Endocrine, *vol. 9, no. 1, 71–77, August 1998* 0969–711X/98/9:71–77/$9.75

Growth Hormone-Releasing Peptide-2 (GHRP-2) Does Not Act via the Human Growth Hormone-Releasing Factor Receptor in GC Cells

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Effect of growth hormone-releasing peptide-2 (GHRP-2) on ovine somatotrophs is abolished by a growth hormone-releasing factor (GRF) receptor antagonist, which raises the possibility that GHRP-2 may act on GRF receptors. In the present study, we used rat pituitary GC cells with or without stable transfection of cDNA coding for the human GRF receptor (GC/R$^+$ or GC/R$^-$) to determine whether or not GHRP-2 acts via the GRF receptor. Northern blot analysis indicated that GRF receptor mRNA was undetectable in GC/R$^-$ cells, whereas a high level of expression occurred in GC/R$^+$ cells that were transfected by GRF receptor cDNA. In GC/R$^-$ cells, incubation with up to 10^{-7}*M* of either hGRF or GHRP-2 did not alter the intracellular cAMP, [Ca^{2+}]i, or GH secretion. In GC/R$^+$ cells, hGRF (10^{-11}–10^{-7}*M*) increased cAMP levels in a concentration-dependent manner up to 20-fold. This increase in cAMP levels was blocked by a GRF receptor antagonist, [Ac-Tyr^1, D-Arg^2]-GRF 1-29, but not by a Ca^{2+} channel blocker, $NiCl_2$ (0.5 m*M*). GH secretion and [Ca^{2+}]i were, however, not increased by hGRF. Incubation of the transfected cells with 10^{-11}–10^{-8}*M* GHRP-2 did not modify intracellular cAMP levels. This result suggests that GHRP-2 does not act through the GRF receptor.

Key Words: cAMP; [Ca^{2+}]i; secretion; pituitary; incubation.

Introduction

Synthetic growth hormone-releasing peptides (GHRP) specifically stimulate growth hormone (GH) release from the pituitary gland both in vitro and in vivo *(1,2)*. GHRP-6 and nonpeptidergic GH secretagogs, which mimic the three-dimensional structure of GHRP-6, act on a receptor that is different from that for the endogenous GH-releasing factor (GRF) *(3)*. The recent molecular cloning of a receptor for GHRP from rat, human, and swine pituitary glands has allowed studies in oocytes expressing this receptor, and it appears that GHRP-6 and nonpeptidergic GH secretagogs (MK-0677) act through this receptor to increase intracellular free Ca^{2+} ([Ca^{2+}]i) levels via the phospholipase C-inositol triphosphate pathway *(4)*. The GHRP-2 was also shown to bind on the receptor with high affinity *(4)*. We have shown previously that GHRP-2 increases the influx of Ca^{2+} in ovine somatotrophs in vitro via the cAMP-protein kinase A pathway and that this correlates with GH release *(2,5,6)*. In addition, the action of GHRP-2 could be blocked by a GRF receptor antagonist, [Ac-Tyr^1, D-Arg^2]-GRF_{1-29} *(6,7)*, which raised the possibility that GHRP-2 might act on GRF receptors. Indirect evidence, however, does not support this notion, because there is no crossdesensitization of the action of GRF and GHRP-2, and the peptides have an additive effect on GH secretion at maximal doses *(6,7)*. On balance, therefore, GHRP-2 does not appear to act through the GRF receptor, but there is no direct evidence for this. In the present experiments, we have utilized a rat pituitary GC cell line with or without overexpression of the hGRF receptor (from S. Melmed, Cedars-Sinai Medical Center, Los Angeles, CA) to test the effect of GHRP-2 and GRF. These cells synthesize and secrete GH, and have been used for the investigation of insulin-like growth factor-1 (IGF-1) receptor function following stable transfection of the gene encoding the IGF-1 receptor into the cells *(8)*. Using a similar technique, the hGRF receptor gene has been stably transfected into the cells for overexpression of hGRF receptors. We used Northern blot analysis to show that hGRF receptor mRNA was very easy to detect in the GC cells transfected with hGRF receptor, but was nondetectable in wide-type GC cells. Accordingly, we were able to compare the effects of hGRF and GHRP-2 in cells that were either not expressing the hGRF receptor (GC/R$^-$) or were overexpressing the receptor (GC/R$^+$). Our data suggest that GHRP-2 does not act through the GRF receptor, except at very high concentrations.

Received February 27, 1998; Revised May 6, 1998; Accepted May 6, 1998.

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Results

Northern Blot Analysis of hGRF Receptor mRNA

Using 15 μg of total RNA, the GRF receptor mRNA was clearly seen in the extract of GC/R$^+$ cells after more than 10

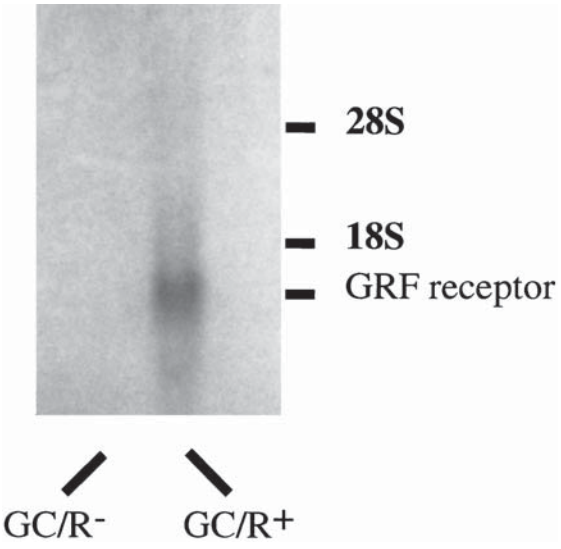

Fig. 1. Expression of transfected GRF receptor in GC/R$^+$ cells. Northern blot analysis was performed using a hGRF receptor mRNA probe. Total RNA (15 μg each lane) from GC/R$^+$ and GC/R$^-$ cells was hybridized to the radiolabeled probe. GRF receptor mRNA was clearly observed in total RNA from GC/R$^+$ cells, but not in GC/R$^-$ cells.

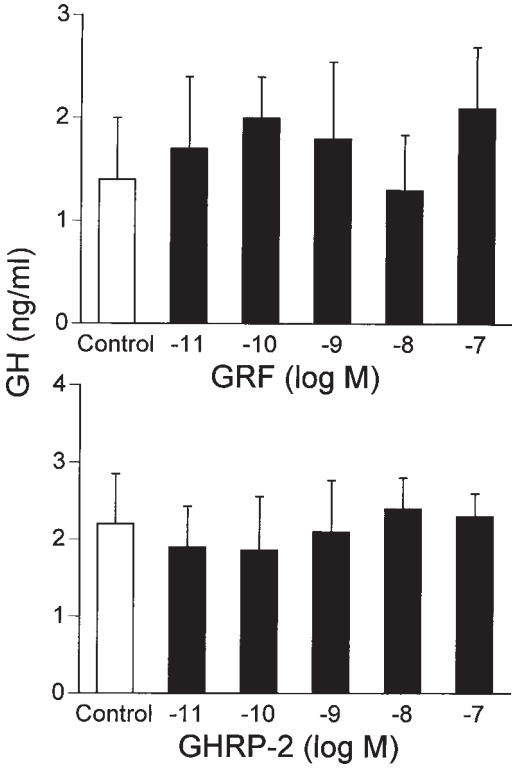

Fig. 2. Effects of GRF and GHRP-2 on the GH secretion in GC/R$^-$ cells. GH levels in culture medium after 30 min of incubation with either vehicle or GRF or GHRP-2 (10^{-11}–$10^{-7}M$). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment.

passages in culture. Under similar conditions, the receptor was not expressed in GC/R$^-$ cells (Fig. 1). This result indicates a stable transfection of hGRF receptor in to GC/R$^+$ cells, whereas the GRF receptor mRNA levels are undetectable in the wild-type GC cells.

Effects of GRF and GHRP-2 on GC/R$^-$ cells

In cultures of the wild-type GC (GC/R$^-$) cells, addition of 10^{-11}–$10^{-7}M$ GRF or GHRP-2 for 30 min did not significantly increase the secretion of GH (Fig. 2). Because we have shown that both GRF and GHRP-2 increase intracellular cAMP levels in ovine somatotrophs in vitro *(6)*, accumulation of cAMP within the cells was used as an index of activation by GRF or GHRP-2. Intracellular cAMP levels were not altered in GC/R$^-$ cells by either GRF or GHRP-2 treatment (Fig. 3). In a suspension of GC/R$^-$ cells, the [Ca^{2+}]i level was 114 ± 9.4 nM (mean ± SEM) before addition of GRF or GHRP-2, and this was not increased by a 10-nM addition of either GRF or GHRP-2 (Fig. 4).

Effects of GRF and GHRP-2 on GC/R$^+$ Cells

In cells that were overexpressing the hGRF receptor (GC/R$^+$), there was a concentration-dependent increase in intracellular cAMP levels in response to hGRF (Fig. 5, lower panel). There was, however, no significant increase in GH secretion in response to same dose range of hGRF (Fig. 5, upper panel). High K^+ solution (55 mM) was tested in these cells, which triggered a marked increase in GH release (data not shown). This indicates that the secretory machineries are functional in GC cells. At a dose of $10^{-7}M$ GRF, a slight (but not significant) increase in GH secretion from the GC/R$^+$ cells was observed. When GHRP-2 was added to the incubation medium, GH secretion was not increased over a dose range of 10^{-11}–$10^{-7}M$ (Fig. 6, upper panel). Intracellular cAMP levels were not increased by GHRP-2 up to a dose of $10^{-8}M$ (Fig. 6, lower panel). The addition of $10^{-7}M$ GHRP-2 significantly ($p < 0.05$) increased mean (±SEM) cAMP levels from 0.019 (±0.001) pmol/10^5 cells to 0.086 (±0.057) pmol/10^5 cells (Fig. 6, lower panel). In comparison, $10^{-7}M$ GRF caused a significantly ($p < 0.01$) greater increase in intracellular cAMP levels than that caused by $10^{-7}M$ GHRP-2 (Figs. 5 and 6). In the other word, $10^{-10}M$ GRF caused a greater increase in cAMP than that caused by $10^{-7}M$ GHRP-2. Neither GRF nor GHRP-2 significantly increased the [Ca^{2+}]i concentration, although $10^{-8}M$ GRF caused an increase in levels from 125 ± 12.5 to 142 + 14.6 nM (mean ± SEM, $p > 0.05$). Figure 7 shows a group of representative [Ca^{2+}]i measurement from both types of the cells.

Effects of Receptor Antagonist or Signaling Blockers on cAMP Response to GRF

Since cAMP levels were increased by GRF in GC/R$^+$ cells, the signaling pathways involved in the modification of cAMP levels were investigated using several pharmacological tools. Incubation with $10^{-7}M$ [Ac-Tyr^1, D-Arg^2] GRF 1-29, a GRF receptor antagonist, totally abolished the effect of $10^{-8}M$ GRF on intracellular cAMP levels (Fig. 8).

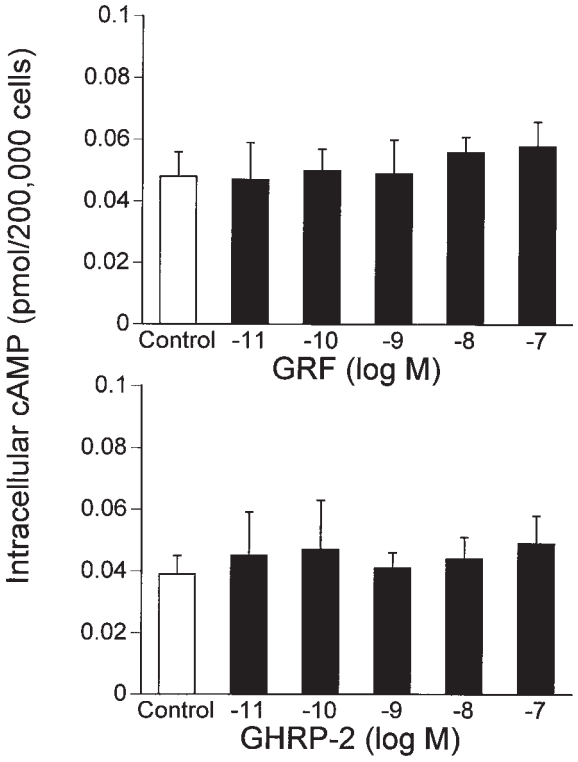

Fig. 3. Effects of GRF and GHRP-2 on the intracellular cAMP concentration in GC/R⁻ cells. Intracellular cAMP levels in cell lysates after 30 min incubation with either vehicle or GRF or GHRP-2 (10^{-11}–10^{-7}*M*). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment.

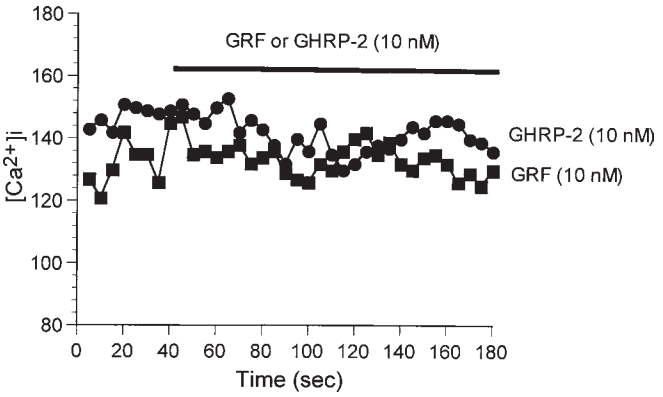

Fig. 4. Effects of GRF and GHRP-2 on the $[Ca^{2+}]i$ in GC/R⁻ cells. $[Ca^{2+}]i$ levels in GC/R⁻ cell suspensions measured by the fura-2 technique. Two representative traces (one group) from six groups of similar experiments are shown to demonstrate the effects of 10 n*M* of GRF (■) or GHRP-2 (●).

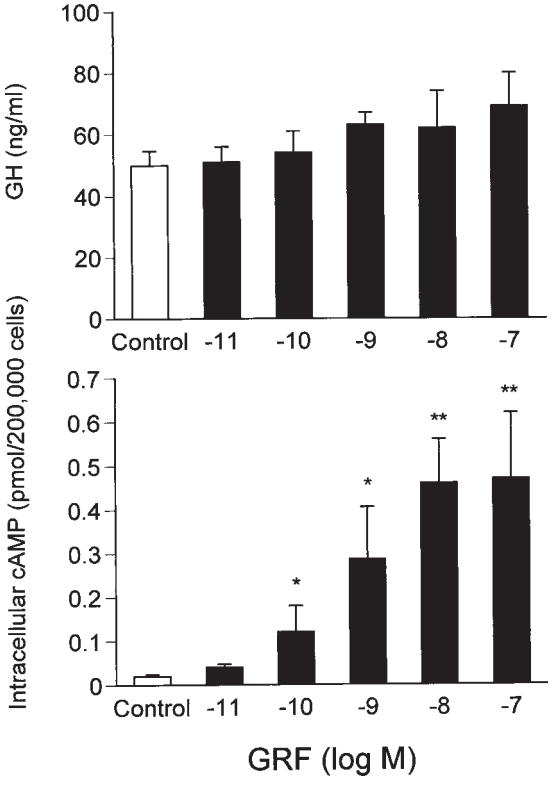

Fig. 5. Effects of GRF on the GH secretion and intracellular cAMP concentration in GC/R⁺ cells. Upper panel: GH levels in culture medium measured after 30 min of incubation with either vehicle or GRF (10^{-11}–10^{-7}*M*). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment. Lower panel: Intracellular cAMP levels in cell lysates measured after 30 min of incubation with either vehicle or GRF (10^{-11}–10^{-7}*M*). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment. *$p < 0.05$ compared to control group.

An adenylyl cyclase inhibitor (MDL 12,330A, 1 μ*M*) and a Ca^{2+} channel blocker ($NiCl_2$, 1 m*M*) partially reduced ($p < 0.01$) the increase in cAMP that was obtained with 10^{-8}*M* GRF (Fig. 8). Neither calphostin C (PKC inhibitor) nor H89 (PKA inhibitor) significantly modified the cAMP responses to 10^{-8}*M* GRF (Fig. 8).

Discussion

The results of the present study strongly suggest that the predominant mode of action of GHRP-2 is not via the GRF receptor. The small amount of cellular activation that is seen with high doses of GHRP-2 may be owing to either a low level of GHRP receptor expression in GC cells or low-affinity binding of GHRP-2 to the GRF receptor. It seems most likely that GHRP-2 acts though a GHRP receptor on rat somatotrophs, such as that which has been recently identified *(4,29)*. It remains an open question regarding why the effect of GHRP-2 on ovine somatotrophs can be blocked by a GRF receptor antagonist *(6,7)*, but it is possible that the antagonist binds to a subtype of the GHRP receptor in ovine somatotrophs that is blocked by the antagonist.

The GC/R⁻ cells used in this experiment did not show any detectable GRF receptor mRNA by Northern blot analysis. In addition, GRF and GHRP-2 did not cause significant GH secretion, intracellular accumulation of cAMP, or $[Ca^{2+}]i$ in these cells, indicating a lack of (or very low level of) GRF and GHRP receptors. Following stable transfection and overexpression of the hGRF receptor, GRF

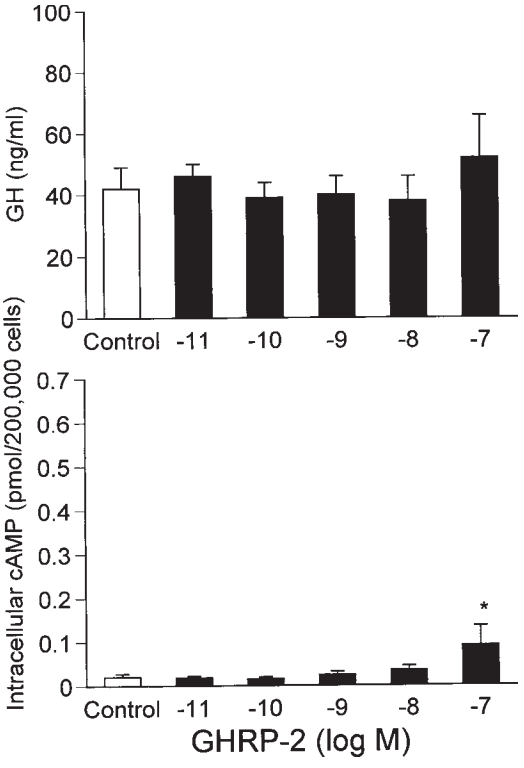

Fig. 6. Effects of GHRP-2 on the GH secretion and intracellular cAMP concentration in GC/R$^+$ cells. Upper panel: GH levels in culture medium measured after 30 min of incubation with either vehicle or GHRP-2 (10^{-11}–$10^{-7}M$). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment. Lower panel: Intracellular cAMP levels in cell lysates measured after 30 min incubation with either vehicle or GHRP-2 (10^{-11}–$10^{-7}M$). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment. *$P < 0.05$ compared to control group.

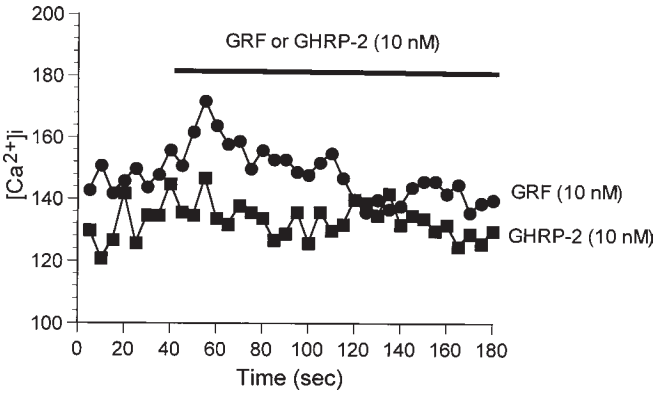

Fig. 7. Effects of GRF and GHRP-2 on the [Ca^{2+}]i in GC/R$^+$ cells. [Ca^{2+}]i levels in GC/R$^+$ cell suspensions (10^6 cells/mL, 2 mL for each measurement) measured by the fura-2 technique. Two representitive traces (one group) from six groups of similar experiments are shown to indicate the effects of 10 nM of GRF (■) or GHRP-2 (●).

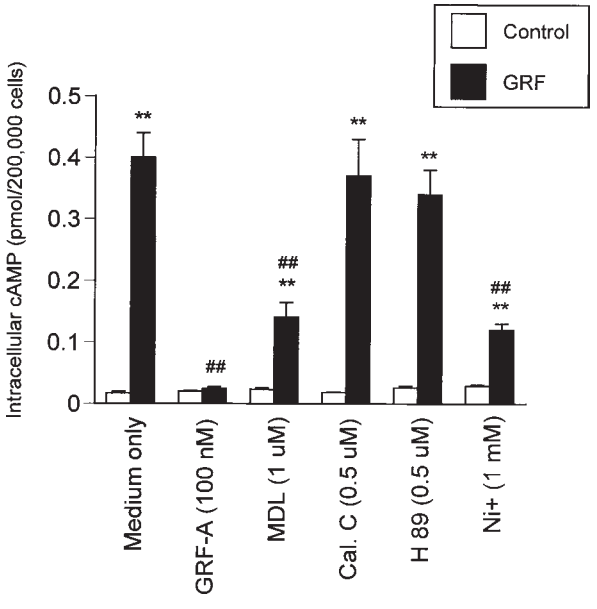

Fig. 8. Effects of GRF with or without pretreatment by a GRF antagonist, MDL 12,330A, calphostin C, H89, and Ni^{2+} on the intracellular cAMP concentration in GC/R$^+$ cells. Intracellular cAMP levels in cell lysates measured after 30 min of incubation with either vehicle (control, open column) or 10 n*M* GRF (GRF, filled column). Data are means (±SEM) of 4 separate experiments with 4 wells/treatment. Pretreatment of the cells by reagents other than GRF was added to the incubation medium 10 min before GRF or vehicle. Abbreviations in the figure: GRF-A ([Ac-Tyr^1, D-Arg^2]GRF 1-29); MDL (MDL 12,330A); Cal. C (calphostin C). **$P < 0.01$ compared to control group. ##$P < 0.01$, compared with GRF response in medium only group.

receptor mRNA was clearly shown by Northern blot analysis as shown in Fig. 1. A clear concentration-dependent alteration in intracellular cAMP levels was also obtained with hGRF, but not with GHRP-2 (except at a very high concentration) in GC/R$^+$ cells. The increase in intracellular cAMP levels that was observed with $10^{-7}M$ GHRP-2 was less than that seen with $10^{-10}M$ GRF. It has previously been demonstrated that GHRP-2 and GRF caused an increase in cAMP and GH release with a similar potency in ovine pituitary cells *(6,7)*. The very small increase in cAMP by $10^{-7}M$ GHRP-2 suggests that GHRP-2 does not act on the GRF receptor or that GHRP-2 has a very low potency to act on the GRF receptor.

GHRP-2 did not modify the [Ca^{2+}]i levels in either GC/R$^-$ cells or GC/R$^+$ cells. The basal [Ca^{2+}]i levels were comparable to those seen in "normal" dissociated somatotrophs in different species *(5,10,11)*, suggesting adequate intracellular Ca^{2+} stores and transmembrane Ca^{2+} channels. GHRP-6 and GHRP-2 have been reported to increase the phosphatidylinositol hydrolysis *(12,13)*, which leads to an increase in inositol triphosphate ($InsP_3$) levels and raised intracellular "free" Ca^{2+} concentrations *(11,14)*. If GHRP-2 had activated phospholipase C, an increase in [Ca^{2+}]i would have been expected in response to GHRP-2 in these cells. The fact that this was not observed further indicates that the GC cells have no (or very few) GHRP receptors.

The increase in intracellular cAMP that was seen in GC/R^+ cells in response to GRF was totally abolished by a GRF receptor antagonist, demonstrating a specificity of action. In normal somatotrophs, GRF-induced elevation of cAMP levels leads to an influx of Ca^{2+} via membrane ion channels *(10)*, and a corresponding increase in $[Ca^{2+}]i$ *(10,15)*, and GH secretion *(6)*. In GC/R^+ cells, however, there was no increase in GH secretion in response to GRF application. GH in GC cells may not be packaged correctly to secret on GRF stimulation. There was, however, no alteration in $[Ca^{2+}]i$ in GC/R^+ cells after GRF administration, indicating that elevation of cAMP levels does not lead to Ca^{2+} influx in these cells. Because high K^+ triggered GH secretion from GC cells, it seems that the lack of increase in $[Ca^{2+}]i$ is the major reason for no GH release in response to GRF in GC/R^+ cells. The reduced cAMP response to GRF of GC/R^+ cells following incubation with a Ca^{2+} channel blocker (Ni^{2+}) suggests that maintenance of basal $[Ca^{2+}]i$ is required for this response, although we have no evidence of Ca^{2+} influx in response to GRF. Blockade of Ca^{2+} channels in ovine somatotrophs reduces the basal $[Ca^{2+}]i$ *(5,10)*, suggesting that the membrane Ca^{2+} channels are integral to the maintenance of $[Ca^{2+}]i$. Whether the GC cells have normal membrane Ca^{2+} channel function has not been determined. The lack of coupling between the rise in intracellular cAMP levels and $[Ca^{2+}]i$ may be owing to a lack of certain types of PKA, which are responsible for the modification of membrane ion channels in normal cells; further experiments need to be performed to clarify this issue. Interestingly, GRF does not cause significant GH secretion in about 50% of human acromegalic tumors *(16)*, although cAMP levels are increased (Chen et al., unpublished data). This lack of coupling is similar to that observed in the GC cells in the present studies and may indicate a fundamental modification in tumor cells.

We have recently shown that both GRF and GHRP-2 activate PKC in cultured ovine pituitary cells, which potentiates the GH release induced by these GH secretagogs *(17)*. The mechanism of this potentiation by PKC activation is via a crosstalk between the PKC and the cAMP-PKA system *(17)*. Accordingly, we sought to determine whether inhibitors of PKC or the cAMP/PKA system would modify the response of GC cells to GRF. The adenylyl cyclase inhibitor (MDL 12,330A) reduced the effect of GRF on the accumulation of cAMP, indicating that activation of GRF receptors stimulates adenylyl cyclase activity leading to an increase in production of cAMP. This confirms that the cAMP/PKA pathway is probably the major second messenger signaling system for GRF as proposed *(17,18)*. The PKC inhibitor (Calphostin C) did not alter the increase in cAMP by GRF which suggests that "crosstalk" between the PKC and cAMP/PKA systems *(19,20)* is not of major significance in GC/R^+ cells. The other possibility is that PKC could act on the elements of cAMP/PKA system distal to the production of cAMP (such as PKA).

In summary, we conclude that:

1. Wild-type GC cells have no (or very few) GRF or GHRP receptors.
2. GHRP-2 does not act on the GRF receptor.
3. hGRF increases cAMP levels in GC cells overexpressing hGRF receptor.
4. The GRF-induced rise in cAMP levels in GC/R^+ cells does not lead to alteration in $[Ca^{2+}]i$ or GH secretion, suggesting the cAMP pathway is not coupled to the intracellular mechanisms regulating $[Ca^{2+}]i$ in these cells.

Materials and Methods

Cell Culture

GC rat pituitary cell lines were grown in 50-mL culture flask (10^7 cell/flask) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. The stable transfectants of the GC cell line overexpressing the hGRF receptor *(21)* were grown under the same culture conditions as the wild-type cells, but with the addition of neomycin (G418) to the culture medium at a concentration of 400 μg/mL. The medium for both cell types was replenished every 72 h. Cells were plated onto 24-well (1 mL/well) culture dishes at a density of 10^5 cells/well 3–5 d before the experiments described.

Northern Blot Analysis

In order to confirm stable transfection and expression of hGRF receptor to the cells, Northern blot analysis of hGRF receptor mRNA from wild-type and transfected cells was performed.

Two oligonucleotides were synthesized based on the published hGRF receptor nucleotide sequence *(21)* as S1: GCA GCC AAG GCT TAC TGA GGC TGG TGG AGG, and A1: ACA GTG GAG AAG CTG CAG TGG TCA GTG TCG. Total RNA from a human somatotrophic pituitary adenoma was reverse-transcribed and amplified on a Perkin Elmer Cetus thermocycler (95°C 20 s, 65°C 30 s,72°C 30 s, 40 cycles, RT-PCR-Kit from Perkin Elmer, Norwalk, CT 06859-0251). DNA residues 2–650 were amplified using S1 and A1 as primers. The PCR product was fractionated and subsequently cloned into pCRII using the TA-Cloning Kit (Invitrogen, San Diego, CA 92121). The insert was labeled with ^{32}P-dCTP using random hexamer primers and the Klenow fragment of *Escherchia coli* DNA polymerase.

Cultured GC cells were homogenized from both wide-type GC cells (GC/R^-) and transfected GC cells overexpressing hGRF receptors (GC/R^+). Total RNA was extracted for Northern blot analysis of hGRF receptor mRNA. The RNA (15 μg/lane) was then loaded onto a denaturing 1% agarose/formaldehyde gel and subjected to Northern gel analysis as previously described *(22)*. The membranes were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Incubation Procedure

Before each experiment, the cells were washed three times with incubation medium (M 199 containing 0.5% BSA) and then preincubated for 1.5 h (1 mL/well). The cells were then treated with control medium or medium containing treatment peptides (1 mL/well), and then incubated for a further 30 min before collection of the medium. Incubation for 30 min was chosen based on our previous observation that a maximal GH response and intracellular cAMP response was obtained using this incubation in our previous experiments *(6)*. In experiments where inhibitors for protein kinases or ion channel blockers were used, the cells were preincubated with the inhibitor blocker for 10 min prior to the addition of either GRF or GHRP-2. The medium was collected for GH assay (stored at –20°C), and the cells were extracted with acid-alcohol. The extracts were dried under vapor overnight and stored at 4°C for the assay of cAMP.

Radioimmunoassay of GH

The concentration of GH in the incubation medium was measured in duplicate, using a double-antibody radioimmunoassay using kits provided by the National Hormone and the Pituitary Program of USA (rGH [RIA] and rGH antisera). The sensitivity of the assay was 0.3 ng/mL and the inter- and intra-assay coefficients of variation were <15 and <8%, respectively ($n = 6$). All samples from one experiment were measured in the same assay and GH values were expressed as ng equivalents rat GH standard (NIH-rGH-s).

cAMP Radioimmunoassay

Cell extracts were assayed for cAMP as described previously *(6)*. Briefly, the cell extracts were reconstituted in 300 μL of assay buffer (1 m*M* theophylline and 0.1% BSA, pH 5.0) and were acetylated by the rapid sequential addition of 10 μL triethylamine and 5 μL acetic anhydride. Duplicate 100-μL aliquots were assayed for cAMP by RIA. Antiserum to cAMP (Ab 5120 kindly supplied by P. Marley, University of Melbourne, Australia) was used at a final dilution of 1:15,000. The cAMP assays were performed in a final volume of 300 μL with 100 μL of sample, tracer (^{125}I-cAMP), and antibodies.

Measurement of [Ca^{2+}]i

Cells in culture were resuspended after a brief trypsinization as described *(10,23)*, washed in loading buffer (138 m*M* NaCl, 6 m*M* KCl, 1 m*M* $MgSO_4$, 1 m*M* Na_2HPO_4, 5 m*M* $NaHCO_3$, 5.5 m*M* glucose, 20 m*M* HEPES, and 0.1% BSA), and then loaded with fura-2-acetoxymethyl ester (1 μ*M*) for 30 min at 37°C with gentle agitation. Cell suspension was then washed and resuspended (0.5×10^6 cell/mL) in measurement buffer (138 m*M* NaCl, 6 m*M* KCl, 1 m*M* $MgCl_2$, 5.5 m*M* Glucose, 20 m*M* HEPES) at room temperature for [Ca^{2+}]i measurement. The cell suspension was injected into a 2-mL chamber of a Hitachi 2000 spectrofluorimeter, and Ca^{2+} was added into the suspension at a final concentration of 1.6 m*M*. After a warming up time of 2 min at 37°C, measurement of [Ca^{2+}]i began. [Ca^{2+}]i was calculated from the recorded fluorescence intensity as previously reported *(10)*.

Materials

DMEM and medium 199 (M199) were obtained from Cytosytems (Castle Hill, Australia), sera and pancreatin from Gibco (Gaithersburg, MD), Collagenase from Worthington Biochemical Corporation (Freehold, NJ), and GRF (hGRF 1-44),[Ac-Tyr^1, D-Arg^2]-GRF$_{1\text{-}29}$ and GHRP-6 from Auspep (Parkville, Australia). GHRP-2 was synthesized and kindly supplied by C. Y. Bowers, and purified using HPLC. Hyaluronidase, DNase, chemicals for cAMP assay, and salts for experimental solutions were purchased from Sigma (St. Louis, MO). Percoll was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). MDL 12,330A was kindly donated by Marion Merrell Dow Research Institute (Cincinnati, OH). PKA inhibitor H_{89} was purchased from CalBiochem (San Diego, CA).

Data Analysis

Data are reported as means ±SEM of 4–5 experiments (4 wells/experiment). Statistical comparisons were made using ANOVA, and significance was taken as $p < 0.05$.

Acknowledgments

We thank P. Marley at Department of Pharmacology, University of Melbourne for supplying cAMP antibodies, S. Melmed at Cedars-Sinai Medical Center for supplying GC cells for this experiment, and C. Y. Bowers at Tulane University, for supplying synthetic GHRP-2 used in this experiment. We also thank T. Taylor for rat GH RIA kits provided by NHPP, NIDDK, NICH, and HD, and S. Panckridge for her assistance with graphic work. Y. Chen and T. Trong have contributed to this work at preliminary stage as vacation scholars. The work was funded by a grant from the Australian NH and MRC.

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