Growth Hormone (GH)-Releasing Peptide Stimulation of GH Release from Human Somatotroph Adenoma Cells: Interaction with GH-Releasing Hormone, Thyrotropin-Releasing Hormone, and Octreotide*

U. RENNER, S. BROCKMEIER, C. J. STRASBURGER, M. LANGE, J. SCHOPOHL, O. A. MÜLLER, K. v. WERDER, AND G. K. STALLA

Max-Planck Institute of Psychiatry, Clinical Institute (U.R., S.B., M.L., G.K.S.); the Departments of Medicine (C.J.S., J.S., O.A.M.) and Neurosurgery (M.L.), University of Munich; and the Medical Department of Krankenanstalt Rotes Kreuz (O.A.M.), Munich; Schlosspark-Klinik, Free University of Berlin (K.v.W.), Berlin, Germany

ABSTRACT

The synthetic hexapeptide GH-releasing peptide (GHRP; His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$) specifically stimulates GH secretion in humans *in vivo* and in animals *in vitro* and *in vivo* via a still unknown receptor and mechanism. To determine the effect of GHRP on human somatotroph cells *in vitro*, we stimulated cell cultures derived from 12 different human somatotroph adenomas with GHRP alone and in combination with GH-releasing hormone (GHRH), TRH, and the somatostatin analog octreotide. GH secretion of all 12 adenoma cultures could be stimulated with GHRP, whereas GHRH was active only in 6 adenoma cultures. In GHRH-responsive cell cultures, simultaneous application of GHRH and GHRP had an additive effect on GH secretion. TRH stimulated GH release in 4 of 7 adenoma cultures; in TRH-responsive cell cultures there was also an additive effect of GHRP and TRH on GH secretion. In 5 of 9 adenoma cultures investigated,

octreotide inhibited basal GH secretion. In these cell cultures, GHRP-induced GH release was suppressed by octreotide. In 5 of 5 cases, the protein kinase-C inhibitor phloretin partly inhibited GHRP-stimulated GH release, but not basal GH secretion. In summary, GH secretion was stimulated by GHRP in all somatotroph adenomas investigated, indicating that its unknown receptor and signaling pathway are expressed more consistently in somatotroph adenoma cells than those for GHRH, TRH, and somatostatin. Our data give further evidence that GHRP-stimulated GH secretion is mediated by a receptor different from that for GHRH or TRH, respectively, and that protein kinase-C is involved in the signal transduction pathway. Because human somatotroph adenoma cell cultures respond differently to various neuropeptides (GHRH, TRH, somatostatin, and others), they provide a model for further investigation of the mechanism of action of GHRP-induced GH secretion. (*J Clin Endocrinol Metab* **78**: 1090–1096, 1994)

GH-RELEASING peptide (GHRP), a synthetic hexapeptide (His-D-Trp-Ala-Trp-D-Phe-Lys-NH$_2$) derived from enkephalins (1, 2), specifically stimulates GH secretion of somatotroph pituitary cells in animals both *in vivo* and *in vitro* (3, 4) as well as in normal humans (5–7), short-statured children (8), and patients with acromegaly (9) *in vivo*. Because in humans GHRP is approximately as active as GH-releasing hormone (GHRH) and, moreover, can be administered orally, it may provide an alternate form of treatment of certain forms of GH deficiency (5–7, 10).

Previous studies demonstrated that GHRP does not stimulate GH release via the GHRH receptor (11, 12), the opiate receptors (13), or the receptor for pituitary adenylate cyclase-activating polypeptide (14). Although the receptor for GHRP is not known, and the mechanism by which GHRP stimulates GH release is not well understood (15), part of the action of

GHRP seems to be mediated via protein kinase-C (PKC) (16). In this case, the action of GHRP is similar to that of phorbol 12-myristate 13-acetate. Previous studies investigating the interaction of GHRP with GHRH and somatostatin (SRIF) have demonstrated that SRIF suppresses GHRP-stimulated GH release (3, 11, 15), and GHRP and GHRH stimulate GH secretion in a synergistic fashion (5, 15, 16). GHRP-binding sites were detected not only in pituitary, but also in hypothalamic membrane fractions (17). Therefore, besides its action at the pituitary level, the possibility of a hypothalamic site of action of GHRP is still under discussion.

Somatotroph pituitary adenomas represent approximately 20% of the pituitary adenomas and cause acromegaly or gigantism due to GH hypersecretion under basal conditions (18). In normal somatotrophs, GH secretion is under control of the stimulatory action of hypothalamic GHRH and the inhibitory effect of its counterpart, SRIF (19). The disturbances in GH secretion of somatotroph adenoma cells, in addition to the quantitative abnormality of basal secretion, may involve qualitative abnormalities in the regulation of GH secretion, such as lack of stimulation by GHRH, paradoxical increase in GH after stimulation with TRH (18), and missing inhibition after administration of SRIF or SRIF analogs such as octreotide in a part of the adenomas (20, 21).

The lack of GH stimulation by GHRH can be observed in about 30–40% of the somatotroph adenomas and was found to be due to the expression of the *gsp* oncogene (22). This oncogene is derived from a single mutation in the α-chain of the regulatory guanosine stimulatory G-protein (G_s) and leads to a constitutive activation of the adenylate cyclase system (23). As a result, cAMP production and associated GH secretion cannot be further stimulated by GHRH; therefore, GH secretion of this tumor type fails to respond to GHRH stimulation (24, 25).

The aim of the present study was to determine whether somatotroph adenomas *in vitro* respond differently to GHRP with respect to GH secretion, as has been found for GHRH, TRH, and somatostatin. In addition, with somatotropic cell cultures, the effect of GHRP on GH release was studied comparatively with GHRH, TRH, and octreotide.

Materials and Methods

Patients

Pituitary adenoma tissue from 12 patients with somatotroph tumors (numbered consecutively ST 1 to ST 12) obtained at transsphenoidal surgery was studied. The presence of pituitary adenomas was confirmed by computerized tomography or magnetic resonance imaging. ST 2 was a recurrent adenoma from a patient who had undergone a first surgery 14 yr previously. ST 4 was from a patient treated with octreotide (three doses; 100 µg daily) over a period of 4 months before surgery. The clinical data of the patients are shown in Table 1.

Cell culture

Cell culture materials and reagents, except where stated, were obtained from Flow Laboratories (Meckenheim, Germany), Gibco Europe (Karlsruhe, Germany), Seromed (Berlin, Germany), Falcon (Heidelberg, Germany), or Nunc (Wiesbaden, Germany).

Human pituitary adenoma cell cultures were performed as described previously (26) with minor modifications. In brief, adenoma tissue was washed four times with preparation buffer [137 mmol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L Na_2HPO_4, 15 mmol/L HEPES (pH 7.3), 10 mmol/L glucose, 2.5 mg/L amphotericin-B, and 10^5 U/L penicillin/streptomycin] and dissected into small pieces. The tissue fragments were mechanically

and enzymatically dispersed in preparation buffer containing 1000 U/mL collagenase (Worthington Biochemical Corp., Freehold, NJ), 4 g/L BSA, 10 mg/L DNAase-II, 1 g/L soybean trypsin inhibitor, and 2 g/L hyaluronidase. Dispersed cells were centrifuged and resuspended in sterile culture medium (Dulbecco's Modified Eagle's Medium, pH 7.3) containing 10% fetal calf serum (FCS), 2.2 g/L $NaHCO_3$, 10 mmol/L HEPES, 2 mmol/L glutamine, 10 mL/L nonessential amino acids, 10 mL/L Minimum Essential Vitamins, 5 mg/L insulin, 5 mg/L transferrin, 2.5 mg/L amphotericin-B, 10^5 U/L penicillin/streptomycin, 20 µg/mL sodium Selenit (Sigma Chemical Co., St. Louis, MO), and 30 pmol/L T_3 (Henning, Berlin, Germany). Between 22–40 × 10^6 cells with a viability of 92–98% (acridine orange-ethidium bromide staining) were isolated from pituitary tumors. The cells were diluted with culture medium and distributed to 24-well tissue culture plates (2 × 10^5 cells/well in 1 mL medium). The cells were grown at 37 C in an atmosphere of 5% CO_2. The dispersed cells attached to the culture dishes within the first 48 h.

Incubation of the cells with test substances

Experiments were conducted on cells 2–3 days after plating. The growth medium was removed by aspiration, and the attached cells were washed several times with phosphate-buffered saline. For stimulation experiments, Dulbecco's Modified Eagle's Medium (pH 7.3) supplemented with 2.2 g/L $NaHCO_3$, 10 mmol/L HEPES, 2 mmol/L glutamine, 1 g/L BSA, and 30 mg/L ascorbic acid (as an antioxidant) was used. GHRP (SmithKline Beecham, King of Prussia, PA), GHRH and TRH (both from Bachem, Bubendorf, Switzerland), the somatostatin analog octreotide (SMS 201–995; Sandoz, Basel, Switzerland), and the PKC inhibitor phloretin (Sigma Chemical Co., St. Louis, MO) (16, 27) were diluted in the same medium and added in the concentrations indicated. For studies in which combinations of the test substances were used, GHRP and GHRH were added to give concentrations of 1, 10, and 100 nmol/L of each; GHRP and TRH or octreotide, respectively, were applied in concentrations of 10 or 100 nmol/L of each. The final volume was 1 mL/well in each case. After an incubation period of 4 h, which was determined to be the optimal incubation time for all of the various test substances, the supernatants were removed and assayed for GH. Due to the limited numbers of cells obtained at some tumor cell preparations, not all studies could be performed in parallel with every somatotroph adenoma.

Determination of human (h) GH, PRL, and insulin-like growth factor-I

hGH levels in cell culture supernatants were determined using a sensitive fluorescence immunometric assay, as described previously (28)

TABLE 1. Clinical characteristics of the 12 patients with somatotroph adenomas (ST 1 to ST 12)

| Tumor no. | Age (yr) | Sex | Duration of illness (yr) | Adenoma extension | Preoperative serum GH (µg/L) | | | | IGF-I (µg/L) | PRL (µg/L) | Pituitary function |
					Basal	After TRH (200 µg, iv)	After OGTT (100 g glucose)	After GHRH (100 µg, iv)			
ST 1	58	F	4	Intra/suprasellar	59	113	123	NE	1507	13.7	Normal
ST 2	54	M	>16	Intra/parasellar	91	123	98	114	1080	15.1	Normal
ST 3	60	F	5	Intrasellar	13	28	17	NE	1264	16.2	SH
ST 4	55	F	3	Intra/parasellar	89	134	109	NE	561	41.8	Normal
ST 5	66	F	3	Intrasellar	12	15	20	NE	1340	4.8	Normal
ST 6	66	F	10	Intra/parasellar	10	27	15	NE	1495	11.9	Normal
ST 7	28	F	5	Parasellar	13	14	8	17	964	6.0	SH
ST 8	34	F	>10	Intrasellar	15	48	13	NE	1343	11.3	Normal
ST 9	27	F	4	Intra/parasellar	83	110	58	147	1146	7.2	SH
ST 10	55	M	1	Intrasellar	36	94	31	137	2259	28.6	Normal
ST 11	41	M	5	Intra/suprasellar	37	62	33	56	2137	12.3	Normal
ST 12	59	F	5	Intra/parasellar	46	53	41	NE	972	10.9	Normal

Clinical characteristics of the patients from which the somatotroph adenomas were obtained. GH (normal, <5 µg/L) was determined with a fluorescence immunoassay, as described in *Materials and Methods*. IGF-I (normal range in adults, 180–480 µg/L) was determined using a kit from Nichols, and PRL (normal, 1–25 µg/L) was determined with a DELFIA kit. F, Female; M, male; NE, nonevaluable; OGTT, oral glucose tolerance test; SH, secondary hypogonadism.

with minor modifications. In brief, anti-hGH monoclonal antibody clone 8B11 was adsorbed to polystyrene microtiter plates (500 ng antibody/well) in 50 mmol/L phosphate buffer, pH 9.6. After 16-h incubation at 4 C, the plates were washed, and 50 μL sample (diluted 1:20 to 1:50) or standard were added. Standards were prepared by serial dilution of recombinant hGH (Genotropin, Kabi, Stockholm, Sweden). Then, 30 ng biotinylated anti-hGH monoclonal antibody clone 7F8 in 150 μL assay buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% BSA, 0.05% (wt/vol) bovine γ-globulin, 0.05% Tween-40, 0.05% NaN$_3$, and 20 μmol/L diethylenetriaminepentaacetic acid, pH 7.75] were added. After 2 h of incubation at room temperature, the plates were washed again, and 10 ng streptavidin-europium conjugate (Kabi-Pharmacia, Freiburg, Germany), dissolved in assay buffer, were added to each well (final volume, 200 μL). After an incubation period of 30 min at room temperature and a final wash step, 200 μL europium fluorescence enhancement solution (Kabi-Pharmacia) were added, and the europium signal was measured in a time-resolved fluorometer (DELFIA, Wallacoy, Turku, Finland). The working range for cell culture samples was 0.12–100 ng/mL, with an intraassay coefficient of variation of less than 10%.

Serum GH levels were measured with the same method, using commercially available hGH standards prepared in ovine serum (Medgenix, Ratingen, Germany). The working range for serum samples is 0.18–110 ng/mL.

Insulin-like growth factor-I serum levels were determined with a RIA kit (Nichols Institute, Bad Nauheim, Germany) after acid-ethanol extraction and cryoprecipitation. Serum PRL was measured with a DELFIA kit (Wallacoy). Both methods were conducted according to the instructions of the manufacturers.

Statistics

All experiments were performed at least in quadruplicate, and values are calculated as the mean ± SD and, with the exception of the data presented in Table 2, are expressed as a percentage of the control values. Statistics were determined by analysis of variance in combination with the Scheffe test. $P < 0.05$ was considered significant.

Results

Basal and stimulated GH secretion

In vitro basal GH secretion varied from 107 (ST 2) to 438 (ST 12) ng GH/2 × 10^5 cells·4 h (Table 2). There was no correlation between basal GH secretion *in vitro* and plasma GH levels *in vivo* (compare with Table 1). As far as comparable, the responsiveness to GHRH and TRH was identical *in vivo* and *in vitro*, with the exception of ST 11, in which GHRH stimulated GH release *in vivo* but not *in vitro* (compare Tables 1 and 2).

GHRP (0.1–1000 nmol/L) stimulated GH release in a dose-dependent manner in all 12 somatotroph adenoma cell cultures (a typical dose-response curve is shown in Fig. 1; see also Table 2 and Fig. 2). In all cases the stimulatory effects became significant at 1 nmol/L (range, 120 ± 2% to 148 ± 9% *vs.* basal secretion) and reached a plateau at 10 nmol/L (range, 126 ± 4% to 184 ± 12%); no further significant stimulation of GH secretion could be observed with either 100 or 1000 nmol/L GHRP (range, 123 ± 4% to 202 ± 8%).

GHRH (0.1–1000 nmol/L) stimulated GH secretion in six adenoma cell cultures dose dependently, but had no significant stimulatory effect in the six other cultures (for typical dose-response curve of a GHRH-responsive culture, see Fig. 1; see also Table 2 and Fig. 2). For GHRH-responsive cultures, stimulatory effects became significant at 1 nmol/L (127 ± 1% and 134 ± 4%) in two cases (ST 1 and ST 10), and at 10

TABLE 2. *In vitro* characteristics of the somatotroph adenoma cell cultures

Tumor no.	Basal GH secretion	Responsive *in vitro* to			
		GHRP	GHRH	TRH	Octreotide
ST 1	293	+	+	ND	ND
ST 2	107	+	−	ND	ND
ST 3	241	+	−	ND	−^{a}
ST 4	176	+	−	ND	ND
ST 5	195	+	−	ND	−
ST 6	392	+	+	+	+
ST 7	117	+	−	−	+
ST 8	208	+	+	+	−^{a}
ST 9	156	+	+	−	+
ST 10	354	+	+	+	−
ST 11	144	+	−	+	+
ST 12	438	+	+	−	+

Characteristics of the 12 somatotroph adenoma cell cultures investigated with respect to basal GH secretion (nanograms of GH per 2 × 10^5 cells/4 h) and responsiveness to stimulation with GHRP, GHRH, TRH, and octreotide. Cell cultures that responded to 1 or 10 nmol/L of the substances with a significant increase or decrease in GH secretion ($P < 0.05$) were considered to be responsive (+). Cell cultures in which GH secretion could not be stimulated by excessively high concentrations (100 or 1000 nmol/L) of the test substances were considered not responsive (−). ND, Not determined.

^{a} Significant ($P < 0.05$ *vs.* control) inhibition of GH secretion only at 1000, but not at 100 nmol/L octreotide.
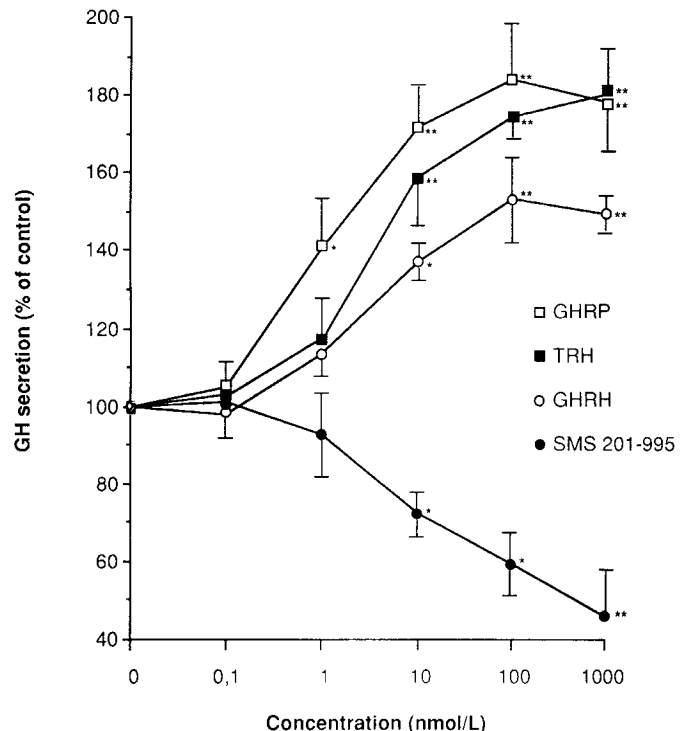

FIG. 1. Influence of increasing concentrations of GHRP, GHRH, TRH, and octreotide (SMS 201–995) on GH secretion of cultured cells of the somatotroph adenoma ST 6. *In vitro*, this adenoma responded to all of the substances tested, and significant stimulation of GH secretion could be observed after treatment with GHRP, GHRH, and TRH as well as significant inhibition of GH secretion after application of octreotide. ★, $P < 0.05$; ★★, $P < 0.01$ [*vs.* basal GH secretion (100%)].

nmol/L (range, 124 ± 3% to 135 ± 7%) in four cases (ST 6, 8, 9, and 12). Maximal stimulation was obtained with 100
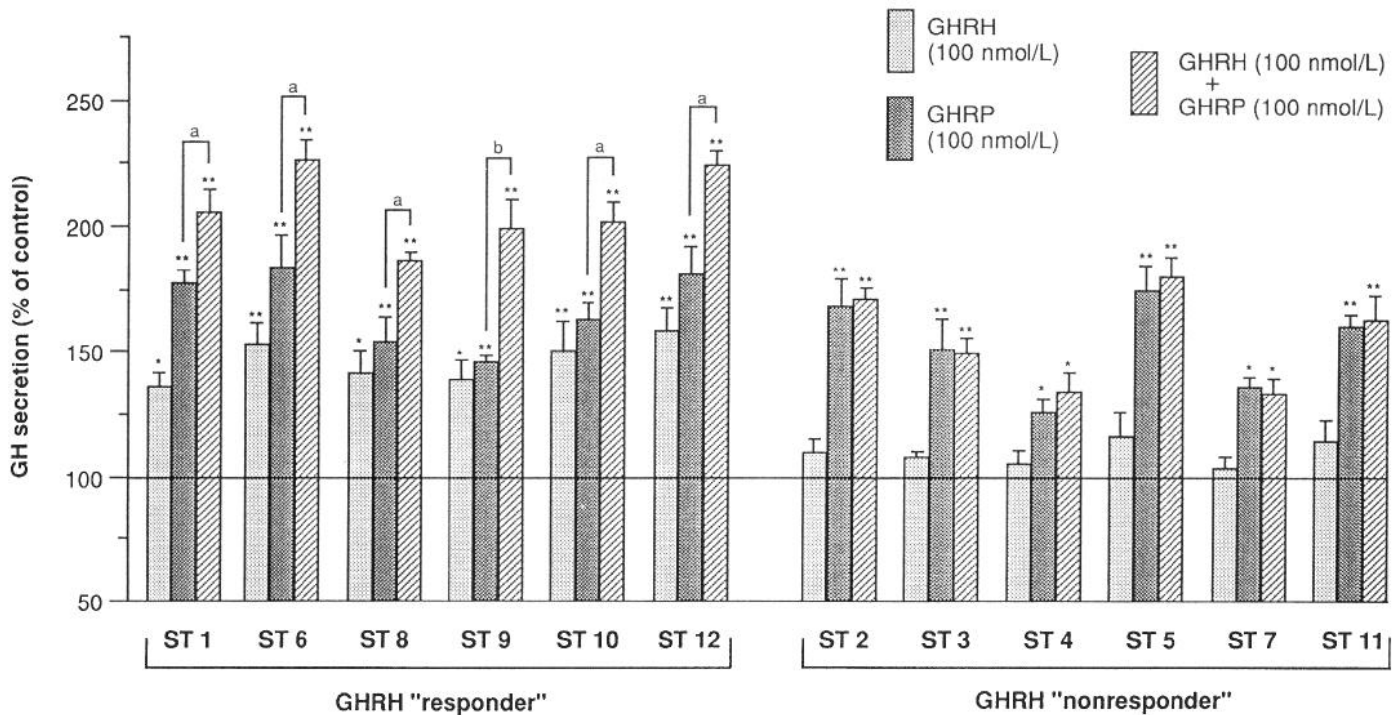
FIG. 2. Comparison of the effects of GHRH (100 nmol/L) and GHRP (100 nmol/L), either alone or in combination, on the GH secretion of 12 individual adenoma cell cultures (ST 1 to ST 12). Basal secretion of the adenoma cultures was set at 100%, and the results of the stimulation experiments are expressed as a percentage of the control value. ★, $P < 0,05$; ★★, $P < 0,01$ (vs. control). In GHRH responders, simultaneous application of GHRH and GHRP led to a further significant increase in GH secretion (a, $P < 0.05$; b, $P < 0.01$) compared to stimulation with GHRP alone.

nmol/L (range, 136 ± 4% to 158 ± 9%). No further significant increase could be observed with 1000 nmol/L GHRH (range, 130 ± 7% to 163 ± 7%).

The effect of TRH (0.1–1000 nmol/L) on GH secretion was tested in seven somatotroph adenoma cell cultures. In three of them, TRH had no significant effect, but stimulated GH release dose dependently in the other four cultures (Fig. 1; see also Table 2 and Fig. 3). In the TRH responders the stimulatory effect became significant at 10 nmol/L (range, 129 ± 5% to 159 ± 9% vs. basal secretion) and reached

maximum values at 100 nmol/L (range, 138 ± 7% to 174 ± 4%), with the exception of one case (ST 8) in which a further significant stimulatory effect of TRH was observed with 1000 nmol/L (161 ± 8% vs. 138 ± 7% at 100 nmol/L).

Although the use of 10-fold increments was critical, we calculated the approximate ED_{50} values for GHRP, GHRH, and TRH for the individual somatotroph adenomas to obtain information about the relative GH-stimulating potency of these substances. The ED_{50} for GHRP ranged from 1.1–3.4 nmol/L, the ED_{50} for GHRH (in GHRH responders) from
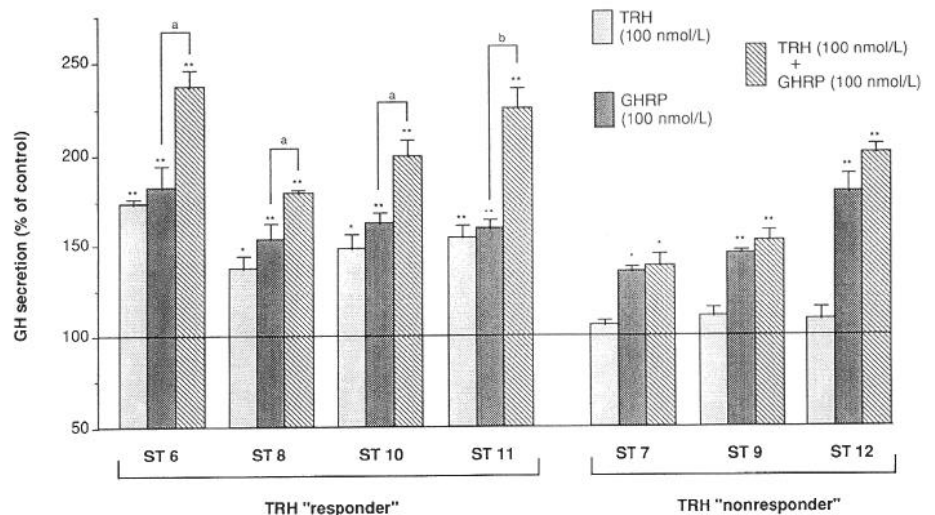

FIG. 3. Comparison of the effects of TRH (100 nmol/L) and GHRP (100 nmol/L), either alone or in combination, on the GH secretion of seven individual somatotroph adenoma cell cultures (ST 6 to ST 12). Results are expressed as a percentage of the control value (100% = basal secretion). ★, $P < 0.05$; ★★, $P < 0.01$ (vs. control). In TRH responders, simultaneous application of TRH and GHRP significantly stimulated GH secretion (a, $P < 0.05$; b, $P < 0.01$) compared to stimulation with GHRP alone.

2.9–9.1 nmol/L, and the ED_{50} for TRH (in TRH responders) from 6.4–12.5 nmol/L.

The influence of octreotide (0.1–1000 nmol/L) on basal GH release was studied with nine somatotroph adenoma cell cultures (Fig. 1; see also Table 2 and Fig. 4). Four adenoma cultures (ST 3, 5, 8, and 10) were considered to be not or only slightly responsive to octreotide, because 1–100 nmol/L octreotide had no significant inhibitory effect on GH secretion. In only two cases (ST 3 and ST 8) was a slight, but significant, inhibition ($P < 0,05$) of basal GH release observed after application of 1000 nmol/L octreotide (79 ± 4% and 83 ± 1% of basal secretion). In contrast, five adenomas responded very well to octreotide. In one of these (ST 6), basal GH secretion was significantly inhibited by 1 nmol/L octreotide (80 ± 2%); GH was stimulated in all cases by 10 nmol/L (range, 69 ± 6% to 81 ± 3%). Basal GH release was further decreased by 100 nmol/L octreotide (range, 51 ± 9% to 70 ± 4%), but 1000 nmol/L had no further significant inhibitory effect (range, 48 ± 4% to 65 ± 8%).

Interaction of GHRH and GHRP on GH secretion

In all somatotroph adenoma cultures the effects of GHRP and GHRH were investigated simultaneously (Fig. 2 summarizes the results for the stimulation with 100 nmol/L of each compound). In the six adenomas in which GH secretion could not be stimulated with GHRH, simultaneous application of GHRP and GHRH had the same stimulatory effect as GHRP alone. In the GHRH-responsive cell cultures, simultaneous stimulation with 100 nmol/L GHRP and GHRH had an additive effect on GH release (GHRP plus GHRH, 186 ± 3% to 225 ± 7%; GHRP alone, 146 ± 2% to 181 ± 10% *vs.* basal secretion). This additive effect was also observed when combinations of the substances were used at lower concentrations (1 and 10 nmol/L; data not shown).

Interaction of TRH and GHRP on GH secretion

In 7 of 12 adenoma cell cultures we studied the effects of GHRP and TRH on the GH release (Fig. 3 summarizes the

effects for 100 μmol/L of each substance). In the 3 TRH nonresponders, simultaneous application of GHRP and TRH resulted in the same stimulatory effect as application of GHRP alone. In TRH-responding somatotroph adenoma cultures, simultaneous application of 100 nmol/L GHRP and TRH had an additive effect on GH secretion (GHRP plus TRH, 181 ± 2% to 239 ± 9%; GHRP alone, 154 ± 9% to 184 ± 12% *vs.* basal secretion). The additive effect could also be seen, but was less pronounced, when 10 nmol/L TRH or GHRP were used (data not shown).

Effect of octreotide on GHRP-stimulated GH secretion

In 9 of 12 somatotroph adenoma cell cultures, the effect of the long-acting somatostatin analog octreotide (10 and 100 nmol/L) on GHRP-stimulated GH release was investigated. In Fig. 4, the effects of 100 nmol/L octreotide on basal and GHRP-stimulated GH secretion are summarized (the effects of 10 nmol/L octreotide were similar, but less pronounced). In the 4 adenoma cell cultures that failed to respond or showed only a moderate response to octreotide, GHRP-induced GH secretion could not be significantly suppressed. In the adenoma cultures that responded to octreotide, however, significant decreases in GHRP-stimulated GH secretion could be observed after application of 100 nmol/L octreotide (GHRP alone, 136 ± 3% to 184 ± 12%; GHRP plus octreotide, 75 ± 8% to 106 ± 4% *vs.* basal secretion).

Influence of phloretin on GHRP-stimulated GH release

In 5 of 12 cases (ST 8 to ST 12), the effects of phloretin (10 and 100 μmol/L), an inhibitor of PKC, on basal and GHRP-stimulated GH release were investigated (Table 3). Phloretin had no cytotoxic effect on the cells, as demonstrated by viability staining using ethidium bromide and acridine orange. In all cases, even the high concentration of phloretin had no significant inhibitory effect on basal GH secretion (range, 91 ± 8% to 98 ± 6% *vs.* basal secretion). GHRP-stimulated GH secretion was significantly inhibited

FIG. 4. Effect of 100 nmol/L octreotide (SMS 201-995) on basal and GHRP (100 nmol/L)-stimulated GH secretion of nine individual somatotroph adenoma cell cultures (ST 3 and ST 5 to ST 12). Basal GH secretion was set at 100%, and results are expressed as a percentage of the control value. ★, $P < 0.05$; ★★, $P < 0.01$ (*vs.* control). In cell cultures in which basal GH secretion could be significantly suppressed by octreotide, GHRP-stimulated GH secretion was also inhibited significantly (a, $P < 0.01$; b, $P < 0.001$).
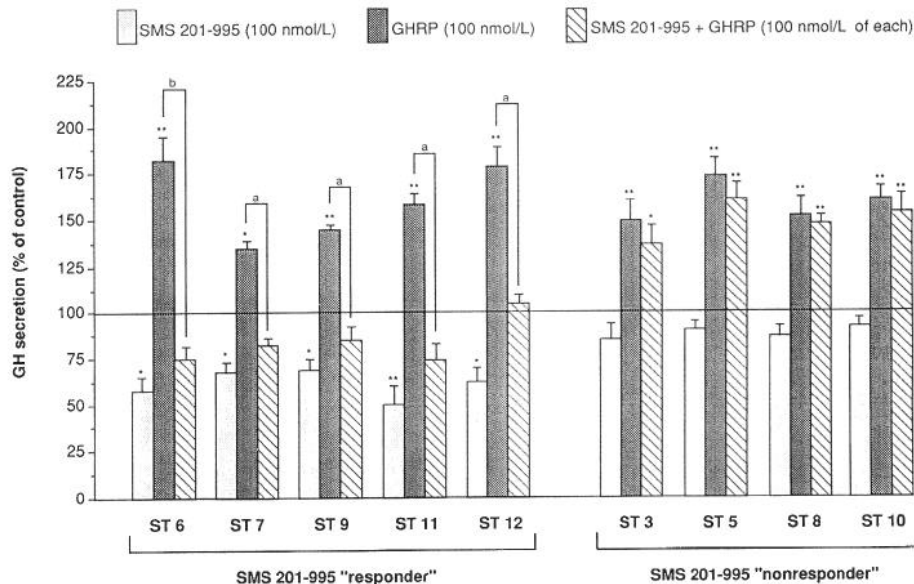
TABLE 3. Effect of phloretin on GHRP-stimulated GH secretion

Tumor no.	ST 8	ST 9	ST 10	ST 11	ST 12
Basal	100 ± 7	100 ± 4	100 ± 5	100 ± 5	100 ± 7
10 μmol/L phloretin	94 ± 3	96 ± 5	100 ± 3	102 ± 3	94 ± 5
100 μmol/L phloretin	92 ± 6	96 ± 1	97 ± 3	98 ± 6	91 ± 8
100 nmol/L GHRP	154 ± 9	146 ± 2	163 ± 6	160 ± 5	181 ± 10
GHRP + 10 μmol/L phloretin	143 ± 6	137 ± 7	138 ± 1[a]	133 ± 4[a]	169 ± 10
GHRP + 100 μmol/L phloretin	128 ± 7[b]	121 ± 4[a]	129 ± 4[a]	119 ± 1[c]	145 ± 1[a]

The effect of the PK-C inhibitor phloretin was investigated on both basal and GHRP (100 nmol/L)-stimulated GH secretion of five individual somatotroph adenoma cell cultures. Each value represents the mean ± SD of quadruplicate determinations and is expressed as a percentage of the control value (basal secretion). Significance was determined vs. GH stimulation with 100 nmol/L GHRP.

[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.001$.

by 10 μmol/L phloretin in 2 of 5 cases and in all cases by 100 μmol/L phloretin. With the latter concentration, the maximum stimulatory effect of GHRP was reduced by 50–60%.

Discussion

The abnormal regulation of GH secretion in somatotroph adenoma cells involves basal GH hypersecretion and, in some of the adenomas, missing response to GHRH, paradoxical response to TRH, and missing inhibition response to somatostatin or its analogs (18–21). Here we report that in vitro, GHRP stimulated GH secretion in all 12 somatotroph adenoma cell cultures investigated, whereas GHRH stimulated GH release in only 6 of 12 cases and TRH increased GH secretion in 4 of 7 cases. Our data confirm the results of a recent in vivo study in which it was shown that GHRP stimulated GH secretion in all 11 patients with acromegaly investigated (9). Our observation that GHRP was active in GHRH and TRH nonresponders confirms previous studies that GHRP acts through a receptor and a mechanism on GH secretion distinct from those of GHRH and TRH. This was further supported by our observation that the combined stimulation with GHRP and either GHRH or TRH had an additive effect on GH secretion, even in concentrations at which each of the substances alone had its maximal effect (5–7, 11).

Comparing the ED$_{50}$ values of GHRP and GHRH for GHRH-responsive somatotroph adenoma cultures, GHRP is even more potent than GHRH in stimulating GH secretion. This is in agreement with in vivo findings, where it has been demonstrated that in most patients with acromegaly GHRP was more effective than GHRH in stimulating GH release (9). Previous studies have also shown that, in general, GHRP is nearly as potent as GHRH in stimulating GH secretion in humans, whereas GHRP is about 100-fold less active than GHRH in rats (3–5). There is still no explanation for this phenomenon; human somatotroph adenoma cell cultures could provide a model for further studies on this topic.

In GHRH nonresponders, the missing effect of GHRH on GH secretion has been correlated with the expression of the gsp oncogene (22, 23). The expression of this oncogene leads to a constitutive activation of the adenylate cyclase system,

and because GHRH mediates GH secretion via this second messenger system, the GH secretion is not or is only poorly stimulated by GHRH (24, 25). However, because the correlation between gsp expression and GHRH nonresponsiveness is not 100%, and exceptions were found (24, 25), we cannot be sure that all of our GHRH nonresponders are gsp positive. On the other hand, because about 40% of the somatotroph adenomas express the gsp oncogene, by extrapolation, it would seem that GHRP is able to stimulate GH secretion in both gsp-negative and gsp-positive adenoma cells.

The mechanism of the paradoxical response of GH secretion to TRH stimulation observed in some somatotroph adenomas is not yet completely understood, but there is evidence that the TRH receptor is expressed in TRH responders, and the phosphoinositol pathway and PKC may be involved (29, 30). It is known from studies in rats that GHRP-stimulated GH secretion is also in part mediated via PKC (16). We could confirm this by the observation that in five somatotroph adenoma cultures investigated, the PKC inhibitor phloretin inhibited GHRP-stimulated GH secretion. On the other hand, we could show that GHRP and TRH in TRH-responsive adenoma cultures exerted an additive effect on GH secretion even at maximal stimulatory concentrations of each alone. Therefore, although the stimulation of GH by GHRP and TRH may share a common intermediate substance, namely PKC, the availability of PKC does not seem to be the rate-limiting step in the combined action of TRH and GHRP on GH secretion.

GH hypersecretion in acromegaly can often be reduced by medical treatment with somatostatin analogs, like the long-acting analog octreotide. On the other hand, in some cases GH secretion is not influenced by octreotide (18, 19, 21). The different responses of the somatotroph adenomas to somatostatin analogs have recently been recognized to be associated with the different expression of various somatostatin receptor subtypes that differ in their affinity to somatostatin or somatostatin analogs (21). Thus, tumors that do not respond to octreotide do not express the appropriate receptor. In our study, basal GH secretion of five of nine somatotroph adenoma cell cultures could be inhibited by octreotide. In these responders, GHRP-stimulated GH secretion was also suppressed significantly by octreotide. In contrast, octreotide had no inhibitory effect on GHRP-induced GH release in

those adenoma cultures in which it was not able to suppress basal GH secretion. Therefore, independent from its mechanism, GHRP-stimulated GH secretion seems to be under the control of somatostatin, as was also shown in rats (3, 11, 15), and can be inhibited in somatotroph adenoma cells that respond to octreotide.

In summary, GHRP was able to stimulate GH secretion dose dependently in all 12 human somatotroph adenoma cell cultures investigated. Thus, the expression of both the still unidentified receptor of GHRP and its unknown signaling pathway seems to be more consistent in somatotroph adenomas than in those for GHRH, TRH, and somatostatin, in which marked variations in different somatotroph adenoma subtypes with respect to stimulation or inhibition of GH secretion are observed. The action of GHRP is different from that of GHRH and TRH, but can be blocked by octreotide in octreotide-responsive adenomas. Although we could confirm the participation of PKC in GHRP-stimulated GH secretion, much more work is necessary to identify the mechanism of action of GHRP and to characterize its receptor.

Acknowledgments

References

1. Momany FA, Bowers CY, Reynolds GA, Chang D, Hong A, Newlander K. 1981 Design, synthesis, and biological activity of peptides which release growth hormone *in vitro.* Endocrinology. 108:31–39.
2. Momany FA, Bowers CY, Reynolds GA, Hong A, Newlander K. 1984 Conformational energy studies and *in vitro* and *in vivo* activity data on growth hormone releasing peptides. Endocrinology. 114:1531–1536.
3. Bowers CY, Momany FA, Reynolds GA, Hong A. 1984 On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology. 114:1537–1545.
4. Sartor O, Bowers CY, Reynolds GA, Momany FA. 1985 Variables determining the growth hormone response of His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 in the rat. Endocrinology. 117:1441–1447.
5. Bowers CY, Reynolds GA, Durham D, Barrera CM, Pezzoli SS, Thorner MO. 1990 Growth hormone (GH)-releasing peptide stimulates GH release in normal man and acts synergistically with GH-releasing hormone. J Clin Endocrinol Metab. 70:975–982.
6. Robinson BM, Friberg RD, Bowers CY, Barkan AL. 1992 Acute growth hormone (GH) response to GH-releasing hexapeptide in humans is independent of endogenous GH-releasing hormone. J Clin Endocrinol Metab. 75:1121–1124.
7. Penalva A, Carballo A, Pombo M, Casanueva FF, Dieguez C. 1993 Effect of growth hormone (GH)-releasing hormone (GHRH), atropine, pyridostigmine, or hypoglycemia on GHRP-6-induced GH secretion in man. J Clin Endocrinol Metab. 76:168–171.
8. Bowers CY, Alster DK, Frentz JM. 1992 The growth hormone-releasing activity of a synthetic hexapeptide in normal men and short statured children after oral administration. J Clin Endocrinol Metab. 74:292–298.
9. Alster DK, Bowers CY, Jaffe CA, Ho PJ, Barkan AL. 1993 The growth hormone (GH) response to GH-releasing peptide (His-DTrp-Ala-Trp-DPhe-Lys-NH2), GH-releasing hormone, and thyrotropin-releasing hormone in acromegaly. J Clin Endocrinol Metab. 77:842–845.
10. Walker RF, Codd EE, Barone FC, Nelson AH, Goodwin T, Campbell SA. 1990 Oral activity of the growth hormone releasing peptide His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 in rats, dogs, and monkeys. Life Sci. 47:29–36.
11. Blake AD, Smith RG. 1991 Desensitization studies using perifused rat pituitary cells show that growth hormone-releasing hormone and His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 stimulate growth hormone release through distinct receptor sites. J Endocrinol. 129:11–19.
12. Bowers CY, Sartor AO, Reynolds GA, Badger TM. 1991 On the actions of the growth hormone-releasing hexapeptide, GHRP. Endocrinology. 128:2027–2035.
13. Codd EE, Yellin T, Walker RF. 1988 Binding of growth hormone-releasing hormones and enkephalin-derived growth hormone-releasing peptides to mu and delta opiod receptors in forebrain of rat. Neuropharmacology. 27:1019–1025.
14. Goth MI, Lyons CE, Canny BJ, Thorner MO. 1992 Pituitary adenylate cyclase activating polypeptide, growth hormone (GH)-releasing peptide, and GH-releasing hormone stimulate GH release through distinct pituitary receptors. Endocrinology. 130:939–944.
15. Cheng K, Chan WWS, Barreto A Jr, Convey EM, Smith RG. 1989 The synergistic effects of His-D-Trp-Ala-Trp-D-Phe-Lys-NH2 on growth hormone (GH)-releasing factor-stimulated GH release and intracellular adenosine 3′,5′-monophosphat accumulation in rat primary pituitary cell culture. Endocrinology. 124:2791–2798.
16. Cheng K, Chan WWS, Butler B, Barreto Jr A, Smith RG. 1991 Evidence for a role of protein kinase-C in His-D-Trp-Ala-Trp-D-Phe-Lys-NH2-induced growth hormone release from rat primary pituitary cells. Endocrinology. 129:3337–3342.
17. Codd EE, Shu AYL, Walker RF. 1989 Binding of a growth hormone releasing hexapeptide to specific hypothalamic and pituitary binding sites. Neuropharmacology. 28:1139–1144.
18. Melmed S, Braunstein GD, Horvath E, Ezrin C, Kovacs K. 1983 Pathophysiology of acromegaly. Endocr Rev. 4:271–290.
19. Reichlin S. 1987 Control of GH secretion: an overview. In: Lüdecke DK, Tolis G (eds) Growth hormone, growth factors, and acromegaly. New York: Raven Press; 1–11.
20. Frohman LA. 1991 Therapeutic options in acromegaly. J Clin Endocrinol Metab. 72:1175–1181.
21. Lamberts SWJ, Krenning EP, Reubi J-C. 1991 The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocr Rev. 12:450–482.
22. Vallar L, Spada A, Giannattasio G. 1987 Altered Gₛ and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature. 330:566–568.
23. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L. 1989 GTPase inhibiting mutations activate the a chain of Gₛ and stimulate adenylyl cyclase in human pituitary tumours. Nature. 340:692–696.
24. Landis CA, Harsh G, Lyons J, Davis RL, McCormick F, Bourne HR. 1990 Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant Gₛ protein. J Clin Endocrinol Metab. 71:1416–1420.
25. Spada A, Arosio M, Bochicchio D, et al. 1990 Clinical, biochemical, and morphological correlates in patients bearing growth hormone-secreting pituitary tumors without or with somatic mutation in the alpha-chain of the stimulatory regulatory protein of adenylyl cyclase (Gₛ). J Clin Endocrinol Metab. 71:1421–1426.
26. Arzt E, Stelzer G, Renner U, Lange M, Müller OA, Stalla GK. 1992 Interleukin-2 and interleukin-2 receptor expression in human corticotrophic adenoma and murine pituitary cell cultures. J Clin Invest. 90:1944–1951.
27. Richardson SB, Twente S, Newman CB. Growth hormone secretion *in vitro* and *in vivo* is inhibited by flavonoids [Abstract]. Proc of the 69th Annual Meet of The Endocrine Soc. 1987;107.
28. Strasburger CJ, Barnard G, Toldo L, et al. 1989 Somatotropin as measured by a two-site time-resolved immunofluorometric assay. Clin Chem. 35:913–917.
29. Martin FTJ. 1983 Thyrotropin-releasing hormone rapidly activates the phosphodiester hydrolysis of polyphosphoinositides in GH3 pituitary cells. J Biol Chem. 238:14816–14822.
30. Le Dafniet M, Garnier P, Bression D, Brandi AM, Racadot J, Peillon F. 1985 Correlative studies between the presence of thyrotropin-releasing hormone (TRH) receptors and the *in vitro* stimulation of growth hormone secretion in GH-secreting adenomas. Horm Metab Res. 17:476–479.