Chromatographic evidence for high-molecular-mass galanin immunoreactivity in pig and cat adrenal glands

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Galanin was measured by radioimmunoassay in extracts of pig, cat and rat adrenals using non-C- and mid to C-terminally directed antibodies. The extracts were fractioned by gel chromatography and HPLC. The non-C-terminal galanin immunoreactivity in pig was 92.8 ± 11.7 pmol/g, in cat 9.1 ± 0.9 pmol/g and in rat < 1 pmol/g. Two higher molecular forms of galanin have been identified in both pig and cat adrenal. One major large form behaves as if it was N-terminally extended (K_{av} pig 0.58, cat 0.48) and the other, a very high-molecular-mass form (K_{av} pig 0.10, 0.24, cat 0.10), as if it had both N- and C-terminal extensions.

Galanin *Molecular form* *Adrenal gland* *Radioimmunoassay* *Chromatography*

1. INTRODUCTION

Galanin is a newly discovered gastrointestinal peptide originally isolated from porcine intestine [1]. It contains 29 amino acids and its name is derived from the fact that its N- and C-terminal residues are glycine and alanine. The amino acid sequence of galanin is different from other peptides and therefore it does not belong to a known peptide family. At the C-terminal end of the molecule galanin shares four amino acids with gonadotropin releasing hormone (GnRH), three with physalaemin (Phys, a frog skin peptide) and two with substance P (SP, a mammalian neuropeptide) [2]. Galanin shows potent biological actions increasing smooth muscle contractility in the gastrointestinal and genitourinary tract of several species [1,3,4] and inhibiting insulin secretion and producing hyperglycaemia in dogs [2].

So far, galanin immunoreactivity has been demonstrated in the central [5–9] and peripheral nervous system, in the intestine [5,10,11], respiratory [12] and genitourinary tract [13] of different mammalian species including man. An abstract report states that galanin-like immunoreactivity is present in cells of the adrenal medulla [14]. As yet, preliminary chromatographic characterisation of galanin immunoreactivity has only been reported in the central nervous system [5,6] and the gastrointestinal tract [5,15] of several mammalian species.

Here we investigated the localisation and molecular heterogeneity of galanin in pig, cat and rat adrenal using radioimmunoassay, gel-permeation chromatography and reverse-phase high performance liquid chromatography (HPLC).

2. MATERIALS AND METHODS

2.1. *Specimens*

Fresh whole adrenals were collected from pigs ($n = 6$), cats ($n = 8$) and rats ($n = 20$). The animals were killed with an overdose of pentobarbitone (pig and cat) or by cervical dislocation (rat).

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2.2. *Tissue extraction*

Fresh pre-weighed tissue samples were plunged into pre-heated polypropylene tubes containing 0.5 M acetic acid (approx. 10 ml/g tissue) and boiled at 100°C for 15 min in a water bath and then cooled and stored at −20°C until assay.

2.3. *Radioimmunoassay*

Galanin was measured using a specific and sensitive radioimmunoassay [15]. The antisera used were produced in rabbits immunised with unconjugated porcine galanin. Two antibodies (designated Gal-8 and Gal-9) had sufficient affinity for assay and were employed at a final dilution of 1/480000 and 1/48000, respectively. For both antibodies the assay was capable of detecting changes between adjacent tubes of 2 fmol/tube with 95% confidence. This corresponds to 1 pmol/g tissue. There was no cross-reactivity of the antibodies with GnRH, Phys, SP, vasoactive intestinal polypeptide (VIP) or adrenocorticotropin hormone (ACTH) in peptide concentrations up to 10 pmol/tube. Regional specificity studies using a C-terminal galanin 10–29 fragment suggested that Gal-8 is non-C- and Gal-9 mid to C-terminally directed. Dose response curves of tissue extracts of several mammalian species showed parallel displacement using both antibodies with the exception of the rat [15]. The non-parallel displacement of rat extracts has been confirmed by others [5].

The ^{125}I-labelled porcine galanin tracer was prepared by the modified iodogen method [16] and the iodinated peptide purified by HPLC on a 5 μm Techsil C-18 column eluted isocratically with 28% acetonitrile/0.05% trifluoroacetic acid. The specific activity of the label was 72.7 Bq/fmol (1.96 mCi/fmol). Galanin tracer (1.5 fmol) and galanin antiserum were added to each tube to a total volume of 800 μl and tissue extracts (20 μl) were assayed in duplicate.

The intra and inter assay variability showed a coefficient of variation of 6.0 ± 0.3 and 11.2 ± 0.3%, respectively (X ± SE).

2.4. *Gel-permeation chromatography*

Galanin immunoreactivity in adrenal extracts of pig (n = 5) and cat (n = 4) were chromatographed on a 1.5 × 100 cm column of Sephadex G-50 superfine (Pharmacia, Uppsala) eluted with 0.06 mol/l phosphate buffer, pH 7.4, containing 7.5 mmol/l sodium azide, 0.01 mol/l EDTA and 0.15 mol/l bovine serum albumin at a flow rate of 6 ml/h. Fractions of 2.0 ml were collected for subsequent radioimmunoassay. Internal column markers run with each sample were dextran blue (V_o), horse heart cytochrome c (CC) and $Na^{125}I$ (V_t). The elution co-efficient (K_{av}) was calculated according to Laurent and Killander [17]. The recovery of all runs was 80–106%. Standard and sample pretreatment using SepPak cartridges did not influence the elution position.

2.5. *High performance liquid chromatography*

Tissue extracts of pig (n = 5) and cat (n = 4) adrenals and porcine galanin standard were absorbed onto SepPak C-18 cartridges (Waters Associates, Milford, USA) and eluted with 1.5 ml of 60% acetonitrile/0.05% trifluoroacetic acid before being loaded for HPLC (Waters Associates) on a 5 μm Techsil, C-18 column, 4.6 × 250 mm (HPLC Technology, Macclesfield, England). Initially the column was run isocratically at 25% acetonitrile for 10 min then a linear gradient was developed from 25 to 32% acetonitrile/0.05% trifluoroacetic acid over 70 min and from 32 to 50% over 54 min. The flow rate was 1 ml·min^{-1} and 2 ml fractions were collected. The recovery of all runs was 78–104%.

3. RESULTS

3.1. *Radioimmunoassay*

The adrenal tissue concentration of galanin immunoreactivity in pig, cat and rat are shown in table 1. The highest concentrations using the non-C-terminal antibody (Gal-8) were found in pig (92.8 ± 11.7 pmol/g) and cat (9.1 ± 0.9 pmol/g). The C-terminal antibody (Gal-9) only detected 71.4 ± 8.1 pmol/g in the pig and none in the cat. Neither antibody detected any galanin-IR in rat adrenals (<1 pmol/g).

3.2. *Chromatography*

Representative gel-permeation chromatographic profiles of galanin immunoreactivity in pig and cat adrenal are shown in fig.1. No elution profile could be demonstrated for rat adrenal extracts. Using the non-C-terminally directed antibody (Gal-8) porcine adrenals showed four peaks at K_{av} (peak)

Table 1

Galanin immunoreactivity in pig, cat and rat adrenals using an non-C-(Gal 8) and C-(Gal 9) terminal directed antibody

	Gal 8	Gal 9
Pig adrenal (n = 6)	92.8 ± 11.7	71.4 ± 8.1
Cat adrenal (n = 8)	9.1 ± 0.9	<1
Rat adrenal (n = 20)	<1.0	<1

Results expressed as pmol/g wet wt of tissue (mean ± SE)

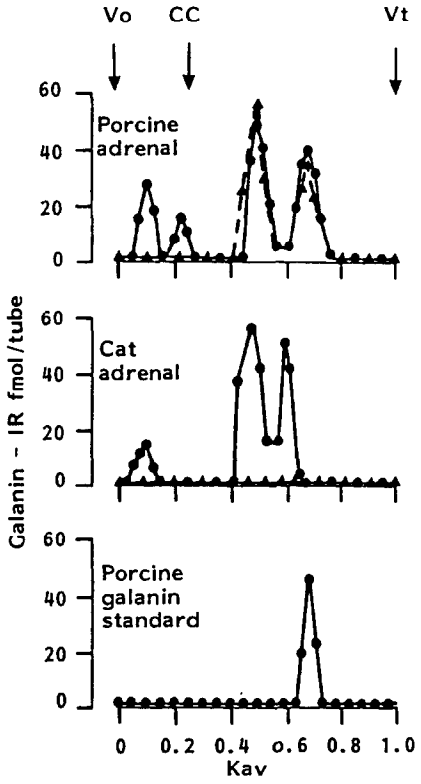

Fig.1. Representative gel-permeation chromatographic profiles of pig and cat adrenal extracts on a column (1.5 × 100 cm) of Sephadex G-50 superfine using a non-C-(Gal-8) (●) and C-(Gal-9) (▲) terminal directed antibody. Arrows indicate elution position of dextran blue (V_o), horse heart cytochrome c (CC) and $Na^{125}I$ (V_t). The pig extracts eluted in four peaks at K_{av} 0.10, 0.24, 0.48 and 0.68 (porcine standard position). The cat extracts emerged in three peaks at K_{av} 0.10, 0.48 and 0.57.

0.10 (1), 0.24 (2), 0.48 (3) and in the position of the porcine galanin standard at K_{av} 0.68 (4). The C-terminally directed antibody (Gal-9) did not detect peaks 1 and 2 but detected peaks 3 and 4 in an equimolar ratio compared to Gal-8. In cat adrenals using Gal-8, 3 peaks of galanin eluted at K_{av} (peak) 0.10 (1), 0.48 (2) and 0.57 (3). In contrast to porcine adrenal, the C-terminal directed antibody (Gal-9) did not detect galanin immunoreactivity in the cat adrenal.

Fig.2 shows representative HPLC profiles from pig and cat adrenals. Four peaks could be shown in both species using Gal-8. In pig adrenal galanin eluted at retention times (peak) of 44 (1), 56 (2), 82 (3) and 114 (4) min. In cat adrenals the retention times (peak) were 40 (1), 56 (2), 72 (3) and 124 (4) min. The porcine standard emerged in a single peak at 56 min. The corresponding peaks of subsequent gel-permeation chromatography of each HPLC peak in both species are shown in table 2.

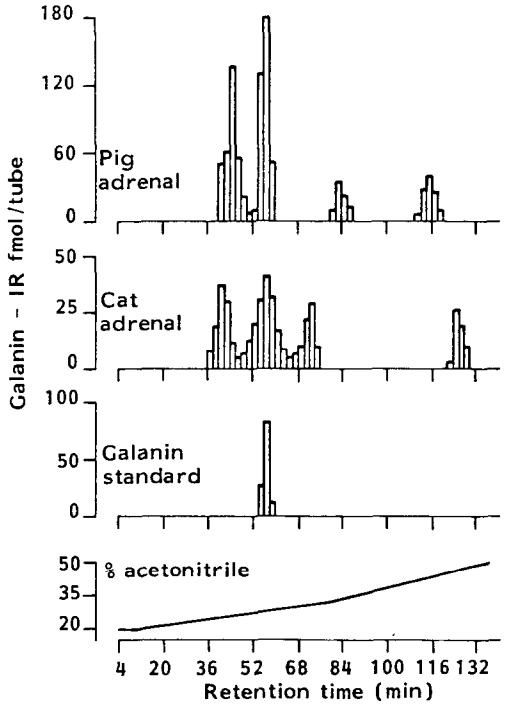

Fig.2. Representative reverse-phase HPLC profiles of pig and cat adrenal extracts (Gal-8). In pig galanin immunoreactivity eluted in four peaks at retention times of 44, 56, 82 and 114 min. In cats galanin eluted also in four peaks at retention times of 40, 56, 72 and 124 min. The porcine standard emerged in a single peak at 56 min.

Table 2

Subsequent gel-permeation chromatography of each reverse-phase HPLC peak and its corresponding peak of galanin immunoreactivity in gel-permeation chromatography of adrenal extracts of pig and cat using the non-C-terminal antibody galanin 8

	HPLC		G-50 column	
	Peak no.	Retention time (min)	Peak no.	K_{av}
Pig	1	44	3	0.48
	2	56	4	0.68
	3	82	2	0.24
	4	114	1	0.10
Cat	1	40	2	0.48
	2	56	2	0.48
	3	72	3	0.57
	4	124	1	0.10

4. DISCUSSION

Galanin immunoreactivity has been shown in substantial quantities in pig and cat adrenals but in rat adrenals the galanin content, as measured by these antibodies, was very low.

Different extraction procedures (0.5 M acetic acid, pH 2.77, water, pH 7.0, Tris buffer, pH 8.5) for several species showed much greater recoveries in acetic acid and no molecular form differences could be shown on gel chromatography of extracts from the three extraction regimes [15]. Thus it seems unlikely that the molecular pattern is a mere artefact of the extraction procedure.

Using gel chromatography porcine galanin eluted in four peaks. The two largest were detected only by the non-C-terminal antibody, whereas the other two peaks, present in larger amounts, were detected by both antibodies. The HPLC elution profile also showed four peaks of porcine galanin and subsequent gel chromatography of the HPLC peaks confirmed each gel chromatographic peak. Using the non-C-terminal antibody on gel chromatography the cat adrenal extracts eluted in three peaks. The C-terminal antibody did not detect any galanin in the cat. On HPLC four peaks of cat galanin immunoreactivity could be shown. Subsequent gel chromatography of the HPLC peaks also confirmed each gel chromatographic peak but HPLC peaks one and two eluted on gel chromatography at the same position (K_{av} 0.48). The last eluting HPLC peak corresponded to the largest size molecular form on gel chromatography.

In both species the results could be explained by extensions at the N- and C-terminal end of the different molecular forms as shown in fig.3. The inability of the C-terminal antibody to detect the porcine G-50 peak 1 and 2 (K_{av} 0.10 and 0.24, respectively) and all cat G-50 peaks indicates the possibility of C-terminal extensions of the galanin molecule in both species. In cat extracts it would also suggest species sequence differences compared to the porcine standard. In contrast to this the detection of porcine G-50 peak 3 (K_{av} 0.58) by the non-C- and C-terminal antibody and cat G-50 peak 2 (K_{av} 0.48) only by the non-C-terminal antibody suggests an N-terminal extension in both species. Furthermore, this finding may indicate that the non-C-terminal antibody is N- to mid-terminal rather than exclusively N-terminal. The lack of cross-reaction of cat galanin with the C-terminal antibody would also be in accordance with C-terminal amino acid differences possibly accompanied by N- and/or C-terminal extensions.

Thus, two higher-molecular-mass forms of galanin have been shown. One major large form, apparently extended at the N-terminal end (K_{av}: pig 0.58, cat 0.48), and another very high-molecular-mass form (K_{av}: pig 0.10, 0.24, cat 0.10), apparently with N- and C-terminal extensions. It is possible that the latter is a galanin precursor from which the peptide itself is cleaved

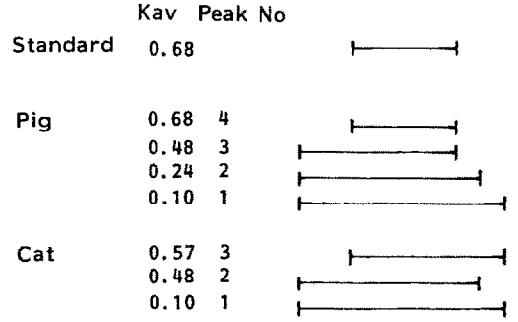

Fig.3. Theoretical scheme of possible multi-molecular form model of galanin in the pig and cat adrenal.

by post-translational processing. However, an aggregate of galanin or of galanin with other proteins cannot be excluded. For VIP and NPY, which are also present in the adrenal gland high-molecular-mass material has been shown in phaeochromocytomas [18,19] and ganglioneuroblastomas [19]. Further investigations are needed to clarify the nature and activity of the high-molecular-mass forms of galanin.

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