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Nucleotide sequence analysis of cDNAs encoding a bovine galanin precursor protein in the adrenal medulla and chemical isolation of bovine gut galanin

Åke Rökaeus[+] and Mats Carlquist[°]

Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD, USA and Depts of [+] Biochemistry I and [°] Biochemistry II, Karolinska Institutet, Stockholm, Sweden

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A description is given of the primary structure of a bovine adrenal medullary precursor protein (123 amino acids), containing a nonrepetitive galanin sequence, different in four amino acid positions from pig galanin, structural information deduced from the nucleotide sequence analysis of two cDNA clones. Pairs of lysine and arginine residues separate the galanin sequence N-terminally from the initiating methionine and a 29 amino acid leader peptide and C-terminally from a 59 amino acid peptide. The deduced amino acid composition was confirmed by the isolation and determination of the amino acid composition for bovine intestinal galanin. Northern blot analysis revealed the presence of an apparent single galanin mRNA species, of approx. 850–900 bases in size, which was present in a population of chromaffin cells scattered throughout the bovine adrenal medulla.

Chromaffin cell; Galanin message-associated peptide; Hybridization histochemistry; Peptide isolation; RNA hybridization

1. INTRODUCTION

Galanin is a 29 amino acid residue peptide, isolated from the upper small intestine of pig, which was named from its glycine and amidated alanine residues in the N- and C-terminal positions, respectively [1]. We have recently demonstrated that pig galanin is processed from a larger 123 amino acid precursor protein by cloning of its mRNA from the adrenal medulla [2]. The development of antibodies against porcine galanin has allowed its mapping by radioimmunoassay and immunohistochemistry, showing its presence in discrete areas in the central nervous system (CNS) as well as in the peripheral nervous system; galanin-like immunreactivity (GAL-LI) is localized in the enteric nervous system of the gastrointestinal tract, in the urogenital tract and the pancreas, as well as in the adrenal medulla (review [3]). The widespread distribution of GAL-LI in the peripheral and central nervous system suggests multiple roles for galanin; in the periphery, galanin may be involved in the regulation of processes such as gastrointestinal motility [1,3] and in the regulation of endocrine pancreatic activity by inhibiting insulin release [1,3,4]. The specific localization of GAL-LI, within certain areas of the CNS, suggests a possible involvement in processes such as pituitary hormone release and neuronal transmission – supported by increase of plasma growth hormone [5] and inhibition of potassium evoked acetylcholine release in the ventral hippocamus of the rat [6].

The aim of the present study was to learn more about the molecular structure of the mRNA encoding the galanin precursor protein in the bovine adrenal medulla, since data obtained by radioimmunoassay suggested that bovine galanin was structurally different from that of porcine; extracts from bovine intestine do not serially dilute along the porcine galanin standard curve in the radioimmunoassay (unpublished observations). This paper

Correspondence (present) address: Å. Rökaeus, Dept of Biochemistry I, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

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reports the screening of a bovine cDNA library, and the isolation and nucleotide sequence analysis of two galanin-positive cDNA clones. The structural analysis revealed that the bovine adrenal medulla precursor protein for galanin, hereafter referred to as preprogalanin, had the same principal organization and size (123 amino acids) as the pig preprogalanin but differed in structure at several amino acid positions. The amino acid composition of bovine galanin was confirmed by the isolation and determination of the amino acid composition for bovine intestinal galanin and its tryptic fragments. Northern blot analysis revealed that preprogalanin was encoded for by an apparent single species of mRNA which was present in a population of chromaffin cells, in the adrenal medulla, as shown by in situ hybridization histochemistry.

2. MATERIALS AND METHODS

2.1. *Screening of the cDNA library*

A cDNA library derived from poly(A)$^+$ selected RNA from cultured bovine adrenal chromaffin cells, kindly provided by Ms Anna Iacangelo [7], was screened with a porcine galanin cDNA probe, spanning the entire coding region of the preprogalanin molecule and some of its flanking sequences. The procedure was essentially that described [2] with the modification that hybridization was performed at 60°C with 50% formamide (v/v) included in the hybridization solution to increase the stringency [2]. The probe, a 465-base-long fragment (nucleotides −56 to 409) obtained from the pGAL 5A2 clone by cleavage with *Sma*I and *Sst*I restriction enzymes [2], was nick-translated [8] and labelled with [α-^{32}P]dCTP.

2.2. *Nucleotide sequencing*

Nucleotide sequencing was carried out as described [2], by the dideoxy method of Sanger [9] using single-stranded DNA templates obtained by subcloning DNA fragments into the M13/18 or M13/19 vectors [10].

2.3. *Preparation and characterization of galanin mRNA*

Bovine adrenal glands, transported on wet ice to the laboratory from the slaughter house, were obtained from newly killed cows. Upon arrival at the laboratory, the adrenal medullas were dissected free of cortices and frozen on solid CO_2 and kept at −70°C until further processing. Porcine adrenal glands were obtained within 30 min from newly killed NIH minipigs and the adrenal medullas were prepared and frozen as described [2]. Total RNA and poly(A)$^+$ RNA were prepared as described [2]. Poly(A)$^+$ RNA from cow and pig adrenal medulla was electrophoresed on a 1.3% agarose-formaldehyde gel, electrically transferred onto GeneScreen (New England Nuclear) membrane paper [11] which was baked at 80°C for 90 min, and hybridized in 50% formamide as described above, at 65°C. Hybridization was performed overnight with two ^{32}P-labelled probes, approx. 1×10^6 cpm·ml^{-1} of each. The probes were made by cloning, a 468 base fragment from the bGAL 1.21 clone (nucleotides −50 to 418), i.e. between the *Pst*I site and the *Hgi*AI site (fig.1), and a 465 base fragment from the pGAL 5A2 clone (see above and [2]), into the gemini and riboprobe vectors, respectively, and single-stranded complementary cRNA^{bGAL} and cRNA^{pGAL} were synthesized according to the instructions of the manufacturer (Promega Biotec).

In situ hybridization histochemistry was performed as described in detail [12,13] using approx. $1\text{–}2 \times 10^6$ dpm of a ^{35}S-labelled (specific activity of about 5000 Ci/mmol) synthetic deoxyribonucleotide probe complementary to nucleotides 97–144. The 48 mer was synthesized on an Applied Biosystems

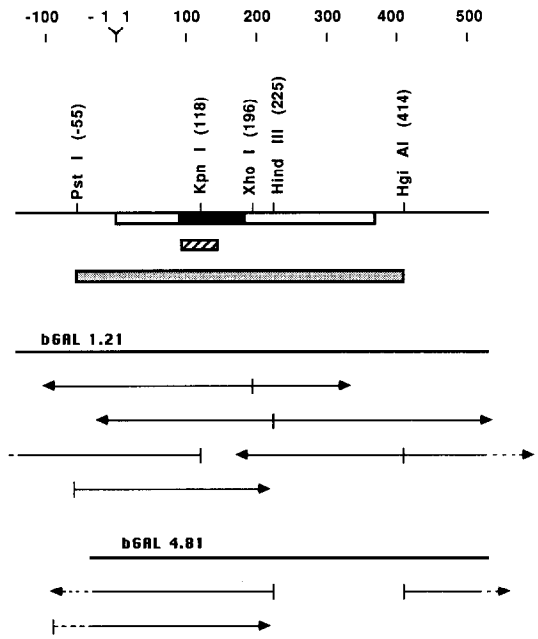

Fig.1. Sequencing the cDNA inserts in clones bGAL 1.21 and bGAL 4.81. Numbers at the top of the figure refer to bases; the first base in the initiating methionine codon is numbered 1 (see fig.2). The restriction map displays only relevant restriction endonuclease sites, the 5′ ends of which are identified by numbers in brackets after the name of the enzyme. (▭) Sequence corresponding to the coding region of preproGAL. (▬) Coding region of GAL. (▭) Sequence corresponding to the insert used in the preparation of a probe for Northern blot analysis (see fig.3). The box (▨) directly below the coding region of GAL indicates the hybridization site and relative size of the oligonucleotide used for in situ hybridization histochemistry (see section 2). The directions and extents of sequence determinations by the Sanger technique are shown by horizontal arrows under each clone used; the priming site on the single-stranded DNA template is indicated by a short vertial line at the beginning of the arrow. Dashed portions of arrow-shafts indicate that the starting point of sequencing was within the original cloning vector or that the sequencing extended into the poly(dA) or poly(dG) tails.

DNA synthesizer (courtesy of Dr M.J. Brownstein, Laboratory of Cell Biology/NIMH) and was purified on an 8% denaturing acrylamide gel.

2.4. *Isolation of bovine intestinal galanin*

The starting material, bovine intestine, and the initial purification steps have recently been described in connection with the isolation of bovine glucose-dependent insulinotropic peptide (GIP) and porcine brain vasoactive intestinal polypeptide (VIP) [14,15]. The methanol-soluble fraction was subjected to gel-filtration on Sephadex G-25 (fine) in 0.2 M acetic acid. The fractions containing GAL-LI, as determined by radioimmunoassay [16] were further chromatographed first on CM-cellulose, eluted by a step-wise increase of ammonium bicarbonate concentration at pH 8.0, and then on a HPLC cation-exchanger column (TSK 535 CM) with a gradient of NaCl in a 0.02 M Na-phosphate buffer at pH 6.4. Finally, highly purified bovine GAL was obtained after reverse-phase HPLC on a TSK ODS-120T column [15]. Digestion with trypsin was performed in 1% ammonium bicarbonate with an enzyme to substrate ratio of 1:100 by weight, at 37°C for 3 h. Fragments obtained were isolated by HPLC [15]. Acid hydrolysis was performed in evacuated tubes with 6 M HCl, containing 0.5% phenol, at 110°C for 24 h and total compositions were determined on a Beckman 121M amino acid analyzer.

3. RESULTS AND DISCUSSION

3.1. *Screening of the cDNA library and sequence analysis*

Two posive clones were isolated after screening approx. 120 000 ampicillin resistant transformants. The two clones were subjected to restriction enzyme analysis and subsequent cloning of suitable restriction fragments into the M13 sequencing vectors [10]. The strategy of sequencing the two clones (bGAL 1.21 and bGAL 4.81) by the method of Sanger is indicated in fig.1.

The primary structure of the two bovine cDNA inserts (fig.2) was determined from the 674 and 560 nucleotide insert (excluding the poly(dG) and poly(dA) tails at the 5′ and 3′ ends, respectively) of the bGAL 1.21 and bGAL 4.81 clones. The nucleotide sequence of residues 97–183 in the cow clones corresponds exactly to the position of the nucleotides encoding porcine galanin in its precursor protein, although it has four amino acid substitutions, i.e. in the amino acid positions 16, 18, 23 and 26 of galanin. In addition, the amino acid composition encoded for by these nucleotides is in perfect agreement with that of bovine intestinal galanin (see below) taking into consideration that nucleotides 184–186 correspond to glycine which upon processing contributes the amide group [17] on the C-terminal alanine residue of galanin. Pairs of lysine and arginine residues separate the galanin molecule and the C-terminal glycine residue from a 59 amino acid galanin message-associated peptide which is followed by a termination codon TGA, nucleotides 370–372. The differences in amino acid sequence between porcine and bovine galanin may explain why antibodies raised against pig galanin, and directed towards the region of the galanin molecule where these amino acid substitutions occur, do not detect bovine galanin in the same manner as porcine galanin, e.g. give rise to non-parallel serial dilution curves in the radioimmunoassay (unpublished). In preliminary studies we have also shown that these differences in amino acid composition effect the biological activity for galanin, i.e. bovine galanin has less than one-tenth the potency of porcine galanin in inhibiting basal insulin release from a rat insulinoma cell line [18].

The translation initiation site is tentatively assigned to the methionine codon ATG at nucleotide positions 1–3, which is the first initiating triplet in the reading frame upstream from galanin (fig.2). The size and hydrophobicity of the peptide following this tentatively assigned initiating methionine is 29 amino acids, in agreement with the size usually found in leader peptides [19,20] and identical in length to that found in the pig [2] but different at three amino acid positions (fig.2). Two of these three amino acid substitutions are changes between hydrophobic amino acids.

C-terminally from the deduced sequence for bovine galanin and its amide donor glycine and separated from the latter by a Lys-Arg sequence, is a 59 amino acid residue peptide, galanin-message-associated-peptide (GMAP), which is notably conserved from the pig [2] in its 15 N-terminal amino acids (residues 65–79). An acidic arrangement is formed by the amino acid residues 65–71, i.e. every other amino acid in this region is a glutamic acid (fig.2). Another relatively conserved region of GMAP is that between amino acid residues 89 and 106, i.e. an area of GMAP which is flanked by single basic (Arg) residues, which upon processing of the precursor protein may be cleaved off by an endopeptidase recognizing single basic amino acids [21], and later released. However, the overall number of amino acid substitutions in the bovine versus the porcine GMAP is 18 (fig.2).

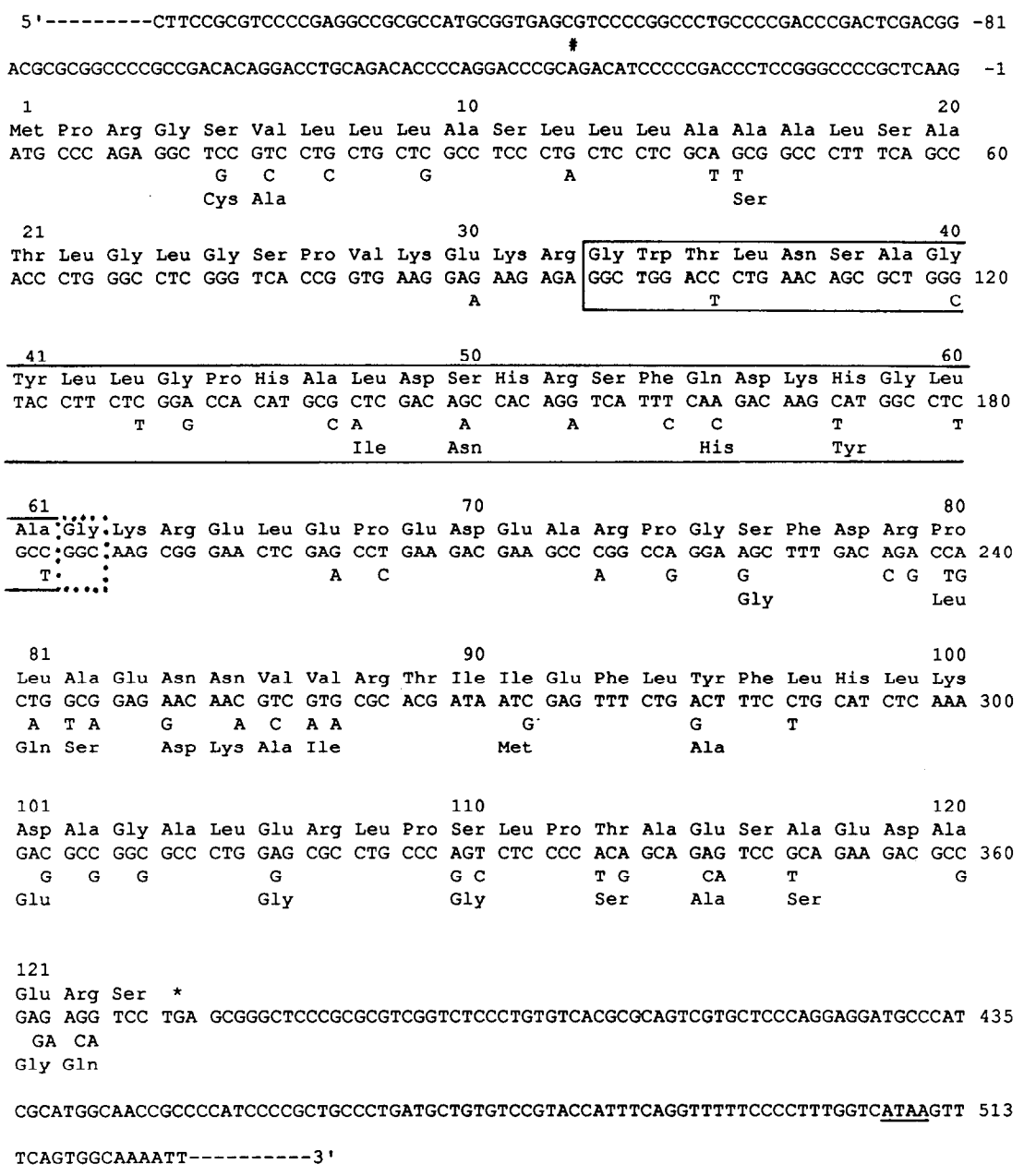

Fig.2. Structure of bovine preprogalanin cDNA. The nucleotide sequence of preprogalanin cDNA is based on the sequences of inserts in clone bGAL 1.21 and pGAL 4.81. The insert in bGAL 1.21 clone includes the entire sequence found in the bGAL 4.81 clone (# indicates the 5′ end of the latter clone). Nucleotides are numbered in the 5′-3′ direction, beginning with the first base in the ATG triplet, which corresponds to the AUG triplet in the mRNA, encoding the initiating methionine. Nucleotides located on the 5′-side of base 1 are indicated by negative numbers. The predicted amino acid sequence of preproGAL is depicted above the nucleotide sequence; amino acid residues are numbered beginning with the initiating methionine. Below the bovine nucleotide sequence the porcine differences are indicated as well as the amino acid changes. The GAL sequence is outlined; the amide on the C-terminal alanine residue of galanin contributed by the glycine [17] residue is next to it (dotted box). The ATAA sequence (bases 507–510) thought to be involved in polyadenylation [22] is underlined. The cDNA inserts in bGAL 1.21 and bGAL 4.81 clones have poly(A) tails of approximately 52 and 34 bases, respectively.

The 3′-untranslated region in the cDNA clones encoding for bovine preproGAL do not contain a full length polyadenylation signal of AATAAA [22] as in the pig, but have a truncated ATAA sequence in the corresponding position of the pig signal (nucleotides 507–510), probably subserving the same function of polyadenylation, and located 17 nucleotides upstream from the poly(A) tract (fig.2).

3.2. *Characterization of the galanin mRNA*

Northern blot hybridization to poly(A)$^+$ selected adrenal medullary RNA obtained from frozen bovine and porcine adrenal medullas visualized an apparent single species of bovine mRNA (approx. 850–900 bases in size, fig.3). This is in agreement with the length of the sequenced cDNA insert in the longest clone (bGAL 1.21) assuming a mean length of 150–200 bases for the poly(A) tail [23]. The bovine adrenal medullary mRNA seemed to be slightly shorter than the pig mRNA; it remains to be shown conclusively whether this finding represents a true difference in sizes of the mRNAs encoding the porcine and bovine precorsor protein, respectively, or is merely an indication that the bovine mRNA, upon transport of the adrenals to the laboratory and subsequent isolation, had been slightly more degraded.

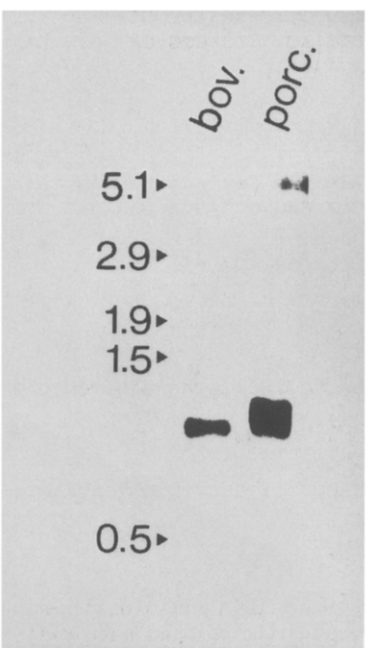

Fig.3. Northern blot-hybridization analysis of poly(A)$^+$ RNA from bovine (bov.) and porcine (porc.) adrenal medulla. RNA was electrophoresed on a 1.3% agarose-formaldehyde-gel, transferred to GeneScreen membrane paper and hybridized to ^{32}P-labelled bovine and porcine galanin cRNA probes. Kodak XAR-2 film was exposed 24 h at −80°C with intensifying screens. Sizes are shown in kb.

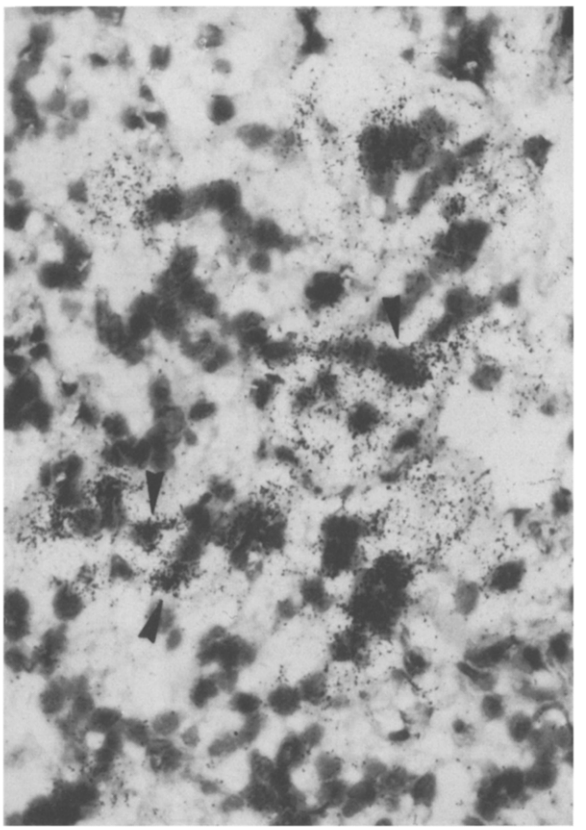

Fig.4. High power bright-field photomicrograph showing several labelled chromaffin cells which express galanin mRNA in an area of a bovine adrenal medulla. The in situ hybridization histochemistry was performed with a ^{35}S-labelled bovine galanin deoxyribonucleotide probe (48 mer) complementary to the coding region for galanin [1–16]. Arrow-heads indicate three heavily labelled cells. Cells were background stained with toluidine blue.

The localization of mRNA encoding bovine galanin in the adrenal gland was further characterized by in situ hybridization histochemistry, demonstrating it to be present in a population of chromaffin cells scattered throughout the adrenal medulla (fig.4), in agreement with an earlier demonstration of GAL-LI in cultured bovine chromaffin cells by immunohistochemistry [24].

3.3. *Isolation of bovine intestinal galanin*

In table 1 the amino acid composition of bovine intestinal galanin and its tryptic fragments are indicated and compared with the composition of pig galanin and the amino acid composition deduced from the cDNA structure obtained above, thus extending and confirming the composition of bovine galanin being one and the same in both these organs. Upon isolation of bovine intestinal galanin we could detect in some chromatographic system as many as four chromatographically separable peaks of GAL-LI, of varying amounts, when screened with the radioimmunoassay system used in this study (unpublished; not shown) which is in agreement with earlier findings in the rat and cat [3,16,25]. These data, together with the cloning data and the finding of only one type of galanin encoded for by the galanin mRNA, suggest that the other forms must be fragments of the precursor protein which are found naturally or are formed upon extraction; galanin may undergo an internal rearrangement upon extraction, i.e. a β-aspartic shift [26] which also may give rise to additional forms of GAL-LI being detected.

In conclusion these experiments have revealed the structure of an mRNA encoding a galanin precursor protein - preprogalanin - in the bovine adrenal medulla, as well as given the confirmatory information of bovine intestinal galanin composition, in perfect agreement with the cloning data. The deduced information of the structure for the bovine precursor protein, in combination with similar information about the porcine precursor protein, will allow studies of the processing of preprogalanin to be planned and performed. Furthermore, the isolated clones are being used as probes to detect mRNAs encoding preprogalanins from other species, to study the regulation of mRNA encoding galanin, as well as being used to detect and characterize the galanin gene, studies which are in progress.

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Table 1

Amino acid compositions of bovine galanin and its tryptic fragments

Amino acid	Porcine galanin	Bovine galanin				
		Sum of DNA sequence	1–29	1–20	21–25	26–29
Asp	4	3	3.1(3)	2.0(2)	1.0(1)	–
Thr	1	1	1.0(1)	1.0(1)	–	–
Ser	2	3	2.8(3)	2.3(2)	1.0(1)	–
Glu	–	1	1.1(1)	–	1.0(1)	–
Pro	1	1	1.1(1)	1.1(1)	–	–
Gly	4	4	3.9(4)	3.1(3)	–	1.0(1)
Ala	3	3	3.0(3)	2.0(2)	–	1.0(1)
Ile	1	–	–	– –	–	–
Leu	4	5	5.0(5)	3.6(4)	–	1.0(1)
Tyr	2	1	1.0(1)	1.0(1)	–	–
Phe	1	1	1.0(1)	–	0.9(1)	–
Trp	1	1	ND(1)	ND(1)	–	–
Lys	1	1	1.1(1)	–	1.0(1)	–
His	3	3	2.8(3)	2.0(2)	–	0.9(1)
Arg	1	1	1.1(1)	1.1(1)	–	–
Sum	29	29	29	20	5	4

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