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Human galanin: primary structure and identification of two molecular forms

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From acid/ethanol extracts of surgical specimens of human large intestine we isolated two peptides, in approximately equal amounts, that reacted with an antiserum against porcine galanin. By amino acid analysis, sequence analysis and mass spectrometry, the larger of the two peptides was found to consist of 30 amino acid residues, the sequence of which was identical to that of porcine galanin except for the following substitutions: Val^{14}, Asn^{17}, Asn^{24}, Thr^{29} and Ser^{30}. Unlike porcine galanin, the carboxy-terminus was not amidated. The smaller peptide corresponded to the first 19 amino acid residues counted from the N-terminus of the 30 residue peptide (again without amidation). The structural analysis was repeated on another batch of tissue with identical results. By HPLC analysis of extracts of specimens from a further 4 patients, the same peptides were identified. Thus, human galanin includes two peptides of 19 and 30 amino acids that share the sequence of the N-terminal 15 residues with other mammalian galanins, but exhibit characteristic differences in the remaining part of the molecules.

Progalanin; Posttranslational processing; Neuropeptide; Amidated peptide

1. INTRODUCTION

Galanin was originally identified as a neuropeptide of 29 amino acids in extracts of pig small intestine by the use of a chemical detection method [1]. Galanin-like immunoreactivity (GAL-LI) is present in the central nervous system, the peripheral nervous system, pancreas and adrenals [2].

Consistent with its widespread localization galanin controls various biological activities: it regulates growth hormone release, it contracts smooth muscle of the gastrointestinal and genitourinary tract, it modulates insulin release and may be involved in the control of adrenal secretion [2,3]. By means of molecular biology techniques the structures of rat and bovine galanin have also been deduced [4,5].

The three animal forms are identical with respect to the first 15 residues but differ at several positions in the C-terminal part. The sequence of human galanin is not known, but the molecule does not react with antisera against the C-terminal region of porcine galanin, indicating that also human galanin differs from its porcine counterpart in this region [6].

This difference may have important functional implications. Thus porcine galanin and rat galanin inhibit insulin secretion in rats and dogs, but has no effect on glucose-induced insulin secretion in man and augments insulin secretion from isolated perfused pig pancreas [7–9]. These results illustrate the importance of studying the effect of the regulatory peptides in the autologous species. It is therefore essential that the structure of human galanin is known, allowing synthesis of the peptide and its subsequent use in functional studies.

Here we report the isolation and structure of two galanin immunoreactive forms from normal human colon.

2. MATERIALS AND METHODS

2.1. *Galanin radioimmunoassay*

In all purification steps GAL-LI was monitored by radioimmunoassay based on a non-C-terminally directed antiserum (RAGS) produced in rabbits [10]. Synthetic porcine galanin (Bachem, Bubendorf, Switzerland) was used as standard and as tracer, labeled according to the stoichiometric chloramine-T method [9]. The purification of the labeled material was performed as described [3]. Incubation conditions and separation were as described in [9].

2.2. *Extraction procedure*

Pieces of normal human colon (14 specimens of varying sizes, 880 g total) were obtained during surgery on the gastrointestinal tract (approved by the Local Ethical Committee), cleaned from the fat and the

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Abbreviations: GAL-LI, galanin-like immunoreactivity; HPLC, high performance liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; PTH, phenylthiohydantoin; PDMS, plasma desorption mass spectrometry

serosa and immediately frozen. Acid ethanol extracts were prepared according to the method II in [11]. Briefly, frozen tissue was homogenized in 4 vols of acid/ethanol and centrifuged. Five volumes of ice-cold diethyl ether were added to the supernatant and the aqueous phase was isolated at −50°C. The precipitate was then dissolved in distilled water containing 1 M urea and subjected to chromatography.

A total of 840 g of human colon was subjected to a series of chromatographic procedures for purification. The fractions eluted from the different columns were dried with the use of a vacuum centrifuge, except for a small aliquot that was assayed after reconstitution in assay buffer. Immunoreactive fractions were subjected to further chromatography. Extracts of specimens from four patients, each weighing 10 g, were subjected separately to high-performance liquid chromatography (HPLC) identification studies as described below.

2.3. *Purification*

The tissue extracts were subjected to gel permeation chromatography either on 50 × 1000 mm (K50/100) or on 16 × 1000 mm K16/100 glass columns packed with Sephadex G-50 fine grade (Pharmacia, Uppsala, Sweden), equilibrated and eluted with 0.5 M acetic acid at a flow rate, respectively, of 1 ml/min and 0.4 ml/min. Trace amounts of ^{125}I-labeled albumin and ^{22}NaCl were added to all samples prior to gel filtration for internal calibration. K_d, the coefficient of distribution, was then calculated for all fractions using the equation: $K_d = (V_e - V_o)/V_i$ where V_e is the elution volume of ^{125}I-albumin, and V_i the available inner volume of the column, determined as the difference between the elution volumes of ^{22}Na and ^{125}I. Fractions containing GAL-LI were then subjected to reversed phase HPLC, separately for the initial run, then as a pool of coeluting fractions with GAL-LI. The HPLC columns were packed with Nucleosil 300-7 μm C_6H_5, 120-7 μm CN, and 300-5 μm C-18 (Macherey-Nagel, Duren, Germany) and eluted (employing LKB HPLC equipment) with gradients of varying steepness of either acetonitrile/water/trifluoracetic acid (ACN/water/TFA) (A, 0:99.9:0.1; and B, 99.9:0:0.1) or acetonitrile/water/heptafluorobutyric acid (ACN/water/HFBA) (A, 0:99.85:0.15; and B, 99.85:0:0.15) (ACN HPLC grade, Rathburn, Walkerburn, Scotland) (TFA and HFBA from Pierce Europe BV, The Netherlands). The immunoreactive material was chromatographed with increasingly shallow gradients until two resulting immunoreactive peptides seemed pure as judged by absorbance at 226 nm (Fig. 1). The final run was performed with ACN/water/HFBA gradients from 30% to 36% of B in 30 min for one form and from 28% to 34% of B in 30 min for the other one. Prior to further analysis the purified peptides were concentrated on a narrow-bore Vydac C8 column (code no. 208TP5215, 2.1 × 100 mm) at a flow rate of 200 μl/min using a Hewlett Packard HP 1090 system equipped with diode array detector. The absorbance was monitored at 214 nm and the gradient was ACN/water/TFA.

2.4. *HPLC identification*

The extracts of 4 pieces of human colon were separately injected (after addition of 10% ACN) onto a column with 300-5 μm C18 as stationary phase and eluted with gradients as above. The retention times of the immunoreative components were then compared with those of the isolated forms.

2.5. *Amino acid analysis*

Analysis of 10% of the purified peptides was performed after hydrolysis for 20 h in 6 N HCl in gas phase at 110°C. The hydrolysates were analyzed on an Aminoquant system (Hewlett-Packard). All chemicals were analytical grade and all solvents were HPLC-grade.

2.6. *Sequence analysis*

The sequences of the purified peptides were determined on an automatic protein sequencer (Model 475A, ABI (Applied Biosystems Instruments)) equipped with on-line HPLC detection for the PTH-derivatives (AB 120A). For this chromatography a C-18 3 μm column (5-7943, Supelco, PA, USA) was used. Solvents were those recommended by ABI (A: 16 mmol/l NaAc, pH 4.0 in 5% tetrahydrofuran; B: 5 mmol/l dimethylphenylthiourea in acetonitrile). With this column it was possible to completely separate Trp from the sequencer byproduct *N,N'*-diphenyl urea.

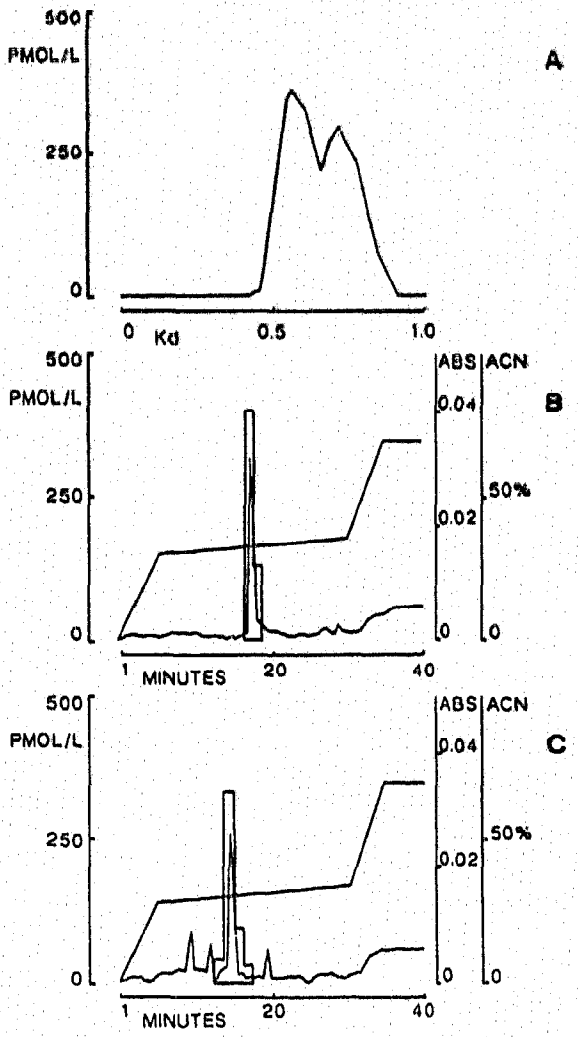

Fig. 1. Isolation of GAL-LI from human colon. The concentration of GAL-LI was monitored by radioimmunoassay (left ordinate scale) using a non-C-terminally directed antiserum. (A) Shows the IR profile at gel filtration: the concentration is plotted against K_d, the coefficient of distribution, calculated as described in the text. In (B) and (C) are shown the last reversed phase HPLC runs (on a C18 column) of the first and the second peak identified by the gel filtration (the peaks were subsequently used for sequence determination and PDMS). The immunoreactive material was eluted with a gradient of ACN/water/HFBA. The concentration of GAL-LI (in histograms) is plotted against the percentage of acetonitrile (ACN, extreme right ordinate scale) and the absorbance (ABS, on the right ordinate scale) expressed in arbitrary units.

2.7. *Mass spectrometry (PDMS)*

Molecular mass determination was carried out on a Bio-Ion Bin 10K plasma desorption time of flight mass spectrometer (Bio-Ion AB, Uppsala, Sweden). Approximately 50 pmol of each peptide were applied to aluminized mylar foil coated with nitrocellulose in 2 μl of 0.1% TFA and dried as described in [12]. After insertion of the sample in the mass spectrometer it was bombarded with fission fragments

from a 10-μCi ^{252}Cf source. The spectra were recorded for 1×10^6 primary ions. The M_r of the peptide was calculated as the mean of the values obtained for single- and double-charged molecular ions.

Methyl esterification [13] was carried out on approximately 50 pmol of peptide (see below), and the mass of the modified peptide determined as described above.

3. RESULTS

Initially 600 g of freshly obtained tissue was extracted, and peptides(s) with GAL-LI were purified and further analyzed by amino acid sequencing, amino acid analysis and PDMS. Because of the unexpected results a further batch of 240 g of freshly obtained human colon was submitted to the same procedure with identical results.

Table I

Sequence analysis of the galanin IR peptides eluting at the reversed phase HPLC at 32% and 30% of ACN

Yields are corrected for background as well as lag. In both peptides amino acid 2 was identified both as genuine PTH-Trp and as a derivative of PTH-Trp that eluted midway between *N,N'*-diphenylthiourea and *N,N'*-diphenylurea.

Cycle	PTH-derivative	32% ACN form Yield (pmol)	30% ACN form Yield (pmol)
1	Gly	106	181
2	Trp	12	80
3	Thr	151	218
4	Leu	86	96
5	Asn	50	82
6	Ser	83	150
7	Ala	63	91
8	Gly	44	57
9	Tyr	60	54
10	Leu	52	73
11	Leu	51	68
12	Gly	36	46
13	Pro	28	72
14	His	13	26
15	Ala	30	68
16	Val	29	53
17	Gly	21	29
18	Asn	10	45
19	His	*	4
20	Arg	12	
21	Ser	29	
22	Phe	23	
23	Ser	21	
24	Asp	18	
25	Lys	2	
26	Asn	5	
27	Gly	7	
28	Leu	10	
29	Thr	10	
30	Ser	13	

*Not found in this run due to injection failure, but His^{19} was unambiguously identified in a preliminary run on a smaller amount of peptide.

The steps of the isolation procedure of the human GAL-LI forms are shown in Fig. 1. The elution patterns of the GAL-LI at the gel filtration and the first HPLC run were identical for the different extracts. At gel filtration, the GAL-LI eluted in two peaks, one at $K_d = 0.58$, another at 0.72 (Fig. 1A). At the final HPLC run on a C18 support and a shallow ACN/water/HFBA gradient, the $K_d = 0.58$ form eluted at 32% ACN and the $K_d = 0.72$ form at 30% ACN (Fig. 1B,C).

The HPLC identification study of extracts prepared freshly from four specimens of colon and directly applied to HPLC revealed for all extracts two immunoreactive forms with retention times that were identical to those of the two purified forms (not shown).

The purified peptides were further studied by amino acid sequencing, amino acid analysis and mass spectrometry.

The form eluting at 32% ACN consisted of 30 amino acids, the sequence of which is shown in Table I. The amino acid composition appears in Table II and the PDMS spectrum in Fig. 2A. The presence of Trp was confirmed by the second derivative of the spectrum recorded during the concentration procedure of the sample, showing a minimum at 290 nm [14]. The average mass of the peptide, 3157.8 ± 3.2 Da, is in agreement with the calculated theoretical value of 3157.4 Da. Because the precision of the PDMS in this mass range is insufficient to determine whether the peptide is COOH-terminally amidated or not, a sample was methyl-esterified by a procedure which methylates all carboxyl groups (and thus add 14.02 atomic mass unit for each) in the molecule [13]. The resulting mass spectrum (Fig. 2B) showed a methylated average mass of 3186.1 ± 3.2 Da. This mass increase corresponds to two

Table II

Amino acid analysis of the two human galanin forms, 1-30 and 1-19

For the analysis, 50 and 45 pmol, respectively, were hydrolyzed. Trp was not determined.

Number of residues:	Expected	Galanin 1-30	Expected	Galanin 1-19
Aspartic acid	4	3.9	2	2.2
Glutamic acid	0	0.2	0	0.5
Serine	4	3.3	1	1.1
Histidine	2	2.1	2	2.0
Glycine	5	5.0	4	4.3
Threonine	2	1.7	1	1.1
Alanine	2	1.9	2	2.0
Arginine	1	1.1	0	0.2
Tyrosine	1	0.9	1	0.9
Valine	1	1.0	1	1.3
Isoleucine	0	0.1	0	0.2
Methionine	0	0.0	0	0.1
Phenylalanine	1	1.0	0	0.5
Leucine	4	3.9	3	3.3
Lysine	1	1.3	0	0.4
Proline	1	1.0	1	1.2

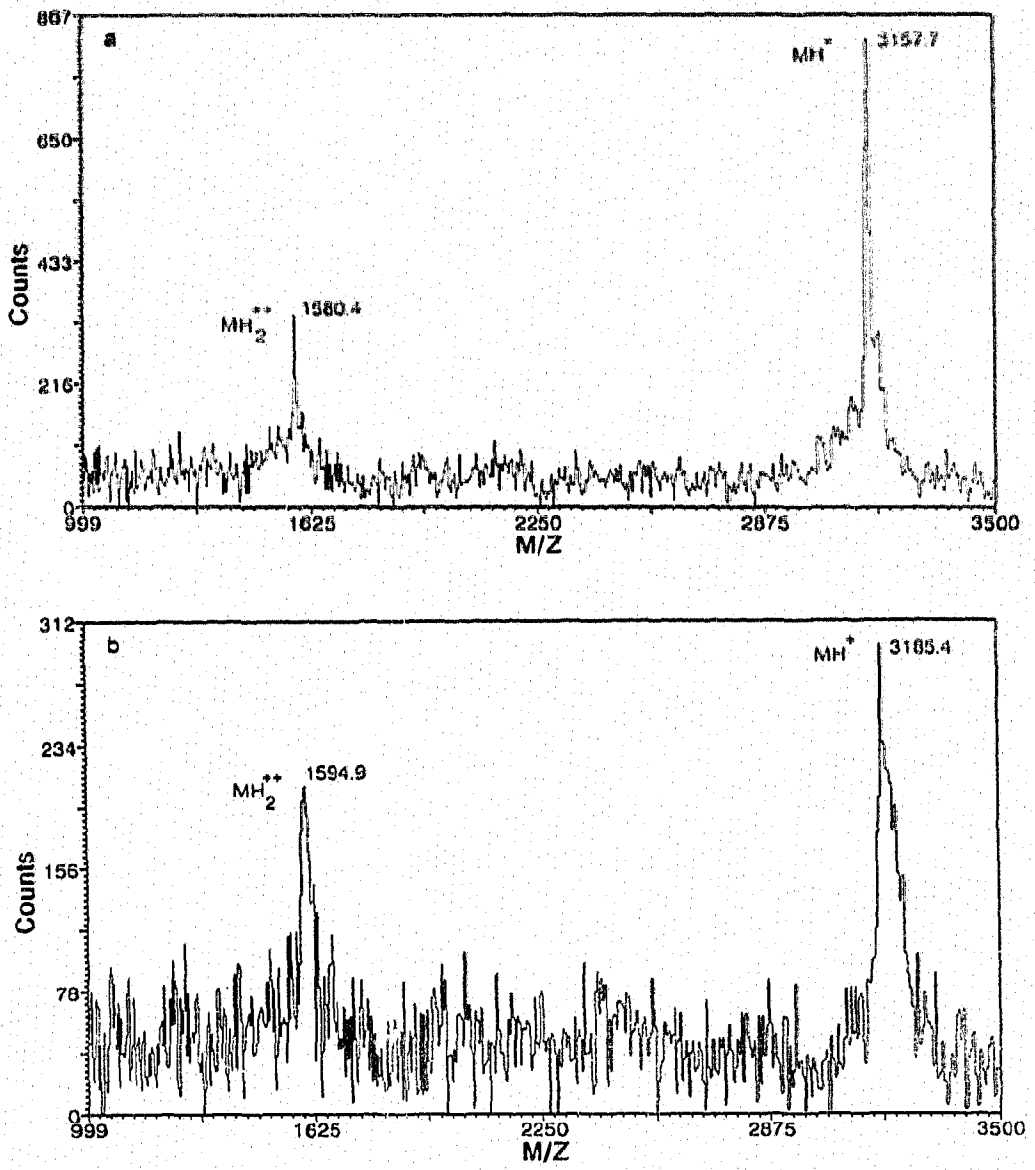

Fig. 2. (A) PDMS spectrum of intact human galanin 1–30. Approximately 50 pmol was applied to a nitrocellulose-covered aluminized mylar target. (B) PDMS spectrum of human galanin after methyl esterification. Both spectra have been background subtracted.

methyl groups indicating the presence of two non-derivatized carboxyl groups. When compared with the sequence (a single acidic side chain in addition to the C-terminal α-carboxyl group) it may be concluded that none of them are blocked, i.e. the peptide is not carboxy-amidated at its C-terminus.

The peptide eluting at 30% ACN consisted of 19 amino acids (Table II), the sequence of which is reported in Table I. From the PDMS spectrum (not shown) an average mass of 1964.4 ± 2.0 Da was obtained, in agreement with the calculated theoretical value of 1964.2 Da for this peptide. By methyl esterification a single free carboxyl group was identified, i.e. the C-terminal one (not shown).

4. DISCUSSION

Galanin was first isolated as a 29 amino acid residue peptide from extracts of pig intestine by the use of a chemical method detecting amide groups [1]. The finding in the cDNA of rat and bovine species of a signal for Gly in position 30 led to the assumption that, in general, galanin is a 29 amino acid residue, C-terminally amidated peptide [4,5].

By the use of a radioimmunoassay based on an antiserum against the N-terminal part of porcine galanin we now isolated two peptides with galanin-like immunoreactivity from extracts of human colon and determined their primary structure. The two peptides consisted of

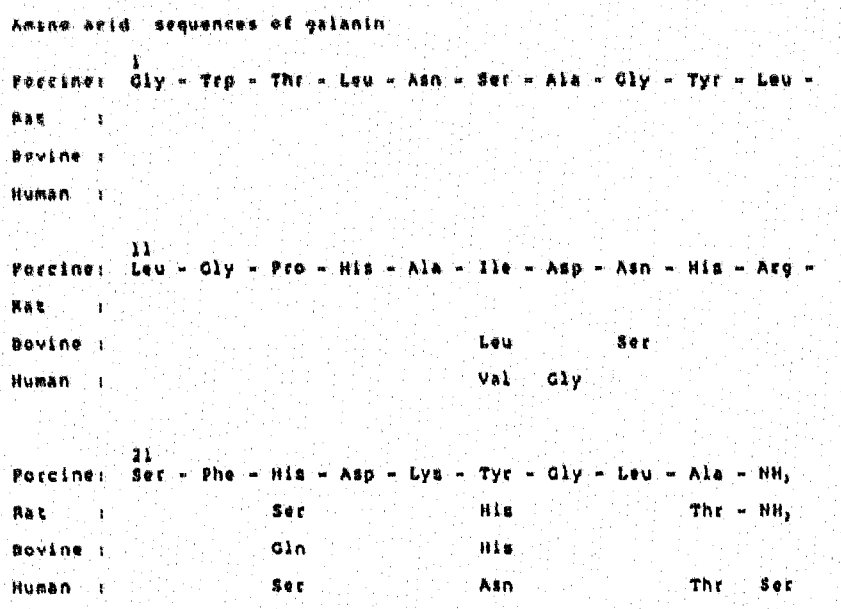

Fig. 3. Comparison of mammalian galanin sequences.

30 and 19 amino acids and occurred in almost equal amounts as judged from the immunoreactive signal obtained by gel filtration and HPLC. Upon sequence analysis it turned out that the 19 amino acid peptide corresponded to the 1-19 sequence of the larger peptide. The first 15 amino acids of the peptides are identical to those of the porcine, rat and bovine galanins, but several substitutions characterize the C-terminal part of the sequence (Fig. 3). Compared with porcine galanin there are changes at positions 16, 17, 23, 26 and 29. Surprisingly, the human galanin is not a 29 amino acid residue, C-terminally amidated peptide. It has a Ser in position 30, substituting the Gly that functions as an amide donor in the other species. The other variable amino acids occur at the same positions in all species and can be explained by single base substitutions for the changes at positions 16, 17, 26, 29 and 30; the change in 23 is more extensive, involving two substitutions when the couples pig-man, pig-rat, cow-man and cow-rat are compared. A single base substitution characterizes the difference between pigs and cows at this position.

The existence of more than one molecular form of galanin in humans was suspected from gel filtration studies by Bauer et al. [6], who also found close to equal amounts of the two forms. Our data show that the smaller form is identical to the 1-19 N-terminal fragment of the 30 amino acid peptide. A posttranslational processing of the precursor to give rise to this peptide could occur as an initial cleavage between residues 20 and 21 (Arg and Ser), a mono-basic cleavage, known to occur in many peptide precursors [15], followed by removal of Arg^{20} by a carboxypeptidase-B-like mechanism [15].

Both the 19- and the 30-amino acid residue peptide may be biologically active. Structure/activity studies have shown that, although less potent than galanin 1-29, N-terminal fragments of galanin as small as the 1-15 fragment may bind to the galanin receptors of rat brain and pancreatic islet tissue and to receptors of insulinoma cell lines (16-19), and the 1-20 fragment was reported to be equipotent with full-length galanin with respect to the effect on intestinal motility in dogs [20]. On the other hand, changes in the N-terminal region of the molecule were generally associated with complete loss of biological activity. However, biological effects of C-terminal fragments have also been described [19,20] and it therefore remains possible that the 1-19 and the 1-30 amino acid molecules differ with respect to their biological activity. Future studies with synthetic peptides prepared according to the sequences reported here should provide answers to these questions and may also throw some light on the question of the physiological actions of human galanin(s) in humans.

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