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SUPER-ACTIVE ANALOGS OF GROWTH HORMONE-RELEASING FACTOR(1-29)-AMIDE

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Human pancreatic growth hormone releasing factor (1-29)-amide [hpGRF(1-29)-NH_2] and the following analogs: [D-Tyr-1]-hpGRF(1-29)-NH_2, [D-Ala-2]-hpGRF(1-29)-NH_2, [D-Asp-3]-hpGRF(1-29)-NH_2, and [N-Ac-Tyr-1]-hpGRF(1-29)-NH_2 were synthesized using solid phase methodology and tested for their ability to stimulate growth hormone (GH) secretion in the rat and the pig in vivo. [D-Ala-2]-hpGRF(1-29)-NH_2 was approximately 50 times more potent than the parent molecule in eliciting GH secretion in the rat. The other analogs were less active, but all were more potent than the 1-29 amide in the rat. [D-Tyr-1]-hpGRF(1-29)-NH_2 was 10 times more potent, [D-Asp-3]-hpGRF(1-29)-NH_2 7 times more potent, and the acetylated molecule approximately 12 times more potent than hpGRF(1-29)-NH_2.

Growth hormone releasing factors (GRFs) have been isolated from human pancreatic tumors (hpGRF)(1,2) and from rat hypothalamic tissue (rhGRF) (3). In both rats and humans the amidated 44 amino acid peptide hpGRF(1-44)-NH_2(4-6), and the free acid of the 40 amino acid residue, hpGRF(1-40)-OH,(7,8) have been shown to specifically release growth hormone (GH) at physiological doses. Full agonist activity is present in shorter fragments containing the first 37, 32 and 29 amino acids (1). With the limited amount of human growth hormone available

ABBREVIATIONS: hpGRF(1-24)-NH_2, hpGRF(1-29)-NH_2 and hpGRF(1-44)-NH_2, the amidated forms of human growth hormone releasing factor containing 24, 29 and 44 amino acids respectively. hpGRF(1-40)-OH, human growth hormone releasing factor with a free carboxy terminal consisting of 40 amino acids, rhGRF, rat hypothalamic growth hormone releasing factor. GH, growth hormone, GIP, gastric inhibitory polypeptide, VIP, vasoactive intestinal peptide, and PHI, porcine intestinal peptide having N-terminal histidine and C-terminal isoleucine.

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for the treatment of dwarfism, the potential therapeutic value of GRF and its analogs, especially in patients with a hypothalamic defect in GH release has recently been remarked upon (9-11). The GRFs isolated from human pancreatic tumors and rat hypothalamic tissue exhibit considerable sequence homologies with PHI, VIP, glucagon, secretin and GIP and are thus classed as members of the secretin-glucagon family of peptides (3). The N-terminal region of all of these peptides has been shown to be relatively more important than the C-terminal region in determining biological activity (12,13,14). The core of intrinsic biological activity in hpGRF is believed to reside between residues 4 and 20 (15). With these observations in mind, we prepared a series of analogs of hpGRF(1-29)-NH_2 in which amino acid residues in the N-terminal region were replaced by their D-isomers. We report here on analogs of hpGRF(1-29)-NH_2 which exhibit biological activities up to fifty times that of the parent compound when injected s.c. in rat. These analogs show similar potencies when injected i.v. in the anaesthetized pig.

MATERIALS AND METHODS

hpGRF(1-29)-NH_2 and the analogs listed in Table 1 were synthesized using solid phase methodology and preparative medium and high performance reverse-phase liquid chromatography as previously described (12,13). Yields of 5-10% with greater than 95% peptide purity were routinely achieved. Peptides were tested using a four point assay design (16,17) in anaesthetized rats (sodium pentobarbital, 6 mg/100g BW) by injecting the peptide s.c in a volume of 0.5ml, taking a blood sample from the jugular vein exactly 15 min after injection and assaying for GH by RIA using materials supplied by the NIADDKD.

Peptides were also tested in mixed breed male and female pigs weighing between 30 and 70kg. The pigs were tranquilized with a 500mg intramuscular injection of ketamine and maintained at a surgical plane of anesthesia with 4% halothane-oxygen at a flow rate of 3 l/min. A 20 gauge cannula was placed in the facial vein at its insertion into the external jugular and a slow drip of 30 IU sodium heparin per ml of isotonic saline was infused to maintain patency of the catheter. Peptide was administered and blood samples drawn via a three way stopcock in the line at the indicated times. Each peptide dose was given as a single 2ml bolus over a period of 20 sec followed by a flush of 2ml heparin-saline. Plasma samples were analyzed for GH

using the NIADDKD kit for rat GH. Serial dilution of pig plasma produced a displacement curve parallel to that of rat GH. Pig plasma growth hormone is expressed as ng/ml equivalents of the NIAMDKD rat GH standard.

RESULTS AND DISCUSSION

The results of the *in vivo* injections of hpGRF(1-29)-NH_2 and analogs in the rat are shown in Table 1. The parent molecule of this series, hpGRF(1-29)-NH_2, is approximately one half as active as hpGRF(1-40)-OH in this *in vivo* assay (8), but is equal in potency to the 40 residue molecule in the *in vitro* rat pituitary cell culture assay (Heiman, M., D.C., W.M., V.L. in preparation). The hpGRF(1-24)-NH_2 fragment (Table 2) has about 2.0% the activity of hpGRF(1-29)-NH_2 *in vivo*. This loss of biological activity in shorter fragments of hpGRF has previously

Table 1. Bioassay data for hpGRF(1-29)-NH_2 and analogs in the rat

Analog	Dose (ug/100g BW)	Plasma GH* (ng/ml)	
Saline	-	541 ± 64	(13)
hpGRF(1-29)-NH_2	10	1627 ± 380	(6)
"	25	2520 ± 420	(5)
[D-Tyr^1]-hpGRF(1-29)-NH_2	1.2	1463 ± 263	(6)
"	3.0	3247 ± 622	(6)
Saline	-	454 ± 62	(6)
hpGRF(1-29)-NH_2	10	2057 ± 187	(5)
"	25	4033 ± 284	(6)
[D-Ala^2]-hpGRF(1-29)-NH_2	0.08	1118 ± 158	(6)
"	0.20	2122 ± 350	(5)
Saline	-	299 ± 39	(8)
hpGRF(1-29)-NH_2	10	1368 ± 330	(7)
"	25	2638 ± 366	(7)
[D-Asp^3]-hpGRF(1-29)-NH_2	1.0	1029 ± 182	(7)
"	2.5	1962 ± 280	(7)
Saline	-	342 ± 53	(9)
hpGRF(1-29)-NH_2	7.2	1686 ± 255	(6)
"	18	2842 ± 295	(6)
[N-Ac-Tyr^1]-hpGRF(1-29)-NH_2	1.2	2518 ± 598	(6)
"	3.0	4899 ± 557	(6)

*Values are the mean ± SEM (n).

Table 2. Bioassay data for hpGRF(1-24)-NH_2 in the rat.

Analog	Dose (ug/100g BW)	Plasma GH* (ng/ml)
Saline	-	278 + 37 (6)
hpGRF(1-24)-NH_2	10	461 + 43 (6)
"	100	557 + 135 (6)
"	500	1673 + 318 (6)

*Values are the mean ± SEM (n).

been reported on in an *in vitro* system (1,2), however, Wehrenberg and Ling (18) were unable to demonstrate any bioactivity of hpGRF(1-24) in the rat *in vivo*. It was not stated however, if the peptide they were testing had a free or amidated carboxy terminal. [D-Tyr^1]hpGRF(1-29)-NH_2 was 10 times more active than the native sequence in the rat (Tables 1 and 3). This result is at variance with data reported by Ling and Brazeau (15) and Rivier *et al.* (19) which indicated decreased potencies of similar analogs of hpGRF(1-44)-NH_2 tested in monolayer pituitary cell cultures. Similarly, the high activity of the N-terminally acetylated hpGRF(1-29)-NH_2 itself is at odds with the reported *in vitro* results. This suggests that the increased *in vivo* potencies of these analogs might be a result of increased biological half-life. However, time course studies in this laboratory (unpublished observations) do not reveal any prolonged GH-releasing activity.

Table 3. Estimated in vivo potency of hpGRF(1-29)-NH_2.

Analog	% Activity	C.L.*
hpGRF(1-29)-NH_2	100	
[D-Tyr^1]-hpGRF(1-29)-NH_2	993	(556-1800)
[D-Ala^2]-hpGRF(1-29)-NH_2	5146	(3365-7868)
[D-Asp^3]-hpGRF(1-29)-NH_2	670	(392-1146)
[N-Ac-Tyr^1]-hpGRF(1-29)-NH_2	1224	(487-2050)

*95% confidence limits.

[D-Ala^2]hpGRF(1-29)-NH_2 exhibits extremely high GH-releasing activity both in the pig (Fig. 1) and the rat (Tables 1 and 3) and is also 50 times as potent as the parent peptide when tested in rat pituitary cell monolayer culture (M. Heiman, V.L., W.M., D.C., in preparation). Although, again, increased biological stability may be a factor, the *in vitro* data and time course experiments indicate that this high activity is probably the result of increased receptor affinity.

In the pig hpGRF(1-29)-NH_2 at a dose of 250 ng/kg BW typically caused a significant rise in GH for over 60 min (Fig. 2). This is similar to that seen in humans injected with a dose of 1000 ng/kg BW of hpGRF(1-44)-NH_2 (5,6) or hpGRF(1-40)-OH (7,10). There was however, more individual variation in the response to the peptides in the pig compared to the rat. Similar variability in the response to GRFs has been noted in healthy human subjects (5-7). The 29 amino acid amide and the free acid of the 1-40 peptide are equipotent in humans (G. M. Besser, D.C. *et al.*, in preparation). As can be seen (Fig. 2) there does not appear to be any priming effect of GRF in the

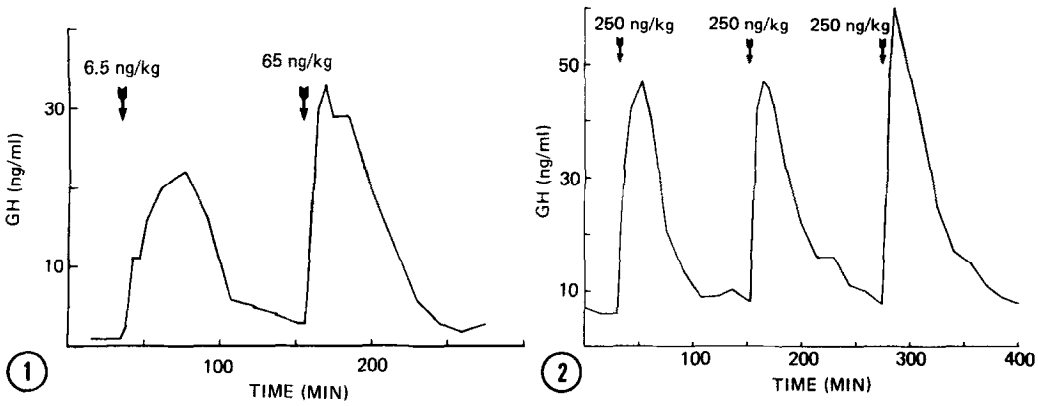

Figure 1. Response in the anaesthetized pig to intravenous injections of [D-Ala-2]-hpGRF(1-29)NH_2. Plasma growth hormone levels are expressed as ng/ml equivalents of the NIADDKD rat standard.

Figure 2. Response in the anaesthetized pig to three sequential intravenous doses of hpGRF(1-29)NH_2.

pig, repeated doses of the peptide giving similar responses. A similar lack of a priming effect has been reported following repeated injections of hpGRF(1-44)-NH_2 in the conscious rabbit (20). When higher doses of the 29 amino acid peptide were tested in the pig the rise in GH was greater (up to 100 ng/ml), and elevated hormone levels persisted for more than 2 hours (data not shown). The difference in sensitivities between pig and human may be due to species differences or could be due to an effect of the anaesthetic.

Similarly, the [D-Asp^3]-analog was also superactive being 7 times more active than hpGRF(1-29)-NH_2. A summary of the estimated potencies of the analogs tested with respect to hpGRF(1-29)-NH_2 is presented in Table 3. Analogues with D-amino acid substitutions in positions 4 to 7 did not exhibit increased activity (manuscript in preparation).

The reason for the increased biological activity of these analogs of GRF is not known. However, an analog of glucagon in which Gly^4 is replaced by D-Phe has about 7 times the glycogenolytic activity of glucagon in the rat (12). It was suggested (12) that substitution of D-Phe resulted in stabilization of a B-bend conformation centered around the Gly residue. Indeed, a similar strategy has been found with several other peptides, notably LH-RH (21) and enkephalin (22) in which insertions of D-Ala in positions 6 and 2 respectively, has resulted in analogs of far greater potency than the parent compound.

The fact that the D-Ala^2 analog is almost as potent in the pig as in the rat suggests that this analog may be equally potent in humans. Combinations of the modifictions described above in a single molecule and substitutions of D-amino acids at

other regions of the molecule are currently being investigated to see if analogs of even greater potency can be developed.

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