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NEW GALANIN(1-15) ANALOGUES MODIFIED IN POSITIONS 9, 10 AND 11 ACT AS GALANIN ANTAGONISTS ON GLUCOSE-INDUCED INSULIN SECRETION

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Galanin (GAL) is a 29-amino-acid residue peptide originally isolated from porcine upper small intestine. GAL exhibits various physiological activities, such as effects on hormones release, smooth muscles contractions, gastric acid secretion, neurons degeneration and feeding. One of the biological actions of GAL is the inhibition of insulin secretion from the pancreatic β-cells. In our studies we have designed several new 15-amino-acid-residue galanin fragment analogues modified in positions: 6, 8, 9, 10, 11 and tested for their effects on glucose-induced insulin secretion from isolated rat pancreatic islets of Langerhans. *In vitro* insulin secretion was studied during static incubation. All peptides were tested at two concentrations: 0.1 μM and 1 μM. Among the analogues derived from GAL(1-15)NH_2 peptide: [Phe^9]GAL(1-15)NH_2 and [Pro^{11}]GAL(1-15)NH_2 were found to be the potent antagonists against the inhibitory effect of GAL on glucose-induced insulin secretion from the isolated rat pancreas. These analogues block the GAL-mediated inhibition of insulin secretion. The present studies have shown that analogues: [Phe^9]GAL(1-15)NH_2 and [Pro^{11}]GAL(1-15)NH_2 may be a key compounds for developing a more potent GAL antagonists.

Key words: *galanin, galanin antagonists, insulin secretion*

INTRODUCTION

Galanin (GAL) is a 29-amino-acid-residue neuropeptide, originally isolated from porcine intestine in 1983 (1 - 6). Its name derives from the first and last amino acids in its porcine sequence, glycine and alanine. Nowadays GAL was isolated from a variety of species. The first 15 N-terminal amino acids in GAL sequence are highly conserved across species. Only the human species of GAL contains 30 amino acids and lacks C-terminal amidation. This neuroendocrine peptide is widely distributed and has several biological functions in the endocrine system as well as in the central and peripheral nervous systems (1, 6). It coexists with several neurotransmitters: acetylocholine, serotonin, dopamine, calcitonin-gene-related peptide, vasoactive intestinal peptide and with many others in different parts of gastrointestinal system.

As one of the most recently discovered neuropeptides, GAL shows many interesting physiological and behavioral actions, which are mediated through its interactions with distinct G-protein-coupled membrane receptor subtypes (GALR1, GALR2 and GALR3), subsequently activating several signalling pathways (1, 4, 6 - 9). Exogenously administered GAL produces several major biological actions, including inhibitory effects on memory and learning, inhibition of acetylcholine and glutamate release, stimulation of feeding and pituitary hormone release, inhibition of insulin release and spinal nociceptive reflexes (1, 4, 6, 10 - 14). Expression of GAL is regulated by steroids. GAL is dramatically overexpressed after neuronal injury and in Alzheimer's disease.

One of the most interesting effect of GAL on the endocrine pancreas is the inhibition of insulin secretion (1 - 3, 5, 8, 10, 11, 15 - 26). *In vitro* and *in vivo* studies have shown that GAL (porcine) inhibits insulin secretion in pigs (19), dogs (20), perfused rat pancreas (16, 17, 21, 22), mouse islets (23), rat insulinoma cells (3, 10, 19) and human islets (5, 15). GAL can regulate insulin secretion by direct inhibition of the L-type Ca^{2+} channels (18, 24) and the activation of the K_{ATP} channels (18, 25), important in the control of the β-cell membrane potential and the influx of Ca^{2+}. The other possible mechanism is the inhibition of adenylate cyclase, leading to the reduction of intracellular Ca^{2+} concentration (2, 18). Moreover the mechanism of insulin secretion inhibition, that may be exerted by the inhibition of exocytosis at a very late stage in the stimulus-secretion coupling is also suggested (18, 26).

The aim of our present studies is researching effects of new GAL analogues on insulin secretion. We have chosen the 15-amino-acid-residue N-terminal fragment of GAL because this fragment is conserved across species and is recognized by its own receptors. In our studies we have designed and synthesized several GAL analogues modified in positions: 6, 8, 9, 10 and 11 respectively (*Table 1*). In next step we characterized the biological properties of GAL analogues, investigating their action on glucose-induced insulin secretion from rat isolated islets of Langerhans. We expected, that such modifications may provide more information

Table 1. The primary structures of the synthesized peptides.

Peptide	Amino Acid Sequence
GAL (porcine)	GWTLNSAGYLLGPHAIDNHRSSFHDKYGLA-NH_2
GAL(1-15)NH_2	GWTLNSAGYLLGPHA-NH_2
[D-Ser^6]GAL(1-15)NH_2	GWTLN-***D-Ser***-AGYLLGPHA-NH_2
[D-Ser^6,D-Trp^8]GAL(1-15)NH_2	GWTLN-***D-Ser***-A-***D-Trp***-YLLGPHA-NH_2
[Sar^8]GAL(1-15)NH_2	GWTLNSA-***Sar***-YLLGPHA-NH_2
[Phe^9]GAL(1-15)NH_2	GWTLNSAG-***Phe***-LLGPHA-NH_2
[Tyr(PO_3H_2)^9]GAL(1-15)NH_2	GWTLNSAG-***Tyr(PO_3H_2)***-LLGPHA-NH_2
[Trp^9]GAL(1-15)NH_2	GWTLNSAG-***Trp***-LLGPHA-NH_2
[D-Leu^{10}]GAL(1-15)NH_2	GWTLNSAGY-***D-Leu***-LGPHA-NH_2
[Val^{10}]GAL(1-15)NH_2	GWTLNSAGY-***Val***-LGPHA-NH_2
[Nva^{10}]GAL(1-15)NH_2	GWTLNSAGY-***Nva***-LGPHA-NH_2
[Ile^{11}]GAL(1-15)NH_2	GWTLNSAGYL-***Ile***-GPHA-NH_2
[Pro^{11}]GAL(1-15)NH_2	GWTLNSAGYL-***Pro***-GPHA-NH_2
[Nle^{11}]GAL(1-15)NH_2	GWTLNSAGYL-***Nle***-GPHA-NH_2

GAL – galanin; Nle – norleucine (L-2-aminocaproic acid); Nva – norvaline (L-2-aminovaleric acid); Sar – sarcosine (*N*-methylglycine); Tyr(PO_3H_2) – L-phosphotyrosine;

about molecular mechanisms of peptide-receptor interaction. Research may also lead to finding high-affinity antagonist of GAL receptors.

MATERIALS AND METHODS

Synthesis of peptides

All peptides (*Table 1*) were synthesized by the solid phase peptide synthesis with the use of a Labortec AG model SP 650 peptide synthesizer and 9-fluorenylmethoxycarbonyl (Fmoc) strategy (27). TentaGel S RAM resin for peptide amides (capacity 0.25 mmol/g) was used as the starting material. All amino acids were coupled as active derivatives in a 3-fold molar excess with the use of the *O*-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate with addition of *N*-hydroxybenzotriazole (1:1) coupling method. Deprotection of the Fmoc group was carried out with 20% piperidine in *N*,*N*-dimethylformamide. After synthesis had been completed, the peptides were cleaved from resin with trifluoroacetic acid/phenol/triisopropylsilane/water (88:5:2:5 v/v/v/v) mixture. Crude peptides thus obtained were purified by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) on preparative Vydac C18 column (32×240 mm, 15 - 20 μm particle size) with several linear gradients of acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA). Eluates were fractioned and analyzed by the analytical RP-HPLC. Purity of the peptides (greater than 98%) was checked by an analytical Beckman „System Gold" chromatograph with a Beckman C18 column (4.6×250 mm, 5 μm particle size) with several linear gradients of ACN in 0.1% TFA. Identities of peptides were confirmed by amino acid analysis and MALDI-TOF mass spectrometry.

Animals and tissue preparation

All procedures were designed in accordance with the generally accepted ethical standards for animal experimentation and accepted by the Local Ethical Committee of the Medical University of Gdańsk. Male Wistar rats (weighting 180 - 220g) were kept under standard laboratory conditions (a

natural light-dark cycle) with unrestricted access to food (Labofeed B, Kcynia, Poland) and tap water. Forty eight animals were used in all experiments. Pancreatic rat islets of Langerhans were isolated by collagenase digestion using an intraductal injection technique (28). After the decapitation, a midline laparotomy was performed and 7 - 10 ml of the collagenase type XI solution (1mg/ml) was injected into the duct system of the pancreas. The inflated pancreas was removed from the animal and placed in a water bath at 37°C. Digestion of the pancreas was completed after 10 min and the tissue was rinsed three times with ice-cold Krebs buffer to remove exocrine cells and collagenase. The composition of Krebs buffer (pH 7.38) was (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24, bovine serum albumin (0.1%). The islets of comparable size were hand picked under a stereomicroscope and preincubated in Krebs buffer containing 15 mM of glucose. The buffer was bubbled with carbogen (O_2/CO_2 95/5). Selected islets were allowed to equilibrate for 60 min and then were rinsed with Krebs buffer. Next, they were divided into groups of 3, and each was transferred to separate test tube containing Krebs buffer and placed in a water bath at 37°C.

Determination of insulin secretion

In vitro insulin secretion from the isolated rat pancreatic islets was studied during static incubation. All peptides were tested at two concentrations: 0.1 μM and 1 μM. Each test tube contained Krebs buffer supplemented with glucose at a concentration of 10 mM (control) or 10 mM of glucose with the addition of one peptide tested. The antagonistic/agonistic activities of GAL analogues were checked by the simultaneous application of GAL and one of the galanin analogues, used at the same concentration. The isolated islets were incubated for 60 min in a water bath at 37°C and in the atmosphere of carbogen. After 1h, samples were collected and frozen at –20°C for subsequent analysis of insulin. The concentration of insulin in the incubation medium was determined radioimmunologically (29) using anti-rat insulin antibody and ^{125}I-labelled rat insulin (DRG, USA).

Statistical analysis of the data

Values of insulin level [ng/60min/islet] induced by peptides in the presence of 10 mM glucose are presented as means±SEM (standard error of mean). Student's *t*-test and one-way ANOVA were used to test the degree of significance. Two tailed P values of less than 0,05 were interpreted as indicating a significant difference.

RESULTS

Porcine GAL, GAL(1-15)NH_2 and its twelve new analogues modified in positions 6, 8, 9, 10 and 11 were tested for their effect on glucose-induced insulin secretion from rat pancreatic islets of Langerhans. Our studies have shown that GAL and its analogues modulate glucose-induced insulin release from rat pancreatic islets in a concentration-dependent manner. Porcine GAL and its fragment GAL(1-15)NH_2 at all concentration tested inhibit glucose-induced insulin secretion. However the N-terminal fragment of GAL inhibited insulin secretion with lower potency in comparison to native GAL.

We have shown that the substitution of Ser^6 in the N-terminal part of GAL(1-15) fragment with its stereoisomer, D-Ser, results in increase of the affinity towards GAL receptors. Analogue [D-Ser^6]GAL(1-15)NH_2 lowered the glucose-induced insulin level by about 63% at a concentration of 0.1 μM and by about 61%

at a concentration of 1 μM. It also significantly increased the inhibitory action of GAL even repeatedly at both concentrations used. The simultaneous substitution of Ser^6 with D-Ser and Gly^8 with D-Trp in GAL(1-15) structure also caused a decrease in the insulin secretion by about 40% and 47% (at a lower and higher concentration, respectively). The simultaneous application of analogue [D-Ser^6,D-Trp^8]GAL(1-15)NH_2 and GAL (at the same concentrations) caused an increase in the inhibitory action of GAL, similar to that for analogue [D-Ser^6]GAL(1-15)NH_2. In this case we observed repeated increase in inhibitory activity of GAL. The substitution of Gly^8 in GAL(1-15) structure with its *N*-methylated form, sarcosine, also caused an increase in the affinity towards GAL receptors. The analogue [Sar^8]GAL(1-15)NH_2 showed the inhibitory activity on glucose-induced insulin secretion as it lowered the insulin level by about 21% and 39% (at a lower and higher concentration, respectively). It also significantly increased the inhibitory action of GAL. Similarly like two mentioned above analogues, it extended repeatedly inhibitory action of GAL at both concentrations used.

We showed that the substitution of Tyr^9 with Phe caused a substantial change in the galanin effect on glucose-induced insulin secretion. Analogue

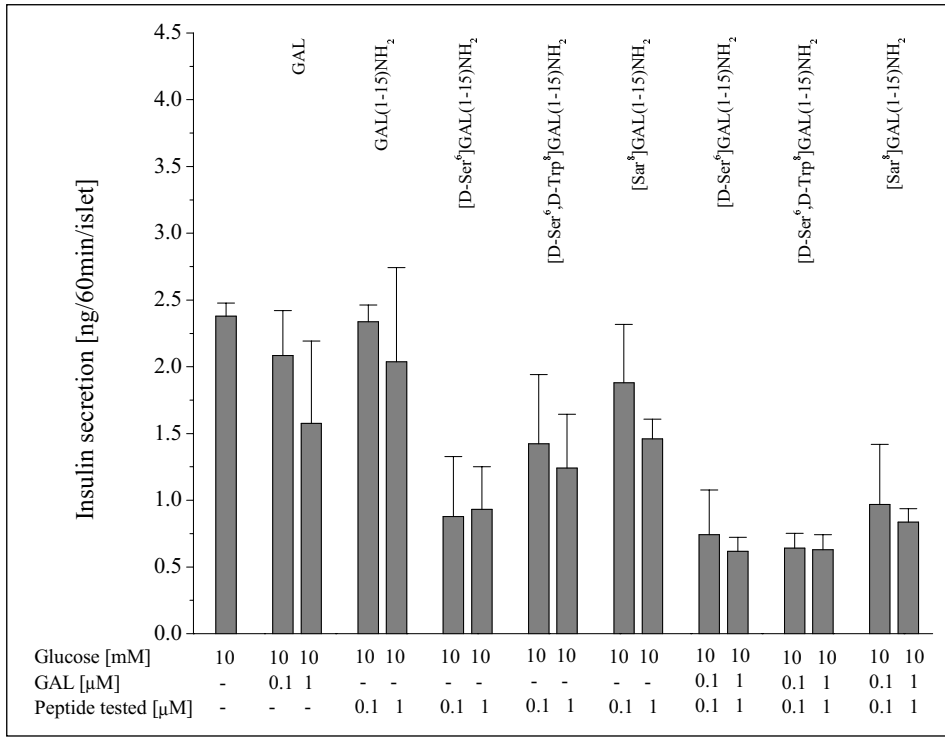

Figure1. Effects of GAL(1-15) analogues modified in positions 6 and 8 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM glucose.

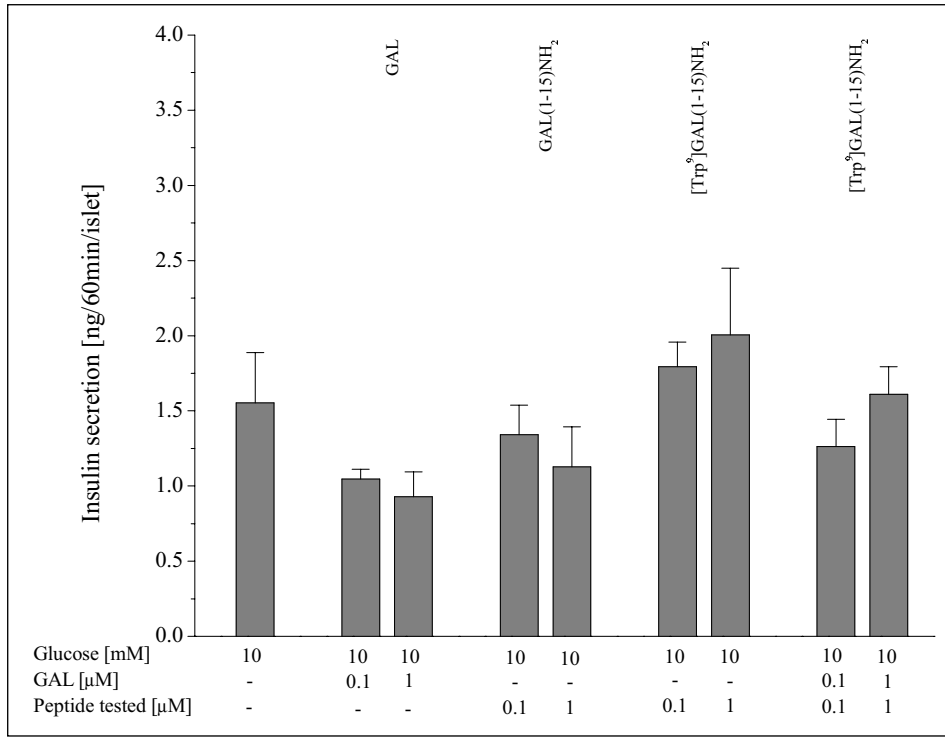

Figure 2. Effect of GAL1-15) analogue modified in position 9 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM glucose.

[Phe^9]GAL(1-15)NH_2 showed insulinotropic activity as compared to GAL or GAL(1-15)NH_2. It caused an increase in the insulin secretion by about 66% and 48% at a concentration of 1 μM and 0.1 μM, respectively. Our studies have shown that it completely abolished inhibitory effect of GAL (when simultaneously used with GAL at the same concentrations) and still showed the insulinotropic activity. However the replacement of Tyr^9 in GAL(1-15) structure with its phosphorylated form, analogue [Tyr(PO_3H_2)^9]GAL(1-15)NH_2, affects the glucose-induced insulin secretion only at a higher concentration. It caused an increase in the insulin level by about 12%. This peptide used at a both concentrations tested abolished inhibitory action of GAL, although its effect was stronger at higher concentration. Also the analogue [Trp^9]GAL(1-15)NH_2, in which Tyr^9 was replaced with Trp, another aromatic residue, showed insulinotropic activity as compared to GAL or GAL(1-15)NH_2. It caused an increase in the insulin secretion by about 15% and 29% (at a lower and higher concentration, respectively). This peptide, used at a concentration of 0.1 μM, also reduced inhibitory effect of GAL by about 14%, but at a concentration of 1 μM completely abolished the inhibitory action of GAL.

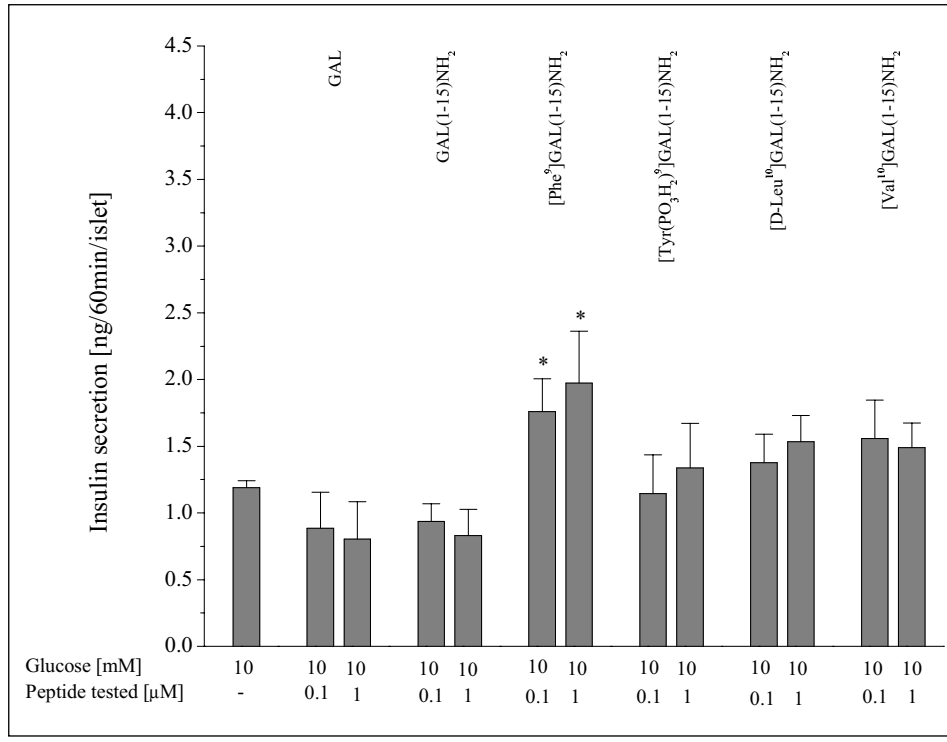

Figure 3. Effects of GAL(1-15) analogues modified in positions 9 and 10 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM glucose (*P<0.05 *vs*. GAL).

The insulinotropic activity also showed the analogue GAL(1-15) in which Leu[10] was replaced with its D-stereoisomer. Analogue [D-Leu[10]]GAL(1-15)NH_2 used at a concentration of 1 μM caused an increase in the insulin secretion by about 29% and by about 16% at a concentration of 0.1 μM. Moreover this peptide used at a concentration of 0.1 μM reduced the inhibitory effect of GAL and at higher concentration slightly stimulated secretion of insulin by about 14%. The substitution of Leu[10] with its homologue, Val, gave in consequence the peptide which stimulated the insulin secretion in the tests on glucose-induced insulin secretion from isolated rat pancreatic islets of Langerhans. Analogue [Val[10]]GAL(1-15)NH_2 increased the insulin level by about 25% and 31% (at a concentration of 0.1 μM and 1 μM, respectively). Simultaneously this peptide abolished the inhibitory action of GAL and slightly increased the insulin level by about 17% and 13% (at a lower and higher concentration, respectively). Whereas the analogue, in which Leu[10] was substituted with Nva have partly shown the insulinotropic activities, only at a concentration of 0.1 μM. Analogue [Nva[10]]GAL(1-15)NH_2 completely abolished the inhibitory action of GAL at both concentrations used.

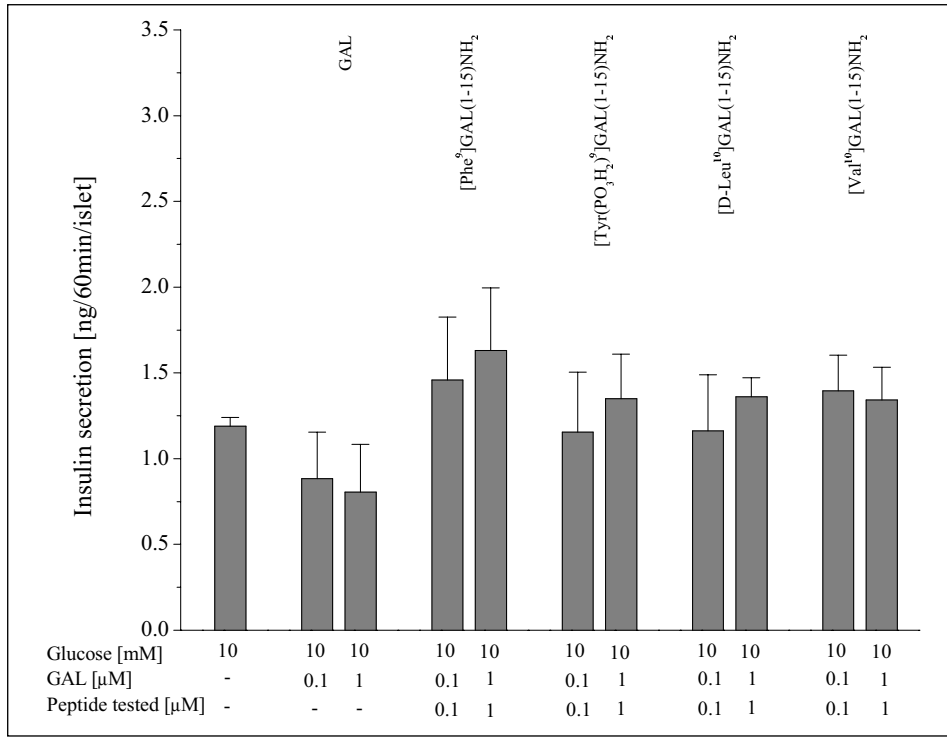

Figure 4. Effects of GAL(1-15) analogues modified in positions 9 and 10 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM glucose in the presence of appropriate concentration of GAL.

The lack of inhibition of insulin secretion was observed in the case of analogue in which Leu[11] was replaced with Pro. This peptide increased insulin secretion by about 21% (at lower concentration) and 26% (at higher concentration). Moreover the analogue [Pro[11]]GAL(1-15)NH_2 showed strong antagonistic activity. At the both concentrations used, it completely abolished the inhibitory action of GAL. Two another analogues, in which Leu[11] was substituted with its structural isomer Ile or Nle, caused a decrease in the insulin secretion by about 19% (at a concentration of 0.1 μM) or 26% (at a concentration of 1 μM) and by about 14% (at a concentration of 0.1 μM) and 20% (at a concentration of 1 μM), respectively. Whereas analogues: [Ile[11]]GAL(1-15)NH_2 and [Nle[11]]GAL(1-15)NH_2 only slightly reduced the GAL activity with a lower potency than [Pro[11]]GAL(1-15)NH_2.

DISCUSSION

Galanin is a 29/30 amino-acid-residue neuropeptide which is implicated in the treatment of Alzheimer's disease, depression, feeding disorders, prevention of

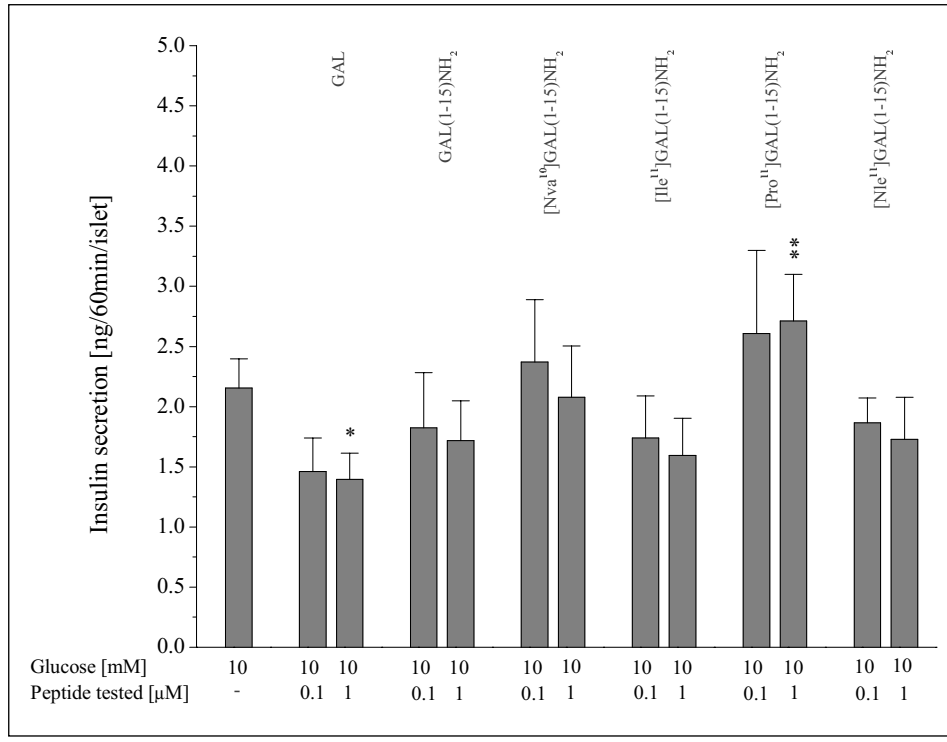

Figure 5. Effects of GAL(1-15) analogues modified in positions 10 and 11 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM glucose (*P<0.05 *vs*. 10 mM glucose, **P<0.05 *vs*. GAL).

ischaemic damage and the treatment of chronic pain (1, 4, 6, 12 - 14, 30 - 37). The N-terminus of GAL is highly conserved between different species, and the first 15 amino acids were found to be sufficient for agonistic receptor binding. Full-length GAL has a high cross-reactivity towards receptors from other species with similar affinity, although the agonistic effect may not be preserved (4, 6 - 10, 13). It has been shown that the one of major action of GAL is inhibition of insulin secretion (1 - 3, 5, 6, 15 - 26). GAL decrease adenylate cyclase activity and intracellular cAMP production via a pertussis-toxinsensitive pathway, involving an inhibitory GTP-binding protein (2, 18). In addition, GAL was reported to activate ATP-sensitive potassium channels in β-cells (18, 25).

Nowadays numerous GAL fragments, chimeras and analogues with point mutation were synthesized and their activities on the insulin secretion were studied (3, 10, 11, 17, 21, 22, 30 - 33, 38 - 45). Structure-activity studies have shown that the activity of GAL is connected with the N-terminal 1-15 fragment, needed for full activation of GAL receptors and the inhibitory effect of GAL on insulin secretion. The study with the use of GAL analogues indicate that Gly^1 and

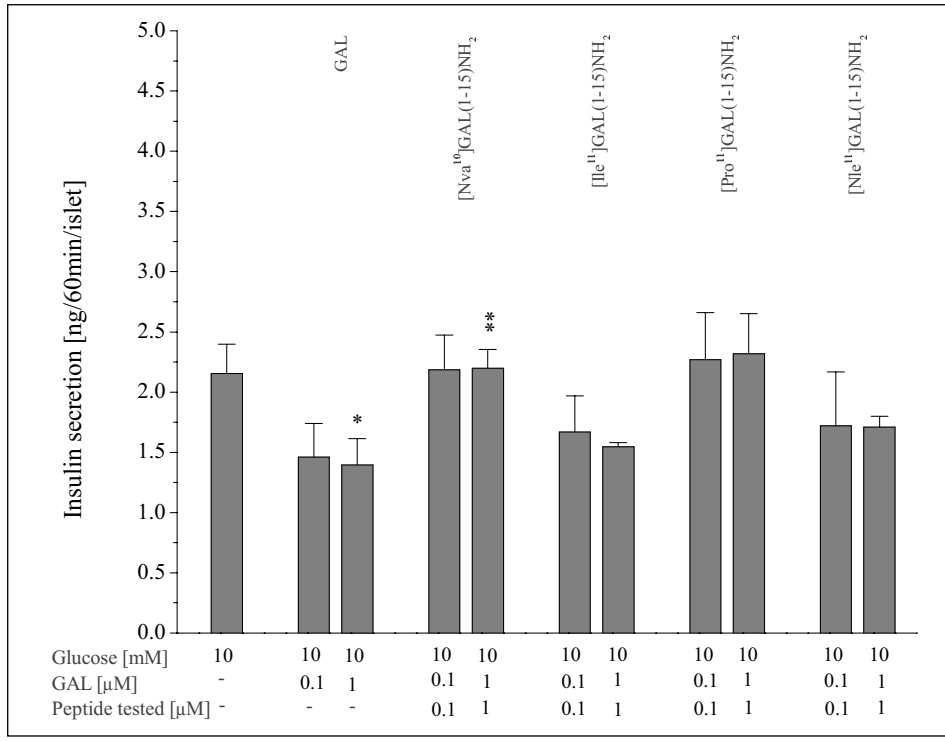

Figure 6. Effects of GAL(1-15) analogues modified in positions 10 and 11 on insulin secretion from the rat pancreatic islets of Langerhans induced by 10 mM in the presence of appropriate concentration of GAL (*P<0.05 *vs*. 10 mM glucose, **P<0.05 *vs*. GAL).

Trp^2 in GAL structure are of great importance in the binding to GAL receptors (7 - 9). It is known that GAL(2 - 29) fragment have substantially lower affinity to GAL receptors than does full-length GAL molecule. Trp^2 residue is the most important pharmacophore in GAL structure and it is though to interact with a pair of histidines placed in trans-membrane domain VI of the GALR1 receptor (7). All attempts to modify or replace the indole structure of Trp have resulted in analogues with very low or lack of affinity. The important pharmacophores are also Asn^5, Tyr^9, Leu^{10} and Leu^{11} (13, 39).

In our studies we have designed twelve new GAL analogues modified in positions: 6, 8, 9, 10, 11 and studied their action on glucose-induced insulin secretion. Our observations confirmed that GAL and its N-terminal fragment GAL(1-15)NH_2 are full GAL receptor agonists in the isolated rat pancreatic islets of Langerhans. They lowered glucose-induced insulin secretion, but fragment GAL(1-15)NH_2 was about 30% less potent inhibitor than GAL. It suggests that the C-terminal fragment GAL 16-29 is required for full activations of GAL receptors in the rat pancreatic islets.

However studies with the use of the GAL(1-15)NH_2 analogues have shown diverse activities of such modified peptides as compared to GAL or its non-modified N-terminal fragment 1-15. Analogues: [D-Ser^6]GAL(1-15)NH_2, [D-Ser^6,D-Trp^8]GAL(1-15)NH_2 and [Sar^8]GAL(1-15)NH_2 have been found to be very strong GAL receptor agonists. They lowered the insulin level in the tests on glucose-induced insulin secretion and significantly increased the inhibitory effect of GAL. Two other analogues: [Nle^{11}]GAL(1-15)NH_2 and [Ile^{11}]GAL(1-15)NH_2 also showed the agonistic activities, but they were less active than mentioned above three peptides.

More significant changes in the biological activity of GAL(1-15)NH_2 resulted from modifications of the amino acid sequence of GAL, in which Tyr^9 was replaced by Tyr(PO_3H_2) or Trp and Leu^{10} was substituted with D-Leu, Val or Nva. Such modified GAL analogues used at a concentration of 1 μM, were able to stimulate the insulin secretion from rat pancreatic islets with the following order of insulinotropic potency: [Trp^9]GAL(1-15)NH_2 ≈ [D-Leu^{10}]GAL(1-15)NH_2 > [Val^{10}]GAL(1-15)NH_2 > [Tyr(PO_3H_2)^9]GAL(1-15)NH_2 > [Nva^{10}]GAL(1-15)NH_2. However the analogue [Nva^{10}]GAL(1-15)NH_2 showed stronger insulinotropic activity when used at a lower concentration than at a higher concentration. Nevertheless, all mentioned above peptides did not significantly affect the insulin secretion.

We have shown that the strongest antagonistic properties owned two GAL analogues: [Phe^9]GAL(1-15)NH_2 and [Pro^{11}]GAL(1-15)NH_2. They stimulated glucose-induced insulin secretion and antagonized the inhibitory effect of GAL in a concentration-dependent manner. Relatively the strongest antagonistic activities showed the analogue [Phe^9]GAL(1-15)NH_2 used at a concentration of 1 μM. Our observations showed that the modification of the amino acid sequence of GAL(1-15)NH_2, in which Tyr^9 was replaced with aromatic ring of Phe is the most advantageous for its antagonistic properties. We suggest that aromatic phenyl, not hydroxyl, group is involved in the interaction with GAL receptors.

In our studies we showed that residues in positions: Ser^6, Gly^8, Tyr^9, Leu^{10} and Leu^{11} within GAL structure may play an important role in the interaction of GAL with its receptors presented in rat pancreas. The modifications of GAL structure in positions 6 and/or 8 resulted in increase of the affinity towards GAL receptors and led to finding analogues with stronger agonistic activities than non-modified GAL(1-15) fragment. However the modifications of GAL structure in positions 9, 10 or 11 gave in consequence peptides which showed insulinotropic activities and antagonized the inhibitory action of GAL in a concentration-dependent manner. We expect that these findings provide more information about molecular mechanisms of galanin-receptor interaction and may be a useful tool for developing a new more potent GAL receptor antagonists or agonist in pancreas.

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