calculated by substracting the amount of LH and FSH released during I_5 and I_6 from that released during I_4, and is expressed by the mean Δμg/ml value.

The results in Table II show that addition of 3 to 50 μg/ml medium, but not

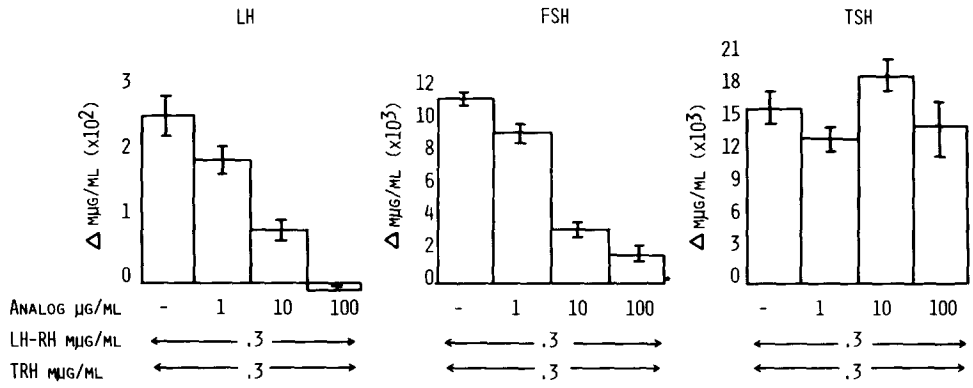

Figure 1.

EFFECT OF [Chl^1, Leu^2, Leu^3, D-Ala^6]-LHRH on the LHRH and TRH RESPONSE, IN VITRO

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1 μg/ml medium of the Chl-analog, inhibited the LH and FSH release induced by LHRH (0.3 mμg), and that the inhibitory effect was dose related.

Of particular note is the observation that the antagonistic activity of [Leu^2, Leu^3, D-Ala^6]-LHRH and [des-His^2]-LHRH, in this same in vitro system, is rapidly reversible (9). When the above analogs and LHRH were present in the medium at the same time, the LHRH response was inhibited. In contrast, the Chl-analog still inhibited the LHRH response after being added only once at P_1 and after the medium was changed 3 times.

Additional results have shown that multiple, rather than single additions (P_1 and P_2 or P_1, P_2 and I_3) of the Chl-analog were even more effective in inhibiting the LHRH response, and again the antagonistic activity was dose related.

In another in vitro study for evaluation of specificity of the antagonistic activity of the Chl-analog, the analog was added only at P_1 and TRH and LHRH was added at I_5 and I_6. Fig. 1 shows that LH and FSH, but not TSH release was significantly inhibited by the Chl-analog. This result again indicates the specificity of the inhibitory action of the Chl-analog.

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