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Growth hormone-releasing hormone stimulates cAMP release in superfused rat pituitary cells

(somatostatin/desensitization/mechanism of action of growth hormone–releasing hormone)

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ABSTRACT **The release of growth hormone (GH) and cAMP was studied in superfused rat pituitary cells by infusing growth hormone-releasing hormone (GHRH) at different doses or a combination of GHRH and somatostatin 14 (SS-14). Three-minute pulses of GHRH caused a dose-dependent GH and cAMP release (effective concentration of 50% of the maximal biological effect is 0.21 nM and 52.5 nM, respectively). The lowest effective doses of GHRH in the superfusion system were 0.03 nM for GH release and 0.3 nM for cAMP discharge when 3-min pulses were applied. The amount of cAMP liberated from the cells was not proportional to GH release: cAMP responses to low doses of GHRH were disproportionally small, and the gradual increase in the release of cAMP after high doses of GHRH was not followed by a parallel rise in GH release. The desensitization induced by repeated pulses or prolonged infusion of GHRH resulted in a greater reduction in GH release than in cAMP liberation. A simultaneous infusion of SS-14 completely blocked GH release stimulated by GHRH but did not inhibit the immediate release of cAMP caused by GHRH. An abrupt decrease in GHRH-stimulated GH release induced by SS-14 was followed by only a minimal reduction in cAMP liberation 9 min later. Our findings indicate that a discharge of cAMP is stimulated after a GHRH pulse, but this effect alone cannot maintain the release of GH. Other steps of the signal transduction mechanisms that are independent of the cAMP route may participate in the process of GH release. The nature of the mechanisms involved in the mediation of GH release may vary with the doses of GHRH used.**

The secretion of growth hormone (GH) from the somatotrophs of the pituitary gland is controlled primarily by two hypothalamic peptide hormones, growth hormone-releasing hormone (GHRH) and somatostatin 14 (SS-14). GHRH not only stimulates the secretion of GH from the anterior pituitary, but it also stimulates adenylate cyclase activity and cAMP production. Numerous studies support the hypothesis that the effect of GHRH is mediated by cAMP (1–4). Previous investigations on the role of cAMP in the GHRH-induced GH release were carried out in static incubation systems by using freshly dispersed or cultured pituitary cells. In most cases only the intracellular changes in cAMP were detected at different intervals after stimulation. Extracellular discharge of cAMP was determined in a few cases (4–6); however, the release of cAMP in a dynamic system has not been previously examined in parallel with GH release. The aim of our study was to investigate the time course of both GH and cAMP secretion in a superfusion system after infusing GHRH or a combination of GHRH and SS-14 at different doses.

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MATERIALS AND METHODS

Peptides. Human GHRH-(1–29)NH_2 (DBO-2-048-1) was synthesized and purified in our laboratory. SS-14 was obtained from Wyeth.

Superfusion. The preparation of the cells and the superfusion system have been described (7, 8). Two or three columns, each containing pituitary cells from three male Sprague–Dawley rats (180–220 g) were perfused simultaneously. Sample collection was started immediately with 9-min samples in the first 90 min of the experiments and 3-min samples, thereafter. The flow rate was 20 ml/h. The test samples were prepared from the stock solutions immediately before use. The void volume of the system was calibrated to 1 ml. The cells were stimulated with 3-min pulses of GHRH (from 0.01 nM to 10 μM). The desensitizing effect of GHRH was examined during 2-h infusions of 0.1 nM or 1 nM GHRH. To avoid the oxidation of GHRH in the tissue culture medium, test samples were freshly diluted every 15 min during the 2-h period. The results are based on 10 independent experiments, and similar experimental designs were repeated 2–5 times.

RIAs. GH was determined by RIA with materials provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (ratGH-RP-2/AFP-3910B/, ratGH-I-6/AFP-5676B/, and anti-ratGH-RIA-5/AFP-411S/). For cAMP determination, the samples were acetylated with triethylamine/acetic anhydride (2:1; 25 μl per 500 μl of sample), and 2′-*O*-monosuccinyl-cAMP tyrosyl methyl ester (Sigma) was used for the iodination. The antiserum for cAMP was obtained from the NHPP (CV-27). cAMP from Sigma was used as the standard.

Data Analysis. GH and cAMP releases were analyzed by a special computer program (7).

RESULTS

Three-min pulses of GHRH caused a dose-dependent GH release from the superfused rat pituitary cells [effective concentration causing 50% of the maximal biological effect (EC_{50}), 0.21 nM; Fig. 1]. The lowest dose of GHRH that released significant amounts of GH was 0.03 nM. When increasing doses of GHRH were applied, the maximal response was obtained with 1 nM GHRH. Augmenting the doses of GHRH increased the duration of the GH response but decreased its amplitude. We also observed a dose-dependent reduction in the amount of GH released during the first 6 min of the responses (Fig. 2).

Parallel determinations of cAMP showed that low doses of GHRH that liberated clearly detectable amounts of GH did

Abbreviations: GH, growth hormone; GHRH, growth hormone-releasing hormone; SS-14, somatostatin 14; EC_{50}, effective concentration causing 50% of the maximal biological effect.

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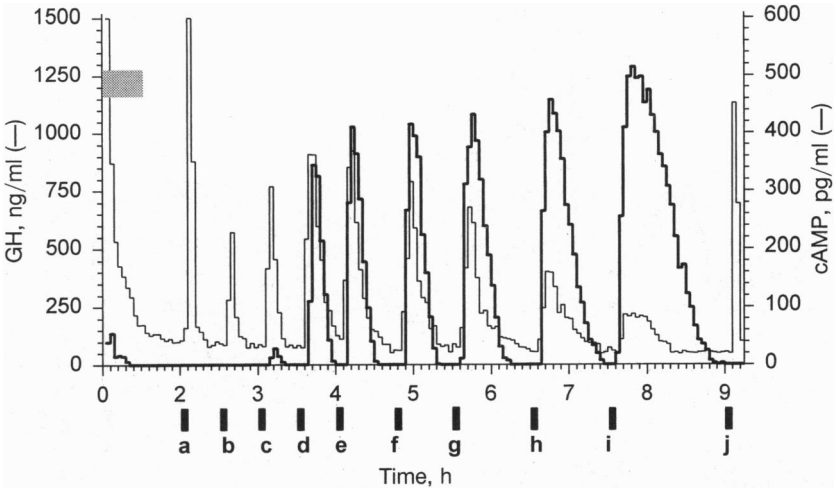

FIG. 1. GH (thin line) and cAMP (thick line) secretion after stimulation with increasing doses of GHRH. Shaded box shows 9-min samples collected in the first 90 min of the superfusion. Vertical filled bars below the abscissa represent stimulation for 3 min with the following substances: a and j, 50 mM KCl; b, 0.1 nM GHRH; c, 0.33 nM GHRH; d, 1 nM GHRH; e, 3.3 nM GHRH; f, 10 nM GHRH; g, 33 nM GHRH; h, 100 nM GHRH; and i, 1 μM GHRH.

not release cAMP from the cells. The lowest effective dose for cAMP release was 0.3 nM, when 3-min pulses of GHRH were applied. The EC_{50} of GHRH for total cAMP release was 52.5 nM. The greatest cAMP release during the first 6 min of the response was triggered by 3 nM GHRH. Higher doses of GHRH released slightly less cAMP in the first 6 min; however, the decrease was not as marked as that seen in the GH responses, and total cAMP response was continuously amplified as increasing doses of GHRH were infused.

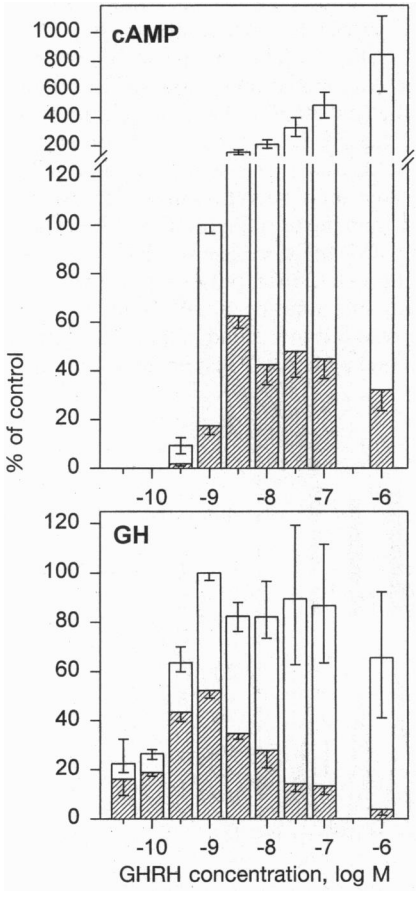

FIG. 2. cAMP and GH release after 3-min pulses of GHRH at different doses. cAMP and GH values were compared to the respective cAMP and GH responses to 1 nM GHRH, which was set at 100%. Hatched areas represent cAMP and GH secreted in the first 6 min of the response. Positive error bars show SEM of the total responses. Negative error bars show SEM of the secretion in the first 6 min on hatched areas and from min 7 to the end of the response in empty areas.

By infusing 1 μM or 10 μM doses of GHRH immediately after a single pulse of 1 nM GHRH, we found that the response was prolonged, and the total GH release was 5–5.5 times greater than that liberated by the single 1 nM dose (Figs. 3 and 4). As in our other experiments, where infusions of 0.3–100 nM GHRH preceded the 1 μM pulse, the immediate GH response decreased within the first 6 min of the peak. GH responses to subsequent 1 nM and 10 μM GHRH pulses showed definite signs of desensitization. Analysis of cAMP release in these experiments showed that the total peak area of the response to 10 μM GHRH did not increase substantially compared with that elicited by 1 μM dose, but the cAMP release was prolonged and its amplitude was lower. Although the subsequent responses to 1 nM GHRH were not reduced, the 10 μM dose of GHRH induced the release of less cAMP than the first application of 1 μM or 10 μM GHRH.

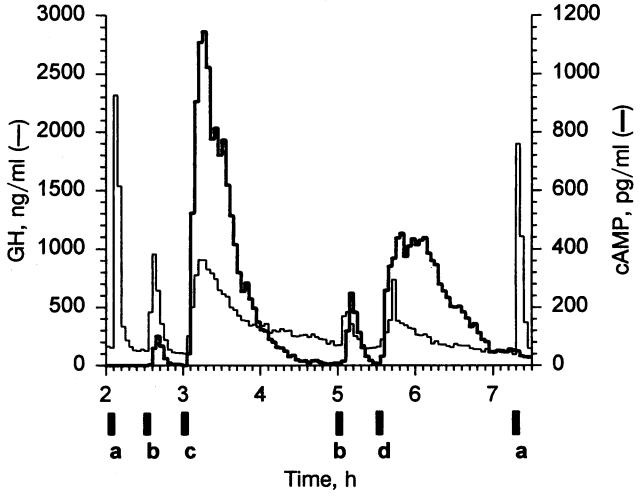

FIG. 3. GH (thin line) and cAMP (thick line) responses to 1 μM GHRH after one pulse of 1 nM GHRH. Vertical filled bars below the abscissa represent 3-min stimulation with the following substances: a, 50 mM KCl; b, 1 nM GHRH; c, 1 μM GHRH; and d, 10 μM GHRH.

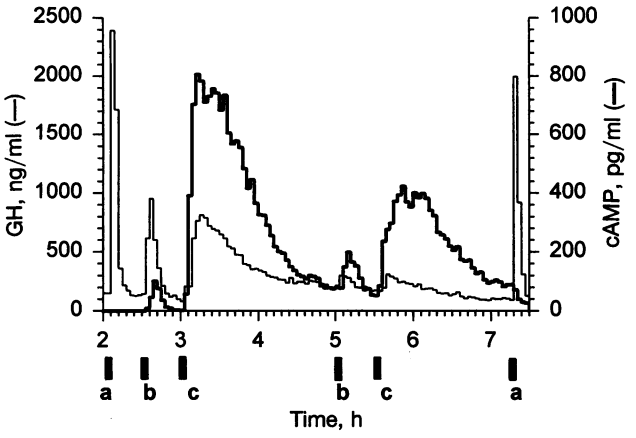

FIG. 4. GH (thin line) and cAMP (thick line) responses to 10 μM GHRH after one pulse of 1 nM GHRH. Vertical filled bars below the abscissa represent 3-min stimulation with the following substances: a, 50 mM KCl; b, 1 nM GHRH; and c, 10 μM GHRH.

Fig. 5 shows an experiment in which repeated 3-min infusions of 1 nM GHRH were applied. Compared with the first response, a subsequent decrease in the GH responses was found in the course of this experiment. However, the cAMP responses did not mimic this decrease and were maintained at a relatively stable level (Fig. 5, *Inset*). When 1 nM GHRH and 1 nM SS-14 were infused simultaneously for 3 min, followed by an additional 6-min infusion with SS-14 alone, the GH response was delayed by 9 min, i.e., for the total duration of the SS-14 infusion (Fig. 5, peak c); however, the cAMP response was not delayed. After coinfusion of 1 nM GHRH and SS-14 for 15 min, the GH response was postponed for 15 min, but cAMP release was already detected during the infusion (Fig. 5, peak d).

The inhibitory effect of SS-14 on cAMP release can be clearly seen in Fig. 6. During a 27-min infusion of 1 nM GHRH, SS-14 was coinfused from min 10 to min 18. The GH response was completely and abruptly blocked during the SS-14 infusion. When the SS-14 was washed from the system, an immediate rebound in the release of GH was observed. The secretion of cAMP was also reduced by the SS-14 infusion; however, this decrease was minimal and followed the decline in GH levels with only a 9-min delay.

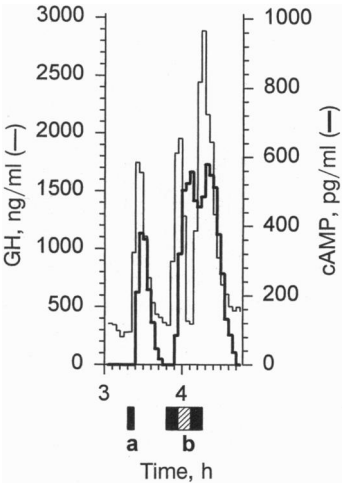

FIG. 6. Effect of SS-14 on GHRH-induced GH (thin line) and cAMP (thick line) release. a, 1 nM GHRH for 3 min; b, 1 nM GHRH for 27 min; 1 nM SS-14 was coinfused from the 10th to the 18th min (hatched area).

Signs of desensitization induced by prolonged GHRH infusion could already be observed during the treatment. After an initial high-amplitude GH response, the GH release declined and returned to nearly basal levels when 1 nM GHRH was applied for 2 h (Fig. 7). The amount of GH released in the last 30 min of the 2-h incubation period was less than 20% of that detected in the first 30 min. A similar pattern was also seen in the cAMP released during the 2-h treatment; however, the magnitude of decrease was less than that seen in the GH release. The amount of secretion in the last 30-min period was only 40% less than the initial secretion. When GHRH was used for 2 h at 0.1 nM, the decrease in GH release was only 50% (Fig. 8). Although 3-min pulses of 0.1 nM GHRH never released detectable amounts of cAMP in our experiments, the 2-h infusion resulted in a steady albeit low level liberation of cAMP. Both GH and cAMP responses were reduced when the

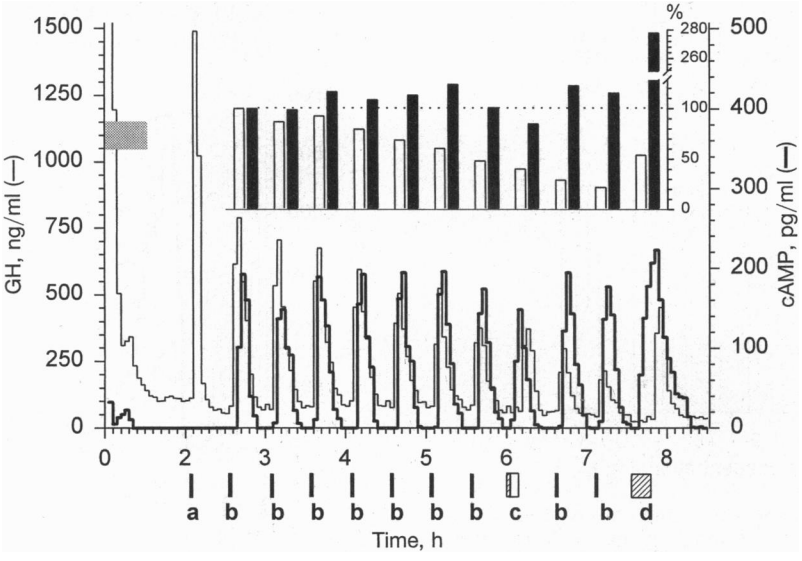

FIG. 5. GH (thin line) and cAMP (thick line) responses to repeated 3-min pulses of 1 nM GHRH and effect of SS-14 on GHRH-stimulated GH and cAMP release. Shaded box shows 9-min samples collected in the first 90 min of the superfusion. Vertical filled bars below the abscissa represent 3-min stimulation with the following substances: a, 50 mM KCl; and b, 1 nM GHRH; c, 3-min simultaneous infusion of 1 nM GHRH; and 1 nM SS-14 (hatched bar) followed by a 6-min infusion of 1 nM SS-14 alone (empty bar); and d, 15-min simultaneous infusion of 1 nM GHRH and 1 nM SS-14. (*Inset*) GH (empty columns) and cAMP (filled columns) responses expressed as percent of the response to the first 1 nM GHRH stimulus.

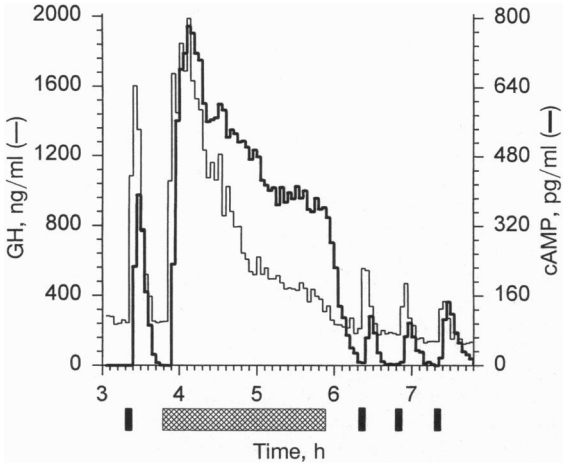

FIG. 7. Effect of 2-h infusion of 1 nM GHRH on GH (thin line) and cAMP (thick line) release. Vertical filled bars represent 3-min stimulation with 1 nM GHRH. Horizontal crosshatched bar indicates 2-h infusion of 1 nM GHRH. GH and cAMP responses during the 2-h infusion were 604% and 1727% of the respective responses to the first 3-min pulse of 1 nM GHRH.

pituitary cells were challenged again with 3-min pulses of 1 nM GHRH after the 2-h treatment with 0.1 nM or 1 nM GHRH.

DISCUSSION

The evidence for the involvement of cAMP as a second messenger in GH secretion was obtained before the isolation of GHRH and resulted from the GH-releasing effects of theophylline and cAMP analogues (see ref. 1). Since GHRH has become available, it has been shown that the GH release induced by GHRH is coupled with a stimulation of adenylate cyclase activity and cAMP production in the somatotrophs (2, 9, 10). Intracellular cAMP levels are elevated within 1–5 min of the addition of GHRH to cultured pituitary cells (4, 5, 11), and extracellular cAMP accumulation is also enhanced. In previous studies, cAMP production and adenylate cyclase activity were reported to be attenuated by SS-14 (3–5, 12–14). It was also stated that the presence of extracellular Ca^{2+} is necessary for the mechanism of action of GHRH and that GHRH elicits a rapid increase in the intracellular Ca^{2+}

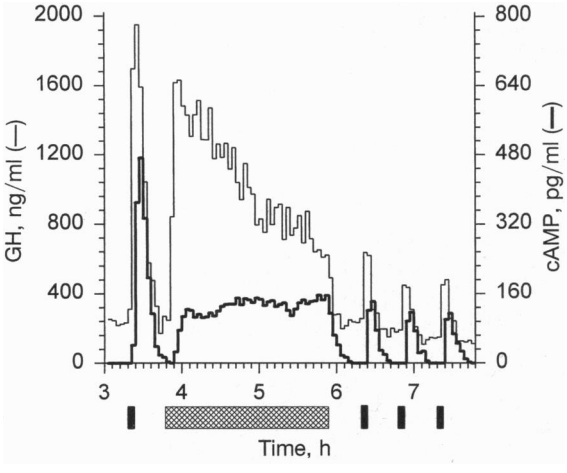

FIG. 8. Effect of 2-h infusion of 0.1 nM GHRH on GH (thin line) and cAMP (thick line) release. Vertical filled bars represent 3-min stimulation with 1 nM GHRH. Horizontal crosshatched bar indicates 2-h infusion of 0.1 nM GHRH. GH and cAMP responses during the 2-h infusion were 558% and 335% of the respective responses to the first 3-min pulse of 1 nM GHRH.

concentration, with peak levels occurring within 30 sec (9, 15–17). However, the precise temporal relationship between Ca^{2+} and other second messengers—i.e., cAMP—and their intracellular effectors is unknown.

The intracellular accumulation and release of cAMP into the tissue culture medium have been examined previously in static incubation systems. The superfusion method allows a dynamic follow-up of the effects of a stimulation. Our results show that the amount of cAMP released from the cells is not proportional to GH release: low doses of GHRH liberate very little or no cAMP, and the steadily increasing release of cAMP in response to high doses of GHRH is not followed in a parallel manner by GH release. We found that the EC_{50} values for GH and cAMP releases differ by two orders of magnitude. Others reported that in static incubation systems, doses of GHRH 10 times higher were required to obtain half-maximal stimulation of cAMP production than for GH secretion (4, 5, 18, 19), while the difference in the EC_{50} for activation of adenylate cyclase was 100-fold (20). It was also found that the minimal dose of GHRH for releasing GH was 1/10th of that needed for producing a detectable intracellular cAMP accumulation or adenylate cyclase stimulation (4, 5, 20). Our results are in agreement with these previously published data. These findings also signify that the extracellularly detected cAMP mirrors the intracellular changes. The fact that 3-min pulses of GHRH at low concentrations did not release cAMP may also mean that cAMP is not involved in the GH release as a second messenger. On the other hand, large amounts of cAMP released by high concentrations of GHRH reflect the view that cAMP alone is not sufficient to release GH.

In the superfusion system, GH release in response to 1 nM GHRH starts less than 30 sec after stimulation, but this is followed only 3 min later by an increase in cAMP liberation. This immediate onset of GH response is in good agreement with the reported rapid increase in the intracellular Ca^{2+} level. On the other hand, the changes that we found in the pattern of GH and cAMP responses to high doses of GHRH indicate that the mechanism(s) required for the release of GH, especially that for the immediate liberation, is blocked by the increase in the dose, while cAMP release is continually stimulated. Although GH and cAMP release might be influenced by the effects of previous stimuli, the phenomenon that high doses of GHRH decrease the initial amplitude of the GH response cannot be explained solely by desensitization or by lack of GH available for immediate release, because similar changes could be also observed when 1 μM or 10 μM GHRH was given after a single 3-min pulse of 1 nM GHRH. In addition, even after repeated stimulation with high or increasing doses of GHRH, when GH responses lacked the initial high amplitude component, the cells still responded well to K^+.

It was reported previously that SS-14 partially inhibited adenylate cyclase activity and intracellular cAMP levels (3, 20) induced by GHRH; however, the effects of SS-14 cannot be explained only on the basis of inhibition of adenylate cyclase activity. SS-14 prevents the GHRH-induced increase in intracellular Ca^{2+} within seconds of exposure by hyperpolarizing the somatotrophs (21). In the superfusion system, SS-14 completely blocks the release of GH when infused simultaneously with GHRH, but the ultimate GH response is not diminished and is only deferred for the duration of the SS-14 infusion (7, 22, 23). Changes in Ca^{2+} levels do not influence cAMP stimulation (11). In our experiments, GH release was delayed by the SS-14 infusion, but the release of cAMP was not deferred. This finding supports the view that SS-14 may influence signal transduction pathways other than cAMP—i.e., Ca^{2+}. However, when SS-14 was introduced during a prolonged GHRH infusion, we found that SS-14 had a small but definite inhibitory action also on the cAMP release that occurred 9 min later than the decrease in GH release. The complete blockade of GH release can be well explained by the

immediate inhibition of Ca^{2+} influx. The 9-min phase difference in the inhibition of GH release and cAMP liberation indicates that the release of cAMP is not influenced by hyperpolarization of the membrane that is an immediate effect of SS-14. This difference could reflect the time needed for the action of SS-14 to decrease cAMP release by inhibiting adenylate cyclase.

The discrepancy in the effect of GHRH on GH and cAMP release was also observed in experiments in which desensitization was studied. Repeated pulses of 1 nM GHRH resulted in decreasing GH responses; however, cAMP responses remained in the same range. During a 2-h infusion of 1 nM GHRH, the GH secretion rapidly declined in spite of the accompanying high cAMP levels. When 0.1 nM GHRH was infused, cAMP release never reached the high magnitude observed with 1 nM GHRH, but the decline in GH secretion was smaller than that seen in response to 1 nM GHRH. The amount of GH secreted in the last 30 min of the 2-h incubation with 0.1 nM GHRH was twice as large as that secreted during the same period with 1 nM GHRH. The total GH release during the 2-h incubation was nearly the same with 0.1 nM or 1 nM GHRH, but the cAMP release was 5 times higher with the 1 nM dose. These results show that the pattern of GH secretion after prolonged stimulation depends on the dose of GHRH: (*i*) 0.1 nM GHRH induces a lower amplitude GH response but with a smaller decline than the 1 nM dose; (*ii*) the amount of GH secreted after a 2-h stimulation is not proportional to the accompanying cAMP secretion. This also implies that GH release is strongly influenced by other steps in the signal transduction mechanism, beside cAMP. After the 2-h incubation with either a 0.1 or 1 nM dose, the GH and cAMP responses to 1 nM GHRH were greatly reduced. This could be explained by the loss of GHRH receptors. It was previously reported that a 2- to 4-h treatment with doses lower than 1 nM GHRH results in a 50% reduction of GHRH-binding sites and intracellular cAMP accumulation (24, 25).

In conclusion, our findings indicate that although cAMP is released after a GHRH pulse, this effect alone cannot maintain the release of GH. Other steps of the signal transduction mechanisms that are independent of the cAMP route may participate in the process of GH release. The nature of the mechanisms involved in the mediation of GH release may vary with the doses of GHRH used.

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