Volume 288, number 1,2, 151–153 FEBS 010061 August 1991

ADONIS 001457939100769C

Chemical detection of natural peptides by specific structures

Isolation of chicken galanin by monitoring for its N-terminal dipeptide, and determination of the amino acid sequence

Åke Norberg[1], Rannar Sillard[1], Mats Carlquist[3], Hans Jörnvall[2] and Viktor Mutt[1]

Departments of [1]Biochemistry II and [2]Chemistry I, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden and *[3]Karo Bio AB, P.O. Box 4032, S-141 04 Huddinge, Sweden*

Received 4 June 1991

We have isolated galanin from chicken intestine by monitoring for the N-terminal glycyltryptophan, which constitutes a conserved part characteristic of the peptide. This monitoring method complements that previously used for C-terminal amide detection and proves chemical monitoring of specific structures to be useful. The isolation allowed determination of the structure, which was found to be unidentical to any of the known galanins. However, N-terminal pentadecapeptide parts are identical, showing this segment to be of special importance. In addition to common substitutions at positions 16, 18, 23, 26 and 29, chicken galanin has phenylalanine at position 28, where all known mammalian galanins have leucine.

Chemical assay; Neuropeptide; Galanin; Peptide amide; Chicken

1. INTRODUCTION

Galanin was originally isolated from pig intestine, using the C-terminal alanine amide to monitor the isolation [1]. It was later found that rat galanin has a C-terminal threonine amide instead [2,3] and that C-terminal variations consequently occur, formally even violating the name galanin (peptide with N-terminal glycine and C-terminal alanine [4]). However, all known mammalian galanins, the two above and that of cow [5], have an identical N-terminal sequence Gly-Trp, which should be releasable with chymotrypsin. We therefore decided to screen for chicken galanin by using this chymotryptic dipeptide for recognition. As starting material, we used a side-fraction from the previous isolation of chicken secretin [6] and obtained the pure galanin. Determination of the amino acid sequence shows that chicken galanin is not identical to any of the known galanins, has a unique residue at position 28, several common variations in the C-terminal part, and a strictly conserved N-terminal part of probable functional significance.

2. MATERIALS AND METHODS

The starting material for the purification was a methanol-insoluble fraction of chicken intestinal peptides previously described [6] and stored frozen at −20°C since then [6].

Correspondence address: V. Mutt, Department of Biochemistry II, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden. Fax: (46) 8 30 51 93.

Bovine TLCK-treated chymotrypsin, glycyl-L-tryptophan and amino acids were from Sigma Chemical Company (St. Louis, MO), 4-dimethyl-aminoazobenzene-4′-sulfonyl (DABSYL) chloride was from Pierce (Rockford, IL) and carboxypeptidase Y from Boehringer-Mannheim Skandinavia AB (Bromma, Sweden). Amino acid amides were from Cyclo Chemical (Los Angeles, CA) and silica-gel coated glass plates for thin-layer chromatography from Riedel-de Haën AG (Seelze, Germany).

Carboxymethyl cellulose (CM22 from Whatman, UK) was pretreated as described [7] until the wash with 0.2 M HCL, after which it was poured into a tube (2.5 × 22 cm), washed with 0.1 M ammonia and equilibrated with 0.02 M ammonium bicarbonate.

Degradation with chymotrypsin was carried out for 2 h at 37°C, in 1% ammonium bicarbonate at a substrate to enzyme ratio of 25:1.

For C-terminal amino acid amide determination, chicken galanin (12 μg) was dissolved in 6 μl 0.1 M pyridine/acetic acid (pH 6.0) in a siliconized Eppendorf tube, carboxypeptidase Y solution (1 μl of 1 mg/ml in water) was added and the mixture was incubated at 37°C for 1 h. Amino acids and amino acid amides were derivatized as described [8]. The sample was dissolved in 40 μl 0.2 M $NaHCO_3$/NaOH (pH 9.0), and 160 μl DABSYL chloride (2 nmol/ml in acetone) was added, and the sample was incubated with constant shaking at room temperature for 2 h, and thereafter kept at 60°C for 10 min. After cooling, 800 μl of 25 mM phosphate buffer (pH 6.5)/ethanol (1:1) were added and 10 μl of the solution was injected for high performance liquid chromatography (HPLC) analysis on a Spherisorb S5 ODS2 column (4.6 × 150 mm) at 50°C using the elution system as in [8] and a linear gradient of 25–65% acetonitrile over 40 min. The peaks were identified using DABSYL-derivatized amino acids and amino acid amides as standards.

Peptide hydrolysis was carried out with 6 N HCl containing 0.5% phenol in evacuated tubes at 110°C for 20 h.

3. RESULTS AND DISCUSSION

300 mg starting material (cf.[2]) was dissolved in 5 ml 0.02 M ammonium bicarbonate and applied to the

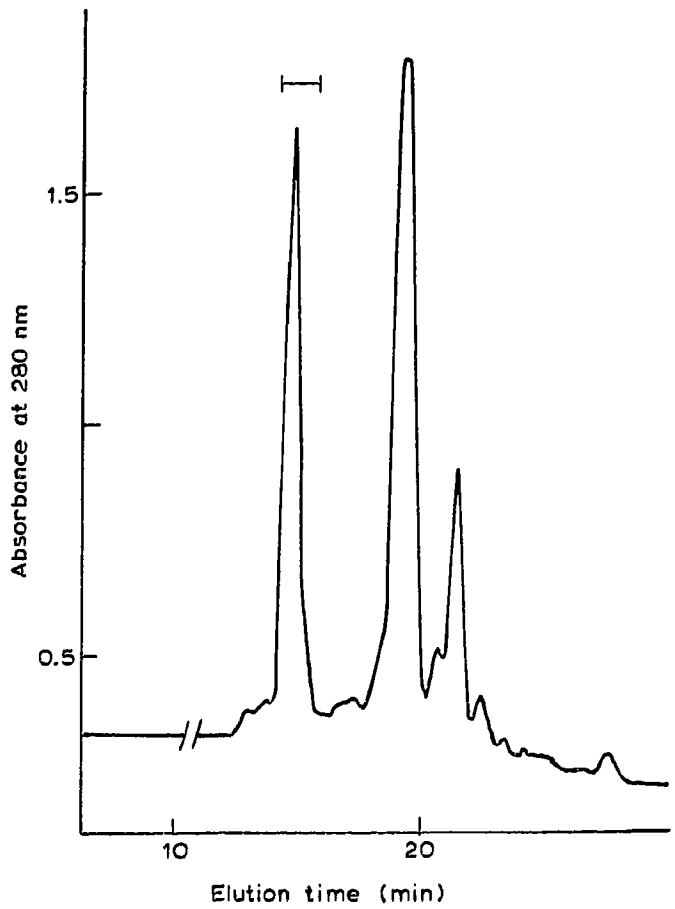

Fig. 1. The final purification of chicken galanin on Ultropac TSK ODS-2 column (7.8×300 mm; LKB, Bromma, Sweden). Gradient: 30–40% acetonitrile in 0.1% trifluoroacetic acid over 30 min. Flow rate: 1.5 ml/min. The fraction denoted by the bar was subjected to structural analysis.

CM22 column in 0.02 M ammonium bicarbonate with monitoring of the absorbance at 280 nm. The first 55 ml of the effluent contained no peptide material and was discarded. The next 70 ml was collected as fraction I (107 mg lyophilized material), the following 120 ml as fraction II (70 mg) and the subsequent 70 ml as fraction III (12 mg).

Table I

Total amino acid composition of the isolated chicken galanin

Amino acid	Mol/mol
Ala	1.96 (2)
Arg	0.89 (1)
Asp	5.04 (5)
Gly	4.05 (4)
His	2.90 (3)
Leu	3.41 (3)
Lys	1.36 (1)
Phe	2.13 (2)
Pro	0.70 (1)
Ser	1.96 (2)
Thr	1.76 (2)
Trp	0.62 (1)
Tyr	0.89 (1)
Val	1.00 (1)

The values represent molar ratios after acid hydrolysis, and those from the sequence analysis are in parenthesis.

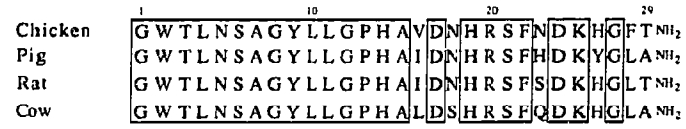

Fig. 2. Amino acid sequences of galanins from different species. Regions conserved in all 4 species are boxed. For references see text.

Aliquots (50 μg) of each fraction were degraded with chymotrypsin, lyophilized, redissolved in water, and chromatographed in parallel with glycyl-tryptophan using the system butan-1-ol/acetic acid/water/pyridine 15:3:12:10 [9]. Tryptophan-containing peptides were visualized with the *p*-dimethylamino-benzaldehyde reagent of Ehrlich [10]. The digest of fraction III contained material that migrated indistinguishably from glycyltryptophan, the N-terminal dipeptide of galanin.

Non-degraded material of fraction III was subjected to reverse-phase HPLC as shown in Fig. 1. An aliquot of the fraction, indicated by the bar, gave a yellow color with cadmium-ninhydrin [11], typical of peptides with N-terminal glycine [10]. That fraction gave also a positive answer in the glycyltryptophan assay performed as described above. The peptide was degraded in an Applied Biosystems 470A gas-phase sequencer, and phenylthiohydantoin derivatives were analyzed with HPLC on Nucleosil C18 with an acetonitrile gradient in sodium acetate, as described [12]. The result was unequivocal showing the sequence Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Val-Asp-Asn-His-Arg-Ser-Phe-Asn-Asp-Lys-His-Gly-Phe-Thr. Total amino acid composition of the isolated peptide is given in Table I.

To determine whether the C-terminal amino acid in chicken galanin was amidated, proteolytic digestion of the peptide with carboxypeptidase Y was carried out followed by derivatization with DABSYL-chloride and subsequent HPLC analysis. This identified threonine amide as the amidated C-terminus. Phenylalanine from the penultimate position was also detected, in accordance with the sequence analysis of chicken galanin.

A comparison of the 3 hitherto known mammalian galanins with the avian form now reported (Fig. 2) shows that all 4 galanins have identical N-terminal pentadecapeptides in keeping with observations on the importance of the N-terminal half of galanin for pharmacological activity (reviewed in [13]). Interestingly, though, chicken galanin has phenylalanine at position 28, where all earlier known galanins have leucine.

The work described in this paper lends support to earlier work [14,15,16] in illustrating the feasibility of using specific structural details in searching for hormonal peptides.

Acknowledgements: This work was supported by grants from the Swedish Medical Research Council (1010 and 3532), The Swedish Cancer Society (1806), Karolinska Institutet, KabiGen AB, Skandigen AB and the Wenner-Gren Center Foundation.

REFERENCES

[1] Tatemoto, K., Rökaeus, Å., Jörnvall, H., McDonald, T.J. and Mutt, V. (1983) FEBS Lett. 164, 124–128.
[2] Kaplan, L.M., Spindel, E.R., Isselbacher, K.J. and Chin, W.W. (1988) Proc. Natl. Acad. Sci. USA 85, 1065–1069.
[3] Vrontakis, M.E., Peden, L.M., Duckworth, M.L. and Friesen, H.G. (1987) J. Biol. Chem. 262, 16755–26758.
[4] Rökaeus, Å. (1987) TINS 10, 158–164.
[5] Rökaeus, Å. and Carlquist, M. (1988) FEBS Lett. 234, 400–406.
[6] Nilsson, A., Carlquist, M., Jörnvall, H. and Mutt, V. (1980) Eur. J. Biochem. 112, 383–388.
[7] Said, S.I. and Mutt, V. (1972) Eur. J. Biochem. 28, 199–204.
[8] Chang, J.-Y. (1984) J. Chromatogr. 295, 193–200.
[9] Waley, S.G. and Watson, J. (1954) Biochemistry, 57, 529–538.
[10] Allen, G. (1981) Laboratory Techniques in Biochemistry and Molecular Biology Vol. 9 (Work, T.S. and Burdon, R.H. eds), North Holland, Amsterdam, New York.
[11] Heilmann, J., Barrollier, J. and Watzke, E. (1957) Hoppe-Seyler's Zeitschr. Physiol. Chem. 309, 219–220.
[12] Kaiser, R., Holmquist, B., Hempel, J., Vallee, B.L. and Jörnvall, H. (1988) Biochemistry 27, 1132–1140.
[13] McDonald, T. (in press) in: Galanin: A Multifunctional Peptide in the Neuroendocrine System, Macmillan, Basingstoke.
[14] Tatemoto, K. and Mutt, V. (1978) Proc. Natl. Acad. Sci. USA 75, 4115–4118.
[15] Håkanson, R., Sundler, F. and Ekman, R. (1980) TIPS 1, 337–341.
[16] Eng, J., Andrews, P.C., Kleinman, W.A., Singh, L. and Raufman, J.-P. (1990) J. Biol. Chem. 265, 20259–20262.