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EFFECT OF SUBSTANCE P/BOMBESIN ANTAGONISTS ON THE RELEASE OF GROWTH HORMONE BY GHRP AND GHRH

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The substance P(SP)/bombesin (Bn) antagonists [DArg^{1}DTrp^{7,9}Leu^{11}] SP(P-7482), [DArg^1-DPro^{2}DTrp^{7,9}Leu^{11}]SP (P-7483), [DArg^{1}DPhe^{5}DTrp^{7,9}Leu^{11}]SP(P-7492), and the growth hormone releasing hormone (GHRH) antagonist [DArg^{2}Ala^{8,9,15}]GHRH(1-29)(DC21-366) were tested for their *in vitro* effects on the release of growth hormone (GH) in the presence of GHRH and growth hormone releasing peptide, HisDTrpAlaTrpDPheLysNH$_2$(GHRP). P-7492, P-7483, and P-7482 decreased, dose-dependently, the release of GH by GHRP (IC_{50}=0.2μM, 0.85 μM, and 6 μM, respectively). These antagonists had only a 10-15%inhibitory effect on the stimulated GH release of GHRH even at high dosage. DC21-366 decreased the stimulated release of GH by GHRH (IC_{50}=0.16 μM) but not by GHRP. Neither SP nor Bn had GH releasing or inhibitory effects in this system.

Starting with an enkephalin analog that has a weak but specific action in releasing GH *in vitro*, GHRP was developed and found to specifically release growth hormone (GH) *in vitro* and in a wide variety of animal species *in vivo* (1). Of special importance has been the potent and specific GH releasing activity of GHRP in humans as well as the finding that GHRP releases GH more efficaciously than GHRH in humans (2). Additionally, *in vivo* results indicate GHRP and GHRH act independently of each other to release GH because administration of these two peptides together to both humans (2) and animals (1) synergistically releases GH. In contrast to GHRH, the small partially-protected GHRP hexapeptide releases GH after oral administration. Oral GHRP increases GH not only in adult men but also in short stature children with various degrees of GH deficiency thus indicating a new potential oral therapy to increase the rate of body growth in short stature children with GH deficiency (3).

Studies have demonstrated that membrane fragments from the rat hypothalamus and anterior pituitary both contain binding sites at which GHRP is saturably and reversibly bound (4,5). Houben et al (6) found that bombesin and ranatensin-like peptides release GH and PRL from rat pituitary cells *in vitro*, thus studies have been performed

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to assess whether bombesin (Bn) and/or substance P(SP) antagonists might inhibit the GH induced action of GHRP *in vitro*. Since our current objective is to characterize the GHRP receptor and to detect, isolate and identify the putative endogenous natural ligand for GHRP in porcine and rat hypothalamic extracts, the present findings of the inhibitory effects of SP/Bn antagonists on the GHRP GH receptor support the possibility that the putative endogenous ligand may be related to the SP and /or Bn or a closely related family of peptides and thus may have special relevance to these objectives.

All of the present studies have been performed *in vitro* by utilization of monolayer cultures of dispersed anterior pituitary cells and the assessment of changes in GH release by measurement of GH levels in the incubation media by GH RIA.

MATERIALS AND METHODS

ANIMALS

Adult male rats of the Sprague-Dawley strain (200-300 g BW; Charles River Laboratory, Wilmington, MA) were used as a source of pituitaries.

PEPTIDES AND PROTEINS

P-7482, P-7483 and P-7492 as well as 8068, rGHRH 1-43 OH and 8686, Nle^{27} GHRH (1-29)NH_2 were purchased from Peninsula Labs, Inc. (Belmont, CA). Bn and SP were obtained from Bachem Fine Chemicals (Torrance, CA). Bovine serum albumin (fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO).

CHEMICALS

All organic and inorganic chemicals were of the highest purity obtained from Sigma Chemical Co., American Scientific Products, or otherwise as indicated.

SOLUTIONS AND BUFFERS

Gey's balanced salt solution (referred to as Gey's) was obtained from Gibco, Grand Island, N.Y., and was supplemented with 1% BSA and 0.15% glucose, as well as 2% (v/v) non-essential amino acid. The cell dispersion medium was Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, N.Y.) to which was added 0.35% glucose, 0.37% $NaHCO_3$, and 0.6% HEPES, pH 7.3. For dispersing the pituitary cells, DMEM was supplemented with 2% (v/v) antibiotic-antimycotic (Gibco) solution, 0.32% (v/v) fungizone (Gibco), 0.1% (v/v) non-essential amino acids (Gibco), 2.5% (v/v) fetal calf serum (Gibco), 3% (v/v) horse serum (Gibco), 0.0066% 3,3',5'-triiodothyronine (Sigma) and 0.01% (v/v) dexamethazone. The stimulation medium was Gey's supplemented with 1% BSA, 0.2% glucose, and 0.6% HEPES, pH 7.4.

DISPERSION

In most experiments, 24 adult male rats were sacrificed by decapitation and anterior pituitaries removed as quickly as possible. They were minced into small pieces on

a glass slide, and placed in two different small erlenmeyer flasks each containing 6.50 mg trypsin (Worthington) in 3.25 ml of Gey's buffer. The flasks were placed in a 37°C constant temperature water bath and gassed with 5% CO_2 for 35 minutes. The cells were washed four times with Gey's and suspended in 3.25 ml of Gey's containing 2.5 mg deoxyribonuclease I (Sigma Chemical Co.), and gassed (5% CO_2) for four more minutes at 37°C. The cells were washed three more times with Gey's, and triturated with a lima bean trypsin inhibitor solution (2.5mg/5 ml Gey's) (Worthington), centrifuged at 1600 rpm, suspended in DMEM, and adjusted to a concentration of $2.6x10^5$ cells/ml. The cells were plated in 24 well plates (Flow Laboratories, McLean, VA).

STIMULATION OF PITUITARY CELLS AND RIA

After 3 days at 37°C and 8% CO_2, cells were washed three times with stimulation buffer (Gey's with 1% BSA). One ml of the buffer was added and cells incubated for an additional hour. The solution was discarded and 0.5 ml of fresh stimulation buffer was added. The appropriate concentration of peptide in about thirty microliter of 0.1% gelatin, or in some cases 0.5% 2-mercaptoethanol was used to stimulate the cultured pituitary cells. Stimulation was for 15 minutes at 37°C with 5% CO_2. Each peptide was tested in triplicate, and results are expressed as the mean ± SEM. The rat RIA reagents were distributed by the NIADDK program. The GH levels were determined in terms of nanogram/ml of the rat GH standard and are recorded as % change.

RESULTS AND DISCUSSION

Results in Figure 1 show that the 3 SP/Bn antagonists, P-7482, P-7483 and P-7492, inhibited the GH response of GHRP but not GHRH in a dose related manner. The IC_{50} values were 6, 0.85 and 0.2 μM, respectively. The most potent inhibitor was [DArg^1,DPhe^5,DTrp^{7,9},Leu^{11}]SP (P-7492). Since the inhibitory dose-response curves of these 3 antagonists were parallel to each other, the results indicate that each antagonist was probably acting at the same receptor site. Additionally, the recent results of Sethumadhaven et al (5) support this conclusion because they found that the ^{125}I-labeled TyrAlaGHRP was displaced by 7483 and 7492 in a rat anterior pituitary GHRP radioreceptor membrane assay. The IC_{50} values of the antagonists in this study were 2.4 and 0.4 μM, respectively. As previously reported by Bowers et al (1), the results in Figure 2 again demonstrate that the GHRH antagonist, [DArg^2,Ala^{8,9,15}]GHRH (1-29)NH_2 of Coy inhibits the GH response of GHRH but not that of GHRP. All of the above results strongly support that GHRP and GHRH mediate their effects on GH release via different receptors. The results of Cheng et al (8) indicate that unlike GHRH, the effect of GHRP on GH is not mediated via a cAMP intracellular pathway.

Since SP and Bn had no effect in this assay system on release of GH alone or in combination with GHRP or GHRH (Figure 3), it can be concluded that the action of the antagonists utilized in this study was at the GHRP and not the SP/Bn receptor level

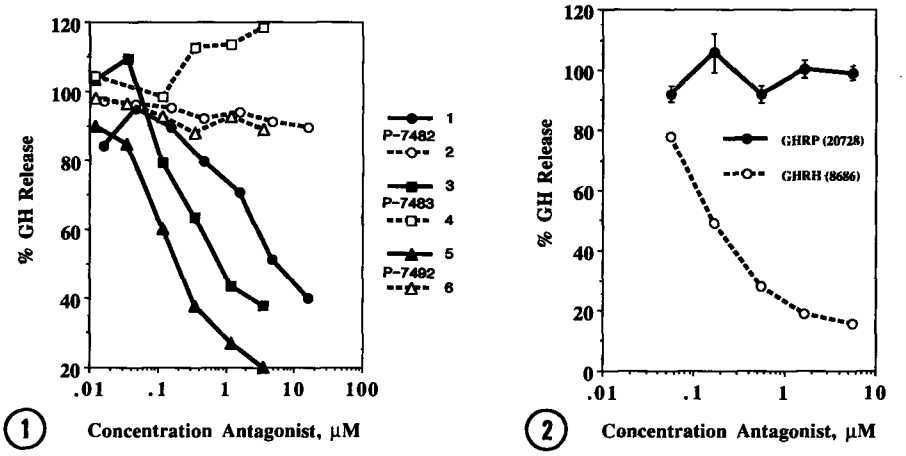

Figure 1. Effect of increasing concentrations of P-7482 (1 and 2), P-7483 (3 and 4), and P-7492 (5 and 6) on the GH stimulated release of GHRP (solid symbols) and rGHRH (open symbols). Dispersed rat anterior pituitary cells were stimulated with 2.08×10^{-8} M GHRP or 3.71×10^{-9} M rGHRH in the presence or absence of the antagonist. GH release was measured after a 15-minute incubation period at 37° C with 5% CO_2 by a RIA method and is expressed as the percentage of GH released in the absence of the antagonists. Samples were run in triplicate and results are the mean ± SEM. Details are as described in Materials and Methods.

Figure 2. Effect of increasing concentrations of [DArg^{2}Ala^{8,9,15}]GHRH (1-29)NH_2 on the GH stimulated release of GHRP and Nle^{27} GHRH (1-29)NH_2. Experimental conditions are as described in Figure 1. Results are the mean of 3 ± SEM.

even though Larsen et al (9) recently demonstrated and characterized SP binding sites on rat pituitary cells. Also, in unpublished studies, neither the SP-related peptides neurokinin A nor neurokinin B altered the GH response of GHRP or GHRH. These results indicate that inhibitory effects of the antagonists observed in the present study were not mediated via the neurokinin A or neurokinin B receptor.

Examples exist in the literature which demonstrate that the SP/Bn antagonists may act on more than one receptor type. For example, Jensen et al (10) found in pancreatic studies that the [DArg^{1}DPro^{2}DTrp^{7,9}Leu^{11}] SP antagonist inhibited at both the SP and Bn receptors with the highest affinity being on the Bn receptor. Nevertheless, in the present study, since the GH response of GHRP was much more effectively inhibited by the SP antagonist than by the Bn-specific antagonist, [DPhe^{6}Leu^3-(ΨCH$_2$NH)DPhe^{14}]Bn(6-14)NH_2 of Coy et al (11) as recorded in Figure 4, the action of GHRP seemingly appears to be more likely mediated via a SP-like rather than a Bn-like pituitary receptor.

Even though it is obvious that the antagonist results of our present study can not be interpreted specifically, they do seem to impart some insight into the possible type of

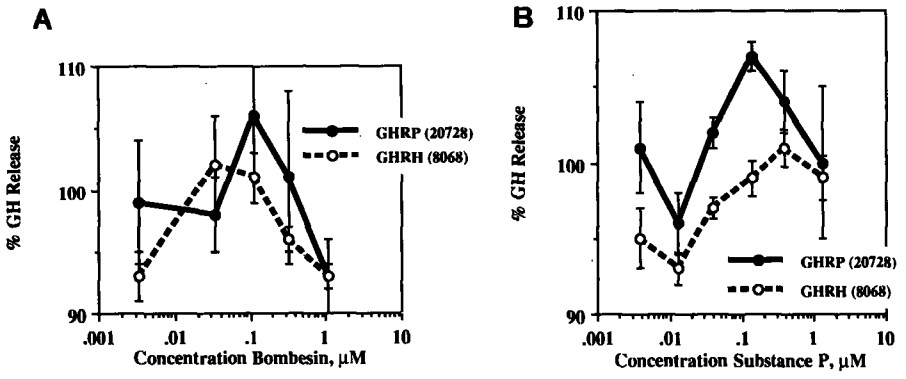

Figure 3. Effect of increasing concentrations of bombesin (A) and substance P (B) on the GH stimulated release of GHRP and rGHRH. Experimental conditions are as described in Figure 1. Results are the mean of 3 ± SEM.

natural ligand and receptor that the action of GHRP on GH release may reflect. Also the SP antagonists, especially the most potent one, [DArg^{1}DPhe^{5}DTrp^{7,9},-Leu^{11}]SP, will become a new tool for characterizing new GHRP-like receptors and peptides as well as become a basis for designing more potent GHRP antagonists. Furthermore, our results suggest that the 3-dimensional structure of the SP/Bn antagonists and GHRP may not only be related but they also support the tentative hypothesis that the putative endogenous natural GHRP ligand may belong to the SP or possibly to a related SP/Bn hybrid family of peptides.

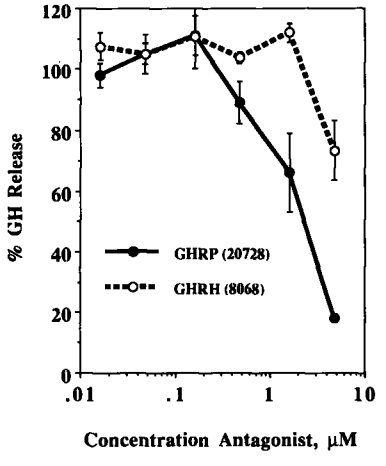

Figure 4. Dose-response curves of the effect of the synthetic and specific Bn receptor antagonist DPhe^{6}Leu^{13}(ΨCH$_2$NH)DPhe^{14}Bn(6-14)NH$_2$ on the GH stimulated release of GHRP and rGHRH. Experimental conditions are as described in Figure 1. Results are the mean of 3 ± SEM.

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