CHARACTERIZATION OF AN AVIAN GASTRIC (PROVENTRICULAR) PEPTIDE HAVING SEQUENCE HOMOLOGY WITH THE PORCINE GASTRIN-RELEASING PEPTIDE AND THE AMPHIBIAN PEPTIDES BOMBESIN AND ALYTESIN

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1. Introduction

Bombesin, a peptide with 14 amino acid residues, was isolated from the skin of an European frog [1] and has been the subject of many recent investigations due to its diverse and potent pharmacological effects on the mammalian gastro-intestinal tract, pancreas, and central nervous system (reviewed [2,3]). We have reported the sequence of a 27 residue porcine gastrin-releasing peptide (GRP) with marked C-terminal homology with bombesin [4]. Subsequent investigations indicate that natural porcine GRP and synthetic bombesin on intravenous administration, share the property of elevating plasma gastro-intestinal-pancreatic hormone levels [5] and that a synthetic replicate of GRP and bombesin on intracranial administration produce similar pharmacological effects [6]. Immunological evidence indicated that the avian proventriculus was an abundant source of bombesin-like immunoreactivity [7–9]. We therefore began an investigation of extracts of the chicken proventriculus and report here the isolation and amino acid sequence of a 27 residue chicken proventricular peptide having marked sequence homology with the porcine GRP and the amphibian peptides bombesin and alytesin.

2. Experimental

2.1. *Purification procedures*

Chemicals, materials, details of chromatography [10], isotachophoretic procedures [11] and the in vivo guinea pig gall bladder contracting assay [12] have been described. The criteria for an increase in purity of material during isolation was an increase in potency of gall bladder contracting activity and of immunoreactive bombesin content [13].

Boiled chicken proventricular tissue (86 kg) was extracted with 0.5 M acetic acid at 4°C for 16–18 h. The peptides in the filtrate were adsorbed to alginic acid, eluted with cold 0.2 M HCl and precipitated by NaCl at saturation. The precipitate was dissolved in 66% ethanol, the pH adjusted to 7.2 and a precipitate removed. The soluble peptides were resubmitted to the alginic acid and NaCl steps. The precipitate obtained (19.1 g) was extracted into methanol as in [14]. The acid and neutral soluble fraction (1.2 g) was chromatographed on a Sephadex G-25 (fine) column (2.5 × 95 cm) in 0.2 M acetic acid. The bioactive fraction emerged at 340–390 ml and weighed 591 mg after lyophilization. This fraction was chromatographed on a carboxymethylcellulose column (5 × 21 cm) equilibrated with 0.02 M ammonium bicarbonate (pH 8.0) and initially eluted with 3460 ml of the same buffer. The active peptide was subsequently recovered on elution with 0.04 M ammonium bicarbonate between the elution volumes 4480–5420 ml and weighed 11.4 mg after lyophilization. Final purification was by reverse phase high-performance liquid chromatography (HPLC) on a C-18 μBondapak column (7.8 × 300 mm) as in [4] using the solvent system 70% 5 mM ammonium acetate–acetic acid buffer (pH 4.2): 30% ethanol. The active peptide was eluted at retention time 13 min.

2.2. *Structural analysis*

C-terminal amide structures were determined by enzymatic hydrolysis, dansylation, and subsequent

identification of amides on polyamide layers as in [15]. N-Terminal amino acids were identified by the dansyl method and manual sequence analysis was by the dansyl-Edman procedure [16]. Tryptic digestion and separation of fragments by high-voltage paper electrophoresis [16] as well as by silica gel thin-layer chromatography (TLC) [4] were performed as described. Amino acid compositions were analysed on a Beckman 121 M analyzer after hydrolysis at 110°C for 24 h in evacuated tubes containing 6 M HCl with 0.5% phenol. Liquid-phase sequencer degradation in a Beckman 890 C instrument using a 0.1 M quadrol peptide program was performed in the presence of polybrene added together with glycine and degraded for 2 cycles before peptide application [17]. PTH amino acids were determined on a Hewlett-Packard 1084B HPLC using an acetonitrile: 0.01 M sodium acetate (pH 4.5) gradient system [18]. Samples were also analysed on silica gel with fluorescent indicator by TLC and subsequent staining with collidine–ninhydrin [19].

3. Results

3.1. *Isolation*

The final yield of active peptide was 1.3 mg and this had an immunoreactive bombesin content of 188 pmol/μg. Isotachophoretic analysis (fig.1b) was consistent with a high degree of homogeneity as was silica gel TLC which demonstrated a single fluorescence-, ninhydrin- and chlorine-positive spot which had a mobility (R_F = 0.54) slightly greater than secretin (R_F = 0.50).

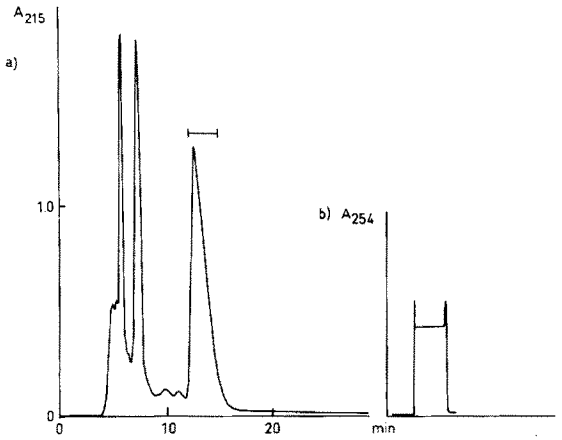

Fig.1. (a) Reverse-phase HPLC of 950 μg of the active fraction from the carboxymethylcellulose chromatography (see text for details). The bioactive material was collected as shown by (⊢⊣). (b) The isotachophoretic analysis of 12 μg of the bioactive material collected from HPLC as shown in (a).

3.2. *End groups, amino acid composition*

Analysis by the dansyl method showed that the N-terminus was alanine and that lysine and tyrosine were present in the peptide since α-dansyl-alanine, ε-dansyl-lysine and *O*-dansyl-tyrosine were demonstrated with virtually no contamination by other derivatives. Analysis for C-terminal amides demonstrated a methionine amide after chymotrypsin incubation, and a leucyl–methionine-amide dipeptide after thermolysin incubation, showing this to be the C-terminal structure of the peptide.

Amino acid analysis of the peptide demonstrated the presence of 26 residues in good integer values (table 1) after acid hydrolysis (destroying trypto-

Table 1
Amino acid compositions

Residue	Intact peptide	T_1	T_2	T_3
Asx	(0)	(0)	(0)	(0)
Thr	1.0 (1)	0.9 (1)	(0)	(0)
Ser	2.0 (2)	0.9 (1)	(0)	1.0 (1)
Glx	1.0 (1)	1.1 (1)	(0)	(0)
Pro	3.7 (4)	2.6 (3)	1.1 (1)	(0)
Gly	4.0 (4)	2.3 (2)	(0)	1.9 (2)
Ala	2.9 (3)	1.9 (2)	(0)	1.2 (1)
Val	0.9 (1)	(0)	(0)	1.0 (1)
Met	0.8 (1)	(0)	(0)	0.9 (1)
Ile	1.0 (1)	(0)	0.9 (1)	(0)
Leu	3.1 (3)	2.0 (2)	(0)	1.1 (1)
Tyr	1.0 (1)	(0)	1.0 (1)	(0)
Phe	(0)	(0)	(0)	(0)
Trp	(1)	(0)	(0)	(1)
Lys	1.0 (1)	1.0 (1)	(0)	(0)
His	1.9 (2)	(0)	(0)	1.9 (2)
Arg	1.0 (1)	(0)	1.0 (1)	(0)
Total	27	13	4	10
N-Terminus	Ala	Ala	Ile	Gly
Electrophoretic mobility at pH 6.5 in relation to Asp		−0.25	−0.40	−0.50

The first values indicate molar ratios of acid hydrolysates (values below 0.1 omitted), values in parentheses the sum from sequence analysis. Values for the hydrolysis of the intact peptide are the mean of two determinations

phan). In addition, the presence of tryptophan was detected by fluorescence on silica gel TLC of the intact peptide as well as in one of the tryptic fragments and was confirmed by sequence analysis (below). Hence the chicken peptide like the porcine GRP is a heptacosapeptide. Consistent with the presence of 1 lysine and 1 arginine residue, silica gel TLC of a tryptic digest of the peptide demonstrated 3 spots positive to cadmium-ninhydrin; one spot acquiring a gold colour consistent with the presence of an N-terminal glycine residue. The 3 tryptic peptides were isolated by high voltage electrophoresis. Their amino acid compositions and N-termini are given (table 1). T_1, having an N-terminal alanine, is the N-terminal peptide. T_3, having no arginine or lysine residue, is the C-terminal peptide. Hence, T_2, is the middle peptide.

3.3. *Sequence determination*

Liquid phase sequencer analysis of 100 nmol peptide identified residues up to position 25. The repetitive yield in the initial steps was 94% which successively fell to 88% at cycle 22. The structure of the chicken proventricular peptide (tentatively called chicken GRP due to its similarity to porcine GRP) is given in fig.2a. All residues except the tryptophan in position 21 were confirmed by manual sequence analysis performed on the intact peptide, the isolated tryptic fragments and an unseparated tryptic digest. The residues in position 26 and 27 were identified by C-terminal amide analysis as well as by direct manual sequence analysis. Results of the sequencer analysis, and the different manual sequence analyses, are in agreement with the amino acid composition of the intact peptide as well as with the composition and electrophoretic mobilities of the tryptic fragments.

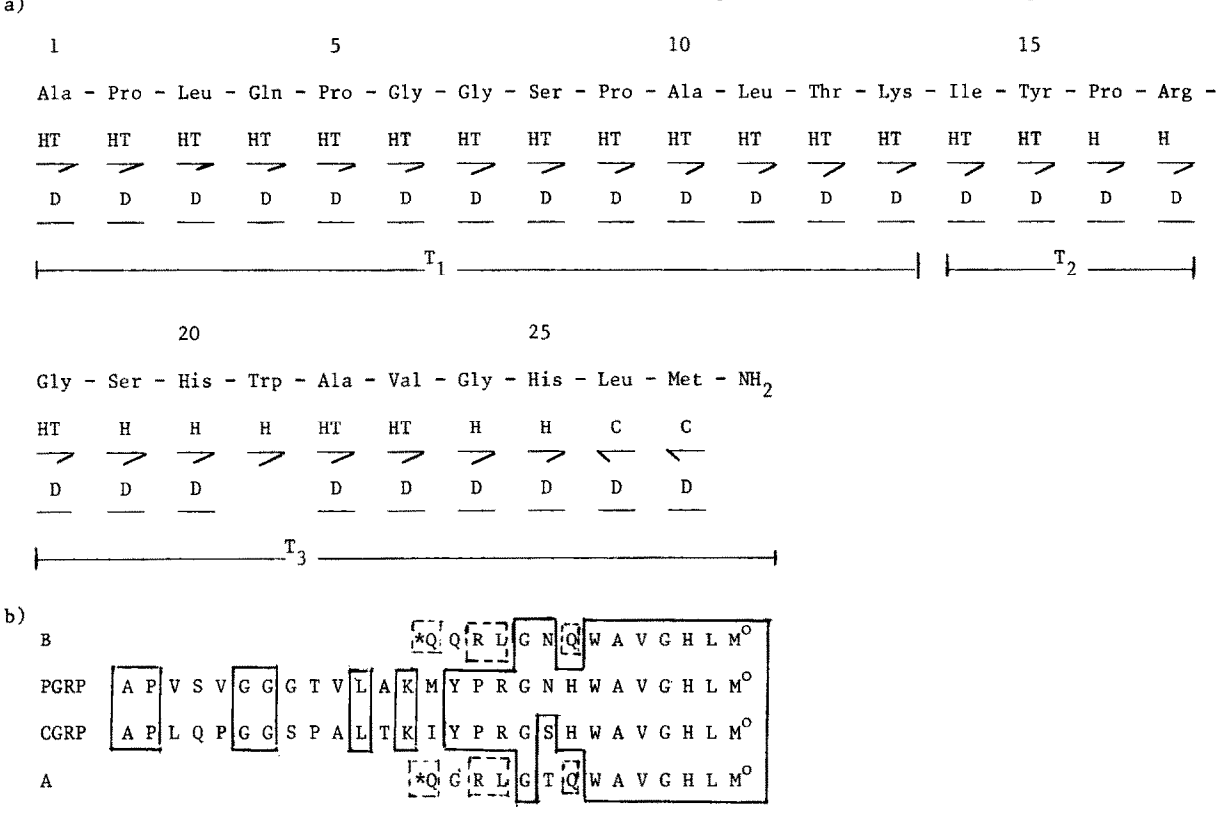

Fig.2. (a) Summary of the sequence analysis on the isolated chicken proventricular peptide (CGRP). The positions of the 3 tryptic fragments (T_1, T_2, T_3) are shown. Residues identified by sequencer degradation are shown by (—); PTH amino acid analysis by HPLC by (H); and by TLC by (T). Residues identified by manual dansyl-Edman sequence analysis are shown by (D) and by C-terminal amide analysis by C. (b) Comparison of the structures of the chicken (CGRP) and porcine (PGRP) peptides with bombesin (B) and alytesin (A) using one letter notation for amino acids. Identical residues in the PGRP and CGRP and with B and A are boxed with solid lines. Residues identical in B and A but not with CGRP or PGRP are boxed with dotted lines. *Q denotes a pyroglutamyl residue and ^{O} an amidated C-terminus.

4. Discussion

In 1971, Anastasi et al. reported the sequences of bombesin and alytesin [1], which differ in only 2 of their 14 residues. At least in certain biological assay systems, these peptides have been reported to have similar biological potencies [20,21]. The porcine GRP, recently isolated from porcine gastric tissue [4], and bombesin have 9 identities in their 10 C-terminal residues (fig.2b). These homologous regions undoubtedly account for their similar pharmacological effects seen on peripheral intravenous [5] and intracranial [6] administration to mammals.

This study reports the isolation and chemical characterization of an avian proventricular peptide with similar bioactivity to porcine GRP and bombesin and which reacts with anti-sera raised against bombesin. Fig.2b shows the structures of porcine and chicken GRP as well as bombesin and alytesin aligned at their C-termini. The chicken and porcine peptides have identical N- and C-termini, identical tryptic cleavage points and 18 identities in their 27 residues. Eight of the 9 amino acid substitutions occur in the N-terminal 14 residues and only one in the C-terminal 13 residues. The sole deviation in the 13 C-terminal residues occurs at position 19, being an asparagine residue in the porcine and a serine residue in the chicken peptide. Interestingly, bombesin like the porcine peptide contains an asparagine residue at this position but alytesin, like the chicken peptide, has a hydroxyl containing residue (threonine) at this position (fig.2b). The chicken and porcine peptides have a histidine residue at position 20 while the two amphibian peptides have a glutamine residue at the equivalent position. Thus the two non-amphibian peptides show homology throughout their molecules and both show marked C-terminal homology with the amphibian peptides bombesin and alytesin; the C-terminal regions being reported [20,21] as essential for the recognized bioactivities. The sequence identities suggest that the chicken and porcine GRP belong, as non-amphibian members, to what has been termed the family of bombesin-like peptides [2]. The well-conserved amino acid sequence in the region responsible for the bioactivity of this group of peptides and their diverse but potent biological activities suggest that they play an important physiological role in many species.

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