NOTE

Effect of Growth Hormone (GH)-Releasing Peptide (GHRP) on the Release of GH from Cultured Anterior Pituitary Cells in Cattle

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Abstract. The effect of His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂ (GHRP) on growth hormone (GH) release from cultured bovine anterior pituitary (AP) cells was studied *in vitro* with the interactive effects of GH-releasing factor (GRF: hpGRF (1–29)-NH₂) and somatostatin (SRIF). The AP cells (5×10^4 cells per well) were incubated with media, and the media were changed 3 days after plating. After 3.5 days in culture, cells were incubated for 2 h with the peptides. GHRP stimulated GH release from cultured cells in a dose-related manner. At doses from 10^{-11} to 10^{-7} M GHRP, the amount of GH released was significantly greater than the controls ($P < 0.05$ to $P < 0.001$). The amounts of GH released at lower doses of GHRP (10^{-14} to 10^{-12} M) were not significantly different from the controls. GH concentrations after treatment with 10^{-11} and 10^{-7} M GHRP were 3.98 ± 0.27 and 4.81 ± 0.16 ng/ml, respectively. In experiments performed similarly, the 10^{-7} M GHRH, GHRP, and combined treatment with GHRP plus GHRH increased GH 126, 57, and 139%, respectively ($P < 0.001$). The GH releasing effects of either GHRH alone or GHRP plus GHRH were significantly more potent than that of GHRP alone ($P < 0.001$). The additive effect was not significant when compared with GHRH alone. GH release induced by either GHRH or GHRP was significantly inhibited by SRIF ($P < 0.01$) compared with the untreated control. The inhibitory effect of SRIF in combined treatment with GHRP plus GHRH was significantly less than that of SRIF with GHRH or with GHRP ($P < 0.01$). The present study suggests that GH-releasing peptide (GHRP) induced GH release in cattle via a direct action on anterior pituitary cells *in vitro*.

Key words: Growth hormone, Growth hormone-releasing peptide, Growth hormone-releasing factor, Somatostatin, Pituitary cell

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SECRETION of growth hormone (GH) from pituitary somatotrophs is regulated by two neuroendocrine hormones, GH-releasing hormone (GHRH) and somatostatin (SRIF). These two hypothalamic peptides are released from nerve endings in the median eminence and reach the anterior pituitary via the hypophyseal-portal circulation [1–3]. More-

over, it has been reported that GHRH and SRIF regulate mitotic activities of GH-producing cells and that the effect of GHRH is possibly mediated by cyclic AMP [4]. A hexapeptide, His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂ (GHRP) which belongs to a series of synthetic GH-releasing pentapeptides and hexapeptides (GHRPs), has been shown to specifically stimulate GH release, both *in vivo* and *in vitro* in rats [5–8]. GHRPs were designed and derived from enkephalin through computer-modeling techniques [9]. Nonopioid receptors for GHRP have been identified in the hypothalamus and pituitary [10]. A previous study has demonstrated

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that GHRP synergizes with GHRH to increase GH release from rat pituitary cells [11]. It has been reported, however, that GHRP mediates its effect through a receptor and mechanisms which are distinct from those of GHRH [11]. Moreover, the effects of GHRP on GH release are at least partially mediated via the activation of protein kinase-C [12]. In cattle, only *in vivo* studies have shown that GHRP increased serum GH levels in steers [13], and the rate of milk production in cows [14]. The effect of GHRP on the release of bovine GH from pituitary cells *in vitro* has not yet been reported. The present study was designed to examine the effect of GHRP on the release of bovine GH with the interactive effects of GHRH and SRIF using cultured anterior pituitary cells *in vitro*.

Materials and Methods

Preparation of bovine anterior pituitary

The anterior pituitary glands were removed from 8 Holstein steers (17–18 months of age) at a local slaughterhouse and were transported to the laboratory in Krebs-Ringer bicarbonate solution to which was added 1.8 mg glucose/ml, 0.5 mg bacitracin/ml, 1.0 mg bovine serum albumin/ml, 0.1 mg streptomycin/ml and 100 IU penicillin/ml (modified KRB solution) at 5°C within 2 h. The pituitary glands were sagittally sectioned before being immersed in modified KRB solution.

Preparation and incubation of dispersed cells

Bovine anterior pituitary cell cultures were prepared as described previously by Vale *et al.* [15] as modified in our laboratory. Anterior pituitary (AP) tissues were diced into small pieces (less than 1 mm³) and the pieces were rinsed 4 times with sterile HEPES buffer (NaCl, 137 mM; KCl, 5 mM; Na₂HPO₄, 0.7 mM; n-2-hydroxyethyl piperazine ethanesulfonic acid (HEPES), 25 mM; glucose, 10 mM; CaCl₂ • 2 H₂O, 36 µM). The AP pieces were then placed in a siliconized glass vial with HEPES buffer containing 0.4% collagenase (219 units/mg, *Clostridium histolyticum*), and 1% bovine serum albumin (BSA-Fraction V). The AP tissues were incubated with agitation in a water bath incubator at 37°C for 45 min. The AP tissues were dispersed by gentle drawings in and out of a siliconized

pasteur pipette every 15 min throughout the entire procedure. After 45 min, the AP cell suspension was transferred to a centrifuge tube and centrifuged at 450 × g for 5 min. The supernatant was discarded and the cells were resuspended in Ca²⁺-free HEPES buffer containing 0.25% pancreatin (4 ×), and then returned to the water bath incubator and incubated for an additional 10 min. The cells were then resuspended with Dulbecco modified Eagle's medium (DMEM) containing 10% horse serum, 2.5% newborn calf serum, 1% MEM non-essential amino acids (100 ×), 100 IU/ml penicillin, and 50 µg/ml streptomycin. The resuspended cells were washed 4 times in the medium by repeated gentle agitation and centrifugation at 450 × g for 5 min. Following the final wash, the cells were counted and suspended in an appropriate volume of the plating medium to a concentration of 5 × 10⁴ cells per ml, and the cells were then plated in Corning 24-well dishes and grown for 3.5 days at 37°C in a humidified atmosphere of 5% CO₂. Total cell yield was more than 15.7 × 10⁶ cells per one AP. Cell viability as determined by trypan blue-exclusion was greater than 90%.

For studies of GH secretion using the cell cultures, media were changed 3 days after plating. After 3.5 days in culture, cells were washed 2 times and the media were replaced with fresh modified KRB solution containing the peptides to be tested; the cells were then incubated for 2 h as short-term secretion experiment. The effect of GHRP on bovine GH release was examined in time-courses of 15, 30, 60, and 120 min incubation and a significant GH release was observed only at 120 min as shown in Table 1. At the end of incubation the media

Table 1. Effect of short-term incubation length on GHRP-stimulated bovine GH release

Time (min)	GH in medium		
	Control (ng/ml)	GHRP (10 ⁻⁷ M)	
		ng/ml	Percent of control
15	2.62 ± 0.20	2.48 ± 0.11	94.7
30	2.05 ± 0.18	2.30 ± 0.36	112.2
60	2.15 ± 0.31	2.93 ± 0.65	136.3
120	2.04 ± 0.21	3.32 ± 0.15**	162.7

Each value represents the mean of five to eight determination ± SEM from two anterior pituitaries. Significant difference from the control is indicated by asterisks (** *P* < 0.01).

were removed from the cells and stored at -20°C until assayed for GH.

Peptides

The following peptides were used in this study: GHRH (hpGRF(1-29)-NH₂) (a generous gift from Dr. S. Ohashi, National Institute of Bioscience and Human Technology, Tsukuba, Japan), His-D-Trp-Ala-Trp-D-Phe-Lys-NH₂ (GHRP) was purchased from Peninsula laboratories (Belmont, CA, USA), and somatostatin (SRIF) from Peptide Institute Inc. (Osaka, Japan).

Radioimmunoassay (RIA)

Bovine GH concentrations in media were measured by a validated RIA method as described previously [16]. The GH standard preparation and hormone for iodination were bGH-B1. The hormone was supplied by the USDA Reproduction Laboratory, Beltsville, MD. Antiserum to bovine growth hormone (bGH) prepared in monkeys was supplied by Dr. T. Johke (National Institute of Animal Industry, Japan) and goat-monkey IgG serum

(2nd antibody) was supplied by Dr. K. Wakabayashi (Institute of Endocrinology Gunma University, Japan). In this assay, the displacement curve for increasing volumes of the bovine serum pool was parallel to the standard curve. Recovery of standard GH from a pool of bovine serum averaged $116 \pm 11\%$. The intra- and inter-assay coefficients of variation were 5.8% and 11.9%, respectively. The least detectable value was 1.97 ng/ml.

Statistical analysis

All data were presented as the mean $\pm$ SEM. The statistical significance of differences in GH concentrations were determined by Student's *t*-test after Bartlett's test for uniformity of the variances [17].

Results

Effects of GHRH and SRIF on GH release were examined to validate the procedures of bovine pituitary cell culture and dispersion. Fig. 1 shows that GHRH 10^{-7} M significantly stimulated GH release (29.6 ± 1.0 ng/ml, $P < 0.001$) compared with the controls (17.3 ± 0.9 ng/ml). The stimulatory effects of GHRH on GH were significantly inhibited by SRIF 10^{-7} M (17.6 ± 0.9 ng/ml, $P < 0.001$) compared with the GHRH alone.

As shown in Fig. 2, GHRP released GH in a dose-related manner from bovine pituitary cells. At doses from 10^{-11} to 10^{-7} M GHRP, GH levels in the media were much higher than in the controls ($P < 0.05$ to $P < 0.001$). The GH concentrations after treatments with GHRP 10^{-11} and 10^{-7} M were 3.98 ± 0.27 and 4.81 ± 0.16 ng/ml, respectively. The amounts of GH release in response to GHRP 10^{-12} to 10^{-7} M were in a dose-related manner as tested by means of a linear regression test ($P < 0.05$). GHRP at concentrations of 10^{-14} to 10^{-12} M failed to release any significant amount of GH.

The combined effect of GHRH and GHRP on GH release from bovine anterior pituitary cells is shown in Fig. 3. GHRH (10^{-7} M), GHRP (10^{-7} M), and combined treatment with GHRP (10^{-7} M) plus GHRH (10^{-7} M) stimulated GH release significantly ($P < 0.001$) compared with the controls. The rates of increase in GH of GHRH, GHRP, and GHRP plus GHRH were 126, 57, and 139%, respectively. The amounts of GH release induced by either GHRH alone ($226.0 \pm 7.7\%$) or GHRP plus

Fig. 1. Effect of SRIF on GHRH-stimulated GH release from cultured bovine anterior pituitary cells *in vitro*. Pituitary cells (5×10^4 per well) were cultured for 3.5 days, and then incubated with media containing GHRH (10^{-7} M) and GHRH plus SRIF (10^{-7} M) for 2 h. GH release is expressed as ng/ml medium. Each bar represents the mean of 11 to 12 determinations $\pm$ SEM from two anterior pituitaries a: Significantly different from b ($P < 0.001$).

Fig. 2. Effect of GHRP on the release of GH from cultured bovine anterior pituitary cells *in vitro*. The cells (5×10^4 per each well) were cultured for 3.5 days, and then incubated with media containing GHRP (10^{-14} to 10^{-7} M) for 2 h. GH release is expressed as ng/ml medium. Values represent the mean of 7 to 8 determinations $\pm$ SEM in two separate experiments. Significant differences from the control are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. 3. The combined effect of GHRP (10^{-7} M) and GHRH (10^{-7} M) on bovine GH release for 2 h *in vitro*. GH concentrations in the controls were averaged and the mean value was set at 100%. GH values were expressed as the percent of controls. Each value represents the mean of 12 determinations $\pm$ SEM from three anterior pituitaries. The different letters (a, b, c) on the bar denote significant differences ($P < 0.001$).

GHRH ($239.0 \pm 8.9\%$) were significantly greater than that by GHRP alone ($157.0 \pm 7.6\%$, $P < 0.001$); but the additive effect was not significant when compared with GHRH alone.

The effects of SRIF on GH secretions that were induced by GHRH, GHRP, and GHRP plus GHRH were investigated. As shown in Fig. 4, bovine GH releases induced by either GHRH or GHRP were significantly inhibited by 10^{-7} M SRIF compared with the untreated control group ($P < 0.001$). The inhibitory effect of SRIF in combined treatment with GHRP plus GHRH was significantly less than that of SRIF with either GHRH or GHRP ($P < 0.01$).

Discussion

The present study demonstrated that GHRP induced bovine GH release via a direct action on anterior pituitary cells *in vitro*. The effect of GHRP with the interactive effects of GHRH and SRIF on the release of GH from pituitary cells *in vitro* in cattle has not, as yet, been reported.

In the present study, we have shown that GHRP is able to stimulate GH release in a dose-related manner in cattle using anterior pituitary cells *in vitro*, and that GHRP-stimulated bovine GH releas-

Fig. 4. The combined effects of GHRH and GHRP with SRIF (10^{-7} M) on bovine GH release for 2 h of incubation *in vitro*. GH concentrations in the untreated control group were averaged and the mean value was set at 100%. GH values were expressed as the percent of controls. Each value represents the mean of 10 to 12 determinations $\pm$ SEM from three anterior pituitaries. The different letters (a, b) on the bar denote significant differences ($P < 0.01$).

ing effects could be seen at doses as low as 10^{-11} M. GHRP has been reported to release GH in a dose-response and specific manner in a number of different species, including the rat, sheep, calf, and chicken [6, 19]. The activity of GHRP in releasing GH was observed when administered directly into the gastrointestinal tract in rats, dogs and monkeys [27]. In man, GHRP infusion resulted in a selective and dose-dependent increase in serum GH concentrations [28]. Only in *in vivo* studies it was reported that GHRP stimulated GH release in steers [13], and increased serum GH levels and milk production in lactating dairy cattle [14]. In addition, GH release was induced by GHRP from rat pituitary cell cultures in a time-dependent manner at 0.5, 1, 2, 3 and 4 h [8]. GH release from rat pituitary cells induced by GHRP was observed after 15 min, but not after 4 h [11]. Concerning the direct action of GHRP in inducing GH release from cultured pituitary cells, the present results in cattle are in agreement with the previous reports in rats [6, 8, 11, 12, 21].

This study showed that GHRH (10^{-7} M) was significantly more potent than GHRP (10^{-7} M) in stimulating bovine GH release *in vitro*. The potency of the GHRH-stimulated GH releasing effect over GHRP in the present study coincided with reports of previous studies on rat pituitary incubates [8], or in the perfusion of dispersed anterior pituitary cells in male rats [20]. Moreover, the bovine GH releasing effects could be seen at doses as low as 10^{-11} M GHRH in perfused adenohypophysis as reported by Hashizume and Kanematsu [22], or at doses as low as 10^{-12} M GHRH from bovine pituitary cells [29]. The bovine GH releasing effects of combined treatment with GHRP and GHRH were more effective than that with GHRP alone. However, the additive effect of GHRP with GHRH was not clear when compared with GHRH alone. Cheng *et al.* [11] reported that GHRP and GHRH produced a synergistic effect on GH release in rat pituitary cell cultures whereas in three different experiments on the *in vitro* system in the rat, Bowers *et al.* [18], demonstrated that a synergistic effect of GHRP and GHRH on GH release is only small at best or nonexistent. But in the present study, only one dose (10^{-7} M) was used in the combined treatment with GHRP and GHRH. Further studies with various doses of these peptides are therefore necessary to clarify their synergistic effects on bovine GH release.

In regard to the mechanism by which GHRP stimulates GH release, evidence provided by previous studies in rats indicates that GHRP stimulates GH secretion from pituitary cells through a receptor and a mechanism which are different from those of GHRH [8, 11, 13]; and Cheng *et al.* [11] reported that this conclusion is based on the observation that a GHRH antagonist inhibited GHRH-stimulated GH release but had no effect on that induced by GHRP.

On the other hand, the present study indicated that the responses of bovine GH release to either GHRH or GHRP were inhibited by SRIF, and that the inhibitory effect of SRIF in combined treatment with GHRP and GHRH was significantly less than that of SRIF with either peptide alone. This could be an additive effect of GHRP and GHRH to release GH. SRIF inhibited the stimulation of GH responses to both GHRH and GHRP by perfused pituitary cells of adult male rats *in vitro* [18]. Moreover, GH secretion induced by GHRH, GHRP, and PACAP from rat pituitary cells was inhibited by SRIF *in vitro* [11, 23]. Furthermore, the inhibitory effect of SRIF on GHRH-induced GH secretion has been reported in *in vitro* studies in rats [2, 24], in chickens [25], and also *in vivo* in young bulls [26]. The present study is in agreement with the demonstration that GHRP may be a useful probe to use in investigating the GH secretory mechanisms as reported by Badger *et al.* [20], in their study on rat pituitary cells.

In conclusion, our results suggest that GHRP induced GH release in cattle via a direct action on anterior pituitary cells *in vitro*. Further studies on the combined effects of GHRP with other peptides are necessary.

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