Exocytosis sensitivity to growth hormone-releasing hormone in subsets of GH cells in rats under different corticosterone conditions. Ultrastructural study using microwave irradiation for fixation and immunocytochemistry

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Abstract

Growth hormone (GH) cells in the rat anterior pituitary have been morphologically classified into three subtypes: type I (mature) containing large secretory granules about 350 nm in diameter, type II (intermediate) containing a mixture of large and small granules, and type III (immature) containing small granules about 150 nm in diameter. However, the functional implications of morphological heterogeneity, especially the different sensitivities to growth hormone-releasing hormone (GRH) under different corticosteroid conditions have not been elucidated to date.

In the present study, by application of microwave irradiation (MWI) for fixation and immunocytochemistry, new findings of the exocytotic response have been revealed among the subsets of GH cells following adrenalectomy (ADX), corticosterone treatment and/or GRH treatment.

The MWI gave effective results for fixation, especially for the permeability of the fixative, and showed good results for immunoelectron microscopy using the protein-A gold method. Moreover, the use of MWI

greatly shortened the fixation, processing and immunolabeling times without compromising the quality of ultrastructural preservation and the specificity of labeling.

The number of exocytotic figures was low in all subtypes of GH cells in the sham-operated control rats. GRH treatment induced a significant increase in exocytosis in each subtype of GH cells, particularly in type I (mature) and type II (intermediate) GH cells in the control rats. GRH injection to rats for 4 days after ADX also showed an increase in exocytosis, but the degree was significantly less in comparison with the GRH injection in the control group. Corticosterone replacement given to ADX rats induced a clear recovery of the exocytotic response to GRH to the control level. Serum GH content measured by radioimmunoassay correlated with these morphological results.

These results suggest that the secretion of GH stimulated by GRH is closely related to corticosteroids, and that the sensitivity to GRH differs among the three subtypes of GH cells.

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Introduction

Since the introduction of immunocytochemistry, various anterior pituitary hormone-producing cells have been classified into subtypes based on the size of secretory granules and the development of cell organelles (Nogami 1984, Kurosumi *et al.* 1986, Ozawa & Kurosumi 1989). Three subtypes of growth hormone (GH) cells have been identified, type I containing only large secretory granules about 350 nm in diameter (mature), type II having a mixture of large and small granules (intermediate) and type III containing only small granules about 150 nm in diameter (immature) (Kurosumi *et al.* 1986). According to a study on ontogeny of GH cells, it has been suggested that

the function of the subtypes of GH cells is related to the developmental condition; type I (mature) GH cells are preferentially observed in adult animals, while the occurrence of the frequency of type III (immature) GH cells is higher in the perinatal stage than in adult animals. Type II (intermediate) GH cells are observed through all stages, but they are preferentially observed in adult female rats (Kurosumi & Tosaka 1988). However, a more detailed observation related to the functional implication of morphological heterogeneity, such as the different sensitivities to the regulating factors for GH secretion, including growth hormone-releasing hormone (GRH) and somatostatin (SRIF), has not been carried out to date.

Recent work has identified that glucocorticoids are important physiological regulators of GH synthesis and secretion, and, in particular, glucocorticoids regulate the expression of GRH receptors (Thakore & Dinan 1994, Lam *et al.* 1996, Miller & Mayo 1997, Miller *et al.* 1999, Nogami *et al.* 1999). Our study showing the expression of glucocorticoid receptor in GH cells also supported a direct action of glucocorticoid on GH cells (Ozawa *et al.* 1999). In the present study, we investigated the sensitivity to GRH of each type of GH cell under different glucocorticoid conditions, as estimated by the frequency of exocytosis induced by GRH injection.

To obtain constant and high quality detection of exocytotic figures and immunolabeling it is necessary to fix the tissues quickly. Some previous studies indicated that post-embedding methods applying microwave irradiation (MWI) to the fixation and immunolabeling of thin plastic-embedded sections give good results (Zondervan *et al.* 1988, Login *et al.* 1995, Rangell & Keller 2000). In the present study, we applied microwave technology to processing and immunolabeling of plastic-embedded tissue for the analysis of exocytosis in GH cells stimulated by GRH under different corticosteroid conditions.

Materials and Methods

Animals

Male Wistar rats (weighing 180–200 g, Shimizu Laboratory Supplies Co., Ltd, Kyoto, Japan) were housed under artificial illumination (12 h light:12 h darkness) at a controlled temperature ($22 \pm 1^\circ\text{C}$) with food and water (or physiological saline for adrenalectomized (ADX) rats) available *ad libitum*. Thirty rats were divided into 3 groups of 10 as follows: group 1, sham-operated rats as controls; group 2, rats for 4 days after ADX; group 3, rats treated with corticosterone (CORT; 30 µg/ml) in physiological saline available *ad libitum* for 4 days following ADX (Hu *et al.* 1997). The rats were treated in accordance with the guidelines issued by the US National Institutes of Health for the humane treatment of experimental animals, and the experiments were approved by the experimental committee of the Kyoto Prefectural University of Medicine.

Treatment of GRH

To avoid interference due to endogenous hypothalamic GRH secretion, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), which is thought to block the endogenous release of both hypothalamic somatostatin and GRH (Wehrenberg *et al.* 1982). Synthetic human GRH (Peptide Institute, Osaka, Japan) diluted in physiological saline (10 µg/kg body weight) was injected into the left femoral vein of five rats of each

group. Three minutes after GRH injection, rats were killed by decapitation. After decapitation, blood from the trunk was collected for GH radioimmunoassay, and pituitaries were quickly removed for ultrastructural study. Five rats of each group were injected with physiological saline alone as a control for the GRH treatment group.

MWI fixation and processing for plastic embedding of tissue

Pituitary glands were removed quickly and cut into small blocks of about 1 mm³ in the fixative. They were fixed with microwave irradiation (200 W, 5 s intermittent irradiation, Azumaya Co. Ltd, Tokyo, Japan) for 10 min in 4% paraformaldehyde (PFA), 1% glutaraldehyde (GLA), and 0.3% tannic acid in 0.1 M sodium cacodylate buffer (pH 7.2) in a cool spot located by using the neon bulb display method with the temperature probe in the fixative and temperature feedback limit set to 37 °C. The use of tannic acid delineated the detailed ultrastructure of filaments and membranes, such as microtubules and omega-shaped exocytosis figures (Pow & Morris 1989, Shimada & Tosaka-Shimada 1989, Wang *et al.* 1995). The tissues were then postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at 4 °C. Following dehydration in a graded ethanol series and treatment with propylene oxide, the tissues were embedded in Quetol 812 at 45 °C for 1 day, and then at 60 °C for 2 days. The embedded tissues were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Heidelberg, Germany).

Immunolabeling of plastic-embedded sections by MWI

Ultrathin sections were mounted on nickel grids. All sections were processed for immunocytochemical reaction by the protein-A gold technique as follows. The sections were treated with 3% hydrogen peroxidase for 10 min in room temperature (RT), rinsed with phosphate-buffered saline (PBS), microwaved for 5 min with normal goat serum diluted with PBS to 1:20, irradiated three times for 5 min with rabbit anti-rat GH serum (kindly supplied by Prof. K. Wakabayashi, Gunma University, Maebashi, Japan) diluted with PBS to 1:2000. The specificity of the antibody has already been tested and described elsewhere (Wakabayashi & Tanaka 1988). The sections were then rinsed with PBS, irradiated for 5 min with protein-A gold (15 nm particles, BBI International, Cardiff, UK), diluted 1:50 with PBS, rinsed with PBS and distilled water (DW), and dried. They were stained with 8% uranyl acetate and lead citrate, and rinsed with DW and dried. The sections were observed and photographed with a JEM-1220 transmission electron microscope (JEOL, Tokyo, Japan).

Enzyme cytochemistry

The pituitaries were cut into 1 mm³ cubes. The specimens were fixed in a mixture of 2% GLA and 2% PFA in

0.1 M sodium cacodylate buffer (pH 7.4) containing 8% sucrose for 10 min with MWI (200 W, 5 s intermittent irradiation). After washing in the same buffer, 50- μ m thick sections were cut with a microslicer (TDK-3000, Dosaka EM Co. Ltd, Osaka, Japan) and washed in the same buffer. The sections were microwaved for 5 min in freshly prepared acid phosphatase activity-detecting medium, with β -glycerophosphate as the substrate, in 0.2 M Tris-maleate buffer (pH 5.5) (Barka & Anderson 1962, Ozawa 1991). The specimens were dehydrated through an ascending ethanol series, and embedded in the plastic-embedding medium, Quetol 812. Ultrathin sections were made using an ultramicrotome (Ultracut E, Reichert-Jung, Heidelberg, Germany) and mounted on nickel grids. All mounted sections were lightly stained with lead citrate and washed with DW and dried. The specimens were observed and photographed as described above. For cytochemical controls, some specimens were incubated in a substrate-free medium. The reaction was negative in these control sections.

Morphometry

Twenty micrographs for each subtype ($\times 15\,000$) of immunoreactive GH cells representing each experimental group, in which the whole cell membrane and nucleus could be seen, were selected. The exocytotic figure was counted on the micrographs, and the circumferential length of micrographed GH cells displayed on a computer screen was measured by NIH-image software (version. 1.63, NIH, Bethesda, MD, USA). Finally, we calculated the number of exocytotic figures per unit length (μ m) and estimated the frequency of exocytosis in each type of GH cell for each experimental group.

All data are expressed as means $\pm$ S.E.M. The Mann-Whitney U-test (non parametric test) was used to determine significant differences between any two experimental groups.

Radioimmunoassay

The serum GH content in each experimental group was evaluated by radioimmunoassay. Blood from the trunk was collected after decapitation and assayed for serum GH levels. A double-antibody radioimmunoassay was carried out to determine serum GH levels. The standard hormone used was RP-2 (AFP-3190B) for rat GH, supplied by NIDDK (Bethesda, MD, USA). The Mann-Whitney U-test was performed to determine statistical significance.

Results

The effects of MWI for tissue fixation and immunolabeling

Using MWI, the processing times for fixation and immunocytochemistry were substantially shortened. The

ultrastructure processed by MWI fixation was suitable for electron microscopic observation. The quality of MWI fixation was as good as that of conventional fixation. Cell organelles, and the membrane structure in particular, were well prepared. Moreover, the penetrability of the fixative buffer was better than with the conventional procedure. The MWI fixation showed a constant fixative effect throughout the depth of fixed materials. The immunolabeling obtained with MWI showed sufficient quality (Figs 1–5).

Classification of GH cells in the rat anterior pituitary

As reported by others (Kurosumi *et al.* 1986, Kurosumi & Tosaka 1988, Takahashi 1991), the present study confirmed the subsets of GH cells of rat anterior pituitary according to the size of secretory granules and the developmental state of cell organelles.

Type I (mature) GH cells contained relatively large secretory granules (about 350 nm in average diameter), which were mostly uniform in size. Cisternae of rough endoplasmic reticulum (rER) were moderately dilated (Fig. 1A). Type II (intermediate) GH cells contained a mixture of large and small secretory granules. The cell organelles such as the Golgi apparatus and rER were moderately developed (Fig. 1B). On the other hand, type III (immature) GH cells contained small secretory granules (about 150 nm in diameter) (Fig. 1C).

The effect of GRH on the exocytosis of GH cells under different corticosteroid conditions

In the control (sham-operated) condition, very few exocytotic figures were observed for each subtype of GH cells (Fig. 2A–C, see also Fig. 7). In contrast, GRH injection to control rats caused a significant increase in exocytosis in each subtype of GH cells. Especially, types I (mature) (Fig. 2D) and II (intermediate) (Fig. 2E) GH cells showed a significant increase in exocytosis in comparison with type III (immature, Fig. 2F) GH cells (see also Fig. 7). Following ADX, vacuolar structures were often observed (Fig. 3A) in the GH cells. Enzyme cytochemistry revealed that these structures reacted with acid phosphatase cytochemistry (Fig. 3B), indicating that these vacuolar structures caused by ADX in GH cells were lysosomes. It was very rare to observe exocytosis in any of the subtypes of GH cells, and the immunolabeling density was diminished in the secretory granules (Fig. 3C).

GRH injection to the ADX rats caused an increase in the exocytosis of each subtype of GH cells (Fig. 4); however, the increase was significantly lower than that in GRH-injected control rats (see also Fig. 7).

Corticosterone (CORT) replacement given to ADX rats induced a recovery of the immunogold labeling density. There was only slight exocytosis in each subtype

Figure 1 Electron micrographs of three types of growth hormone (GH) cells: (A) type I (mature), (B) type II (intermediate), and (C) type III (immature). Bar = 1 μ m.

of the GH cells as well as in the control and the ADX group (Fig. 5, see also Fig. 7). In contrast, the number of exocytosis findings was significantly increased following GRH injection to CORT-treated ADX rats similar to the

case of GRH injection to controls (Fig. 6A, 6B, see also Fig. 7). Not only exocytosis, but also endocytosis was often observed near the exocytotic area (Fig. 6A).

A histogram showing the number of exocytotic figures found in the plasma membrane per unit length (μ m) in each subtype of GH cells in each experimental group is shown in Fig. 7.

Radioimmunoassay

Serum GH levels were calculated by radioimmunoassay. In the control condition, the serum GH level was about 50 ng/ml; GRH injection induced a significant increase in serum GH to about 250 ng/ml. This high level of serum GH was also observed after GRH injection to the CORT-treated ADX rats. In contrast, ADX induced a clear reduction of serum GH; however, GRH injection to ADX rats and to CORT-treated ADX rats maintained serum GH at levels similar to those of the controls (Fig. 8).

Discussion

Effect of MWI on fixation and immunolabeling of plastic-embedded sections

The MWI has been widely utilized to advance biological processing: tissue fixation, histochemistry, antigen retrieval, and *in situ* hybridization (Shi *et al.* 1991, Login *et al.* 1995, Van Ginneken *et al.* 1998, Rangell & Keller 2000, Mitchell *et al.* 2001, Paupard *et al.* 2001). The great increase in molecular motion induced by MWI enables fixatives to soak rapidly and deeply into cells and tissues (Boon *et al.* 1990, Rangell & Keller 2000). In the present study, we applied MWI for fixation and immunocytochemistry using rat GH antibody. The fixative effect of MWI seemed to be clearly better than that of conventional chemical fixation. Indeed, with MWI fixation, the ultrastructures at 50 μ m from the tissue surface showed consistent good quality in comparison with conventional chemical fixation, which often showed some insufficiently fixed figures.

On the other hand, MWI did not show significantly better results for the immunolabeling density using the protein-A method on plastic-embedded sections. Some recent papers have indicated preferential immunolabeling density by MWI (Rangell & Keller 2000, Paupard *et al.* 2001). These results were obtained using acrylic resin (such as LR Gold, LR White) or frozen sections. In contrast, we used a plastic embedding resin and also performed the post-fixation with osmium tetroxide. These differences might have influenced the present results. However, the immunolabeling using MWI was no less effective than the conventional method for the identification of GH cells. In the present study, MWI was found to provide an effective method for immunoelectron

Figure 2 High magnification electron micrographs of type I (mature) (A, D), type II (intermediate) (B, E), and type III (immature) (C, F) GH cells under control conditions (A, B, C) and after growth hormone-releasing hormone (GRH) injection to control rats (D, E, F). The GRH injection induced frequent exocytosis (arrows), especially in type I (D) and type II (E) GH cells. Arrowheads indicate the basement membrane. Bar=1 μ m.

microscopy that can be applied to the processing and immunolabeling of plastic-embedded sections.

The effect of GRH on the exocytosis of each subtype of GH under different corticosteroid conditions

Rat GH cells in the anterior pituitary are immunoelectron microscopically classified into three subtypes, distinguished as type I (mature), type II (intermediate) and type III (immature) GH cells (Kurosuni *et al.* 1986,

Kurosuni & Tosaka 1988, Takahashi 1991). In spite of being morphologically heterogeneous, the functional implication of these subtypes has not been precisely elucidated.

It is well known that GH secretion is regulated by two hypothalamic peptides, GH-releasing hormone (GRH) (Guillemin *et al.* 1982, Rivier *et al.* 1982, Soya & Suzuki 1990), and the inhibiting peptide, somatostatin (SRIF) (Brazeau *et al.* 1973, 1974, Soya & Suzuki 1990). Intravenous GRH injection to control (normal) rats has been

Figure 3 Electron micrographs of type II (intermediate) GH cells 4 days after adrenalectomy (ADX). (A) Many vacuolar bodies (arrows) appeared in the cytoplasm. (B) Enzyme cytochemical reaction for acid phosphatase was observed in these vacuoles, indicating that these vacuoles were lysosomes. (C) High magnification micrograph of a GH cell indicated that few exocytotic figures were observed and the protein-A gold immunolabeling of secretory granules was reduced after ADX. M, mature type GH cell. IT, intermediate type GH cell. Bar=1µm.

reported to induce a clear increase in both serum GH content and the number of exocytotic findings in GH cells within 4 min under pentobarbital anesthesia (Shimada & Tosaka-Shimada 1989, Shimada *et al.* 1990). However, these studies did not mention the difference in response in each subtype of GH cell. In the present study, we found that each of the subtypes of GH cells reacted to GRH, and

Figure 4 A type II (intermediate) GH cell following GRH injection to ADX rats showing few exocytotic cells (arrow). Arrowheads indicate the basement membrane. Bar=1µm.

Figure 5 A type II (intermediate) GH cell following corticosterone (CORT) treatment of ADX rats showing immunoreactivity restored to the control level. Arrowheads indicate the basement membrane. Bar=1µm.

showed an increase in exocytosis. However, the degree of reaction differed between the three subtypes. Type I (mature) and type II (intermediate) GH cells clearly showed a greater increase in exocytosis after GRH stimulation compared with type III (immature) cells, suggesting that the morphological heterogeneity of GH cells is associated with the difference in sensitivity to GRH, i.e. to the functional heterogeneity.

GH cells are known to be regulated by glucocorticoids (Miller & Mayo 1997, Dean & Porter 1999, Nogami *et al.* 1999, Porter *et al.* 2001). Recently, we immunohistochemically indicated the expression of glucocorticoid receptor (GR) in adrenocorticotrophic hormone cells, thyroid stimulating hormone cells, folliculo-stellate cells and GH cells in the rat anterior pituitary (Ozawa *et al.* 1999). This indicated that GH cells were directly regulated by glucocorticoids via GR. The present study showed that the effect of GRH on GH secretion was decreased by ADX, although the corticosterone replacement given to the

Figure 6 GRH injection to CORT-treated ADX rats showed a clear increase in exocytosis (arrows), particularly in type I (mature) (A) and type II (intermediate) (B) GH cells. Endocytosis was sometimes observed near the exocytosis (A, double arrows). Bar = 1 μ m.

Figure 8 Histogram of serum GH content (ng/ml) measured by radioimmunoassay in each experimental group. * $P < 0.001$ compared with the control group. Cont, control; ADX, adrenalectomy; GRH, growth hormone-releasing hormone; CORT, corticosterone.

ADX rats restored the response of GH cells to GRH to a level similar to that in controls, suggesting that a role of the glucocorticoids is regulation of the sensitivity to GRH and/or the expression of GRH receptor (GRH-R) in GH cells.

It has been reported that glucocorticoids regulate the expression of GRH-R in the pituitary cells *in vivo* and *in vitro* (Lam *et al.* 1996, Miller & Mayo 1997, Nogami *et al.* 1999). Previously, it was indicated that GRH binding sites were down-regulated in dispersed pituitary cells from ADX rats, and that replacement with the synthetic glucocorticoid, dexamethasone, was able to restore GRH binding (Seifert *et al.* 1985a,b). It was also reported that these changes in GRH binding were not due to alteration in the binding affinity, suggesting that the difference in the

Figure 7 Histogram of the number of exocytotic figures found in the plasma membrane per unit length (μ m) in each subtype of GH cells - type I (mature, black bars), type II (intermediate, gray bars) and type III (immature, open bars) - in each experimental group. * $P < 0.0001$ compared with the same subtype of the control group. ** $P < 0.001$ compared with another subtype in the same experimental group. Cont, control; ADX, adrenalectomy; GRH, growth hormone-releasing hormone; CORT, corticosterone.

number of receptors was responsible for the corticosteroid effects (Seifert *et al.* 1985a,b).

The cDNA for the GRH-R has been cloned and characterized from several species (Mayo 1992, Gaylinn *et al.* 1993, Hsiung *et al.* 1993). Glucocorticoid responsive elements have been identified in the 5' flanking region in the rat GRH-R gene as well as in the human GRH-R gene (Miller *et al.* 1999, Nogami *et al.* 2002). These findings suggest that the effect of glucocorticoids on GH secretion is mediated by the binding of activated GRs to GRH in the GRH-R gene and by the expression of GRH-R.

Miller and Mayo (1997) demonstrated that glucocorticoids modulated the GRH-R mRNA level in both *in vivo* and *in vitro* pituitary cells, and that the induction required new RNA synthesis, indicating that glucocorticoids act to regulate gene transcription, rather than mRNA stability. They also demonstrated that ADX decreases GRH-R mRNA expression, and corticosterone treatment increases GRH-R mRNA levels in the pituitary gland of ADX and normal rats. Nogami *et al.* (1999) also reported the possibility of the induction of GRH-R mRNA by glucocorticoids mainly through stimulation of mRNA transcription using MtT-S cells, a clonal strain of mouse GH cells. These chemical and molecular biological studies strongly support the hypothesis of the present study that secretion of GH stimulated by GRH is closely related to the circulating corticosteroid condition.

In conclusion, the present study indicates that differential sensitivity to GRH in each subtype of GH cells is closely related not only to the morphological heterogeneity but also to the functional heterogeneity among these subtypes. Further study on the differences in molecular regulation between glucocorticoids and the expression of GRH-R in each GH cell subtype is necessary.

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