Effect of Galanin(15-29)-related Peptides on Glucose-induced Insulin Release from the Isolated Perfused Rat Pancreas

FUMIYASU KAJIYAMA, SATOMI MUKAIYAMA, YUKO WATANABE, MASAKI OGATA, KAZUAKI IGUCHI, TOHRU MOCHIZUKI and MINORU HOSHINO

Laboratory of Bioorganic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

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ABSTRACT

In the present study, we examined the effects of the C-terminal fragments and analogues of rat and porcine galanin on 13.9 mM glucose-induced insulin release from the isolated perfused rat pancreas. The results revealed clearly that [His23, Tyr29] rat galanin (15-29) and [His23, Leu29] rat galanin (15-29) at 10⁻⁸ M as well as porcine galanin (15-29) exhibit insulinotropic activity on glucose-induced insulin release from the isolated perfused pancreas, whereas rat galanin (15-29) exhibited little effect on glucose-induced insulin release. The secondary structural analysis of the 15-29 sequence of rat and porcine galanins suggests that the conformation of the 15-29 sequences may be of importance for the insulinotropic activity of the C-terminal fragments.

Galanin has been known to cause a significant suppression of glucose-induced insulin release from the isolated perfused rat pancreas (20). Subsequent structure-function studies using synthetic porcine galanin fragments indicate that galanin (2-29) exhibited a complete loss of the inhibitory action, and further shortening of the peptide chain gave peptides having insulinotropic effects, and the maximum potency occurred at the 15-29 sequence (16). This is the first demonstration of a neuropeptide and its fragments that possess opposite effect in action on a single biological system such as insulin release.

In addition, the modified peptides, [D-Trp²]-porcine galanin and [Phe²]-porcine galanin, did not cause a significant change in the effect on glucose-induced insulin release, while [Ala²]-porcine galanin was found to exhibit a complete loss of the suppressing effect on insulin release, similar to that seen with shorter porcine galanin fragments lacking the N-terminal amino acid sequence.

Based on the secondary structures of the analogues and fragments as predicted by the Chou-Fasman method (3, 4), a high incidence of β-sheet structure in the amino terminal region of galanin was indicated to be essential for the inhibitory effect of galanin on glucose-induced insulin release. Both galanin (1-15)-OH and galanin (1-15)-ol showed little effect on glucose-induced
insulin release from the isolated perfused rat pancreas (16), although both N-terminal fragments showed a significant inhibitory effect on neurally evoked circular muscle contraction (12), suggesting that the active site of galanin for suppression of neurally-evoked circular muscle contractions resides in the amino-terminal half of the molecule.

To date, three types of cloned galanin receptors, GALR1 (7, 15), GALR2 (8, 9, 21) and GALR3 (19, 22), have been reported and showed expression on hippocampas. In the present study, we have synthesized various fragments and analogues in the C-terminal portions of rat and porcine galanin and the effect of these synthetic peptides were examined on glucose-induced insulin release from the isolated perfused rat pancreas. In addition, we performed receptor binding assay for these synthetic peptides on rat hippocampal membrane.

MATERIAL AND METHODS

Materials. Organic solvent such as DCM, DMF used for the peptide synthesis were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). DIEA and TFA were purchased from Peptide Institute, Inc. (Osaka, Japan). Boc-Ala, Boc-Gly, Boc-Ile, Boc-Phe and Boc-Val were purchased from Eiw Weiss Chemical Co. (Yokohama, Japan). Boc-Asn, Boc-Arg(Tos), Boc-Asp(OcHex), Boc-His(Bom), Boc-Leu, Boc-Lys(2-Cl-Z), Boc-Ser (Bzl), Boc-Thr(Bzl), Boc-Tyr(Cl,Bzl) and Boc-Val were purchased from Peptide Institute, Inc. p-Methylbenzhydrylamine resin (0.49 meq NH₂/g) was purchased from Peptide Institute, Inc. BOP reagent was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan).

Peptide Synthesis. Rat and porcine galanin(15-29), four C-terminal fragments of rat galanin, and nine analogues of rat galanin(15-29) were synthesized manually by solid-phase technology according to the method previously described (16). These peptides were rat galanin(15-29), rat galanin(16-29), rat galanin(21-29), rat galanin(23-29), rat galanin(25-29), [His²³]-rat galanin(15-29), [Tyr²⁶]-rat galanin(15-29), [Ala²⁹]-rat galanin(15-29), [His²³, Tyr²⁶]-rat galanin(15-29), [Tyr²⁶, Ala²⁹]-rat galanin(15-29), [His²³, Ala²⁹]-rat galanin(15-29), [His²³, Val²⁹]-rat galanin(15-29), [His²³, Leu²⁹]-rat galanin(15-29) and [His²³, f-Leu²⁹]-rat galanin(15-29), as shown in Fig. 1.

In case of [Tyr²⁶, Ala²⁹]-rat galanin(15-29), a starting material was t-butyloxy-carbonyl (Boc)-Ala linked to p-methylbenzhydrylamine-polystyrene-1% divinyl-benzene copolymer resin (Boc-Ala-NH-resin, 1% cross linked 100-200 mesh, 1.5 g, -NH₂ 0.74 mmol). Two molar excess of Boc amino acids were activated in the presence of benzotriazol-1-yl-oxytris(dimethylamino) phosphonium hexafluorophosphate (BOP) (2 eq. mol excess) and diisopropylethylamine (DIEA) (2 mol excess) (13). The coupling was performed for elongation of these peptide chain on a Boc-Ala-NH-resin (0.5 mmol amide content). After the final coupling of N-terminal amino acid, the protected peptide-resin was treated with liquid HF (10 mL) in the presence of anisol (1 mL) at 0°

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<th>15</th>
<th>23</th>
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<td>rat galanin(15-29)</td>
<td>Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-Ser-Asp-Lys-His-Gly-Leu-Thr *</td>
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<td>porcine galanin(15-29)</td>
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<tr>
<td>[His²³]-rat galanin(15-29)</td>
<td>- - - - - - - His - - - - Thr *</td>
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<td>[Tyr²⁶]-rat galanin(15-29)</td>
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<td>[Ala²⁹]-rat galanin(15-29)</td>
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<td>[His²³, Tyr²⁶]-rat galanin(15-29)</td>
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Fig. 1 Porcine and rat galanin(15-29) related peptides. Dashes denote the same amino acid residues. * represents amide.
C for 60 min. After removal of HF under reduced pressure, the residual products were washed with ethyl acetate (3×10 mL) and extracted with 3 M AcOH (3×10 mL). The extracts were lyophilized and crude peptide was purified by reverse-phase HPLC on YMC-Pack R-ODS-5 column (4.6×25 cm) (YMC Inc., Kyoto, Japan) using linear gradient of CH₃CN from 0 to 60% in 0.01 N HCl over 30 min. Purity of the product was assessed by analytical HPLC with linear gradient of CH₃CN from 20 to 40 in 0.01 N HCl (30 min), amino acid analysis of acid hydrolysates (Hitachi model 835) and FAB-MS spectrometer (JMX-SX100, JEOL, Tokyo).

Pancreatic perfusion. Male Wistar rats weighing 250-300 g that were fasted overnight with free access to water, were anesthetized with pentobarbital (50 mg/kg). Perfusion of the isolated rat pancreas was done according to a slight modification of Grodsky and Fanska (6, 11). Briefly, the inlet for the vascular perfusion was the celiac artery, and the outlet was the portal vein. Other vessels were ligated. The pancreas was perfused with a modified Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.4) containing 0.2% (w/v) bovine serum albumin, 5 mM glucose and 4% (w/v) Dextran T-70 (Meito Sangyo, Nagoya, Japan). It was gassed with a 95% O₂-5% CO₂ mixture at 37°C. The flow rate was maintained at 1.9 mL/min and the pancreas was preperfused for 30 min. After this preperfusion, sampling was started. After the initial basal period (10 min), the perfusate with or without (controls) galanin-related peptides (10⁻⁸ M final) was then infused for 30 min at the rate of 0.1 mL/min. Following the infusion for 10 min, the perfusate was changed to a 5 to 13.9 mM glucose concentration for 20 min. Total period of 13.9 mM glucose stimulation was 20 min. After 10 min, portal effluents were collected every 1 min into chilled glass tubes and stored at −20°C until assayed for insulin content.

Insulin assay. Insulin release in the perfusate was measured by radioimmunoassay (RIA) using a rat insulin (Novo Research Institute, Copenhagen, Denmark) as standard. ¹²⁵I-porcine insulin and anti-porcine insulin serum GP-2 (final dilution 1:200,000). Porcine insulin (SIGMA) was radioiodinated by the chloramine T method (10) and separated from free iodine by gel filtration (Sephadex G-10) using 1 M AcOH as an eluent. Standard diluent for RIA was 0.01 M phosphate buffer (pH 7.4) containing 0.14 M NaCl, 25 mM EDTA and 0.5% (w/v) BSA. Free and bound ¹²⁵I-porcine insulin were separated by adding normal guinea-pig serum (1:300, 0.1 mL), goat anti-guinea-pig γ-globulin serum (1:40, 0.1 mL) and 10% (w/v) polyethylene glycol 6,000 (0.5 mL). After the centrifugation at 3,000 rpm for 30 min at 4°C, the supernatant was aspirated and the precipitate was counted in a gamma-counter. The detection limit was 40 pg/tube.

Statistical Analysis. For each experiment, the insulin release was graphed and integrated during the 13.9 mM glucose stimulation. Results presented are means ± SEM. Statistical analysis was done with the Student's t-test. A probability level of 0.05 or less was taken as statistically significant.

Receptor Assay in Rat Hippocampal Membrane. Radioreceptor assay was performed by the method according to Lindskog et al. (14). Rat hippocampal membrane fraction was incubated with peptide and ¹²⁵I-rat galanin for 60 min at 4°C in 50 mM Tris-HCl buffer (pH 7.4) containing 0.05%(w/v) BSA. The incubation was terminated by adding 1 mL ice cold incubation buffer. The mixture was then filtered on a Whatman glass filter GF/B (Maidstone, U.K.) which was previously soaked with 0.1%(w/v) polystyrenemine (SIGMA Chemicals, St.Louis, MO, U.S.A.). The mixture was filtered and measured the radioactivity using in a gamma-counter. Specific binding was calculated as the difference between ¹²⁵I-rat galanin binding in the absence (total binding) and the presence (non-specific binding) of 10⁻⁸ M rat galanin.

RESULTS

Effect of galanin-related peptides on insulin secretion.

Rat Gal(15–29) showed little, if any, effect on the 13.9 mM glucose-induced insulin release in the isolated and perfused rat pancreas perfusion, although porcine Gal(15–29) enhanced the first, second, and entire phase of the glucose-induced IR-Ins release (8). [His³⁵, Ala²⁹] -rat Gal(15-29), [Ala²⁹]-rat Gal(15-29) and [His³⁵, Leu²⁹]-rat Gal(15-29) potentiated the first, second, and entire phase in the same preparation. The potentiating effects of the three synthetic peptides were significant (423.15 ng IR-Ins; P < 0.05,
384.72 ng IR-Ins; \( P < 0.05 \), 356.73 ng IR-Ins; \( P < 0.005 \), respectively). Total insulin releases of these analogues were 1.5–1.7-fold greater than that of the control (242.62 ng IR-Ins). On the other hands, little, if any, potentiation of the glucose-induced insulin release were detected when the preparations were incubated with the analogues displaced with Thr, Val and t-Leu at a position of 29; [Thr\(^{29}\)]-porcine Gal(15–29), [Ser\(^{31}\), Thr\(^{29}\)]-porcine Gal(15–29), [His\(^{26}\), Thr\(^{29}\)]-porcine Gal(15–29), [His\(^{26}\), Val\(^{29}\)]-porcine Gal(15–29) and [His\(^{26}\), t-Leu\(^{29}\)]-porcine Gal(15–29) which correspond to [His\(^{23}\), Tyr\(^{26}\)]-rat Gal(15–29), [Tyr\(^{26}\)]-rat Gal(15–29), [His\(^{23}\)]-rat Gal(15–29), [His\(^{23}\), Val\(^{29}\)]-rat Gal(15–29) and [His\(^{23}\), t-Leu\(^{29}\)]-rat Gal(15–29) (Fig. 2). Fig. 3 shows the dose-dependent insulintropic action of porcine Gal(15–29), [Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Ala\(^{29}\)]-rat Gal(15–29) and [His\(^{23}\), Leu\(^{29}\)]-rat Gal(15–29).

![Fig. 3 Total IR-Insulin release by 13.9 mM glucose in the presence of 10\(^{-4}\)–10\(^{-7}\) M rat and porcine galanin (15–29) analogues. Results are expressed as mean ± SEM (n = 5–6).](image)

**Binding affinity of galanin related peptides with the rat hippocampal membranes.**

Fig. 4 shows the displacement curves of galanin related peptides estimated in the membrane prepared from rat hippocampal. There was no displacement of membrane binding by any of the galanin related peptides; rat gal(15–29), porcine gal(15–29), rat gal(15–29), [His\(^{23}\)]-rat gal(15–29), [Tyr\(^{26}\)]-rat gal(15–29), [Ala\(^{29}\)]-rat gal(15–29), [His\(^{23}\), Tyr\(^{26}\)]-rat gal(15–29), [Tyr\(^{26}\), Ala\(^{29}\)]-rat gal(15–29), [His\(^{23}\), Val\(^{29}\)]-rat gal(15–29), [His\(^{23}\), t-Leu\(^{29}\)]-rat gal(15–29), and [His\(^{23}\), t-Leu\(^{29}\)]-rat gal(15–29). In this preparation rat galanin showed different binding, IC\(_{50}\) 0.9 ± 0.1 nM.

**DISCUSSION**

It is well-established that porcine galanin causes significant suppression of glucose-induced insulin release in the isolated perfused rat pancreas. In sharp contrast to this suppressing effect, the potentiating effects of C-terminal fragments of porcine galanin was demonstrated on the glucose-induced insulin release in the same preparations. Among the C-terminal fragments examined, Gal(15–29) showed the maximum potentiating
Fig. 4 Displacement curves of peptides related to galanin(15–29) estimated in the membrane prepared from rat hippocampus. ●, Rat galanin; ■, including all data obtained with folwging peptide; rat Gal(15–29), porcine Gal(15–29), [His\(^{23}\)]-rat Gal(15–29), [Tyr\(^{26}\)]-rat Gal(15–29), [Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Tyr\(^{26}\)]-rat Gal(15–29), [Tyr\(^{26}\), Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Leu\(^{29}\)]-rat Gal(15–29), and [His\(^{23}\), t-Leu\(^{29}\)]-rat Gal(15–29); △, [His\(^{23}\), Val\(^{29}\)]-rat Gal(15–29).

effect (16). We have further studied the potentiating effects of synthetic five fragments and 10 analogues of Gal(15–29). In the present study, structure-function relations in the C-terminal 15–29 sequence of porcine and rat galanin were investigated using synthetic peptides related to porcine and rat Gal(15–29).

Porcine Gal(15–29) showed insulinotropic activity on glucose-induced insulin release from the isolated perfused rat pancreas. The present study provided substantial evidence, for the first time, that rat Gal(15–29) has no effect on the glucose-induced insulin release. The primarily structure of rat Gal(15–29) is different from porcine Gal(15–29) only at the three residues; 23, 26 and 29, which may certainly be responsible for the controversial effect. Accordingly, we synthesized six analogues with different amino acid at these three residues; [His\(^{23}\)]-rat Gal(15–29), [Tyr\(^{26}\)]-rat Gal(15–29), [Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Tyr\(^{29}\)]-rat Gal(15–29) and [Tyr\(^{26}\), Ala\(^{29}\)]-rat Gal(15–29). Using these synthetic peptides, we examined the effects of peptides on glucose-induced insulin release. Effect enhancing the glucose-induced insulin release was obtained when the analogues substituted Thr in position 29 by Ala; [Ala\(^{29}\)]-rat Gal(15–29), [Tyr\(^{26}\), Ala\(^{29}\)]-rat Gal(15–29) and [His\(^{33}\), Ala\(^{29}\)]-rat Gal(15–29), [His\(^{23}\), Tyr\(^{26}\)]-rat Gal(15–29) and [His\(^{23}\), Tyr\(^{26}\)]-rat Gal(15–29), but, caused the complete loss of insulinotropic activity. These results led us to conclude that His in position 26 of rat Gal(15–29) and Ala in position 29 of porcine Gal(15–29) may play an essential role in the insulinotropic activity of Gal and its analogues.

Other analogues, such as [His\(^{23}\), Val\(^{29}\)]-rat Gal(15–29) and [His\(^{23}\), t-Leu\(^{29}\)]-rat Gal(15–29) did not show any effect on the glucose-induced insulin release. [His\(^{23}\), Leu\(^{29}\)]-rat Gal(15–29) exhibited marked insulinotropic activity in the present study.

It may be worth to mention that a chimeric galanin analogue, galpan was stimulated insulin release in the isolated perfused rat islets in a recent report (17). The insulinotropic activity of the analogue, however, was confirmed at as high as 10\(^{-5}\) M. An explanation for the high dose stimulation may be due to lower sensitivity of the preparation they used. In the isolated and vascularly perfused pancreas, we showed significant potentiating effect of the rat galalin analogues, such as [Ala\(^{29}\)]-rat galalin(15–29), [His\(^{23}\), Ala\(^{29}\)]-rat galalin(15–29) and [His\(^{23}\), Leu\(^{29}\)]-rat galalin(15–29) at a concentration as low as 10\(^{-6}\) M. The present study revealed that the synthetic analogues may provide useful clues to further analysis of the inslinotropic mechanism.

Binding of fragments and synthetic analogues of galanin with the galanin receptors was further analyzed in the membrane prepared from rat hippocampus. Result obtained showed that rat galalin(15–29), porcine galalin(15–29) and rat galalin(15–29) analogues had no detectable binding activity even at a high concentration, 10\(^{-6}\) M. Previous studies showed that porcine galalin(3–29) had little binding affinity for rat GALR1, GALR2 (21) and GALR3 (19) which showed on an expression on rat hippocampus (1, 5, 19). On the present and previous results, it has been concluded that binding affinity of porcine galalin(3–29) was lost binding affinity to cloned rat GALRs when the first two N-terminal residues were deleted. N-terminal residues of galanin may play important role in binding with rat cloned GALRs. These results harmonize with a view that rat galalin, porcine galalin(15–29) and rat galalin(15–29) analogues had no binding affinity for rat GALRs. The present study showed further that [Ala\(^{29}\)]-rat galalin(15–29),
[His2, Ala29]-rat galanin (15–29) and [His2, Leu29]-rat galanin (15–29) caused the strong potentiation of the glucose-induced insulin release, suggesting existence of either GALRs diversity or differences in rat pancreas.

Prediction according to the Chou-Fasman method indicated that synthetic rat Gal (15–29) analogues with His in position 23 and Ala in position 29 had an incidence of α-helical structure. The α-helical structure may be a structural basis for the strong insulinotropic activity of analogues possessing His in position 23 and Ala in position 29, suggesting that α-helical structure may play an important role in recognizing putative GALR in rat pancreas.

Results obtained in the previous studies carried out on the cloned galanin receptor may hardly be harmonized with the result obtained by analyzing the structure-activity relation. Following results have been reported: (a) Although porcine Gal and porcine Gal (1–10) caused a concentration-dependent decrease in the resting tension of internal anal sphincter and an augmentation of the percent decrease in the resting tension with electrical field stimulation, porcine Gal (15–29) produced an increase in the resting tension of the internal anal sphincter and had no effect on the electrical field stimulation-induced decrease in the resting tension (2); (b) Rat Gal (3–29) stimulated prolactin releasing activity when compared to rat Gal from dispersed anterior pituitary cells (23). These results may have substantial basis if studies on receptor cloning as well as analysis of selective agonist and antagonist receptors can be developed in future.

In conclusion, it is important of [Ala29]-rat galanin (15–29), [His23, Ala29]-rat galanin (15–29) and [His23, Val29]-rat galanin (15–29) for providing tools to study insinotropic action.

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