Endocrine, *vol. 17, no. 2, 91–102, March 2002* 0969–711X/02/17:91–102/$13.00

Differential Secretion of Chicken Growth Hormone Variants After Growth Hormone–Releasing Hormone Stimulation In Vitro

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Variants of growth hormone (GH) are present in most vertebrates. Chicken GH (cGH) undergoes posttranslational modifications that contribute to its structural diversity. Although the 22-kDa form of GH is the most abundant, some other variants have discrete bioactivities that may not be shared by others. The proportion of cGH variants changes during ontogeny, suggesting that they are regulated differentially. The effect of growth hormone–releasing hormone (GHRH) on the release of cGH variants was studied in both pituitary gland and primary cell cultures, employing sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blotting, and densitometry. GHRH (2 n*M*, 2 h) stimulated the secretion of most of the size variants of cGH although the amplitude of increase was not equal for each one. A differential effect on the secretion of GH size variants, particularly on the 22- (monomer) and 26-kDa (putatively glycosylated) cGH isoforms was found in both systems. In the whole pituitary culture, the proportion of the 26-kDa immunoreactive cGH increased 35% while the 22 kDa decreased 31% after GHRH treatment in comparison with the controls. In the primary cell culture system, the proportion of the glycosylated variant increased 43% whereas the monomer and the dimer decreased 22.26 and 29%, respectively, after GHRH stimulation. Activators of intracellular signals such as 1 m*M* 8-bromo-cAMP and 1 µ*M* phorbol myristate acetate had a similar effect to that obtained with GHRH. The data support the hypothesis that GH variants may be under differential control and that GHRH promotes the release of a glycosylated cGH variant that has an extended half-life in circulation.

Key Words: Growth hormone; growth hormone–releasing hormone; secretion; variants.

Received October 12, 2001; Revised December 18, 2001; Accepted December 19, 2001.

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Introduction

Pituitary growth hormone (GH) has many functions in all vertebrates. It has been suggested that the functional diversity of this hormone may reflect its molecular heterogeneity. Although monomeric (22 kDa) GH is the major form of the hormone in the pituitary, several structural variants are present in most species *(1–4)*. This molecular heterogeneity results from gene duplication, alternative splicing of GH mRNA, and/or posttranslational modifications of the proteins (e.g., glycosylation, phosphorylation, proteolytic cleavage, amidation). Biologic activities in some of these forms have been established, e.g., those described for the product of the hGH-V gene *(5–8)*, the 20-kDa hGH *(9–16)*, the product of GH-II gene *(17–19)*, and the 15-kDa fragment of chicken GH (cGH) *(20)*. Some forms of GH have also been shown to exhibit differential bioactivities in humans, bovines, and birds *(2,20–23)*. In the chicken, two different charge variants share equipotent growth-promoting activities, whereas they show a differential effect on lipid metabolism *(23)*. The presence of GH variants in the circulation has also been established *(24,25)*, which suggests that they are released from the pituitary and hence may have physiologic roles. Furthermore, there are some data indicating that the expression, processing, and release of some GH variants might be under differential control in diverse physiologic states *(26,27)*, analogous to the differential regulation described for some prolactin (PRL) variants in rodents and turkeys *(28–31)*.

Pituitary GH synthesis and release is regulated by an array of interacting, multihierarchical factors that dynamically modify somatotroph function. The influence of these factors may differ during ontogeny, sexual maturation, and senescence according to physiologic state and extrinsic and intrinsic stimuli *(32)*. As in most vertebrate species, pituitary GH release in chickens is controlled by stimulatory and inhibitory factors from the hypothalamus. Growth hormone–releasing hormone (GHRH) and thyrotropin-releasing hormone (TRH) are thought to be the primary physiologic GH secretagogues (GHSs) in birds *(33)*, while somatostatin (SRIF) decreases GH secretion, possibly by antagonizing the effects of GHRH and TRH. In vivo studies have shown that mammalian GHRH is an effective GHS

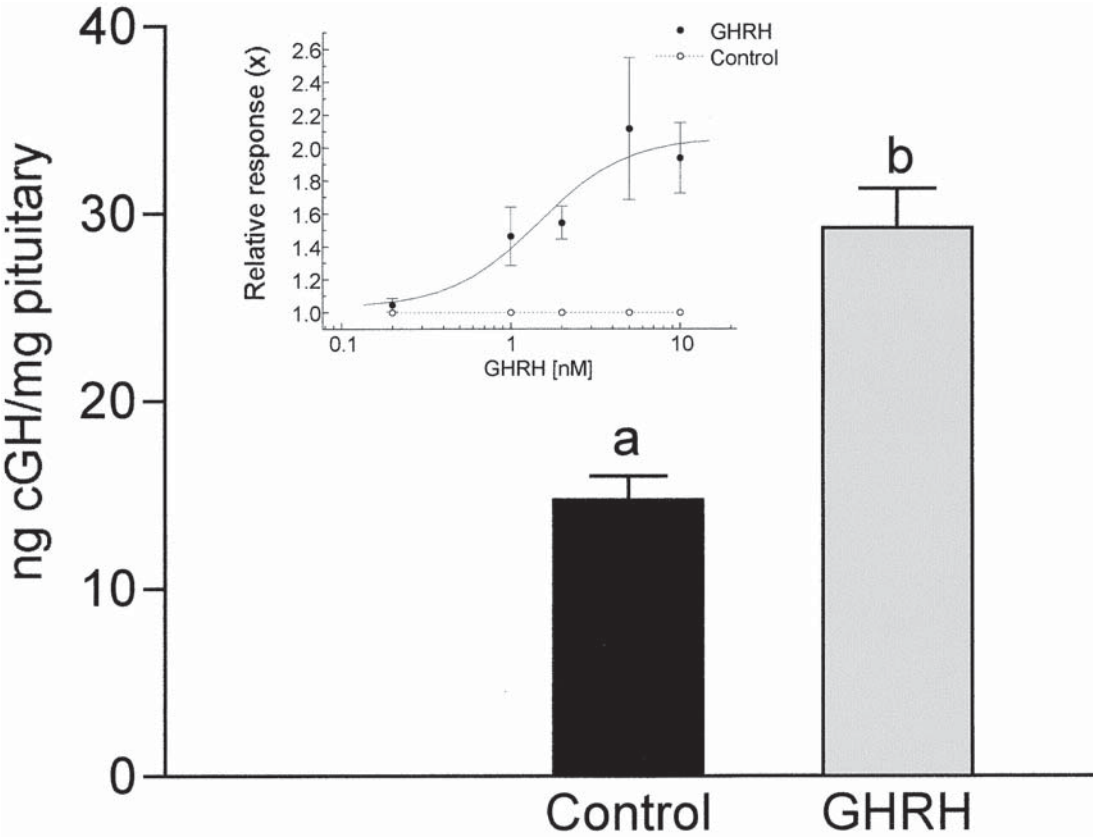

Fig. 1. Determination of total ir-cGH by ELISA in pituitary culture media after stimulation with 2 n*M* GHRH incubated for 2 h in normal Earle's solution. (**Inset**) Dose-response evaluation of activity of GHRH on release of ir-cGH. Bars correspond to 10 different experiments done in triplicate while the inset corresponds to seven experiments done also in triplicate (mean ± SEM). Different letters mean a statistical significance ($p < 0.001$).

in both young and adult birds *(34–37)*. TRH is also a very effective GHS in young chicks but rarely stimulates GH secretion in adult chickens *(33,35,38)*. Both GHRH and TRH stimulate GH release from chicken pituitary glands and adenohypophyseal cells in vitro *(34,39,40)*. The influence of these secretagogues on the release of different GH moieties is, however, uncertain.

To understand further the physiologic significance of GH molecular heterogeneity, we have assessed the release of GH size variants from pituitary tissue and primary pituitary cell cultures in response to GHRH and secretagogue analogs that stimulate the cyclic adenosine monophosphate (cAMP) and the protein kinase C (PKC) routes involved in the GHRH signal transduction pathway.

Results

Whole Pituitary Culture

The effect of GHRH on the release of total immunoreactive (ir)-cGH, measured by enzyme-linked immunosorbent assay (ELISA), is shown in Fig. 1 (inset). A dose-dependent response was observed when the tissue was exposed for 2 h to different concentrations (0.2, 1, 2, 5, and 10 n*M*) of the secretagogue (EC_{50} = 1.5 ± 0.8 n*M*). At a dose of 2 n*M*, GHRH increased cGH release 1.9 times higher (29.2 ± 1.8 vs 14.7 ± 1.1 ng/mg of pituitary, respectively; $p < 0.001$) (Fig. 1); this dose was used in subsequent studies.

The ir-cGH size variants in the culture media were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (NRC) and then immunostained with a polyclonal antibody after Western blotting. Figure 2A shows that several ir-cGH bands were detected, with the following apparent mol wt: 66, 60, 50, 44, 26, 22, and 15 kDa. It is also clear that the immunoreactivity of the cGH bands was increased in the media obtained after stimulation with 2 n*M* GHRH for 2 h in comparison with that observed in the control experiment, since the densitometric analysis of these bands showed that all were stimulated by this secretagogue (Fig. 2B). However, the increment in the immunoreactivity of each molecular form was different. The predominant monomeric form (22 kDa) increased 2.2 times in the GHRH-stimulated media in relation to the control conditions, whereas the 26- and 15-kDa bands were 4.4 and 5.7 times higher after GHRH stimulation than the control, respectively (Fig. 2C). To analyze the relative concentration of each cGH size variant released by the pituitary, data were expressed as a

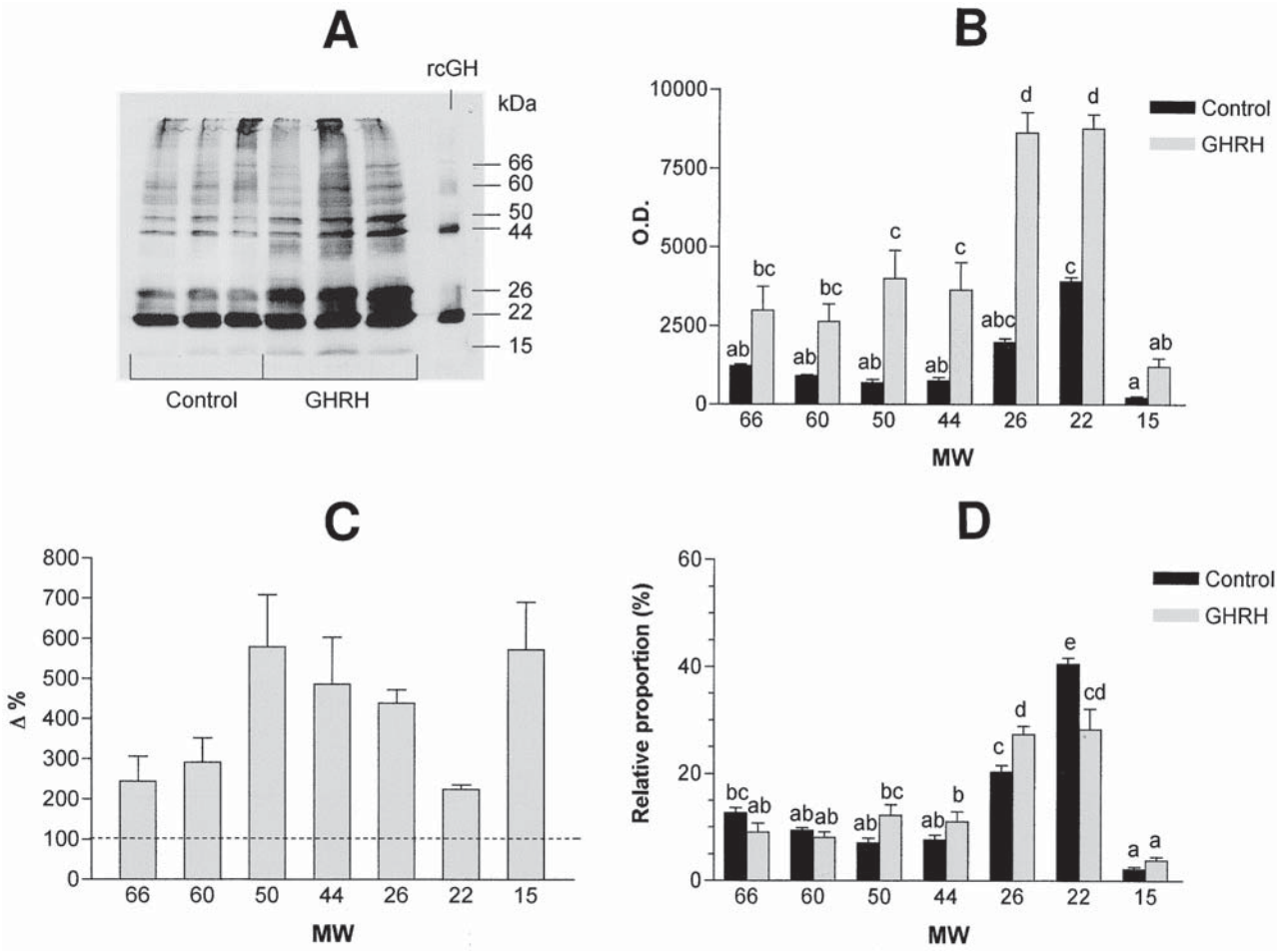

Fig. 2. Electrophoretic analysis of the ir-cGH size variants after stimulation with 2 n*M* GHRH for 2 h in pituitary culture media. (**A**) Media were analyzed by SDS-PAGE (NRC) and Western blotting and immunostained with a primary antibody anti-cGH. Development was performed by chemiluminescence with enhanced chemiluminescence (ECL) substrate (Amersham). (**B**) Densitometric analysis of the Western blot luminogram was done in an HP scanner using IPLabGel 2.2 software (Scanalytics, Fairfax, VA). The optical density (OD) of each ir-cGH band was plotted. (**C**) Differential release of each ir-cGH band after GHRH stimulation compared with the basal values in controls, expressed as percentage change, is shown. (**D**) Relative proportion of each ir-cGH variant released by the pituitary, expressed as percentage of the total cGH immunoreactivity, is shown. The data show a representative experiment of three replicates (mean ± SEM; $n = 3$). MW, molecular weight. Different letters mean a statistical significance.

percentage of the total cGH immunoreactivity (Fig. 2D). The monomeric form (22 kDa) in the control group represented 40.5 ± 1.6%, whereas in the presence of GHRH it represented 28.3 ± 3.9%. On the other hand, the proportion of the 26-kDa variant corresponded to 20 ± 1.2% in the control, and it increased to 27 ± 1.5% after GHRH stimulation. In the case of the oligomeric forms (44, 50, 60, and 66 kDa), each represented <12% of the total immunoreactivity and the 15-kDa variant corresponded to about 2% in the control and 4% in the presence of GHRH.

Figure 3 shows the SDS-PAGE analysis (reducing conditions [RC]) of the culture media after isolating the ir-cGH forms by immunoprecipitation with the monoclonal immunosorbent. The Western blot membrane was stained for carbohydrate detection and for immunoreactivity. It was apparent that the 29-kDa band showed a positive reaction for the

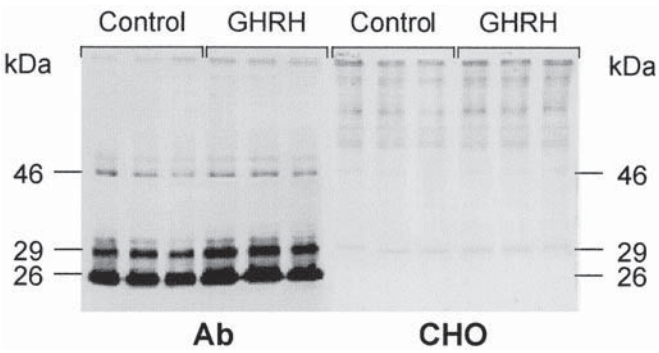

Fig. 3. Electrophoretic analysis of cGH size variants after stimulation with 2 n*M* GHRH for 2 h in pituitary culture media. Media were analyzed by SDS-PAGE (RC) and Western blotting and developed by immunostaining with a primary antibody anti-cGH and chemiluminescence (Ab) and with the Bio-Rad kit for glycoprotein detection (CHO).

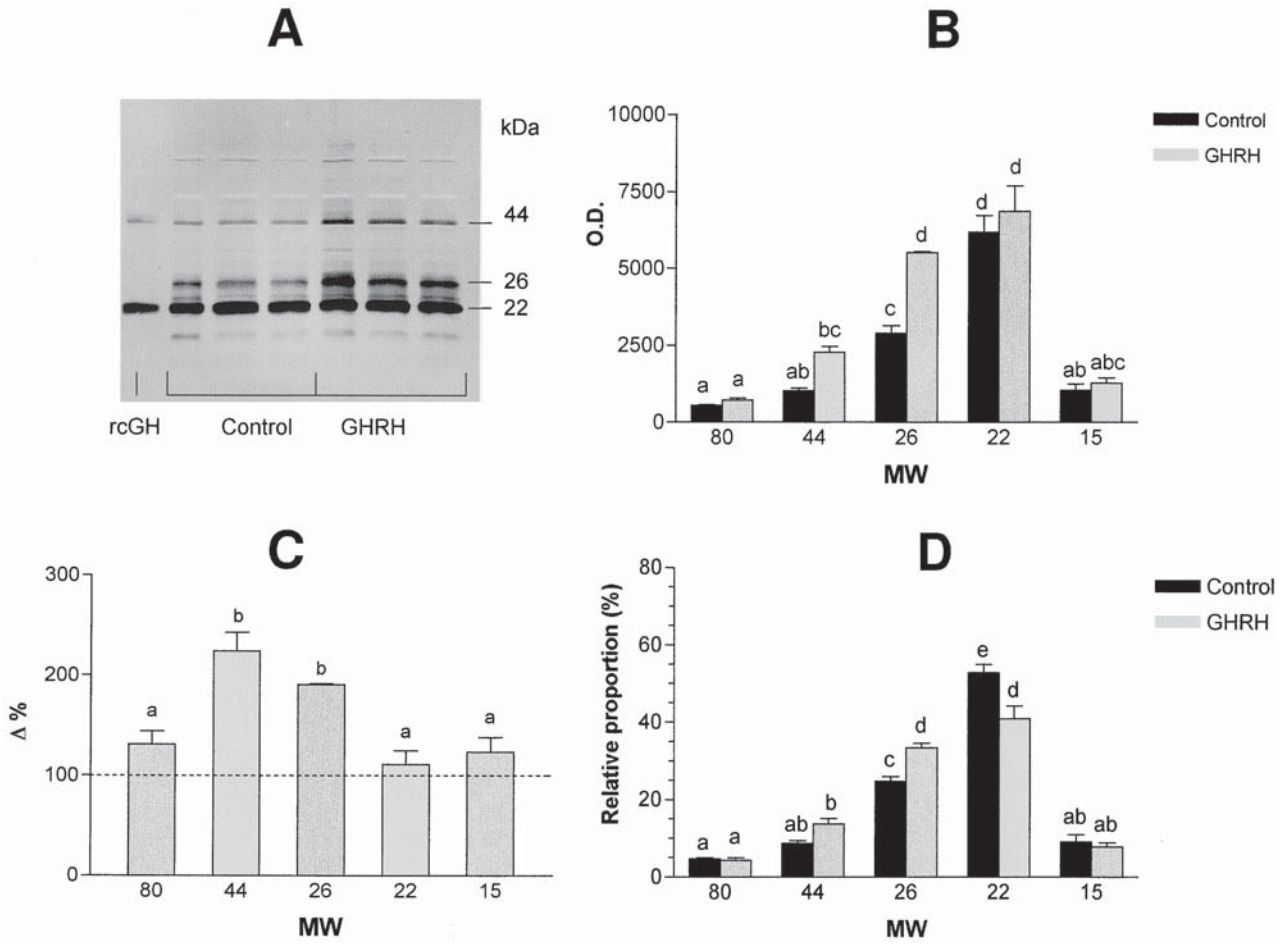

Fig. 4. Electrophoretic analysis of ir-cGH size variants after stimulation with 2 n*M* GHRH for 2 h in pituitary gland tissue. (**A**) Extracts were analyzed by SDS-PAGE (NRC) and Western blotting and immunostained with a primary antibody anti-cGH. Development was performed by chemiluminescence with ECL (Amersham). (**B**) Densitometric analysis of the Western blot luminogram was done in an HP scanner using IPLabGel 2.2 software (Scanalytics). The OD of each ir-cGH band was plotted. (**C**) Differential release of each ir-cGH band after GHRH stimulation compared with the basal values in controls, expressed as percentage change, is shown. (**D**) Relative proportion of each ir-cGH variant released by the pituitary, expressed as percentage of the total cGH immunoreactivity, is shown. The data show a representative experiment of three replicates (mean ± SEM; $n = 3$). MW, molecular weight. Different letters mean a statistical significance.

presence of carbohydrate and was also recognized by the polyclonal anti-cGH antibody, indicating that this ir-cGH variant is glycosylated.

The total ir-cGH content in the pituitary tissue after GHRH was not significantly different from that in the controls, when analyzed by ELISA (300.8 ± 38.0 vs 252.7 ± 7.1 ng/mg of pituitary, respectively). The relative proportion of the ir-GH variants in the pituitary gland was, however, changed in the presence of GHRH (Fig. 4A) when analyzed under NRC. Densitometric analysis (Fig. 4B) showed that the monomeric form (22 kDa) was unaltered by GHRH treatment (OD units = 6168.6 ± 550 in the control vs 6844 ± 843.2 in the stimulated tissue), but the 44- and 26-kDa ir-GH variants were 2.3 and 1.9 times higher (Fig. 4C) after GHRH incubation than in the control values, respectively (44 kDa: OD = 2296 ± 184.8 vs 1012 ± 92.5; 26 kDa: OD = 5509 ± 26.0 vs 2890 ± 247.7; $p < 0.001$). The relative proportion analysis of each variant showed that the 44- and 26-kDa forms increased significantly ($p < 0.05$) in the presence of GHRH over the control conditions, whereas the monomeric form (22 kDa) decreased significantly after GHRH (the relative proportion of 26 kDa represented 25 ± 1.0% in the control group and 33 ± 1.2% in the treated group, whereas the 22-kDa variant represented 53 ± 2.2% in the control and 41 ± 3.4% after treatment, respectively) (Fig. 4D).

Primary Pituitary Cell Culture Study

When pituitary cells in primary cultures were treated with 2 n*M* GHRH for 2 h, the total ir-cGH released to the culture media was significantly increased (2.8 times higher) over the values obtained by ELISA in control conditions (75.8 ± 14.7 vs 26.7 ± 6.1 ng/mL, respectively; $p < 0.001$). The culture media were immunoprecipitated with a mono-

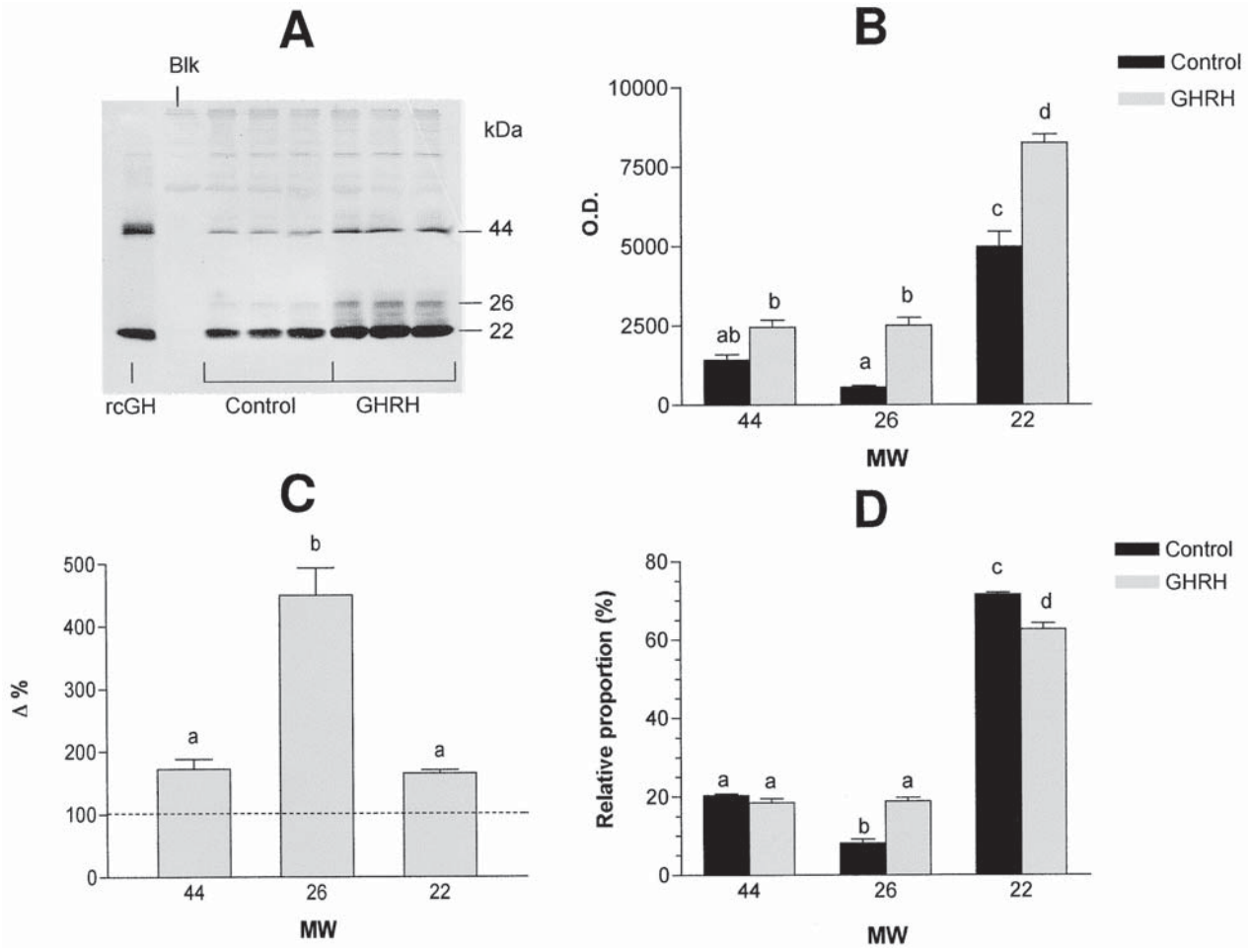

Fig. 5. Electrophoretic analysis of ir-cGH size variants after stimulation with 2 n*M* GHRH for 2 h in primary pituitary cell culture media. (**A**) Media were analyzed by SDS-PAGE (NRC) and Western blotting and immunostained with a primary antibody anti-cGH. Development was performed by chemiluminescence with ECL (Amersham). (**B**) Densitometric analysis of the Western blot luminogram was done in an HP scanner using IPLabGel 2.2 software (Scanalytics). The OD of each ir-cGH band was plotted. (**C**) Differential release of each ir-cGH band after GHRH stimulation compared with the basal values in controls, expressed as percentage change, is shown. (**D**) Relative proportion of each ir-cGH variant released by the pituitary, expressed as percentage of the total cGH immunoreactivity, is shown. The data show mean ± SEM; $n = 9$. MW, molecular weight. Different letters mean a statistical significance.

clonal immunosorbent before electrophoretic separation to eliminate interferences by the media salts. This system increased the specificity of the analysis because two sets of antibodies were employed: a monoclonal anti-cGH for immunoprecipitating the ir-cGH variants in the media and a polyclonal anti-cGH for detection of ir-cGH in the Western blot membrane. With this system, three ir-cGH bands were observed under NRC (22, 26, and 44 kDa) (Fig. 5A). The densitometric analysis showed that, as observed in the experiment using whole pituitary tissue, all the ir-cGH variants increased with GHRH treatment (Fig. 5B). However, again, the particular increase of each molecular form was different. As shown in Fig. 5C, the increments were 1.6, 4.5, and 1.7 times higher for the 22-, 26-, and 44-kDa bands, respectively, in the treated cultures than in their corresponding controls. Figure 5D shows the results obtained when the relative proportion of each band was analyzed. It was observed that the proportion of the 26-kDa band increased importantly, apparently at the expense of the decrease observed in the 22-kDa monomeric form, when the GHRH treatment was applied. The relative proportion of the 26-kDa ir-cGH band was almost 130% higher in the GHRH-treated sample than in the controls.

To characterize the nature of the 26-kDa variant, the experiments were analyzed under RC, to rule out the possibility of a cleaved cGH that could migrate very closely (25 kDa). After disulfide bonds were broken with reduction, differential changes were also observed in the number and absorbance of the ir-cGH bands. At least seven immunoreactive bands were observed (46-, 29-, 26-, 20-, 18-, 17-, and 15-kDa forms). The 20-, 18-, 17-, and 15-kDa bands correspond to submonomeric forms, while the 26-kDa band corresponds to the unfolded monomer, the 29-kDa band to the putatively unfolded glycosylated cGH (G-cGH), and the

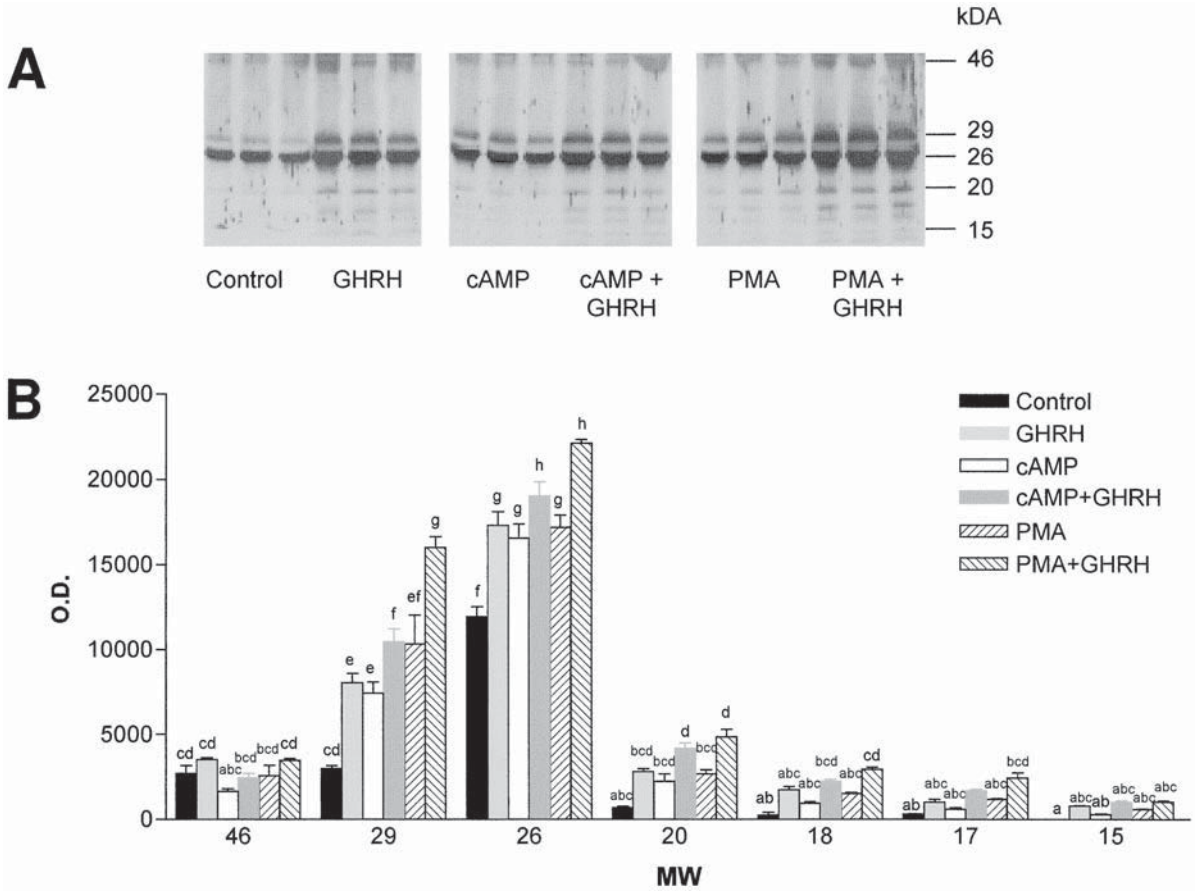

Fig. 6. Electrophoretic analysis of ir-cGH size variants after stimulation with 2 n*M* GHRH, 1 m*M* 8-bromo-cAMP, or 1 µ*M* phorbol myristate acetate (PMA) for 2 h in pituitary cell culture media. (**A**) Media were analyzed by SDS-PAGE (RC) and Western blotting and immunostained with a primary antibody anti-cGH. Development was performed by chemiluminescence with ECL (Amersham). (**B**) Densitometric analysis of the Western blot luminogram was done in an HP scanner using IPLabGel 2.2 software (Scanalytics). The OD of each ir-cGH band was plotted. Data show mean ± SEM; $n = 9$. MW, molecular weight. Different letters mean a statistical significance.

46-kDa band probably to a reduction-resistant dimer, which has been described earlier *(2)* (Fig. 6A). After GHRH stimulation, the OD of the monomeric (26-kDa) band increased 1.4 times and that of the 29-kDa band 2.7 times over the controls ($p < 0.05$). In terms of their relative proportion, the monomer (26 kDa) represented 63.3 ± 0.8% in the control group and 49.2 ± 1.3% after GHRH treatment, whereas the 29-kDa band represented 15.9 ± 0.8 and 22.8 ± 0.9%, respectively (Fig. 7).

Very similar results were observed when the cell cultures were treated with 8-bromo-cAMP, as shown in Figs. 6 and 7. The effect of this membrane-permeable substitute of cAMP on the monomeric (22-kDa) and the 29-kDa forms was analogous to that of GHRH. The combination of 8-bromo-cAMP and GHRH showed a higher increment on the release of both variants. However, these changes were not as clear when the relative proportion of the variants was determined (Fig. 7). Treatment with PMA had a similar effect on the secretion of the 29- and 26-kDa ir-cGH bands than treatments with GHRH or cAMP. However the combination of GHRH and PMA had a potentiating effect over these two variants (Figs 6B and 7).

Discussion

It has been proposed that the functional diversity of some pituitary hormones, such as PRL and GH, may be explained by their molecular heterogeneity. Much evidence has been reported for the existence of structural variants of these hormones. The sources of such heterogeneity include gene duplication, mRNA alternative splicing, and posttranslational modifications of the mature proteins. In addition, some reports indicate that the molecular variants of these hormones may express differential bioactivities *(2,5–23)*. On the other hand, the presence of some of the structural variants of PRL and GH has been documented in the circulation *(24,25)*. All these data, taken together, indicate that these proteins constitute a family of proteins, structurally related, whose known actions may be the result of the sum of the particular bioactivities of its members. If this is true,

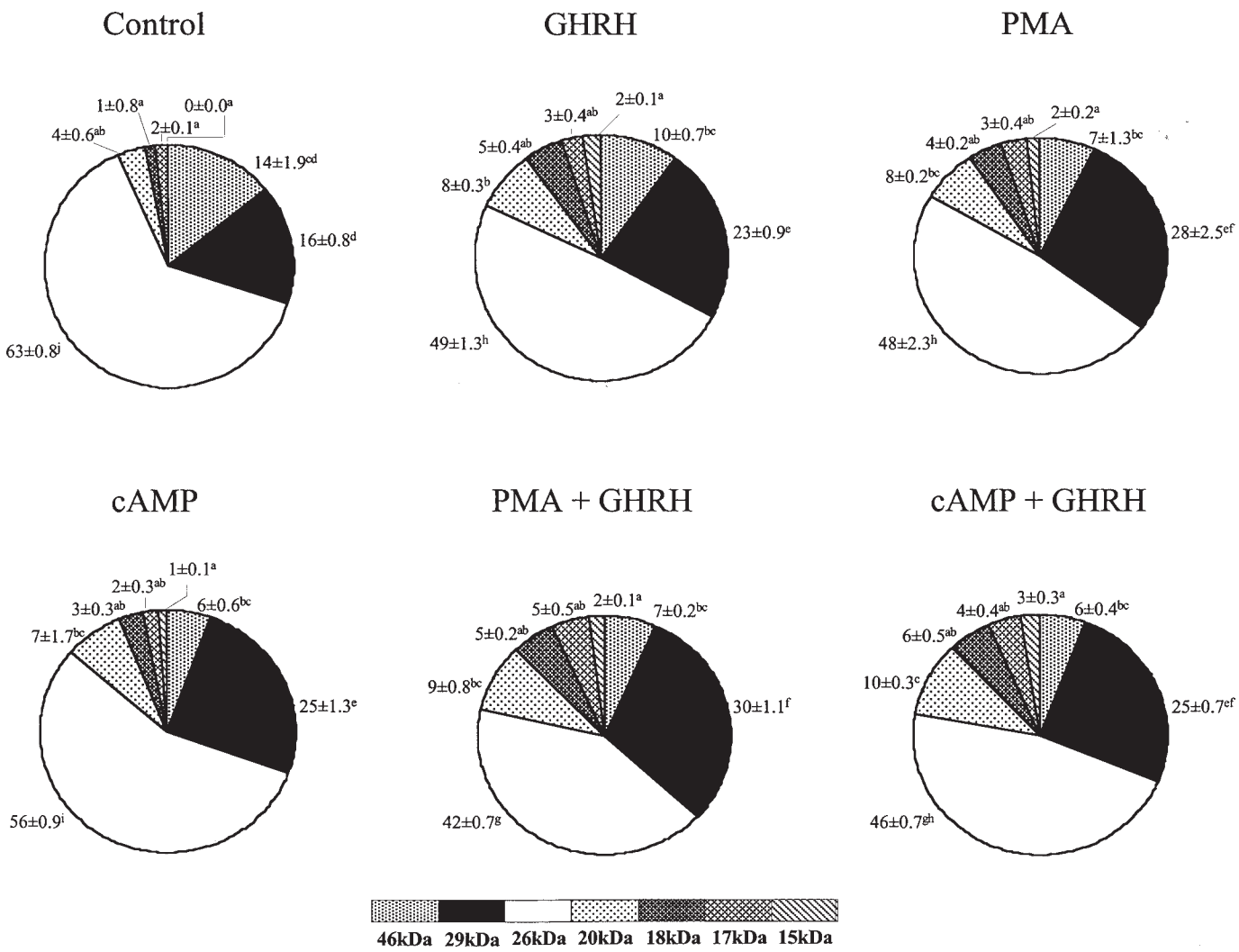

Fig. 7. Relative proportion (%) of ir-cGH variants in primary pituitary cell culture media, as analyzed by SDS-PAGE (RC) and Western blotting after different treatments. Different letters mean a statistical significance ($p < 0.05$, mean ± SEM; $n = 9$).

then the functional relevance of each variant should be evaluated to understand its participation in the overall effect related to the parental hormone. For GH variants to have a physiologic role as hormones, they must be released and their secretion must be under some kind of control.

In the chicken, the existence of posttranslational modifications of pituitary GH has been previously demonstrated, and several variants have been described: phosphorylated cGH *(41,42)*; glycosylated cGH *(43,44)*; various oligomeric forms (dimer, trimer, tetramer, pentamer *[2, 23]*); cleaved cGH; and submonomeric variants of cGH, especially a 15-kDa fragment of the hormone *(20)*. We have also shown that some of the charge variants of cGH (relative mobility [Rf] = 0.23 and 0.3, named after the relative mobility of each variant in nondenaturing PAGE) express a differential effect on lipid metabolism *(23)* and that the 15-kDa amino-terminal fragment of cGH is bioactive *(20)*. Some of the size and charge variants of cGH are found in plasma *(25)*, and it has been reported that the clearance rate of the G-cGH is lower than the major form of the hormone, thus increasing its half-life in circulation *(45)*. Additionally, the number and relative proportion of pituitary cGH variants change during ontogeny *(27)*.

In the present work, we analyzed the effect of an established GHS to determine whether the GH variants in the pituitary were affected similarly or whether they were differentially affected. Two in vitro systems were employed: whole pituitary tissue culture, to keep the integrity of the gland architecture and thus preserve cell interactions; and disperse primary pituitary cell culture, to ensure the availability of the secretagogue(s) to all cells.

The stimulatory effect of GHRH on GH secretion, both in vivo and in vitro, has been extensively documented in mammals and birds *(33–35,40,46–48)*. In most of the reports,

released GH has been measured by a radioimmunoassay (RIA), which accounts for the overall GH present in the plasma or culture media, without differentiating among variants. Here, we employed a method that allows for the separation (SDS-PAGE) and identification (Western blotting) of the major immunoreactive size variants of cGH. Furthermore, the combined use of a chemiluminescent assay and densitometry gave a semiquantitative tool to compare differences in concentration of each band in response to the treatments, since we have previously established that the assay is linear *(27)* in the range used.

Initially, we studied the response of chicken pituitary glands to different concentrations of GHRH (0.2, 1, 2, 5, and 10 m*M*) in order to determine the best dose for experimentation. A dose-dependent effect was observed, and we decided to use 2 n*M* GHRH for these studies since it was close to the EC50. This concentration is similar to that determined by other investigators *(40)* using cultured pituitary cells.

In the first study, the total amount of ir-GH released to the culture media following GHRH stimulation was almost double (1.9 times) that in the controls, as determined by a specific and homologous ELISA. When the culture media were analyzed by SDS-PAGE under NRC and Western blotting, seven clear ir-cGH bands (mol wt: 66, 60, 50, 44, 26, 22, and 15 kDa) were observed in both the control and the stimulated conditions. These bands have previously been described in the pituitary *(2,27)* and in the circulation *(25)*. Although all these ir-cGH variants were increased by GHRH stimulation (Fig. 2), the magnitude of the response differed for different ir-GH moieties. For instance, the OD of the band corresponding to the monomeric form (22 kDa) was 2.2 times higher in the treated samples than in the control, whereas that of the 26-kDa band was 4.4 times higher, respectively. In addition, the 15-kDa band was 5.5 times higher in the GHRH-stimulated media than in the control. To study further the effect of the secretagogue on the release of each variant, we decided to analyze the results in terms of the proportion (percentage) of each variant in relation to the total immunoreactivity of the hormone in each sample (Fig. 2D). The results indicated that the proportion of the 26-kDa band increased significantly whereas the monomeric (22-kDa) band decreased significantly after the tissue was incubated with GHRH. The proportion of the 26-kDa form was 35% higher and that of the monomer was 31% lower than their corresponding values under control conditions. The increment in the proportion of the 26-kDa band was therefore at the expense of the monomeric form. These data showed that although all the immunoreactive forms of cGH are stimulated by GHRH, they are not released at the same extent. Interestingly, the analysis of what occurred in the tissue indicated that there was also a differential effect of the secretagogue in the content of the stored cGH pituitary variants. Although the total cGH content stored in the pituitary did not show any significant difference between treatments, the analysis of the variants showed that the proportion of the 26-kDa band was significantly increased, again at the expense of the 22-kDa band, which was significantly reduced (Fig. 4).

The results of these experiments suggested that treatment of the pituitary with GHRH causes a preferential release of the 26-kDa ir-GH moiety. We have shown previously that the 26-kDa band may correspond to the G-cGH when analyzed under NRC *(27,42)* and that it shifts to a 29-kDa band when studied under RC. To determine whether this was the case, samples were analyzed for the presence of carbohydrates. A positive reaction for carbohydrate was found in the 29-kDa band, which was also recognized by the polyclonal anti-cGH antibody, indicating that this ir-cGH variant is glycosylated (Fig. 3). The reason that this variant was investigated under RC was to avoid the possible interference with a 25-kDa ir-cGH that corresponds to a cleaved form of the hormone *(20)*, which might be observed under NRC. This form, however, disappears under RC when the disulfide bonds are reduced and gives rise to 15- and 7-kDa cGH fragments. Under RC, the monomeric form shifts its apparent mol wt from 22- to 26-kDa (owing to the unfolding of the molecule and an increase in its Stoke's radius), and the G-cGH shifts from 26 to 29 kDa. The recognition of the 29-kDa band by two sets of specific antibodies (monoclonal during immunoprecipitation and polyclonal in Western blot) indicates that it is a cGH variant, and that treatment with GHRH increased its proportion in comparison with controls (Fig. 3).

GHRH caused a great release of total ir-cGH into the media of pituitary primary cell cultures as determined by ELISA (almost three times that of the control values), thus showing that the system was responsive. The analysis performed under NRC with the primary cell cultures rendered a similar result to that observed with the whole pituitary in culture; that is, GHRH stimulated a differential release of the main ir-cGH variants. Again, it was shown that the relative proportion of the putatively glycosylated variant (26 kDa under NRC) was differentially increased apparently at the expense of the monomeric form (Fig. 5).

When the media of cell cultures were studied under RC, several changes were apparent, mainly that several submonomeric forms were now present and that all the variants were stimulated, although in different proportions; that is, the changes were 1.3, 2.7, 1.5, 4.1, 6.8, and 3.2 times higher for the 46-, 29-, 26-, 20-, 18-, and 17-kDa bands, respectively, in the treated cultures than in the corresponding controls (Fig. 6). In the case of the 15-kDa ir-cGH band, it was not detectable in the controls but was apparent in the GHRH-treated sample. Again, the relative proportion of the monomeric ir-cGH form (26 kDa under RC) decreased from $63.3 \pm 1.4\%$ in the controls to $49.2 \pm 2.2\%$ in the stimulated media, whereas the putatively glycosylated form (29 kDa

under RC) increased from 15.9 ± 1.3 to 22.8 ± 1.5%, respectively. Additionally, there were substantial increments in the relative proportions of the submonomeric forms (Fig. 7).

The molecular changes observed in monomeric ir-cGH as well as in the putatively glycosylated variant and in the submonomeric forms were also observed when the cell cultures were stimulated with secretagogue analogs known to be involved in the intracellular signaling pathways elicited by GHRH, namely the cAMP and PKC pathways. As shown in Fig. 6A, both 8-bromo-cAMP and PMA (an activator of PKC) stimulated the release of ir-cGH variants when analyzed under RC. Again, there was a differential increase in the release of the variants after stimulating with the analogs, and as before, the increase in the 29-kDa band and those corresponding to the submonomeric forms were higher than that produced in the monomer (Fig. 6B). When the relative proportion was analyzed, it was clear that both secretagogues favored the release of the 29-kDa ir-cGH band and had an effect similar to that obtained with GHRH, apparently at the expense of the monomeric form. In this experiment, it was shown that the proportion of the 46-kDa band decreased with the treatments (Fig. 7). On the other hand, the combined effect of GHRH and PMA seemed to exert a synergistic effect on the proportion of the 29-kDa ir-cGH band released as well as on the magnitude of the decrease in the proportion of the monomer. The results obtained are consistent with reports proposing that cAMP is the major signaling pathway employed by GHRH, but that the activation of PKC may potentiate the cAMP signaling via pathway route *(49)*.

The overall results of the experiments performed in our study suggest that GHRH stimulates the conversion of monomeric cGH to a form of 26-kDa band (29 kDa under RC), which presumably corresponds to a glycosylated variant of cGH and stimulates its differential release. In addition, GHRH (and its surrogate secretagogues) stimulate the release of the submonomeric forms, suggesting that limited proteolysis of the hormone is induced by this treatment.

Glycosylated variants of pituitary GH have been described in several mammals and birds *(26,42,43)*. In the chicken, as well as in other nonmammalian species, the primary structure of GH has an N-glycosylation consensus sequence in the C-terminus side ($N_{188}C_{189}T_{190}$). Human GH lacks this site for N-linked sugar. However, there are reports showing a content of 1–5% for G-hGH in relation to total pituitary GH. Furthermore, it has been shown that the metabolic clearance rate of G-cGH was 20% lower compared with nonglycosylated cGH *(45)*. According to our results, it is possible that GHRH favors the glycosylation of cGH and thus increases its half-life in circulation. If this is the case, then a differential control could be established. This is interesting in view of some evidence that has been reported for the glycosylated variant of turkey PRL, which is released in different proportions according to different physiologic changes *(30)*. Glycosylated turkey PRL is mainly released when the total PRL secretion is increased during incubation behavior. Perhaps GHRH favors the synthesis and secretion of a longer half-life variant of cGH that may be of importance during some critical physiologic stage.

In conclusion, our data suggest that the pattern of basal secretion of cGH variants can be modified by GHRH stimulation. They also support the hypothesis that the GH variants may be under differential control and in some physiologic states can change the proportion in which they are synthesized and secreted. The regulation of GH secretion is very complex, and more research is needed to understand the intracellular and intercellular mechanisms involved in the regulation of the synthesis/secretion of GH variants.

Materials and Methods

Animals

Male 4-wk-old domestic chickens were obtained from Pilgrim's Pride of Mexico farms and maintained on food and water available ad libitum for 1 d in the laboratory before sacrifice.

Pituitary Tissue Culture

Anterior pituitaries were removed immediately following decapitation. They were longitudinally bisected through the caudal and cephalic lobes, weighed, and each half was placed in a culture tube (polypropylene, 13 × 30 mm; Nalgene, Rochester, NY) containing 0.3 mL of normal Earle's salt solution (NESS) (Gibco, Grand Island, NY). The tubes were gassed with 95% O_2–5% CO_2, sealed with rubber stoppers, and incubated at 37ºC in a metabolic shaker (Labline, Melrose Park, IL). After 60 min, the media were removed and the glands were incubated with fresh NESS containing 2 n*M* human $GHRH_{1–44}$ (Sigma, St. Louis, MO) under the same conditions, a further 2 h. At the end of this incubation period, the media and the tissues were collected for analysis and stored at –70ºC until assayed.

To determine the dose of GHRH to be used in the experiments, dose-response relationships were obtained by fitting the data in the Hill equation:

$$S_{GHRH} = S_{max} \times EC_{50}{}^{nH}/[GHRH]^{nH} + EC_{50}{}^{nH}$$

in which S_{GHRH} is the cGH released in the presence of GHRH, S_{max} is the control maximum secretion, EC_{50} is half effect concentration of GHRH, and *nH* is the Hill coefficient.

Primary Pituitary Cell Culture

Anterior pituitaries from 4-wk-old cockerels were removed immediately after decapitation and placed in a Petri dish containing Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) (HBSS-CMF) (Gibco). Glands were washed three times with the same solution and then diced in approx 2-mm^3 cubes. They were transferred following incubation to a conical test tube (15 × 75 mm) containing 0.35% Type II

collagenase (Worthington, Freehold, NJ) in HBSS-CMF. Adenohypophyseal cells were dispersed in this medium for 60 min at 37ºC in a water bath with agitation, and periodic mechanical trituration with a sterile Pasteur pipet facilitated dispersion. Following dispersion, cells were washed twice with medium 199 and placed in culture medium: M199 (Gibco) containing 10% fetal bovine serum, 2.0 m*M* glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Aliquots of 1 mL (3.0×10^5 cells) were placed in a 24-well culture plate (Costar, New York, NY) and cultured for 48 h in a humidified chamber under 5% CO2 and air. Following culture, the media were removed, and the attached cells were washed once with fresh M199 prior to incubation with culture medium containing the test agent. The cells were incubated for 2 h with 2 n*M* GHRH, or 1 m*M* 8-bromo-cAMP or 1 µ*M* PMA (both from Sigma) since these treatments are known to stimulate GH release from culture of chicken pituitary cells *(40)*. The media and cells were collected and then stored at –70ºC until assayed.

The total concentration of GH was determined in duplicate by a specific and homologous ELISA, whereas the ir-cGH size variants that secreted to the medium and remaining in the tissue were analyzed by SDS-PAGE followed by Western blotting and densitometry. Culture media of primary cell cultures were immunoprecipitated before SDS-PAGE.

Enzyme-Linked Immunosorbent Assay

A specific and homologous competitive ELISA was employed. Plates (Immulon 2HB, 96 wells, Dynex Technologies, Chantully, VA) were coated with 12 ng of recombinant chicken GH (rcGH) in 100 µL of 1 *M* carbonate buffer, pH 10.3, for 16 h at 4ºC. A polyclonal GH antiserum raised in rabbit against purified chicken GH *(2)* (diluted 1:100,000) was incubated with samples or serial dilutions of rcGH (5120 to 5 ng/mL, for the standard curve) in phosphate buffer with Tween-20 (TPBS) and nonfat milk (0.01 *M* phosphate, 0.15 *M* NaCl, 0.05% Tween-20, and 1% [w/v] nonfat milk) for 16 h at 4ºC. The samples and standards were added to the coated wells for 2 h at room temperature (20ºC). Horseradish peroxidase (HRP)–conjugated anti–rabbit IgG (Bio-Rad, Hercules, CA) was then added (diluted 1:3000 in TPBS with 1% nonfat milk and incubated at 2 h at room temperature). Bound secondary antibodies were developed by reaction with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] substrate (Roche, Mannheim, Germany), and the OD in each well was determined at 405 nm in an automatic ELISA reader (Bio-Rad). This assay has a sensitivity of 2 ng/well (20 ng/mL), with inter- and intrassay coefficients of variation 3.27 and 3.91%, respectively. Other pituitary hormones had no crossreactivity in this assay (<0.001%).

Immunoprecipitation

To avoid interference in SDS-PAGE with salts present in the culture media, the samples were immunoprecipitated with a specific monoclonal immunosorbent prepared as described by Berghman et al. *(50)*. Monoclonal antibodies (MAbs) were produced in the laboratory using specific anti-cGH hybridoma cells generously provided by Dr. Berghman (Texas A&M University). The MAbs were purified from ascites fluid using a Protein A–Sepharose matrix (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, 6 mL of ascites fluid was mixed with 0.75 g of protein A–Sepharose in glycine buffer (1.5 *M* Gly; 3 *M* NaCl, pH 8.9) and incubated with agitation at room temperature. The IgG fraction was obtained by elution with citrate buffer (0.05 *M* citrate, pH 3.5) and then dialyzed against coupling buffer (0.05 *M* phosphate, 0.5 *M* NaCl, pH 7.6). The immunosorbent was prepared by incubating the IgG fraction (15 mg) with activated CNBr–Sepharose 4B (1 g) in coupling buffer for 3 h in an end-over-end shaker, at room temperature. The unoccupied remaining active sites in the resin were blocked with 1.5 *M* Gly, 3 *M* NaCl (pH 8.0) overnight at room temperature. Finally, the immunosorbent was washed alternately with TBS (0.05 *M* Tris; 0.15 *M* NaCl, pH 7.6) and glycine (0.05 *M* Gly; 0.15 *M* NaCl, pH 3.0) buffers. The total cGH present in the culture media was isolated by immunoaffinity precipitation: aliquots (250 µL) of media were incubated with 1 mL of TBS and 100 µL of immunosorbent suspension (1:2 with TBS) in Eppendorf tubes in an end-over-end shaker, overnight at room temperature. The resin with the bound ir-cGH was recuperated by centrifugation (2365*g*) and then washed with TBS (three times) and with 0.020 *M* Tris, pH 7.6. Immunoreactive cGH was then isolated after resuspending the pellet in SDS-PAGE sample buffer, boiling for 5 min, and centrifuging in a microfuge at 2365*g*. The supernatants were collected and submitted to SDS-PAGE electrophoretic analysis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Culture media of both primary cultures and whole pituitary incubations, as well as gland or cell extracts, were analyzed by SDS-PAGE on 1-mm-thick 12.5% gels (Mini-Protean; Bio-Rad) under NRC or under RC, in the presence of 5% β-mercaptoethanol, using the buffer system of Laemmli *(51)* and run at 150 V in the separating gel. A control, corresponding to 50 ng of rcGH, was included in each gel. Prestained molecular weight markers (Bio-Rad) were employed to determine the relative mobility of proteins.

Western Blotting

After the electrophoresis, the gels were equilibrated in transfer buffer (25 m*M* Tris, 192 m*M* Gly, 20% methanol) for 15 min, and the proteins were electrotransferred to nitrocellulose sheets (Bio-Rad) at 200 mA for 1 h and then immunostained. Briefly, the membranes were blocked (5% nonfat milk in 0.02 *M* Tris, 0.5 *M* NaCl), and after 2 h they were incubated with polyclonal anti-cGH rabbit serum *(2)* at a dilution of 1:10,000. The membranes were washed and

incubated for 2 h with a secondary antibody (horseradish peroxidase-conjugated anti–rabbit IgG, 1:3000; Bio-Rad). Finally, the ir-cGH variants were visualized with a chemiluminescent substrate (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

A positive reaction for the presence of carbohydrate in the ir-GH was determined by the blot kit for glycoprotein detection (Bio-Rad), which employs a specific carbohydrate oxidation reaction to label the carbohydrate with biotin, and then the subsequent detection is done with streptavidin–alkaline phosphatase.

Densitometric Analyses

The number, amount, and relative proportion of ir-cGH size variants were determined by densitometric analysis after digitalization of the luminograms in an HP scanner using IPLabGel 2.2 software (Scanalytics, Fairfax). In both, the whole-pituitary systems and in the primary cell culture system, the values obtained in the absence of the test agents (GHRH, 8-bromo-cAMP, PMA) were taken as control values.

Statistical Analyses

Data (OD of each GH variant or the proportion of the variants as percentage of total GH immunoreactivity) were analyzed by analysis of variance. Differences between treatments were determined by Tukey test and considered significant at $p < 0.05$.

Acknowledgments

We thank Dr. Luc R. Berghman for his generous donation of the anti-cGH hybridoma cells. We also thank Pilgrim's Pride of Mexico for the donation of chickens. We acknowledge the efficient work of Pilar Galarza-Barrios (librarian); Martín García-Servín (vivarium); Alberto Lara and Omar Hernández (computing unit); Lourdes Lara, Leopoldo González, and Rafael Favila (image analysis unit); and Gerardo Courtois (laboratory attendant). This work was partially supported by grants from DGAPA-UNAM (PAPIIT IN206196 and PAPIIT IN227399) and CONACyT (3455-PN and 31817-N). H.M.-C. received a doctoral fellowship from CONACYT.

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