The Time Course and Extracellular Ca$^{2+}$ Involvement of Growth Hormone (GH) Releasing Factor-induced GH Secretion in Perifused Dispersed Rat Pituitary Cells

Masakatsu KATO and Mitsuo SUZUKI

Department of Physiology, Institute of Endocrinology, Gunma University, Maebashi, Gunma, 371 Japan

Abstract  The time course of GH secretion in response to hpGRF and its dependency on the extracellular Ca$^{2+}$ concentration were studied in perifused dispersed anterior pituitary cells. The onset of GH secretion in response to 1 nM hpGRF was relatively rapid (within 5 s) but removal of hpGRF after 10-min application further increased the rate of secretion (off-response). The threshold and maximum concentrations of hpGRF in stimulatory secretion were $10^{-12}$ and $10^{-8}$ M respectively. Between these two concentrations, the responses showed dose dependency. A reduction in the extracellular Ca$^{2+}$ concentration to 0.25 mM or to nominally zero reduced hpGRF-induced GH secretion to 64.4% or to 1.9%, respectively, of the control response in the presence of 2.5 mM Ca$^{2+}$. Two mM Co$^{2+}$, known as a strong calcium channel blocker, completely suppressed hpGRF-induced GH secretion. The removal of Ca$^{2+}$ from the perifusion buffer immediately after the offset of 1 min-applied 1 nM hpGRF accelerated the falling phase of GH secretion, which is parallel to the decline in [Ca$^{2+}$]o in the perifusion chamber. Under nominal Ca$^{2+}$-free conditions, hpGRF produced no increase in GH secretion. However, 10 min after the offset of 1 min-applied hpGRF under Ca$^{2+}$-free conditions, the introduction of normal buffer containing 2.5 mM Ca$^{2+}$ substantially restored GH secretion, although after 20 min the introduction of normal buffer produced only a slight increase in GH secretion. In perifusion experiment of 10$^6$ cells, intracellular cyclic AMP (cAMP) content was raised from the basal value of 4 to 26 pmol by 2-min application of 1 nM hpGRF. After cessation of hpGRF application, cAMP content decreased to 8.7 pmol at 11 min and returned to the basal value by 20 min. The same tendency was observed in Ca$^{2+}$-free buffer. In conclusion, the extracellular Ca$^{2+}$ was essential for hpGRF-induced GH secretion. This indicates the importance of the influx of Ca$^{2+}$ in response to hpGRF. The time course of hpGRF-induced rise and fall in cAMP content was roughly parallel to the GH secretion. The possible explanations of the off-response and the re-
storation of GH secretion by reintroducing normal buffer were discussed.

Key words: extracellular calcium ion, anterior pituitary cells, perifusion, growth hormone, growth hormone releasing factor.

The growth hormone (GH) release from somatotrophs of the adenohypophysis is mainly regulated by two hypothalamic peptides, somatostatin (SRIF), and growth hormone releasing factor (GRF) (Arimura and Culler, 1985; Jansson et al., 1985). The first GH-specific peptide isolated was an inhibitory one, SRIF (Brazeau et al., 1973). The stimulatory peptide GRF was purified and characterized from human pancreatic tumor (Guillemin et al., 1982; Rivier et al., 1982). We used synthetic human pancreatic GRF-44 (hpGRF) in the experiments presented here.

hpGRF stimulates both the cAMP production and GH secretion in the in vitro static incubation method and SRIF inhibits both processes. Moreover, without affecting cAMP production verapamil inhibits GH release in response to hpGRF, suggesting that cAMP plays a role as an intracellular mediator of GRF action in somatotrophs and that Ca\(^{2+}\) is required for the release process (Bilezikjian and Vale, 1983). An adenylate cyclase dually regulated by SRIF and hpGRF is found in normal anterior pituitary cells and in human GH-secreting pituitary adenomas (Spada et al., 1984). Thus, the adenylate cyclase system seems to be a major intracellular machinery for the hpGRF-induced GH release process. On the other hand, pulsatile GH secretion following bolus injections of hpGRF and the desensitization of somatotrophs caused by continuous infusion of hpGRF were observed (Badger et al., 1984), but the precise time course of GH secretion in response to hpGRF was not studied.

The involvement of calcium in the release process is generally accepted, and there are two main theories concerning the source of calcium. One is the theory of the stimulus-secretion coupling, which stated that the influx of Ca\(^{2+}\) from the extracellular space was important in the release process (Douglas, 1968). The other is the theory that the concentration of intracellular Ca\(^{2+}\) is raised by mobilizing intracellular stored calcium. The latter was formulated in the case of thyrotropin releasing hormone (TRH) action on GH\(_3\) cells (Gershengorn, 1985) in which inositol 1,4,5-tris phosphate, a hydrolytic product of phosphatidilinositol 4,5-bisphosphate, mobilizes endoplasmic reticulum-bound calcium.

In the present series of experiments, the relatively fine time course of GH secretion in response to hpGRF and the involvement of calcium and cAMP in that process were studied. We report here that the GH release and the rise in cAMP content are initiated within 5 s in response to hpGRF and that the influx of Ca\(^{2+}\), rather than the mobilization of intracellular stored calcium, may be essential in the hpGRF-induced GH secretion from the perifused dispersed anterior pituitary cells. 

*Japanese Journal of Physiology*
METHODS

Animals. Male Wistar rats (Imai Animal Farm, Saitama, Japan), weighing 180–220 g, were maintained under conditions of controlled light (light: 0700–
1,900 h) and temperature (25 ± 2°C). Food and water were available ad libitum.

Mediums for perfusion. The perifusion medium A was prepared aseptically
with Eagle’s Minimum Essential Medium (Nissui Pharmaceutical Co., Ltd., Tokyo,
Japan) by adding L-glutamine (2 mM), 0.25% bovine serum albumin (Fraction V,
Boehringer Mannheim, W. Germany), and HEPES (10 mM, Dojin Chemical
Laboratory, Kumamoto, Japan) to adjust to pH 7.4.

Normal perifusion medium B contains 137.5 mM NaCl, 5 mM KCl, 2.5 mM
CaCl₂, 0.8 mM MgCl₂, 10 mM glucose, 10 mM HEPES (pH adjusted to 7.4 by 4 N
NaOH), and 0.25% bovine serum albumin. Ca²⁺-free medium was prepared by
removing CaCl₂ from normal medium B, so that 10–20 μM Ca²⁺ was contained. An
excess K⁺ solution was made by adding KCl and removing the same amount of
NaCl from normal medium B. Perifusion medium was sterilized by 0.45-μm
filtration and oxygenated just before and during the perifusion experiment. As a
secretagogue, human pancreatic growth hormone releasing factor (hpGRF-44,
Peptide Institute Inc., Osaka, Japan) was used.

Preparation. Dispersion of adenohypophysial cells was performed (INOUE et
al., 1985). The perifusion system (Fig. 1, inset) was a modification of that previously
described (LOUGHLIN et al., 1981; EVANS et al., 1983).

The animals were anesthetized with pentobarbital (50 mg/kg body weight) and
perfused transcardially first with 25 ml of phosphate buffered saline without Ca²⁺
and Mg²⁺ (PBS), then with 15 ml of 0.5% trypsin (Sigma, bovine pancreas, Type
III) in PBS. The anterior pituitary glands were then harvested from the animals and
placed in 0.5% trypsin solution at room temperature. Trypsinized pituitaries were
mechanically dispersed by pipetting, incubated with 0.5 ml DNase (Sigma, de-
oxiribonuclease I, 5 μg·ml⁻¹) for 5 min, then further incubated with 0.5 ml of
trypsin inhibitor (Sigma, type II, 2 μg·ml⁻¹). After pipetting, cell suspensions were
centrifuged for 5 min at 400 g and the precipitates were resuspended in perifusion
medium A. The numbers of viable cells were determined by counting those cells
excluding 0.4% trypan blue in a hemocytometer. The cell viability was more than
95% throughout the experiments. The cells were mixed with preswollen Bio-Gel P2
(Bio-Rad Laboratory, Calif. U.S.A., 200–400 mesh) and aliquoted into columns
(0.9 cm in diameter). Each column contained 1.5–2 pituitaries with a packed bed
volume of 0.5 ml and a head volume of 100 μl. The numbers of cells per column in
each group were equalized and are shown in the figure legends. The columns were
connected to a peristaltic pump and continuously perifused with medium A for the
first 3 h, and then with medium B for the next 1 h. The flow rate was 1.2 ml·min⁻¹
and the fraction was collected over periods of 5 s to 10 min, so that the maximum
time resolution of the system was 5 s. Figure 1 shows the time course of the
concentration change in perifusion column, when phenol red solution was applied.
M. KATO and M. SUZUKI

for the first 1 min. No phenol red was detected in the effluent fractions for the first 1 min and the maximum concentration was obtained within 2 min. Then the concentration gradually declined and the phenol red had almost disappeared by 4 min. Thus, any change in the perifusion medium, i.e., drug application or a change in ionic composition, followed the time course shown in Fig. 1.

Radioimmunoassay. Rat GH in the effluent fractions was determined with a double-antibody radio-immunoassay method using materials supplied by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (KATO et al., 1986). Purified rat GH (rGH-I-5) was used for radiolabeling with $^{125}$I and rGH-RP-2 as the reference standard. Separation of antibody-bound and free hormones was accomplished with goat antimonkey immunoglobulin G (IgG). Intracellular cAMP was extracted by adding 0.25 ml of 1.5 N perchloric acid and neutralized by KOH. After centrifugation at 1,500 g for 15 min, the supernatants were assayed for cAMP with a radioimmunoassay kit (Yamasa Shoyu Co., Choshi, Japan) (HATTORI and WAKABAYASHI, 1983).

Student’s $t$-test was used for a statistical analysis. When the probability of error was less than 0.05, the difference was considered to be statistically significant.

RESULTS

Dose response relationship of hpGRF

Various concentrations of hpGRF applied for the first 1 min increased the GH secretion in a dose dependent manner (Fig. 2). The time course of the increase in the GH secretion paralleled that of the concentration change in hpGRF. The peak GH secretion was, however, observed by 3.5 min for the applied hpGRF concentration.

Fig. 1. The dynamic profile of the perifusion system and its schematic drawing (inset). Phenol red was applied for the first 1 min and the effluent fractions were collected over 0.5 min for 30 min with a flow rate of 1.2 ml min$^{-1}$. No phenol red was detected for the first 1 min. The maximum absorbance was obtained by 2 min. The phenol red concentration declined rapidly and had reached zero, by 4 min. The perifusion chamber was constructed with a 2.5-ml plastic syringe.
up to $10^{-10}$ M and by 4.5 min for $10^{-8}$ M. As shown in Fig. 1, the concentration of hpGRF in the column is thought to reach its maximum by 2 min and to decline almost to zero by 4.5 min. Even after 5 min, at which time no hpGRF remained in the column, the GH secretion was still high and this was prominent at higher doses of hpGRF. The total increases in GH secretion due to a 1-min application of hpGRF were calculated for the collection period of 30 min (Fig. 3).

Figure 4 shows the responses for the 10-min applications of 1 nM hpGRF and of 30 mM KCl. As in the case of 1-min application, the change in the phenol red concentrations should reflect concentration changes of either hpGRF or KCl. The increase in GH secretion was parallel to the concentration change in hpGRF in the column. No apparent latencies were observed with this 5 s time resolution. In a static incubation of $10^6$ cells, 1 nM hpGRF raised cAMP content from 4.8 ± 0.3 pmol (mean ± S.E.) to 6.7 ± 0.7 pmol within 5 s. By 3.5 min after the onset of hpGRF application, the rate of GH secretion slowed down as the concentration of hpGRF in the column reached a plateau, although the GH secretion gradually increased during this plateau period, which continued by 11 min. The hpGRF concentration started to decline at 11 min and had reached zero by 13 min. The GH secretion, however, rapidly increased during this period (11–13 min) and reached its maximum by 13 min at which time no hpGRF remained in the column. Thus hpGRF elicited not only on-response but also prominent off-response. Both on- and off-responses in GH release probably occurred when the various concentrations of hpGRF were applied for 1 min as shown in Fig. 2. If so, the delay in the appearance of the peak in GH release induced by a 1-min application of hpGRF.
could be explained by this off-response. Perifusion with excess K⁺ medium (30 mM KCl) also induced GH secretion. The rising phase of GH secretion paralleled the increase in the K⁺ concentration and the secretion reached the maximum by 2 min. Even when the KCl concentration in the column remained constant, the GH secretion began to decline (Fig. 4). The falling phase after 11 min was parallel to the change in the K⁺ concentration in the column.

**Extracellular Ca²⁺ and GH release**

GH secretions induced both by 1 nM hpGRF and by 30 mM KCl were dependent on extracellular Ca²⁺. Figure 5 shows the responses induced by hpGRF. One nM hpGRF failed to elicit the GH release in Ca²⁺-free medium. Extracellular Ca²⁺ was essential for both on- and off-responses. A higher concentration of extracellular Ca²⁺ (10 mM) also suppressed hpGRF-induced GH release possibly by its binding effect on the negative surface charges of the cell membrane (KIDOKORO et
Fig. 4. GH secretion induced by 1 nM hpGRF or by 30 mM KCl in 10-min application. The secretagogue was applied for the first 10 min, indicated by downward and upward arrows. Phenol red concentration was determined by absorbance and is shown as a normalized value. Data points with error bars represent the means and the S.E.'s of the mean (n = 4). In the case of the phenol red, the S.E. was too small to show here. Each column contained 1.5 x 10^6 cells. The time scale is expanded between 1 and 2 min. When phenol red absorbance starts to rise at 1.2 min (5th closed square from the left), the augmentation of GH secretion also begins (5th closed circle from the left).

Fig. 5. The effect of the extracellular Ca^{2+} concentration on hpGRF-induced GH secretion. One nM hpGRF was applied between downward and upward arrows. There was a 30-min equilibration period before the application of hpGRF for each medium. In the case of 0.0 mM Ca^{2+}, the perfusion medium contained 10-20 μM Ca^{2+} since no chelating agents were added. Data points represent the means for 3 samples. Each column contained 10^6 cells.
al., 1979; Sorimachi et al., 1986). The same tendency was observed in the case of 30 mM KCl (Fig. 6). Figure 7 shows integrated values for GH induced by 1 nM hpGRF (open column) and by 30 mM KCl (hatched column). With 2.5 mM Ca\(^{2+}\) in the perifusion medium (control), 1 min-application of 1 nM hpGRF induced the release of 220 ng GH in 30 min. One tenth the amount of Ca\(^{2+}\) (0.25 mM) reduced GH release to 141.8 ng/30 min (64.4% of control) and in Ca\(^{2+}\)-free medium no actual response took place. With 30 mM KCl stimulation, the GH release for 5 min was 31.1 (2.5 mM Ca\(^{2+}\)), 10.3 (0.25 mM Ca\(^{2+}\)), and 0 ng (0 mM Ca\(^{2+}\)).

Two mM Co\(^{2+}\), which is known as a strong Ca\(^{2+}\) channel blocker, also suppressed GH release induced by 1 nM hpGRF (Fig. 8). In this case, Co\(^{2+}\) suppressed not only hpGRF-induced GH release but also basal release of GH.

In order to study the timing of Ca\(^{2+}\) involvement in hpGRF-induced GH secretion, two separate experiments were carried out. First, when the perifusion medium was changed to Ca\(^{2+}\)-free medium immediately after the 1-min application of 1 nM hpGRF, the falling phase of GH secretion was much steeper than that of the control (Fig. 9). The reductions in GH secretion were prominent from 3.5 min till 10 min. Since it took at least 2 min for the change of perifusion medium in the chamber to occur, the time course of the reduction in GH secretion was parallel to that of removal of Ca\(^{2+}\) from the perifusion column. The results of the second experiment are shown in Fig. 10. In Ca\(^{2+}\)-free medium, 1 nM hpGRF was applied for 1 min, and then after 10 min, normal medium containing 2.5 mM Ca\(^{2+}\) was introduced. This introduction of Ca\(^{2+}\) rapidly increased GH secretion for a period of 3 min. The time to the peak of this response was 2 min. Another thing to note here is the timing of Ca\(^{2+}\) reintroduction. Ca\(^{2+}\) reintroduction produced substantial increase in GH secretion at 11 min period around which time GH secretion by 1 nM hpGRF in the presence of Ca\(^{2+}\) was reduced to a low level. However, at 20 min period only a weak response was produced by the introduction of Ca\(^{2+}\).

In a same perifusion experiment of 10\(^6\) cells, 2 min-applied 1 nM hpGRF raised

---

**Fig. 6.** The effect of the extracellular Ca\(^{2+}\) concentration on 30 mM KCl-induced GH secretion. Excess K\(^+\) was applied between downward and upward arrows. Conditions and experimental procedures are the same as for Fig. 5.
in intracellular cAMP content from 3.9 ± 0.2 pmol (mean ± S.E.) at 0 min to 25.5 ± 1.5 pmol at 2.5 min and even at 11 min cAMP content showed 8.7 ± 0.5 pmol in normal medium (Fig. 11), then cAMP content returned to the basal value at 20 min (data not shown). A similar tendency was observed in Ca\(^{2+}\)-free medium, although cAMP content at 2.5 min was higher (39.7 ± 1.6 pmol) than that in normal medium (Fig. 11).
The time course of GH secretion in response to hpGRF and its dependence on the extracellular Ca^{2+} concentration were studied in perfused dispersed anterior pituitary cells. Since the proportion of somatotrophs in the male rat anterior pituitary is more than 50\% (Surks and Defesi, 1977), the use of purified somatotrophs would be unnecessary for these experiments. The dose dependence of GH secretion in response to hpGRF showed a similarity to the results of the static incubation experiment (Bilezikjian and Vale, 1983). The threshold concentration of hpGRF was 10^{-12} M and its maximum concentration was 10^{-8} M. The time course of GH secretion and the rise of cAMP content in response to hpGRF were relatively rapid (within 5 s) as shown in Figs. 2 and 4.

The first question is whether extracellular Ca^{2+}, i.e., the influx of Ca^{2+}, is essential for hpGRF-induced GH secretion. In excess K^+ -stimulated GH secretion, the importance of the Ca^{2+} influx is relatively easy to accept if the involvement of voltage-dependent calcium channels is supposed. The 30 mM KCl-stimulated GH secretion depends on the concentration of extracellular Ca^{2+} (Fig. 6). Ten times less Ca^{2+} (0.25 mM) produced 33.3\% of the control response, and in nominal Ca^{2+}-free medium no substantial increase in GH secretion took place. A similar tendency was
observed in hpGRF-induced GH secretion on the change in the extracellular Ca\(^{2+}\) concentration. Since there was a 30 min equilibration period, the intracellular calcium might be diffused away from the cells under Ca\(^{2+}\)-free condition. In order to exclude this possibility, the equilibration period was shortened to 5 min but no substantial changes occurred in the suppression of the hpGRF-induced GH secretion (data not shown). Furthermore, a strong calcium channel blocker, Co\(^{2+}\), completely suppressed the hpGRF-induced GH secretion (Fig. 8). Thus the extracellular Ca\(^{2+}\), i.e., influx of Ca\(^{2+}\), seems to be essential for the GH secretion in response to hpGRF. There is, however, still another possibility: Ca\(^{2+}\), which enters from the extracellular space, mobilizes calcium stored in intracellular organelles, i.e., endoplasmic reticulum. As shown in Fig. 9, the removal of Ca\(^{2+}\) after the offset of hpGRF did reduce the GH secretion, especially in its falling phase. This observation does not accord with the possibility of the triggering action of Ca\(^{2+}\) entered from the extracellular space. Besides on-response, hpGRF elicited prominent off-response i.e., the increase in GH secretion after the offset of its application (Fig. 4). This off-response also required extracellular Ca\(^{2+}\) since the removal of extracellular Ca\(^{2+}\) or addition of Ca\(^{2+}\) antagonist Co\(^{2+}\) completely suppressed this response. Similar “off” response was observed in mouse pancreatic β-cells by application of theophylline with 7 mM glucose which was blocked by Ca\(^{2+}\).
antagonist D600 (Henquín and Meissner, 1984). Moreover, the \(\beta\)-cells were depolarized by theophylline and the greater depolarization took place after the offset of theophylline. Thus the "off" response in \(\beta\)-cells is probably caused by a transient increase of \(\text{Ca}^{2+}\) influx (Henquín and Meissner, 1984). Although the mechanism of off-response in somatotrophs remains to be elucidated, the regulation of \(\text{Ca}^{2+}\) influx seems to be an important point.

Figure 10 showed the relatively long life of hpGRF-activated \(\text{Ca}^{2+}\) channels especially under \(\text{Ca}^{2+}\)-free condition. This experiment showed that, without extracellular \(\text{Ca}^{2+}\), hpGRF stimulates cAMP production (Fig. 11; Barinaga et al., 1985) and probably activates calcium channels. This augmented level of cAMP gradually decreased but at 11 min period it was still higher than the basal level (Fig. 11), at which time the introduction of \(\text{Ca}^{2+}\) into the perfusion medium elicited substantial GH release (Fig. 10). But only a small increment of GH secretion occurred by introducing \(\text{Ca}^{2+}\) at 20 min period, at which time cellular cAMP level returned to the basal value. Both cAMP and extracellular \(\text{Ca}^{2+}\) seem to be necessary for hpGRF-induced GH secretion (Kraicer and Chow, 1982; Bilezikjian and Vale, 1983), although the mechanisms of \(\text{Ca}^{2+}\) and cAMP actions.

**Fig. 10.** The restoration of GH secretion by introducing 2.5 mM \(\text{Ca}^{2+}\) after 1 min-application of 1 nM hpGRF under \(\text{Ca}^{2+}\)-free conditions. \(\text{Ca}^{2+}\)-free medium was introduced at 30 min before the hpGRF application. Ten min after the offset of hpGRF (upward arrow), normal medium, which contains 2.5 mM \(\text{Ca}^{2+}\), was reintroduced (downward arrow at 11 min) which restored substantial GH secretion. After 20 min of hpGRF application, reintroduction of 2.5 mM \(\text{Ca}^{2+}\) (downward arrow at 20 min) produced a slight increase in GH secretion. Data points with error bars represent the means and S.E.'s of the mean (\(n=4\)). Each column contained \(2.5 \times 10^6\) cells. *\(p<0.05\), **\(p<0.02\), ***\(p<0.001\) vs. basal value.
GH secretion and extracellular Ca\(^{2+}\) in hpGRF-induced GH secretion remain to be solved. Moreover in the present experiments two processes, i.e., the interaction between hpGRF and its receptor and GH secretion, were separated. This kind of manipulation may help in studying the secretory process per se and the mechanism of drug actions, e.g., the effect of SRIF. In order to further clarify the mechanism of GH secretion, biochemical and electrophysiological approaches (Israel, 1983; Ozawa, 1985) are necessary in combination with the perifusion experiment.

In conclusion, the time course of GH secretion and the rise in cAMP content in response to hpGRF were relatively rapid, i.e., within 5 s, and extracellular Ca\(^{2+}\) was essential for hpGRF-induced GH secretion.

The authors wish to thank Dr. A. F. Parlow of the NIADDK, the U.S. National Hormone and Pituitary Program for providing rGH RIA kits. The authors are also grateful to Drs. K. Wakabayashi and M. Hattori for their assistance in radioimmunoassay and to Dr. K. Sekimoto for his help in measuring calcium concentration. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by the Foundation for Growth Science (59570072 and 60570076).

Vol. 36, No. 6, 1986
REFERENCES


Japanese Journal of Physiology
GH SECRETION AND EXTRACELLULAR Ca\(^{2+}\)

adenosine 3',5'-monophosphate, and somatostatin. *Endocrinology*, **111**: 1173–1180.


