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DEMONSTRATION AND CHARACTERIZATION OF THE SPECIFIC BINDING OF GROWTH HORMONE-RELEASING PEPTIDE TO RAT ANTERIOR PITUITARY AND HYPOTHALAMIC MEMBRANES

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Since the growth hormone-releasing peptide (GHRP), His-D-Trp-Ala-Trp-D-Phe-Lys-NH2, was found to specifically release growth hormone by a complementary but yet not clearly defined action on the pituitary as well as the hypothalamus, *in vitro* studies have been performed to demonstrate and characterize GHRP binding sites on peripheral membranes of both the rat anterior pituitary and hypothalamus. Optimum binding assay conditions were established using [^{125}I]Tyr-Ala-GHRP as the radioligand. The membrane binding sites were specific, reversible, saturable and time, temperature, pH and concentration dependent. Computerized analyses of competition experiments suggested two classes of binding sites in both pituitary and hypothalamic membranes. The maximum specific binding was observed at pH 5.0 than the physiological pH in both tissues. Pretreatment of the membranes with trypsin prevented specific binding. The increase in B*max* was statistically significant and showed a 2.0- to 8.9-fold and 5.8- to 11.2-fold in pituitary and hypothalamus, respectively, whereas the affinity constants (Kds) were not significant. Of the synthetic and natural neuropeptides that influence the release of GH from somatotrophs, only (D-Lys^3)GHRP, substance P antagonists and growth hormone-releasing factor analog were potent inhibitors of GHRP binding in both tissues.

Growth hormone-releasing peptide (GHRP) was developed using a closely integrated theoretical, biological and synthetic approach (1, 2) to release growth hormone (GH) from somatotrophs. Its action has been demonstrated in many species including rats (2), lambs (2), monkeys (2, 3), chimpanzees (3), steers (4), pigs (5), cows (6) and humans (7, 8). Chronic administration in rats enhanced body weight gain (2) and in cows enhanced milk production (6, 9). Studies from our laboratory also indicated that GHRP acts at both pituitary and hypothalamus (10) and probably stimulates GH-releasing factor (GRF) from hypothalamus without influencing somatostatin (SRIF) release (11-12). Both GHRP and GRF act directly on pituitary somatotrophs and several observations suggest that the two compounds stimulate release of GH through different intracellular mechanisms (13-18). Previous study indicated the presence of specific binding sites in the anterior pituitary and hypothalamus (19). Therefore, in this paper, we examine the optimum binding conditions using [^{125}I]Tyr-Ala-GHRP as a radioligand with special reference to the influence of pH on the binding characteristics and compare the binding efficiency of various natural and synthetic neuropeptides that influence the release of GH.

Abbreviations used: GHRP, growth hormone-releasing peptide; GH, growth hormone; SRIF (somatostatin), somatotroph release inhibiting factor; GRF, growth hormone releasing factor.

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MATERIALS AND METHODS

Rat: Adult male rats of the Sprague Dawley strain (200-250 g BW) were purchased from Charles River laboratory (Wilmington, MA) and maintained in a constant temperature (25° C) and humidity environment with a 14-h light and 10-h dark cycle. Purina rat chow was fed ad libitum.

Peptides: The peptides used in these studies were GHRP, (Nle^{27})-hpGRF(1-29)-NH_2 (GRF analog), SRIF (Peninsula Laboratories Inc., Belmont, CA), (D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11})-substance P, (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11})-substance P,(D-Pro^4, D-$Trp^{7,9}$)-substance P (4-11) (Bachem Fine Chemicals Inc., Torrance, CA) and substance P, bombesin, neuromedin C (Bachem Bioscience Inc., Philadelphia, PA). Tyr-Ala-GHRP and (D-Lys^3)-GHRP were synthesized and purified by Dr. D. Coy in our department.

Radioiodination: The addition of Tyr-Ala to the GHRP molecule made it possible to obtain a high specific active radioligand. Experiments carried out in our laboratory revealed that this iodinated [^{127}I] and noniodinated octapeptide released GH similarly to GHRP in monolayer cultures of dispersed rat pituitary cells. Tyr-Ala-GHRP was iodinated by lactoperoxidase method (20). Briefly, 10 μg of the peptide in 30 μl sodium acetate buffer (pH 5.6) was mixed with 100 ng lactoperoxidase (10 μl), 1 mCi Na[^{125}I] (Amersham, Ill.) and 100 ng (5 μl) hydrogen peroxide. The reaction was allowed for 9 min at room temperature and further continued for 9 min after the addition of 160 ng (8 μl) of hydrogen peroxide. The iodinated peptide was separated from the free iodine by a disposable C_{18}-cartridge (Bond Elut, Analytichem) and purified by HPLC using reverse phase C_{18} column (Phenomenex, 250 x 4.6 mm, pore size 300° A, 1 ml/min, 10-40% acetonitrile containing 0.1% trifluoroacetic acid for 50 min). After purification, the peak fraction was dried under nitrogen and suspended in 50 mM Tris-HCl (pH 7.4) containing 2 mM EGTA and 0.1% bacitracin (buffer A). The specific activity of the radioligand was ~ 2070 Ci/mmol.

Membrane preparation: The rats were decapitated and the anterior pituitary and hypothalamus removed, rinsed and homogenized (Teflon-glass homogenizer, 8 strokes) in ice cold buffer (one adenopituitary or hypothalamus/ml). The homogenate was centrifuged at 500 x g for 10 min and the resulting supernatant was then centrifuged at 20,000 x g for 20 min to pellet the membranes. The membranes were resuspended in 20 volumes of buffer using a polytron (PT 3000, Brinkman) at low speed (setting at 4 for 4 secs.) and centrifuged to collect the membranes. This procedure of washing the membranes was repeated once more. Protein concentration was estimated by the method of Lowry et al (21) using bovine serum albumin as standard.

Binding experiments: All reaction vessels and pipette tips used to handle GHRP were siliconized before use. The borosilicate glass assay tubes (12 x 75 mm from Chase Instruments, Corp., Poultney, VT) were coated with 0.1% bacitracin overnight to reduce nonspecific adsorption of peptides. Pituitary and hypothalamic membranes (200 μg protein) were incubated in a total volume of 400 μl with 57 pM radioligand and increasing concentrations of unlabelled GHRP in buffer A. After the incubation period, the reaction was terminated by filtration over Whatman GF/B filters presoaked in 0.5% polyethylenimine using Brandel Harvester. The amount of [^{125}I]Tyr-Ala-GHRP bound to the membranes were counted by a gamma counter. Nonspecific binding was determined in the presence of 1.68×10^{-4} M GHRP.

Data analysis: The binding parameters were analyzed using computer assisted Ligand program (Biosoft/Elsevier, NJ). Values are the mean ± SEM of triplicates unless otherwise indicated.

RESULTS

Optimum binding conditions: Initially binding of GHRP to rat anterior pituitary and hypothalamic membranes was determined. Under the experimental conditions outlined, the maximal specific binding was 15% of the total counts added while the nonspecific binding was 4%. The binding of GHRP was linear at low protein concentrations (up to 730 μg for pituitary and up to 1600 μg for hypothalamus) and pre-treatment of the membranes with 5 μg trypsin for 15 min at 25° C removed the specific binding sites (results not shown).

Specific binding was time and temperature dependent (Fig 1a and b). At 22° C specific binding reached equilibrium after 20 min in both pituitary and hypothalamus and was stable for one hour. At 37° C maximum specific binding was reached at 40 min with pituitary membranes followed by a

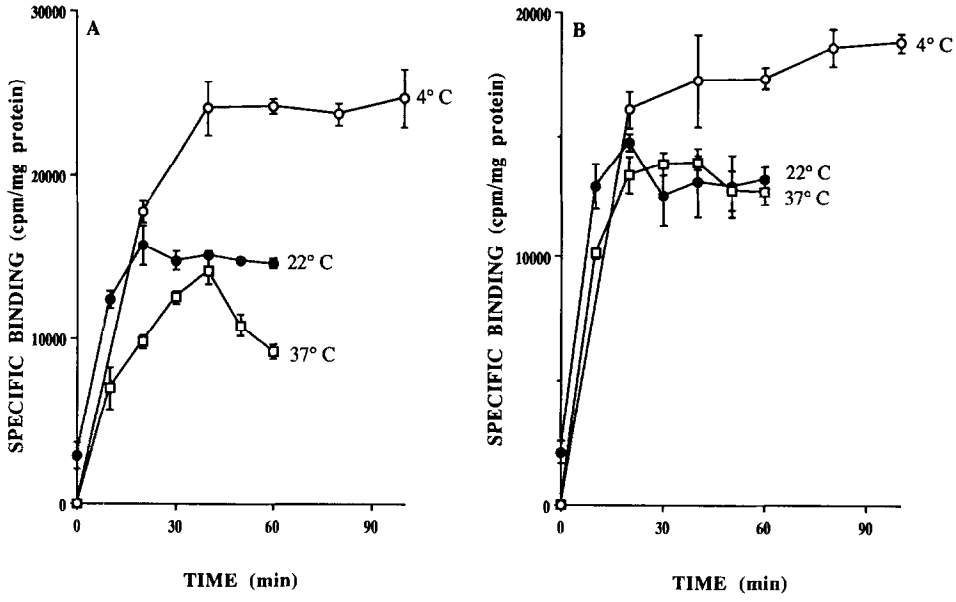

Fig. 1. Effect of incubation time and temperature on GHRP binding to rat anterior pituitary (A) and hypothalamic (B) membranes. Membranes (200 µg/ml) were incubated at 4° C, 22° C and 37° C with 57 pM tracer in binding buffer (Tris-HCl, pH 7.4 buffer containing 2 mM EGTA and 0.1% bacitracin) for various time intervals. The specific binding measured at the indicated time were mean ± SEM triplicate determinations from a single experiment.

rapid fall whereas with hypothalamus equilibrium was reached at 20 min followed by a slow decline. At 4° C association occurred slowly and was stable only after 90 min in both tissues. Therefore, all our binding experiments were carried out at 22° C since the maximum specific binding was reached at 20 min and the standard error of the mean obtained were minimum. Also, the peptides used in these experiments were stable during the incubation period.

Influence of pH on GHRP binding: The effect of pH on the binding of GHRP to anterior pituitary and hypothalamic membranes are shown in Fig. 2a. It is observed that the maximum specific binding occurred at pH 5.0 for both membranes. There was a 3- to 4-fold increase of specific binding at pH 5.0 compared to pH 7.4. Time course experiments done at 22° C with pituitary membranes at both pHs (Fig. 2b) showed that the equilibrium reached at 20 min and after that a constant state was observed over time at pH 5.0 . Similar results were observed with hypothalamic membranes (data not shown).

The binding of GHRP at pH 7.4 and 5.0 was reversible (Fig. 3). Addition of 1.68×10^{-4} M GHRP to pituitary (Fig. 3a) and hypothalamic (Fig. 3b) membranes preincubated for 20 min with 57 pM radioligand resulted in dissociation of bound radioligand down to nonspecific levels within 30 min.

GHRP binding to pituitary and hypothalamic membranes: The displacement curves of GHRP by unlabeled GHRP suggested that the labeled octapeptide bound to a high affinity (Kd1) and low affinity (Kd2) classes of sites at pH 7.4 and 5.0 (Table 1). The Kd1 obtained at pH 7.4 were 152

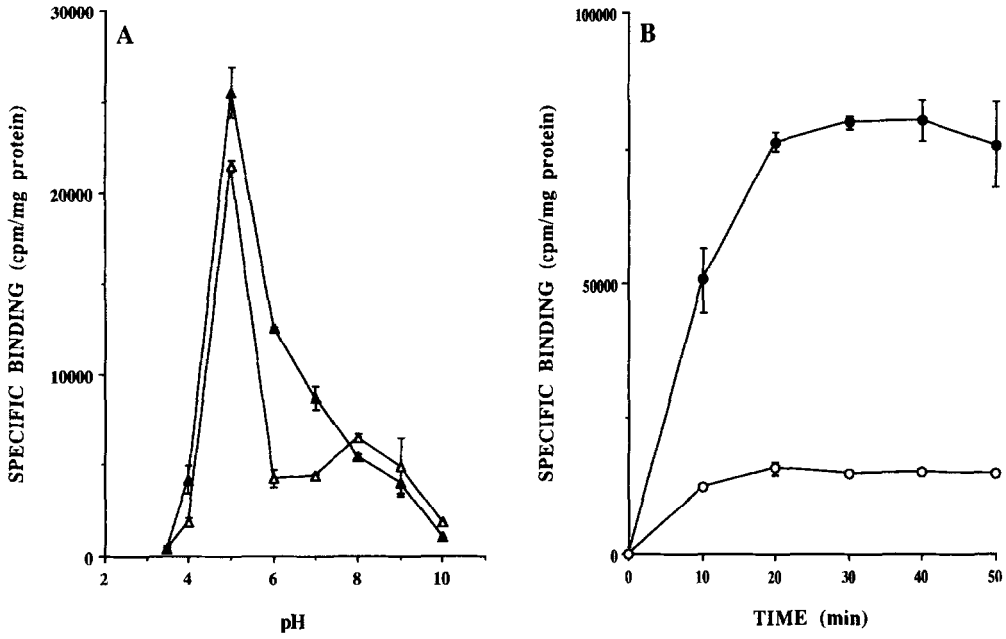

Fig. 2. A) Effect of pH on specific GHRP binding to rat anterior pituitary (Δ) and hypothalamic (▲) membranes. The incubations were carried out with 57 pM radioligand at 22° C for 20 min. The buffers used were 50 mM acetate (pH 3.5-5.0) phosphate (pH 6.0-7.0), and Tris-HCl (pH 7.0-8.0) and glycine-HCl (pH 9.0-10.0) B) Time course of association of GHRP to rat anterior pituitary membranes at pH 7.4 (O) and 5.0 (●). In both experiments, each point is the mean ± SEM of triplicate determinations from a single experiment.

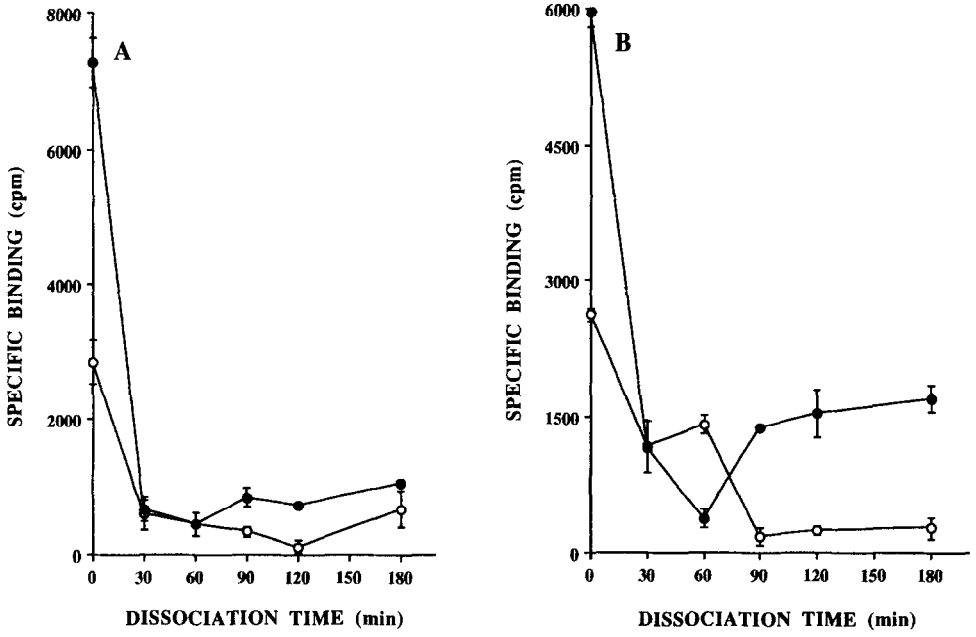

Fig.3. Dissociation of GHRP binding from rat anterior pituitary (A) and hypothalamus (B). Membranes (200 μg) were preincubated for 20 min at 22° C with 57 pM radioligand and dissociation was initiated by the addition of 1.68 x 10^{-4} M GHRP at pH 7.4 (O) and pH 5.0 (●). The points represent the means ± SEM of triplicate determinations of specific binding at the times indicated.

Table 1. Dissociation constants and maximal binding capacities of GHRP binding sites from scatchard plot analysis

	Pituitary		Hypothalamus	
	pH 5.0	pH 7.4	pH 5.0	pH 7.4
Kd1 (nM)	125 ± 50	152 ± 20^{NS}	162 ± 40	80 ± 5^{NS}
Kd2 (μM)	43 ± 13.5	38 ± 19.0^{NS}	17 ± 2.4	15 ± 1.8^{NS}
Bmax1 (pmol/mg)	13.3 ± 6.20	8.6 ± 2.7^{NS}	42.8 ± 12.0	3.8 ± 0.15†
Bmax2 (pmol/mg)	5000 ± 500	560 ± 300††	3700 ± 350	640 ± 100††

The affinity and binding sites at pH 5.0 and 7.4 were compared using unpaired student's-t test. NS = Not significant; $^{\dagger}p < 0.01$; $^{\dagger\dagger}p < 0.001$.

nM and 80 nM for pituitary and hypothalamus whereas Kd2 were 38 μM and 15 μM. The changes observed for Kds at both pHs were not statistically significant for the pituitary or hypothalamus. However, the binding sites in both classes increased 2- to 11-fold) at pH 5.0 when compared to pH 7.4. There was no change in the nonspecific binding levels when incubations were done at pH 7.4 or 5.0.

To investigate various neuropeptides that have influence on the release of GH from somatotrophs by various mechanisms, GRF analog, SRIF, substance P, substance P antagonists, bombesin and neuromedin C were added in competition with GHRP at pH 7.4 (Table 2). In both pituitary and hypothalamus, GRF analog, (D-Lys^{3})-GHRP, (D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11})-substance P, (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-substance P competed very well in a concentration dependent process whereas bombesin, neuromedin C and substance P didn't influence these binding sites. The IC_{50}, values observed with (Nle^{27})-hPGRF(1-29)-NH2, (D-Lys^{3})-GHRP, (D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11})-substance P, (D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-substance P were 1.4, 0.4, 0.4, and 2.4 μM in pituitary and 10, 5.0, 5.0, and 4.1 μM in hypothalamus, respectively.

Table 2. Inhibition of GHRP binding to rat anterior pituitary and hypothalamic membranes by various natural and synthetic neuropeptides

Peptides	IC_{50} (μM)	
	Pituitary	Hypothalamus
GHRP	2.0	3.0
(Nle^{27})-hpGRF(1-29)-NH2	1.4	10.0
Somatostatin	16.0	90.0
D-Lys^{3}-GHRP	0.4	5.0
Substance P	>100.0	>100.0
(D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11})-substance P	0.4	5.0
(D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11})-substance P	2.4	4.1
(D-Pro^{4}, D-Trp^{7,9})-substance P (4-11)	50.0	>100.0
Bombesin	>100.0	>100.0
Neuromedin C	>100.0	>100.0

DISCUSSION

In the present study, we have demonstrated and characterized the properties of GHRP binding to rat anterior pituitary and hypothalamus. The radioligand with a specific activity of over 2000 Ci/mmol allowed us to use 30-40 times lesser amount of tissue compared to earlier study (19) to measure the membranes associated specific binding. The influence of hydrogen ion concentration on the binding kinetics for GHRP were surprising but not unusual since several receptors have been reported to be influenced by pH (22-26). The significant increase in specific binding with no affinity change at low pH observed in our study is similar to angiotensin receptor (22) and colony stimulating factor-1 receptor (23). When the monolayer pituitary cells were incubated at pH 5.0, we noticed that the cells were normal (viability ~96%) and GH release in response to GHRP stimulation was unaffected even after several hours. The increase in GHRP specific binding at pH 5.0 may be due to the stabilization of receptor as observed for CSF-1 receptor or the synthetic peptide GHRP can exist in a protonated form enhancing binding.

The presence of binding sites in pituitary indicated that GHRP acts through these sites to release GH from somatotrophs. Our earlier studies (2, 8) as well as recent study (27) confirmed that GHRP acts directly on pituitary to release GH. The exact role for the binding sites of hypothalamus is not clear at this time. However, the enhanced GH release by GHRP from pituitary was observed in the presence of hypothalamic fragments (10), suggesting a possible role for GHRP to stimulate GRF release and/or unknown factors from hypothalamus which in turn specifically increase GH release from the pituitary. Several earlier studies suggested that GHRP and GRF act directly on pituitary somatotrophs via different receptors and different intracellular mechanisms (13-17).

The present study with various synthetic peptides suggest that the GHRP antagonist, (D-Lys^3)-GHRP, is a potent inhibitor of GHRP binding to receptor. We have observed earlier that the *in vitro* GH release to GHRP but not GRF is inhibited by (D-Lys^3)-GHRP supporting the presence of a specific GHRP receptor. We have obtained evidence which supports that the GHRP receptor may be species specific (28).

In vitro studies with monolayer pituitary cells indicated that GH release by GRF but not GHRP is mediated via cAMP (29). It is also observed that GRF activates adenylate cyclase (30) whereas GHRP does not (unpublished data). The substance P antagonists compete strongly with GHRP binding and, surprizingly, so did the GRF analog. Our *in vitro* studies with substance P antagonists also confirmed that the antagonists are potent inhibitors of the GHRP GH response (31). Though GHRP receptor is different from GRF receptor, it is not clear whether substance P antagonists have structural similarity to the GHRP binding sites or whether GHRP binding sites are related to a substance P receptor subpopulation. Work is in progress to further understand these seemingly confusing but most interesting relationships.

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