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CHARACTERIZATION OF A GASTRIN RELEASING PEPTIDE FROM PORCINE NON-ANTRAL GASTRIC TISSUE

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SUMMARY. A heptacosapeptide with potent gastrin releasing activity has been isolated from porcine non-antral gastric and intestinal tissue. The amino acid sequence suggested from a preliminary study on the gastric peptide is: Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2. Striking homology in the C-terminal region is seen with bombesin, accounting for the similar bioactivities of the two peptides. Some structural resemblance with porcine cholecystokinin in the N-terminal region is noted.

INTRODUCTION. Bombesin, a tetradecapeptide isolated from frog skin (1), has been reported to have gastrin releasing and cholecystokinin(CCK)-like activities on administration to mammals (2,3). A peptide with similar biological properties has been demonstrated in extracts of porcine non-antral gastric and intestinal tissue (4,5). We report here the isolation of this gastrin releasing peptide (GRP) from porcine gastric and intestinal tissue and the results of an amino acid sequence analysis of the gastric peptide. Structural similarities with bombesin and certain mammalian gut hormones are discussed.

MATERIAL AND METHODS. Details of tissue extraction (4,6,7), radioimmunoassays (4), and in vivo bioassays for gastrin release (4) and gall bladder contraction (8) have been described. Synthetic bombesin was purchased from Bachem and Calbiochem. Reverse phase high pressure liquid chromatography (HPLC) (Waters Associates apparatus, M6000A solvent delivery system, U6K injector, model 450 variable wavelength detector) was performed on a 300 x 3.9 mm μBondapack C_{18} column (Waters Associates) using a solvent of 65% ammonium acetate 0.005M, pH 4.55 and 35% ethanol at a flow rate of 1 ml per minute. Elution profiles were followed at 215 nm.

For purification, the gastric tissue starting material was the active carboxymethylcellulose (CMC) fraction previously described (4). This was dissolved (10% w/v) in the lower phase of the system 0.1M ammonium bicarbonate: n-butanol (with 0.05% v/v ethanethiol) and extracted 4 times with equal volumes of the upper phase. The bulk of the active peptide was recovered from the organic phase by adsorption to alginic acid (9) and then subjected to countercurrent distribution in a 1 x 2 ml glass apparatus as described for purification of secretin (9). After 60 passes the active fraction was recovered from tubes 11-28, the bulk of the contaminants remaining in the initial tubes. Final purification was by CMC chromatography on a 0.6 x 59 cm column equilibrated and eluted with

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0.04M ammonium bicarbonate pH 8.0 (0.02% v/v ethanethiol). A symmetrical peak containing the active peptide was eluted between 12-15 column volumes.
The starting material for purification of the intestinal peptide was a side fraction obtained from purification of the vasoactive intestinal polypeptide (VIP) (10), and this was subjected to the procedure outline above. For countercurrent distribution a 10 x 10 ml all glass apparatus was used and an additional reverse phase HPLC after the CMC chromatography step was performed.
Thin layer chromatography (TLC) was performed on silica gel plates (Riedel-de-Haen, Seelze, Hannover, Germany) in the solvent system, n-butanol:pyridine: acetic acid:water (15:10:3:12) (11) and peptides were identified by UV-fluorescence, ninhydrin and chlorination (12).
Tryptic digestion and separation of fragments by high voltage electrophoresis at pH 6.5 and 3.5 was performed as described (13). Amino acid analyses were performed on a Beckman model 121M analyzer after hydrolysis at 110°C for 24 h in 6M HCl containing 0.5% phenol. Manual sequence analysis was by the dansyl-Edman procedure and dansyl amino acids were identified on polyamide layers in four solvent systems (13). C-terminal amide structures were determined by enzymatic hydrolysis followed by dansylation (14).
Analysis in a Beckman 890C sequencer using a 0.1M quadrol peptide program was performed in the presence of polybrene that had been added together with glycine and degraded for 2 cycles before peptide application (15). PTH amino acids were determined on a Hewlett-Packard 1080B HPLC using an acetonitrile:0.01M sodium acetate pH 4.5 gradient system (16). Samples were also analysed by TLC in xylene: isopropanol (7:2 v/v) on silica gel with fluorescent indicator and subsequent development with collidine-ninhydrin (17).

RESULTS.

ISOLATION. Figure 1 outlines the procedure and results obtained from purification of the gastric peptide. An apparently identical intestinal peptide was isolated using the same procedure with an additional reverse phase HPLC step but a much lower yield was obtained (65 µg of final product from 2500 mg of starting material). Purification was followed by measuring the increasing potency of gastrin releasing activity but concomitant increases in gall bladder contracting activity and immunoreactive bombesin were seen (fig. 1). Sequential

Purification Step	Weight (mg)	Immunoreactive Bombesin Content (ng/µg)
Starting material	177	
↓		
n-butanol Extraction	31.5	12.4
↓		
Countercurrent Distribution	2.2	41.2
↓		
CMC chromatography	0.43	122.8

Fig. 1. Outline of purification scheme and results obtained on purification of the gastric extracts.

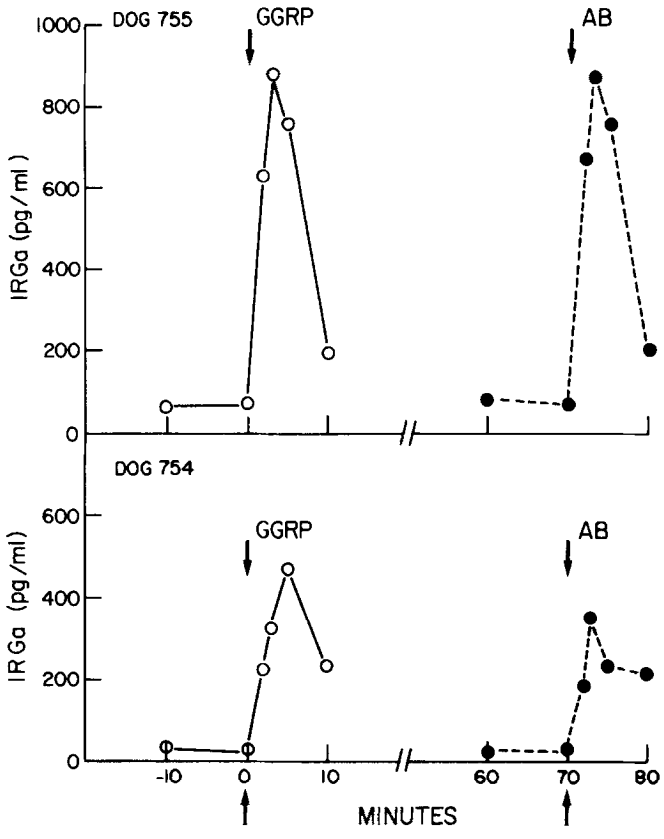

Fig. 2. Plasma IRGa levels in pg/ml before and after intravenous bolus injection (arrows) of 150 ng·kg^{-1} of the gastric GRP (GGRP) and of 50 ng·kg^{-1} of bombesin (AB) into 2 dogs.

bolus injections of 150 ng·kg^{-1} gastric GRP and 50 ng·kg^{-1} bombesin into 2 dogs resulted in the rapid and large changes in plasma immunoreactive gastrin (IRGa) shown in fig. 2.

Analytical reverse phase HPLC (fig. 3) of the gastric GRP demonstrated a dominant peak at retention time 9.0 min.; the intestinal GRP showing a similar dominant peak with an identical retention time. Silica gel TLC of both intestinal and gastric GRP gave UV-fluorescent and ninhydrin positive spots with identical migrations midway between the positions of porcine secretin and bombesin. No additional spots were seen after chlorination and development with a tolidine/KI reagent.

END GROUPS AND TOTAL COMPOSITION. Analysis by the dansyl method showed that in both the intestinal and gastric GRP the N-terminus is alanine and that lysine and tyrosine are present in the peptide since α-dansyl-alanine, ε-dansyl-lysine, and O-dansyl-tyrosine were demonstrated. Analysis of both preparations for C-

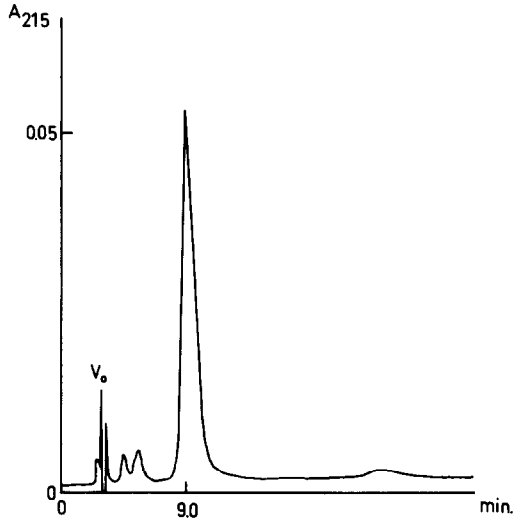

Fig. 3. Reverse phase HPLC of 5 μg of the gastric GRP injected at time 0. V_o is the void volume. Conditions of operation are given in the text.

terminal amides demonstrated a methione amide after chymotrypsin incubation and, in addition, a leucine-methionine amide after thermolysin incubation, showing this to be the C-terminal structure of the peptide.

The amino acid composition of the intestinal GRP preparation is shown in Table 1. Minor contaminants are the cause of some deviation from integer values but the data suggest the peptide has 27 residues with Ile, Phe, and Glx being absent. The amino acid composition of the gastric GRP was identical within experimental variation but greater contamination apparently caused relatively higher amounts of serine and glutamic acid and lower amounts of histidine. Silica gel TLC of a tryptic digest of the gastric peptide demonstrated 3 ninhydrin positive spots consistent with the presence of 1 lysine and 1 arginine residue. Two of the tryptic fragments were isolated after high voltage electrophoresis; B1 from the N-terminus and B2 from the middle part of the peptide. Their amino acid compositions are given in Table 1. The C-terminal fragment was not recovered.

STRUCTURAL ANALYSIS. Liquid phase sequencer analysis of 60 nmoles of the gastric peptide identified residues up to the penultimate residue 26. The repetitive yield in the initial steps was 96% which successively fell to 87% around cycle 20. The structure obtained is given in fig. 4A. All residues except those in positions 16,20 and 27 were identified by HPLC and TLC. The recoveries of the residues in positions 16 and 20 were low. Consequently, the assignments of proline and histidine to these positions respectively, are somewhat tentative but consistent with the total compositions of the peptide and

Table 1 : Amino acid analyses.

	Intestinal GRP		Gastric GRP Tryptic Fragment B1		Gastric GRP Tryptic Fragment B2	
Residue	Acid Hydrolysis	Sum of Sequence Analysis	Acid Hydrolysis	Sum of Sequence Analysis	Acid Hydrolysis	Sum of Sequence Analysis
Asp	1.0	1	0.1	0	0.1	0
Thr	1.2	1	1.0	1	0	0
Ser	1.3	1	1.1	1	0.2	0
Glu	0.2	0	0.3	0	0	0
Pro	2.3	2	1.1	1	1.0	1
Gly	5.3	5	2.9	3	0.3	0
Ala	2.9	3	2.0	2	0	0
Val	4.3	4	2.9	3	0	0
Met*	1.4	2	0	0	+	1
Ile	0.2	0	0	0	0	0
Leu	2.0	2	0.9	1	0	0
Tyr	1.1	1	0	0	1.0	1
Phe	0	0	0	0	0	0
Trp**	+	1	0	0	0	0
Lys	1.2	1	1.0	1	0.1	0
His	1.8	2	0	0	0	0
Arg	1.4	1	0	0	1.0	1
Total		27		13		4

* non-quantitatively recovered due to oxidative losses

** non-quantitatively recovered due to hydrolytic losses

the tryptic fragments (table 1) as well as with the results of a manual sequence analysis for 3 cycles on the unseparated tryptic digest. Overlapping between the sequencer analysis and the C-terminal analysis is via a single residue of leucine in position 26. Although this overlap is not yet proven by additional analysis, it is considered reliable as the total composition of the peptide indicates the presence of 2 leucines, both of which are accounted for in the structure determined (fig. 4A). Therefore, additional leucine residues around position 26 are unlikely.

DISCUSSION.

This report outlines the structural characterization of a heptacosapeptide from porcine non-antral gastric tissue which has bioactivities consistent with it being a releasing factor for gastrin and possibly CCK (4.5). An appar-

A) GGRP

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 1           5              10              15
Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-
|-------------------B1-------------------| |------B2------|
    20          25
-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH2
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B)

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AB                               *Q Q R L [G N] Q [W A V G H L Mº]
GGRP   [A P] - V S [V] G G G T V [L] A K M Y [P] R [G N] H [W A V G H L Mº]
CCK  K [A P] S G R [V] S M I K N [L] Q S L D [P] S H R I S D R D Y M G W M D Fº
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Fig. 4. A) Summary of the structural determination of the porcine gastric gastrin releasing peptide (GGRP). Residues in positions 16 and 20 (underlined) are tentative. The positions of the tryptic fragments B1 and B2 are also shown.

B) Comparison of the structures of the GGRP with amphibian bombesin (AB) and porcine CCK. For ease of comparison one letter notation for amino acids is used (Eur.J.Biochem. (1968) 5, 151-153). Identical residues are boxed. The blank position in 3 of the GGRP is an assumed deletion, o signifies an amidated C-terminus and *Q a pyroglutamyl residue.

ently identical peptide was also isolated from the porcine intestine. Alignment of the C-terminal residues of the mammalian GRP and amphibian bombesin reveals 9 identities in the 10 C-terminal positions (fig. 4B). The single deviation (at position 8 from the C-terminus) being a histidine for a glutamine residue which is compatible with a one base difference in the genetic code, This deviation needs to be confirmed, but no evidence was found for the presence of a glutamine residue at this position in the mammalian peptide. This striking homology must account for the similar bioactivity and the cross reaction of the mammalian peptide with anti-sera to bombesin. Hence, the prediction, based on immunological evidence (18,19,20), that a bombesin-like peptide would be found in the mammalian gut is fulfilled.

The comparison of this newly characterized mammalian peptide and bombesin is analogous to that between porcine CCK and amphibian caerulein (21); the C-terminal regions having striking homology and the mammalian peptides being greatly extended at the N-terminus. The significance of this N-terminal extension is unknown. Noticeably, however, the N-terminal region in the newly characterized heptacosapeptide shows some similarity to CCK (fig. 4B). If the N-terminus of the GRP is aligned with the alanine in position 2 of CCK and a one-residue deletion is assumed, 5 identities are noted and the differences seen in some of the other residues are comparatively small. Hence, it is possible that an additional relationship exists with the CCK family of peptides, although the resemblance is considerably less striking than the obvious homology with bombesin.

Similarities between bombesin and the porcine VIP have been suggested (22), but using the suggested alignment there is less similarity between the GRP and VIP. A comparison between porcine glucagon and bombesin has been made (23) and using this alignment 4 identities in the C-terminal region are also seen with the mammalian GRP.

Whether or not this newly characterized heptacosapeptide functions as a physiological releasing factor is unknown, but its potent bioactivities and presence in the mamalian gut suggest an important functional role.

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