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Influence of chronic treatment with the growth hormone secretagogue Ipamorelin, in young female rats: somatotroph response *in vitro*

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Summary. Growth hormone (GH) is secreted in the anterior pituitary gland by the somatotroph cells. Secretion is regulated by growth hormone releasing hormone (GHRH) and somatostatin. Morever, GH secretagogues (GHS) can exert a considerable effect on GH secretion. In order to determine the effects of chronic treatment with the GHS Ipamorelin on the composition of the somatotroph cell population and on somatotroph GH content, an *in vitro* analysis was performed of the percentage of somatotroph cells (% of total), the ratio of different GH cell types (strongly/weakly-staining) and individual GH content, in pituitary cell cultures obtained from young female rats receiving Ipamorelin over 21 days (Ipamorelin group) and the effects were compared with those of GHRH (GHRH group) or saline (saline group). The ultrastructure of somatotroph cells did not change, but the volume density of secretion granules was increased ($P<0.05$) by previous *in vivo* Ipamorelin or GHRH treatment. In 3-day basal pituitary cell monolayer cultures, the percentage of somatotroph cells showed no modifications between groups, nor was there any change in the ratio of strongly/weakly immunostaining GH cells. In the Ipamorelin group alone, *in vitro* treatment with Ipamorelin (10^{-8} M), or GHRP 6 (10^{-8} M), or GHRH (10^{-8} M) for 4 hours, increased the percentage of somatotroph cells, without modifying the ratio of strongly/weakly immunostained GH cells. Basal intracellular GH content in somatotroph cells over 4 hours was lower in the Ipamorelin group and the GHRH group than in the saline group. Only in the Ipamorelin group did Ipamorelin (10^{-8} M), GHRP 6 (10^{-8} M) and GHRH (10^{-8} M) prompt increased intracellular GH content. These data suggest that, at least in the young female rat, the GHS Ipamorelin is able to exert a dynamic control effect on the somatotroph population and on GH hormone content.

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Introduction

The biosynthesis and secretion of growth hormone (GH) in the anterior pituitary gland is under complex hormone regulation. It is regulated by two hypothalamic hormones, somatostatin and growth hormone-releasing hormone (GHRH), which oppose one another and act through distinct membrane receptors. Somatostatin inhibits GH release via a family of GTP-binding protein-coupled membrane receptors (Jakobs et al., 1983; Yamada et al., 1992; Buscail et al., 1994) and GHRH activates GH release through a stimulatory G-protein-coupled receptor (Dhanasekaran et al., 1995). GH regulates its own release through negative feedback loops acting at the level of the hypothalamus, via GH receptors (Burton et al., 1992; Bennet et al., 1995), and at pituitary level (Jiménez-Reina et al., 2000; Peng et al., 2001).

In addition to GHRH and somatostatin, other neuropeptides can exert important modulatory effects on GH secretion, by acting either directly on the pituitary gland and/or at hypothalamic level. Major neuropeptides that affect GH release are the synthetic molecules termed GH secretagogues (GHS) (Giustina and Veldhuis, 1998). GHS act through membrane receptors that increase intracellular concentrations of inositol triphosphate, and enhance the activity of protein kinase C (Cheng et al., 1991; Herrington and Hille, 1994; Lei et al., 1995). Recently, an endogenous peptide ligand for the GHS receptor, ghrelin, was purified from the rat stomach and subsequently cloned (Kojima et al., 1999)

Synthesis and release of GH in the pituitary takes place in somatotroph cells, a cell population traditionally reported as heterogeneous on the basis of both physical cell-separation methods (Snyder et al., 1977; Hall et al., 1982; Lindstrom and Savendahl, 1996), and ultrastructural features (Kurosumi, 1986; Takahasi,

1991). Changes take place both in cell morphology and in the relative proportions of the different types of somatotrophs under different physiological and experimental conditions, such as hormone treatment (González-Parra et al., 2000), chronic IGF-1 administration (Pellizas et al., 2000), GH autoregulation (Asa et al., 2000; Jiménez-Reina et al., 2000), pituitary hyperplasia (Vidal et al., 2001), and in genetic and transgenic models with perturbations of individual components of the GH regulatory system (Frohman et al., 2000).

Since GHRH plays an essential part in regulating the functional and proliferative activity of somatotroph cells (Giustina and Veldhuis, 1998; Mayo et al., 2000, Frohman et al., 2000), thus affecting the morphology and composition of the somatotroph cell population (Dobado-Berrios, 1996a), it may be hypothesized that GHS also play a major role in the dynamic control of the somatotroph cell population.

The present study aimed to ascertain, *in vitro*, the influence of chronic administration of a recently-synthesized GHS, Ipamorelin (Raun et al., 1998), on the composition of the somatotroph cell population and on the individual GH content of these cells in young female rats.

Materials and methods

Animals

Sixty-day-old female Wistar rats were used. Animals were given free access to rat chow (IPM R-20, Letica S.A., Hospitalet, Barcelona, Spain) and tap water. They were housed individually and kept under conventional conditions (temperature: 22±2 °C 12:12 h light/dark cycle with lights on at 06.30) in the laboratory animal center of the School of Medicine in Córdoba. The rats were cared for, and used, in accordance with European Council directive 86/609/ EEC (24/11/1987).

Experiment design

The duration of the experiment was 21 days, and rats were weighed daily. They were divided into three groups (n=6): the Ipamorelin-pretreated group (Ipamorelin group); the GHRH-pretreated group (GHRH group); and the saline-pretreated group (saline group). In the *Ipamorelin-pretreated group*, animals were injected subcutaneously (sc) daily at 9 am with 100 μg/kg body weight of diacetate salt of Ipamorelin (Novo-Nordisk, Denmark). In the *GHRH-pretreated group*, animals were injected sc daily at 9 am with 10 μg/kg body weight of $GHRH_{1-29}$ (Serono Labs., Spain). The *Saline-pretreated group* received the same treatment as the above groups, but received saline serum instead of Ipamorelin or GHRH.

After the last injection, animals were decapitated at 1 pm and pituitaries were removed. The posterior pituitaries were discarded, and the anterior pituitaries diced into small pieces for cell dispersion or for ultrastructural study.

Ultrastructural and stereological analysis

The small pieces were fixed with fixative solution (1% glutaraldehyde and 2% formaldehyde in 0.2 M cacodylate buffer) for 2 hours, and postfixed with 1% osmium tetroxide for 1 hour. Following dehydration with ethanol they were embedded in Durcupan, ACM. Sections of approximately 300 nm were collected on 300-mesh nickel grids. GH cells were identified on ultrathin sections by immunogold (Roth, 1983). After etching with a saturated aqueous solution of sodium metaperiodate, rabbit serum anti-rat GH (UCB Bioproducts, diluted 1:1000, 20h at 4 °C) as the first antiserum, and goat anti-rabbit IgG conjugated with colloidal gold (15 nm in diameter) (Janssen Life Sci, Glen, Belgium; diluted 1:10) were used. For washing and dilution of sera, tris buffer (0.05 M, pH 7.4) (TRIS) and 1% albumin bovine serum (BSA) in TRIS were used, respectively. After immunostaining, grids were stained with uranyl acetate and lead citrate. Preparations were examined using a Phillips CM 10 electron microscope and 40 micrographs were taken per animal (20 at x1950 and 20 at x6400). The immunoreaction specificity for rat GH was analysed by omission of the specific antiserum, replacing the antiserum with normal rabbit serum, and adsorption of the specific antiserum with its homologous (rat GH) or heterologous (rat prolactin) hormone.

The volume density of nucleus (VvNu), cytoplasm (VvCy) and secretion granules (VvGr; % cytoplasm), calculated by the formula of Weibel and Bolender (1973), were selected as stereological parameters.

Cell dispersion and cell culture

Pituitary cell cultures were prepared according to Jiménez-Reina et al. (2000) Briefly, pituitaries were diced into small pieces and dispersed in Erlenmeyer flasks with a mixture of 0.02 g/l collagenase (Type V, Sigma Chemical Co.) and 0.01 g/l trypsin 1:250 (Sigma Chemical Co.) in Dulbecco Modified Eagle's Medium (DMEM) (Sigma Chemical Co.). The Erlenmeyer flask was placed in a humidified atmosphere of 5% CO_2 in air, at 37 °C, for 60 minutes. The cell suspension obtained was gently pipetted for ten minutes, washed in DMEM, and centrifuged (100 g for 10 min) twice. The cellular pellet was washed again, centrifuged, and resuspended in 10% fetal bovine serum (FBS)-DMEM. Cell yield and viability were checked by the trypan blue exclusion method: cell yields were $1.64\pm0.12\times10^6$/pituitary, and cell viability was over 90% in all cell dispersions. The cellular suspension was dispensed into tissue culture consisting of 96 wells (2×10^4 cells/well/200 μl 10% FBS-DMEM) and incubated in a humidified atmosphere of 5% CO_2 in air, at 37 °C, for 3 days. Monolayer cultures were washed twice with DMEM and fresh

serum-free-DMEM was added. After 4 hours' incubation at 37 °C and 5% CO_2, with DMEM, or with Ipamorelin (10^{-8} M), or with GHRH (10^{-8} M), or with GHRP-6 (10^{-8} M), the medium was decanted. Treatments were performed in sextuplicate. Monolayer cultures were fixed with Bouin fluid (saturated solution of picric acid, 71.4 ml/100 ml; formaldehyde 35%, 8.8 ml/100 ml; and acetic acid, 4.8 ml/100 ml) for 30 minutes, and washed three times with PBS.

Immunocytochemistry

Monolayer cultures were immunostained for GH, using the extravidin-peroxidase method (EXTRA-3, Sigma Chemical Co). Anti-rat GH rabbit serum (UCB Bioproducts, 1:1000), was used. Endogenous peroxidase was blocked with H_2O_2 (3%). For washing and dilution of sera, phosphate buffer (PBS) was used. The reaction was developed in freshly prepared 3,3' - diaminobenzidine (Sigma, 0.025% in PBS buffer containing 0.03% H_2O_2). The immunoreaction specificity for rat GH was measured by omission of the specific antiserum by replacing the antiserum with normal rabbit serum, and by preadsorption of the specific antiserum with its homologous (rat GH) or heterologous (rat prolactin) hormone. The proportion of immunostained somatotroph cells was then calculated in each well.

Cell immunoblot assay (CIBA)

Pieces (1.5x1.5 cm) of polyvinylidene difluoride transfer membrane (IMMOBILON™ Millipore) were placed in multiwell plates. Three-day pituitary cell monolayer cultures were incubated with 0.01 g/l trypsin 1:250 (Sigma Chemical Co.) at 37 °C 95% air-5% CO_2 for 30 minutes, in order to obtain pituitary cell suspensions, which were placed on membranes ($4x10^3$ cells/50μl DMEM) and preincubated at 37 °C 95% air-5% CO2 for 30 minutes. Then, either 50 μl DMEM alone, or with Ipamorelin (10^{-8} M), or with GHRH (10^{-8} M), or with GHRP6 (10^{-8} M), was added and incubated for 4 hours. Thereafter, the transfer membranes were fixed with Bouin fluid for 30 minutes, and then immediately washed three times with PBS.

The standard curve and the estimated amount of GH content from each isolated somatotroph cell were recorded following the method of Dobado-Berrios et al., (1992). Briefly, various concentrations of purified rat GH (GH-RP-2, NIADK-NIH) dissolved in bicarbonate buffer-ClNa (1 μl drops), containing between 0.78 ng and 25 ng of rat GH (increase x2), were fixed on IMMOBILON™ membranes.

Transfer membranes were immunostained using the extravidin-peroxidase method (EXTRA-3, Sigma Chemical Co) modified from the method described by Kendall and Hymer (1987). Anti-rat GH serum (Biogenesis Ltd.) was used at a final dilution of 1:1000. The specificity of CIBA for rat GH was examined by removing the antiserum, replacing the antiserum with normal rabbit serum, and preadsorbing the antiserum with rat GH (Biogenesis Ltd.) at 4 °C for 24 hours. Crossreactivity of the primary antiserum to prolactin was also checked by incubating, in the presence of anti-rat GH, Immobilon membranes in which 1 μl droplets contained 25 ng/μl of purified rat prolactin.

Image analysis

Image analysis was performed as described previously (Dobado-Berrios et al., 1992; Jiménez-Reina et al., 2000). GH immunostained cell blots were measured using a conventional Nikon microscope equipped with a light source stabilized at 5 volts and connected via a Hitachi television camera to an image-analysis system consisting of a computer (Pentium/Intel) with a digital card equipped with VISILOG 4.1 software (Noesis, Orsay, France) belonging to the University of Córdoba Cell Biology Department, an additional monitor (Fujitsu, Japan) and a digital board (KURTA). In each membrane, the cellular area (in μm^2) and optical density (OD, in arbitrary units of 60 GH-immunostained cell blots) were measured. Measurements of standard intensities define a variable that expresses the amount of hormone immobilized per unit area or immobilization density (pg/μm^2) (Dobado-Berrios et al., 1992); this enabled determination of GH content (in terms of pg/cell) in each somatotroph cell.

Statistical analysis

Results were expressed as a mean ± standard error of the mean (SEM). A minimum of six animals were used in each experimental and control group. Experiments were repeated three times with different groups of animals. The statistical significance of inter-group differences was determined by ANOVA, and was accepted at P<0.05. The Student-Newman-Keuls test was used after ANOVA. When the normalized test failed, the Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney Rank Sum Test, were performed.

Results

Body weight

Table 1 shows the percentage gain in body weight

Table 1. Body weight gain (%) in 60-day old female rats, due to subcutaneous treatment for 21 days with saline serum, Ipamorelin (100 μg/Kg/day) or with GHRH (1μg/Kg/day).

	DAY 7	DAY 14	DAY 21
Saline group	4.13	9.81	18.10
Ipamorelin group	7.72	13.93	22.94
GHRH group	6.67	13.07	21.49

(BW) with respect to the start of treatment. From the third day, the effect of Ipamorelin and GHRH on BW gain was immediately apparent and more marked than in the saline group ($P<0.05$); this difference was maintained up until day 21. The greatest gain in BW due to Ipamorelin and GHRH occurred during the first week of treatment; BW in the second and third weeks was similar for all three groups studied (saline group, Ipamorelin group and GHRH group).

Ultrastructure and stereology

Ultrastructural examination of somatotroph cells showed that these cells were similar in each group (saline, Ipamorelin and GHRH groups). Based on the size of the secretion granules, two types of somatotroph cells could be seen: cells with large secretory granules (diameter, 250-350 nm) and cells with large and small secretory granules (diameter, 250-350 and 100-150 nm). In both cases, cells were round or oval in shape, with rounded nuclei. GH cells with larger secretion granules were densely granulated, while somatotroph cells with smaller secretion granules were sparsely granulated (Fig. 1). Therefore, somatotroph cells were similar to type I and type Il/Ill cells, respectively, as described by Kurosumi (1986) and Takahashi (1991).

Values for VvNu were greater in the Ipamorelin group than in the saline and GHRH groups (36.53±1.43 vs 29.06±2.68 and 27.64±2.49, in %; $P<0.05$), and VvCy was therefore lower (63.46±1.43 vs 70.93±2.68 and 72.35±2.49, in %; $P<0.05$) (Fig. 2A).

Values for VvGr (as a % of cytoplasmic volume) were greater in the Ipamorelin and GHRH groups than in the saline group (20,89±1.57 and 23.03±2.16 vs 16.45; ($P<0.05$) (Fig. 2A). Stereological analysis of type II/III somatotrophs showd that VvGr did not exceed 14% of cytoplasmic volume. A VvGr vaue of 14% was thus randomly taken as the limit for separating strongly-staining from weakly-staining somatotroph cells. *In vivo* treatment led to an increase in the percentage of strongly-staining somatotroph cells. In the saline group, a ratio of 33% weakly-staining to 67% strongly-staining somatotrophs was observed, compared with a ratio of 24%/76% for the Ipamorelin and GHRH groups (Fig. 2B).

Monolayer cultures

After 3 days, immunocytochemical analysis of 4-hour monolayer cultures revealed somatotroph cells as either strongly (type I-like GH cells of Kurosumi) or weakly (type Il/Ill-like GH cells of Kurosumi) immunostained (Fig. 3). The proportion of immunostaining somatotroph cells is shown in Fig. 4. In control treatment, the percentage of GH-immunostained cells was lower in the Ipamorelin group than the saline group ($P<0.05$) (Fig. 4). *In vitro*, Ipamorelin, GHRP 6, and GHRH prompted no variation in the somatotroph population in either the saline or the GHRH groups, but variation was observed in the Ipamorelin group, where the % of somatotroph cells increased ($P<0.05$) (Fig. 4). No differences were observed in the ratio of strongly/weakly staining somatotrophs in any of the groups (Fig. 4).

Cell immunoblot assay

Basal intracellular GH content in somatotroph cells over 4 hours in IMMOBILON™ was lower in the Ipamorelin group and GHRH group than in the saline

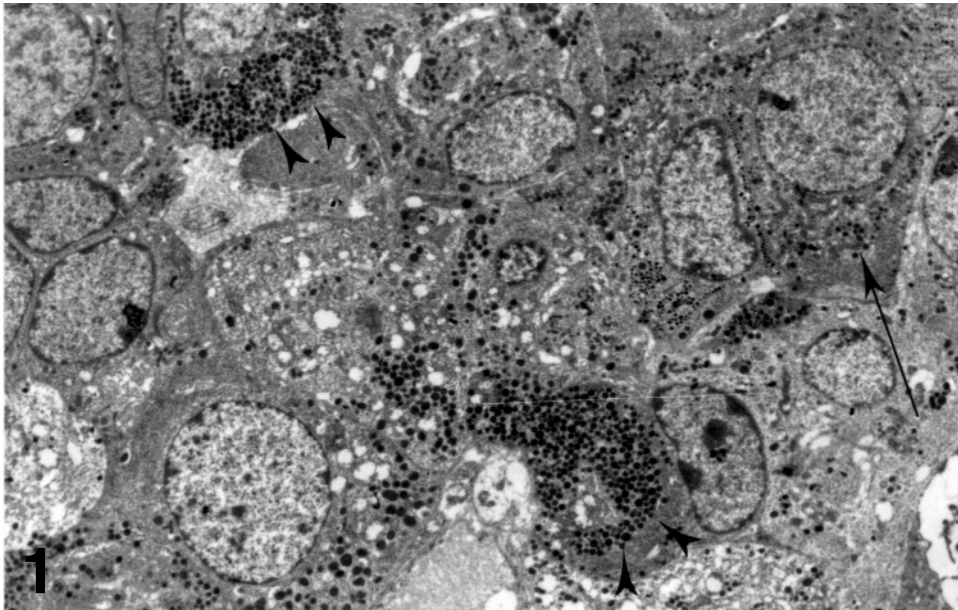

Fig. 1. Ultrastructural aspects of rat pituitary cells (Ipamorelin group). Type I (arrowheads) and type II/III (arrow) somatotroph cells. x 3,000

group (6.05±0.76 and 5.75±0.63 vs 10.4±1.23 pg GH/cell, respectively; $P<0.05$) (Fig. 5). Only in the Ipamorelin group did Ipamorelin (10^{-8} M), GHRP 6 (10^{-8} M) and GHRH (10^{-8} M) prompt increased intracellular GH content (6.05±0.76 -control- vs 9.41±1.15 -Ipamorelin-, 10.27±0.94 -GHRP 6- and 10.99±1.13 -GHRH-, pg GH/cell; $P<0.05$) (Fig. 5).

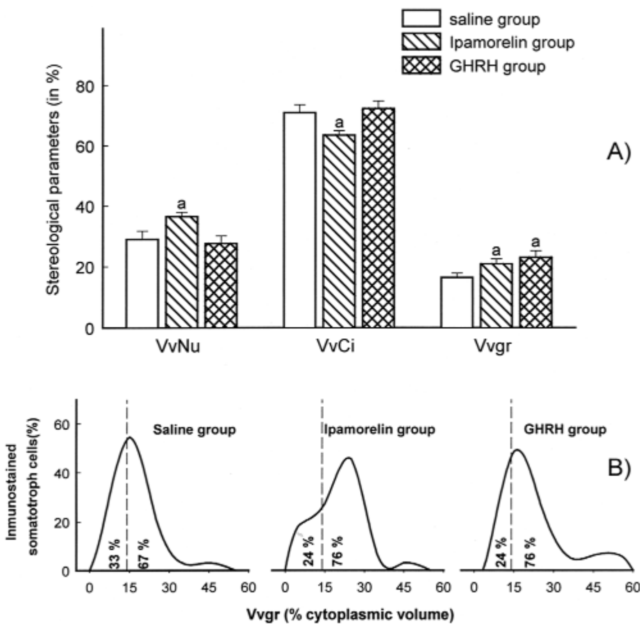

Fig. 2. A. Stereologic parameters of young female rat somatotroph cells. VvNu: Volume density of nucleus; VvCy: Volume density of cytoplasm; VvGr: Volume density of secretory granules. a: $P<0.05$ vs saline group. **B.** Histogram of VvGr for all groups. The vertical line is set randomly at 14% of cytoplasmic volume. This line separates weakly-staining (left of line) from strongly-staining somatotroph cells (right of line).

Discussion

Composition of the somatotroph cell population is heterogeneous, and chiefly comprises two GH cell types, distinguished either by cell separation techniques (Snyder et al., 1977; Hall et al., 1982; Lindstrom and Savendahl, 1996) or in terms of ultrastructural features (Kurosumi, 1986; Takahasi, 1991). Intracellular GH content therefore varies depending on the type of somatotroph cell involved, and is also modified by the action of the hormones controlling GH secretion, by the age of the animal (Dobado-Berrios et al., 1996a,b) and under different physiological and experimental conditions (Giustina and Veldhuis, 1998). It has not, as yet, been established whether these somatotroph cell subpopulations represent different phases of the same cell (Kazemzadeh et al., 1992; Dobado-Berrios et al., 1996b) or whether they are in fact distinct, differently-regulated cell types. If they are indeed a single cell type, then a dynamic process must be involved (Dobado-Berrios et al., 1996b), in which GHRH wields particular influence due to its proliferative and stimulative effects on somatotroph cells (Giustina and Veldhuis, 1998; Mayo et al., 2000). Although some studies have analyzed the effect of GHRP 6 on GH release in somatotroph subpopulations (Lindstrom and Savendahl, 1996; Mitani et al., 1996), little is known about the influence of chronic GH secretagogue administration on either the somatotroph cell population or on cell GH content.

This study showed that chronic administration of the GHS Ipamorelin in young female rats, increased

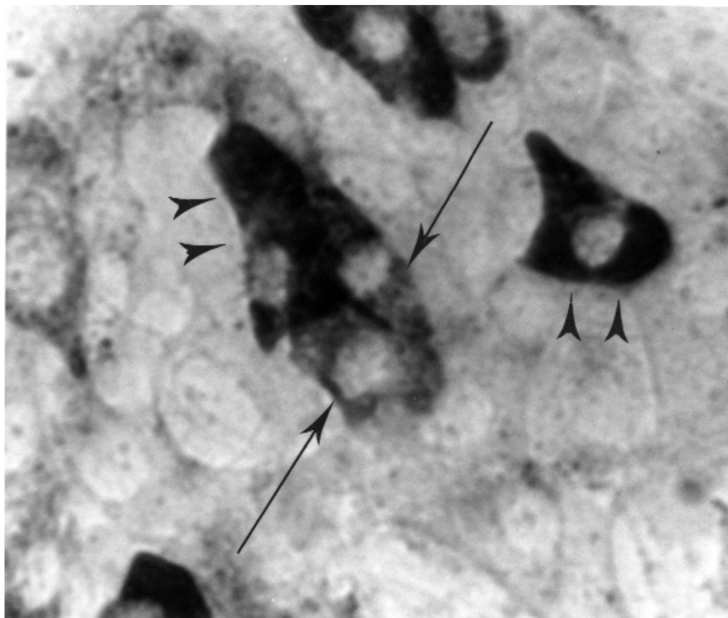

Fig. 3. GH-immunostained cells in 3-day young female rat pituitary cell monolayer cultures (Ipamorelin group). The GH-immunostain was strong (arrowheads) or weak (arrows). x 915

somatotroph cell GH content, and that, *in vitro*, the proportion of somatotroph cells was not modified. However, pretreatment with Ipamorelin led to an increase in somatotroph cell numbers following *in vitro* stimulation with GHRH, GHRP 6 or Ipamorelin, although the ratio of strongly/weakly immunostained GH cells was not modified.

Chronic *in vivo* treatment with Ipamorelin or GHRH did not modify the ultrastructure of somatotroph cells in young female rats, since two cell types, type I and type II/III, similar to those described by Kurosumi (1986) and Takahashi (1991) were found. However, both Ipamorelin and GHRH prompted an increase in the amount of GH cell secretory granules and thus an increase in the percentage of strongly immunostaining somatotroph cells. These findings differ from those reported by Stefaneanu et al. (1993) and Torres et al. (1995) following treatment with GHRH, who report the presence of sparsely granulated somatotroph cells and the prominence of organelles involved in hormone synthesis. The difference in findings may be due to the fact that these authors used higher GHRH doses (up to 35 times higher) over shorter periods (maximum 7 days).

The present study used 3-day pituitary cell cultures, since GH release is similar to that produced by recently scattered somatotroph cells (Wilfinger et al., 1979) and, morever, responsiveness to GHRH is reported to be greatly enhanced after 3 days' culture (Ohlsson et al., 1988). After three days *in vitro*, under baseline conditions, chronic pretreatment with GHRH was not found to modify either the percentage of somatotroph cells or the ratio of strongly/weakly-staining somatotroph cells, while chronic pretreatment with Ipamorelin led *in vitro* to continued stimulation of GH release and, consequently, to a smaller proportion of weakly immunostained GH cells; some somatotroph cells contained so few secretory granules that they were not observable by immuncytochemistry.

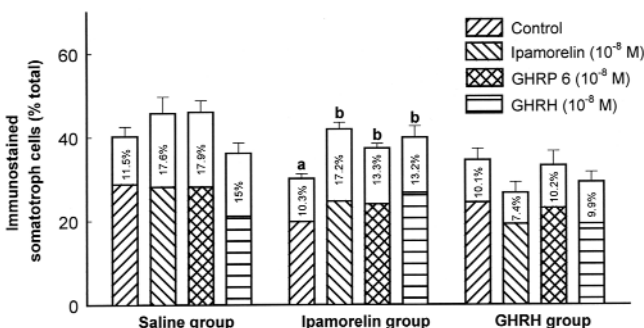

Fig. 4. GH-immunostained cells (% total) in chronic pretreated young female rat pituitary cell monolayer cultures. Chronic pretreatments were administered using saline serum (saline group), or with Ipamorelin (100 μg/kg/day -Ipamorelin group-), or with GHRH (10 μg/kg/day -GHRH group-). After 3 days, monolayer cultures were treated with Ipamorelin, GHRP6 or GHRH for 4 hours. Staked bars show the ratio of strongly (lower bar) to weakly (upper bar) GH-immunostained cells. a: $P<0.05$ vs control of saline group. b: $P<0.05$ vs control of Ipamorelin group.

In animals pretreated with Ipamorelin, but not with GHRH, somatotroph cells were able to increase this percentage *in vitro* after stimulation both with GHS (Ipamorelin or GHRP 6) and with GHRH. This increase is achieved at the expense of weakly immunostained somatotroph cells in the case of GHS stimulus, and of strongly immunostained somatotroph cells in the case of GHRH stimulus. It would therefore appear that the different intracellular routes of GHS (Cheng et al., 1991; Herrington and Hille, 1994; Lei et al., 1995) and GHRH (Barinaga et al., 1985) favor the proliferation of one or other cell type. Strikingly, pretreatment with GHRH failed to prompt an increase in the percentage of somatotroph cells, despite its well-documented ability to stimulate GH cell proliferation (Bertherat et al., 1995), probably because the dosage and frequency of administration used here caused a certain degree of desensitization of these cells (Kovacs et al., 1994; Sato et al., 1994).

Although *in vivo* administration of Ipamorelin and GHRH leads to an increase in the amount of secretory granules in somatotroph cells, after three days' culture intracellular GH content (measured by CIBA) is lower in these two groups than in animals pretreated with saline. Since releasing activities have a significant linear dependence on intracellular GH contents (Dobado-Berrios et al., 1996a,b) and there is data to show that pituitary cells *in vitro* display a program of secretory activity previously predefined in vivo (Castaño et al.,1994; Jiménez-Reina et al., 2000), our results suggests that chronic treatment with the GHS Ipamorelin is able to preserve the *in vitro* responsiveness of somatotroph cells to both GHS and GHRH.

In conclusion, the present results show that chronic treatment of young female rats with the GHS Ipamorelin maintains somatotroph responsiveness *in vitro* to GHS (Ipamorelin or GHRP 6) or to GHRH. This responsiveness is eliminated in animals chronically pretreated with GHRH. These results may be due to activation of weakly GH-immunostained cell proliferation following pretreatment with Ipamorelin, or strongly GH-immunostained cell proliferation following

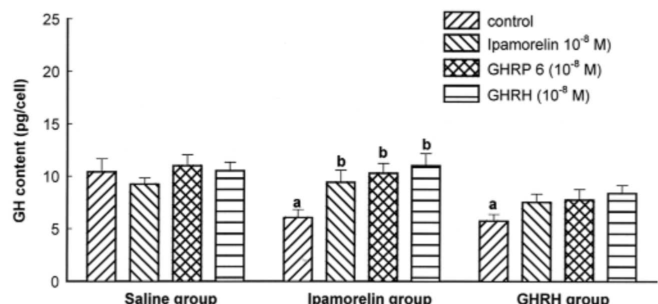

Fig. 5. Individual GH content of young female rat pituitary cell monolayer cultures. After 3 days, monolayer cultures were treated with Ipamorelin, GHRP6 or GHRH for 4 hours. a: $P<0.05$ vs control of saline group. b: $P<0.05$ vs control of Ipamorelin group.

pretreatment with GHRH: the GHS Ipamorelin thus exerts a positive dynamic control of the somatotroph population.

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