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Role of N-terminal active sites of galanin in neurally evoked circular muscle contractions in the guinea-pig ileum

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Abstract

Synthetic galanin-related peptides and several galanin-(1–15)ol analogs were used to examine structure–function relationships of the N-terminal active site of galanin in more detail, using the guinea-pig ileum. The synthetic peptides examined showed their inhibitory activity on the neurally evoked circular muscle contractions with the following order of potency: rat, human and tuna galanin, galanin-(1–15)ol and [D-Trp^8]galanin-(1–15)ol > N^{α}-acetylated galanin-(2–15)ol, [Ala^6,D-Trp^8]galanin-(1–15)ol, galanin-(1–15) > tuna galanin-(1–15), [D-Ala^8]galanin-(1–15)ol, N^{α}-acetylated galanin-(1–15)ol. In contrast, [D-Thr^6]galanin-(1–15)ol, [D-Tyr^9]galanin-(1–15)ol and [D-Trp^9]galanin-(1–15)ol were ineffective and showed no antagonistic activities to galanin. These results suggest that the L-configuration at positions 6 and 9 seems to be important for the inhibitory action of galanin on the neurally evoked guinea-pig circular muscle contractions. © 1997 Elsevier Science B.V.

Keywords: Galanin, analog; Galanin-(1–15)ol; Ileum contraction, neurally evoked, guinea-pig

1. Introduction

The enteric nervous system contains a variety of neuropeptides and classical neurotransmitters such as acetylcholine and norepinephrine. Galanin is a 29-residue peptide originally isolated from porcine upper small intestine (Tatemoto et al., 1983). The mammalian forms so far isolated have in common the first 15 residues, but differ at several positions in the carboxy-terminal part. On the other hand, tuna fish galanin, which was recently isolated from the hypothalamus, differs at two positions in the N-terminal 1–15 sequence (Habu et al., 1994). Galanin was reported to be widely distributed in the central and peripheral nervous system (Melander et al., 1985; Skofitsch and Jacobowitz, 1986). In the gastrointestinal tract, galanin-containing nerve cell bodies have been localized principally in the submucosal and myenteric plexus. Intracellular microelectrode studies on myenteric neurons of the guinea-pig small intestine have revealed that galanin shows an inhibitory action on myenteric neurons (Tamura et al., 1988). Galanin binding sites have also been found on rat and guinea-pig gastric smooth muscle membranes as well as rat jejunal and canine ileal smooth muscle membranes (Rossowski et al., 1990; Chen et al., 1994a) and canine circular muscle synaptosomes (Chen et al., 1994b).

The primary structure of the galanin receptor has recently been shown to be a member of the seven-transmembrane domain receptor superfamily by hydropathy analysis (Habert-Ortoli et al., 1994; Lorimer and Benya, 1996). In most cases, N-terminal fragments of galanin show a high affinity to galanin receptors (Amiranoff et al., 1989). In addition, specific binding sites for the N-terminal fragment, galanin-(1–15), were found in the rat brain (Hedlund et al., 1992). Galanin-(1–15) and galanin-(1–16) also exert a wide range of biological actions (Fisone et al., 1989; Narváez et al., 1994; Yanaihara et al., 1991).

Our previous studies have revealed that porcine galanin inhibited neurally evoked circular muscle contraction in

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the guinea-pig ileum (Kuwahara et al., 1989). Although porcine galanin did not affect the basal activity of longitudinal or circular muscle, galanin inhibited neurally evoked circular muscle contraction even in the presence of atropine. The neurally evoked circular muscle contractions were blocked by tetrodotoxin. These results indicated that galanin plays an inhibitory role in circular muscle contractions via myenteric neurons involved in the non-cholinergic excitation of the guinea-pig ileum.

In the previous structure–function studies on galanin, the N-terminal 1–15 sequence of galanin, especially Gly^1 and Trp^2, was shown to be essential for the inhibitory effect on glucose-induced insulin release from the isolated perfused rat pancreas (Mochizuki et al., 1992; Yanaihara et al., 1991) and on the neurally evoked circular muscle contraction in guinea-pig ileum (Yanaihara et al., 1991; Kuwahara et al., 1990). Galanin-(1–15)ol, which was prepared as a stable analog against carboxypeptidase, was found to be nearly equipotent to galanin for inhibitory activity on the neurally evoked circular muscle contraction (Yanaihara et al., 1991). Recently, we found that [D-Trp^8]galanin-(1–15)ol, in which Gly was replaced by D-Trp at position 8, showed an affinity higher than that of galanin on human small cell lung carcinoma cell membranes, suggesting the importance of D-Trp^8 at position 8 for binding to the receptor (Kakuyama et al., 1995).

The aim of this study was to investigate the structure–function relationships of synthetic galanin-(1–15)ol analogs and galanins of several species as they affect intestinal motility.

2. Materials and methods

2.1. Synthesis of peptides

Peptides were synthesized with the solid phase technique using an automatic peptide synthesizer (MilliGen/Biosearch 9600, UK) in our laboratory. Boc-alanino-succinyl-methyl-benzyl-hydrylamine-resin (1.5 g, Boc-alaninol content 0.81 mmol/g) was used for the synthesis of galanin-(1–15)ol analogs. An appropriate amount of Boc-amino acid (3-fold excess) was successively coupled to the peptide resin. After construction of the desired protected peptide resin, deprotection was carried out as described previously (Mochizuki et al., 1994b).

2.2. Purification of crude peptides

The crude peptides (100 mg each) thus obtained were purified by reverse phase high performance liquid chromatography (HPLC) on a YMC-Pack D-ODS-5 column (20×250 mm) in a linear gradient with a solvent system of 0.01 M HCl/CH_3CN over 30 min at a flow rate of 10 ml/min. The purity of each peptide was assessed according to a number of analytical criteria: analytical HPLC on a YMC-Pack R-ODS-5 column (4.6×250 mm) in 0.01 M HCl/CH_3CN over 30 min at a flow rate of 1.0 ml/min, amino acid analysis using an automatic amino acid analyzer (Beckman system 7300) after hydrolysis in 6 M HCl containing 1% (v/v) phenol and 1% (v/v) mercapto-

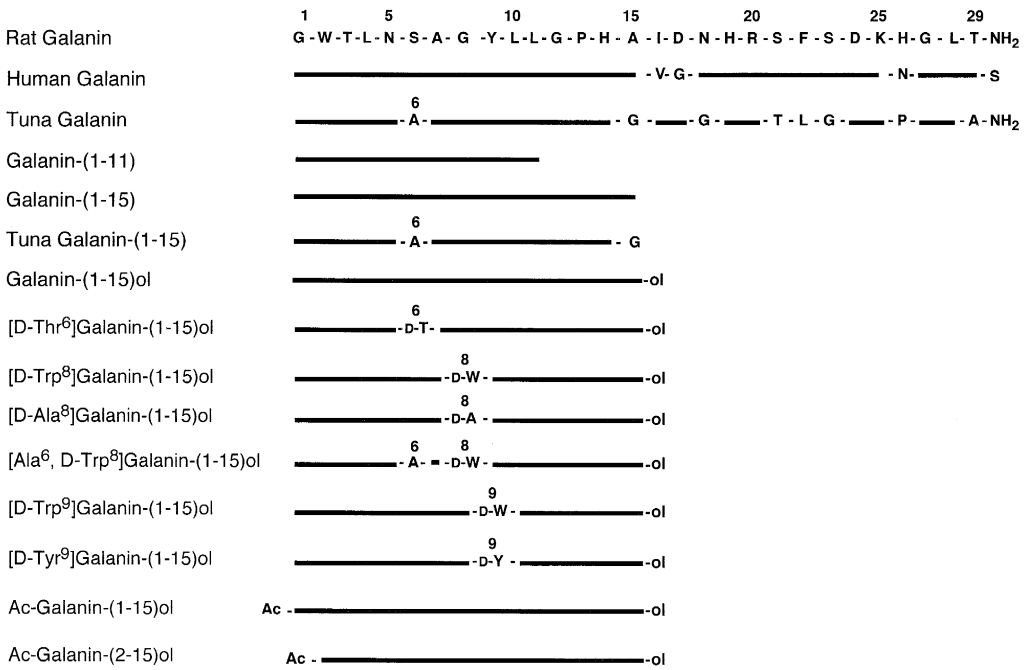

Fig. 1. Synthetic galanin-related peptides and galanin-(1–15)ol analogs. Bars indicate the same amino acid residues as those of rat galanin. Only substituted or modified residues are presented. Ac: N^{α}-acetylated. D at the position 6 through 9 means D-form.

ethanol at 110°C for 24 h, and FAB-MS. Peptides used in this study are listed in Fig. 1.

2.3. Tissue preparation and neurally evoked muscle contraction

Albino guinea-pigs (300–600 g) were used and were allowed food and water ad libitum prior to the experiment. L-shaped muscle strips were made for the simultaneous recording of longitudinal and circular muscle contractile activity in the guinea-pig ileum as described previously (Kuwahara et al., 1990). Briefly, a strip was cut first 2–4 mm wide and 10–15 mm long along, then similarly rectangular to, the oral-anal axis to make an L-shaped smooth muscle preparation. The preparation contained all elements of the gastrointestinal tract including enteric nervous system, muscle layers and mucosa. The part along the oral-anal axis was used for recording longitudinal contraction, and the other rectangular part for circular contraction. The strip was suspended horizontally in a chamber filled with modified Krebs-Ringer solution which was kept at 37°C and gassed with 95% O_2/5% CO_2. The modified Krebs-Ringer solution consisted of (mM): NaCl, 117; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; glucose, 11. The junctional corner of the muscle strip was fixed and the free ends were connected to force-displacement transducers (M.C. Commercial, Tokyo, Japan) for recording isotonic regulation of mechanical activity. Representative volley electrical stimulation (0.2 ms duration, 20–30 pulses at 10–20 Hz, 10–20 V strength) was used to evoke muscle contraction: one electrode was immersed in the solution bath, which was situated under the junction of the strip.

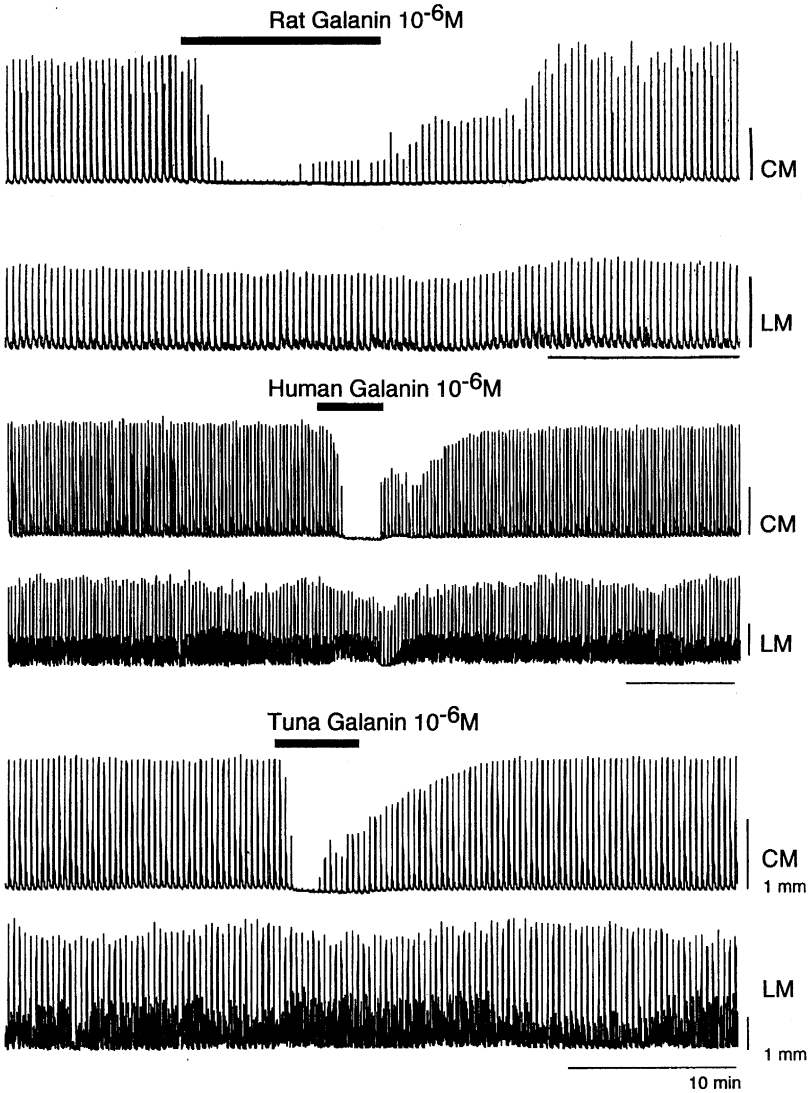

Fig. 2. Effect of rat, human and tuna galanin on neurally evoked muscle contractions in guinea-pig ileum. Repetitive volley stimulation (20 pulses at frequency of 10–20 Hz derived every 20–30 s with 0.2 ms duration and 15 V strength) evoked contractions in both muscle layers. 1 μM of galanins was added during the period indicated by the bars. Only circular muscle contractions were reduced by the three species of galanin. LM: longitudinal muscle; CM: circular muscle.

The voltage and duration of the pulses were chosen to stimulate the nerves but not the smooth muscle. Volleys were repeated every 20–30 s. All tissues were equilibrated for at least 40 min in warm oxygenated Krebs solution and perfused at 1.5 ml/min before the start of the experiments. Peptides were dissolved in saline immediately before use. While the peptides were being added to the chamber, the perfusion was clamped off for 5 min. Perfusion was then restarted and the buffer was washed out.

2.4. Data analysis

Percent contraction was calculated as a percentage of the maximal contraction induced by electrical stimulation. A dose–response curve for each peptide was made using 5–7 different animals, and the ED_{50} value was calculated from the analysis of dose–response plots using a computer program (Pharmacological Calculation System-Pharm/PCS, version 4.0). The results were expressed as the means ± S.E.M. These data were analyzed by one-way analysis of variance (ANOVA) and statistical significance was assessed with the Bonferroni test; probability values less than 5% were considered to be significant.

3. Results

3.1. Effect of human, rat and tuna galanin and their N-terminal fragments on neurally evoked muscle contractions in guinea-pig ileum

Repetitive volley stimulation evoked contraction in both muscle layers. The rat, human and tuna galanin inhibited the neurally evoked circular muscle contraction in a dose-dependent manner. Fig. 2 shows a representative response of neurally evoked muscle contractions in the presence of 1 μM peptide. The potencies of human, rat and tuna galanin were nearly identical on neurally evoked circular muscle contractions. Both mammalian galanin-(1–15) and tuna galanin-(1–15) significantly inhibited the neurally evoked circular muscle contractions. However, the inhibitory action of both mammalian galanin-(1–15) and tuna galanin-(1–15) was less potent when compared to that of native galanin. On the other hand, a shorter fragment, such as mammalian galanin-(1–11), did not show any inhibitory effect on neurally evoked circular muscle contractions. Table 1 summarizes the ED_{50} values and percent contractions of the peptides examined at 1 μM concentration on neurally evoked circular muscle contractions. No peptide tested affected the neurally evoked contractions of longitudinal muscle.

3.2. Effect of galanin-(1–15)ol analogs on neurally evoked muscle contractions in guinea-pig ileum

The ED_{50} values and percent contractions with galanin-(1–15)ol analogs at 1 μM for neurally evoked circular muscle contractions are listed in Table 2.

Table 1
Comparison of the potencies, % contraction for neurally evoked response in the presence of galanin-related peptides

Peptide	ED_{50} (nM)	% Contraction [a]
Rat galanin	11 ± 2	5 ± 4
Human galanin	15 ± 4	3 ± 1
Tuna galanin	34 ± 13	7 ± 2
Galanin-(1–11)	> 1000	89 ± 3
Galanin-(1–15)	133 ± 34	26 ± 5
Tuna galanin-(1–15)	204 ± 65	50 ± 8

Values are the means ± S.E.M. for 5–6 animals.
[a] % contraction in the presence of 1 μM peptides. Repetitive volley stimulation (20 pulses at a frequency of 10–20 Hz derived every 20–30 s with 0.2 ms duration and 15 V strength) evoked contractions in both circular and longitudinal muscle layers. The potencies of ED_{50} values were calculated from the appropriate dose–response curves (10^{-10} M–10^{-6} M).

Both [D-Trp[8]]galanin-(1–15)ol and galanin-(1–15)ol showed an inhibitory effect nearly identical to that of native galanin. [D-Ala[8]]Galanin-(1–15)ol had a weak effect as compared to that of galanin-(1–15)ol and [D-Trp[8]]galanin-(1–15)ol. [Ala[6],D-Trp[8]]Galanin-(1–15)ol, in which Gly was replaced by Ala at position 6, was six times less potent than [D-Trp[8]]galanin-(1–15)ol. N-terminal-modified analogs such as N^{α}-acetylated galanin-(2–15)ol were three times less potent than galanin-(1–15)ol. However, N^{α}-acetylated galanin-(1–15)ol was approximately eight times less potent than galanin-(1–15)ol. In contrast, [D-Thr[6]]galanin-(1–15)ol, [D-Trp[9]]galanin-(1–15)ol and [D-Tyr[9]]galanin-(1–15)ol did not cause any inhibition of neurally evoked circular muscle contractions, as shown in Fig. 3. The percent contraction induced by neural stimulation in the presence of 1 μM peptide was 98 ± 2% for [D-Thr[6]]galanin-(1–15)ol, 92 ± 5% for [D-Tyr[9]]galanin-(1–15)ol and 101 ± 2% for [D-Trp[9]]galanin-(1–15)ol, respectively. Moreover, these inactive analogs did not act as

Table 2
Comparison of the potencies, % contraction for neurally evoked response in the presence of galanin-(1–15)ol analogs

Peptide	ED_{50} (nM)	% Contraction [a]
Rat galanin	11 ± 2	5 ± 4
Galanin-(1–15)ol	31 ± 7	21 ± 5
[D-Trp[8]]Galanin-(1–15)ol	22 ± 5	17 ± 7
[Ala[6],D-Trp[8]]Galanin-(1–15)ol	124 ± 22	37 ± 8
[D-Ala[8]]Galanin-(1–15)ol	204 ± 58	46 ± 11
[D-Trp[9]]Galanin-(1–15)ol	> 1000	101 ± 2
[D-Tyr[9]]Galanin-(1–15)ol	> 1000	92 ± 5
[D-Thr[6]]Galanin-(1–15)ol	> 1000	98 ± 2
Ac-Galanin-(1–15)ol	253 ± 66	47 ± 6
Ac-Galanin-(1–15)ol	94 ± 34	25 ± 5

Ac: N^{α}-acetylated. Values are the means ± S.E.M. for 5–6 animals.
[a] % contraction in the presence of 1 μM peptides. Repetitive volley stimulation (20 pulses at a frequency of 10–20 Hz derived every 20–30 s with 0.2 ms duration and 15 V strength) evoked contractions in both circular and longitudinal muscle layers. The potencies of ED_{50} values were calculated from the appropriate dose–response curves (10^{-10} M–10^{-6} M).

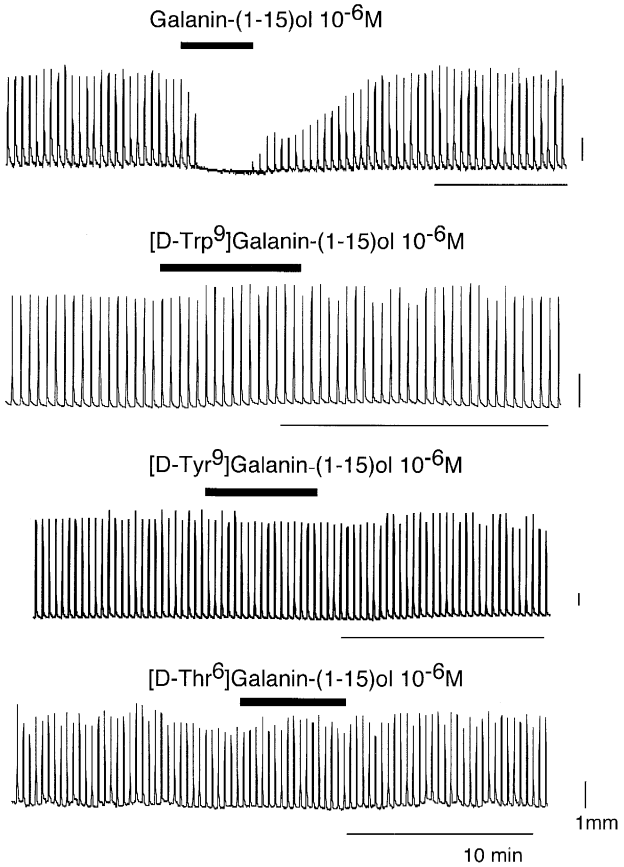

Fig. 3. Effect of galanin-(1–15)ol, [D-Trp^9]galanin-(1–15)ol, [D-Tyr^9]galanin-(1–15)ol and [D-Thr^6]galanin-(1–15)ol on neurally evoked circular muscle contractions in guinea-pig ileum. Repetitive volley stimulation (20 pulses at frequency of 10–20 Hz derived every 20–30 s with 0.2 ms duration and 15 V strength) evoked contractions in both circular and longitudinal muscle layers. 1 μM of peptides was added during the period indicated by the bars.

antagonists of galanin, as shown in Table 3. Also these galanin-(1–15)ol analogs did not affect the neurally evoked longitudinal muscle contractions even at the high concentration examined.

Table 3
% Contractions of neurally evoked response to galanin-(1–15)ol analogs in the presence of rat galanin

Peptide	% Contraction
Rat galanin 10^{-7} M	31 ± 8
[D-Thr^6]Galanin-(1–15)ol 10^{-6} M + rat galanin 10^{-7} M	32 ± 1
[D-Tyr^9]Galanin-(1–15)ol 10^{-6} M + rat galanin 10^{-7} M	23 ± 2
[D-Trp^9]Galanin-(1–15)ol 10^{-6} M + rat galanin 10^{-7} M	34 ± 12

Values are the means ± S.E.M. for 4 animals. Repetitive volley stimulation (20 pulses at a frequency of 10–20 Hz derived every 20–30 s with 0.2 ms duration and 15 V strength) evoked contractions in both circular and longitudinal muscle layers.

4. Discussion

In the present study, we synthesized nine galanin-(1–15)ol analogs including six D-amino acid residue analogs to explore the structure–function relationships of galanin-(1–15) as they affect neurally evoked muscle contractions in guinea-pig ileum in detail. We focused on the importance of the amino acid residue at positions 6, 8 and 9 and N-terminal Gly.

[D-Thr^6]Galanin-(1–15)ol, in which L-Ser was replaced by D-Thr, resulted in a remarkable reduction of the activity. Furthermore, the inhibitory activity of tuna galanin was equipotent to that of mammalian galanin on neurally evoked guinea-pig ileal contraction, although the amino acid sequence of tuna galanin at positions 6 and 15 differs from the 1–15 sequence of mammalian galanin. The two peptides in which Ser^6 was replaced by Ala^6, such as tuna galanin-(1–15) and [Ala^6,D-Trp^8]galanin-(1–15)ol, still retain significant inhibitory activity, although these peptides were less potent than mammalian form peptides such as mammalian galanin-(1–15) and [D-Trp^8]galanin-(1–15)ol on neurally evoked circular muscle contractions. These results confirmed that the replacement of L-Ser^6 by L-Ala^6 retained the inhibitory activity of galanin. Together, the results make it reasonable to speculate that the L-configuration of position 6 is important in the inhibitory activity of galanin.

We have previously reported that [D-Trp^8]galanin-(1–15)ol, in which Gly at position 8 was replaced by D-Trp, showed higher affinity than rat and human galanin in both rat hippocampal and human small cell lung carcinoma cell (SBC-3A) membranes, suggesting the importance of D-Trp at position 8 for receptor binding (Kakuyama et al., 1995). In the present study, the inhibitory effect of [D-Trp^8]galanin-(1–15)ol was shown to be equipotent to that of galanin-(1–15)ol. These two were the most potent of the synthetic galanin-(1–15)ol analogs. The results derived from structure–function studies might be useful for the development of specific galanin antagonists consisting of small-size peptides containing the 1–15 sequence of the active site.

Neither [D-Tyr^9]galanin-(1–15)ol nor [D-Trp^9]galanin-(1–15)ol had any effect on neurally evoked circular muscle contractions in guinea-pig ileum, showing results similar to the loss of binding in rat hippocampus and hypothalamus that resulted from replacement of L-Ala at position 9 (Fisone et al., 1991; Land et al., 1991). Thus, Tyr at position 9 seemed to be essential for the biological activity of galanin. Moreover, [D-Thr^6]galanin-(1–15)ol, [D-Tyr^9]galanin-(1–15)ol and [D-Trp^9]galanin-(1–15)ol, which were inactive in the present system, did not show any antagonistic activity against the inhibitory action of galanin, although [Ala^6,D-Trp^8]galanin-(1–15)ol or [D-Thr^6,D-Trp^{8,9}]galanin-(1–15)ol have shown some antagonistic activity against the inhibitory activity of galanin on insulin release (Yamabe et al., 1996). These results

again indicate clearly the importance of the residues at positions 6 and 9 for binding to the galanin receptor in the guinea-pig ileum.

In order to develop proteolytically stable peptides, N^{α}-acetylated galanin-(1–15)ol and N^{α}-acetylated galanin-(2–15)ol were synthesized. The results showed that N^{α}-acetylated galanin-(2–15)ol exhibits a potent inhibitory activity of nearly the same magnitude as that of mammalian galanin-(1–15), while N^{α}-acetylated galanin-(1–15)ol showed a lower inhibitory effect than did galanin-(1–15)ol in the present system. Similarly, N^{α}-acetylated porcine galanin-(2–29) has been shown to have the same potency as porcine galanin for inhibition of insulin release, while N^{α}-acetylated galanin showed little effect (Mochizuki et al., 1992). Thus, the extension with acetyl groups at the N-terminus of galanin was found to reduce biological activity significantly. This may be due to the conformational change of the intact galanin.

Comparison of the inhibitory activity of rat, human and tuna galanin revealed that these three galanins showed nearly identical potencies in the present system. However, tuna galanin has been demonstrated to cause no significant effects on glucose-induced insulin release from the isolated perfused rat pancreas and basal gastrin release from the isolated perfused rat stomach (Mochizuki et al., 1994a). These findings may have resulted from the different recognition sensitivity of these peptides among the neural and endocrine systems and/or different species.

In conclusion, the present results clearly indicated that (1) the L-configuration at position 6 of galanin seems to be important for retaining inhibitory activity, (2) the L-configuration of the Tyr^{9} residue of galanin may play a crucial role for biological activities, (3) the N^{α}-amino moiety in Gly^{1} is not important for the inhibitory activity of galanin.

The present data indicated that detailed structure–function studies using galanin analogs will be of great importance in elucidating receptor subtypes and their physiological functions.

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