MOTILIN-LIKE IMMUNOREACTIVITY IN PORCINE, CANINE, HUMAN AND RAT TISSUES

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ABSTRACT

Eight antisera were obtained from three rabbits and two guinea pigs immunized with synthetic porcine motilin and from three guinea pigs immunized with [15-Glutamine]-motilin. Specificities of the antisera were characterized and three of them having different specificities were used in radioimmunoassay to examine motilin immunoreactivity in extracts of canine, porcine, human and rat gastrointestinal tissues. Immunoreactive motilin in rat tissue extract showed a very low cross-reaction in all of the assay systems used, suggesting a relatively high degree of variation in the amino acid sequence of rat motilin as compared with that of porcine motilin. In the assay system using the carboxyl-terminal specific antiserum R-1105, the inhibition curves of canine, porcine and human tissue extracts were superimposable on that of synthetic porcine motilin. However, in the assay system using antiserum R-1106 or GP-1103, the inhibition curve of canine tissue extract was not parallel to that of synthetic motilin. Human intestinal tissue extract also showed slightly weak inhibition in the assay system with antiserum R-1106. The motilin concentrations in duodenum extracts of the dog, pig and man measured by four different immunoassay systems were variable depending on the species of the tissue and antiserum used. Gel filtration of human duodenum and jejunum mucosa showed that the tissues contain three major components with motilin-like immunoreactivity. The results suggest either the presence of degradation products of motilin precursor or the existence of a new family of related peptides.

KEY WORDS motilin-like immunoreactivity / radioimmunoassay / tissue extracts

Porcine motilin is a docosapeptide (1, 15): H-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln-OH, which has been isolated from the duodenal mucosa (2). In dogs with a denervated pouch of the fundus of the stomach and Mann-

Bollman fistula, duodenal alkalinization resulted in an increase in serum motilin with a corresponding increase in gastric motor activity in the fundic pouch (3). However, Mitznegg *et al.* (8) reported that duodenal acidification, but not alkalinization resulted in a significant increase in plasma motilin concentration in men. Similarly, Tai and Chey (19) have observed an increase in plasma motilin concentration after duodenal acidification in dogs and no significant change

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in the plasma motilin level following the duodenal alkalization in men or in dogs.

Immunoreactive motilin is localized in endocrine cells of the gastrointestinal tract of various animals. Pearse *et al.* (9, 10, 13, 14) and Tobe *et al.* (20) have shown that motilin is produced in enterochromaffin (EC) cells of the duodenum and upper jejunum in animals. Contrary to these observations, other groups of investigators have demonstrated that motilin and 5-hydroxytryptamine are apparently stored in different cell types (4, 18). This was further confirmed in the human and monkey duodenum and upper jejunum by Helmstaedter *et al.* (6). Polak *et al.* (11) have also found, in contrast to their previous findings, that the C-terminal specific antiserum reacts with non-EC cells.

These variable observations in the physiological and immunohistochemical studies on motilin may be explained by species variation in motilin action and/or motilin immunoreactivity and different specificities of the antiserum used. The availability of a highly purified preparation of synthetic porcine motilin (17) allowed us to produce anti-synthetic porcine motilin sera having different specificities. This communication characterizes the immunoreactive motilin extractable from the gastrointestinal tissues of several mammalian species using these various antisera.

MATERIALS AND METHODS

Motilin and Related Peptides

Pure synthetic porcine motilin, [15-Glutamine]-motilin and the related peptides were synthesized according to the method of Shimizu *et al.* (17). The immunological property of the synthetic porcine motilin was proved to be identical with that of the natural preparation in a radioimmunoassay system using ^{125}I -natural porcine motilin and an antiserum raised against the natural preparation. The assay was kindly performed by Prof. J. C. Brown, University of British Columbia, Vancouver, Canada. [15-Glutamine]-motilin was shown to possess $154 \pm 18.3\%$ activity of that of the synthetic porcine motilin in terms of the gastric motor stimulating activity in dogs (7). Synthetic fragments, motilin (7-22), motilin (12-22) and motilin (1-6) Me were used for the characterization of the antisera used in this study.

Immunization

A solution of synthetic porcine motilin (5.0 mg)

in 0.9% NaCl (0.5 ml) was mixed with 50% aqueous polyvinylpyrrolidone (PVP, MW 25,000-30,000, Merck, Darmstadt) in 0.9% NaCl solution (1.5 ml). The mixture was emulsified with complete Freund's adjuvant (Calbiochem, Calif.) (2.5 ml) and the emulsion was injected subcutaneously at multiple sites on three mixed-bred male rabbits and three male guinea pigs. A total of 1.0 ml of the emulsion was used per rabbit and 0.5 ml per guinea pig. Each of the animals received the 2nd-8th injections in the same manner with reduced amounts of the immunogen (0.5-1.0 mg). The series of immunizations were performed at 2 week intervals, and blood was taken 10 days after the 4th and 5th injections. Finally, the animals were bled 10 days after the last injection. Additional three guinea pigs were immunized with [15-Glutamine]-motilin in a manner similar to that described above.

Labelled Antigen

Iodination of synthetic porcine motilin was performed by a slight modification of the method of Greenwood *et al.* (5). To a solution of synthetic porcine motilin or [15-Glutamine]-motilin (5 μg) in 0.5 M phosphate buffer (pH 7.6) (20 μl) were added [^{125}I]Na (NEN, Boston) (1 mCi) and Chloramine T (25 μg /10 μl 0.05 M phosphate buffer). The solution was mixed for 15 sec, and sodium metabisulfite (150 μg /50 μl 0.05 M phosphate buffer) was added. The mixture was applied to a column of Sephadex G-10 (1.0 $\times$ 25 cm) and the column was eluted with 0.01 M phosphate-saline buffer (pH 7.4). The eluates excluded in the void volume were collected and lyophilized.

Radioimmunoassay

The radioimmunoassay was performed in the manner described previously (21). The standard diluent used was a 0.01 M phosphate buffer (pH 7.4) containing 1% BSA, 0.025 M EDTA and 0.14 M NaCl. To the standard diluent (0.4 ml) in each test tube were added synthetic porcine motilin or unknown sample (0.1 ml) and the 1st antiserum, anti-motilin serum (0.1 ml). The mixture was kept at 4°C for 48 hr and then ^{125}I -motilin (0.1 ml) was added. After 48 hr incubation at 4°C, the 2nd antiserum, anti- γ -globulin serum (0.1 ml) was added and the mixture was incubated at 4°C for 24 hr and then centrifuged at 3,000 rpm for 30 min at 4°C. The supernatant was removed by aspiration and the

precipitate was counted.

Tissue Extraction

Duodenal tissues were obtained from dogs, pigs, and rats after the intravenous injection of lethal doses of sodium pentobarbital. Human autopsied samples were also used. The tissue samples were weighed and immediately frozen on dry ice. Each of the frozen tissues was homogenized and extracted with boiling water for 5 min at a concentration of 0.2 g of wet weight tissue per milliliter of water. After acidifying with acetic acid to 1 M concentration on cooling, the extract was centrifuged at 3,000 rpm for 20 min at 4°C. The supernatant was separated and the precipitate was washed with 1 M acetic acid. The combined supernatant and washing were lyophilized. The dried material was dissolved in 1 M acetic acid and the insoluble material was removed by centrifugation. The extract solution was subjected to gel filtration on Sephadex G-25 (1.5×90 cm) using 1 M acetic acid as eluent. The eluates containing materials of molecular weight over 1,000 were pooled, lyophilized and used for assay. Extraction of human autopsied tissues was performed with 0.1 M acetic acid in a boiling water-bath for 5 min. The extract was centrifuged and the supernatant was separated, lyophilized and used for assay.

Gel Filtration of Extract on Sephadex G-50

Gel filtration of some of the extracts was carried out on Sephadex G-50 (medium) column (1.5×90 cm) using 3 M acetic acid as eluent. Each of the fractions (3 ml each) was lyophilized, reconstituted with the radioimmunoassay buffer and assayed.

RESULTS

Synthetic porcine motilin and [15-Glutamine]-motilin adsorbed to PVP were relatively immunogenic and produced antibodies in three rabbits and five guinea pigs after three or four immunizations, which bound 50% of the labelled antigen at titers ranging from 1:5,000 to 1:20,000. The antisera obtained were anti-motilin sera: R-1104, R-1105, R-1106, GP-1102 and GP-1103, and anti-[15-Glutamine]-motilin sera: GP-2801, GP-2802 and GP-2803. The minimum detectable dose of the present immunoassays was 5 pg of synthetic porcine motilin. Fig. 1 shows a typical standard curve for the motilin immunoassay with antiserum R-1105. The logit plot of the B/B₀ ratios gave a straight line. In the assay using any of the antisera produced in the present study, no detectable cross-reaction was observed with our synthetic preparations of substance P, somatostatin, porcine VIP, porcine secretin and human gastrin I and with the natural preparations of porcine

Fig. 1 A standard curve for motilin radioimmunoassay. Antiserum: R-1105 (final dilution 1:7,000). Labelled antigen: ¹²⁵I-synthetic porcine motilin. Standard: synthetic porcine motilin (NY-FS-3-8). Separation: double-antibody method.

glucagon and insulin (Lilly Research Laboratories, Ind.) at concentrations up to 1 μ g per ml.

Characterization of Antisera

Table 1 summarizes the relative cross-reactivities of synthetic motilin-related peptides to the eight antisera obtained in the present study. The relative cross-reactivities were calculated by comparing the amount of a peptide needed to displace 50% of the antibody-bound labelled antigen with that of the standard motilin. In the systems with antisera R-1104 and R-1105, [15-Glutamine]-motilin displaced the labelled antigen significantly and the shorter carboxyl-terminal fragment, motilin (12-22) still showed considerable cross-reaction. Antiserum R-1105 was found to be especially specific to the C-terminal region of motilin and the major immunologic determinant was located within the 12-22 sequence, while in the system with R-1104, the displacement curves of the shorter carboxyl-terminal fragments, motilin (7-22) and motilin (12-22) were not parallel to that of synthetic motilin. For antiserum R-1106, [15-Glutamine]-motilin cross-reacted nearly identically with the standard motilin, but any of the motilin fragments examined showed very low cross-reactivities or did not react. This antiserum seems to recognize the entire molecule of motilin. In the system with GP-1102 or GP-1103, [15-Glutamine]-motilin showed markedly reduced crossreactivity. Motilin (7-22) cross-reacted significantly with GP-1103, but the displacement curve was not parallel to that of the standard motilin. The major immunologic determinant of GP-1103 seems to be located within the 7-15 sequence. On the other hand, the relative cross-reactivities of the three motilin fragments indicate that the whole molecule of

motilin is required for antigen-antibody interaction in the GP-1102 system. For antisera GP-2801 and GP-2803, which were raised against [15-Glutamine]-motilin, the [15-Glutamine]-analog showed more or less higher cross-reactivity than that of the standard motilin, indicating that the Gln residue in position 15 participates in the cross-reaction in these systems. The reactivity of the amino-terminal hexapeptide was only less than 0.1% in any of the assay systems examined.

Comparison of Immunoreactive Motilin in Tissue Extracts of Various Species

The inhibition curve of the porcine duodenum extract was parallel to that of synthetic porcine motilin in any of the systems using the present antisera. Immunoreactive motilin in the extract from the duodenal tissue of each species was examined in the assay systems using R-1105, R-1106 and GP-1103 (Fig. 2). In the system with the carboxyl-terminal specific antiserum R-1105, the dilution curves of the duodenal tissue extracts from the pig, dog, human and rat were all parallel to that of the standard motilin. For antisera R-1106 and GP-1103, the duodenal tissue extracts from rat and dog showed cross-reaction, but the inhibition curves were not parallel to that of the standard motilin, while the extract from the human tissue showed better parallelism in its curve. The immunoreactive motilin contents in the porcine, canine, and human duodenal tissue extracts were measured in the assay systems using antisera R-1104, R-1105, R-1106 and GP-1103, respectively (Table 2). In any of the assay systems, the highest immunoreactivity was detected in the porcine extract, while the contents in the canine extract were the lowest. It is of particular in-

Table 1 *Relative Cross-Reactivities of Synthetic Motilin-Related Peptides to Various Antisera (%)*

Antiserum	Motilin	Motilin (7-22)	Motilin (12-22)	Motilin(1-6)Me	[15-Glutamine]-motilin
R-1104	100	2.0 (NP)	0.1 (NP)	0.002	66.7
R-1105	100	69.2	115	0.09	96.7
R-1106	100	<0.001	<0.001	0.002	86.7
GP-1102	100	0.81 (NP)	<0.001	0.016	4.5 (NP)
GP-1103	100	46.0 (NP)	3.2 (NP)	0.04	1.7
GP-2801	100	0.001	<0.001	<0.001	111.4
GP-2802	100	0.001	<0.001	<0.001	166.7
GP-2803	100	<0.001	<0.001	0.01	288.9

NP: not parallel

Fig. 2 Dose-response curves of extracts of porcine, canine, human and rat duodenal tissues. Antiserum: R-1105, R-1106 and GP-1103. Labelled antigen: ^{125}I -synthetic porcine motilin.

Table 2 *Motilin-like Immunoreactivities in Extracts of Porcine, Canine and Human Duodenal Tissues*

Species	R-1104	Antiserum used in radioimmunoassay		
		R-1105	R-1106	GP-1103
Porcine	155.56	64.1	111.11	150.00
Canine	6.01	9.08	3.55	7.66
Human	45.92	33.27	25.51	32.65

Unit: pg motilin equivalent/mg wet weight of tissue

terest that the measured values of motilin immunoreactivity varied depending upon the assay system used.

Gel Filtration of Tissue Extract

Gel filtration profiles of motilin immunoreactivity in the extracts of human corpus, duodenum and jejunum are shown in Fig. 3. The assay of each fraction was carried out using the carboxyl-terminal antiserum R-1105. In the extracts of the duodenum and jejunum, a small peak eluting in the region of the void volume (peak I) was observed, while the peak was absent in the case of the corpus extract. A major peak (peak III) was eluted in the position where the marker motilin was detected. Two small peaks were observed just before and after peak III (peak II and peak IV) in all three gel filtration profiles. These peaks were reproducible in repeated gel filtration.

Fig. 3 Gel filtration profiles of motilin immunoreactivity in extracts of human corpus, duodenum and jejunum. Column: Sephadex G-50 (medium) (1.5×90 cm). Antiserum: R-1105. Labelled antigen: ^{125}I -synthetic porcine motilin.

DISCUSSION

The data presented here describe the production of antisera against synthetic porcine motilin. Cross-reactivities with synthetic or natural gastrointestinal peptides indicate that these antisera are specific to porcine motilin. Availability of synthetic fragments of the motilin molecule made it possible to characterize the immunologic determinants of these antisera. Characterization of the specificity of antiserum is indispensable for its use in immunohistochemical study and radioimmunoassay measurement. It

seems to be particularly important in the case of motilin antisera, since the conflicting results presented for the cellular origin of motilin may be interpreted as due to the different specificities of the antisera used. In the present study, we have investigated eight antisera raised in rabbit or guinea pig. All the antisera were shown to possess different specificities. Of them, rabbit antisera R-1105 and R-1106 and guinea pig antiserum GP-1103 were used for characterization of the immunoreactive motilin extractable from the gastrointestinal tissues of the pig, human, dog and rat. Antiserum R-1105

recognizes predominantly the carboxyl-terminal 12-22 sequence and R-1106 requires the presence of the whole motilin molecule for binding. Antiserum GP-1103 is specific to the middle part of the sequence, i.e. the 7-15 sequence. In any of the assay systems using these three different antisera, the porcine tissue extract gave inhibition curves parallel to that of synthetic porcine motilin, whereas the rat tissue extract showed very low cross-reaction. Seino *et al.* (16) reported that motilin-positive cells were not observed in the intestinal tract of the rat, lizard, mudsucker and Pacific hagfish, although the same intestinal cells of the dog and Japanese quail were stained with both anti-synthetic motilin and anti-natural motilin sera. These observations suggest that there may be a high degree of sequence difference in the rat. In the assay system using the carboxyl-terminal specific antiserum R-1105, the inhibition curves of the canine, porcine and human tissue extracts were superimposable on that of synthetic porcine motilin, indicating that there is sufficient similarity in the sequences of these motilins corresponding to the 12-22 sequence of porcine motilin. However, the inhibition curves of the canine tissue extract was not parallel to that of synthetic motilin in the R-1106 or GP-1103 assay system. The human intestinal tissue extract also showed slightly weak inhibition in the assay system using antiserum R-1106 as compared with that of the porcine tissue extract. The motilin concentrations in the duodenum extracts measured by the assay systems using four different antisera were variable depending on species of the tissue and antiserum used. These results clearly indicate the existence of species variation in motilin immunoreactivity. In addition, the present findings also emphasize that well-characterized antisera should be used for the measurement of the motilin concentration in tissue and blood of species other than pig.

Polak *et al.* (12) have recently described the possible presence of multiple forms of motilin-like immunoreactivity. It is likely that motilin, like other peptide hormones, is synthesized in a larger precursor form. We have found that extracts of the human and jejunum mucosa contain three major components with motilin-like immunoreactivity. One of them (peak III) had similar properties on Sephadex G-50 gel filtration as the synthetic motilin. In contrast, the other components (peak I and peak II) emerged earlier than porcine motilin on the gel filtration. A reproducible elution profile of

peaks I and II in repeated gel filtration suggests the presence of larger forms of motilin-like immunoreactivity in the tissues. The present findings may reflect either the presence of degradation products of motilin precursor or the existence of a new family of related peptides.

Further investigation should be performed to answer the questions raised by the present study.

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