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EFFECTS OF PORCINE GALANIN, GALANIN(1-15)NH_2 AND ITS NEW ANALOGUES ON GLUCOSE-INDUCED INSULIN SECRETION

Jarosław Ruczyński[a,#], Magdalena Cybal[b], Czesław Wójcikowski[b], Piotr Rekowski[a]

[a]Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, PL 80-952, Gdańsk, Poland,
[b]Department of Endocrinology, Institute of Obstetrics and Gynaecology, Medical University of Gdańsk, Kliniczna 1a, PL 80-402 Gdańsk, Poland

Effects of porcine galanin, galanin(1-15)NH_2 and its new analogues on glucose-induced insulin secretion. J. RUCZYŃSKI, M. CYBAL, C. WÓJCIKOWSKI, P. REKOWSKI. Pol. J. Pharmocol., 2002, 54, 133–141.

Porcine galanin (pGAL), its 15-amino-acid-residue fragment and five new analogues modified in positions 4, 6 or 14 were tested for their effects on glucose-induced insulin secretion from isolated rat pancreatic islets of Langerhans. *In vitr*o insulin secretion was studied during static incubation. All peptides were tested at two concentrations: 100 nM and 1 μM. The insulin level in the presence of 10 mM of glucose was a reference for all experiments. Our studies have shown that porcine galanin and its fragment GAL(1-15)NH_2 at all concentrations tested inhibit glucose-induced insulin secretion. However, the modifications of the amino acid sequence of galanin caused changes in the interaction of GAL with its receptors, consequently yielding peptides that showed reverse activity as compared to pGAL or GAL(1-15)NH_2. Finally, we have found three analogues: [Cit^{14}]GAL(1-15)-NH_2, [Hse^6]GAL(1-15)NH_2 and [Cle^4]GAL(1-15)NH_2, which were able to stimulate glucose-induced insulin secretion and also antagonized inhibitory effect of pGAL. Other two galanin analogues: [D-Leu^4]GAL(1-15)NH_2 and [des-Leu^4]GAL(1-15)NH_2 showed a rather weak agonistic activity. Our observations suggest that positions: 4, 6 and 14 in the amino acid sequence of galanin may play an important role in the high-affinity binding of GAL to its receptors and biological action in perfused rat pancreas.

Key words: *galanin, galanin analogues, insulin secretion, rat pancreatic islets*

[#] *correspondence*; e-mail: jarecki@chemik.chem.univ.gda.pl

Abbreviations: The symbols of the amino acids, peptides and their derivatives are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [Eur. J. Biochem., 1984, 138, 9]. Other symbols: ACN – *acetonitrile, ATP – adenosine triphosphate,* Cit – *L-citrulline (L-2-amino-5-ureidovaleric acid),* Cle – *cycloleucine (1-amino-1-cyclopentane carboxylic acid),* DIPEA – *N,N-diisopropylethylamine,* DMF – *N,N-dimethylformamide,* ESI-MS – *Electrospray Ionization Mass Spectrometry,* FAB-MS – *Fast Atom Bombardment Mass Spectrometry,* Fmoc – *9-fluorenylmethoxycarbonyl,* GAL – *galanin,* HOBt – *N-hydroxybenzotriazole,* Hse – *L-homoserine (L-2-amino-4-hydroxybutanoic acid),* K_{ATP} chanel – *ATP-sensitive K^+ chanel,* NMP – *N-methylpyrrolidone,* pGAL – *porcine galanin,* RP HPLC – *reverse phase high performance liquid chromatography,* TBTU – *O-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate,* TFA – *trifluoroacetic acid,* TIPS – *triisopropylsilane.*

INTRODUCTION

Galanin (GAL) is a 29-amino-acid C-terminally amidated neuroendocrine peptide, for the first time isolated in 1983 by Tatemoto et al. [38]. Neuropeptide GAL is widely distributed in the central and peripheral nervous system of several vertebrate species, including man [3–5, 7]. It was found in the spinal cord and in the brain: hypothalamus, locus coeruleus and hippocampus. In the peripheral nervous system, GAL occurs in the respiratory tract, gastrointestinal tract and urogenital tract. In all amino acid sequences of GAL, isolated from a variety of species, except for trout, the first 15 N-terminal residues are highly conserved. However, the human form of GAL contains 30 amino acids and lacks C-terminal amidation. GAL displays many types of interesting physiological and behavioral activities, which are mediated through its interaction with distinct G-protein-coupled membrane receptor subtypes (GALR1, GALR2, GALR3), subsequently activating several signaling pathways [7]. It shows an ability to modulate pituitary hormone release and insulin secretion, affects memory, learning, feeding, pain threshold control and sexual behavior [3–5, 7, 17]. In the gastrointestinal tract, GAL can both stimulate and inhibit smooth muscle activity in a concentration-dependent, species-specific and location-specific manner [9–11, 18, 37].

The most interesting and the best recognized effect of GAL on the endocrine pancreas is the inhibition of insulin secretion. This biological action of GAL may play an important role in pathological states and clinical therapeutics. *In vitro* and *in vivo* studies have shown that porcine galanin (pGAL) inhibits insulin secretion in pigs [25], dogs [26], perfused rat pancreas [22], mouse islets [23] and rat insulinoma cells [2, 12, 25]. However, in humans, glucose-stimulated insulin secretion appears not to be inhibited by the infusion of pGAL [13]. pGAL can inhibit glucose-stimulated insulin secretion from human islets *in vitro* [8], but only at concentrations higher than those achieved during intravenous infusion of pGAL in humans. These observations suggest that the human form of GAL may interact more strongly with GAL receptors on human β-cells and perhaps inhibit human insulin secretion with greater relative efficacy [27]. Moreover, the studies have shown that GAL regulates insulin secretion by different mechanisms. These include direct inhibition of the L-type Ca^{2+} channels [14, 34] and the activation of the K_{ATP} channels [34, 36], 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 [1, 34]. Furthermore, the latest studies have shown the growing acceptance for 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 [34, 35].

In present studies, we have designed five new GAL(1-15)NH_2 analogues modified in positions: 4, 6 or 14 and tested their activities on glucose-induced insulin secretion from isolated rat pancreatic islets of Langerhans (Table 1 shows the primary structures of the synthesized peptides). The aim of this work was to compare the direct effects of all synthesized peptides on glucose-induced insulin secretion and to check the antagonistic/agonistic activities of GAL analogues. Thus, we intended to characterize the molecular domains of GAL responsible for its inhibitory effect in rat pancreatic islets. The obtained data may be useful in designing new GAL analogues acting as specific GAL receptor antagonist in the pancreas.

Table 1. The primary structures of the synthesized peptides

PEPTIDE	AMINO ACID SEQUENCE
pGAL	G-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-I-D-N-H-R-S-F-H-D-K-Y-G-L-A-*NH_2*
GAL(1-15)NH_2	G-W-T-L-N-S-A-G-Y-L-L-G-P-H-A-*NH_2*
[Cit^{14}]GAL(1-15)NH_2	G-W-T-L-N-S-A-G-Y-L-L-G-P-***Cit***-A-*NH_2*
[Hse^{6}]GAL(1-15)NH_2	G-W-T-L-N-***Hse***-A-G-Y-L-L-G-P-H-A-*NH_2*
[D-Leu^{4}]GAL(1-15)NH_2	G-W-T-***D-Leu***-N-S-A-G-Y-L-L-G-P-H-A-*NH_2*
[Cle^{4}]GAL(1-15)NH_2	G-W-T-***Cle***-N-S-A-G-Y-L-L-G-P-H-A-*NH_2*
[des-Leu^{4}]GAL(1-15)NH_2	G-W-T- ~ -N-S-A-G-Y-L-L-G-P-H-A-*NH_2*

The symbols of the amino acids, peptides and their derivatives are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [Eur. J. Biochem., 1984, 138, 9]. Other symbols: Cit – L-citrulline (L-2-amino-5--ureidovaleric acid), Cle – cycloleucine (1-amino-1-cyclopentane carboxylic acid), Hse – L-homoserine (L-2-amino-4-hydroxybutanoic acid), ~ – removed amino acid residue, GAL – galanin, pGAL – porcine galanin

MATERIALS and METHODS

Animals and tissue preparation

All procedures were designed in accordance with the generally accepted ethical standards for animal experimentation and accepted by the Local Ethics Committee of the Medical University of Gdańsk. Male Wistar rats (weighting 180–220 g) were kept under standard laboratory conditions (a natural light-dark cycle) with unrestricted access to food (Labofeed B, Kcynia, Poland) and tap water. Sixteen animals were used in all experiments. Pancreatic rat islets of Langerhans (about 280 islets from each pancreas) were isolated by collagenase digestion using an intraductal injection technique [19]. Pilocarpine (30 mg/kg) was intraperitoneally administered to animals 60 min before the experiment. Then, non-fasted animals were sacrificed by decapitation. After the decapitation, a midline laparotomy was performed and 7–10 ml of the collagenase type XI solution (1 mg/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 96/4). 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.

Substances

Collagenase type XI, Krebs buffer, bovine serum albumin, pilocarpine hydrochloride, TBTU, HOBt, DIPEA, DMF, NMP, TFA, TIPS, ACN, diethyl ether, acetic acid and phenol were obtained from Sigma-Aldrich Co. (USA). All amino acid derivatives were purchased from Bachem AG (Switzerland). Peptides (Tab. 1) were synthesized by the solid phase peptide synthesis with the use of a Labortec AG model SP 650 peptide synthesizer and Fmoc strategy [6]. A base-labile 9-fluorenylmethoxycarbonyl group (Fmoc) was used to protect the α-amino function of the amino acids. TentaGel S RAM resin (Rapp Polymere, Germany) for peptide amides (capacity 0.25 mmol/g) was used as the starting material. All amino acids were coupled as active derivatives with the use of a standard coupling protocol for all peptide syntheses. The TBTU/HOBt/DIPEA (1:1:2) in DMF:NMP (1:1 v/v) solution coupling method was used in a 3-fold molar excess. Deprotection of the Fmoc group was carried out with 20% piperidine in DMF. After synthesis had been completed, the peptides were cleaved from resin with TFA/phenol/TIPS/H_2O (88:5:2:5 v/v/v/v) mixture for 2 h. Then, solutions were filtered and peptides were precipitated with

Table 2. Some physicochemical properties of the synthesized peptides

Peptide	Molecular formula	Molecular mass		RP HPLC R_t [min]
		Calculated	Found [M+H]	
pGAL	$C_{146}H_{213}N_{43}O_{40}$	3210.6	3212.4	15.95
GAL(1-15)NH_2	$C_{72}H_{106}N_{20}O_{19}$	1555.8	1556.5	12.62
[Cit^{14}]GAL(1-15)NH_2	$C_{72}H_{110}N_{20}O_{20}$	1575.8	1576.2	13.30
[Hse^{6}]GAL(1-15)NH_2	$C_{73}H_{108}N_{20}O_{19}$	1569.5	1570.2	13.97
[D-Leu^{4}]GAL(1-15)NH_2	$C_{72}H_{106}N_{20}O_{19}$	1555.8	1556.3	12.85
[Cle^{4}]GAL(1-15)NH_2	$C_{72}H_{104}N_{20}O_{19}$	1553.8	1554.6	13.22
[des-Leu^{4}]GAL(1-15)NH_2	$C_{66}H_{95}N_{19}O_{18}$	1442.6	1443.2	11.13

All peptides were characterized by the analytical RP HPLC with linear gradient 20–60% of ACN for 30 min. Trp, Cit, Hse, and Cle were not determined by amino acid analysis

cold diethyl ether. Precipitated peptides were dissolved in water or 25% acetic acid and lyophilized to obtain crude peptides. Crude peptides thus obtained were purified by reverse phase HPLC on preparative Vydac C-18 column (32 × 240 mm, 15–20 μm particle size) with several isocratic systems and linear gradients of ACN in 0.1% TFA. The absorbance of column eluates was monitored at 226 nm (LKB UVICORD SII detector). Subsequently, fractioned eluates were analyzed by the analytical reverse phase HPLC and the homogeneous fractions (purity greater than 98%) were combined and lyophilized. Purity of the peptides were checked by an analytical Beckman "System Gold" chromatograph with a Vydac C-18 column (4.6 × 250 mm, 5 μm particle size) with several isocratic systems and linear gradients of ACN in 0.1% TFA, flow rate was 1 ml/min, absorbance at 226 nm. Identities of the peptides were confirmed by amino acid analysis (Beckman model 121 M amino acid analyzer) and mass spectrometry: FAB-MS (VG Mass Lab Trio-3) or ESI-MS (Finnigan MAT 95 S). For details see Ref. [33]. Table 2 shows some physicochemical properties of the synthesized peptides.

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: 100 nM and 1 μM. Each test tube contained Krebs buffer supplemented with glucose at a concentration of 3, 10 and 20 mM (as 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 pGAL and one of the analogues, used at the same concentration. The isolated islets were incubated for 60 min in a water bath at 37°C and an atmosphere of carbogen. After 1 h, samples of the incubation medium were collected and immediately frozen at –20°C for subsequent analysis of insulin. The concentration of insulin in the incubation medium was determined radioimmunologically [30] using anti-rat insulin antibody and ^{125}I-labelled rat insulin (DRG, USA).

Statistical analysis of the acquired data

Results are expressed as absolute values of insulin level [ng/60 min/island] induced by peptides in the presence of 10 mM glucose. Values were presented as means of all experiments ± SEM (standard error of mean). Student's *t*-test and one-way ANOVA were used to test the degree of significance.

RESULTS

pGAL, GAL(1-15)NH_2 and its five new analogues modified in positions 4, 6 or 14 were tested for their effect on glucose-induced insulin secretion from rat pancreatic islets of Langerhans. The insulin level in the presence of 10 mM of glucose was a reference for all experiments. Our studies have shown that pGAL and its analogues modulate glucose-induced insulin release from rat pancreatic islets in a concentration-dependent manner. Figures 1 and 2 show effects of the peptides on insulin secretion induced by 10 mM glucose. pGAL, its frag-

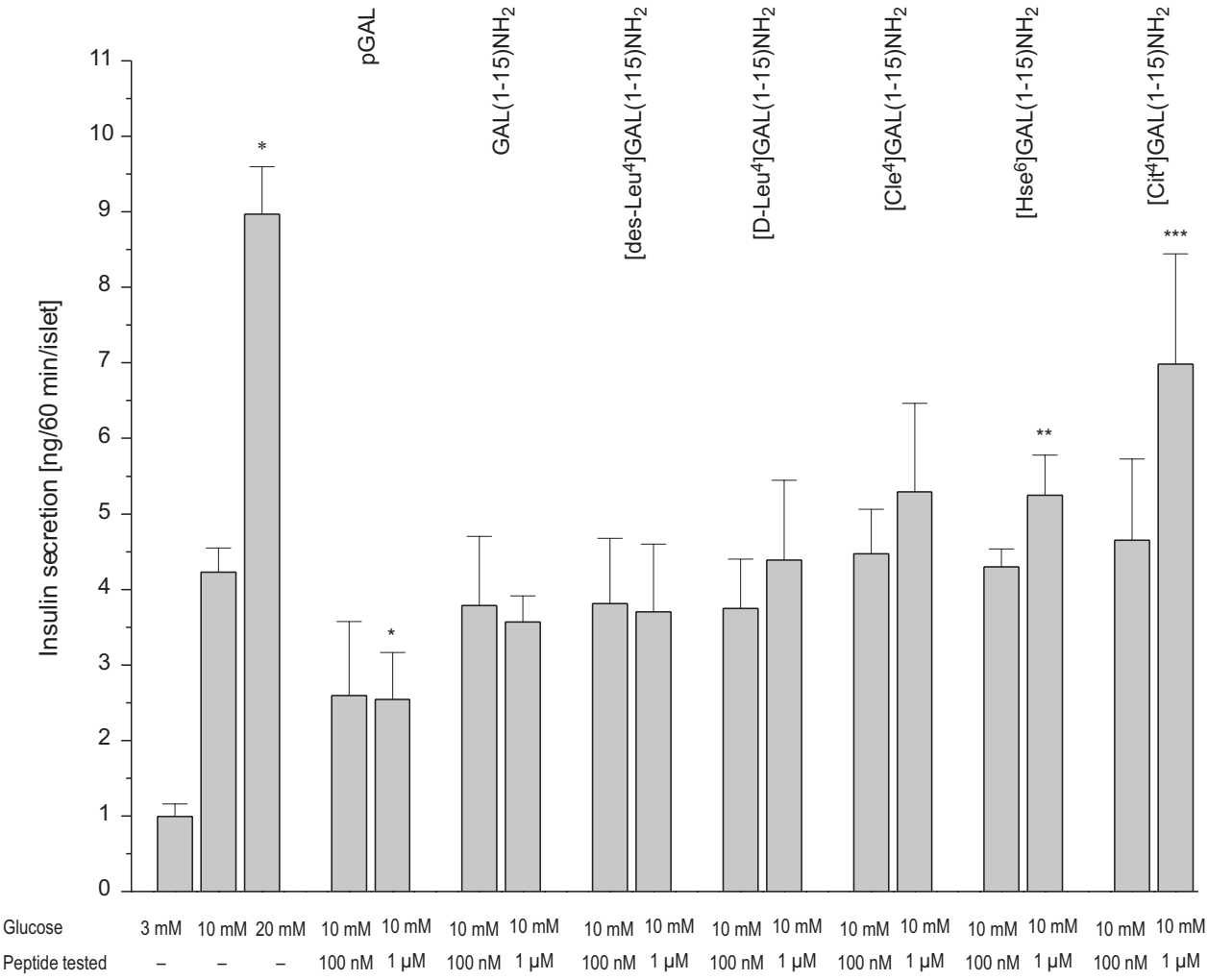

Fig. 1. Effects of peptides on insulin secretion from the isolated rat pancreatic islets induced by 10 mM glucose. Results are presented as means ± SEM of insulin level induced by the tested peptide. The insulin level in the presence of 10 mM of glucose is a reference for all experiments. Asterisks indicate the probability level of random difference *vs*. 10 mM of glucose (* $p < 0.01$), 1 µM of pGAL (** $p < 0.01$), 10 mM of glucose and 1 µM of pGAL (*** $p < 0.01$)

ment GAL(1-15)NH_2 and analogue [des-Leu^4]-GAL(1-15)NH_2 lowered the glucose-induced insulin level. pGAL used at a concentration of 1 µM caused a decrease in the insulin secretion by about 40% (2.54 ng/60 min/islet) and a concentration of 100 nM lowered it by about 38% (2.59 ng/60 min/islet) as compared to the control tissue incubated with 10 mM of glucose. We have also shown that N-terminal fragment of pGAL inhibited insulin secretion with lower potency in comparison to native pGAL. This 15-amino acid fragment of pGAL lowered the insulin level by about 16% (3.56 ng/60 min/islet) at a concentration of 1 µM and by about 11% (3.78 ng/60 min/islet) at a concentration of 100 nM. However, the simultaneous application of pGAL and GAL(1-15)NH_2, used at the same concentration, caused an increase in the inhibitory action of pGAL by about 30 % (at a lower concentration) and 37% (at a higher concentration).

The analogue [des-Leu^4]GAL(1-15)NH_2 also shown the inhibitory activity on glucose-induced insulin secretion as it lowered the insulin level by about 10% (3.81 ng/60 min/islet) at a concentration of 100 nM and about 12% (3.71 ng/60 min/islet) at a concentration of 1 µM. Moreover, the analogue [des-Leu^4]GAL(1-15)NH_2 increased the inhibitory effect of pGAL by about 8% and 17% (at a lower and higher concentration, respectively). The inhibitory activity was partly shown also by analogue [D-Leu^4]GAL(1-15)NH_2. This analogue tested at

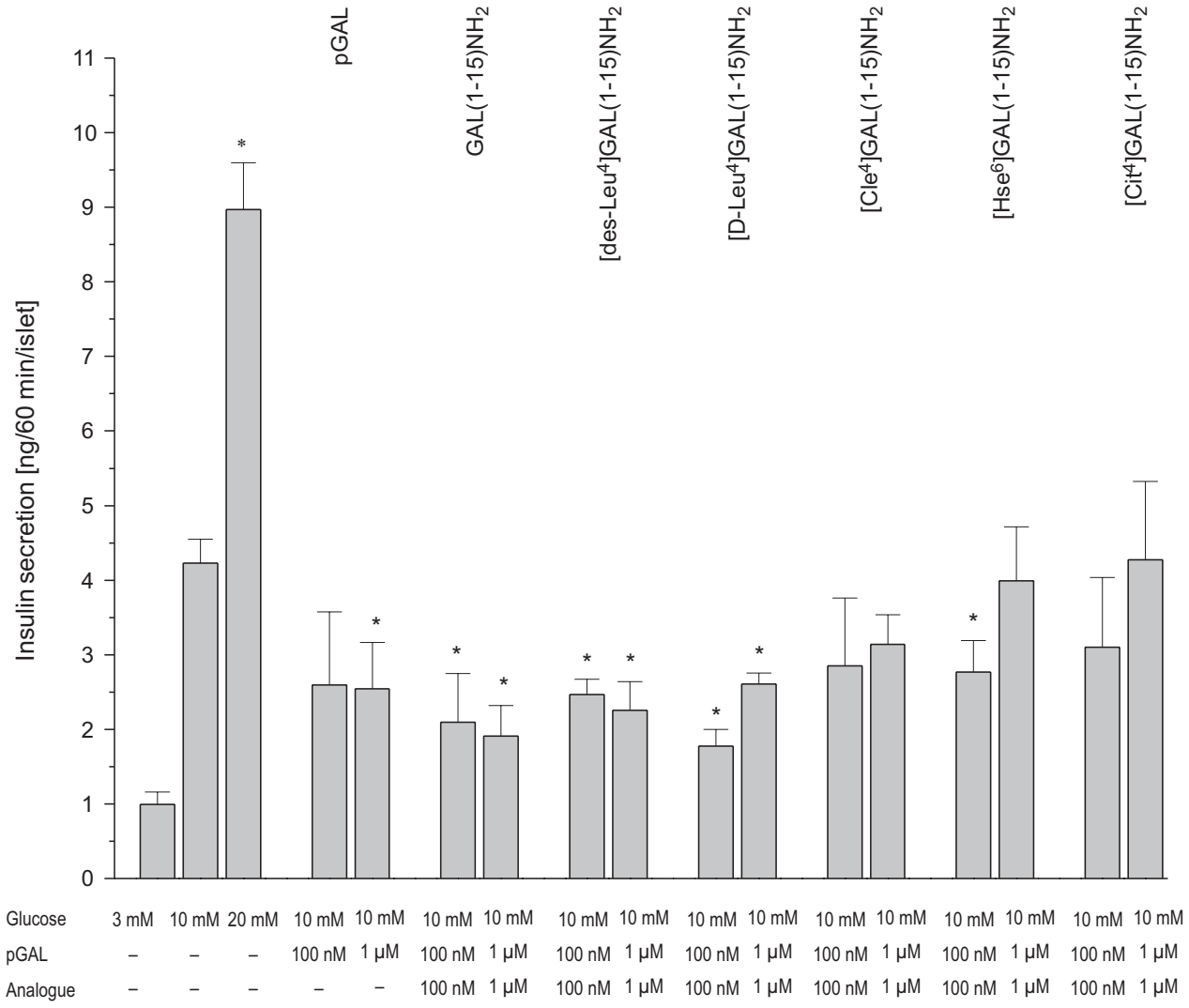

Fig. 2. Effects of GAL(1-15)NH_2 and its analogues on insulin secretion from the isolated rat pancreatic islets induced by 10 mM glucose in the presence of appropriate concentration of pGAL. Results are presented as means ± SEM of insulin level induced by the tested peptide. The insulin level in the presence of 10 mM of glucose is a reference for all experiments. Asterisks indicate the probability level of random difference *vs*. 10 mM of glucose (* $p < 0.01$)

a concentration of 100 nM lowered the insulin level by about 11% (3.75 ng/60 min/islet) as compared to the control incubated with 10 mM of glucose, and significantly increased the inhibitory action of pGAL by about 50%. However, [D-Leu^4]GAL(1-15)NH_2, used at a higher concentration, did not affect the glucose-induced insulin secretion and the inhibitory action of pGAL. The insulin level in the presence of this analogue reached 4.38 ng/60 min/islet.

The lack of inhibition of the insulin secretion was observed in the case of [Cle^4]GAL(1-15)NH_2, [Hse^6]GAL(1-15)NH_2 and [Cit^{14}]GAL(1-15)NH_2, when tested at a concentration of 100 nM. The insulin level in the presence of these peptides reached: 4.47, 4.30 and 4.65 ng/60 min/islet, respectively. At a higher concentration, analogues: [Cle^4]GAL-(1-15)NH_2, [Hse^6]GAL(1-15)NH_2 and [Cit^{14}]GAL-(1-15)NH_2 showed insulinotropic activities as compared to pGAL or GAL(1-15)NH_2. They caused an increase in the insulin secretion by about 25% (5.29 ng/60 min/islet), 24% (5.25 ng/60 min/islet) and 65% (6.98 ng/60 min/islet), respectively. Moreover, our studies have shown that peptides: [Cle^4]-GAL(1-15)NH_2, [Hse^6]GAL(1-15)NH_2 and [Cit^{14}]-GAL(1-15)NH_2, used at a concentration of 100 nM, reduced inhibitory effect of pGAL by about 16% (2.85 ng/60 min/islet), 11% (2.77 ng/60 min/islet) and 31% (3.10 ng/60 min/islet), respectively. Rela-

tively stronger antagonistic activities (at a concentration of 1 μM) were shown by [Cle^4]GAL(1-15)-NH_2 and [Hse^6]GAL(1-15)NH_2, which reduced the pGAL activity by about 36% (3.14 ng/60 min/islet) and 86% (3.99 ng/60 min/islet), respectively. However, [Cit^{14}]GAL(1-15)NH_2, tested at a concentration of 1 μM, completely abolished the inhibitory action of pGAL. The insulin level in the presence of this analogue reached 4.27 ng/60 min/islet.

DISCUSSION

Numerous GAL fragments, chimeras and analogues with point mutations were synthesized and their activity on the insulin secretion was studied [2, 12, 15, 16, 20, 24, 28, 29, 32, 39]. 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. Gly^1 and Trp^2 in the amino acid sequence of pGAL play a crucial role for high-affinity binding to its receptors. Removal of the first two amino acids, Gly^1 and Trp^2, caused a complete loss of the inhibitory effect. Moreover, the results suggested Trp^2 to be the most important residue. The substitution of this residue with D-Trp, L-Tyr, L-Phe, L-Ile or L-Ala caused significant changes in the GAL-mediated inhibitory effect on glucose-induced insulin secretion and yielded peptides that showed no significant inhibitory activity or were inactive. Other important residues are Leu^4, Ser^6, Gly^8 and Tyr^9, which also contribute significantly to the activation of GAL receptors in the pancreas. Moreover, the studies of GAL(1-16) analogues in which single residues were substituted with the L-Ala residue have shown that the substitutions of Leu^4, Ser^6 or His^{14} did not reduce the affinity of these peptides to the GAL receptors in the rat hypothalamus [21]. The peptides thus obtained were able to fully displace ^{125}I-pGAL from the high affinity GAL receptors. Another study in which the N-terminal 1-13 fragment of GAL was extended by coupling different amino acids or nonpeptidic structures via the ε-amino group of a Lys residue, that was introduced in position 14, showed high affinity of such modified peptides to GAL receptors in the rat hypothalamus [31]. Thus, the modifications of GAL in position 4, 6 and 14 seem to be a potent target in the search for a high-affinity GAL receptor agonist or antagonist.

Based on these observations we have designed five new GAL analogues modified in positions 4, 6 or 14 and studied their action on glucose-induced insulin secretion. Our present studies confirmed earlier observations that pGAL 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 pGAL. It suggests that the C-terminal fragment GAL(16-29) is required for full activation of GAL receptors in the rat pancreatic islets. Simultaneous application of pGAL and GAL(1-15)NH_2, used at the same concentration, caused a considerable increase in the inhibitory action of pGAL. However, studies with the use of the GAL(1-15)NH_2 analogues have shown diverse activities of such modified peptides as compared to pGAL or its non-modified N-terminal fragment 1-15. Two GAL analogues: [des-Leu^4]-GAL(1-15)NH_2 and partly [D-Leu^4]GAL(1-15)NH_2, have been found to be very weak GAL agonists. We have shown that [des-Leu^4]GAL(1-15)NH_2 inhibited insulin secretion and slightly increased inhibitory action of pGAL at all concentrations tested, while analogue [D-Leu^4]GAL(1-15)NH_2 inhibited the insulin secretion and markedly increased the inhibitory action of pGAL only at a concentration of 100 nM. Analogue [des-Leu^4]GAL(1-15)NH_2 seems to be a less potent inhibitor than GAL(1-15)-NH_2. However, [D-Leu^4]GAL(1-15)NH_2, used at a concentration of 1 μM, did not influence insulin secretion and inhibitory action of pGAL. Our observations suggest that the removal or replacement of Leu^4 with its stereoisomer D-Leu did not significantly affect the activity of such modified GAL-(1-15)NH_2.

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 L-Leu^4 was replaced with Cle, L-Ser^6 was replaced with L-Hse or L-His^{14} was replaced with L-Cit. 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: [Hse^6]GAL(1-15)-NH_2 [Cle^4]GAL(1-15)NH_2 < [Cit^{14}]GAL(1-15)-NH_2. However, these peptides, tested at a concentration of 100 nM, did not influence the insulin secretion. We have also shown that analogues: [Hse^6]GAL(1-15)NH_2, [Cle^4]GAL(1-15)NH_2 and

[Cit^{14}]GAL(1-15)NH_2 antagonized the inhibitory effect of pGAL in a concentration-dependent manner. [Cle^{4}]GAL(1-15)NH_2 and [Hse^{6}]GAL(1-15)-NH_2, used at a concentration of 100 nM, showed very weak antagonistic activities, while [Cit^{14}]-GAL(1-15)NH_2 displayed relatively strong antagonistic activity. The inhibitory action of pGAL on the glucose-induced insulin secretion was completely abolished by [Hse^{6}]GAL(1-15)NH_2 and [Cit^{14}]GAL(1-15)NH_2, tested at a concentration of 1 μM. Our observations suggest that the modifications of the amino acid sequence of GAL(1-15)-NH_2, in which **Leu^{4}** was replaced with cyclic amino acid Cle, **Ser^{6}** was replaced with its homologue L-Hse or **His^{14}** was replaced with L-Cit, caused changes in the interaction of GAL with its receptors and produced peptides that showed reverse activities as compared to pGAL.

Although the effect of GAL analogues on glucose-induced insulin release is well-known, relatively little is known about the mechanism(s) of its action and how these peptides initiate their action at the molecular level. It is difficult to interpret the behavior of these peptides in complex biological systems, because we are not able to adequately explain in molecular terms how the inhibitory/stimulatory effect of GAL analogues is exerted. We do not know, whether this effect is connected with the activation of several signaling pathways or/and the conformational changes of the GAL structure, responsible for the high-affinity binding to its receptors and biological activity. To understand the action of GAL and its analogues at the molecular level, the knowledge of the 3-dimensional structures that the peptides assume close to or in contact with biomembranes is needed. It is clear now that many peptides, including GAL, use multiple mechanisms at the molecular level to regulate its action. Our observations suggest that GAL and GAL analogues may use different mechanism(s) to regulate their action in rat pancreatic islets.

In conclusion, the modifications introduced into the amino acid sequence of GAL, caused changes in the interaction of GAL(1-15)NH_2 with its receptors and yielded peptides that consequently showed diverse activities as compared to pGAL or GAL-(1-15)NH_2. Finally, we have found three GAL analogues: [Cit^{14}]GAL(1-15)NH_2, [Hse^{6}]GAL(1-15)-NH_2 and [Cle^{4}]GAL(1-15)NH_2, which were able to stimulate glucose-induced insulin secretion and also antagonized inhibitory effect of pGAL. Other two analogues: [D-Leu^{4}]GAL(1-15)NH_2 and [des-Leu^{4}]GAL(1-15)NH_2 showed rather weak agonistic activities. Our observations suggest that positions 4, 6 and 14 in the amino acid sequence of GAL(1-15)NH_2 may play an important role in the high-affinity binding of GAL to its receptors and biological activity in perfused rat pancreas. This conclusion may be useful in designing new GAL analogues acting as specific GAL receptor antagonist. Further modifications of amino acid sequence of GAL, especially in position 14 or 6, seem to be a very potent tool in searching for strong GAL antagonist in the pancreas.

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REFERENCES

1. Amiranoff B., Lorinet A.M., Lagny-Pourmir I., Laburthe M.: Mechanism of galanin-inhibited insulin release. Occurrence of a pertussis-toxin-sensitive inhibition of adenylate cyclase. Eur. J. Biochem., 1988, 177, 147–152.
2. Amiranoff B., Lorinet A.M., Yanaihara N., Laburthe M.: Structural requirement for galanin action in the pancreatic beta cell line RINm5F. Eur. J. Pharmacol., 1989, 163, 205–207.
3. Bartfai T., Fisone G., Langel Ü.: Galanin and galanin antagonists: molecular and biochemical perspectives. Trends Pharmacol. Sci., 1992, 13, 312–317.
4. Bartfai T., Langel Ü.: Galanin receptor ligands as potential therapeutic agents in depression and neurodegeneration. Eur. J. Med. Chem., 1995, 30, 163S–174S.
5. Bedecs K., Berthold M., Bartfai T.: Galanin – 10 years with a neuroendocrine peptide. Int. J. Biochem. Cell Biol., 1997, 27, 337–349.
6. Chan W.C., White P.D.: Fmoc Solid Phase Peptide Synthesis. A Practical Approach. 1st edn., Oxford University Press, Oxford, 2000.
7. Crawley J.N.: Biological actions of galanin. Regul. Peptides, 1995, 59, 1–16.
8. Dunning B., Ar'Rajab A., Bottcher G., Sundler F., Ahrén B.: Galanin (GAL) is present in human pancreatic nerves and inhibits insulin release (IRI) from isolated human islets. Diabetes, 1990, 39, 135A.
9. Ekblad E., Hakanson R., Sundler F., Wahlstedt C.: Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. Brit. J. Pharmacol., 1985, 86, 241–246.
10. Ekblad E., Rökaeus A., Hakanson R., Sundler F.: Galanin nerve fibers in the rat gut: distribution, origin and projections. Neuroscience, 1985, 16, 355–363.
11. Fox J.E.T., McDonald T.J., Kostolanska F., Tatemoto K.: Galanin: an inhibitory neural peptide of the canine small intestine. Life Sci., 1986, 39, 103–110.

12. Gallwitz B., Schmidt W.E., Schwarzhoff R., Creuztzfeldt W.: Galanin: structural requirements for binding and signal transduction in RINm5F insulinoma cells. Biochem. Biophys. Res. Commun., 1990, 172, 268–275.
13. Gilbey S.G., Stephenson J., O'Halloron D.J., Burrin J.M., Bloom S.R.: High-dose porcine galanin infusion and effect on intravenous glucose tolerance in humans. Diabetes, 1989, 38, 1114–1116.
14. Homaidan F.R., Sharp G.W., Nowak L.M.: Galanin inhibits a dihydropyridine-sensitive Ca^{2+} current in the RINm5f cell line. Proc. Nat. Acad. Sci. USA, 1991, 88, 8744–8748.
15. Kakuyama H., Mochizuki T., Iguchi K., Yamabe K., Hosoe H., Hoshino M., Yanaihara N.: [Ala^6,D-Trp^8]-galanin(1-15)ol is a potent galanin antagonist on insulin release. Biomed. Res., 1997, 18, 49–56.
16. Kask K., Berthold M., Bourne J., Andell S., Langel Ü., Bartfai T.: Binding and agonist/antagonist actions of M35, galanin(1-13)-bradykinin(2-9)amide chimeric peptide, in Rin m5F insulinoma cells. Regul. Peptides, 1995, 59, 341–348.
17. Kask K., Langel Ü., Bartfai T.: Galanin – a neuropeptide with inhibitory actions. Cell. Mol. Neurobiol., 1995, 15, 653–673.
18. Kuwahara A., Ozaki T., Yanaihara N.: Galanin suppresses neurally evoked contractions of circular muscle in the guinea-pig ileum. Eur. J. Pharmacol., 1989, 164, 175–178.
19. Lacy P.E., Kostianovsky M.: Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes, 1967, 16, 35–39.
20. Lagny-Pourmir I., Lorinet A.M., Yanaihara N., Laburthe M.: Structural requirements for galanin interaction with receptors from pancreatic beta cells and from brain tissue of the rat. Peptides, 1989, 10, 757–761.
21. Land T., Langel Ü., Löw M., Berthold M., Undén A., Bartfai T.: Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor. Int. J. Peptide Prot. Res., 1991, 38, 267–272.
22. Leonhardt U., Siegel E.G., Köhler H., Barthel M., Tytko A., Nebendhal K., Creutzfeldt W.: Galanin inhibits glucose-induced insulin release in vitro. Hormone Metab. Res., 1989, 21, 100–101.
23. Lindskog S., Ahrén B.: Galanin: effects on basal and stimulated insulin and glucagon secretion in the mouse. Acta. Physiol. Scand., 1987, 129, 305–309.
24. Lindskog S., Ahrén B., Land T., Langel Ü., Bartfai T.: The novel high-affinity antagonist, galantide, blocks the galanin-mediated inhibition of glucose-induced insulin secretion. Eur. J. Pharmacol., 1992, 210, 183–188.
25. Lindskog S., Dunning B.E., MÍrtensson H., Ar'Rajab A., Taborsky G.J., Ahrén B.: Galanin of the homologous species inhibits insulin secretion in the rat and in the pig. Acta Physiol. Scand., 1990, 139, 591–596.
26. McDonald T.J., Dupre J., Tatemoto K., Greenberg G.R., Radziuk J., Mutt V.: Galanin inhibits insulin secretion and induces hyperglycemia in dogs. Diabetes, 1985, 34, 192–196.
27. McKnight G.L., Karlsen A.E., Kowalyk S., Mathewes S.L., Sheppard P.O., O'Hara P.J., Taborsky G.J.: Sequence of human galanin and its inhibition of glucose--stimulated insulin secretion from RIN cells. Diabetes, 1992, 41, 82–87.
28. Mochizuki T., Ishikawa J., Takatsuka N., Hoshino M., Yanaihara N.: Structure-function relationship of galanin. Proc. 28th Symp. Pept. Chem., Osaka, 1990, Ed. Shimonishi Y., Protein Research Foundation, Osaka, 1991, 373–377.
29. Mochizuki T., Sato T., Takatsuka N., Kakuyama H., Yamabe K., Yanaihara C., Yanaihara N.: Synthesis of galanin antagonist. Proc. 31st Symp. Pept. Chem., Osaka, 1993, Ed. Okada Y., Protein Research Foundation, Osaka, 1994, 325–328.
30. Morgan C.R., Lazarow A.: Immunoassay of insulin: two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats. Diabetes, 1963, 12, 115–120.
31. Pooga M., Juréus A., Rezael K., Hasanvan H., Saar K., Kask K., Kjellén P., Land T., Halonen J., Mäeorg U., Uri A., Solyom S., Bartfai T., Langel Ü.: Novel galanin receptor ligands. J. Peptide. Res., 1998, 51, 65–74.
32. Rekowski P., Borowiec A., Drużyńska J., Kusiak E.: New chimeric analogues of galanin: synthesis and effects on the glucose-induced insulin secretion. LIPS, 1998, 5, 417–420.
33. Ruczyński J., Szyk A., Konstański Z., Korolkiewicz R., Rekowski P.: Galanin and its analogues modified in position 14: chemical synthesis and biological activity. Pol. J. Chem., 2000, 74, 1375–1384.
34. Sharp G.W.: Mechanisms of inhibition of insulin release. Amer. J. Physiol., 1996, 271, C1781–C1799.
35. Sharp G.W., Le Marchand-Brustel Y., Yada T., Russo L.L., Bliss C.R., Cormont M., Monge L., Van Obberghen E.: Galanin can inhibit insulin release by a mechanism other than membrane hyperpolarization or inhibition of adenylate cyclase. J. Biol. Chem., 1989, 264, 7302–7309.
36. Straub S.G., James R.F., Dunne M.J., Sharp G.W.: Glucose activates both K_{ATP} channel-dependent and K_{ATP} channel-independent signaling pathways in human islets. Diabetes, 1998, 47, 758–763.
37. Tamura K., Palmer J.M., Wood J.D.: Galanin suppresses nicotinic synaptic transmission in the myenteric plexus of guinea-pig small intestine. Eur. J. Pharmacol., 1987, 136, 445–446.
38. Tatemoto K., Rökaeus A., Jörnvall H., McDonald T.J., Mutt V.: Galanin – a novel biologically active peptide from porcine intestine. FEBS Lett., 1983, 164, 124–128.
39. Yanaihara N., Mochizuki T., Takatsuka N., Iguchi K., Sato K., Kakuyama H., Li M., Yanaihara C.: Galanin analogues: agonist and antagonist. Regul. Peptides, 1993, 46, 93–101.

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