Activity of Growth Hormone-Releasing Peptide-2 in Cultured Human Pituitary Adenoma Cells and Its Interaction with Growth Hormone-Releasing Hormone and Somatostatin

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Experiments on primary cultures of human pituitary adenoma cells producing growth hormone (GH) or GH and prolactin showed that similarly to GH-releasing hormone (GHRH) synthetic hexapeptide GH-releasing peptide-2 (GHRP) directly enhance secretion of GH but not of prolactin by human pituitary cells. The effect of various doses of GHRP and GHRH applied in combination was additive or slightly synergistic in nature. Somatostatin inhibits secretion of GH induced by GHRP, GHRH, or their combination. A dissociation is found between the inhibitory effects of somatostatin on basal and stimulated GH secretion.

Key Words: *growth hormone (GH); GH-releasing peptide-2; GH-releasing hormone; somatostatin; human pituitary; pituitary adenoma; cell culture*

According to the classical scheme, secretion of pituitary growth hormone is controlled by two physiological peptide regulators: a stimulatory GH-releasing factor (GHRH) and somatoliberin and an inhibitory hormone somatostatin [11,14]. Both peptides are produced by neurosecretory hypothalamic cells. Apart from classical GHRH, the GH-releasing peptide (GHRP) family has recently attracted considerable attention as specific stimulators of GH secretion. These peptides were initially synthesized and studied as various remote analogs of enkephalin [4,5]. GHRP are small peptides (6-7 amino acids) and contain D-amino acids or other unusual amino acid residues and aromatic rings in side chains. The effect of GHRP on the endocrine system is now little studied. The most intriguing is the assumption on the existence of GHRP-like endogenous ligands involved in physiological regulation of GH secretion [4,5]. Another important aspect is possible interrelationships between GHRP and classical hypothalamic regulators GHRH and somatostatin. Experimental and clinical data suggest that these peptides stimulate GH release by acting on both the hypothalamus and pituitary [4,6]; they stimulate secretion of human GH and may be of clinical importance [4,10].

Recently, a GHRP hexapeptide (DAla^1-DβNal^2-Ala^3-Trp^4—DPhe^5-Lys^6-NH_2, one of the most active member of the GHRP family [4,15], was synthesized. It has been found that GHRP effectively stimulates the release of GH from the pituitary of healthy young people and children of short stature [3,4]. The aim of the present study was to investigate *in vitro* secretory reaction of human pituitary to GHRP and interaction between GHRP and GHRH and somatostatin. The study was performed on primary culture of pituitary adenoma cells producing GH (somatotropinomas) and GH and prolactin (mixed tumors).

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MATERIALS AND METHODS

Synthetic GHRP was provided by Tulane University Medical Center, New Orleans, USA). Synthetic (1-29)NH_2 GHRH (Kabi Pharmacia) and somatostatin-14 (Serva) were used in the experiments.

Preparation of cell cultures from human pituitary tumors and mixed adenomas was described previously [1,2]. Adenoma tissue obtained from patients with acromegaly and high serum GH levels was minced (<1 mm) and mechanically and enzymatically dispersed in Ca^{2+},Mg^{2+}-free RPMI-1640 medium (Serva) containing 0.25% trypsin (Sigma). The cell suspension was centrifuged at 800*g*, resuspended in medium 199 (Flow) supplemented with 10% embryonic calf serum (Calbiochem) and 50 U/ml penicillin, seeded to 96-well plates (Flow) 10^5 cells per well, and cultured at 37°C and 5% CO_2. After 48 h, the cells formed a monolayer. Culture medium was replaced every 48 h.

The effect of peptides on the secretion of GH and prolactin was studied in 5-6-day cultures. Culture medium was removed, and the cells were twice washed with the same medium. The cells were incubated in the presence of test peptide for 1, 3, or 24 h; then aliquots of conditioning medium were collected, frozen, and stored before hormone assay. Cells cultured in the absence of peptides served as the control.

The contents of GH and prolactin in the incubation medium were measured by radioimmunoassay using test systems with highly purified hormones and monospecific polyclonal antisera obtained at the Endocrinology Research Center (Russian Academy of Medical Sciences). The test systems were calibrated with IRP 66/217 (GH) and IRP 75/504 (Prolactin) international standards. The data were expressed in ng/ml highly purified human GH or prolactin (immunological activity of 2 and 30 U/mg, respectively). The test systems for GH and prolactin had a sensitivity of 0.2 and 0.5 ng/ml, respectively; coefficient of variations being <8% for both systems.

The data were processed statistically using the Student test, the differences were significant at $p<0.05$. Each experimental point was performed in 4-6 replicates.

RESULTS

Activity of GHRP and GHRH was studied on cell cultures of 5 pituitary adenomas (I-V). All cell cultures were characterized by different but relatively high basal GH secretion. Two of these adenomas (II and III) apart from GH produced a measurable amount of prolactin.

The data obtained on adenoma I cells are presented in Table 1. Effects of various doses of GHRP and GHRH (0.1-100 ng/ml medium) on the release of GH into incubation medium during 1-, 3-, and 24-h incubation were studied. All used doses of GHRH considerably stimulated secretion of GH by adenoma I cells. It increased by 53% after 1-h incubation with the lowest dose of GHRH (0.1 ng/ml), while the maximum reaction to GHRP after 1- and 3-h incubation was 151 and 157%, respectively. Under these experimental conditions GHRP (0.1-100 ng/ml) also stimulated the release of GH into incubation medium: the maximum rise of GH secretion after 1- and 3-h incubation constituted 99 and 87%, respectively. Twenty-four-hour incubation in the presence of GHRH and GHRP (30 ng/ml) induced a similar rise of GH secretion (by 67 and 68%, respectively).

Figure 1, *a* shows the effect of GHRH and GHRP on GH secretion in cell cultures of human pituitary adenomas II-V. The cells were incubated for 1 h in the presence of individual peptides or their combinations in different doses. Maximum stimulation of GH release from adenoma II induced by GHRP, GHRH, and their combination were 45, 66, and 128%, the corresponding values from adenoma III were 87, 131, and 209%. Maximum response to individual peptides was observed at their concentration of 1 ng/ml for adenoma II cells and 5 ng/ml for adenoma III cells, the higher dose (10 ng/ml) was less effective.

As seen from Fig. 1, *a*, the effect of combination of GHRP and GHRH was additive and slightly sy-

TABLE 1. Effect of GHRH and GHRP on Secretion of GH in GH-Producing Pituitary Adenoma I Cells (*M±m*, *n*=6)

Peptide, ng/ml		Content of GH in the incubation medium, ng/ml		
		1 h	3 h	24 h
Control		180±12	251±8	1191±36
GHRH	0.1	276±5*	—	—
	1	368±7*	430±52**	—
	10	352±1*	585±2*	—
	30	—	—	1989±50*
	100	452±17*	452±48*	—
GHRP	0.1	330±11*	—	—
	1	244±19*	389±46*	—
	1	259±14*	470±45*	—
	30	—	—	2001±56*
	100	358±20*	468±43*	—

Note. *$p<0.001$; **$p<0.01$ compared with the control.

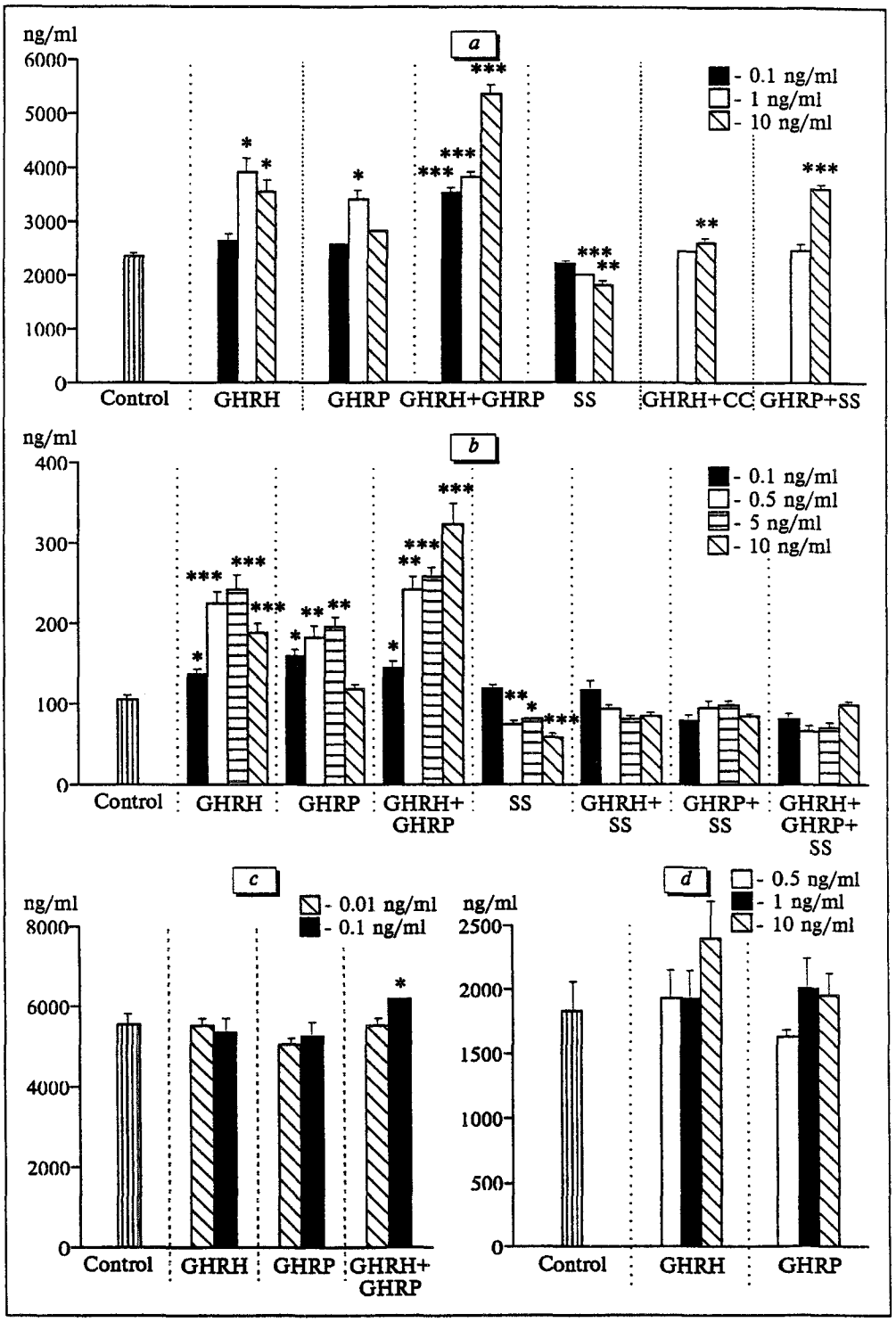

Fig. 1. Secretion of growth hormone in pituitary adenoma cultures II-IV under different conditions. *a*) adenoma II, *b*) adenoma III, *c*) adenoma IV, *d*) adenoma V, SS: somatostatin. Incubation time: 1 h; the same experimental conditions for 4-6 wells. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with the control.

nergistic in nature. In adenoma II cells, individual peptides in low doses (0.1 ng/ml) were inactive, while their combination enhanced secretion of GH by 50% ($p<0.001$). In adenoma II and III cultures, stimulating effect of GHRP+GHRH (10 ng/ml each) combination considerably surpassed that of individual peptides. This attests to some synergism of the effect of GHRP and GHRH on human somatotrophes.

Adenoma IV cultures were treated with low doses of GHRP and GHRH (0.01 and 0.1 ng/ml), which were ineffective. However, in the presence of both peptides in the incubation medium (0.1 ng/ml), a significant stimulation of GH secretion was noted ($p<0.05$).

Adenoma V cells did not react to stimulation with GHRP and GHRH in doses of 0.5, 1, and 10 ng/ml.

Mixed adenomas II and III characterized by basal release of GH and prolactin into incubation medium, GHRP, GHRH, and GHRP+GHRH in doses that stimulated GH secretion had no effect of prolactin secretion during a 1-h incubation. Table 2 illustrates the effect of test peptides on prolactin secretion by adenoma II cells.

For evaluation of the interaction of GHRP and GHRH with somatostatin, adenoma II and III cells were incubated for 1 h with GHRP and GHRH in doses that stimulated the release of GH and different doses of somatostatin. Somatostatin (0.1 ng/ml) had no effect on basal GH secretion by both cultures (Fig. 1, *b*). Surprisingly, being added in combination with GHRP, GHRH, or GHRP+GHRH, somatostatin exerted a pronounced inhibitory effect. All doses of somatostatin (including the lowest dose 0.1 ng/ml, which had no effect on basal secretion of GH) partially (adenoma II cells) or completely (adenoma III cells) abolished stimulating effect of GHRP, GHRH, and their combination.

It should be noted that these findings are the first evidence that GHRP directly stimulates secretion of GH by human pituitary cells *in vitro*. This stimulation is specific for GH, since GHRP had no effect on the release of prolactin in GH or prolactin-producing mixed adenomas. In our *in vitro* experiments GHRP exhibited a pronounced GH-releasing activity; however, its activity standardized to molar concentration was lower than that of GHRH(1-29)NH_2. The latter is inconsistent with the data obtained on sheep pituitary cultures [15], where GHRP and GHRH exhibited similar activity. This discrepancy can be explained by species-specific differences or different culturing conditions, which probably were optimal for GHRH, but not GHRP. Experiments with rat pituitary cell cultures showed that the same cultural condition is optimal for GHRP-6 and not optimal for GHRH [12].

There is no consensus concerning direct effect of different GHRP and natural GHRH on pituitary cells. Many investigators demonstrated an additive effect of GHRP and GHRH in *in vitro* experiments [6,12,15], while others observed synergism in their action [9]. Our experiments clearly showed that these stimulators in combination exert a complimentary effect, which can be additive or slightly synergistic depending on the dose of GHRP and GHRH. This

TABLE 2. Effect of GHRH and GHRP on Secretion of Prolactin in Pituitary Adenoma II Cells Producing GH and Prolactin (1-h Incubation, $M\pm m$, $n=6$)

Peptide, ng/ml		Content of prolactin, ng/ml
Control		190±11
GHRH	1	191±13
	10	167±10
GHRP	1	178±12
	10	161±16
GHRH+GHRP	1+1	213±18
	10+10	195±26

implies that these peptides act on human pituitary through different receptors and postreceptory signal transduction pathways. The existence of specific receptors for GHRP arises the question on the existence of an GHRP-like endogenous ligand.

Since synergism between GHRP and GHRH *in vivo* is more pronounced than *in vitro*, it can be assumed that the effect of GHRP on the organism is realized through the hypothalamus. Acute effect of GHRP, GHRH, and their combination was assessed in children with various clinical forms of short stature [3]. In children with short stature and idiopathic GH deficiency both peptides induced pronounced and approximately equal effects, while the response to their combination 2-fold surpassed the sum of the reactions to individual peptides, which clearly attested to synergism of their effects. Thus, the high synergism of GHRP+GHRH *in vivo* cannot be explained only by interaction between these peptides at the level of the pituitary.

An important phenomenon observed by us is the blockade of GH-releasing effect of GHRP and GHRH by somatostatin in the concentration that had no effect on basal secretion of GH. Pronounced antisecretory effect of somatostatin on human pituitary adenoma cultures is observed only after 3- or even 24-h incubation [1,2]. In our experiments cells were exposed to GHRP and GHRH only for 1 h. Short incubation is probably responsible for low inhibitory effect of somatostatin (even in high doses) on basal secretion of GH. Dissociation between the effects of somatostatin on basal and stimulated secretion and its predominant effect of stimulated secretion can be due to complex mechanism of its action, which includes various intracellular events: cAMP-dependent, cAMP-independent, and Ca^{2+}-mediated reactions [11, 13]. It has been found that somatostatin induces hyperpolarization of plasma membrane in GH-producing pituitary adenoma cells, thus inactivating Ca^{2+} channels and reducing intracellular Ca^{2+} concentration [16,17]. Participation of ion channels and Ca^{2+}

in stimulation of GH secretion [7,8,15] suggests that suppression of GH-releasing effect of GHRP and GHRH in human pituitary adenoma cultures in the presence of somatostatin can result from hyperpolarization of their plasma membrane and inactivation of Ca^{2+} channels.

Thus, our findings suggest the presence of GHRP receptors and specific postreceptory signal mechanisms in human pituitary adenoma cells distinct from those for GHRH. This agrees with the hypothesis [4,5] that GH secretion in animals and men is regulated, apart from GHRH and somatostatin, by an unknown physiological factor similar to GH-releasing peptides.

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REFERENCES

1. A. A. Bulatov, I. S. Komolov, N. B. Smirnova, *et al., Byull. Eksp. Biol. Med.*, **113**, No. 4, 421-424 (1992).
2. A. A. Bulatov, A. V. Martynov, A. L. Grigoryan, *et al., Biokhimiya*, **60**, No. 10, 1251-1257 (1995).
3. A. N. Tuilpakov, A. A. Bulatov, V. A. Peterkova, *et al., Metabolism*, **44**, 1199-1204 (1995).
4. C. Y. Bowers, *J. Pediatr. Endocrinol.*, **6**, 21-31 (1993).
5. C. Y. Bowers, F. Momany, G. A. Reynolds, *et al., Endocrinology*, **106**, 663-667 (1980).
6. C. Y. Bowers, A. O. Sartor, G. A. Reynolds, and T. M. Badger, *Ibid.*, **128**, 2027-2035 (1991).
7. L. Bresson-Bepoldin, M. F. Odessa, and L. Dufy-Barbe, *Endocrine*, **2**, 793-803 (1994).
8. C. Chen, J. D. Vincent, and I. J. Clark, *Trends Endocrinol. Metab.*, **5**, 227-233 (1994).
9. K. Cheng, W. W.-S. Chan, A. Barreto, *et al., Endocrinology*, **124**, 2791-2798 (1989).
10. E. Ghigo, E. Arvat, L. Gianotti, *et al., J. Clin. Endocrinol. Metab.*, **78**, 693-698 (1994).
11. Y. C. Patel, In: *Somatostatin. Basic and Clinical Aspacts of Neuroscience*. Ed. C. Weil et al., Berlin (1992), Vol. 4, pp. 1-16 (1992).
12. O. Sartor, C. Y. Bowers, and D. Chang, *Endocrinology*, **116**, 952-057 (1985).
13. A. Schonbrunn, *Metabolism*, **39**, Suppl. 2, 96-100 (1990).
14. M. L. Vance, *Clin. Chem.*, **36**, 415-420 (1990).
15. D. Wu, C. Chen, K. Katoh, *et al., J. Endocrinol.*, **140**, R9-R13 (1994).
16. N. Yamashita, N. Shibuya, and E. Ogato, *Proc. Nat. Acad. Sci. USA*, **83**, 6198-6202 (1986).
17. N. Yamashita, K. Takano, A. Teramoto, *et al., Endocrinol. Jpn*, **39**, 481-49.