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Behaviour of a somatotroph population under a growth hormone releasing peptide treatment

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Summary

In this investigation, we studied the effects of Momany peptide (GHRP-5), on somatotroph secretory activity. Acute and chronic administration of GHRP-5 provokes a significant release of growth hormone that can be closely correlated with ultrastructural changes in somatotroph populations. After 3, 5 and 7 days of GHRP-5 treatment, two somatotroph cell subpopulations coexist. One of them has an enhanced secretory activity and the other presents a quiescent appearance. Therefore, pituitary growth hormone content was not affected in the first seven days of GHRP-5 treatment. After 14 days, there was a significant depletion of growth hormone pituitary content coincident with the highest levels of serum growth hormone. These results concur with the surge of a new hyperactive somatotroph subtype characterised by numerous immature secretory granules that are discharged bypassing the maturation step. Acute and chronic treatments caused no changes in somatotroph cell density, the area immunostained for growth hormone and the levels of total mRNA for transcription factor pit-1.

The results of pituitary cell cultures incubated with specific blockers for different signalling pathways demonstrated an involvement of the phospholipase C–inositol phosphate system in GHRP-5 stimulated somatotroph secretion.

GHRP-5 treatment enhanced significantly the release of growth hormone, thereby eliciting ultrastructural modifications in somatotrophs that can be correlated with an increased secretory activity devoid of cell density changes.

Introduction

The secretion of growth hormone (GH) is under a complex neuroendocrine control exerted by hypothalamic growth hormone releasing hormone (GHRH) and somatostatin (SRIF). We have previously reported that the stimulation and inhibition of GH secretion induced by GHRH, SRIF, thyroid hormone and insulin-like growth factor I (IGF-I) treatment are closely correlated with ultrastructural and immunocytochemical changes occurring in somatotroph cell populations (Torres *et al.* 1995, Bonaterra *et al.* 1998, Pellizas *et al.* 2000). The main cytological modifications were detected in organelles involved in hormone synthesis and secretion, proliferation and somatotroph cell death and the differentiation of subpopulations of GH-producing cells.

Different neurotransmitters and a vast array of metabolic and hormonal signals originating in peripheral tissues would also influence GH secretion. In addition, small synthetic molecules called GH secretagogues (GHS), such as GH-releasing peptide (GHRP) and non-peptidyl GHS (Smith *et al.* 1993, Tiupakov *et al.* 1995), stimulate GH release from pituitary through a specific receptor (GHS-R) (Howard *et al.* 1996, McKee *et al.* 1997, Smith *et al.* 1997). Recently, an endogenous ligand for GHS-R, called ghrelin, was identified in the rat stomach. Synthetic ghrelin specifically releases GH both *in vivo* and *in vitro* and is involved in a novel system for regulation of GH secretion (Kojima *et al.* 1999).

It has been demonstrated that GHS acts through receptors different from those for GHRH that activates adenylyl cyclase. In turn, this increases intracellular cAMP levels and subsequently provokes an influx of Ca^{2+}, which is fundamental for GH release (Bilezikjian *et al.* 1987, Bowers *et al.* 1991). In addition, GHRH induces a significant proliferation of the somatotroph population (Torres *et al.* 1995). This proliferative activity is mediated in part by cAMP, an important factor in the mitotic activity of somatotrophs (Billestrup *et al.* 1986) and by the proto-oncogene c-fos, that is stimulated at transcription level by GHRH (Billestrup *et al.* 1987). Besides c-fos, another potential relay for the positive effect of cAMP in somatotroph proliferation is the pituitary-specific transcription factor pit-1, because it is stimulated by cAMP and is implicated in the development and proliferation of somatotrophs (Castrillo *et al.* 1991, Bertherat *et al.* 1995).

A variety of intracellular signalling systems have been suggested as mediating the GHS action. These include changes in intracellular free Ca^{2+} cAMP, protein kinases A and C, and phospholipase C (PLC) (Chen *et al.* 1996, Wu *et al.* 1997). Indeed, it has been reported that the same GHRPs (GHRP-1 and GHRP-6) and non-peptidergic GHS activate the PLC–inositol triphosphate (IP3) pathway and the subsequent

mobilisation of intracellular Ca^{2+} in rat somatotrophs (Lei *et al.* 1995, Adams *et al.* 1995).

The search for alternative compounds to either GH or GHRH, endowed with oral bio-availability and clinical effectiveness to enhance GH release, has been relevant in the past few years. Although several reports on various GHRPs provided a wide range of information on the effects of GH secretion and the mechanism of action, no information has been disclosed on the morphological and quantitative immunocytochemical changes of somatotroph population, critical parameters for the interpretation of the effects of GHRPs.

In this paper, we report a multidisciplinary study on Momany peptide (Y-A-W-F-NH_2) (Momany *et al.* 1981), a pentapeptide named as GHRP-5. The effects of acute and chronic treatments with GHRP-5 on pituitary content and secretion of GH, and on the ultrastructural and morphometric variations of somatotroph population are reported. Furthermore, the levels of pit-1 mRNA and the involvement of different intracellular signalling pathways described in rat somatotrophs treated with GHRP-5 are also analysed.

Materials and methods

Adult male rats of the Wistar strain, aged two months, were used in this investigation. They were housed in air-conditioned quarters with a light–dark cycle (14–10 h) and provided with free access to tap water and rodent chow (Nutric, Córdoba, Argentina). Treated rats were injected intraperitoneally with 12 μg GHRP-5 (Sigma, T-5517, purity 96%) in saline per 100 g body weight/day for 3, 5, 7 and 14 days. Control rats were injected with solvent only. Eight rats were used in each experimental trial and the data presented are representatives of at least three independent experiments. The rats were sacrificed 2 h after the last injection. Animals were decapitated avoiding any stress or external stimuli, within 10 sec of the removal of each animal from its cage. Arterial and venous blood drained from head and trunk were collected in a centrifuge tube allowed to clot at 4 °C, spun down and the serum withdrawn and frozen at −20 °C until their GH contents were measured. The pituitary gland was rapidly excised and split into two halves by a medial section with a razor blade. One hemipituitary was processed for electron microscopy, morphometry and immunocytochemistry. The other half was frozen at −20 °C until GH radioimmunoassay was performed. Animal procedures were in compliance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare, and the local Institutional Animal Care Committee.

Electron microscopy

Changes in the ultrastructure of GH cells in GHRP-5 treated rats were studied in three hemipituitaries from each experimental group. The tissues were fixed by immersion in 4% (v/v) glutaraldehyde plus 4% (w/v) formaldehyde in cacodylate buffer (0.1 M), for 2–4 h at room temperature. The fixed tissue was then treated with 1% osmium tetroxide for 2 h at room temperature, dehydrated with increasing concentrations of acetone and embedded in Araldite 6005 (Electron Microscopy Sciences, Fort Washington PA, USA). Thin sections, cut with diamond knives on a Porter-Blum MT2 or a JEOL JUM-7 ultramicrotome, were examined in a Zeiss 109 electron microscope.

Immunocytochemistry and morphometry

Three hemipituitaries obtained from rats treated with GHRP-5 for 3 and 14 days were fixed in 2% (v/v) glutaraldehyde plus 4% (w/v) formaldehyde in 0.1 M cacodylate buffer, pH 7.3, at room temperature for 5–6 h. Each fixed hemipituitary was divided into three zones (anterior, medial and posterior), dehydrated in increasing concentrations of ethanol and embedded in acrylic resins (LR White, London Resin Corporation). Several sections (1 μm thick) were sliced from each zone on a Porter-Blum MT1 microtome, mounted onto clean glass slides and immunostained for GH. Preparations of the immuno-gold complexes and other details of the immunocytochemical procedure are described elsewhere (Maldonado & Aoki 1986). Monkey anti-rat GH (diluted 1 : 4000) was used as primary antiserum (all kindly donated by Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD (NIDDKD). To visualise the immunostaining at light microscope level, the silver enhancement technique of Danscher and Ryter-Norgard (1983) was applied.

To validate the specificity of the immunoreaction, the following controls were performed: (1) adsorption of antibodies to highly purified GH; (2) replacement of the primary antiserum with 1% bovine serum albumin (BSA) in 0.1 M phosphate buffer, pH 7.3 plus 0.15 M sodium chloride (PBS); and (3) replacement of primary antiserum with diluted preimmune serum followed by the protein A–colloidal gold complex.

The morphometric analysis was performed in rats treated with GHRP-5 for 3- and 14-day periods. The somatotroph cell densities and the areas immunostained for GH were assessed on micrographs obtained in a Zeiss Photomicroscope III and printed at a final magnification of ×185. The analyses of scanned sections were processed with an image processor (UTHSCSA ImageTool, program 1.2). On the micrographs, all immunostained somatotrophs cut through the nucleus were counted to evaluate the changes occurring in the total number of somatotroph-producing cells per area unit (cell density: number/μm^2) (Griffiths 1998).

Electron microscope immunocytochemistry was achieved on thin sections of LR-White embedded pituitaries and immunostained for GH with the same specific anti-rat GH serum diluted 1 : 6000. Immunoreactive sites were labelled with anti-monkey IgG adsorbed to colloidal gold particles. The same controls used for light microscope immunocytochemistry were also applied here.

In vitro *studies: Dissociation of anterior pituitary cells and experimental procedures*

Cell suspensions were prepared from anterior pituitaries of 30 male rats. The pituitaries were rapidly excised, posterior and intermediate lobes discarded and anterior pituitaries were pooled, placed in Eagle's Minimal Essential Medium (S-MEM) and dispersed together by means of an enzymatic and mechanical dispersion method which was described in detail previously (De Paul *et al.* 1997). Briefly, glands were minced with a razor blade and then exposed to sequential incubation with 0.4% trypsin (type II-S), 0.4% soybean trypsin inhibitor I-S, and 0.1% of deoxyribonuclease. After a wash in S-MEM at room temperature, the cells were mechanically dispersed with siliconized Pasteur pipettes. The cells were spun down and the pellets resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3% foetal calf serum and 8% horse serum (Gibco). The cell yield was $1.5–2 \times 10^6$ per pituitary and the cell viability, tested with trypan blue exclusion, was always better than 90%.

Whole cultures of dispersed cells were placed in 35-mm sterile culture plates (Corning, New York) at a density of 5×10^5 cells/2 ml DMEM/well and six wells for each treatment were studied. The cell cultures were incubated at 37 °C in a humidified atmosphere of 95% air – 5% CO_2. An additional aliquot of 1 ml fresh culture medium was added to each well 48 h later. At 72 and 96 h of incubation, the media were withdrawn and replaced with 2 ml fresh DMEM.

Experiments were carried out after 4 days of culture. At 2 h preincubation in fresh serum-free DMEM was used to stabilise basal GH secretion before adding test substances: 6 μM GHRP-5, 1 μM GHRH, 0.03 μM TRH, 10 μM U-73122, phospholipase C inhibitor (Smallridge *et al.* 1992) and 100 μM Rp-cAMPS triethylamine (Rp-cAMPS), cAMP antagonist, (Wu *et al.* 1996). Both U-73122 and Rp-cAMPS were incubated for 30 min, followed by addition of GH secretagogues at time 0. Then, the cells were incubated for 120 min. The medium was collected and renewed every 15-min period and stored at −20 °C before GH analysis. Controls with medium DMEM and without the addition of substances tested were performed.

Radioimmunoassay

For full extraction of the GH stored in secretory granules, frozen pituitaries were homogenised (1 mg gland/ml medium) using an all glass Potter–Elvehjem tissue grinder (clearance 0.07–0.127 mm) in Tris–HCl, pH 9.0, and incubated for 3 h at 4 °C (Torres *et al.* 1995). Protein concentrations were determined by the Lowry technique. The pituitary GH contents were expressed in terms of specific activity of GH (μg GH/mg protein).

Serum and pituitary GH were quantified by radioimmunoassay applying a double antibody technique according to the protocol provided by NIDDKD, at two dose levels, and the results expressed in terms of rat growth hormone RP-2 (biological potency equivalent to 2.0 IU/mg) with reagents donated by NIDDKD. All samples were processed simultaneously to avoid interassay variations. The intra-assay coefficient of variation was lower than 10%.

Total RNA extraction

Total RNA was prepared by the one-step acid-guanidinium method described by Chomczynski and Sacchi (1987). In brief, 0.1 g pituitary tissue was homogenised in 1 ml denaturation solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5 N-lauroyl sarcosine and 0.1% β-mercaptoethanol). After extraction with phenol–chloroform–isoamyl alcohol (50 : 49 : 1 by vol.), RNA was precipitated in isopropanol, recovered by centrifugation and washed in 80% ethanol. After a further extraction, precipitation and wash, the RNA was dissolved in diethylpyrocarbonate-treated water, quantified and checked for purity by spectrophotometry at 260 and 280 nm.

Northern blot

The procedure was similar to that described previously (Fourney *et al.* 1988) with slight modifications (Pellizas *et al.* 1998). 20 μg total RNA were electrophoresed in 1% agarose gel containing 0.66 M formaldehyde. The gel was stained with ethidium bromide to visualise ribosomal RNA (rRNA). After electrophoresis, RNAs were transferred to a nylon membrane.

The membranes were incubated in pre-hybridisation solution containing 30% deionised formamide – 5 × Denhart's solution (0.1% Ficoll type 400 – 0.1% albumin – 0.1% polyvinylpirrolidone (PVP) – 5 × SSPE (0.75 M ClNa – 0.05 M NaH_2PO_4 – 5 mM EDTA) – 0.1% SDS – 200 μg/ml DNA from herring testes, for 5 h at 42 °C in hybridisation bags. Hybridisation with the probe was performed for 48 h at the same temperature. The entire PSK pit-1 cDNA linearised with *Hind III* was used as hybridisation probe for Northern blots. The entire cDNA was approximately 3.8 kb and included the full coding sequence. To ensure an even loading, the same blots were hybridised using the entire pBR 322 with 18 S rRNA genomic probe. The probes were labelled by the random primer technique with [(^{32}P)deoxy-ATP(3000 Ci/mmol)]. The specific activity of the labelled probes ranged from 2.6×10^9 to 3.9×10^9 d.p.m. μg DNA. After hybridisation, blots were washed in 2 × SSC (0.3 M NaCl – 0.015 M sodium citrate) – 1% SDS for 20 min at room temperature, followed by 2 × SSC – 1% SDS for 20 min at 55 °C, 1 × SSC – 1% SDS for 20 min at 55 °C and 0.2 × SSC – 1% SDS for 20 min at 55 °C. The membranes were exposed to Kodak X-Omat film at −70°C with intensifying screens, for three days in the case of the pit-1 probe, and for 6 h in the case of the 18S rRNA probe. The bands were quantified densitometrically (Shimadzu Dual-Wavelength Chromato Scanner CS-930) at 500 nm and the levels of pit-1 mRNA expressed as absorbancy of the pit-1 signals normalised with that of the 18S rRNA in the same lane.

Statistical analysis

All the results were analysed by one-way analysis of variance and the differences between means were determined applying Tukey's test. Significance is reported at $p < 0.05$.

Drugs and reagents

All the reagents used in this investigation were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Results

Growth hormone radioimmunoassay

In vivo *studies*

As shown in Figure 1, GHRP-5 treatment for a 3-, 5-, 7- and 14-day period led to a significant increase of the serum GH compared to controls. Serum GH reached its highest levels after 14 days of GHRP-5 treatment, increasing 10-fold compared to the control group level.

In GHRP-5 treated rats, the pituitary GH content remained unchanged at the levels of controls (Figure 2). However, in rats injected with GHRP-5, coincident with the highest level of serum GH, there was a significant diminution in pituitary GH content after 14 days of GHRP-5 treatment in comparison to the 7-day treatment ($p < 0.05$). Although the diminution in relation to control group was 6% lower, it was not statistically significant.

In vitro *studies*

Role of adenylate cyclase–cAMP pathway in GHRP-5 stimulated GH release. The possible contribution of the adenylate cyclase–cAMP pathway in the GH release from isolated pituitary cells incubated with GHRP-5, was evaluated with Rp-cAMPS a competitive antagonist of cAMP on GHRP-5 stimulated GH release. The GHRH elicited the expected upsurge of GH secretion at 15 min (t1) after GHRH addition to the medium (Figure 3). The GHRP-5 also stimulates significantly the GH secretion ($p < 0.001$ vs control), but this action was detected after incubation for 60 min (t4). The pretreatment with cAMP antagonist for 30 min blocked the GH release caused by GHRH (see t1); in contrast, it did

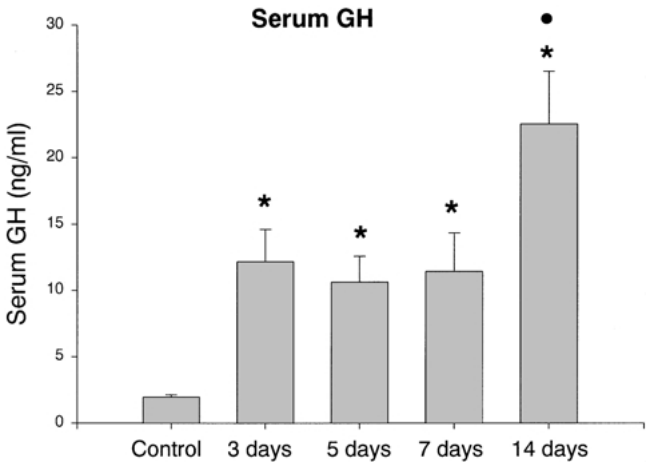


Figure 1. Serum GH in rats treated for 3, 5, 7 and 14 days with 12 µg GHRP-5/100 g body wt./day. Significant increase of GH secretion was observed in all groups compared to controls. After 14 days of treatment with GHRP-5 the serum GH reached the highest values. Data are expressed as mean ± SEM of eight rats of each group, $^{*}p < 0.05$; 3, 5, 7 and 14 days vs control, $^{\bullet}p < 0.05$; 14 days vs 7 days. ANOVA followed by Tukey's test.

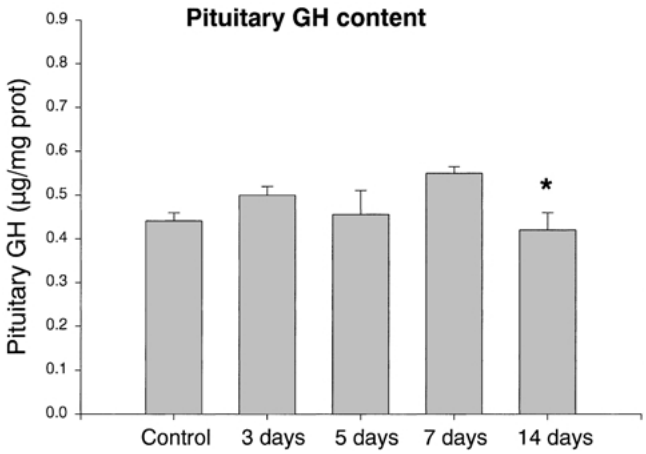


Figure 2. Pituitary GH content in rats treated for 3, 5, 7 and 14 days with 12 µg GHRP-5/100 g body wt./day. The administration of GHRP-5 did not change the pituitary GH content compared to control rats. After 14 days of treatment, there is a significant decrease in this parameter when compared to rats treated for 7 days with GHRP-5. Data are expressed as mean ± SEM for eight rats in each group, $^{*}p < 0.05$; 14 days vs 7 days. ANOVA followed by Tukey's test.

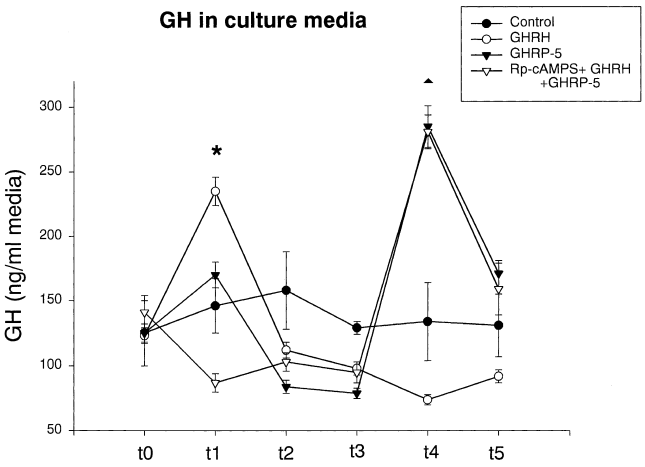


Figure 3. Secretion of GH from pituitary cell cultures treated with GHRH, GHRP-5 and Rp-cAMPS + GHRH + GHRP-5. Striking augmentation of GH levels in culture medium were observed at t1 in cells incubated with GHRH ($^{*}p < 0.01$). The stimulation with GHRP-5 increased significantly ($^{\blacktriangle}p < 0.001$) the GH secretion after 60 min of incubation period (t4). The pretreatment with cAMP antagonist, Rp-cAMPS, blocked the GH release in response to GHRH (see t1), whereas it did not affect the GH release caused by GHRP-5 as observed at t4 ($^{\blacktriangle}p < 0.001$). Means ± SEM of three different experiments (6 wells/treatment/experiment). Significance was reported at $p < 0.05$ vs control wells. ANOVA followed by Tukey's test.

not modify the GHRP-5 effect observed at t4 ($p < 0.001$ vs control).

Role of phospholipase C–IP pathway in GHRP-5 stimulated GH release. As shown in Figure 4, TRH, an activator of PLC–IP/Ca^{+2} pathway produced an early increase of the GH secretion at t1 ($p < 0.001$ vs control). A possible involvement of PLC–IP system in the secretory activity of GHRP-5 on somatotrophs was verified with the specific PLC inhibitor U-73122. The preincubation with this agent prevented stimulation of TRH on GH release occurring at t1. Also, it blocked the GHRP-5 peak of GH observed at t4.

Electron microscopy

In the pituitary gland of control rats, somatotrophs constitute a rather homogeneous population of typical secretory cells characterised by numerous round secretory granules, 200–350 nm in diameter and containing an electron-dense material. Also well-developed Golgi complex and rough endoplasmic reticulum (RER) cisternae were commonly seen in the cytoplasm (Figure 5).

After GHRP-5 treatment for 3, 5 and 7 days, the fine structural characteristics of somatotroph cells were associated with an enhanced biosynthetic activity. The cytoplasmic organelles engaged in synthesis and processing of proteins became prominent, especially the RER and Golgi complexes with a variable number of secretory granules depending on a differential secretory response of somatotroph populations. Some cells showed features of an enhanced secretory activity with diminution of secretory granules and some of them were seen clustered along the plasma membrane or in the process of exocytosis. Others with a quiescent appearance acquired ultrastructural features similar to those of control, with large stores of secretory granules in the cytoplasm (Figure 6).

After 14 days of GHRP-5 treatment, a great deal of heterogeneity in the fine structural organisation of somatotrophs was observed. Predominant somatotrophs exhibited an ultrastructural organisation compatible with increased synthesis and secretion. Ultrastructural characteristics were similar to those observed in rats treated for 3, 5 and 7 days. In addition, after 14 days of GHRP-5 treatment, a different somatotroph subtype appeared the cytoplasm of which contains abundant immature secretory granules. These secretory granules were smaller in size and enclosed a granular material of low electron density (Figure 7). Some other somatotrophs displayed different stages of cell death, recognised in electron micrographs by the high electron density of the condensed cytoplasmic matrix and pyknotic nuclei (Figures 8 and 9). The specific identification of GH cells by ultrastructural immunocytochemistry in all experimental groups was crucial to identify the somatotroph cells with

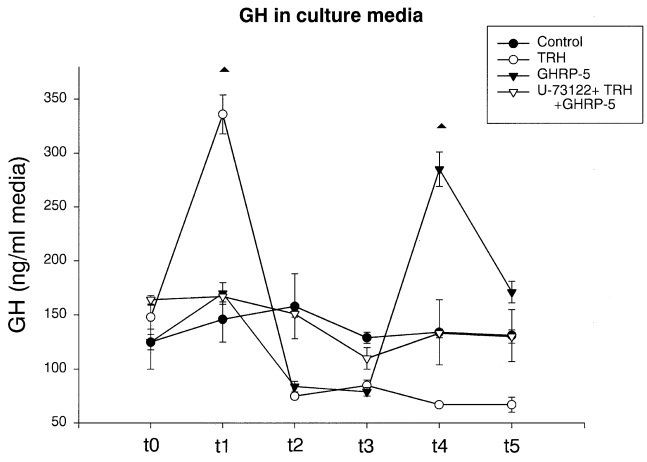
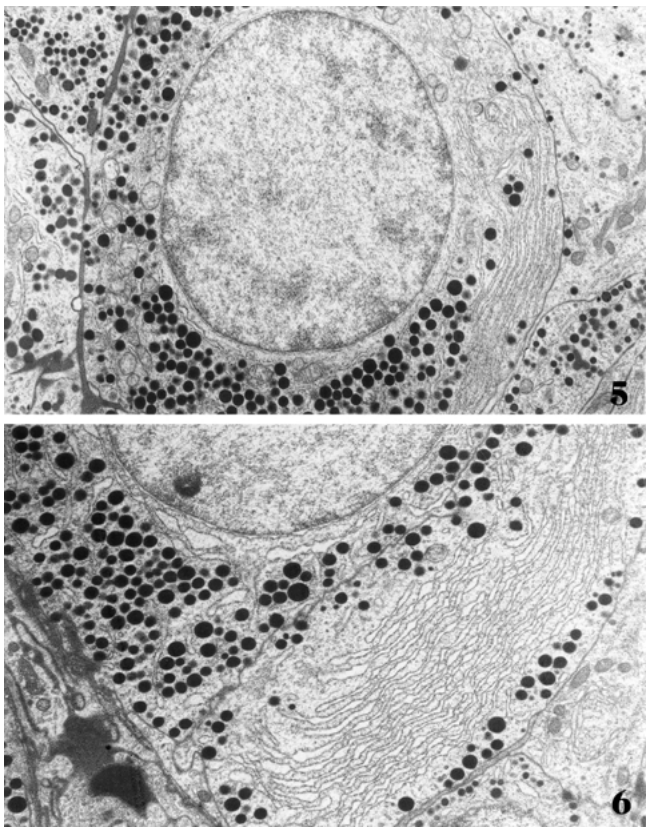
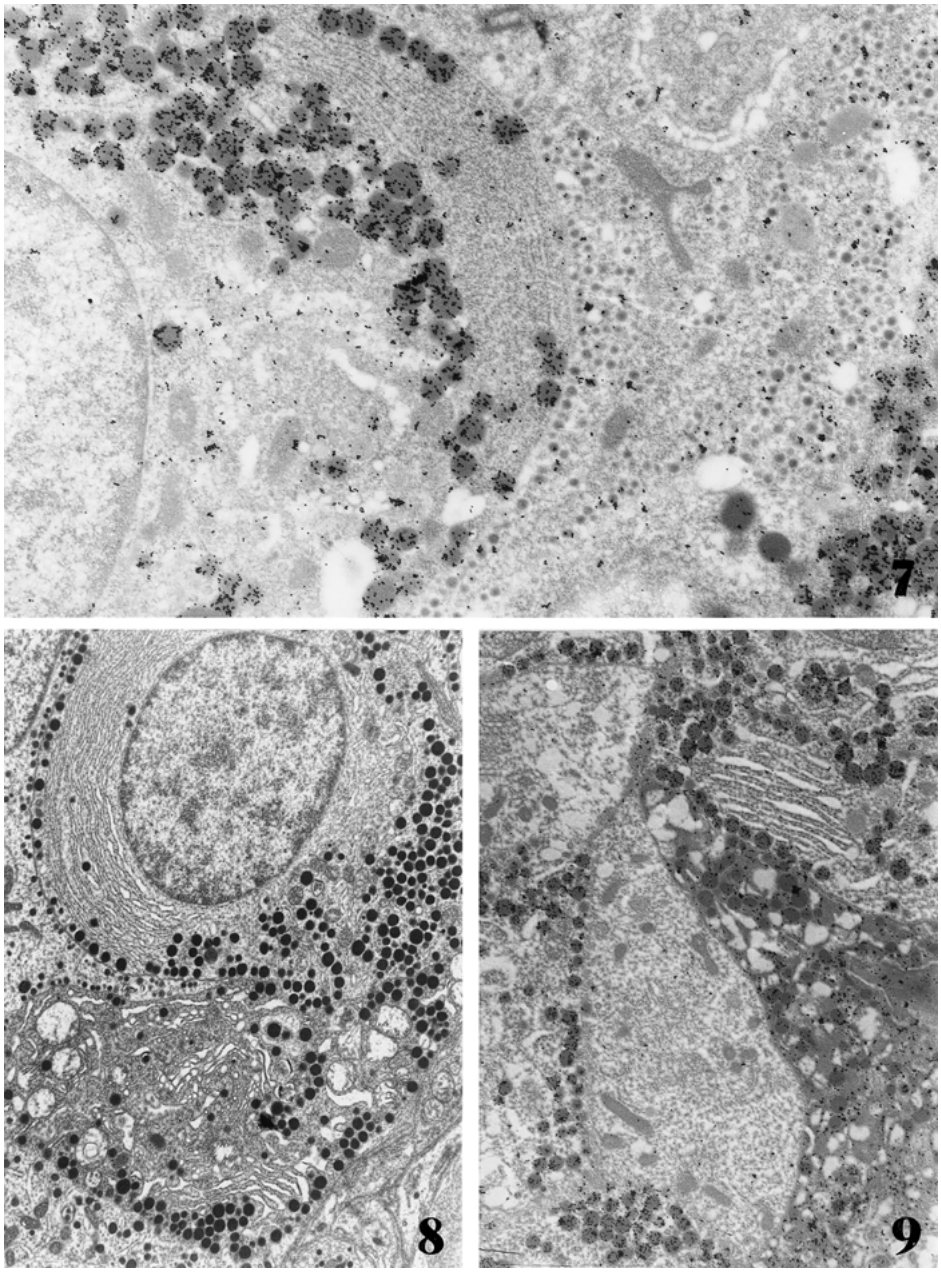


Figure 4. Secretion of GH from pituitary cell cultures treated with TRH, GHRP-5 and U-73122 + TRH + GHRP-5. A significant increase of GH secretion (▲$p < 0.001$) was produced by TRH at t1. The stimulatory effect of GHRP-5 on GH secretion was observed at t4 (▲$p < 0.001$).The preincubation with the PLC inhibitor, U-73122, prevented both stimulatory effect of TRH and GHRP-5 on GH release. Means ± SEM of three different experiments (6 wells/treatment/experiment). Significance was reported at $p < 0.05$ vs control wells. ANOVA followed by Tukey's test.



Figures 5 and 6. (5) Electron micrograph of typical somatotrophs in a control pituitary gland. The cytoplasm contains abundant mature round secretory granules ranging from 200–350 nm in diameter, and moderately developed RER and Golgi apparatus cisternae, ×12,600. (6) Somatotroph from GHRP-5 treated rats for 3 days. Two somatotrophs present a different ultrastructural organisation. On the right, a portion of somatotroph cytoplasm with a marked proliferation of RER membranes. Some granules occurring adjacent to cell membrane are often seen released by exocytosis. On the left, there is a somatotroph with numerous secretory granules stored in the cytoplasm, only few of them are seen adjacent to the cell membrane, ×13,800.



Figures 7–9. (7) Electron microscope immunocytochemistry of a pituitary gland section after 14 days of GHRP-5 treatment. Immunolabelled somatotrophs at different functional states are seen. At the centre of the micrograph, a somatotroph with smaller secretory granules contains granular material with variable electron density. Two cells on the left and on the right containing large acumulus of typical mature secretory granules can be observed, ×24,200. (8) An electron micrograph of a pituitary gland section of GHRP-5 treated rat for 14 days. Two somatotrophs at different functional states are seen. In the upper part, there is a somatotroph with cytological features of high activity characterised by a remarkable RER membrane and a depletion of secretory granules few remaining adjacent to the cell membrane. In the lower part, there is a somatotroph at advanced regression state, with high electron density of the cytoplasmic matrix and organelle swelling, ×9,600. (9) Electron immunocytochemistry of a pituitary gland from GHRP-5 treated rats for 14 days showing immunolabelled somatotrophs with different ultrastructural organizations. On the right, there is a somatotroph at an advanced stage of degeneration coexisting with other somatotrophs with normal ultrastructural characteristics, ×13,120.

dissimilar metabolic activity among other pituitary cell types (Figures 7–9).

Morphometry

Morphometric data of somatotroph populations treated for 3 and 14 days with GHRP-5 are shown in Table 1. The acute and chronic treatment with GHRP-5 did not modify the somatotroph cell density in relation to control rats. The areas of immunostained GH remained unchanged with respect to controls in all experimental groups.

Pit-1 mRNAs

The levels of pituitary pit-1 mRNA isoforms (4.1, 3.1 and 1.2 kb) after administration of GHRP-5 (12 μg/day/3 days)

Table 1. Morphometric data of somatotroph cell population in GHRP-5 treated rats for 3 and 14 days.

Treatment	Cell density ($Nr \times 10^2\ mm^{-2}$)	Immunostained area ($\mu m^2 \times 10^3\ \mu m^{-2}$)
Control	12.0 ± 0.1	5.5 ± 0.8
GHRP-5 (3 days)	9.0 ± 0.1	5.2 ± 0.6
GHRP-5 (14 days)	11.0 ± 0.1	6.1 ± 0.6

The acute and chronic treatment did not modify somatotroph cell density and the GH immunostained area. Results are expressed as mean ± SEM for three rats in each group. ANOVA followed by Tukey's test.

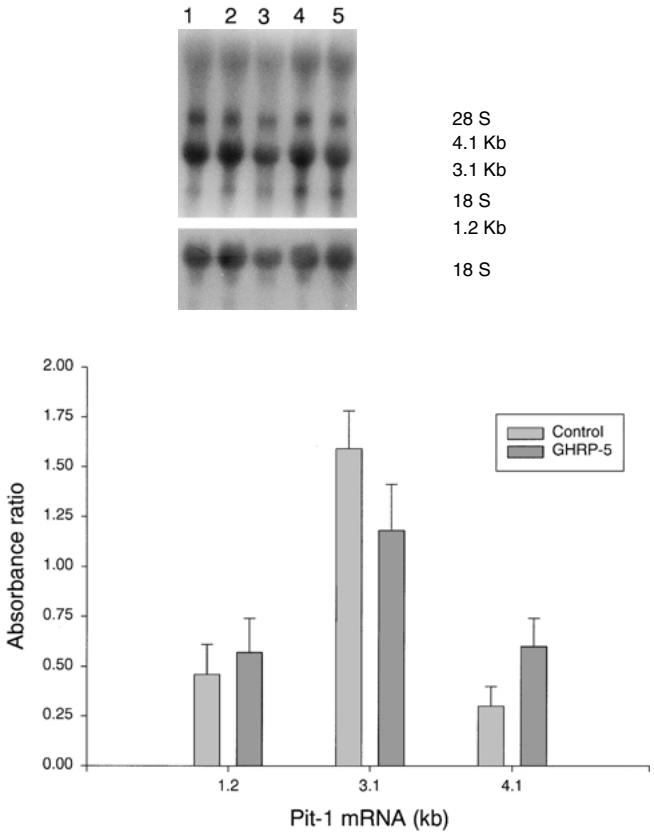


Figure 10. (A) Northern blot of pituitary GHF-1 mRNAs of rats treated during 3 days with GHRP-5. 20 μg total RNA were applied to each lane: Lanes 1 and 2, control; lanes 3–5, GHRP-5. Blots were hybridised with pit-1 (upper panel) and 18 S rRNA probes (lower panel). Arrows indicate the 28 S and 18 S rRNA markers. (B) Densitometric analysis of Northern blots. Data are given as the ratio between the absorbance of each pit-1 isoforms (4.1, 3.1 and 1.2 kb) signal and the absorbance of the 18 S rRNA signal in the same line. Results are expressed as mean ± SEM for eight rats in each group. ANOVA followed by Tukey's test.

are shown in Figure 10. The treatment did not alter the isoforms of pit-1 mRNA and the same effect was found in pit-1 total mRNA.

Discussion

The discovery of GHRPs and their mechanism of action have introduced new concepts in GH secretory control (Adams *et al.* 1998). A range of analogues with variable potency has been synthesised (Korbonits & Grossman 1995). Our interest was focussed in the study of GHRP-5, one of the earliest GHRPs synthesised. Although numerous studies have been reported on the effects of different GHRPs on somatotrophs secretory activity, direct evidence to understand the action of GHRP-5 on both somatotroph population and the mechanism of GH regulation is not available at present.

Treatment with GHRP-5 affected substantially the secretory activity of somatotrophs, yielding a significant increase of GH secretion, the levels of which were augmented significantly after short- and long-term treatment.

The administration of GHRP-5 for 3, 5 and 7 days induced several changes in somatotroph fine structure. A more active somatotroph cell subpopulation exhibited depletion of secretory granules and a marked development of those organelles engaged in protein synthesis and processing. These cells coexisted with somatotrophs with a lower secretory activity, characterised by a large pool of mature secretory granules stored in the cytoplasm. These results are in agreement with reports suggesting that rat pituitary somatotrophs comprised at least two functionally distinct subpopulations with different secretory response to GH secretagogues (Snyder *et al.* 1977). The coexistence of different somatotroph subpopulations with increased biosynthetic activity and a variable secretory responsiveness may explain the maintenance of pituitary GH content in rats treated with GHRP-5 for 3, 5 and 7 days in comparison to the control group.

In contrast, after GHRP-5 stimulation for 14 days there was a significant depletion of pituitary GH content when compared with rats treated for 7 days. These changes in pituitary GH were synchronous with the highest level of serum GH. The ultrastructural observations of pituitary cells reveals the occurrence of a different hyperactive somatotroph subtype characterised by storage of numerous small secretory granules. This GH cell subtype displayed signs of an enhanced intracellular hormone processing, with scarce mature secretory granules due to the fact that newly synthesised hormone was preferentially released. This alternative pathway of GH release can be extrapolated to what occurred in hyperstimulated lactotrophs where the PRL is synthesised in large amounts and discharged rapidly, bypassing the storage step in mature secretory granules (Torres & Aoki 1987, Diaz Gavier *et al.* 1999). In pituitary glands stimulated for longer periods, a number of somatotrophs were seen at various stages of cell death. The fine structure of degenerating somatotrophs suggested that the death process may reflect a regular cell renewal process in pituitary glands when submitted to a sustained stimulation (Haggi *et al.* 1986). Similar observations have been previously reported with other GH releasers (Soto *et al.* 1995, Bonaterra *et al.* 1998). The cytological modifications observed after GHRP-5 treatment have no strict correlation with morphometry, the results of which showed no significant changes between representative groups. This fact can be interpreted as due to the functional heterogeneity of somatotroph subpopulations in treated rats.

Different forms of GHS appear to have differential activities on GHS-R subtypes, probably following distinctive

signalling pathways in different species (Chen 2001). In cultured ovine somatotrophs, it has been reported that GHRP-2 binds to a putative GHRH-like receptor employing a signal transduction pathway similar to that used by GHRH, both activating adenylate cyclase to increase cAMP levels. However, this effect was not found in isolated rat pituitary cells. GHRP-6 has no direct effect on intracellular cAMP levels in either rat or ovine somatotrophs. (Wu *et al.* 1996). The GHRP-6 activity seems to be expressed on a site different to that used by GHRP-2 (Chen 2001) and the stimulation of GH secretion is induced through PLC activation, IP3 production and increase in intracellular free Ca^{2+} (Lei *et al.* 1995, Herrington & Hille 1994).

In order to evaluate the possible involvement of the adenylate cyclase–cAMP or PLC–IP pathway in the stimulation of GH secretion by GHRP-5, a specific blocker was tested. Preincubation with the specific PLC inhibitor, U-73122, arrested the stimulatory effect of GHRP-5. In contrast, the cAMP antagonist did not inhibit this action of GHRP-5 in cultured pituitary cells. Therefore, we suggest a participation of the PLC–IP pathway in the secretory response of somatotrophs to GHRP-5.

The somatotroph population changes under different experimental conditions. In a previous study from our laboratory, a significant expansion of the somatotroph population under GHRH stimulation was described. This finding correlated well with a marked rise of GH secretion (Torres *et al.* 1995). However, treatment with GHRP-5 for 3 and 14 days did not modify the somatotroph cell density, despite the significant increase in serum GH levels. The differential effects of GHRH and GHRP-5 on somatotroph proliferation may be correlated with a different mechanism of action. It is well-known that GHRPs and GHRH bind to specific receptors and display distinct signal transduction pathways (Casanueva & Dieguez 1999). The GHRH receptor is associated to adenylate cyclase–cAMP, which yielded conveying signals for cell growth and GH gene expression through pit-1 (Radovick *et al.* 1998). It has been suggested that pit-1 appeared to be involved in the cAMP regulation of the rGH promoter. The increase of the pit-1 mRNA levels after GHRH is dose and time dependent (Soto *et al.* 1995). The administration of GHRP-5 did not modify either the levels of the total mRNA for pit-1 or the pit-1 mRNA isoforms (4.1, 3.1 and 1.2 kb). Our results are in keeping with GHRP-6 studies performed in primary pituitary cell cultures where this hexapeptide had no effect on pit-1 mRNA levels (Soto *et al.* 1995). In accordance with what was suggested, GHRP-5 may act through the PLC–IP signalling pathway. Therefore, the different transduction system of both GHRH and GHRP-5 may be responsible for absence of GHRP-5 effect on pit-1 mRNA and somatotroph proliferation.

The importance of GHRPs has renewed interest in novel aspects of the regulation of somatotroph secretion. It is reported that GHRP-5 treatment is a time effective stimulator of GH secretion, inducing changes on somatotroph fine structure compatible with an increased secretory activity without changes in somatotroph cell density.

The benefit of GH as a metabolic modulator at different ages and in certain pathological conditions emphasises the interest in the therapeutical use of GHRPs for clinical deficiencies of GH.

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