Polish J. Chem., **76**, 57–66 (2002)

New Galanin Analogues with Contractile Activities on Rat Gastric Smooth Muscles

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(Received September 3rd, 2001; revised manuscript October 15th, 2001)

We synthesized new 15-amino-acid-residue analogues of porcine galanin modified in positions 2, 6, 8 or 14 and studied their activity on isolated rat gastric smooth muscles. Thus, we intended to characterize the molecular domains of GAL responsible for binding to its receptors and biological activity in the gastric fundus. All peptides were synthesized by the solid phase peptide synthesis with the use of Fmoc strategy. All galanin analogues contracted rat gastric fundus strips in a concentration-dependent manner with significantly increased or decreased activities as compared to GAL(1–15)NH_2. As expected, the modifications introduced into the amino acid sequence of galanin caused changes in the interaction of GAL(1–15)NH_2 with its receptors. Thus, residues: Trp^2, Ser^6, Gly^8 and His^{14} in the amino acid sequence of GAL(1–15)NH_2 play an important roles in the high-affinity binding of GAL to its receptors and biological activity in rat gastric smooth muscle cells.

Key words: galanin, galanin analogues, rat gastric smooth muscles

Galanin (GAL) is a 29-amino-acid-residue C-terminally amidated neuropeptide, widely distributed in the central and peripheral nervous system of several vertebrate species including mammals [1–12]. It has been found in the spinal cord and in the brain: hypothalamus, locus coeruleus, hippocampus. In the peripheral nerve system GAL occurs in the respiratory, gastrointestinal and urogenital tracts. In all amino acid sequences of galanin, isolated from a variety of species [1–8], except for trout [7], the first 15 N-terminal residues are highly conserved. However, the human form of galanin contains 30 amino acids and lacks C-terminal amidation [6]. Galanin displays many types of interesting physiological and behavioural activity, which are mediated through its interaction with distinct G-protein-coupled membrane receptor subtypes (GALR1, GALR2, GALR3), subsequently activating several signalling pathways [9,13]. It has the ability to modulate pituitary hormone release and insulin secretion, affects memory, learning, feeding, pain threshold control and sexual behaviour [9–12,14].

In most of biological actions galanin is well known as an inhibitor, but in the gastrointestinal tract it can both stimulate and inhibit smooth muscle activity in a concentration-dependent, species-specific and location-specific manner [1,15–23]. *In vitro*

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studies have shown that porcine galanin evokes a strong contractile action on rat fundus strip, jejunum, ileum, colon and urinary bladder [1,15], human small intestine [16], human appendix [17] and mouse colon [18]. On the contrary, relaxing action of GAL has been observed in guinea-pig small intestine [19,20], taenia coli [21], canine ileum [22] and human urinary blader [23]. Moreover, the studies have shown that porcine galanin contracted gastric fundus not only by promoting extracellurar Ca^{2+} influx into the cell, but also by stimulating phospholipase C and releasing Ca^{2+} from the intracellular stores [24,25].

Structure-activity studies of galanin, its fragments and analogues (chimeras and peptides with point mutations) showed that the activity of GAL is connected with the N-terminal 1–15 fragment, needed for full activation of galanin receptors [26–30]. The N-terminal fragment of galanin, GAL(1–16), retains full ligand binding affinity whereas the C-terminal fragment, GAL(16–29), does not bind to the receptor, but may display the ability to distinguish between receptor subtypes. The most important residue is Trp^2, which plays a key role in high-affinity binding of GAL to its receptors. The substitution of this residue with L-Ile, L-Phe, L-Tyr or L-Ala yields an essentially inactive peptide. Other important residues are Gly^1, Asn^5 and Tyr^9, which also contribute significantly to the activation of galanin receptors. Moreover, the earlier studies of galanin (1–16) analogues, in which single residues were substituted with the L-Ala residue, have shown that the substitutions in positions 14, 8 or 6 did not significantly reduce the affinity of obtained peptides to the galanin receptors in the rat hypothalamus [26]. These observations have suggested that the modifications of galanin in position 14, 8 or 6 seem to be a potent target in the search of a high-affinity galanin receptor agonist or antagonist.

In present studies we have designed and synthesized some new GAL(1–15)NH_2 analogues, modified in position 2, 6, 8 or 14, and studied their activity on isolated rat gastric fundus strips (Table 1 shows the primary structures of the peptides synthesized). We have also synthesized porcine galanin (pGAL) and its N-terminal fragment GAL(1–15)NH_2. We compared the effects of pGAL, GAL(1–15)NH_2 and its analogues on rat gastric fundus strips and characterized the molecular domains of GAL, responsible for its spasmogenic effect and binding to galanin receptors in isolated rat gastric fundus. We expected that the results obtained may provide more information about relationships between biological activities of galanin and its structure and may be useful in searching for strong galanin receptor ligand, acting as a specific antagonist in the gastrointestinal tract.

Table 1. The primary structures of the peptides synthesized.

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
[Asp^{14}]GAL(1–15)NH_2	G-W-T-L-N-S-A-G-Y-L-L-G-P-***Asp***-A-NH_2
[Nle^{14}]GAL(1–15)NH_2	G-W-T-L-N-S-A-G-Y-L-L-G-P-***Nle***-A-NH_2
[GAL(1–13)-N-ε-L-Lys^{14}]GAL(1–15)NH_2	***(G-W-T-L-N-S-A-G-Y-L-L-G-P)$_2$-Lys***-A-NH_2

Table 1 (continuation)

[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-Ser^6]GAL(1–15)NH_2	G-W-T-L-N-***D-Ser***-A-G-Y-L-L-G-P-H-A-*NH_2*
[D-Ser^6,D-Trp^8]GAL(1–15)NH_2	G-W-T-L-N-***D-Ser***-A-***D-Trp***-Y-L-L-G-P-H-A-*NH_2*
[D-Trp^8]GAL(1–15)NH_2	G-W-T-L-N-S-A-***D-Trp***-Y-L-L-G-P-H-A-*NH_2*
[des-Gly^8]GAL(1–15)NH_2	G-W-T-L-N-S-A-Y-L-L-G-P-H-A-*NH_2*
[Sar^8]GAL(1–15)NH_2	G-W-T-L-N-S-A-***Sar***-Y-L-L-G-P-H-A-*NH_2*
[D-Ala^8]GAL(1–15)NH_2	G-W-T-L-N-S-A-***D-Ala***-Y-L-L-G-P-H-A-*NH_2*
[D-Tic^2]GAL(1–15)NH_2	G-***D-Tic***-T-L-N-S-A-G-Y-L-L-G-P-H-A-*NH_2*
[D-Trp^2]GAL(1–15)NH_2	G-***D-Trp***-T-L-N-S-A-G-Y-L-L-G-P-H-A-*NH_2*

EXPERIMENTAL

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.*, **138**, 9 (1984)]. Other symbols: ACN – Acetonitrile, AcOH – Acetic acid, Boc – t-Butoxycarbonyl, DIPEA – N,N-Diisopropylethylamine, DMF – N,N-Dimethylformamide, EC_{50} – Effective concentration required to produce 50% of the maximum contraction calculated from the respective concentration-response curves, ESI-MS – Electrospray Ionization Mass Spectrometry, FAB-MS – Fast Atom Bombardment Mass Spectrometry, Fmoc – 9-Fluorenylmethoxycarbonyl, HOBt – N-Hydroxybenzotriazole, Hse – Homoserine (2-Amino-4-hydroxybutanoic acid), NMP – N-Methylpyrrolidone, Nle – Norleucine (2-Aminocaproic acid), Pmc – 2,2,5,7,8-Pentamethylchroman-6-sulphonyl, RP HPLC – Reverse Phase High Performance Liquid Chromatography, Sar – Sarcosine (N-methylglycine), tBu – t-Butyl, TBTU – O-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, TFA – Trifluoroacetic acid, Tic – 1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid, TIPS – Triisopropylsilane, Trt – Trityl.

Synthesis of the peptides. All peptides (Table 1) were synthesized by the solid phase synthesis with the use of a Labortec AG model SP 650 peptide synthesizer and Fmoc strategy [31]. A base-labile 9-fluorenylmetoxycarbonyl group (Fmoc) was used to protect the α-amino function group of the amino acids (in case of [GAL(1–13)-N-ε-L-Lys^{14}]GAL(1–15)NH_2 also to protect the ε-amino function group of Lys). Others groups of the side chains were blocked by the acid-labile groups: **Boc** for Lys and Trp, **Pmc** for Arg, **Trt** for Gln, Asn, His, **tBu** for Tyr, Ser, Hse, Thr and Asp. 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 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 hr. Then solutions were filtered and peptides were precipitated with cold diethyl ether. Precipitated peptides were dissolved in water or 25% acetic acid and lyophilized to obtain crude peptides.

Purification and characterization of synthesized peptides. Crude peptides were purified by reverse phase HPLC (High Performance Liquid Chromatography) on preparative Vydac C-18 column (32 x 240 mm, 15–20 μm particle size). All peptides were separated at a flow rate of 18 ml/min, using several isocratic systems and linear gradients of ACN in 0.1% TFA. The absorbance of column eluates was monitored at 226 nm. 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 was checked by an analytical Beckman "System Gold" chromatograph with a Vydac C-18 column (4.6 x 250 mm, 5 μm particle size) with several isocratic systems and linear gradients of ACN in 0.1% TFA, flow rate was 1ml/min, absorbance at 226 nm. Identities of peptides were confirmed by amino acid analysis and mass spectrometry: FAB-MS or ESI-MS. For amino acid analyses, the peptides (0.5 mg) were hydrolyzed with hydrochloric acid (400 μl) containing 1% of phenol in evacuated sealed ampoules heated at 100°C for 24 h. The analyses were performed on a Beckman model 121 M amino acid analyzer. FAB mass spectrometry was performed on a VG Mass Lab Trio-3 quadrupole mass spectrometer.

ESI mass spectrometry was performed on a Finnigan MAT 95 S high resolution, double focusing mass spectrometer with the magnetic and electric field analyzer. Table 2 shows some physicochemical properties of the peptides synthesized.

Table 2. Some physicochemical properties of the peptides synthesized.

Peptide	Molecular formula	Molecular mass		RP HPLC
		Calculated	Found $[M+H]^+$	R_t [min]
pGAL	$C_{146}H_{213}N_{43}O_{40}$	3210.6	3211.7	15.95
GAL(1-15)NH_2	$C_{72}H_{106}N_{20}O_{19}$	1555.8	1556.5	12.62
[Asp^{14}]GAL(1-15)NH_2	$C_{70}H_{104}N_{18}O_{21}$	1533.7	1534.6	13.28
[Nle^{14}]GAL(1-15)NH_2	$C_{72}H_{110}N_{18}O_{19}$	1531.8	1532.8	16.64
[GAL(1-13)-N-ε-L-Lys^{14}]GAL(1-15)NH_2	$C_{135}H_{202}N_{34}O_{36}$	2877.3	2878.0	18.39
[Hse^6]GAL(1-15)NH_2	$C_{73}H_{108}N_{20}O_{19}$	1569.8	1570.3	13.97
[D-Ser^6]GAL(1-15)NH_2	$C_{72}H_{106}N_{20}O_{19}$	1555.8	1556.6	13.35
[D-Ser^6,D-Trp^8]GAL(1-15)NH_2	$C_{81}H_{113}N_{21}O_{19}$	1684.9	1685.4	16.96
[D-Trp^8]GAL(1-15)NH_2	$C_{81}H_{113}N_{21}O_{19}$	1684.9	16.85.7	17.28
[des-Gly^8]GAL(1-15)NH_2	$C_{70}H_{103}N_{19}O_{18}$	1498.7	1499.6	14.02
[Sar^8]GAL(1-15)NH_2	$C_{73}H_{108}N_{20}O_{19}$	1569.8	1571.0	13.24
[D-Ala^8]GAL(1-15)NH_2	$C_{73}H_{108}N_{20}O_{19}$	1569.8	1570.8	14.28
[D-Tic^2]GAL(1-15)NH_2	$C_{71}H_{105}N_{19}O_{19}$	1528.7	1528.9	13.31
[D-Trp^2]GAL(1-15)NH_2	$C_{72}H_{106}N_{20}O_{19}$	1555.8	1556.6	13.41

[1)] All peptides were characterized by the RP HPLC with linear gradient 20–60% of ACN for 30 min.
[2)] Trp, Cit, Dab, Nle, Hse, Sar, Tic were not determined by amino acid analysis.

Animals and tissue preparation. Albino-Wistar rats of either gender (weighing 180–250 g) were housed under standard laboratory conditions (a natural light-dark cycle) with unrestricted access to food and tap water. Animals were fasted overnight before experiments and killed by cervical dislocation. The abdominal cavity was opened, gastric fundus excised and placed in ice-cold Tyrode solution bubbled with carbogen (O_2/CO_2 95/5). The composition of Tyrode solution (pH 7.2–7.4) was (mM): NaCl 136.9, KCl 3.35, $CaCl_2$ 1.46, $MgCl_2$ 1.03, $NaHCO_3$ 11.9, NaH_2PO_4 0.48, glucose 5.0. Longitudinal muscle strips were prepared according to Vane's procedure [32] and mounted vertically in organ baths (volume 15 ml). The organ baths were filled with carbogen-gassed Tyrode solution (37°C). The tissues were kept at a resting tension of 2.0 g. One end of each strip was attached to a fixed support and the free one to a lever connected *via* a spring to PIT 212 force displacement transducers for the isotonic registration of mechanical activity. Transducers were connected to a TZ-4100 line recorder. Tissues were allowed to equilibrate for 90 min before the start of the experiment. The buffer was changed every 5 min, except for the contact time of the tested peptides with the tissues. To eliminate the probable involvement of cholinergic and adrenergic components in the responses investigated, the studies were carried out in the presence of atropine (1 μM), hexamethonium (10 μM) and guanethidine (10 μM). To prevent excessive degradation of peptides the experimental buffer contained amastatin (10 μM) and phosphoramidon (1 μM). Our previous experiments had shown that at the concentration used these inhibitors did not affect the contraction of gastric fundus strips. Viability and contractility of each strip were examined by the addition of carbachol (30 nM).

Concentration-response curves. Experiments were started when reproducible contractile responses to carbachol (30 nM) were obtained. Conventional concentration-contraction curves were constructed in a non-cumulative manner, by adding the increasing concentrations of peptides directly into the organ baths, until the maximum muscular effect occurred (when the contraction could not be further increased by a higher concentration of the peptide). The contact time of the peptide with muscle strips

ranged from 1 to 3 min. When the maximum contraction had developed, the tissues were washed out at a rate of 2.5 ml/s for 2–3 min until the length of the strip returned to basal level. In order to avoid tachyphylaxis in isolated gastric fundus strips, the peptides were applied at 30 min intervals. No more than two complete concentration-response curves were plotted for each strip. Viability and reproducible contractility of each strip were examined at the end of each experimental session by a submaximal response to carbachol, at the same concentration as at the start.

Statistical analysis of the acquired data. Results are expressed as the percentage of the maximum response induced by each peptide. Efficacy, potency (EC_{50}) and the slope of the concentration-response curves are expressed as mean values with 95% confidence limits. Efficacy is expressed as a percentage of the maximum contractile effect of pGAL or GAL(1–15)NH_2 respectively. EC_{50}, the slopes of the dose response curves, relative potencies of galanin analogues and their statistical significance were determined using the Pharmacological Calculation System v. 4 computer programme. Efficacy and EC_{50} were compared using the non-parametric Mann-Whitney, Wilcoxon signed-rank test for pairs or one-way analysis of variance (ANOVA) plus Bonferroni post-ANOVA tests. Hill's coefficients were calculated using a computer program and the equation $\log(E/(E_{max} - E)) = \log[C] - \log K$, where: E – effect evoked by the appropriate concentration of hormone [C], E_{max} – maximum effect, K – dissociation constant of hormone-receptor complex. On the basis of this equation Hill's graph was drawn as the function $f(\log[C]) = \log(E/(E_{max} - E))$ for each peptide. Thus obtained straight lines were characterized with direction numbers (slopes) – Hill's coefficients. Hill's coefficients are expressed as mean ± SEM (standard mean error). To examine whether the value of Hill's coefficients are different from unity a non-parametric Mann-Whitney test was used. Two-tailed P values of less than 0.05 were interpreted as indicating a significant difference.

RESULTS AND DISCUSSION

Isotonic contractions of the isolated gastric fundus strips were measured. Results of this study are presented as a comparison of some pharmacological variables obtained from the respective concentration-response curves. Results were expressed as the maximum response (efficacy) produced by the peptide tested and as the effective concentration required to produce 50% of the maximum contraction (EC_{50}). Efficacies were expressed as a percentage of the maximum contraction to pGAL or GAL(1–15)NH_2 control. Moreover, Hill's coefficients were calculated to examine, whether GAL short analogue-receptor interactions followed the classical receptor theory, according to which the biological effect is proportional to the concentration of the hormone-receptor complex in which one hormone molecule binds to one receptor. In this case, Hill's coefficient equals one. Otherwise (when Hill's coefficient is significantly lower than one) the action of the hormone is not consistent with the classical receptor theory and may either affect secondary activity carriers, such as cyclic nucleotides, or bind to several agonist molecules. Tables 3 and 4 give a comparison of some pharmacological variables of the peptides tested.

Table 3. A comparison of some pharmacological variables of pGAL and GAL(1–15)NH_2

Peptide	Efficacy [%]	EC_{50} (nM)	Relative potency	Slopes of concentration -response curves	Hill's coefficient
pGAL	100	13.39 (6.17–29.05)	1	34.63 (23.72–45.54)	0.96±0.05
GAL(1-15)NH_2	59.64 [a] (53.39–65.32)	174 [a] (105–288)	0.08 (0.04–0.16)	35.57 (27.59–43.54)	0.99±0.08

[a] $P < 0.05$ vs. pGAL

Table 4. A comparison of some pharmacological variables of GAL(1–15)NH_2 and its some analogues.

Peptide	Efficacy [%]	EC_{50} (nM)	Relative potency	Slopes of concentration-response curves	Hill's coefficient
GAL(1–15)NH_2	100	174 (105–288)	1	35.57 (27.59–43.54)	0.99±0.08
[Asp^{14}]GAL(1–15)NH_2	99.34 (76.13–123)	39.96[a] (30.02–50.87)	4.09	26.67 (24.02–29.31)	0.94±0.03
[Nle^{14}]GAL(1–15)NH_2	118 (96.17–139)	47.71[a] (26.32–86.48)	3.29	31.28 (24.23–38.34)	0.99±0.03
[GAL(1–13)-N-ε-L-Lys^{14}] GAL(1–15)NH_2	64.44 (28.12–107.7)	28.42[a] (8.15–99.10)	5.70	31.36 (12.53–50.19)	0.68±0.21[b]
[Hse^6]GAL(1–15)NH_2	65.48 (51.14–105)	235 (168–328)	0.74	35.65 (30.28–41.03)	0.78±0.03[b]
[D-Ser^6]GAL(1–15)NH_2	47.95[a] (35.64–60.26)	425.19[a] (188–961.4)	0.44	31.36 (19.91–42.80)	0.58±0.03[b]
[D-Ser^6,D-Trp^8]GAL (1–15) NH_2	82.89 (65.86–99.92)	9340.01[a] (7219.4–12083.5)	0.02	28.50 (25.50–31.50)	0.73±0.04[b]
[D-Trp^8]GAL(1–15)NH_2	114.11 (76.49–151.73)	161.22 (75.55–344.04)	1.09	36.27 (22.49–50.28)	0.98±0.31
[des-Gly^8]GAL(1–15) NH_2	40.94[a] (25.30–56.57)	100.97 (89.28–114.18)	1.65	32.47 (30.51–34.44)	0.57±0.01[b]
[Sar^8]GAL(1–15)NH_2	31.95[a] (19.91–43.99)	5020[a] (4499.8–5603.1)	0.04	37.78 (35.49–40.06)	0.73±0.06[b]
[D-Ala^8]GAL(1–15)NH_2	89.29 (61.42–117.15)	177.80 (103.54–305.34)	0.99	39.07 (28.29–49.85)	0.69±0.03[b]
[D-Tic^2]GAL(1–15)NH_2	63.76 (33.33–94.24)	4683.78[a] (3579–6129.5)	0.04	38.52 (33.19–43.85)	1.02±0.08
[D-Trp^2]GAL(1–15)NH_2	61[a] (48–74)	229 (169–310)	0.74	31.98 (27.78–36.17)	0.57±0.04[b]

[a] $P < 0.05$ *vs*. GAL(1–15)NH_2, [b] value of Hill's coefficient significantly different from 1.0 at $P < 0.05$.

All synthesized peptides contracted longitudinal rat gastric fundus strips in a concentration-dependent manner. The most active was pGAL, which acts as a full agonist, evoking reproducible contraction at 3 nmol/l and a maximum contraction at 1 μmol/l. The EC_{50} value estimated from the concentration-contraction curves equaled 13.39 nM. Hill's coefficient for pGAL was not different from unity indicating an interaction of one molecule with one receptor, thus fulfilling the criteria of the classical receptor theory. The GAL(1–15)NH_2 fragment was also active, exhibiting about 10% of the native pGAL potency, with a significantly lower maximum response of 59.6% and a higher EC_{50} of 174 nM than pGAL. Hill's coefficient for GAL(1–15)NH_2 was not different from unity. These results confirmed earlier observations that porcine galanin acts as a full galanin receptor agonist in rat gastric fundus [33]. Moreover, our studies have shown the activity of pGAL is connected with its N-terminal fragment 1–15, which plays a key role in high-affinity binding of GAL to its receptors, but the C-terminal fragment 16–29 is required for full activation of galanin receptors in gastrointestinal smooth muscle cells. On the other hand, we have previously shown

that the C-terminal fragment, pGAL(16–29), was inactive and did not act as an antagonist of GAL receptors in the gastric fundus [34].

We have shown that all GAL(1–15)NH_2 analogues, modified in position 14, evoked reproducible concentration-dependent contractions with considerably increased activities as compared to GAL(1–15)NH_2. The most active was: [GAL(1–13)-N-ε-L-Lys^{14}]GAL(1–15)NH_2 with a 5.7-fold higher relative potency and a significantly lower EC_{50} of 28.42 nM than GAL(1–15)NH_2. Relatively strong biological activities were also shown by other two GAL(1–15)NH_2 analogues: [Asp^{14}]GAL(1–15)NH_2 and [Nle^{14}]GAL(1–15)NH_2. Their activities were higher than that of GAL(1–15)NH_2, with relative potency equal to 4.09 (EC_{50} = 39.96 nM) and 3.29 (EC_{50} = 47.71 nM), respectively. Hill's coefficients for [Asp^{14}]GAL(1–15)NH_2 and [Nle^{14}]GAL(1–15)NH_2 were not different from unity, indicating the interaction of one molecule with one receptor. However, Hill's coefficients for [GAL(1–13)-N-ε-L-Lys^{14}]GAL(1–15)NH_2 was significantly lower than unity, indicating that the rules of the classical receptor theory may not apply.

Our previous studies have shown that the substitutions of L-His^{14} in the amino acids sequence of galanin with other basic L-amino acids (L-Lys, L-Orn, L-Dab, L-Dpr, L-Arg, or L-Cit) caused a significant increase in contractile activity [35]. In present studies we substituted His^{14} with hydrophobic amino acid (L-Nle), acidic amino acid (L-Asp) and also extended the GAL(1–15)NH_2 molecule by coupling the N-terminal 1–13 fragment of galanin via the ε-amino group of a L-Lys residue, introduced in position 14. Such obtained galanin analogues clearly showed increased contractile activities as compared to GAL(1–15)NH_2. Our results suggest that position 14 in the amino acid sequence of GAL(1–15)NH_2 may play an important role in high-affinity binding of GAL to its receptors and contractile activity. Moreover, our studies suggest that the basic properties of the fourteenth residue in the amino acid sequence of GAL seem to be less important for this effect. Further changes in position 14 may lead to a discovery of a very strong galanin receptor ligand, acting as specific GAL antagonists in the gastrointestinal tract.

However, studies with the use of the GAL(1–15)NH_2 analogues modified in position 8 have shown diverse activities of such modified peptides as compared to non-modified N-terminal fragment, GAL(1–15)NH_2. Two galanin analogues: [des-Gly^{8}]GAL(1–15)NH_2 and [D-Trp^{8}]GAL(1–15)NH_2 have shown somewhat higher activities than that of galanin 1–15 fragment, with relative potency equal to 1.65 (EC_{50} = 100.97 nM) and 1.09 (EC_{50} = 161.22 nM), respectively. Another galanin analogue [D-Ala^{8}]GAL(1–15)NH_2 has shown activities similar to that of GAL(1–15)NH_2, with relative potency of 0.99 (EC_{50} = 177.80 nM). Only two galanin analogues modified in position 8: [Sar^{8}]GAL(1–15)NH_2 and [D-Ser^{6},D-Trp^{8}]GAL(1–15)NH_2 displayed significantly lower contractile activities than GAL(1–15)NH_2, exhibiting respectively 4% (EC_{50} = 5020 nM) and 2% (EC_{50} = 9340 nM) of the GAL(1–15)NH_2 potency. Hill's coefficients for all galanin analogues modified in position 8, except for [D-Trp^{8}]GAL(1–15)NH_2, for which Hill's coefficient was not different from unity, were significantly lower than unity, indicating that the rules of the classical receptor

theory may not apply. Our studies have shown that the removal or substitution of Gly^8 in the amino acid sequence of GAL(1–15)NH_2 with D-isomers (D-Ala or D-Trp) did not significantly change the biological activities of such modified galanin structure. However, substitution of Gly^8 with N-methylated L-Gly (Sar) or simultaneous substitution of Gly^8 with D-Trp and Ser^6 with D-Ser caused a considerable decrease in contractile activity, which may probably result from significant conformational changes of galanin structure. We concluded that Gly^8 in the amino acid sequence of galanin plays an important role in biological activity, but further changes in position 8 may not lead to the discovery of a strong galanin receptor antagonist in rat gastric fundus.

Lack of significant changes in the biological activities (except for [D-Ser^6, D-Trp^8]GAL(1–15)NH_2 described above) was also shown by galanin analogues in which L-Ser^6 was replaced by its homologue L-Hse (analogue [Hse^6]GAL(1–15)NH_2) or stereoisomer D-Ser (analogue [D-Ser^6]GAL(1–15)NH_2). Such modified galanin analogues reached about 74% (EC_{50} = 235 nM) and 44% (EC_{50} = 425.19 nM) of the GAL(1–15)NH_2 potency, respectively. Hill's coefficients for both peptides were significantly lower than unity, which may indicate either a heterogenity of binding sites or negative cooperativity. These results demonstrate that single substitutions in position 6 did not significantly change the contractile activity of GAL(1–15)NH_2, however, simultaneous substitutions in positions 6 and 8 seem to be a potent target in searching for strong galanin antagonist in rat gastric smooth muscles.

Our previous studies have shown that removal of Trp^2 in the amino acid sequence of GAL(1–15)NH_2 gave analogue that was completely inactive [36]. Moreover, we have shown that this inactive analogue did not act as an antagonist of GAL receptors in the gastric fundus. On the other hand, we have shown that addition of a second L-Trp residue in position 2 reduced the contractile activity of about 50% as compared to GAL(1–15)NH_2 [36]. In present studies we checked contractile activities of new GAL(1–15)NH_2 analogues, in which L-Trp^2 was substituted with its D-isomer (D-Trp) and other hydrophobic D-isomer (D-Tic). Such obtained galanin analogues: [D-Trp^2]GAL(1–15)NH_2 and [D-Tic^2]GAL(1–15)NH_2 displayed different activities. [D-Trp^2]GAL(1–15)NH_2 has shown about 75% (EC_{50} = 229 nM) of the GAL(1–15)NH_2 potency, but [D-Tic^2]GAL(1–15)NH_2 showed only 4% (EC_{50} = 4683.78 nM) of the GAL(1–15)NH_2 potency. In case of [D-Trp^2]GAL(1–15)NH_2, Hill's coefficient for was significantly lower than unity. We concluded that Trp^2 in the amino sequence of GAL plays a crucial role in the recognition and/or stimulation of GAL receptors in rat gastric fundus. However, modifications of amino acid sequence of GAL in position 2 seem not to lead to the discovery of strong galanin receptor antagonists in the gastrointestinal tract.

In conclusion, our observations showed that the N-terminal fragment 1–15 plays a key role in high-affinity binding of GAL to its receptors, but the C-terminal fragment 16–29 is required for full activation of galanin receptors in gastrointestinal smooth muscle cells. We have also shown that all GAL(1–15)NH_2 analogues can

influence their interactions with GAL receptors in gastric fundus. Our results suggest that positions 14, 8 and 2 in the amino acid sequence of GAL(1–15)NH_2 are important for the biological activity of GAL(1–15) in isolated rat gastric fundus. However, position 6 seems to be less important. The data obtained may be useful in designing new galanin analogues acting as specific GAL agonists or antagonists in the gastrointestinal tract.

Acknowledgments

This work was partly supported by the Polish State Committee for Scientific Research, grant 1606/T09/2000/19 and BW-8000-5-0185-1.

REFERENCES

1. Tatemoto K., Rökaeus Å., Jörnvall H., McDonald T.J. and Mutt V., *FEBS Lett.*, **164**, 124 (1983).
2. Kaplan L.M., Spindel E.R., Isselbacher K.J. and Chin W.W., *Proc. Natl. Acad. Sci. USA,* **85**, 1065 (1988).
3. Rökaeus Å. and Carlquist M., *FEBS Lett.*, **234**, 400 (1988).
4. Vrontakis M.E., Peden L.M., Duchworth M.L. and Friesen H.G., *J. Biol. Chem.,* **262**, 16755 (1987).
5. Norberg Å., Sillard R., Carlquist M., Jörnvall H. and Mutt V., *FEBS Lett.*, **288**, 151 (1991).
6. Schmidt W.E., Kratzin H., Eckart K., Drevs D., Mundkowski G., Clemens A., Katsoulis S., Schäfer H., Gallwitz B. and Creutzfeldt W.*, Proc. Natl. Acad. Sci. USA,* **88**, 11435 (1991).
7. Anglade I., Wang Y.X., Jensen J., Tramu G., Kah O. and Conlon J.M.*, J. Comp. Neurol.,* **350**, 63 (1994).
8. Langel Ü. and Bartfai T., *Ann. N. Y. Acad. Sci.*, **863**, 86 (1998).
9. Crawley J.N., *Regul. Pept*., **59**, 1 (1995).
10. Bedecs K., Berthold M. and Bartfai T., *Int. J. Biochem. Cell. Biol*., **27**, 337 (1997).
11. Bartfai T., Fisone G. and Langel Ü., *TIPS*, **13**, 312 (1992).
12. Bartfai T. and Langel Ü., *Eur. J. Med. Chem*., **30**, 163S (1995).
13. Branchek TA, Smith KE, Gerald C and Walker M., *Trends Pharm. Sci.*, **21**, 109 (2000).
14. Kask K., Langel Ü. and Bartfai T., *Cell. Mol. Neurobiol.*, **15**, 653 (1995).
15. Ekblad E., Rökaeus Å., Håkanson R. and Sundler F., *Neuroscience*, **16**, 355 (1985).
16. Maggi C.A., Patacchini R., Santicioli P., Giuliani S., Turini D., Barbanti G., Beneforti B., Misuri D. and Meli A., *Naunyn Schmiedebergs Arch. Pharmacol.*, **339**, 415 (1989).
17. Ekblad E., Arnbjörnsson E., Ekman R., Håkanson R. and Sundler F., *Dig. Dis. Sci.*, **34**, 1217 (1989).
18. Fontaine J. and Lebrun P., *Eur. J. Pharmacol.*, **164**, 583 (1989).
19. Kuwahara A., Ozaki T. and Yanaihara N., *Eur. J. Pharmacol.*, **164**, 175 (1989).
20. Tamura K., Palmer J. M. and Wood J. D., *Eur. J. Pharmacol.*, **136**, 445 (1987).
21. Ekblad E., Håkanson R., Sundler F. and Wahlstedt C., *Br. J. Pharmacol.*, **86**, 241 (1985).
22. Fox J.E.T., Mc Donald T.J., Kostolanska F. and Tatemoto K., *Life Sci.*, **39**, 103 (1986).
23. Maggi C.A., Santicioli P., Patacchini R., Turini D., Barbanti G., Beneforti B., Giuliani S. and Meli A., *Eur. J. Pharmacol.*, **143**, 135 (1987).
24. Ahtaridis S.A., Katoch S.S. and Moreland R.S., *Am. J. Physiol.*, **274**, G306 (1998).
25. Korolkiewicz R., Konstański Z., Rekowski P., Ruczyński J., Szyk A., Szczepańska R., Korolkiewicz K. Z. and Petrusewicz J., *Pharm. Pharmacol. Commun.*, **6**, 415 (2000).
26. Land T., Langel Ü., Löw M., Berthold M., Undén A. and Bartfai T., *Int. J. Pept. Protein Res.*, **38**, 267 (1991).
27. Yanaihara N., Mohizuki T., Takatsuka N., Iguchi K., Sato K., Kakuyama H., Li M. and Yanaihara C., *Regul. Pept*., **46**, 93 (1993).
28. Juréus A., Langel Ü. and Bartfai T., *J. Peptide. Res.*, **49**, 195 (1997).
29. 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. and Langel Ü., *J Peptide. Res.,* **51**, 65 (1998).
30. Carpenter K.A., Schmidt R., Yue S.Y., Hodzic L., Pou C., Payza K., Godbout C., Brown W. and Roberts E., *Biochem.,* **38**, 15295 (1999).

31. Chan WC and White PD: Fmoc Solid Phase Peptide Synthesis: A Practical Approach. Oxford, Oxford University Press, 2000.
32. Vane J.R., *Br. J. Pharmacol,* **12**, 344 (1957).
33. Katsoulis S., Schmidt W.E., Schwörer H. and Creutzfeldt W., *Br. J. Pharmacol.*, **101**, 297 (1990).
34. Śliwiński W., Korolkiewicz R., Rekowski P., Halama A. and Korolkiewicz K.Z., *Fundam. Clin. Pharmacol.,* **10**, 442 (1996).
35. Ruczyński J., Szyk A., Konstański Z., Korolkiewicz R. and Rekowski P., *Polish. J. Chem*., **74**, 1375 (2000).
36. Korolkiewicz R., Konstański Z., Rekowski P., Ruczyński J., Szyk A., Grzybowska M., Korolkiewicz K.Z. and Petrusewicz J., *J. Physiol. Pharmacol.*, **52(1)**, 127 (2001).