IDENTIFICATION AND PURIFICATION OF FACTOR B-GHRH FROM HYPOTHALAMI WHICH RELEASES GROWTH HORMONE

K. Nils-Gunnar Johansson, Bruce L. Currie and Karl Folkers*

Institute for Biomedical Research
The University of Texas at Austin
Austin, Texas 78712

Cyril Y. Bowers

Tulane University
School of Medicine
1430 Tulane Avenue
New Orleans, Louisiana 70112

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SUMMARY

Exploratory purifications and assays of fractions for release of growth hormone (GH) revealed two separable entities each of which unambiguously released GH by radioimmunoassay. They are provisionally designated factor A-GHRH and factor B-GHRH until they are chemically and biologically characterized. After initial steps of isolation from porcine hypothalami, factor B-GHRH was extensively purified by stepwise chromatography using Bio-Gel P-2, Sephadex LH-20, Sephadex G-25 with a partition system of acetic acid-butanol-pyridine, DEAE-Sephadex A-25, and Bio-Gel CM-2. Assays showed that certain fractions were active, in vitro, at levels of ca. 15 μg. Factor B-GHRH is inhibited by somatostatin.

INTRODUCTION

Deuben and Meites[1] in '64 believed they had demonstrated the existence of a factor in the hypothalamus of the rat which released in vitro the growth hormone (GH). They used an acid extract of rat hypothalami. Schally et al.[2] in '69 described the isolation of a peptide from 200,000 porcine hypothalami which released GH in rats at a dosage of 1 ng, and in vitro at 0.1 pg. Schally et al.[3] in '71 described the amino acid sequence of this peptide as Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Gly-Ala and designated it as the growth hormone-releasing hormone. Veber et al.[4] described the synthesis of this decapeptide, and observed the similarity between its sequence and the proposed amino-terminal sequence of the β-chain of porcine hemoglobin (Val-His-Leu-Ser-Ala-Glx-Glx) Lys(Ala,Glx,Val--). A citation in this paper[4] states that neither the synthetic nor natural decapeptide is active by the radioimmunoassay for release of rat GH. Subsequently,

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Schally et al.[5,6] affirmed that the "natural" decapeptide and its synthetic counterpart did not stimulate the release of immunoreactive GH when administered to sheep, monkeys, pigs and rats. The GH released was measured by the tibia method and by the formation of the sulfation factor.[6]

Wilbur et al.[7] in '71 reported on the hypothalamic hormone-releasing activity in extracts from rat and porcine hypothalami. They determined GH released in the medium by a double antibody radioimmunoassay when fractions from Sephadex G-25 were added. Stachura et al.[8] in '72 reported the extraction of ovine stalk-medium emminence with glacial acetic acid followed by gel filtration on Sephadex G-25, and found an increased release of GH from a pool of newly synthesized hormone by such extracts. Malacara et al.[9] in '72 reported that significant elevations of RIA-GH occurred in rats following injection of material from 90% methanol extracts of hypothalamic fragments, and that after filtration upon Sephadex G-10, the activity overlapped the distribution of TRH. Reichlin and Mitnick[10] in '73 observed the biosynthesis of growth hormone releasing factor by measuring the elevation of radioimmunoassayable GH using rat hypothalamic fragments in a special medium, and concluded that GHRF is formed enzymatically by a non-ribosomal mechanism.

Johansson et al.[11] in '73 reported the possible biosynthesis of hypothalamic hormones in mitochondria and, after purification of solvent extracts of mitochondrial fractions by Bio-Gel P-2 and Sephadex G-25, found an activity (GHRH) which released GH measured by radioimmunoassay.

Apparently, a hypothalamic growth hormone releasing hormone has not yet been clearly identified and extensively purified. Our effort toward isolation of GHRH has led to identification of two active entities in separable fractions both of which release, in vitro, GH as measured by a radioimmunoassay. These two entities have been provisionally named factors A-GHRH and B-GHRH; our experience on factor B-GHRH is described herein, and that on factor A-GHRH is described by Currie et al.[12]

METHODS

Preparation of Factor B Fractions from Porcine Hypothalami. - Batches of 5,000-10,000 lyophilized hypothalamic fragments were homogenized in methanolic acetic acid. The homogenate was filtered, and the filter cake was resuspended in fresh methanolic acetic acid with thorough mixing. Filtration and resuspension were repeated several times, and the combined filtrates were evaporated to dryness. The residue was defatted and then lyophilized (Stage SP).

The defatted extract was dissolved in 1 M acetic acid and submitted to gel filtration on Bio-Gel P-2 having an exclusion limit of 1800 Daltons (Stage P-2).

Subsequent purification steps included chromatography on columns of Sephadex LH-20 (Stage LH-20), partition chromatography on Sephadex G-25 with the solvent system A which was 0.1% acetic acid-butanol-pyridine (11:5:3) (Stage A-PC), DEAE-Sephadex A-25 (Stage DEAE-S) and Bio-Gel CM-2 (Stage CM-2). Samples for bioassays were taken after the defatting step, and after pooling appropriate fractions from each chromatographic column. The samples for assay were lyophilized and dissolved in 0.05 M sodium phosphate buffer, pH 7.4. Aliquots of 50 µl were added to the in vitro incubation medium at the beginning of the third hour (I_3) of incubation.

Assay Procedure. - The assays were conducted in vitro with pituitaries which were obtained from 20-day old female rats of the Sprague-Dawley strain. For each assay, two pituitaries were incubated in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10-ml Teflon beakers in a Dubnoff shaker. The incubations were at 37° C, and after pre-incubation for 1 hour, the medium was separated, discarded, and fresh medium was added. After a second hour, pre-incubation (P_2), the medium was separated for assay and replaced. Next, the samples were added to separate beakers, and after the third hour (I_3), the medium was removed and assayed for release of the growth hormone. The radioimmunoassay of Parlow, NIH Hormone Distribution Program, was used to determine the GH released into the medium and was calculated as ng of a GH standard (0.6 IU/mg). The samples for assay were lyophilized and the residues were dissolved in 0.05 M sodium phosphate buffer, pH 7.4. Aliquots of 50 µl were added to the in vitro incubation medium at the start of the third hour (I_3).

RESULTS AND DISCUSSION

Data on stages of fractionation and corresponding assay results are in Table 1. As exploratory fractionation developed, the assays for the release of GH made it increasingly clear that certain fractions at certain stages unambiguously released the growth hormone in the in vitro bioassay. Of unique importance, it was observed that releasing activity reproducibly occurred in two distinct regions of fractions following gel filtration on Bio-Gel P-2.

The bioassays of pooled fractions from six P-2 columns (Table 1) showed a consistent growth hormone releasing activity in the region of fractions 200-600 and higher. The data on these six columns are representative of similar results obtained from about 30 such P-2 columns. These fractions from 200-600 contained one active entity which released GH and was strongly retarded on Bio-Gel P-2. This entity was designated factor B-GHRH. The other active entity which released growth hormone was designated factor A-GHRH as described by Currie et al.[12] Some of the activity of factor B-GHRH can be obscurred in fractions where there

Table 1. DATA ON PURIFICATION AND ASSAY OF THE RELEASE OF GROWTH HORMONE BY FACTOR B-GHRH

Stage of Fractionation	Pooled Fractions	Dose	ng GH/ml (P_2)	ng GH/ml (I_3)
Saline Control	-	-	740	625
Saline Control	-	-	600	225
Saline Control	-	-	700	285
P-2	201-300[a]	210 µg	1130	1600
	301-615	90 µg	1420	1930
P-2	201-300	130 µg	890	>2560
	301-614	110 µg	420	655
P-2	201-300	210 µg	1275	1780
	301-720	150 µg	1460	2200
P-2	201-300	70 µg	1495	1195
	301-600	100 µg	675	800
P-2	201-300	160 µg	583	830
	301-609	90 µg	810	565
P-2	201-300	120 µg	1145	1700
	301-733	40 µg	985	815
Saline Control	-	-	1260	1560
LH-20	76-100[b]	20 µg	232	357
	101-140	10 µg	125	670
	141-350	20 µg	300	1280
Saline Control	-	-	740	625
Saline Control	-	-	195	225
Saline Control	-	-	700	285
A-PC	91-105[c]	50 µg	590	1190
A-PC	56- 72[d]	100 µg	800	1210
A-PC	151-175[e]	300 µg	428	833
	176-200	300 µg	431	782
Saline Control	-	-	725	428
DEAE-S	2 mM-1[f]	100 µg	430	1620
	2 mM-2	15 µg	470	1300
	10 mM	15 µg	670	955
Saline Control	-	-	425	170
CM-2	2 mM[g]	70 µg	200	2170
	10 mM	250 µg	840	2230

a. Each fraction was 14.5 ml, column dimensions 5.0 x 140 cm.
b. Each fraction was 10 ml, column dimensions 1.5 x 90 cm.
c. Each fraction was 4.4 ml, column dimensions 2.0 x 90 cm.
d. Each fraction was 10 ml, column dimensions 2.5 x 95 cm.
e. Each fraction was 4.4 ml, column dimensions 2.5 x 95 cm.
f. Column eluted with NH_4OAc buffers at pH 6.7, column dimensions 1.5 x 25 cm.
g. Column eluted first with 2 mM NH_4OAc, pH 4.5 and then 10 mM NH_4OAc, pH 6.7, column dimensions 1.5 x 25 cm.

is overlapping with the hormone D-GHIH which inhibits the release of growth hormone according to the findings of Greibrokk et al.[13] The behavior of factor B-GHRH on Bio-Gel P-2 indicates that it has a relatively small molecular size.

The data in Table 1 on fractions from Sephadex LH-20 are representative of similar results obtained from four such purifications with this modified Sephadex G-25.

Partition chromatography of fractions of factor B-GHRH in the solvent System A with Sephadex G-25 (Stage A-PC) further and distinctly differentiated factor A-GHRH from factor B-GHRH. Factor B-GHRH had an average R_f of 0.17 in System A. The assay data in Table 1 for Stage A-PC are representative of three typical chromatographic purifications of fractions of factor B-GHRH by this partition chromatography.

Factor B-GHRH was further purified and characterized by ion exchange chromatography. The data in Table 1 show factor B-GHRH is slightly retained on diethylaminoethyl-Sephadex. It was slowly eluted with 2 mM NH_4OAc and completely eluted with 10 mM NH_4OAc. Similar behavior was observed on Bio-Gel CM-2, since factor B-GHRH was slowly eluted with 2 mM NH_4OAc at pH 4.5 and completely eluted with 10 mM NH_4OAc, pH 6.7.

The data in Table 2 show the inhibition by somatostatin of factor B-GHRH induced release of GH at a dose of 1 μg.

It is becoming generally recognized that the hypothalamic peptide hormones can also have more than one biological activity. For example, pGlu-His-Pro-NH_2

Table 2. IN VITRO EFFECT OF PARTIALLY PURIFIED PORCINE FACTOR B-GHRH ON RELEASE OF GH FROM PITUITARIES OF 20 DAY OLD FEMALE RATS AND INHIBITION OF FACTOR B-GHRH BY SOMATOSTATIN

Additions to Incubation Medium	P_2	I_3	Δ	p
Saline Control	287	230	- 57	
" "	272	255	- 17	
" "	641	565	- 76	
	Ave Δ =	-50 ± 17		-
Factor B-GHRH	560	909	+349	
" "	438	734	+296	
" "	478	890	+412	
	Ave Δ =	+352 ± 33		< 0.001[a]
Factor B-GHRH + Somatostatin, 1μg	590	446	-134	
" "	327	433	+106	
" "	526	648	+122	
	Ave Δ =	86 ± 28		< 0.01[b]

a) Factor B-GHRH vs. Saline.
b) Factor B-GHRH vs. Factor B-GHRH + Somatostatin.

releases both thyrotropin and prolactin. pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 releases both the luteinizing and follicle stimulating hormones. In the fractionation described herein and that by Currie et al.[12], two chemically differentiated entities release growth hormone measured by radioimmunoassay and the intrinsic significance of these two entities is uncertain. Consequently, these two entities have been designated factor B-GHRH and factor A-GHRH until such time as they are chemically and biologically characterized for their primary hormonal functions. The linking of GHRH to the letters A and B merely indicates the releasing activity which is presently guiding the isolation of the substance.

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