Peptides, Vol. 12, pp. 569–574. © Pergamon Press plc, 1991. Printed in the U.S.A. 0196-9781/91 $3.00 + .00

GRF Analogs and Fragments: Correlation Between Receptor Binding, Activity and Structure

ROBERT M. CAMPBELL,[1] YUNG LEE, JEAN RIVIER,*
EDGAR P. HEIMER, ARTHUR M. FELIX AND THOMAS F. MOWLES

*Departments of Animal Science and Peptide Research, Hoffmann-La Roche Inc., Nutley, NJ 07110 and *Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA 92037*

Received 2 November 1990

CAMPBELL, R. M., Y. LEE, J. RIVIER, E. P. HEIMER, A. M. FELIX AND T. F. MOWLES. *GRF analogs and fragments: Correlation between receptor binding, activity and structure.* PEPTIDES **12**(3) 569–574, 1991.—GH-releasing activity in vitro was directly correlated with GRF receptor binding affinity for all hGRF analogs examined. hGRF(1–29)-NH_2 analogs with Ala^{15}-substitution (for Gly^{15}) displayed 4–5 times higher affinity for the GRF receptor relative to hGRF(1–44)-NH_2. Replacement of Gly^{15} with Sar^{15} resulted in a dramatic loss of activity and receptor binding. The present data supports the proposal that Ala^{15}-substitution increases receptor affinity, and hence potency, due to increased amphiphilic α-helical interactions. Fragments of hGRF, representative of DPP-IV and trypsin-like cleavage, are inactive as a consequence of greatly diminished GRF receptor binding. These results provide a comprehensive analysis of the structural features required for both GRF receptor binding and activation.

Growth hormone-releasing factor (GRF) GRF analogs Growth hormone (GH) Rat anterior pituitary GRF receptor binding

IT has been proposed that specific regions of peptides (e.g., enkephalins, endorphins, melittin, calcitonin, glucagon, PTH, CRF and GRF) assume amphiphilic secondary structures in suitable environments, such as cell membranes (12). Human GRF is substantially amphiphilic between residues 4–29 (12). Calculations of secondary structure (circular dichroism and molecular dynamics based upon 2-D NMR) indicate that hGRF(1–29)-NH_2 is α-helical between residues 4–29 in 75% methanol/water and between residues 9–14 and 24–28 in water (14). Theoretically, a synthetic GRF peptide with optimized amphiphilic conformation would participate in stronger interactions with the membrane receptor (i.e., increased affinity) and exhibit greater biopotency. Indeed, hGRF(1–29)-NH_2 analogs designed to enhance amphiphilic, α-helical character have been reported to display greater in vitro (4–6, 15, 18, 19) and in vivo GH-releasing activities (3, 13, 22).

As GRF potency may be increased by conformational enhancements, it may also be naturally decreased by plasma enzyme activity or spontaneous oxidation. hGRF(1–44)-NH_2, hGRF(1–29)-NH_2 and [Ala^{15}]hGRF(1–29)-NH_2 are rapidly metabolized in plasma as a result of Ala^2-Asp^3 cleavage (dipeptidylpeptidase IV: DPP-IV) to hGRF(3–44)-NH_2, hGRF(3–29)-NH_2 and [Ala^{15}]hGRF(3–29)-NH_2, respectively (9,10). Other minor metabolites observed are characteristic of trypsin-like degradation between Arg^{11}-Lys^{12} and Lys^{12}-Val^{13} residues (10). Direct tryptic digestion of hGRF(1–44)-NH_2 reveals further susceptibility to degradation between Arg^{20}-Lys^{21} and Lys^{21}-Leu^{22} residues (11). The effect of DPP-IV or trypsin-like enzymes on the receptor binding affinity of resultant hGRF fragments has not been previously studied. hGRF may also be nonenzymatically oxidized at position 27 (Met^{27}→$Met(O)^{27}$) to yield a methionine sulfoxide derivative with reduced bioactivity (16). The effect of Met^{27} oxidation on hGRF receptor binding has yet to be reported.

The objectives of this study were as follows: 1) to design and synthesize novel, highly potent hGRF analogs, 2) to systematically correlate the in vitro GH-releasing activity of hGRF analogs and metabolites with receptor binding affinity, and 3) to characterize the effects of enzymatic cleavage on hGRF activity and receptor binding affinity using representative hGRF fragments. For these experiments, novel hGRF(1–29)-NH_2 analogs have been synthesized with selected amino acid substitutions in an effort to extend and/or stabilize the α-helical region and optimize the amphiphilic secondary structure. Fragments of hGRF(1–44)-NH_2, hGRF(1–29)-NH_2 and [Ala^{15}]hGRF(1–29)-NH_2 analogs, representing plasma dipeptidylpeptidase IV (DPP-IV) and trypsin-like enzyme cleavage products, were also prepared.

[1]Requests for reprints should be addressed to Dr. Robert M. Campbell, Hoffmann-La Roche Inc., Department of Animal Science Research, 340 Kingsland Street/Bldg. 86, Nutley, NJ 07110.

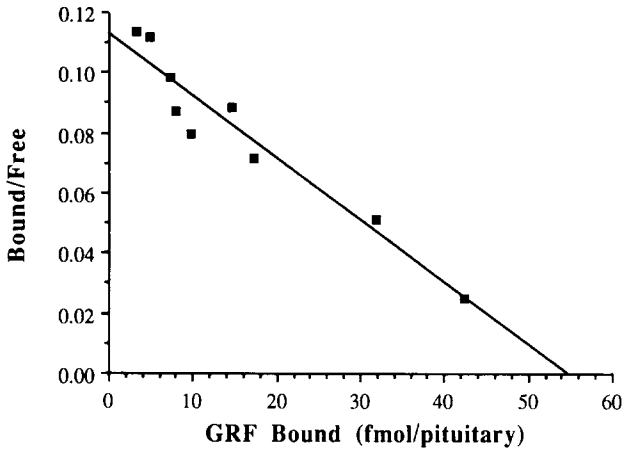

FIG. 1. Scatchard plot of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 binding to rat anterior pituitary homogenates (60 min incubation at 24°C). Increasing concentrations (500–500,000 cpm) of [His^1,^{125}I-Tyr^{10},Nle^{27}] hGRF(1–32)-NH_2 were added in the presence (nonspecific binding) or absence of 1 μM hGRF(1–44)-NH_2. The data is representative of 4 independent experiments, such that each concentration of radioligand was examined in quadruplicate tubes per experiment.

ABBREVIATIONS

GRF, growth hormone-releasing factor; GH, growth hormone; DPP-IV, dipeptidylpeptidase-IV; K_d, dissociation constant.

METHOD

Peptide and Radioligand Synthesis

Growth hormone-releasing factor analogs (human sequence: hGRF) were synthesized by solid-phase methodology (1). Methionine^{27} sulfoxide, [Met(O)^{27}], GRF analogs were prepared by the reaction of 10 mg GRF(1–44)-NH_2 or GRF(1–29)-NH_2 with 3% H_2O_2 in 40 ml H_2O. All GRF peptides were purified by preparative HPLC, determined to be homogeneous (≥99% purity) by analytical HPLC and the structure confirmed by amino acid analysis, fast atom bombardment mass spectroscopy and Edman sequence analysis. Secretin, glucagon, vasoactive intestinal peptide (VIP) and PHI(1–27) were obtained from Sigma Chemical (St. Louis, MO). Somatostatin [SRIF(1–14)] was purchased from Bachem (Torrance, CA). Bovine GH (bGH) was obtained from Dr. A. Parlow (Research and Education Institute, Torrance, CA). Iodogen reagent was purchased from Pierce Biochemical (Rockford, IL). [His^1,Nle^{27}]hGRF(1–32)-NH_2 (Peninsula Labs, Belmont, CA) was radioiodinated by the Iodogen method (8). The radioligand, [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2, was purified by reverse-phase HPLC [C18 column, Vydac, 4.6×250 mm, 5 μ particle size; mobile phase: (A) 0.1% trifluoroacetic acid in H_2O, (B) acetonitrile, in a linear gradient from 34–45% (B) in 30 min; flow rate: 1 ml/min, detection λ=206 nm] and collected in tubes containing 10 mg/ml bovine serum albumin (BSA) and 30 μg/ml bacitracin. Using a 9.5-min Iodogen reaction time, single, well-defined peaks corresponding to unreacted [His^1,Nle^{27}]hGRF(1–32)-NH_2 and [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 were observed at 11 and 14 min retention times, respectively. The specific activity of the monoiodinated radioligand was typically 100–150 μCi/μg and was stable for ≈10 days at 4°C.

GRF Radioreceptor (RRA) Assay

In preliminary experiments, no differences in binding affinity or receptor number were noted between crude rat anterior pituitary homogenates or purified membrane fractions (centrifugation of homogenates at 600×g for 10 min, followed by centrifugation of the supernatant at 44,000×g for 40 min). Therefore, in all studies, rat pituitary homogenates were prepared by the method of Struthers and coworkers (21), with some modifications, and used directly for radioreceptor assay. Anterior pituitaries from male Sprague-Dawley rats (200–220 g b.wt.) were excised, washed 3× with ice-cold RRA buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 20 mg/ml BSA and 30 μg/ml bacitracin, pH 7.4) and homogenized (Tissuemizer SDT-1810, speed 70, 30 s) on ice. For Scatchard analysis/saturation binding (Fig. 1), increasing concentrations of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 (500–500,000 cpm/tube in 100 μl RRA buffer) were added, in the presence (i.e., nonspecific binding) or absence of excess hGRF(1–44)-NH_2 (1 μM), to 0.4 ml pituitary homogenate (0.25 pituitary equivalents) per tube. For competitive experiments (Table 1, Fig. 3a–d), 1.2×10^5 cpm of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 (100 μl in RRA buffer) and varying concentrations of specified nonradioactive analogs ($10^{-3}\rightarrow10^4$ nM) were added to 0.4 ml pituitary homogenate (0.25 pituitary equivalents) per tube. Following

TABLE 1

POTENCIES AND RECEPTOR BINDING AFFINITIES OF HUMAN GRF (hGRF) ANALOGS, hGRF FRAGMENTS AND OTHER PEPTIDES RELATIVE TO hGRF(1–44)-NH_2

hGRF Analog	Relative Potency	Relative Affinity
hGRF(1–44)-NH_2 (standard)	1.00	1.00
[Met(O)^{27}]hGRF(1–44)-NH_2	0.30±0.04	0.17±0.05
[His^1,Nle^{27}]hGRF(1–32)-NH_2	2.24±0.29	2.65±0.20
[His^1,I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2	2.65±0.30	2.46±0.43
hGRF(1–29)-NH_2	0.71±0.23	0.91±0.22
[Met(O)^{27}]hGRF(1–29)-NH_2	0.32±0.06	0.17±0.06
[desNH$_2$Tyr^1]hGRF(1–29)-NH_2	1.24±0.12	0.82±0.15
[D-Ala^2]hGRF(1–29)-NH_2	1.81±0.46	1.98±0.44
[Ala^{15}]hGRF(1–29)-NH_2	3.81±0.30	3.60±0.49
[desNH$_2$Tyr^1,Ala^{15}]hGRF(1–29)-NH_2	4.04±0.75	4.50±0.91
[D-Ala^2,Ala^{15}]hGRF(1–29)-NH_2	4.86±1.21	4.87±0.57
[desNH$_2$Tyr^1,D-Ala^2,Ala^{15}] hGRF(1–29)-NH_2	4.70±0.45	5.17±0.85
[Sar^{15}]hGRF(1–29)-NH_2	<0.001	<0.001
hGRF(3–44)-NH_2	<0.001	<0.001
hGRF(3–29)-NH_2	<0.001	<0.001
hGRF(12–29)-NH_2	<0.001	<0.001
hGRF(13–29)-NH_2	<0.001	<0.001
hGRF(21–29)-NH_2	<0.001	<0.001
hGRF(22–29)-NH_2	<0.001	<0.001
hGRF(1–11)-OH	<0.001	<0.001
hGRF(1–20)-NH_2	<0.001	<0.001
[Ala^{15}]hGRF(1–20)-NH_2	<0.001	<0.001
[Ala^{15}]hGRF(3–29)-NH_2	<0.001	<0.001
[Ala^{15}]hGRF(13–29)-NH_2	<0.001	<0.001
Secretin, glucagon, VIP, PHI(1–27)	<0.001	<0.001
SRIF(1–14), TRH, bGH	<0.001	<0.001

For assessment of biological potency, cultured (96 h) anterior pituitary cells were incubated for 4 h in the presence of hGRF analogs, hGRF fragments or other peptides and compared for GH-releasing ability. Binding affinities were determined by competitive displacement of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 from rat pituitary homogenate receptors (60 min, 24°C). Potencies and binding affinities (mean ± SEM; n≥3 experiments) were determined by a Parallel Line Bioassay computer program (PARATL, Hoffmann-La Roche).

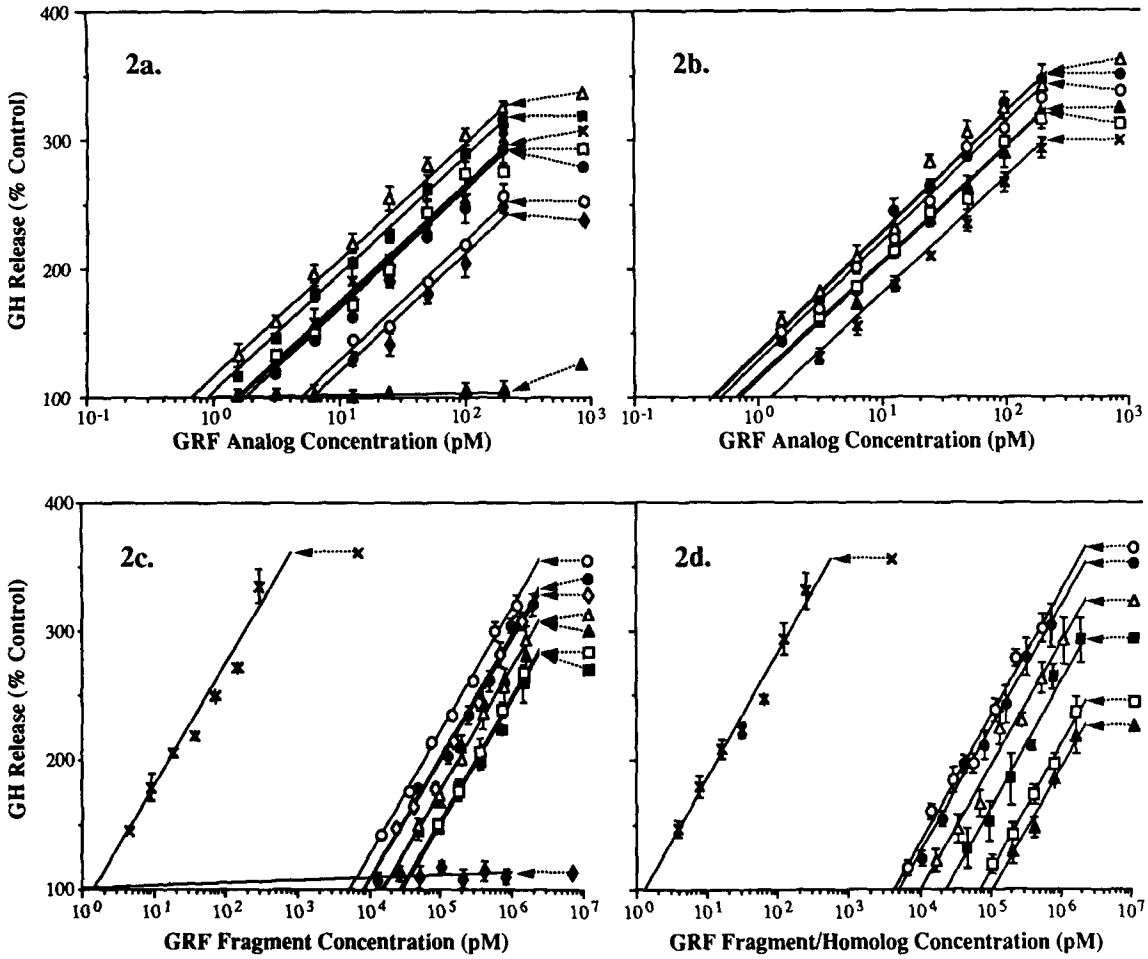

FIG. 2. (a–d). Effect of hGRF analogs or homologous peptides on GH release by cultured rat anterior pituitary cells. The following hGRF analogs were added at the indicated concentrations in serum-free BBM and allowed to incubate for 4 h. Figures illustrate only the linear portion of the log[dose-response] curve, determined (95% confidence limits) by a Parallel Line Bioassay computer program (PARATL: Hoffmann-La Roche) for potency assessment. Computer-generated simple regression lines ($p \geq 0.90$) are shown. All results are presented as mean ± SEM for $N \geq 3$ independent determinations. (a) Effect of monosubstituted hGRF analogs: [Ala^{15}]hGRF(1–29)-NH$_2$ (△), [D-Ala^2]hGRF(1–29)-NH$_2$ (■), hGRF(1–44)-NH$_2$ (X), [desNH$_2$Tyr^1]hGRF(1–29)-NH$_2$ (□), hGRF(1–29)-NH$_2$ (●), [Met(O)^{27}]hGRF(1–44)-NH$_2$ (○), [Met(O)^{27}]hGRF(1–29)-NH$_2$ (◆) and [Sar^{15}]hGRF(1–29)-NH$_2$ (▲). (b) Effect of multisubstituted hGRF analogs: [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)-NH$_2$ (△), [D-Ala^2,Ala^{15}]hGRF(1–29)-NH$_2$ (●), [desNH$_2$Tyr^1,Ala^{15}]hGRF(1–29)-NH$_2$ (○), [His^1,I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH$_2$ (▲), [His^1,Nle^{27}]hGRF(1–32)-NH$_2$ (□) and hGRF(1–44)-NH$_2$ (X). (c) Effect of hGRF fragments: hGRF(1–44)-NH$_2$ (X), hGRF(3–44)-NH$_2$ (○), hGRF(1–20)-NH$_2$ (●), hGRF(3–29)-NH$_2$ (◆), hGRF(12–29)-NH$_2$ (△), hGRF(13–29)-NH$_2$ (▲), hGRF(21–29)-NH$_2$ (□), hGRF(22–29)-NH$_2$ (■) and hGRF(1–11)-OH (◇). (d) Effect of Ala^{15}-substituted hGRF fragments and homologous peptides: hGRF(1–44)-NH$_2$ (X), [Ala^{15}]hGRF(1–20)-NH$_2$ (○), [Ala^{15}]hGRF(3–29)-NH$_2$ (●), [Ala^{15}]hGRF(13–29)-NH$_2$ (△), VIP (■), PHI(1–27) (□) and secretin (▲).

these additions (saturation- and competitive-binding assays) all tubes were vortexed and incubated on an orbital shaker (1500 rpm) at room temperature (24°C). A 450 μl aliquot from each tube was then transferred to polypropylene microcentrifuge tubes, centrifuged (2 min at ≈8000 × g) and the supernatant aspirated. To reduce nonspecific binding, 1 ml/tube of RRA buffer was added and the tubes recentrifuged. This "washing" step was then repeated and the supernatant again aspirated. The tube tip, containing the pellet, was cut off for counting (Micromedic 10/600 PLUS γ-counter). Similar to previous observations (21), equilibrium binding was achieved at ≈60 min and was stable for up to 120 min at 24°C. Subsequently, all GRF saturation- and competitive-binding experiments were conducted at 24°C for 60 min. Under these conditions, total (specific + nonspecific) binding was 11–16% of radioligand added; specific binding accounting for 65–75% of the total cpm bound (i.e., nonspecific binding was 25–35% of total binding).

Pituitary Cell Dispersion and Culture

Anterior pituitary cells from male Sprague-Dawley rats (150–160 g b.wt.) were dispersed with 0.4% collagenase/0.2% dispase (80 min), followed by a brief (10 min) incubation with neuraminidase (4 μg/ml) as previously described (2). After 4 days of culture, cells were incubated in the presence or absence of hGRF analogs for 4 h (37°C) in 1 ml of defined serum-free Dulbecco's MEM (2) per well at concentrations of 3.1→400 pM (1:2 serial dilutions). Peptides with potencies <0.01 relative

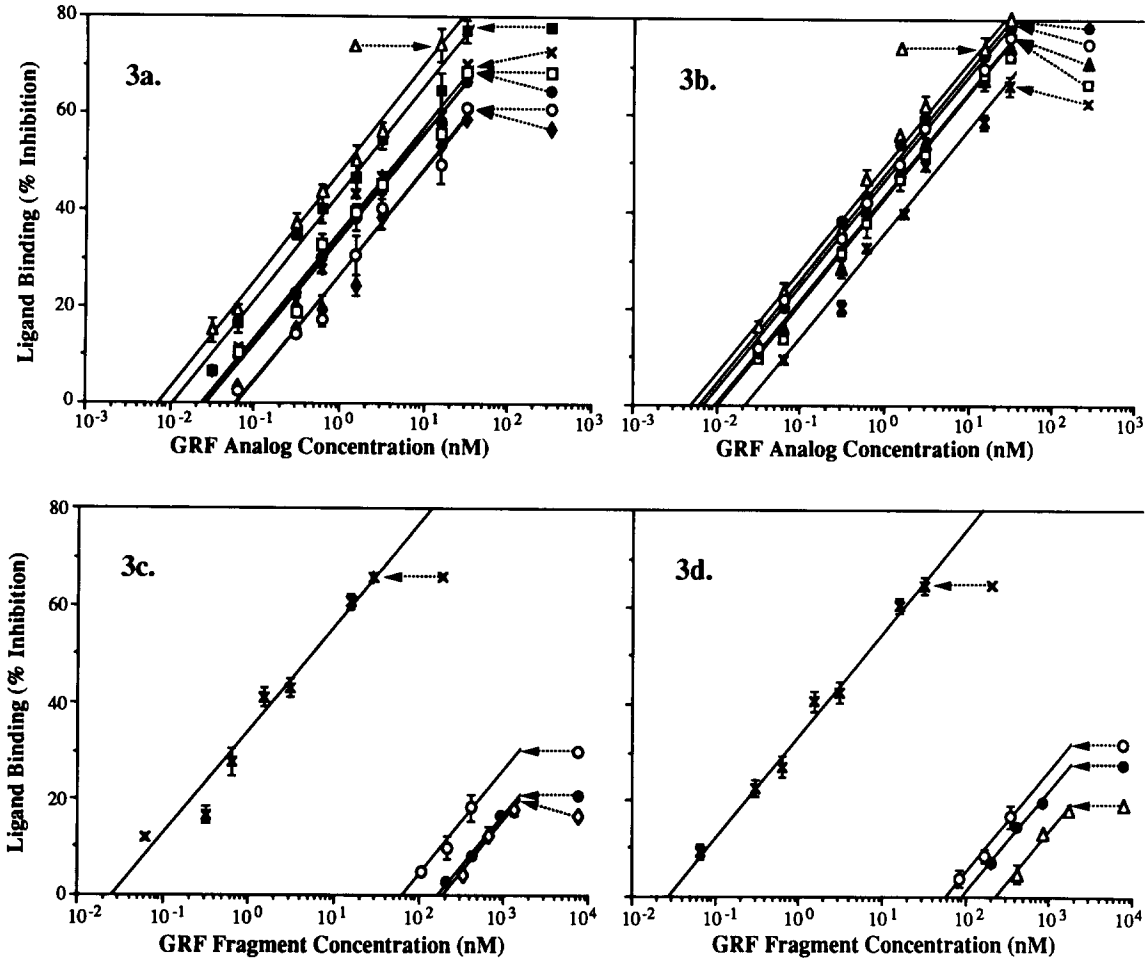

FIG. 3. (a–d) Competitive inhibition of [His^1,^{125}I-Tyr^{10},Nle^{27}]GRF(1–32)-NH_2 binding to rat anterior pituitary homogenates (60 min, 24°C) by hGRF analogs, fragments and homologous peptides. Figures illustrate only the linear portion of the log[dose-response] curve, determined (95% confidence limits) by a Parallel Line Bioassay computer program (PARATL: Hoffmann-La Roche) for affinity assessment. Computer-generated simple regression lines ($p \geq 0.90$) are shown. All results are presented as mean ± SEM for N≥3 independent determinations. (a) Effect of monosubstituted hGRF analogs: [Ala^{15}]hGRF(1–29)-NH_2 (△), [D-Ala^2]hGRF(1–29)-NH_2 (■), hGRF(1–44)-NH_2 (X), hGRF(1–29)-NH_2 (□), [desNH$_2$Tyr^1]hGRF(1–29)-NH_2 (●), [Met(O)^{27}]hGRF(1–44)-NH_2 (○), [Met(O)^{27}]hGRF(1–29)-NH_2 (◆) and [Sar^{15}]hGRF(1–29)-NH_2 (▲). (b) Effect of multisubstituted hGRF analogs: [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]hGRF(1–29)-NH_2 (△), [D-Ala^2,Ala^{15}]hGRF(1–29)-NH_2 (●), [desNH$_2$Tyr^1,Ala^{15}]hGRF(1–29)-NH_2 (○), [His^1,I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 (▲), [His^1,Nle^{27}]hGRF(1–32)-NH_2 (□) and hGRF(1–44)-NH_2 (X). (c) Effect of hGRF fragments: hGRF(1–44)-NH_2 (X), hGRF(3–44)-NH_2 (○), hGRF(1–20)-NH_2 (●) and hGRF(3–29)-NH_2 (◇). No inhibition of [His^1,^{125}I-Tyr^{10},Nle^{27}]GRF(1–32)-NH_2 binding was observed with hGRF(12–29)-NH_2, hGRF(13–29)-NH_2, hGRF(21–29)-NH_2, hGRF(22–29)-NH_2 and hGRF(1–11)-OH. (d) Effect of Ala^{15}-substituted hGRF fragments and homologous peptides: hGRF(1–44)-NH_2 (X), [Ala^{15}]-hGRF(1–20)-NH_2 (○), [Ala^{15}]hGRF(3–29)-NH_2 (●) and [Ala^{15}]hGRF(13–29)-NH_2 (△). No inhibition of [His^1,^{125}I-Tyr^{10},Nle^{27}]GRF(1–32)-NH_2 binding was observed with VIP, PHI(1–27), secretin, glucagon, SRIF(1–14), TRH or bGH.

to hGRF(1–44)-NH_2 were reassayed at higher concentrations (0.8→1600 nM). Each dose of GRF analog was tested in quadruplicate wells. Media aliquots (75 μl, diluted 1:40 in RIA buffer) were later assayed for GH by standard double antibody RIA employing reagents (NIADDK Rat GH standard GH-RP-2) supplied by Dr. A. Parlow (Torrance, CA).

Data Analysis

Relative potencies and receptor binding affinities, representing pooled data from at least three independent experiments, were calculated using a Parallel Line Bioassay Program (PARATL: Hoffmann-La Roche, written for the DEC VAX cluster), such that hGRF(1–44)-NH_2 was assigned a potency of 1.0. PARATL plots log[dose] vs. response (linear regression) and employs the following formula for calculation of relative potency (% stimulation) or affinity (% inhibition):$\ln(\rho) = \ln(z) - \ln(x) = (a_s - a_t)/b$; where ρ = relative potency/affinity, x = standard dose, z = test dose, a_s = y-intercept of the standard, a_t = y-intercept of the test substance, b = parallel slope. PARATL tests (95% confidence) for both parallelism (i.e., to the standard) and linearity (i.e., excluding points significantly departing from linearity, such as doses producing no effect or saturation). Therefore, calculations (Table 1) and plots (Figs. 2 and 3) represent the linear portion of the log[dose]-response curve.

RESULTS

GRF Receptor Saturation Assays

A Scatchard plot of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2

binding to rat anterior pituitary homogenates is shown in Fig. 1. GRF binds to a single high-affinity class of receptors as best fit (Cricket Graph software, Malvern, PA) by simple linear regression ($r^2 = .84$). The mean (±SEM, N = 4 experiments) calculated GRF receptor concentration was 54.40 ± 12.68 fmol/pituitary with a dissociation constant (K_d) of 202 ± 27 pM.

GRF Analogs: Relative Potencies vs. Receptor Affinities

With all hGRF analogs examined, in vitro GH-releasing activity (Fig. 2) was directly correlated (Table 1: $r^2 = .98$, potency vs. affinity) with GRF receptor binding affinity (Fig. 3). Various peptides (≤1 μM) with homologous [secretin, glucagon, VIP and PHI(1–27)] and nonhomologous (SRIF-14, TRH, bGH) amino acid sequences did not displace [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 bound (Fig. 2). The binding affinity of hGRF(1–29)-NH_2 was similar to that of "full-length" hGRF(1–44)-NH_2, employed as a standard for these assays. However, the affinities of both hGRF(1–29)-NH_2 and hGRF(1–44)-NH_2 were greatly reduced (81% and 83%, respectively) by Met^{27} oxidation. Deamination of Tyr^1, [desNH$_2$Tyr^1], did not affect hGRF(1–29)-NH_2 binding, while D-Ala^2 (replacement of L-Ala^2) increased affinity (≈2-fold). hGRF(1–29)-NH_2 analogs with Ala^{15}-substitution (for Gly^{15}) displayed 4–5 times higher affinity for the GRF receptor relative to hGRF(1–44)-NH_2. The effect of [Ala^{15}]-substitution was not significantly altered by additional [desNH$_2$Tyr^1]- and/or [D-Ala^2]-substitution. However, replacement of Gly^{15} with Sar^{15} resulted in a dramatic loss in GH-releasing activity and receptor binding.

The predominant fragments of hGRF(1–44)-NH_2, hGRF(1–29)-NH_2 and [Ala^{15}]hGRF(1–29)-NH_2 observed in plasma due to DPP-IV cleavage between Ala^2 and Asp^3 residues were relatively inactive in vitro [Fig. 2, Table 1: potency <0.001 relative to hGRF(1–44)-NH_2]: hGRF(3–44)-NH_2,hGRF(3–29)-NH_2 and [Ala^{15}]-hGRF(3–29)-NH_2. Some displacement (10–20%) of bound [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 was noted with these fragments at the highest concentrations tested (≤1.6 μM), but was insufficient for assessment of relative affinity (Fig. 3). Fragments accounting for trypsin-like cleavage between residues Arg^{11}-Lys^{12}, Lys^{12}-Val^{13}, Arg^{20}-Lys^{21} and Lys^{21}-Leu^{22} were also biologically inactive (Fig. 2): hGRF(12–29)-NH_2, hGRF(13–29)-NH_2, hGRF(21–29)-NH_2, hGRF(22–29)-NH_2, hGRF(1–11)-OH, hGRF(1–20)-NH_2, [Ala^{15}]hGRF(1–20)-NH_2 and [Ala^{15}]hGRF(13–29)-NH_2. With the exception of hGRF(1–20)-NH_2 and [Ala^{15}]hGRF(1–20)-NH_2 (i.e., 12–16% displacement at 1.4 μM only), none of these fragments displaced the bound [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 up to 1 μM (Fig. 3).

DISCUSSION

In the present studies, [His^1,^{125}I-Tyr^{10},Nle^{27}]hGRF(1–32)-NH_2 was demonstrated to bind specifically to a single class of receptors in rat anterior pituitary homogenates. The receptor number and dissociation constant (K_d) determined from saturation binding (i.e., using increasing amounts of radioligand) (Fig. 1) is comparable to that previously reported using competitive binding (i.e., using fixed radioligand concentrations, but varied nonradioactive GRF analog concentrations) (19,20). Specificity of GRF binding was illustrated by the inability of secretin, glucagon, VIP, PHI(1–27), SRIF(1–14), TRH and bGH to displace the bound radioligand. In all cases, the in vitro GH-releasing activity of hGRF(1–44)-NH_2, hGRF(1–29)-NH_2 analogs, hGRF fragments and other peptides was directly correlated ($r^2 = .98$) with GRF receptor binding affinity.

Several hGRF(1–29)-NH_2 analogs were constructed in an attempt to increase biological potency. Replacement of Gly^{15} (a neutral amino acid) with Ala^{15} (a hydrophobic amino acid) was initially selected for two reasons: 1) to increase regional amphiphilicity via hydrophobic amino acid substitution and 2) to extend and/or stabilize the central α-helical core. When displayed in an α-helical (≈4 residues per turn) Edmundson wheel projection (Fig. 4), Ala^{15} enhances the hydrophobic character of one portion of the helix (Ile^5, Leu^{23}, Ala^{19}, Ile^{26}, Ala^{15}, Ala^4 and Leu^{22}), which is opposite another region of high hydrophobicity (Val^{13}, Phe^6, Leu^{17}, Tyr^{10} and Leu^{14}), and separated by a region of neutral→hydrophilic character (Thr^7, Asp^{25}, Ser^{18}, Arg^{11}, Arg^{29}, Arg^{20}, Ser^9 and Gln^{16}). Substitution of a hydrophobic amino acid such as Ala^{15} would theoretically allow for improved ligand insertion into the amphipathic bilayer of the pituitary membrane receptor. Improved stability/rigidity of the α-helix by Ala^{15}-substitution (a better helix-stabilizing amino acid than Gly by virtue of increased intramolecular interactions) may increase the "tightness of fit" or affinity for the receptor. Physical-chemical measurements (circular dichroism, molecular dynamics calculations based upon 2-D NMR) confirm that Ala^{15}-substitution extends the α-helical segments from residues 9–14 and 24–28 [for hGRF(1–29)-NH_2 in aqueous solution] to residues 9–15 and 21–26 (for [Ala^{15}]hGRF(1–29)-NH_2 in aqueous solution) (14). Conversely, [Sar^{15}]-substitution destabilizes the α-helix, as [Sar^{15}]hGRF(1–29)-NH_2 exhibits little evidence of ordered secondary structure (14). The high receptor binding affinity and potency of Ala^{15}-substituted hGRF(1–29)-NH_2 analogs, combined with the low affinity/activity of [Sar^{15}]hGRF(1–29)-NH_2, provides support for the concept that enhanced amphiphilic, α-helical character produces a "preferred" bioactive conformation.

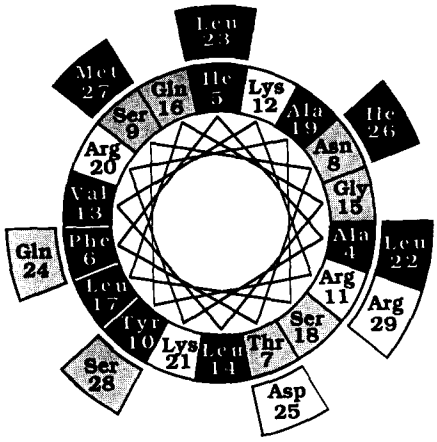

FIG. 4. Edmundson wheel projection of residues 4–29 of hGRF(1–29)-NH_2 in an α-helical conformation (turn frequency ≈4 residues). Replacement of Gly^{15} (a neutral amino acid) with the more hydrophobic Ala^{15} increases the relative amphiphilic character (i.e., relative segregation of hydrophobic and hydrophilic/neutral residues) and stability of the α-helix, resulting in enhanced biopotency. Conversely, Sar^{15}-substitution destabilizes the α-helix (due to unfavorable side-chain interactions), disrupts any ordered secondary structure and, hence, is biologically inactive. Hydrophobic residues are black, neutral residues are shaded, and charged residues are white.

Fragments of hGRF(1–44)-NH_2, hGRF(1–29)-NH_2 and [Ala^{15}]hGRF(1–29)-NH_2 were synthesized, omitting residues Tyr^1 and Ala^2, to simulate DPP-IV cleavage products. The extremely low affinity of hGRF(3–44)-NH_2, hGRF(3–29)-NH_2 and [Ala^{15}]hGRF(3–29)-NH_2 (Table 1), similarly noted with hGRF(2–32)-NH_2(19,20), indicates an absolute requirement of amino-terminus residues 1 and 2 for GRF binding to rat pituitary receptors. The terminal amino group of [Tyr^1] is not re-

quired since [desNH$_2$Tyr^1]-substitution does not affect GRF receptor binding. However, replacement of [D-Ala^2] for [L-Ala^2] moderately increased hGRF(1–29)-NH$_2$ affinity (1.98 ± 0.44) in the rat GRF RRA. The relative affinity of [D-Ala^2]hGRF(1–29)-NH$_2$ is in agreement with previous reports, where postreceptor effects were determined: GH release (4, 5, 15) or adenyl cyclase activity (17). The mechanism of the in vitro effects of [D-Ala^2]-substitution is unclear, but speculatively may involve resistance to membrane peptidases (if they exist) and/or more favorable orientation of side-chain moieties.

Various fragments representing trypsin-like cleavage between Arg^{11}-Lys^{12}, Lys^{12}-Val^{13}, Arg^{20}-Lys^{21} and Lys^{21}-Leu^{22} residues also displayed low affinity and activity in vitro. These observations suggest that plasma DPP-IV and trypsin-like enzymes convert hGRF to fragments which are incapable of binding to the receptor (i.e., rendering it biologically inactive). A comprehensive survey of DPP-IV and trypsin-like cleavage (of hGRF) occurring in vivo has not been conducted.

Based upon the present data, the GRF analog of choice for potential clinical and/or veterinary application would exhibit enhanced potency and resistance to enzymatic degradation. Substitution of [desNH$_2$Tyr^1] and/or [D-Ala^2] has been previously shown to inhibit plasma DPP-IV degradation of hGRF(1–29)-NH$_2$ analogs in vitro (10), potentially conferring longer duration of action in vivo. When combined with [Ala^{15}]-replacement, the resulting trisubstituted analog, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}] hGRF(1–29)-NH$_2$ (6), is highly potent (Table 1: 4–5-fold that of hGRF) and resistant to plasma DPP-IV and trypsin-like degradation in vitro (10). The high in vivo activity of this analog reported recently in pigs (3) and dairy cows (13) may indeed be the combined result of high receptor affinity and increased resistance to enzymatic degradation.

ACKNOWLEDGEMENTS

The authors wish to thank R. S. Struthers for helpful discussions, P. Stricker for GH RIA, T. Egan for HPLC, and also Drs. C. Scanes and C. Campen for their critical review of this manuscript.

REFERENCES

1. Barany, G.; Merrifield, R. B. Solid-phase peptide synthesis. In: Gross, E.; Meienhofer, J.; eds. The peptides: Analysis, synthesis, biology. vol 2. New York: Academic Press 1980:1–284.
2. Brazeau, P.; Ling, N.; Böhlen, P.; Esch, F.; Ying, S.-Y.; Guillemin, R. Growth hormone-releasing factor, somatocrinin, releases pituitary growth hormone *in vitro*. Proc. Natl. Acad. Sci. USA 79:7909–7913; 1982.
3. Dubreuil, P.; Petitclerc, D.; Pelletier, G.; Gaudreau, P.; Farmer, C.; Mowles, T. F.; Brazeau, P. Effect of dose and frequency of administration of a potent analog of growth hormone-releasing factor on hormone secretion and growth in pigs. J. Anim. Sci. 68:1254–1268; 1990.
4. Felix, A. M.; Heimer, E. P.; Mowles, T. F.; Eisenbeis, H.; Leung, P.; Lambros, T. J.; Ahmad, M.; Wang, C.-T.; Brazeau, P. Synthesis and biological activity of novel growth hormone releasing factor analogs. In: Theodoropoulus, D., ed. Peptides 1986. New York: Walter de Gruyter & Co; 1987:481–484.
5. Felix, A. M.; Wang, C.-T.; Heimer, E.; Fournier, A.; Bolin, D. R.; Ahmad, M.; Lambros, T. J.; Mowles, T. F.; Miller, L. Synthesis and biological activity of novel linear and cyclic GRF analogs. In: Marshall, G. R., ed. Peptides: Chemistry and biology. Leiden: ESCOM; 1988:465–467.
6. Felix, A. M.; Heimer, E. P.; Wang, C.-T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Webrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. Synthesis, biological activity and conformational analysis of cyclic GRF analogs. Int. J. Pept. Prot. Res. 32:441–454; 1988.
7. Fournier, A.; Wang, C.-T.; Felix, A. M. Applications of BOP reagent in solid phase synthesis. Int. J. Pept. Prot. Res. 31:86–97; 1988.
8. Fraker, P. J.; Speck, J. C. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril. Biochem. Biophys. Res. Commun. 80:849–857; 1978.
9. Frohman, L. A.; Downs, T. R.; Williams, T. C.; Heimer, E. P.; Pan, Y.-C. E.; Felix, A. M. Rapid enzymatic degradation of growth hormone-releasing hormone by plasma in vitro and in vivo to a biologically inactive product cleaved at the NH$_2$ terminus. J. Clin. Invest. 78:906–913; 1986.
10. Frohman, L. A.; Downs, T. R.; Heimer, E. P.; Felix, A. M. Dipeptidylpeptidase IV and trypsin-like enzymatic degradation of human growth hormone-releasing hormone in plasma. J. Clin. Invest. 83:1533–1540; 1989.
11. Heimer, E. P.; Felix, A. M.; Ahmad, M.; Chang, M.; Hulmes, J.; Pan, Y.-C. E. Characterization of the tryptic digest of human growth hormone-releasing factor GRF(1–44)-NH$_2$. Fed. Proc. 45: 1715; 1986 (abstract).
12. Kaiser, E. T.; Kézdy, F. J. Amphiphilic secondary structure: Design of peptide hormones. Science 223:249–255; 1984.
13. Lapierre, H.; Pelletier, G.; Petitclerc, D.; Gaudreau, P.; Dubreuil, P.; Mowles, T. F.; Brazeau, P. Effect of a growth hormone-releasing factor analog on growth hormone, insulin-like growth factor 1 and milk production in dairy cows. Can. J. Anim. Sci. 70:525–535; 1990.
14. Madison, V.; Berkovitch-Yellin, Z.; Fry, D.; Greeley, D.; Toome, V. Conformation of three peptides deduced from experiments and molecular energetics. In: Tam, J.; Kaiser, E. T., eds. Synthetic peptides: Approaches to biological problems. vol. 86. New York: Alan R. Liss Inc; 1989:109–123.
15. Murphy, W. A.; Coy, D. H. Potent long-acting alkylated analogs of growth hormone-releasing factor. Pept. Res. 1:36–41; 1988.
16. Rivier, J.; Spiess, J.; Vale, W. Isolation of two human hypothalamic growth hormone releasing factors. In: Munekata, E., ed. Peptide chemistry 1983. Osaka: Protein Research Foundation; 1984:11–17.
17. Robberecht, P.; Waelbrook, M.; Coy, D.; De Neef, P.; Camus, J.-C.; Christophe, J. Comparative structural requirements of thirty GRF analogs for interaction with GRF- and VIP receptors and coupling to adenyl cyclase in rat adenopituitary, liver and pancreas. Peptides Suppl. 7:53–59; 1986.
18. Sato, K.; Hotta, M.; Kageyama, J.; Chiang, T.-C.; Hu, H.-Y.; Dong, M. H.; Ling, N. Synthesis and *in vitro* bioactivity of human growth hormone-releasing factor analogs substituted with a single D-amino acid. Biochem. Biophys. Res. Commun. 149:531–537; 1987.
19. Seifert, H.; Perrin, M.; Rivier, J.; Vale, W. Growth hormone releasing factor binding sites in rat anterior pituitary membrane homogenates: Modulation by glucocorticoids. Endocrinology 117:424–426; 1985.
20. Seifert, H.; Perrin, M.; Rivier, J.; Vale, W. Binding sites for growth hormone releasing factor on rat anterior pituitary cells. Nature 313:487–489; 1985.
21. Struthers, R. S.; Perrin, M.; Vale, W. Nucleotide regulation of growth hormone-releasing factor binding to rat pituitary receptors. Endocrinology 124:24–29, 1989.
22. Tou, J. S.; Kaempfe, L. A.; Vineyard, B. D.; Buonomo, F. C.; Della-Ferra, M. A.; Baile, C. A. Amphiphilic growth hormone releasing factor (GRF) analogs: Peptide design and biological activity *in vivo*. Biochem. Biophys. Res. Commun. 139:763–770; 1986.
23. Velicelebi, G.; Patthi, S.; Kaiser, E. T. Design and biological activity of analogs of growth hormone releasing factor with potential amphiphilic carboxyl termini. Proc. Natl. Acad. Sci. USA 83:5397–5399; 1986.