Galanin – a novel biologically active peptide from porcine intestine

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The isolation of a novel biologically active peptide, designated galanin, is described. The peptide was discovered by the detection of its C-terminal amide structure in porcine intestinal extract using a chemical method. It was found that galanin consists of 29 amino acids and the complete amino acid sequence is: Gly–Trp–Thr–Leu–Asn–Ser–Ala–Gly–Tyr–Leu–Leu–Gly–Pro–His–Ala–Ile–Asp–Asn–His–Arg–Ser–Phe–His–Asp–Lys–Tyr–Gly–Leu–Ala–NH_2. Galanin was found to contract smooth muscle preparations from the rat and to cause a mild and sustained hyperglycemia in dog.

Gastrointestinal polypeptide *Amino acid sequence* *C-terminal amide* *Biological activity*

1. INTRODUCTION

Many biologically active peptides contain a C-terminal amidated structure [1,2]. Peptides with such a structure may be assayed by using a chemical technique in which the C-terminal of the peptide is cleaved off by a proteolytic enzyme, converted into the dansyl derivative, extracted selectively and identified by thin-layer chromatography [3]. Using this chemical technique, it was found that extracts of porcine intestine and brain contain many previously unknown peptides with the C-terminal amidated structure [3,4]. Efforts to isolate and identify some of these peptide amides have resulted in the finding of previously unknown peptides, peptide HI (PHI) [4,5], peptide YY (PYY) [4,6] and neuropeptide Y (NPY) [7,8]. Subsequent studies indicated that these peptides may be hormonal and neural peptides [9]. These results therefore suggest that new hormonal and neural peptides may be found by a systematic search for peptide amides from tissue extracts using the chemical assay technique.

During the study of purification of PHI and PYY, it was noticed that extracts of porcine intestine contained a peptide with C-terminal alanine amide. We report here the isolation, amino acid sequence and some of the biological activities of this novel peptide amide which we have designated galanin from the N- and C-terminal residues, glycine and alanine.

2. MATERIALS AND METHODS

Alanine amide was obtained from Vega Biochemicals and dansyl alanine amide was prepared from alanine amide by reaction with dansyl chloride [10]. Reagents for sequence analysis were of sequanal grade and acetonitrile was of HPLC grade. Other reagents were of analytical grade. Polyamide thin layer sheets were obtained from Schleicher & Schüll, thermolysin (EC 3.4.24.4) from Daiwa Kasei K.K., Osaka, and trypsin (EC 3.4.21.4; L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated) from Worthington Biochemical Co.

Galanin was determined by the amount of alanine amide released after treatment of the sample with thermolysin in a chemical assay method [3]. Reversed-phased high performance liquid chroma-

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tography (HPLC) was performed in a Waters instrument using a μBondapak C-18 column under conditions described in the legends to fig.2,3. N-terminal amino acids were determined as in [10] by using dansyl chloride. Amino acid compositions were determined with a Beckman 121 M automatic amino acid analyzer after hydrolysis of samples in 5.7 M HCl/0.5% phenol at 110°C for 24 h. The tryptophan content was determined after hydrolysis in 3 M mercaptoethanesulfonic acid [11]. Liquid-phase sequencer degradation was performed in a Beckman 890 D instrument, in the presence of glycine, precycled polybrene using a 0.1 M quadrol peptide program [12]. Phenylthiohydantoin amino acids were determined by HPLC [13] and thin-layer chromatography [14]. Manual sequence analysis was performed by a modified dansyl-Edman method [8].

Biological actions of galanin was tested on a fundus strip, ileum, proximal colon and urinary bladder from rat. The isolated tissues were prepared as in [15]. The tissues, obtained from non-fasted and decapitated animals, were immediately suspended in a siliconized organ bath which contained a modified Krebs–Henseleit solution (5 ml) with glucose 2 g/l. The solution was bubbled with 95% O_2 and 5% CO_2 at 37°C. The tissues were allowed to equilibrate for about 40 min and regular contractions were elicited by acetylcholine (Roche) before the experiments. Dose intervals for galanin were at least 10 min. The contractions of the rat urinary bladder were registered using a Harvard isotonic force transducer (no. 385) and the contractions of the other organs were registered using a Grass isometric force transducer FT 03C. The twitches were expressed in mN except for the rat urinary bladder where the twitches were measured in mm. The effect of galanin on plasma glucose levels was studied using conscious dogs as in [16].

3. RESULTS

3.1. *Isolation procedures*

Galanin was purified from a side fraction obtained during the isolation of PYY. Briefly, procine upper intestines (4000 kg) were boiled in water for 10 min, frozen, minced and extracted with 0.5 M acetic acid for 24 h. Peptides in the extract were adsorbed onto alginic acid, eluted with 0.2 M HCl and precipitated with NaCl at saturation. They

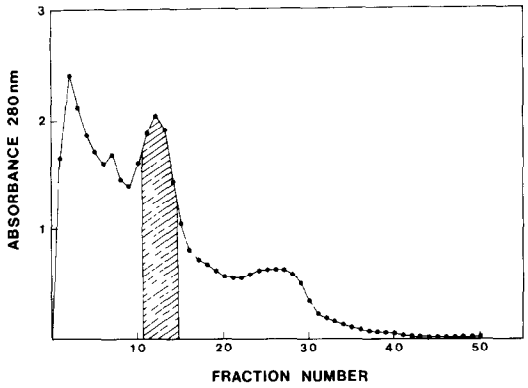

Fig.1. Ion exchange chromatography of the starting material on CM cellulose (1.6 × 15 cm) in 0.02 M NH_4HCO_3 (pH 8) at a flow rate of 0.55 ml/min. Fractions (5 ml) were lyophilized and an aliquot from each was subjected to the chemical assay, the shaded area indicates fractions containing galanin.

were further purified by extraction with 66% ethanol, gel chromatography on Sephadex G-25 and extraction with methanol. The methanol-soluble fraction was subjected to chromatography on a CM–cellulose column. It was first eluted with 0.02 M NH_4HCO_3 (adjusted to pH 6.5 by CO_2) and then with 0.2 M NH_4HCO_3 (pH 8). The fraction eluted with 0.2 M NH_4HCO_3 was further purified by gel chromatography on Sephadex G-25 [6]. The fractions (starting material 125 mg) containing PYY and galanin were purified further by rechromatography on CM cellulose in 0.02 M NH_4HCO_3 (pH 8) as shown in fig.1. Fractions 11–14 contained galanin and were pooled and lyophilized. The preparation (18 mg) was further purified by reversed-phase HPLC on μBondapak C-18 (fig.2). This step yielded a preparation (2.5 mg) found to be homogeneous as judged by TLC, HPLC, determination of N-terminal residue and amino acid analysis.

3.2. *Structural studies*

The results of amino acid analysis suggested that the peptide consisted of 29 amino acid residues as shown in table 1. Analyses for the terminal groups revealed that it had N-terminal alanine amide. The name galanin was given to this peptide based on its N- and C-terminal amino acids (glycine and alanine). Treatment of galanin with trypsin yielded four fragments (T-1 to T-4) which were separated

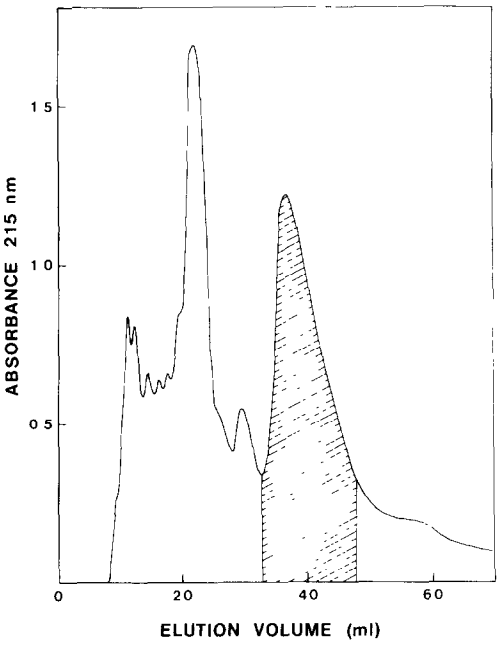

Fig.2. Reversed-phase HPLC separation of fractions 11–14 from fig.1. An aliquot (3 mg) of the fraction (18 mg) was applied to a μBondapak C-18, 7.8 × 300 mm column and isocratically eluted with 35% ethanol/5 mM NH_4OAc/0.2% HOAc at a flow rate of 2 ml/min. The major peak (oblique lines) contained galanin.

by HPLC (fig.3). The complete amino acid sequences of T-2–T-4 were determined by a manual sequence analysis as shown in fig.4. These results, together with the results from the amino acid analyses (table 1) indicate that one of the fragments, T-4, is composed of T-2 and T-3 fragments and produced because of a slow hydrolysis at a Lys–Tyr bond (position 25–26). The whole molecule of galanin (20 nmol) was subjected to Edman degradation in a Beckman 890 D liquid-phase sequencer which revealed the amino acid sequence up to residue 28 and traces of the last residue. The repetitive yield was 96% and an initial coupling 75%. From these results the complete amino acid sequence of galanin is deduced to be as in fig.4.

3.3. *Biological studies*

Galanin induced dose-dependent contractions of the fundus strip, ileum, colon and urinary bladder from the rat at concentrations above 20 ng/ml (fig.5). Data indicate that the rate ileum may be a suitable bioassay organ for galanin.

The effect of galanin on plasma glucose levels was also tested in dogs. Four dogs, conscious and overnight fasted, received 2 h peripheral vein infusions of galanin at a dose of $2\mu g \cdot kg^{-1} \cdot h^{-1}$ which produced no evidence of distress or discom-

Table 1

Amino acid compositions of galanin and its isolated tryptic fragments

Amino acid	Galanin	T-1	T-2	T-3	T-4
Ala	3.1 (3)	2.0 (2)		1.0 (1)	1.0 (1)
Arg	1.1 (1)	0.9 (1)			
Asx	4.0 (4)	2.9 (3)	1.0 (1)		1.1 (1)
Gly	4.0 (4)	3.0 (3)		1.0 (1)	1.1 (1)
His	3.0 (3)	2.2 (2)	1.0 (1)		1.1 (1)
Ile	1.1 (1)	0.9 (1)			
Leu	4.1 (4)	3.0 (3)		0.9 (1)	0.9 (1)
Lys	1.0 (1)		1.1 (1)		1.1 (1)
Phe	1.0 (1)		0.9 (1)		1.0 (1)
Pro	1.0 (1)	1.1 (1)			
Ser	2.0 (2)	0.8 (1)	1.0 (1)		0.8 (1)
Thr	1.0 (1)	0.9 (1)			
Trp	0.7 (1)	0.7 (1)			
Tyr	2.1 (2)	1.0 (1)		1.0 (1)	1.1 (1)
Sum	29	20	5	4	9
N-terminus	Gly	Gly	Ser	Tyr	Ser

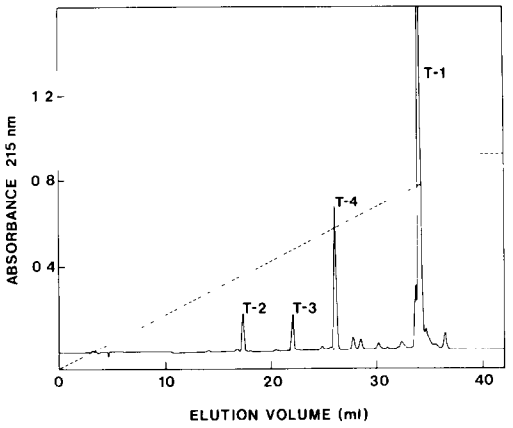

Fig.3. HPLC separation of tryptic fragments of galanin (T-1 to T-4). Galanin (70 μg) was treated with trypsin (3 μg) in 35 μl of 1% NH_4HCO_3 for 1 h at room temperature. The lyophilized digest was applied to a μBondapak C-18 column (3.9 × 300 nm) and separated at a flow rate of 1 ml/min by using a linear gradient system of 0.12% CF_3COOH/H_2O (solvent A) and 0.1% CF_3COOH/CH_3CN (solvent B, 0–50%).

fort. During the infusions, plasma glucose levels gradually increased becoming statistically significantly (Student's *t*-test) elevated from basal values at 20 min after initiation of infusion and attaining plateau values at 60 min which were then sustained during the remainder of the infusion (table 2). On cessation of the infusion, plasma glucose levels gradually fell and reattained basal values 40 min post-infusion. Hence, intravenous infusions of galanin result in a mild (but statistically significant) sustained hyperglycemia.

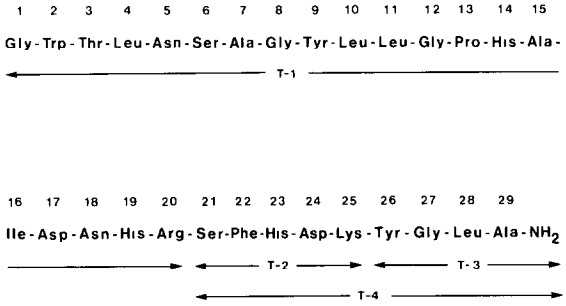

Fig.4. The complete amino acid sequence of galanin. T-1 to T-4, tryptic fragments.

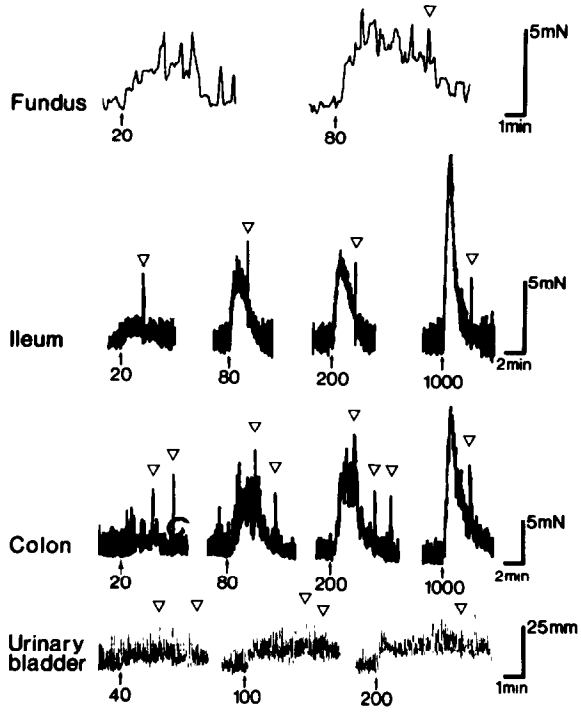

Fig.5. Responses of isolated rat fundus strip, rat ileum, rat proximal colon and rat urinary bladder to galanin (20 ng·ml^{-1} and higher concentrations as indicated). Arrow below the registration shows the time for administration of galanin and the triangle (▽) above the curve shows the time for washing.

Table 2

Effect of galanin on plasma glucose levels

Time (min)	Plasma glucose level (mean ± SEM, mg·dl^{-1})	
Basal	107.9 ± 4.5	
20	118.1 ± 6.4	($p < 0.05$)
60	134.0 ± 9.5	($p < 0.02$)
90	134.0 ± 7.7	($p < 0.01$)
120	130.5 ± 9.5	($p < 0.01$)

4. DISCUSSION

The C-terminal tetrapeptide amide of galanin, -Tyr-Gly-Leu-Ala-NH_2, is structurally similar to the corresponding part of physalaemin, -Tyr-Gly-Leu-Met-NH_2 [17] and substance P, -Phe-Gly-Leu-Met-NH_2 [18] and to the sequence of

gonadoliberin, Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg- Pro-Gly-NH_2 [19]. No structural similarities were found between galanin and corticotropin-releasing factor [20] in spite of the fact that both peptides contain the C-terminal alanine amide. Galanin contains the N-terminal sequence, Gly-Trp-Thr-Leu-Asn-Ser, similar to that of propiomelanocortin, Gly-Trp-Cys-Leu-Glu-Ser- [21]. Furthermore, a middle part, -Gly-Tyr-Leu-Leu-Gly- appears similar to that of the phosphate acceptor peptide, -Gly-Tyr-Ser-Leu-Gly- [22]. Despite these partial similarities, however, the amino acid sequence of galanin is not identical to any known peptides and galanin may belong to a hitherto unknown peptide family. The C-terminal structural similarities of galanin to other well-known neuropeptides suggest that it may also be a novel neuropeptide, which is supported by preliminary immunohistochemical findings (in preparation).

Galanin was found to contract isolated preparations from rat fundus, ileum, colon and urinary bladder and to induce a mild and sustained hyperglycemia.

The results indicated that galanin is a novel mammalian gut peptide with various biological actions. Its physiological roles in the gastrointestinal tract and other organs, as well as in the central and peripheral nervous systems, remain to be determined.

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