Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 360-366

SYNTHETIC ANALOGS OF GROWTH HORMONE-RELEASING FACTOR WITH ANTAGONISTIC ACTIVITY IN VITRO

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Received January 26, 1990

Analogs of human and rat growth hormone-releasing factor (hGRF and rGRF), related to [D-Arg²]hGRF(1-29)NH_2, were synthesized by solid phase methodology. Their capacity to inhibit growth hormone secretion stimulated by hGRF(1-44)NH_2 was tested on rat anterior pituitary cells in monolayer culture. Among the analogs of hGRF, [D-$Arg^{2,29}$,Arg^{30}]hGRF(1-30)NH_2 showed the highest antagonistic potency of 3.64 relative to [D-Arg^{2}]hGRF(1-29)NH_2 = 1. However, the most potent analog synthesized thus far was [N-Ac-His^{1},D-Arg^{2},Ala^{15}]rGRF(1-29)NH_2, which showed a relative potency of 27.7. © 1990 Academic Press, Inc.

Growth hormone-releasing factor (GRF) was first isolated and characterized from two tumors of the pancreas (1,2) and later from the hypothalamic tissues of rat (3), porcine (4), bovine (5), caprine (6), and human species (7). The structure of the tumor-derived GRF was found to be identical to that of the human hypothalamic GRF (7). Moreover, the amino acid sequences of porcine, bovine, caprine and ovine GRFs are closely related to that of the human because they differ from human GRF by only 3-6 amino acid substitutions (7). Thus it is not surprising that all these GRFs have more or less the same potencies to release growth hormone in the rat anterior pituitary cell culture assay (8). Rat GRF, on the

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Abbreviations: hGRF, human growth hormone-releasing factor; rGRF, rat growth hormone-releasing factor; GH, growth hormone.

0006-291X/90 $1.50
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other hand, is rather unique because it contains 15 amino acid substitutions from the human counterpart, which might account for its three times higher potency than human GRF in the in vitro bioassay (8).

Since the characterization of GRF, we have been interested in the preparation of potent GRF agonists and antagonists for clinical applications. In an earlier paper (9), we reported that a C-terminal amidated analog of human GRF containing the first 29 residues has approximately one-half the growth hormone releasing potency of the parent compound. Subsequently, we prepared many GRF analogs based on the hGRF(1-29)NH_2 sequence and have reported recently that [D-Arg^2]hGRF(1-29)NH_2 showed some antagonistic property in the in vitro bioassay (10). In order to increase the antagonistic activity of this peptide, we have incorporated other substitutions in specific positions, which had previously been shown to increase the affinity of hGRF(1-29)NH_2 to the GRF receptor (10,11). In addition, a series of similarly modified analogs of rat GRF was also prepared. The synthetic peptides were assayed for their intrinsic activity to release growth hormone as well as their capacity to antagonize the release of growth hormone induced by 10^{-9} M hGRF(1-44)NH_2 in the rat anterior pituitary cell culture system.

MATERIALS AND METHODS

All the hGRF(1-29)NH_2 analogs synthesized were based on the parent amino acid sequence, Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg, whereas the rat GRF(1-29)NH_2 analogs were based on the parent sequence, His-Ala-Asp-Ala-Ile-Phe-Thr-Ser-Ser-Tyr-Arg-Arg-Ile-Leu-Gly-Gln-Leu-Tyr-Ala-Arg-Lys-Leu-Leu-His-Glu-Ile-Met-Asn-Arg. They were prepared by solid phase methodology (12) and purified by cation-exchange and partition chromatography as described previously (9,11). The structure and purity of the synthetic peptides were verified by amino acid analysis and high performance liquid chromatography as described (9,11).

Their capacity to stimulate growth hormone release above the control level was evaluated in a dose-dependent manner in adult male rat anterior pituitary culture (13). Since most of the synthetic analogs have very low potencies, their intrinsic activity was expressed as the quotient derived from dividing the highest level of growth hormone released into the medium induced by the analog at the dosage ranging from 10^{-9} M to 10^{-5} M by the level of growth hormone elicited by 10^{-9} M hGRF(1-44)NH_2.

The antagonistic activity of the analogs was determined by the addition of an increasing dosage of the analogs (10^{-9} to 10^{-5} M) to a constant concentration of hGRF(1-44)NH_2 (10^{-9} M) present in every culture well and measuring the inhibition of growth hormone release induced by the hGRF. The antagonistic potency of the analogs was calculated using the computer program BIOPROG with [D-Arg^2]hGRF(1-29)NH_2 taken as the reference standard (14).

RESULTS AND DISCUSSION

The relative intrinsic activities and antagonistic potencies of the synthetic GRF analogs are summarized in Table 1. As reported in our previous paper (10), replacement of the alanine at position-2 of hGRF(1-29)NH_2 with D-arginine (analog no. 2) resulted in a compound with antagonistic property. But substitution with an L-arginine at the same position (analog no. 3) yielded a compound with almost full intrinsic activity, but no antagonistic property, implicating the importance of the D-configuration for the arginine residue. Moreover, replacement of the

Table 1: Biological Potencies of GRF Analogs

Analogs	Intrinsic Activity	Antagonistic Potency (95% Confidence Limits)	
1. hGRF(1-29)NH_2	1	0	
2. [D-Arg^2]hGRF(1-29)NH_2	0.11	1	
3. [Arg^2]hGRF(1-29)NH_2	0.9	0	
4. [D-Arg^4]hGRF(1-29)NH_2	1.0	0	
5. [N-Ac-Tyr^1,D-Arg^2]hGRF(1-29)NH_2	<0.1	0.46	(0.27-0.79)
6. [Des-amino-Tyr^1,D-Arg^2]-hGRF(1-29)NH_2	1.0	0	
7. [Arg^1,D-Arg^2]hGRF(1-29)NH_2	<0.1	0.06	(0.04-0.07)
8. [D-$Arg^{2,8}$]hGRF(1-29)NH_2	0.1	0	
9. [D-Arg^2,D-Asn^8,Ala^{15}]hGRF(1-29)NH_2	0.3	1.10	(0.59-1.80)
10. [N-Ac-Tyr^1,D-Arg^2,D-Asn^8,Ala^{15}]-hGRF(1-29)NH_2	<0.1	0.63	(0.55-0.74)
11. [D-Arg^2,D-Asp^3,D-Asn^8,D-Tyr^{10}, D-Arg^{11},Ala^{15}]hGRF(1-29)NH_2	0.1	0	
12. [D-$Arg^{2,29}$,Tyr^{30}]hGRF(1-30)NH_2	0.13	1.67	(1.28-2.20)
13. [N-Ac-Tyr^1,D-$Arg^{2,29}$,Tyr^{30}]-hGRF(1-30)NH_2	<0.1	0.22	(0.13-0.37)
14. [D-$Arg^{2,29}$,Arg^{30}]hGRF(1-30)NH_2	0.14	3.64	(2.66-5.01)
15. [N-Ac-Tyr^1,D-$Arg^{2,29}$,Arg^{30}]-hGRF(1-30)NH_2	<0.1	0.63	(0.44-0.90)
16. [D-Arg^2]hGRF(1-44)NH_2	0.10	1.54	(1.04-2.29)
17. [D-Arg^2]rGRF(1-29)NH_2	0.12	1.29	(1.00-1.67)
18. [D-Arg^2,Gln^{15},Nle^{27}]rGRF(1-29)NH_2	0.12	1.89	(1.31-2.72)
19. [N-Ac-His^1,D-Arg^2,Gln^{15},Nle^{27}]-rGRF(1-29)NH_2	<0.1	22.6	(16.0-31.9)
20. [D-Arg^2,Ala^{15}]rGRF(1-29)NH_2	0.12	2.52	(1.52-4.21)
21. [N-Ac-His^1,D-Arg^2,Ala^{15}]-rGRF(1-29)NH_2	<0.1	27.7	(19.4-39.5)

alanine at position-4 with D-arginine (analog no. 4) also resulted in a compound with full intrinsic activity, but no antagonistic property, suggesting the absolute requirement of the [D-Arg^{2}] configuration.

Although [D-Arg^{2}]hGRF(1-29)NH_2 possesses antagonistic activity, it still retains about 10% of the intrinsic activity to release growth hormone at high concentrations. Acetylation of the N-terminal (analog no. 5) reduced the intrinsic activity of [D-Arg^{2}]hGRF(1-29)NH_2. However, the concomitant antagonistic activity was also reduced, implying that N-terminal acetylation might interfere with the binding of the analog to the receptor. [N-Ac-Tyr^{1},D-Arg^{2}]hGRF(1-29)NH_2, first prepared by Coy et al. (15) has also been shown by Robberecht and coworkers (16) to be able to antagonize the GRF-induced adenylate cyclase activity in rat anterior pituitary membrane preparations and by Lumpkin et al. (17,18) to inhibit the spontaneous pulsatile secretion of growth hormone in conscious, freely moving rats.

To determine whether the N-terminal amino group was necessary for antagonistic activity, [des-amino-Tyr^{1},D-Arg^{2}]hGRF(1-29)NH_2 (analog no. 6) was synthesized. However, the des-amino analog showed full intrinsic activity without any antagonistic property, suggesting that the acetylated N-terminal was necessary for the [D-Arg^{2}] modification to express the antagonistic activity. Imparting more basic characteristics to the N-terminal by substituting the tyrosine at position 1 with arginine to yield [Arg^{1},D-Arg^{2}]hGRF(1-29)NH_2 (analog no. 7), actually decreased the antagonistic potency. Replacement of the asparagine at position 8 with another D-arginine to give [D-Arg^{2,8}]hGRF(1-29)NH_2 (analog no. 8) also decreased the antagonistic potency.

Previously, we have reported that hGRF analogs with [D-Asn^{8}] or [Ala^{15}] substitution possessed higher agonistic activities than the parent compound (10,11), implicating that these two substitutions increased the affinity of the analogs to the GRF receptor. To determine whether such substitutions could also increase the affinity and thus the potency of the [D-Arg^{2}] analogs, [D-Arg^{2},D-Asn^{8},Ala^{15}]hGRF(1-29)NH_2 (analog no. 9) and [D-Arg^{2},D-Asp^{3},D-Asn^{8},D-Tyr^{10},D-Arg^{11},Ala^{15}]hGRF(1-29)NH_2 (analog no. 11) were prepared. Although the [D-Arg^{2},D-Asn^{8},Ala^{15}] analog showed higher antagonistic potency than [D-Arg^{2}]hGRF(1-29)NH_2, it also had higher intrinsic activity, whereas [D-Arg^{2},D-Asp^{3},D-Asn^{8},D-Tyr^{10},D-Arg^{11},Ala^{15}]hGRF(1-29)NH_2 suffered a total loss of antagonistic activity. This finding suggests that additional replacements with D-amino acids in the [D-Arg^{2}]hGRF(1-29)NH_2 sequence may not be effective to increase the antagonistic activity. However, N-terminal acetylation was still able to decrease the intrinsic activity as well as lowering the affinity, since [N-Ac-Tyr^{1},D-Arg^{2},D-Asn^{8},Ala^{15}]hGRF(1-29)NH_2 (analog no. 10) has less

intrinsic activity and also less antagonistic potency than [D-Arg^{2},D-Asn^{8},Ala^{15}]hGRF(1-29)NH_2.

To determine whether the [Tyr^{1}-D-Arg^{2}] configuration was unique in imparting the molecule with antagonistic activity, we added this configuration to the C-terminal of the reference compound to yield [D-Arg^{2,29},Tyr^{30}]hGRF(1-30)NH_2 (analog no. 12). In addition, the N-terminal acetylated derivative was also prepared (analog no. 13). The former compound showed a slight increase of antagonistic activity compared to [D-Arg^{2}]hGRF(1-29)NH_2 but its intrinsic activity was also higher, whereas the latter caused a drop in both intrinsic activity and antagonistic potency. Replacement of the [Tyr^{30}] in these two analogs with [Arg^{30}] to yield [D-Arg^{2,29},Arg^{30}]hGRF(1-30)NH_2 (analog no. 14) and [N-Ac-Tyr^{1},D-Arg^{2,29},Arg^{30}]hGRF(1-30)NH_2 (analog no. 15) resulted in an increase in antagonistic activity and [D-Arg^{2,29},Arg^{30}]hGRF(1-30)NH_2 was the most potent antagonist in the human GRF series. Increasing the C-terminal sequence of [D-Arg^{2}]hGRF(1-29)NH_2 to the full length of hGRF (analog no.

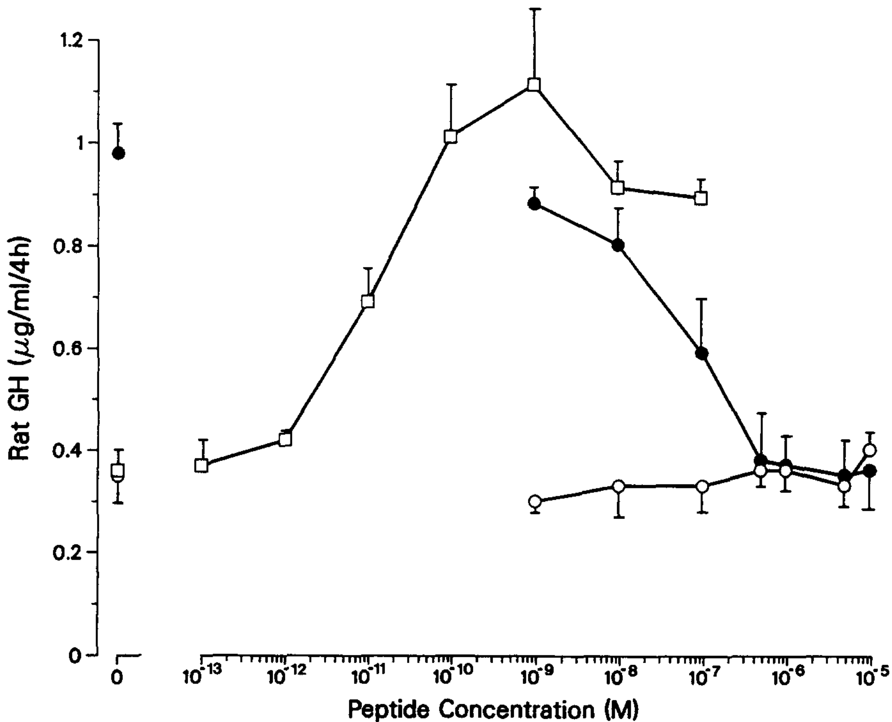

Figure 1: Dose-response curves of hGRF(1-44)NH_2 and its antagonist on the release of pituitary GH. □ :hGRF(1-44)NH_2; ○:[N-Ac-His^{1},D-Arg^{2},Ala^{15}]-rGRF(1-29)NH_2; ● :[N-Ac-His^{1},D-Arg^{2},Ala^{15}]rGRF(1-29)NH_2 + 10^{-9} M hGRF(1-44)NH_2.

16) also potentiated the antagonistic activity. This result is consistent with the higher activity of hGRF(1-44)NH_2 than hGRF(1-29)NH_2 (9).

Since we had reported that rat GRF was more potent than human GRF to release growth hormone in the rat pituitary cell culture assay (8), we also introduced the [D-Arg^2] substitution in the rGRF(1-29)NH_2 sequence to determine if the resulting analog is more potent than the human counterpart as an antagonist. [D-Arg^2]rGRF(1-29)NH_2 (analog no. 17) indeed showed a slight increase in antagonistic potency than [D-Arg^2]hGRF(1-29)NH_2. Contrary to the results of the hGRF analogs, substitution of glycine at position-15 with L-amino acids to yield [D-Arg^2,Gln^{15},Nle^{27}]rGRF(1-29)NH_2 (analog no. 18) and [D-Arg^2,Ala^{15}]rGRF(1-29)NH_2 (analog no. 20) improved the antagonistic activity compared to [D-Arg^2]rGRF(1-29)NH_2. Furthermore, N-terminal acetylation was more effective not only to decrease the intrinsic activity, but also to increase the antagonistic potency. So far, [N-Ac-His^1,D-Arg^2,Gln^{15},Nle^{27}]rGRF(1-29)NH_2 (analog no. 19) and [N-Ac-His^1,D-Arg^2,Ala^{15}]rGRF(1-29)NH_2 (analog no. 21) are the most potent antagonists in the series of analogs that has been synthesized. A dose-response curve of the agonistic and antagonistic activities of analog no. 21 is presented in Figure 1. Further modifications based on this structure are in progress to increase its affinity to the GRF receptor.

ACKNOWLEDGMENTS

We wish to express our appreciation to D. Angeles, V. Bonora, M. Regno, R. Schroeder and C. Wong for their excellent technical assistance, and to E. Exum for secretarial assistance. This research was supported by program project grants from NIH (P01-HD-09690 and P01-DK-18811) and the Robert J. and Helen C. Kleberg Foundation.

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