Evidence for a physiological role of hypothalamic gastrin-releasing peptide to suppress growth hormone and prolactin release in the rat

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ABSTRACT Gastrin-releasing peptide (GRP) is localized to hypothalamic neurons and is a potent inhibitor of basal and growth hormone (GH)-releasing factor-induced GH secretion in the rat. It also acts similarly to inhibit opiate- and stress-induced prolactin (PRL) release. To determine the physiological significance of the peptide in the control of the release of these two hormones, a highly specific antiserum against GRP was injected into the third brain ventricle to immunoneutralize hypothalamic GRP. The injection of the antiserum initially did not alter levels of the hormones; however, both PRL and GH levels in the plasma began to increase within 3 and 3.5 hr, respectively. They were still significantly elevated 24 hr after the injection. There was no change in the plasma levels of either hormone in animals injected intraventricularly with a similar volume of normal rabbit serum (NRS). Mean plasma GH levels 24 hr after antiserum injection were more than twice those of the NRS-injected controls, whereas the PRL concentrations were 14-fold higher in the antiserum injected as compared to the control NRS-injected animals. A second similar injection of antiserum 24 hr after the first administration resulted in a slight and transient further increase in both GH and PRL levels so that they were both significantly (P < 0.001) higher than those of the animals given a second injection of NRS. The anti-GRP antiserum was highly specific for GRP by radioimmunoassay procedures and this antiserum produced positive immunostaining of GRP neuronal perikarya and terminals within discrete hypothalamic nuclei. Beaded fibers and terminals were observed in the suprachiasmatic nucleus (SCN) and the area lateral and dorsal to the SCN in the region of the periventricular nucleus (PeVN). GRP-positive perikarya were observed in the parvocellular neurons of the paraventricular nucleus. In addition, GRP-positive cell bodies were observed in the PeVN in close proximity to the third ventricle. Furthermore, the median eminence displayed no immunostaining for GRP, and all traces of positive staining were abolished by preabsorption of the antiserum with GRP-27 (30 μg/ml), confirming the specificity of the antiserum. The combined results with immunoneutralization of GRP and the immunostaining of GRP neuronal elements in the hypothalamus support the physiological role of this peptide in the inhibitory control of both GH and PRL release.

Gastrin-releasing peptide (GRP) is one of the many peptides now found to be present in hypothalamic neurons. It is a 27-residue molecule originally isolated from porcine nonantral stomach (1). It is related to the amphibian skin peptide bombesin and has been localized in tissues from various mammalian species including the human (2–5). The highest detectable concentrations of GRP are found in tissue extracts from brain (6) and gut (7, 8). Within the brain, the highest concentrations of GRP-like peptides can be found within the rostral hypothalamus (6). The anatomical distribution of GRP suggests a role for this peptide in the control of anterior pituitary function.

Intravenous administration of GRP stimulates the secretion of several gastroenteropancreatic hormones, among them gastrin, somatostatin, cholecystokinin (9), and insulin (10). Central administration of GRP and related peptides via the brain ventricles produces profound physiological and behavioral effects, including a significant decrease in gastric acid secretion (11), poikilothermy (12), grooming behavior (13), and decreased food intake (14).

In previous studies, we have demonstrated the ability of GRP to inhibit growth hormone (GH) release in vivo. Within 10 min after intraventricular (i.v.t.) injection of minute doses (10−12–10−9 mol) of GRP, basal GH levels were significantly depressed and all spontaneous GH pulses were abolished for periods in excess of 90 min. GRP also blocked the GH surge in response to GH-releasing factor (15, 16). We have further shown that this GH release inhibitory activity resides within the C-terminal heptapeptide by testing both amino- and carboxyl-terminal fragments (17). It is within the C-terminal decapetide that GRP resembles bombesin, the 14-amino acid amphibian peptide, which also possesses GH inhibitory activity (17).

Matsushita et al. (18) reported an inhibitory action of GRP on opiate-induced prolactin (PRL) secretion. We have also observed that GRP can inhibit the increase in plasma PRL induced by ether stress (unpublished data). We have further shown that somatostatin is an important mediator in the inhibitory response of GRP on GH release (19) and that GRP acts via a dopaminergic mechanism to block the stress-induced release of PRL.* In this study, we have examined the physiologic significance of hypothalamic GRP in the control of GH and PRL release by immunoneutralizing the endogenous peptide with a highly specific antiserum. This antiserum was also used in immunohistochemical studies that reveal a GRP neuronal system of GRP-like immunostaining in the rat hypothalamus.

MATERIALS AND METHODS

In Vivo Experiments. All experiments were performed on female Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) weighing 260–280 g. The rats were ovariectomized at least 3 weeks prior to each experiment so that the animals would be in a comparable hormonal state to those used previously (15) and to eliminate ovarian steroid feedback

Abbreviations: GRP, gastrin-releasing peptide; GH, growth hormone; i.v.t., intraventricularly; PRL, prolactin; NRS, normal rabbit serum; SCN, suprachiasmatic nucleus; PeVN, periventricular nucleus; PVN, paraventricular nucleus;

Lyophilized antiserum to porcine GRP (anti-GRP, serum 7188) was obtained from Peninsula Laboratories (San Carlos, CA). This antiserum was shown to crossreact 100% with porcine GRP(1–27), (14–27), -Ac(20–27), and bombesin in radioimmunoassay. It cross-reacted only 0.1% with substance P and did not cross-react at all with motilin, secretin, vasoactive intestinal polypeptide, gastrin, or cholecystokinin(26-33). The lyophilized antiserum was brought to its original volume with distilled water for i.v.t. injection. All other reagents were obtained from Sigma unless otherwise specified.

In the first experiment, a 23-gauge stainless steel cannula was implanted into the third brain ventricle of each rat 1 week prior to the experiment (20). Cannulae were implanted in the external jugular vein 24 hr prior to the experiment (21). Animals received a preliminary 3-μl dose of either anti-GRP or normal rabbit serum (NRS) i.v.t. at this time. On the following day, the rats received a second injection of anti-GRP or NRS (3 μl, i.v.t.). The protocol of antiserum injection was the same as that used successfully in earlier studies. Blood samples of 400 μl (heparinized) were drawn at 10-min intervals and replaced with an equal volume of physiological saline throughout the procedure to maintain blood volume. After the experiment, blood samples were centrifuged at low speed and the plasma was decanted and frozen for RIA of GH and PRL. The protocol of the second experiment was virtually the same except that the animals received a single dose of anti-GRP or NRS (3 μl, i.v.t.) on the day of the experiment and plasma GH and PRL levels were measured every 30 min over a 4.5-hr period to determine the time course of action of the antiserum.

Plasma samples were assayed in duplicate for GH and PRL concentrations and results were expressed in terms of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) rat-RP-1 standards. Statistical probabilities were calculated by analysis of variance with repeated measures (Newman-Keuls test). Student's t test was used for comparison of two means.

**Immunohistochemistry.** Animals were divided into two groups of three animals each. The first group received colchicine (40 μg per 2 μl, i.v.t.) 24 hr prior to sacrifice to facilitate identification of neuronal perikarya. All six animals were anesthetized with Equithesin, flushed via cardiac perfusion with physiological saline, and fixed with Zamboni's fixative. A block of tissue consisting of the preoptic area, hypothalamus, and median eminence was isolated from each brain and post-fixed in the same fixative for 24 hr. Transverse serial sections 50 μm thick were cut from each tissue block and stained for GRP by the indirect immunoperoxidase technique of Sternberger (22). All reagents for the peroxidase-antiperoxidase procedure were prepared in phosphate-buffered saline (PBS) (pH 7.4) containing 2% normal sheep serum. The tissue was first incubated at 4°C for 48 hr in a 1:400 dilution of the same rabbit anti-porcine GRP used earlier followed by incubation at 25°C for 30 min in a 1:100 dilution of sheep anti-rabbit IgG (Miles). The tissue was then placed in a solution of 3,3'-diaminobenzidine tetrachloride (7.5 mg per 50 ml of PBS) to which 50 μl of 3% H2O2 had been added. The sections were incubated at room temperature for 5–10 min with gentle agitation, after which they were thoroughly washed in PBS (pH 7.4), dehydrated with alcohol and xylene, and covered with glass cover slides. To verify the specificity of the primary antiserum, 1.0 ml of the anti-GRP (1:100 dilution) was preabsorbed with 30 μg of synthetic porcine GRP 24 hr prior to incubation of control sections.

**RESULTS**

GH. Twenty-four hours after the first i.v.t. injection and just prior to the second injection, plasma GH levels in GRP antiserum-injected animals were significantly (P < 0.001) elevated above those in the NRS-injected controls (63.3 ± 15.9 vs. 28.4 ± 3.1 ng/ml, respectively) (Fig. 1). After the second i.v.t. injection of anti-GRP antiserum, there was a slight but not significant further increase in plasma GH and the levels remained elevated for the rest of the sampling period of 50 min. In contrast, there was a slight but nonsignificant decline to lower levels of plasma GH in the NRS-injected control animals so that the difference between plasma GH in the two groups remained highly significant (Fig. 2A). The mean increase in plasma GH in the antiserum-
FIG. 3. GH levels monitored for 4.5 hr after a single 3-μl injection of GRP antiserum into the third brain ventricle (i.v.t.). GH levels demonstrated a significant (P < 0.001) increase by 3 hr after injection. Values shown are means ± SEM of seven measurements.

injected animals was significantly different from the decrease observed in the NRS-injected controls (P < 0.001) (Fig. 2A).

Since the plasma GH levels were already elevated 24 hr after the first injection of antiserum, it appeared of interest to examine the time course of the effect of a single injection of antiserum. After this single injection, plasma GH concentrations remained virtually unchanged for 150 min and then gradually increased so that they were significantly elevated by 210 min after the injection (P < 0.001) (Fig. 3). GH levels continued to increase throughout the remainder of the sampling period to a peak level of 102 ± 12 ng/ml at 270 min.

Prolactin. Results with PRL were similar to those just described for GH, but the effects of the antiserum were even more pronounced. Initial prolactin levels were highly significantly elevated in animals that had received anti-GRP antiserum i.v.t. 24 hr previously when compared to the values in the NRS-injected controls (P < 0.001) (Fig. 4). After the second injection of anti-GRP i.v.t., there was a slight further increase in plasma prolactin and a slight decrease in the NRS-injected controls so that there was a further significant difference in the behavior of prolactin in these two groups within the 20 min following the second injection (P = 0.05) (Fig. 2B).

Consequently, the time course of the effect of a single i.v.t. injection was determined. Values remained virtually unchanged after i.v.t. injection of either antiserum or NRS and began to increase at 150 min in the antiserum-injected

animals (P < 0.001) (Fig. 5). Plasma PRL levels continued to increase and reached the peak at the termination of sampling at 270 min. Values of PRL remained low and stable throughout the sampling period in the NRS-injected control animals.

Immunohistochemistry. Examination of the preoptic area and the entire rostrocaudal extent of the hypothalamus and median eminence revealed that GRP-like immunoreactivity was confined to the rostral hypothalamic area. No GRP-positive fibers or perikarya were detected in the preoptic area in either colchicine-treated or non-colchicine-treated animals. Beaded fibers and terminals were observed in the suprachiasmatic nucleus (SCN), the area lateral to SCN, and dorsally in the region of the periventricular nucleus (PeVN) in non-colchicine-treated animals. A sparse population of GRP-positive fibers was observed in the PeVN region of the anterior hypothalamus; however, no fibers were detected caudal to this area, nor were any fibers observed in the median eminence. GRP-positive perikarya were restricted to the rostral hypothalamus, specifically to the PeVN and the parvocellular neurons at the ventral border of the paraventricular nucleus (PVN) in rats injected i.v.t. with colchicine (Fig. 6). Preabsorption of the primary antiserum with GRP-27 completely abolished all evidence of immunostaining in the areas previously described.

FIG. 4. A single 3-μl injection of GRP antiserum into the third ventricle 24 hr prior to blood sampling resulted in a highly significant (P < 0.001) increase in basal PRL levels as shown here. Subsequent administration of 3 μl of GRP antiserum at time 0 produced a transient increase in plasma PRL levels within 20 min. Values shown are means ± SEM of seven measurements.

DISCUSSION

Growth Hormone. Our earlier studies have established the fact that GRP can inhibit GH release following its i.v.t. injection via a dopaminergic mechanism that results in stimulation of somatostatin release (17, 18). The somatostatin induces a decline in plasma GH, which is associated with blockade of the response to GH-releasing factor. The present results indicate that this action is physiologically significant since immunoneutralization of hypothalamic GRP with highly specific GRP antiserum resulted in an increase in plasma GH. This highly specific antiserum directed against the carboxyl terminus of GRP was used to immunostain GRP-containing neuronal structures in the hypothalamic region. The anti-GRP-staining fibers and terminals were intermingled with fibers and perikarya of anti-somatostatin-staining neurons, which provides an anatomical basis for the observed stimulation of somatostatin release by GRP.

Previous immunoneutralization studies of this type have involved a double injection of the antiserum because measure-
ment of hormones shortly after injection of antisera had failed to reveal any effect (23). In the present study, it was noticed that plasma GH levels were increased 24 hr after the first injection and showed little further change in response to a second injection of antisera, suggesting that the immunoneutralization of hypothalamic GRP was nearly complete even 24 hr after the first injection. Consequently, we evaluated the time course of the effect in the present experiments and showed that plasma GH began to increase 3.5 hr after injection and reached the highest level at sampling 270 min after injection.

The time required for the elevation to take place probably represents the time required for the antisera to be absorbed from the third ventricle either passively or by some active mechanism involving the ependymal cells. The remaining interval probably represents the time to diffuse to the active sites of interaction of GRP with somatostatin. We speculate that the longer the latency for the effect, the further from the ventricle are the sites. In the case of i.v.t. injection of antisera directed against galanin, the response can occur within 30 min, which suggests that the interaction is taking place very close to the ventricular wall (A. Ottlecz and S. M. M., unpublished data).

GH release is pulsatile in the rat and GRP was capable of blocking the pulses, probably via stimulation of the release of somatostatin, which then blocked the response of the pituitary to the release of GRF, which initiates the pulses (24). It is possible, however, that GRP may inhibit the release of GRF as well. We have no data to answer this question.

PRL. The report of Matsushita et al. (18) has established the inhibitory action of GRP on opiate-induced PRL release in vivo. Tache et al. (25) reported similar results with stress-induced PRL release with the related peptide bombesin. We have also seen an inhibitory response using GRP itself (unpublished observations). The effect of GRP antisem to elevate plasma PRL levels was more rapid in onset (3 hr) and more pronounced than that observed for GH. The more rapid onset suggests that the structures involved may be closer to the ventricle or more sensitive to the regulatory effects of GRF in the case of PRL than in the case of the GH-controlling system. These results suggest that hypothalamic GRP plays an important role in the tonic inhibition of PRL release.

Our previous studies have implicated the neurotransmitter dopamine in the inhibitory response of GH to GRP. Furthermore, Widerlov et al. report that bombesin, a GRP-like peptide, increases dopamine turnover in vivo in the hypothalamus (26). On the basis of this information, it seems reasonable to suggest that GRP may act to inhibit PRL release via a dopaminergic mechanism. This may also provide a mechanism whereby GRP inhibits GH release, since dopamine has also been shown to stimulate somatotropin release inhibiting factor release in vivo (27, 28).

Finally, we have examined the specificity of this antisem in immunohistochemical studies and found GRP-positive immunostaining in the SCN and PVN as described by Roth et al. (2). In addition, we observed GRP-positive cell bodies in the PVN in close proximity to the third ventricle. Furthermore, all labeling disappeared when the antibody was preabsorbed with GRP-27, indicating the specificity of the antisem. We have noted that GRP-containing neurons and perikarya in the hypothalamus occur solely in nuclei surrounding the third ventricle. GRP-like immunostaining in the rat hypothalamus is confined to a few discrete nuclei including the SCN, PVN, and parvocellular part of the PVN. The close association of these elements to the third ventricle allows for rapid uptake and action of the antisem in the appropriate tissues. In contradistinction, Kita et al. (29) reported that in the rabbit hypothalamus the highest concentrations of GRP-like immunoreactivity are found in the ventromedial and infundibular nuclei followed by PVN, SCN, and PeVN. Their results concur with ours in the absence of GRP-like immunoreactivity in the median eminence (29). These findings suggest species variability with respect to GRP localization and control in the mammalian brain.

The present study has enabled us to appreciate the profound effect endogenous GRP has on both plasma GH and prolactin levels. The results presented here in association with previous studies suggest that hypothalamic GRP is an important neuromodulator of anterior pituitary function with a primary action to inhibit GH and PRL secretion.

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