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# Distribution, Content and Molecular Heterogeneity of Gastrin-Releasing Peptide in Rat Pancreas

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Although importance of intrapancreatic neurons containing gastrin-releasing peptide (GRP) in control of exocrine secretion has been raised, the nature of GRP in the pancreas is unclear. Thus, the present study was undertaken to see distribution, content and molecular heterogeneity of immunoreactive GRP in the rat pancreas. Content of immunoreactive GRP in the rat pancreas was 2.99 ± 0.66 ng/g wet tissues determined by radioimmunoassay. Immunoreactive GRP was most abundantly expressed in the duodenal part among 3 parts of the pancreas; duodenal, body and splenic part. Vagotomy failed to change the content of immunoreactive GRP in the pancreas. Three distinct forms of immunoreactive GRP, very identical to GRP-27, bombesin-24 and neuromedin C, were observed in the rat pancreas by using reversed phase $C_{18}$ HPLC and Sephadex G-50 superfine column chromatography. Cell bodies of neurons containing immunoreactive GRP were scattered in pancreatic connective tissues and their nerve fibers innervated pancreatic acini and large ducts as determined by immunohistochemistry. The present results suggest that three distinct forms of GRP exist in intrapancreatic GRPergic neurons, which exert a stimulatory role in pancreatic exocrine secretion in rats.

Key Words: Intrapancreatic neuron, Peptidergic neuron, GRPergic neurons, GRP antibody, Pancreatic exocrine secretion

## INTRODUCTION

Gastrin-releasing peptide (GRP) is a neuropeptide with 27-amino acid residues firstly isolated from the porcine stomach (McDonald et al, 1979). It has been demonstrated that immunoreactive GRP exists in pancreatic neuronal elements of several mammalian species (Moghimzadeh et al, 1983; Ghatei et al, 1984; Knuhtsen et al, 1987; De Giorgio et al, 1992a; De Giorgio et al, 1992b; Shimosegawa et al, 1993). The results suggest that endogenous GRP may exert a physiological role in pancreatic exocrine secretion. The suggestion has been verified by showing that stimulation of the vagus or intrapancreatic neurons increases exocrine secretion as well as GRP release from the pancreas (Knuhsten et al, 1987; Holst et al, 1989; Park et al, 1999). The effect of neural stimulation on pancreatic exocrine secretion is reduced by a GRP antagonist (Holst et al, 1989) or an anti-GRP serum (Park et al, 1999).

Molecular forms of GRP in the pancreas are unclear at the present time although heterogeneous forms of GRP exist in the gastrointestinal tract. Two forms of immunoreactive GRP have been reported in the pancreas of several animal species (Ghatei et al, 1984). However, together with GRP with 27-amino acid residues, GRP with 23- and 10-amino acid residues are also isolated from the canine intestine (Reeve et al, 1983). Since peptides belonging to the GRP family, such as bombesin-14 and neuromedin C have immunological cross-reactivity with an GRP antibody (Kwon et al, 1998), careful studies should been taken to identify molecular heterogeneity of a peptide. Thus, the present study was undertaken to determine content and molecular forms of GRP in the

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pancreas of rats. Localization and innervation of the GRP-containing neuronal cell body were also examined.

## METHODS

*Animals and tissue preparations*

Experiments were performed on male Sprague-Dawley rats, weighing 250~300 g, which were supplied by the Hallym Laboratory Animal Center. Each animal was anesthetized with Ketamine (0.4 mg/kg, i.p.) and xylazine hydrochloride (0.2 mg/kg, i.m.) after a 24 hour-fasting with free access to water. After resection of the pancreas, rats were sacrificed by an intravenous overdose of Ketamine and saturated potassium chloride. Pancreata were trimmed to eliminate fat tissue and divided into duodenal, body, and splenic part. After weighing, each part of the pancreas was immediately boiled for 10 min and then homogenized in 0.5 M acetic acid (10 ml/g wet tissue) to extract GRP. Supernatant of the pancreatic extract was stored at −70°C until determination of GRP by radioimmunoassay (Kwon et al, 1998). For immunohistochemical studies, rats were perfused via the heart with ice-cold solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The pancreas was kept in the fixative overnight and thoroughly rinsed in the buffer solution containing 30% sucrose. Frozen sections with thickness of 20 $\mu$m were mounted on chromalum gelatin-coated slides and stored at −20°C until staining of GRP neurons by the streptavidin-biotin peroxidase method.

*Production of anti-GRP serum and radioimmunoassay of GRP*

An anti-GRP serum was produced in a New Zealand White rabbit by multiple injections of an antigen, which was prepared by conjugation of synthetic porcine GRP-27 to bovine serum albumin (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) (Boem et al, 1974). A tracer was prepared by lactoperoxidase (Calbiochem, La Jolla, CA, USA)-catalyzed iodination of synthetic [Tyr$^4$]-bombesin (Sigma) with $^{125}$I (Amersham, Buckinghamshire, UK), which had a specific radioactivity of 2,644 $\mu$Ci/nmole as determined by the self-displacement method (Stadil & Rehfeld, 1972). Radioimmunoassay of GRP was carried out according to the method described previously (Kwon et al, 1998).

*Tissue content and molecular heterogeneity of immunoreactive GRP*

Content of immunoreactive GRP in pancreatic extract was determined by radioimmunoassay (Kwon et al, 1998). Bilateral subdiaphragmatic vagotomy was carried out 48 h before the experiment to see an effect of the vagus nerve on pancreatic content of immunoreactive GRP. Molecular heterogeneity of pancreatic GRP was observed according to the method described previously using reversed phase HPLC and gel filtration (Park et al, 1992; Kwon et al, 1998). Pancreatic extract was injected to a $C_{18}$ analytical HPLC column (4.6×250 mm; ODS-120 T, LKB, Uppsala, Sweden) equilibrated with solution containing 0.1% trifluoroacetic acid and 10% acetonitrile. The column was eluted with a linear gradient from 10% to 21.5% of acetonitrile, at a flow rate of 0.6 ml/min. The column was calibrated with GRP-27, bombesin-14 and neuromedin C. Concentration of GRP in each fraction was measured by radioimmunoassay. Pancreatic extracts were also fractionated using Sephadex G-50 superfine column (1.6×112 cm) at 4°C with 600 mM sodium chloride in 10 mM phosphate buffer at a flow rate of 4.2 ml/h. The column was calibrated with GRP-27, bombesin-14, neuromedin C, blue Dextran and Na $^{125}$I as molecular size markers. Concentration of GRP in each fraction was also determined by radioimmunoassay.

*Immunohistochemistry of GRP neurons*

Streptavidin-biotin peroxidase method was employed for immunostaining of the pancreatic tissue in this study. Immunoreaction of tissue sections with the anti-GRP serum at a dilution of 1 : 1,000 was produced in a moist chamber over 12 h at 4°C. Biotinylated-goat anti-rabbit IgG (Zymed, USA), at a dilution of 1 : 200, was applied to tissue sections for 2 h at room temperature and then peroxidase conjugated streptavidin (Zymed), at a dilution of 1 : 200, was applied to tissue sections for 1 h at room temperature. Immunoreaction was rendered observable by soaking the tissue sections in 0.05% 3,3-diaminobenzidine tetrahydrochloride/0.005% $H_2O_2$ in 0.01 M phosphate buffered saline for 2~4 min at room temperature. The antiserum preabsorbed with bombesin-

14 (1 nmol/ml) gave no staining reaction, whereas the antiserum preabsorbed with substance P, gastrin cholecystokinin, vasoactive intestinal polypeptide or secretin gave results identical to those with the unabsorbed antiserum.

*Statistical analysis of data*

All results were illustrated as mean±S.E. The data were statistically analyzed using the Student's *t* test. The difference was considered significant when *P* value was less than 0.05.

## RESULTS

*Characterization of produced anti-GRP serum*

The antiserum had a titer of 1 : 66,000. The effective affinity constant and total binding sites of the antiserum, determined by using the Scatchard equation (Scatchard, 1949), were $1.07\times10^{11}$/M and $0.71\times10^{11}$/M, respectively. The heterogeneity index and the average affinity constant, obtained by using the Karush's linear regression analysis and the Split's equation (Split 1948), were 0.76 and 0.62 M, respectively. The half saturation concentration to GRP was 19.1 pM. As shown in Fig. 1, the antiserum showed full cross-reactivity with bombesin but very low cross-reactivity with cholecystokinin, secretin, gastrin, substance P, vasoactive intestinal polypeptide, neuromedin B or neuromedin U.

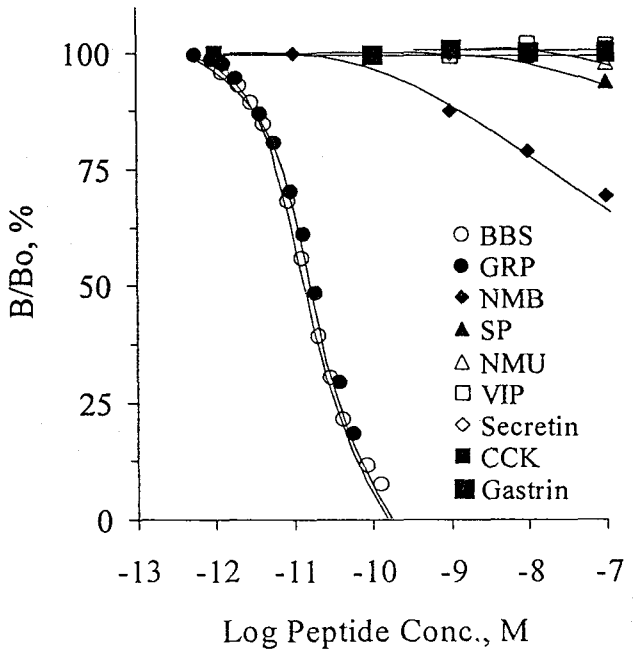


**Fig. 1.** Cross-reactivity of an anti-GRP serum raised in a rabbit. Extent of the cross-reactivity was expressed as tracer binding ratio at various peptide concentrations comparing with that of zero concentration (Bo). Abbreviation: GRP, gastrin-releasing peptide; BBS, bombesin; NMB, neuromedin B; SP, substance P; NMU, neuromedin U; VIP, vasoactive intestinal polypeptide; S, secretin; CCK, cholecystokinin; G, gastrin. The anti-GRP serum has low cross-reactivity with other peptides except GRP and bombesin.

*Pancreatic contents of immunoreactive GRP*

The mean content of immunoreactive GRP in the whole pancreas was 2.99±0.66 ng/g wet tissue. Among 3 parts of the pancreas, the duodenal part (4.94±0.76 ng/g wet tissue) contained the largest amount of immunoreactive GRP while the splenic part (1.37±0.33 ng/g wet tissue) had the least amount (Fig. 2A). Subdiaphragmatic vagotomy failed to change the mean content (2.75±0.35 ng/g wet tissue) of immunoreactive GRP in the whole pancreas (Fig. 2B).

*Molecular heterogeneity of pancreatic immunoreactive GRP*

The reversed phase $C_{18}$ HPLC profile of pancreatic extracts showed two distinct peaks of GRP immunoreactivity. The first peak was eluted at the position of synthetic neuromedin C with 42.3% of a relative amount of immunoreactive GRP, while the second peak was eluted at the position between those of synthetic bombesin-14 and synthetic GRP-27 with 57.7% of a relative amount of immunoreactive GRP (Fig. 3). The gel filtration profile using a Sephadex G-50 superfine column showed three distinct peaks. The first peak (Kav 0.56) was eluted at the position of GRP-27. The second peak (Kav 0.82) was eluted at the position of bombesin-14. The third peak had an identical Kav (0.92) with that of neuromedin C (Fig. 4).

*Neuronal cell body and nerve fibers of GRP in rat pancreas*

Neuronal cell bodies containing GRP immunoreactivity and its nerve fibers were localized in interlobular connective tissues (Fig. 5A). As shown in Fig.

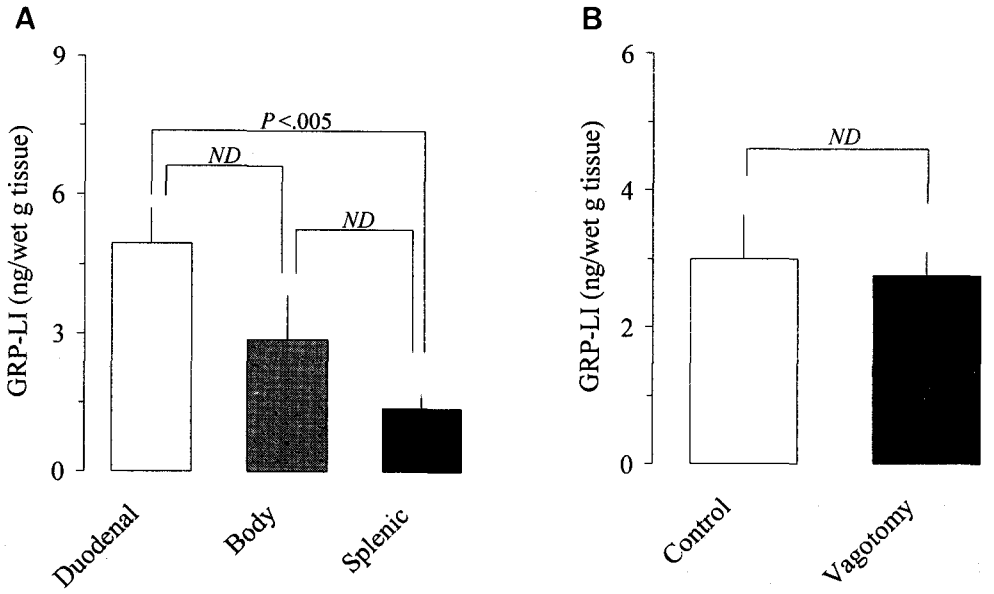


**Fig. 2.** Contents of GRP-like immunoreactivity in the pancreas of rats. A; Contents of immunoreactive GRP in the duodenal, body and splenic part of the pancreas. Each bar represents the mean ± S.E. of 6 experiments. B; Effects of subdiaphragmatic vagotomy on the content of immnoreactive GRP in the pancreas. Each bar represents the mean ± S.E. of 6 and 9 experiments for control and vagotomy, respectively. ND means no statistical difference.

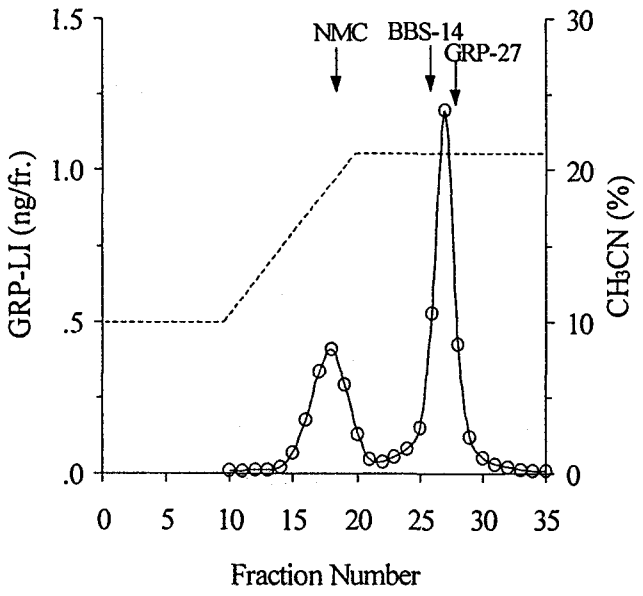


**Fig. 3.** A reversed phase $C_{18}$ HPLC profile of GRP-like immunoreactivity extracted from the pancreas of rats. Abbreviations: NMC, neuromedin C; BBS, bombesin; GRP, gastrin-releasing peptide. Pancreatic extract was eluted with a linear gradient of acetonitrile from 10% to 21.5%.

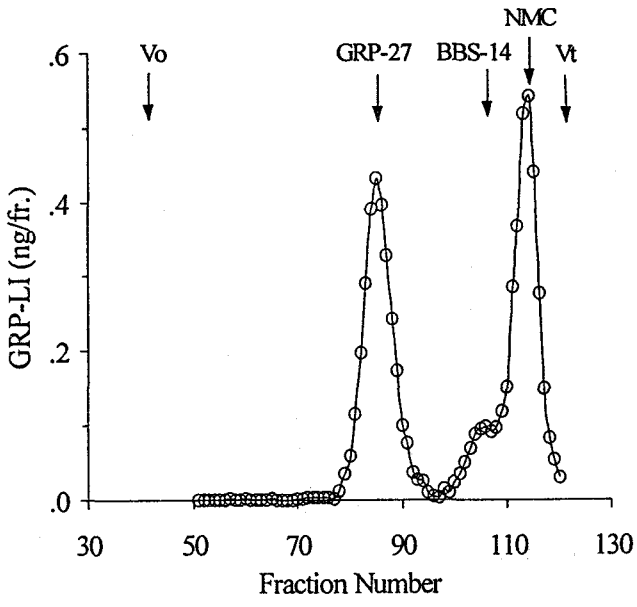


**Fig. 4.** A Sephadex G-50 superfine gel-filtration chromatogram of GRP-like immunoreactivity extracted from the pancreas of rats. Blue Dextran and $^{125}I$ were used for determination of the void volume (Vo) and the total volume (Vt). Abbreviations: NMC, neuromedin C; BBS, bombesin; GRP, gastrin-releasing peptide.

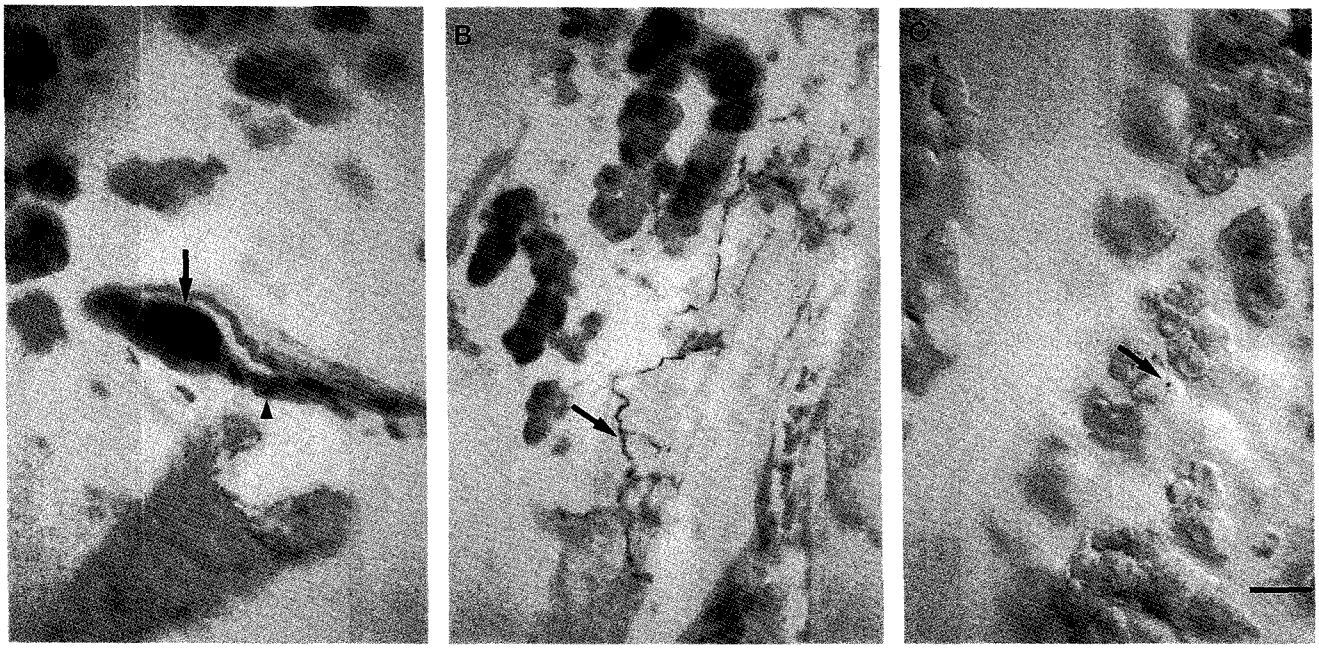


**Fig. 5.** GRP-containing neuronal cell body and nerve fibers in the rat pancreas. A; A GRP-immunoreactive neuronal cell body (arrow) and its nerve fiber (arrow head) in interlobular pancreatic connective tissue. B; GRP-immunoreactive nerve fibers innervating the pancreatic duct with bead-like shape (arrow). C; GRP-immunoreactive nerve terminals in periacinar spaces (arrow). Calibration bar: 50 $\mu$m.

5B and 5C, GRPergic nerve fibers innervated the pancreatic duct with bead-like shape and also detected in periacinar spaces.

## DISCUSSION

The rat pancreas contains immunoreactive GRP at a concentration of 2.99 ng/g wet tissue. The content of immunoreactive GRP in the pancreas was not changed even if the vagal input had been eliminated in this study. The result suggests that pancreatic synthesis of GRP is not under a vagal influence. When the pancreatic extract was fractionated by reversed phase $C_{18}$ HPLC, two distinct peaks of immunoreactive GRP were identified. However, the gel filtration profile with a Sephadex G-50 superfine column clearly showed three distinct peaks of immunoreactive GRP. In terms of molecular size, the first peak is identical with GRP-27 while the second one and the third one are similar to bombesin-14 and neuromedin C, respectively. The results of this study indicate that three forms of GRP exist in the rat pancreas. The major forms were GRP-27-like and neuromedin C-like immunoreactivity, which were determined at a similar concentration. The content of bombesin-14-like immunoreactivity was very low. In other study (Ghatei et al, 1984), however, only two peaks of immunoreactive GRP were observed in rat pancreas extract. The discrepancy may be explained if difference of a fraction volume is considered. Since three forms of GRP have been already reported in the canine gastrointestinal tract (Reeve et al, 1984) and porcine pancreatic juice (Knuhtsen et al, 1987), characteristics of the two GRP antibodies should be also compared in a future study.

GRP seems to be synthesized in neurons since GRP immunoreactivity is only detected in intrapancreatic neurons located in the interlobular connective tissue in this study. GRP-containing neurons has been already observed in the pancreas of rats (Ghatei et al, 1984; De Giorgio et al, 1992a), cats (De Giorgio et al, 1992b), pigs (Moghimzadeh et al, 1983; Knuhtsen et al, 1987) and humans (Shimosegawa et al, 1993). Innervation of GRPergic nerves does not seem consistent among species. GRPergic neurons could be detected in exocrine and endocrine compartment except pancreatic ducts in pigs (Moghimzadeh et al, 1983). However, we observed that GRPergic nerve fibers were in contact with pancreatic ducts and acini

in rats. The immunohistochemical findings suggest that intrapancreatic GRPergic neurons of rats may play a more significant role in exocrine secretion than those of other species. It has been reported that pancreatic protein secretion evoked by electrical vagal stimulation is inhibited by a GRP antagonist and desensitization of the pancreas for GRP but not by GRP antibodies (Holst et al, 1989). In a previous our study (Park et al, 1999), however, we observed that excitation of intrapancreatic neurons resulted in increases in exocrine secretion and GRP release in the isolated rat pancreas. Tetrodotoxin inhibited the GRP release induced by the intrapancreatic neuronal excitation. An anti-GRP serum reduced the effect of intrapancreatic neuronal excitation on exocrine secretion. Thus, we concluded that intrapancreatic GRPergic neurons play an important role in pancreatic exocrine secretion of rats.

In summary, three heterogeneous forms of GRP are contained in intrapancreatic neurons of which nerve fibers innervate pancreatic duct and acini. The results provide a strong evidence that intrapancreatic GRPergic neurons play a stimulatory role in exocrine secretion of the rat pancreas.

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