Gastroenterologia Japonica
Vol. 22, No. 2

Printed in Japan

—Original Article—

DISTRIBUTION OF GASTRIN-RELEASING PEPTIDE (GRP)-LIKE IMMUNOREACTIVITY IN THE RAT

Atsushi TAKAGI, M.D.*, Motoyuki MORIGA, M.D.*, Haruto UCHINO, M.D.* and Mitsuru AONO, M.D.**

**First Department of Internal Medicine and **Department of Clinical Nutrition, Kyoto University Faculty of Medicine, Kyoto 606, Japan*

Summary

Rat tissues were examined for gastrin-releasing peptide (GRP)-like immunoreactivity, using carboxy-terminus-specific antibody made against GRP (19–27), which is thought to be the biologically active site and to be common among species. The distribution of GRP-like immunoreactivity in the rat was similar to that of bombesin-like immunoreactivity. Sephadex G 50 chromatography revealed two peaks of GRP-like reactivity in the rat stomach.

Immunohistochemical studies using the antiserum to GRP (19–27) revealed numerous nerve fibers in the mucosa of the rat stomach. In the antral mucosa, GRP-containing nerves were present mainly around the base of the antral glands. Some GRP-containing nerves were in contact with gastrin-containing cells and somatostatin-containing cells. GRP-containing nerve fibers were numerous in the middle portion of the oxyntic gland, where somatostatin-containing cells were also detected. None of the endocrine cells stained positively with anti-GRP serum. These results support the hypothesis that GRP is a neurotransmitter in the stomach and that the peptide plays a physiological role in the gastrointestinal tract.

Key Words: *Bombesin-like peptide, Gastrin-containing cells, GRP, Immunohistochemistry, Radioimmunoassay, Somatostatin-containing cells.*

Introduction

Gastrin-releasing peptide (GRP) was initially isolated from porcine non-antral gastric tissue[1]. GRP and bombesin are very similar in their C-terminal amino-acid sequences as well as in their actions on the gastrointestinal tract[2]. Bombesin-like immunoreactivity has been described in mammalian tissues, but the level of reactivity is low[3–5]. It is not clear whether the relatively low bombesin-like immunoreactivity in mammalian tissue reflects poor cross-reactivity with frog bombesin radioimmunoassays or a low amount of the peptide.

We have studied the distribution of immunoreactive GRP in rat tissues, using a heterogenous antibody to porcine GRP (1–27) that recognizes both the C-terminus and the N-terminus of GRP, and have found that the level of GRP-like immunoreactivity in rat tis-

Received November 19, 1986. Accepted December 8, 1986.

Address for correspondence: Atsushi Takagi, M.D., First Department of Internal Medicine, Faculty of Medicine, Kyoto University, 54 Kawaramachi, Shogoin, Sakyo-ku, Kyoto 606, Japan.

This study was supported in part by grant in aid for scientific research from the Japanese Ministry of Education, Science and Culture (No. 59570300). We thank Mr. D. Mrozek for critical reading of the manuscript. We also thank Miss N. Shimizu for her assistance during these studies.

sues is similar to that of bombesin-like immunoreactivity[6]. Recently differences of the N-terminal sequence of GRP found in various species have been reported[7]. Therefore, the possibility existed that the concentrations of immunoreactive GRP in rat stomach might have been underestimated in our previous study[6], because of our use of the heterogenous antibody.

Although several studies have proposed GRP as a neurotransmitter for gastrin release[8–10], its precise role in the regulation of gastric secretion has not yet been fully defined. We reported that GRP suppressed secretagogue-stimulated gastric secretion in spite of its stimulation of gastrin release in the rat[11], and speculated that the inhibitory effect of GRP on secretagogue-stimulated gastric secretion is due to co-released somatostatin[11].

In the present study, we measured the levels of immunoreactive GRP in rat tissues using antiserum to porcine GRP (19–27), which is thought to be the biologically active site[12] and to be common among species[13]. In addition, we studied the localization of GRP-like immunoreactivity in the rat stomach by immunohistochemistry using the antiserum, and assessed the anatomical relationship among gastrin-containing cells, somatostatin-containing cells and GRP-like immunoreactivity.

Materials and Methods

Peptides

Synthetic porcine GRP (1–27)[14], synthetic porcine GRP (19–27)[14] and synthetic chicken GRP[15] were generous gifts from Dr. Yajima. Bombesin, substance P, VIP, and CCK were purchased from Peninsula Laboratories Inc (San Carlos CA. U.S.A.).

Production of antibody

C-terminal specific antisera were made using GRP (19–27). Synthetic GRP (19–27)[14] was coupled to bovine serum albumin (BSA) with carbodiimide. GRP-BSA conjugates were prepared by adding 0.145 μmol BSA, 260.9 μmol carbodiimide, and 1.3 mmol dimethylformamide to 1.65 μmol GRP (19–27) dissolved in 1 ml of 0.04 M phosphate buffer (pH 7.4). The mixture was stirred overnight at 22°C. Unreacted GRP (19–27) and carbodiimide were removed by dialysis against 0.04 M phosphate buffer. The antigen was emulsified with complete Freund's adjuvant for immunization. Japanese white rabbits were immunized by multiple intradermal injections at intervals of three weeks. Each rabbit received 1.0 ml of the emulsion, containing approximately 15–30 nmol GRP (19–27).

Evaluation of antibody

The optimal titer of antibody was determined by testing serial dilutions of antisera with 15,000 cpm of ^{125}I-GRP (20 fmol/ml). The titer was expressed as the dilution of the antisera at which 50% of ^{125}I-GRP was bound. The antisera were characterized for sensitivity and specificity. Sensitivity was expressed as the final concentration of peptide that caused 50% inhibition of the initial B/F ratio under optimal conditions (ID_{50}). The specificity of the antibodies was determined by the ability of VIP, CCK, substance P, chicken GRP and bombesin to compete with ^{125}I-GRP for the binding sites of the antibodies. The antigenic sites of these antisera were characterized by the reactivity with synthetic GRP fragments.

Radioiodination

Iodination of GRP was carried out by the chloramine T-method[16]. The reagents were added to a small glass tube in the following order; 20 μl of 0.15 M phosphate buffer, pH 7.4, 0.16 nmol GRP in 20 μl of 0.15 M phosphate buffer, 0.8 mCi (0.46 nmol) Na ^{125}I in 10 μl of solvent and 83.4 nmol chloramin T in 10 μl of H_2O. After 3 minutes at 22°C, 166.7 nmol sodium metabisulfite in 20 μl of phosphate buffer was added to terminate the reaction.

The reaction mixture was diluted with 203 nmol of BSA in 200 μl of 0.04 M phosphate buffer, pH 7.4, to which 100 μl of 1.2 μmol potassium iodide had been added. The final solution was applied to a 1 × 88 cm column of Sephadex G 50 superfine and eluted with 0.75 M ammonium acetate containing 72.5 μmol BSA, pH 6.5, and fractions of 1.0 ml were obtained. The radioactivity of labeled peptide was calculated from radiopaper chromatography and expressed as mCi/mg.

Assay procedure

GRP radioimmunoassay was performed in 0.04 M phosphate buffer with 0.1% BSA, pH 7.4. Each tube contained 100 μl of GRP in a standard, or unknown solution, 100 μl of antibody at a dilution sufficient to bind 40 to 50% of the labeled marker, 100 μl of ^{125}I-labeled GRP (15000 cpm), and 100 μl of 1% solution of bovine gamma-globulin. The tubes were incubated for 48 h at 4°C. Separation of the bound and free labeled peptide was performed by adding samples with polyethylene glycol-6000 (PEG). After mixing with 500 μl of 25% PEG solution, the tubes were centrifuged at 3500 rpm for 30 minutes. The radioactivity of the precipitate was determined. The detection limit was assessed by measuring pure buffer solution and buffer solution to which small amounts of the peptide had been added. Intraassay variation was estimated by measuring the same sample in quadruplicate in the same assay. The interassay variation was estimated by measuring the same sample in different assays.

Samples

Rat: Tissues were obtained from male Wistar rats. Brains were dissected following the method of Glowinski[17]. Each sample was homogenized by polytron PE-10, and extracted for 10 minutes in boiling 0.1 N acetic acid. The supernatants were neutralized to pH 7.0 by addition of 0.1 N NaOH and stored at −20°C prior to radioimmunoassay of immunoreactive GRP.

Immunohistochemical staining procedure

Rat gastric mucosa was fixed for 24 h at 4°C in Zamboni's fixative (4% formaldehyde in 0.01 M potassium phosphate buffer, pH 7.4, containing 15% saturated picric acid), dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Four micron sections of gastric mucosa were deparaffinized in xylene, hydrated in graded ethanol, and rinsed in 0.05 M phosphate-0.1 M NaCl buffer pH 7.4 (PBS). The sections were stained by the peroxidase anti-peroxidase (PAP) method[18], using a PAP kit (DAKO, Inc. CA. U.S.A.) for immunocytochemical localization of GRP. The sections were treated with 0.5% H_2O_2 for 20 min, rinsed for 20 min in PBS, and incubated for 20 min with 3% normal swine serum which was then shaken off. The sections were incubated with GRP (19–27) antiserum (1:800) for 48 h at 4°C and then rinsed gently three times for 5 min each time in PBS. They were incubated with swine antiserum to rabbit immunoglobulin for 20 min and then rinsed three times for 5 min each in PBS. They were incubated with PAP complex solution for 20 min and rinsed for 15 min in PBS. The slides were stained for 3 to 8 min in a freshly prepared solution of 0.01% 3.3′-diaminobenzidine tetrahydrochloride (Sigma St. Louis, MO, U.S.A.) containing 0.001% H_2O_2 in 0.05 M Tris-buffer, pH 7.6, to yield a reddish-brown immunoreactive color. Immunohistochemical localization of GRP and the second peptide (gastrin or somatostatin) in the same tissue section of rat gastric mucosa was performed using the method of Nakane[19]. After localization of GRP, the sections were washed with 0.1 M Glycine-HCl buffers (pH 2.2). To eliminate nonspecific staining, they were incubated with 5% non-immune swine serum for 20 min. In order to localize the second peptide (gastrin

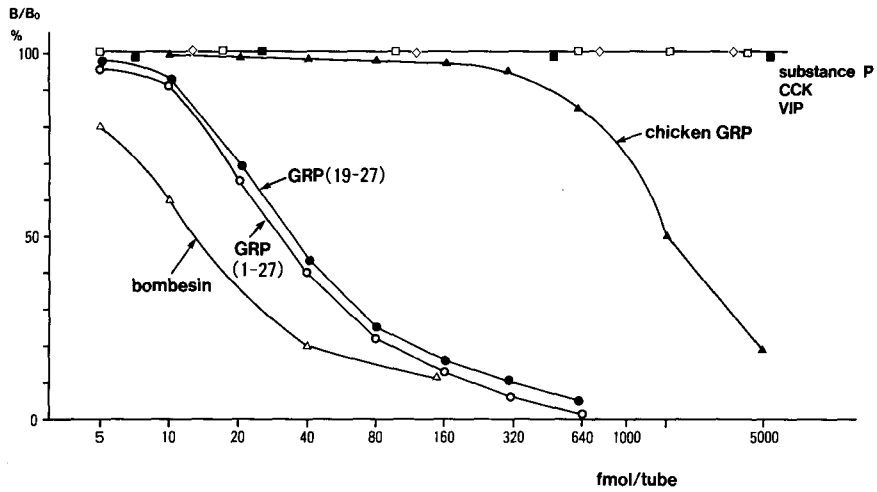

Fig. 1. Inhibition of ^{125}I-GRP binding to GRP antibody produced by various concentrations of gastrointestinal regulatory peptide.

or somatostatin) they were incubated with antiserum to gastrin (Dako) or antiserum to somatostatin (Dako) for 24 h at 4°C. They were incubated with swine antiserum to rabbit gamma-globulin and PAP solution as well as in the initial staining step, and stained with 4-Cl-1-Naphtol (Nakarai, Kyoto, Japan). Gastrin or somatostatin-containing cells were stained blue with 4-Cl-1-Naphtol. For controls normal non-immune rabbit serum or diluted GRP antiserum preincubated with GRP (10 nmol/ml) was substituted for the active antiserum.

Results

RIA: The final dilution of antibody to GRP (19–27) (code name R_3), which was most useful was 1:6000. The ID_{50} for antiserum R_3 was 63 pmol/L. The specificity of antiserum R_3 is indicated in **Fig. 1**. GRP, GRP (19–27) and bombesin demonstrate almost identical inhibitory potency with antiserum R_3. The binding affinity for antiserum R_3 of chicken GRP, which has c-terminal homology with porcine GRP except at position 19, was 36%. Judging from the cross reactivity with chicken GRP, asparagine/serine, at position 19, might be involved in the antigen-antibody binding of antiserum R_3. The antiserum did not show any inhibition of binding with VIP, CCK or substance P.

The calculated specific acttivity of labeled GRP ranged from 328 to 391 mCi/mg.

Reliability of the assay

The minimum detection limit for antiserum R_3 to porcine GRP was 5 pmol/L. The intra- and inter-assay variation with rat tissue extract were 13.2% and 18.1%, respectively.

Immunoreactive GRP in rat tissue extract

Table 1 shows the distribution of immunoreactive GRP in various rat tissues. In the central nervous system, higher levels of GRP-like immunoreactivity were seen in the mid brain. The highest concentration was seen in the stomach, with lower levels in other portions of the gastrointestinal tract. Fractionation of the extracts of rat stomach on the Sephadex G-50 revealed two components of immunoreactive GRP (**Fig. 2**). The larger MW peak was about the same size as that of GRP (1-27). The smaller MW peak corresponded to GRP (19–27).

Table 1. Distribution of immunoreactive GRP in various rat tissues

Tissue	pg/mg wet weight
Brain	
Hypothalamus	21.7± 5.5
Mid brain	33.4±13.4
Cerebral cortex	5.5± 1.3
cerebellum	6.5± 0.6
Esophagus	4.1± 1.5
Stomach	160.5±16.6
Duodenum	14.6± 8.9
Jejunum	44.0±12.9
Ileum	12.6± 1.2
Colon	52.5±15.4
Pancreas	5.6± 1.8

All values represent the means ± SEM of three rats.

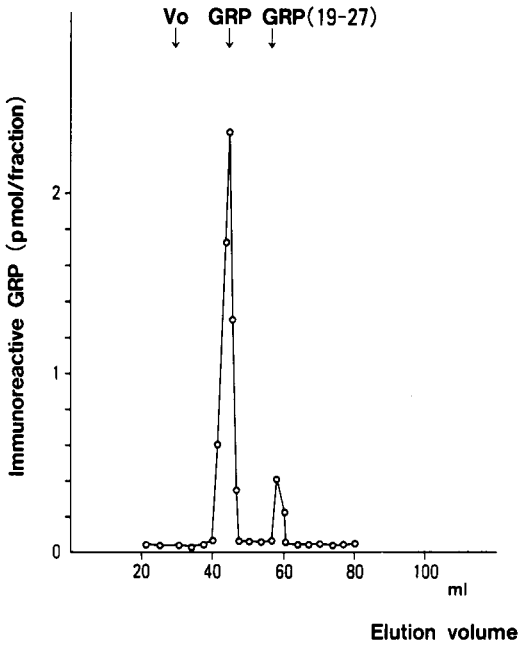

Fig. 2. Sephadex G50 gel chromatography of rat stomach extract.

Immunohistochemical staining of GRP

GRP-like immunoreactivity was detected in the nerve fibers of the rat mucosa. In the rat antral gland area, GRP-containing nerve fibers were present mainly around the base of

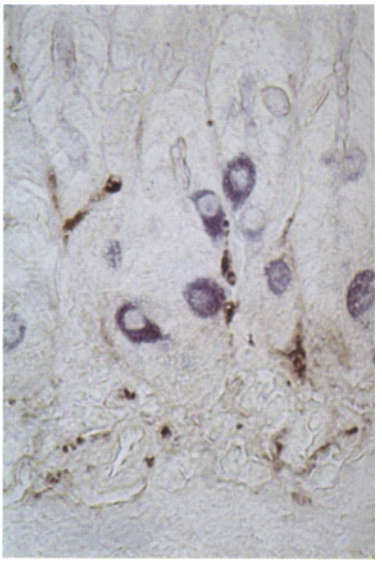

Fig. 3. Cross-section of rat antral mucosa showing GRP immunoreactive nerve fibers (brown) running between glands, and making close contact with gastrin-containing cells (blue); ×600.

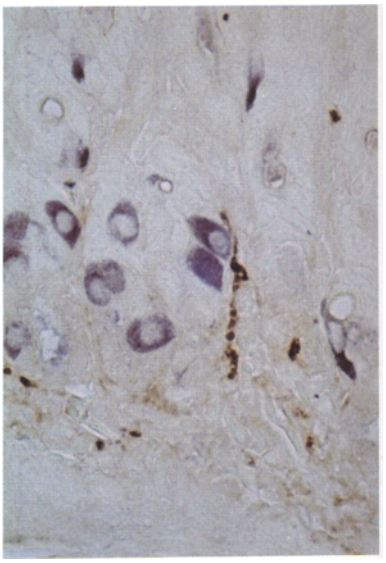

Fig. 4. Cross-section of rat antral mucosa showing GRP immunoreactive nerve fibers (brown), and making close contact with somatostatin-containing cells (blue); ×600.

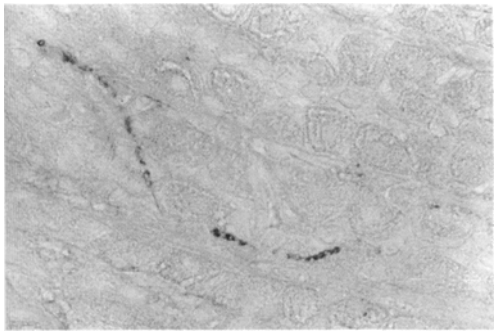

Fig. 5. GRP-containing nerve fibers in the oxyntic mucosa of a rat; ×600.

the antral gland, where gastrin-containing and somatostatin-containing cells were also detected. Some GRP-containing nerves were in contact with the gastrin-containing (**Fig. 3**) and somatostatin-containing cells (**Fig. 4**). In the oxyntic gland area, GRP-containing fibers were numerous in the middle portion of the gland (**Fig. 5**), where somatostatin-containing cells were also detected (data not shown). Because of the inadequate density of somatostatin-containing cells at this site, close contact between GRP-containing nerves and somatostatin-containing cells could not be demonstrated. No GRP-like reactivity was observed in studies in which the GRP antiserum was replaced by normal rabbit serum or antiserum absorbed with GRP (10 nmol/ml).

Discussion

This study demonstrated the presence of GRP-like immunoreactivity in rat tissues. The distribution of immunoreactive GRP confirms previous reports[3,20] using a bombesin radioimmunoassay in rat. In the central nervous system, higher levels of GRP-like immunoreactivity were seen in the mid brain. Centrally administered GRP has been reported to affect gastric secrretion[21], thermoregulation[22], and behavior changes[23], so endogenous GRP may have an active role in the central nervous system.

The fact that the highest level of GRP-like immunoreactivity was shown in the stomach, suggests that GRP plays an important role in the regulation of gastric function. The level of GRP-like immunoreactivity measured with c-terminal specific anti-GRP serum, was relatively higher than the reactivity measured by heterogenous antiserum to porcine GRP[6]. These results suggest that the rat stomach contains peptides that are more similar to porcine GRP than bombesin but not identical to porcine GRP. The same speculation was raised by Roth et al.[7], who showed that the GRP-like peptide from the rat brain might be different from authentic GRP, using high performance liquid chromatography analysis.

Gel chromatography of the rat stomach extract showed two peaks of GRP-like immunoreactivity, one eluting with GRP (1–27) and the second with GRP (19–27). A molecular heterogeneity of GRP-like peptides has been reported in mammalian brain and gut[3,20], but the proportions of the two forms vary. Further study with species-specific antisera is required to determine the actual level of GRP in each species.

The present immunohistochemical study demonstrated that GRP-like immunoreactivity is present in the nerve fibers of the rat gastric mucosa, and some GRP-containing nerves in the antrum made contact with gastrin-containing cells. These findings confirm earlier results[24–26], and support the hypothesis that GRP plays a role as a neurotransmitter of gastrin release[8–10].

Studies on the effect of GRP on gastric secretion have shown conflicting results[11,27,28]. GRP stimulates gastric secretion in dogs[27] and man[28]. On the other hand, our study shows GRP suppresses secretagogue-stimulated gastric secretion in the rat[11]. Since GRP stimulates somatostatin release in vitro[9], it is specu-

lated that the inhibitory effects of GRP on secretagogue-stimulated gastric secretion is due to co-released somatostatin[11]. In this study, we showed that some GRP-containing nerves made contact with somatostatin-containing cells. These findings suggest that GRP may stimulate somatostatin release directly.

GRP-containing nerves were also detected in the fundic portion of the stomach. We could not demonstrate an anatomical relationship between the GRP-containing nerves and the somatostatin-containing cells in the oxyntic gland area. The role of the GRP-containing nerves in the fundus remains unclear.

This study is the first to show that GRP-containing nerves made close contact with somatostatin-containing cells as well as gastrin-containing cells in the antrum. Further study is necessary to define the complex function of GRP in the gastrointestinal tract.

References

1) McDonald TJ, et al: Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. Biochem Biophys Res Commun 1979, 90: 227
2) Brown M, et al: Is gastrin releasing peptide mammalian bombesin? Life Sci 1980, 27: 125
3) Brown M, et al: Bombesin-like immunoreactivity: Radioimmunologic assessment in biological tissues. Life Sci 1978, 23: 2721
4) Walsh JH, et al: Bombesin-like peptides, In: Jaffe BM, Behrman HR, eds. Methods of Hormone Radioimmunoassay. Academic Press, New York, 1979: 581
5) Yanaihara C, et al: Bombesin-like immunoreactivity in mammalian tissues. Biomed J 1980, 1: 96
6) Takagi A, et al: Distribution of gastrin-releasing peptide in mammalian tissues, In: Miyoshi A, ed. Gut Peptides and Ulcer. Biomed Res Found, Tokyo, 1983: 33
7) Roth KH, et al: Identification of gastrin releasing peptide-related substances in guinea pig and rat brain. Biochem Biophys Res Commun 1983, 112: 528
8) Grossman MI: Vagal stimulation and inhibition of acid secretion and gastrin release, In: Reheld JJ, Amdrup E, eds. Gastrin and the Vagus. Academic Press, London, 1979: 105
9) Duval JW, et al: Stimulation of gastrin and somatostatin secretion from the isolated rat stomach by bombesin. Am J Physiol 1981, 241: G242
10) Nishi S, et al: Vagal stimulation of GRP, gastric somatostatin, and gastrin secretion in vitro. Am J Physiol 1985, 248: E425
11) Takagi A, et al: Paradoxical effects of gastrin releasing peptide on gastrin release and gastric secretion in the rat. Gastroenterol Jpn 1986, 21: 573
12) Rivier JE, et al: Bombesin, bombesin analogues, and related peptide: effects on thermoregulation. Biochemistry 1978, 17: 1766
13) Spindel ER, et al: Cloning and characterization of cDNAs encoding human gastrin-releasing peptide. Proc Natl Acad Sci USA 1984, 81: 5699
14) Akaji K, et al: Studies on peptide CII synthesis of a heptacosapeptide amide corresponding to the entire amino acid sequence of gastrin-releasing peptide. Int J Peptide Protein Res 1981, 18: 180
15) Akaji K, et al: Studies on peptides CXI. synthesis of chicken gastrin-releasing peptide. Int J Peptide Protein Res 1982, 20: 276
16) Hunter WM, et al: Preparation of ^{131}I-labelled human growth hormone of high specific activity. Biochem J 1963, 89: 114
17) Glownski J, et al: Regional studies of catecholomines in the rat brain-1. J Neurochem 1966, 13: 655
18) Sternberger LA, et al: The unlabelled antibody-enzyme method of immunohistochemistry. J Histochem Cytochem 1970, 18: 315
19) Nakane PK, et al: Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method. J Histochem Cytochem 1968, 16: 557
20) Walsh JH, et al: Bombesin-like peptides in mammals. Fed Proc 1979, 38: 15
21) Tache Y, et al: Central nervous system inhibition of gastric secretion in the rat by gastrin-releasing peptide, a mammalian bombesin. Gastroenterology 1981, 81: 298
22) Francesconi R, et al: Thermoregulatory effects of centrally administered bombesin, bradykinin, and Methionine-Enkephalin. Brain Res Bulletin 1981, 7: 63
23) Kalkosky PJ, et al: Feeding suppression and grooming repeated by elicited by intraventricular bombesin. Brain Res 1982, 242: 194
24) Dockray GJ, et al: The neural origin of bombesin-like immunoreactivity in the rat gastrointestinal tract. Neuroscience 1979, 4: 1561
25) Iwanaga I: Gastrin-releasing peptide (GRP)/bombesin-like immunoreactivity in the neurons and paraneurons of the gut and lung. Biomed Res 1983, 4: 93
26) Jain DK, et al: Functional and anatomical relationships between antral gastrin cells and gastrin-releasing peptide neurons. Histochemistry 1985, 82: 463
27) Hirschwitz BI, et al: Relation of gastric acid and pepsin secretion to serum gastrin levels in dogs given bombesin and gastrin 17. Am J Physiol 1983, 244: G546
28) Knigge U, et al: Gastrin-releasing peptide: Pharmacokinetics and effects on gastro-entero-pancreatic hormones and gastric secretion in normal men. J Clin Metab 1984, 59: 310