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SPECIFIC BINDING OF SYNTHETIC HUMAN PANCREATIC GROWTH HORMONE RELEASING FACTOR (1-40-OH) TO BOVINE ANTERIOR PITUITARIES

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SUMMARY We have studied the specific binding of a synthetic 40 amino acid, free carboxy terminus analog of human pancreatic growth hormone releasing factor (hp GRF-40-OH) to partially purified homogenates of bovine anterior pituitaries. The binding of hpGRF-40-OH to pituitary receptors at 4°C reached maximal level in 4 hours and remained steady for the next 18 hours. Specific binding increased linearly with the amount of protein present in the assay. ^{125}I-hpGRF-40-OH binding to pituitary homogenates was competitively inhibited by hpGRF-40-OH but not by unrelated hormones. The competition curve and Scatchard analysis suggest the presence of single class of receptors with a K_d≅3nM and binding capacity of ~200 fmoles/mg protein. This is the first demonstration of specific receptors for GRF on anterior pituitary cells.

Introduction The secretion of growth hormone by the pituitary is under positive and negative control of peptides secreted by the hypothalamus. One of the positive effectors is a 44-amino acid peptide, GRF, which has been purified and characterized from several sources including human pancreatic tumors (1-3). Furthermore, it has been reported recently that human hypothalamic GRF has the same amino acid sequence as ectopically produced hp GRF (4). It has been demonstrated that GRF and its various synthetic analogs, including hpGRF (1-40)OH, can stimulate GH secretion both *in vivo* and *in vitro* (3, 5-10).

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The abbreviations used are: GRF, growth hormone releasing factor; GH, growth hormone; hpGRF-40-OH, 40 amino acid peptide of human pancreatic GRF with free carboxy terminus; KRG, Krebs-Ringer bicarbonate buffer pH 7.4; KRBG, KRB containing 0.011 M D-glucose; PMSF, phenyl methyl sulfonyl fluoride; Buffer A, 0.001 M $NaHCO_3$, 0.005 M NaF and 0.005 M β-mercaptoethanol; BSA, bovine serum albumin; TEAF, triethylammoniumformate.

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The molecular mechanism by which GRF stimulates GH release is unknown. Existing information regarding the molecular basis of peptide hormone action would suggest that there are receptors for GRF on the surfaces of pituitary cells and that the binding of the peptide to its receptor(s) would trigger molecular events resulting in the secretion of GH by the pituitary.

The homology between the amino acid sequences of hpGRF40-OH and bovine GRF (11) encouraged us to use hpGRF40-OH to search for putative GRF receptors on bovine anterior pituitary cells. We report here preliminary results characterizing the specific binding of synthetic hpGRF-40-OH to partially purified homogenates of bovine anterior pituitaries.

MATERIALS AND METHODS

Materials Synthetic hpGRF-40-OH was prepared and supplied by the Clayton Laboratories. The synthesis is described elsewhere (3). $Na^{125}I$ was purchased from New England Nuclear, chloramine T, sodium metabisulfite and aprotinin from Sigma, crystalline BSA from Miles and Bond Elut columns from Analytichem International. All solvents were HPLC grade.

Preparation of ^{125}I-GRF-40-OH hpGRF-40-OH was iodinated using chloramine T following the protocol developed by Vale and co-workers (12). Briefly, the peptide was iodinated using $Na^{125}I$ and chloramine T and the reaction was stopped by adding sodium metabisulfite. Subsequently, the peptide was loaded on a Bond-elut column and eluted by 50% 2-propanol in TEAF. Next, the eluted material was purified by HPLC (Beckman) on a Vydac C_{18} column by reverse phase chromatography. HPLC fractions that demonstrated the best receptor binding were pooled and used in binding experiments.

Preparation of Pituitary Homogenates Twenty bovine pituitary glands were collected fresh at the slaughterhouse and placed in ice-cold KRBG pH 7.4 containing 1 mM PMSF and 1% (v/v) aprotinin to inhibit proteolytic degradation. They were brought to the laboratory within 2 hours of their removal. Anterior pituitaries were removed, cleaned, minced and weighed. (~2.3 g wet tissue/pituitary).

Homogenates were partially purified according to the method of Labrie et al (13). 5 volumes of Buffer A was added per g of original wet tissue, and was blended at low speed for 30 minutes in a Waring blender. Subsequently, 25 ml of buffer was added per g of wet tissue and homogenized using 3 strokes with a motor-driven Teflon glass homogenizer. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 2000 x g for 30 min. The pellet was resuspended in Buffer A (4 ml/g of original tissue) using 3 strokes of loose pestle in a Dounce homogenizer. The homogenate was then centrifuged at 1220 x g for 20 min. The supernatant obtained at this stage was tested for binding of [^{125}I]hpGRF-40-OH to its receptors. Protein concentrations were determined by Lowry assays (14) using BSA as standard. The material was stored at -70°C after quick freezing in 1 ml aliquots.

Binding Assays To measure the binding of hpGRF-40-OH to its receptors, frozen pituitary homogenates were thawed and resuspended in KRB containing 2 mg/ml BSA and 1% (v/v) aprotinin. In all cases, binding assays were carried out in a total volume of 1.2 ml at 4°C by gentle rocking. Approximately 100,000 cpm of ^{125}I-GRF-40-OH was added to each assay tube in concentrations below 100 pM. The control tubes contained 4 μM unlabeled GRF-40-OH in addition to the labeled peptide in order to determine

nonspecific binding. At the end of the incubation, duplicate 500 µl aliquots were centrifuged at 10,000 X g in a Beckman microfuge for 3 minutes at 4°C. The supernatant was removed and the pellets were washed with ice-cold buffer. The tip of the tube was cut off and counted for [^{125}I] counts in a γ-counter. Nonspecifically bound counts were subtracted from total bound to determine the extent of specific binding. Nonspecific binding was less than 30% of total binding in all cases.

Competition Binding and Scatchard Analysis The inhibition of the specific binding of [^{125}I]GRF-40-OH was measured as a function of increasing GRF-40-OH concentration under the assay conditions described above. The incubation was carried out for 6 hours at 4°C in the presence of 400 µg/ml of protein. The data was first plotted as a competition curve and subsequently was analyzed by the Scatchard method (15) to determine the binding capacity of the preparation.

In Vitro Biological Assays Primary cultures of rat anterior pituitary cells were prepared exactly as described by Vale et al. (16). Three to four days after plating, the cells were washed free of medium containing fetal calf serum. They were incubated for one hour in medium containing 0.1% crystalline BSA and no serum. At the end of the incubation, the media was replaced by fresh media containing a specific dose of hpGRF-40-OH. The incubation was continued for 4 hours at 37°, and the media was subsequently removed and GH levels were assayed by RIA using the NIADDDK kit obtained from Dr. A. Parlow through The National Hormone and Pituitary Program. Each dose of hpGRF-40-OH was tested in quadruplet.

RESULTS AND DISCUSSION

Binding to Pituitary Homogenates Specific binding of [^{125}I]GRF-40-OH to bovine pituitary homogenates at 4°C reached half-maximal by 4 hours and remained steady for the next 18 hours (Fig. 1). The binding of ^{125}I-hpGRF-40-OH to pituitary receptors increased linearly with added protein over the range 100-600 µg/ml total protein

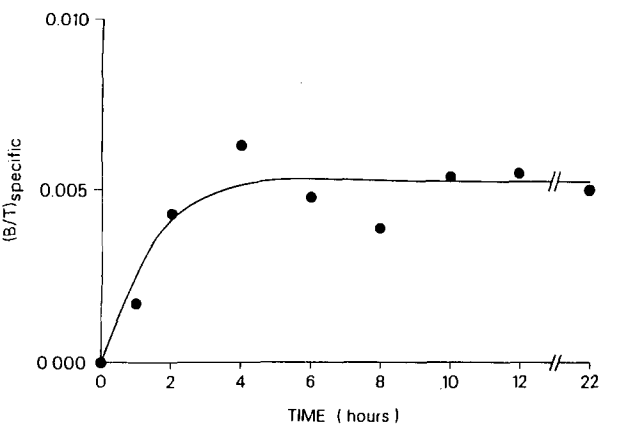

Figure 1: Binding of ^{125}I-GRF-40-OH to Bovine Anterior Pituitaries: Time Course. 200 µg/ml of homogenate protein was incubated with ^{125}I-GRF-40-OH (100,000 cpm) in the presence and absence of 4 µM ^{125}I-GRF-40-OH at 4°C. Two 500 µl aliquots were withdrawn at the indicated time points and centrifuged. The pellets were washed with ice-cold buffer, recentrifuged, cut and counted. Specific binding was calculated by subtracting the counts bound in the presence of 4 µM hpGRF-40-OH from counts bound in the absence of excess unlabeled peptide.

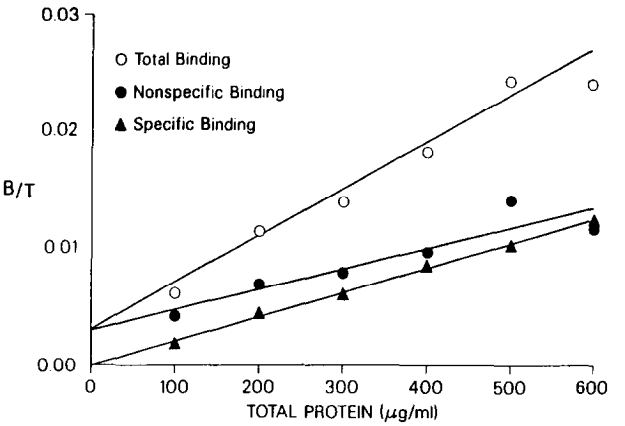

Figure 2: Binding of ^{125}I-hpGRF-40-OH to Bovine Pituitary Homogenates: Dependence on Protein Concentration. Binding assays were performed as described under Materials and Methods. The assay incubation was carried out for 6 hrs at 4°C. Increasing amounts of homogenate protein was added. Nonspecific binding was determined by measurement of binding in the presence of 4 μM hpGRF-40-OH and was subtracted from total binding at each concentration to determine specific binding.

concentration (Fig. 2). Thus, we carried out the subsequent binding assays in the presence of 400 μg/ml pituitary protein with 6 hour incubation at 4°C.

Specificity and Affinity of Binding To determine the specificity and affinity of the GRF receptor for GRF-40-OH, we studied the ability of GRF-40-OH to displace bound ^{125}I-GRF-40-OH from pituitary homogenates. The specific binding of labeled peptide was inhibited increasingly by increasing concentration of unlabeled GRF-40-OH but was unaffected by unrelated peptides such as bovine insulin or rat growth hormone added in excess amounts (Fig. 3). The competition curve was sharply sigmoidal with a K_d~3nM of hpGRF-40-OH. Scatchard analysis of the data (Fig. 3, inset) revealed the binding capacity of the preparation to be ~200 fmoles/mg protein.

Correlation of Receptor Binding with Biological Activity The biological activity of hpGRF-40-OH was measured in terms of secretion of GH from primary cultures of rat anterior pituitary cells as described under Materials and Methods. GH secretion was stimulated in a dose-dependent fashion with increasing hpGRF added to the media (Fig. 4). The maximal level corresponds to 4 μg/ml GH secreted into the media, which represents an 8-fold stimulation above control levels. Biological potency curve was compared with the extent of receptor occupancy calculated from competition binding

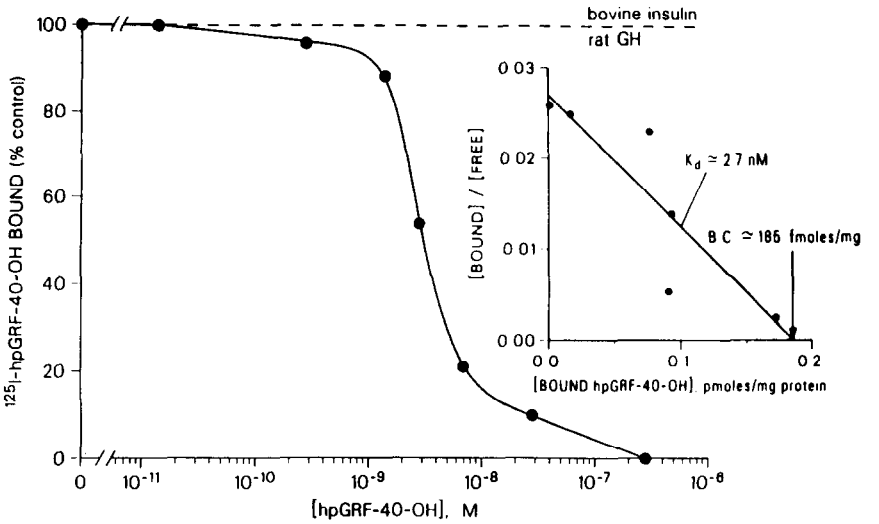

Figure 3: Competition of Specifically Bound ^{125}I-hpGRF-40-OH by hpGRF-40-OH and Other Hormones. 400 µg/ml bovine anterior pituitary homogenate protein was incubated with ^{125}I-hpGRF-40-OH for 6 hrs at 4°C. Increasing amounts of unlabeled hpGRF-40-OH was added to individual assay tubes. Nonspecific binding in the presence of 4 µM unlabeled peptide was subtracted from total binding at each hpGRF-40-OH concentration. Rat GH and bovine insulin was each added at a concentration of 2 µM.
INSET: Scatchard Analysis of the Same Data: The ratio of specifically bound hormone to free hormone (Bound/Free) was calculated from the competition binding data and plotted against pmoles of specifically bound hormone per 1.0 mg of total protein.

data. The results shown in Figure 4 indicate that the amount of hpGRF-40-OH necessary to half-maximally stimulate GH secretion in vitro is approximately one-tenth the amount needed to half-maximally inhibit the binding of ^{125}I-hpGRF-40-OH

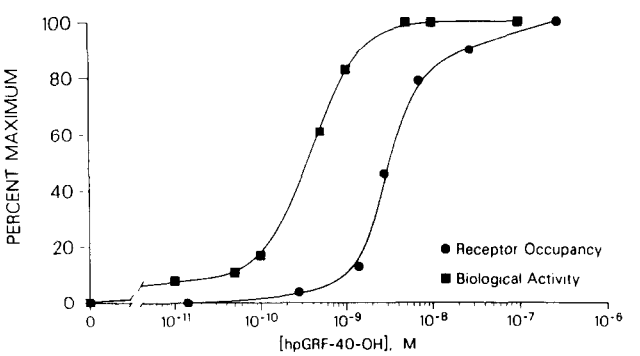

Figure 4: Correlation of Receptor Occupancy with Biological Activity.

●——● Percent of receptor occupancy determined from competition binding data shown in Figure 3:

■——■ hpGRF-40-OH dependent secretion of GH in primary cultures of anterior pituitary cells represented as percent of maximal response.

to pituitary receptors. This result suggests that not all of the receptors need be occupied to elicit maximal biological response. Similar correlations between biological activity and receptor occupancy have been reported for other peptide hormone-receptor systems. An example is the finding that only 10% of insulin receptors are occupied for maximal stimulation of glucose transport by insulin in adipocytes (17-20).

CONCLUSIONS

Our preliminary results reported here clearly demonstrate the presence of specific receptors for GRF on anterior pituitaries. The binding of hpGRF to these preparations is specific, saturable and correlates with biological activity. The data suggests a single class of receptors with an overall Kd of ~3 nM for binding of hpGRF-40-OH analog.

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