Acta Chemica Scandinavica, 1994: 48: 434–438
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Analogs of Galanin(1–16) Modified in Positions 1–3 as Ligands to Rat Hypothalamic Galanin Receptors*

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Rivera Baeza, C., Kask, K., Langel, Ü., Bartfai, T. and Undén, A., 1994. Analogs of Galanin(1–16) Modified in Positions 1–3 as Ligands to Rat Hypothalamic Galanin Receptor. – Acta Chem. Scand. 48: 434–438 © Acta Chemica Scandinavica 1994.

Structure–activity relationship (SAR) studies have revealed that the first three residues of galanin (Gly^1-Trp^2-Thr^3) are of critical importance for high-affinity binding to the galanin receptor. Furthermore degradation studies have shown that galanin is easily cleaved to yield inactive fragments in rat hypothalamus ($t_{1/2}$ = 100 min). To obtain galanin receptor ligands with long-lasting biological activity the amino-terminus of galanin must be protected. We have therefore synthesized analogs of rat galanin(1–16) carrying modifications at the three amino-termini of galanin. All modifications of the peptide backbone flanking Trp^2 as in the analogs [*N*-Me-Trp^2]-galanin(1–16), [Tcc^2]-galanin-(1–16), (Trp^2-Ψ[CH_2NH]-Thr^3)-galanin-(1–16) produced a dramatic loss of affinity toward the galanin receptor. [*N*-Me-Thr^3]-galanin(1–16) was the most active of the peptide backbone modified analogs (K_D = 997 ± 1 nM). Modifications of the indole ring in Trp^2 {[For-Trp^2]-galanin-(1–16), [Tcc^2]-galanin-(1–16)} yielded analogs which, at concentrations up to 10 μM, did not displace [^{125}I]galanin binding. *N*-Methylation of Gly^1 by the introduction of sarcosine {[Sar^1]-galanin(1–16)} did not significantly affect the ligand-binding properties of galanin(1–16) (K_D = 8.7 ± 0.1 nM).

Galanin,[1] a 29/30 amino acid long carboxy-terminally amidated peptide and its receptors are widely distributed in the central and peripheral nervous system. The distribution of galanin binding sites has been mapped by autoradiographic techniques in the central nervous system[2] and is in good agreement with the distribution of the endogenous peptide.[3,4] Galanin exerts a number of biological and pharmacological actions.[5,6] It inhibits the noradrenaline-stimulated accumulation of cyclic AMP in slices of the rat cerebral cortex[7] and blocks dopamine release from the rat median eminence.[8] It inhibits the insulin secretion from isolated human islets.[9] Microdialysis studies on rat striatum have shown that galanin stimulates acetylcholine release in rat striatum.[10] It also acts as a physiological antagonist of substance P in the spinal cord attenuating chronic pain,[11] and induces growth hormone (GH) release from pituitary in man.[12]

Structure–activity relationship studies on synthetic galanin fragments show that the amino-terminal fragment galanin(1–16) is sufficient for high-affinity binding to the rat hippocampal[13] and hypothalamic receptor[14] and for exerting agonist-like properties in different systems such as rat hippocampus[15] and the pancreatic β-cell line Rin m5F.[16] Only in the case of galanin receptors from smooth muscle are both amino- and carboxy-termini of galanin required for full biological activity.[17] Analysis of the peptidolytic products of the high affinity receptor agonist, galanin(1–16) demonstrates a short half-life of this amino-terminal galanin fragment in rat hypothalamus (28 min).[18] Gly^1, Trp^2, Asn^5 and Tyr^9 are important amino acid residues for high-affinity binding of galanin(1–16). Among these, Trp^2 is the most important residue for receptor recognition as shown by ligand-binding studies of L-Ala^2 and D-Trp^2 analogs[14,16,19] and for pharmacological effects such as the inhibition of the scopolamine-induced release of acetylcholine from rat ventral hippocampus.[20] Replacement of Trp^2 with its enantiomer D-Trp^2 results in a loss of affinity towards the rat hippocampal galanin receptor of more than three orders of magnitude.[13,19] Replacement of the same residue with L-Ala leads to a loss of affinity for the rat hypothalamic galanin receptor of more than five orders of magnitude.[14]

Because of the apparently essential role of Trp^2 in the interaction of galanin with the galanin receptor as well as in the putative proteolytic inactivation of galanin in the hypothalamus, it is of interest to study further the importance of the functional groups and peptide bonds flanking Trp^2 in galanin–galanin receptor interactions. In the present report we describe a more detailed structure–

* *Abbreviations:* Bom, *p*-benzyloxymethyl; 2-BrZ, 2-Bromobenzyloxycarbonyl; For, Formyl; Tcc, 2,3,4,5-tetrahydro-β-carboline-4-COOH; OBzl, benzyl ester; Sar, sarcosine; TFA, trifluoroacetic acid; DMS, dimethyl sulfide.

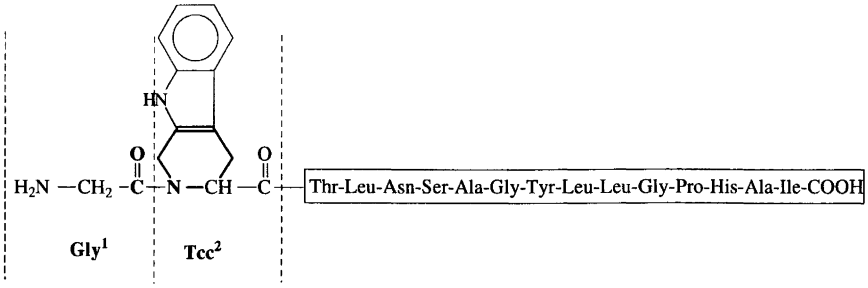

Fig. 1. Structure of [Tcc^2]Galanin(1–16).

activity relationship study focused on the peptide bonds between Gly^1-Trp^2-Thr^3, the indole ring of Trp^2 and the amino-terminus of galanin(1–16).

Results and discussion

The bioavailability and physiological activity of bioactive hormones appear to be partly dependent upon the time of exposure to membranes or barriers, which in turn is highly dependent on the proteolytic stability of the hormone. The most successful approach today to increase the proteolytic stability of bioactive peptides is to modify the peptide backbone.[21] In this study we have introduced modifications to the peptide backbone in the region, known from degradation studies,[18] to be a critical target for cleavage of galanin.

In order to achieve proteolytically stable peptides, modifications of the peptide bond, like N^{α}-methylation, and reduced peptide bonds have been introduced into analogs of several peptide hormones[21] with variable success. A good example, in line with the results of the present work, is a study carried out on bradykinin where among several N^{α}-methylated analogs, only [N-Me-Arg^1]-bradykinin was nearly equipotent with its parent peptide.[22]

We introduced two modifications into the side chain of Trp^2: the formulation of the indole nitrogen and the incorporation to the galanin(1–16) sequence of a tryptophan analog 2,3,4,5-tetrahydro-β-carboline-4-COOH (Tcc)[23] (Fig. 1). This modification results both in a modified side chain and in an alkylated N^{α} and strongly reduces the rotational freedom of the side chain. N^{α}-methylation of peptide bonds in the vicinity of the central pharmacophore(s) can lead to analogs with dramatically reduced affinities as ligands to receptors. This has been previously reported for substance P where replacement of N-Me-Gly^7 in the analog [Glp^6,MeGly^7]-substance P(6–11) results in 93% reduction of activity in the guinea-pig ileum assay as compared with [Glp^6]-substance P(6–11).[24]

Tcc and similarly rigid phenylalanine analogs have recently been successfully employed in the study of other peptide hormones like endothelin and bombesin.[25,26] In the present study the introduction of Tcc resulted in a galanin(1–16) analog that did not bind to the galanin receptor in concentrations of up to 10 μM (analog **8**, Table 1, Fig. 2). The question why this modified analog did not bind to the galanin receptor cannot be answered entirely on the basis of existing data, but it is noteworthy that the analog in which N^{α} was methylated (analog **6**, Table 1, Fig. 2) also showed no measurable affinity towards the galanin receptor. It is therefore reasonable to assume that the introduction of the methylene bridge in the rigid Tcc^2-carrying galanin(1–16) analog is sufficient to explain the large reduction in the affinity of this analog. Thus loss of affinity of [Tcc^2]galanin(1–16) may result from the introduction of the methylene bridge as well as

Table 1. Galanin(1–16) analogs containing N^{α}-methylated and reduced peptide bonds as ligands to galanin receptor in rat hypothalamus.

Peptide	K_D/nM	Relative affinity (%)
1. Galanin-(1–29) (*native peptide*)	0.98±0.07[b]	673
2. Galanin-(1–16) (*full agonist*)	6.6±0.1[b]	100
3. [AcGly^1]-Galanin-(1–16)[a]	500±100	1.30
4. Galanin-(2–16)[a]	100±100	0.70
5. [Sar^1]-Galanin(1–16)	8.7±0.09[b]	76
6. [N-Me-Trp^2]-Galanin(1–16)	$>10^4$	>0.0001
7. [N-Me-Thr^3]-Galanin(1–16)	997±1[b]	0.66
8. [Tcc^2]-Galanin-(1–16)[c]	$>10^4$	>0.0001
9. (Trp^2-Ψ[CH$_2$NH]-Thr^3)-Galanin-(1–16)	$>10^4$	>0.0001
10. [For-Trp^2]-Galanin-(1–16)	$>10^4$	>0.0001

[a]Data from Ref. 13. [b]Hill coefficients calculated by non-linear last-squares fitting to the Hill equation were close to unity. [c]See Fig. 1.

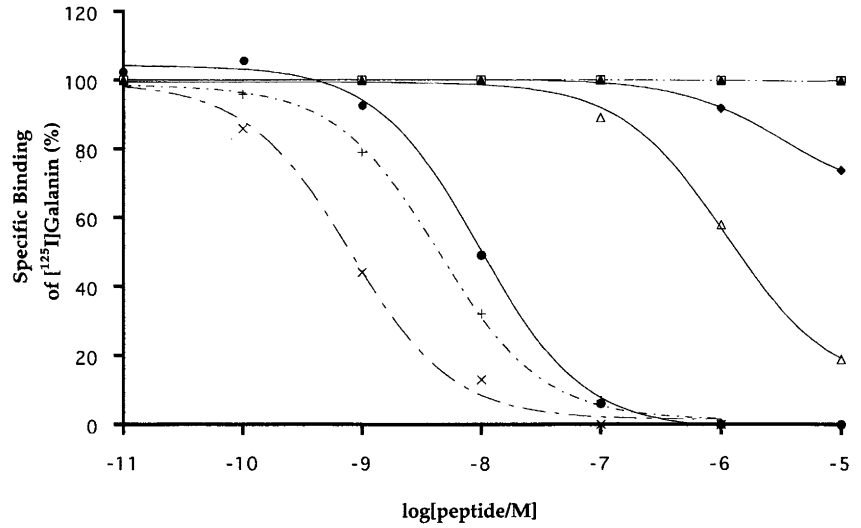

Fig. 2. Displacement of [^{125}I]galanin in membranes from rat hypothalamus by porcine galanin, galanin(1–16) and modified galanin analogs: (●) [Sar^1]galanin(1–16); (◆) (Trp$^2\Psi$[CH$_2$NH]Thr^3)galanin(1–16); (△) [N-Me-Thr^3]galanin(1–16); (×) galanin(1–29); (+) galanin(1–16); (□) [N-Me-Trp^2]galanin(1–16); (▲) [Tcc^2]galanin(1–16).

from reduction of the mobility of the indole ring. Furthermore, binding of galanin(1–16) to galanin receptors is sensitive to modifications in the indole ring itself as illustrated by the large reduction of the affinity of the N^{in}-formylated analog **10** (cf. Table 1, Fig. 2).

Susceptibility of binding properties to any kind of modification at the peptide bond between Trp^2 and Thr^3 of galanin(1–16) was further demonstrated by the finding that the galanin(1–16) analog carrying the so-called reduced peptide bond Ψ[CH$_2$NH] (analog **9**, Table 1, Fig. 2) had almost no measurable affinity to galanin receptors in the rat hypothalamus. Interestingly, N^{α}-methylation of Thr^3 (analog **7**, Table 1, Fig. 2) yielded an analog with less than 1% of the affinity of galanin(1–16) ($K_D = 997 \pm 1$ nM), suggesting a seemingly greater importance of the carbonyl oxygen as a hydrogen-bond acceptor.

N-Methylation of Gly^1 decreased only slightly the binding affinity for the galanin receptor compared with galanin(1–16). Comparison of this result with the previously reported[14] dramatic decrease in affinity of the des-Gly^1 and amino-terminally acetylated (Ac-Gly^1) analogs of galanin(1–16) (analogs **3** and **4**, Table 1), suggests the possible requirement of a hydrophilic or charged amino-terminus at a distance corresponding to one glycine residue from Trp^2. Furthermore, methylation of the amino-terminus may protect the peptide against exoproteases as demonstrated in the case of angiotensin.[27]

The present study clearly corroborates the theory that Trp^2 is the central pharmacophore in galanin(1–16) in its interaction with the rat hypothalamic galanin receptor (Table 1 and Fig. 2). All the modifications introduced: *N*-methylation of the peptide bond, reduction of the carbonyl group to a methylene group in the peptide bond, formylation of the indole nitrogen and the previously reported[14] change of chirality of Trp^2, lead to a loss in affinity of several orders of magnitude. No other residue in the whole peptide hormone galanin(1–29) displays similar sensitivity to chemical modification as demonstrated in previous studies where the individual amino acids in galanin(1–16) were replaced by alanine.[14] The same is supported by similar findings of the present study where the modification of Gly^1 and Thr^3 by N^{α}-methylation (analogs **5** and **7** with K_D 8.7 ± 0.1 nM and 997 ± 1 nM, respectively) resulted in far less dramatic changes in affinity than modification of Trp^2.

The results of the present study also show that an attempt to modify the apparently proteolytically sensitive peptide bond *C*-terminal to Trp^2 will be a difficult strategy for the development of proteolytically stable galanin analogs. A possible alternative which will be investigated is whether different kinds of *N*-alkylation of the amino terminal Gly^1 may be introduced in order to reduce the sensitivity of galanin to proteases while retaining its high-affinity binding to the galanin receptor.

Experimental

Materials. Na^{125}I (2500 Ci mmol^{-1}) was purchased from NEN (Boston, MA, USA), amino acid derivatives were from Bachem (Basel, Switzerland) and Nova (UK), amino methyl resin from Bachem. All other reagents were from Sigma (St. Louis, MO, USA). Porcine ^{125}I-galanin (specific activity 1800–2000 Ci mmol^{-1}) was prepared by iodination using the chloramine-T method as reported previously.[28]

Synthesis of t-*Boc-L-tryptophanal and* t-*Boc*-N-*methyl amino acids.* *t*-Boc-L-Tryptophanal was synthesized from the corresponding *N*-methylhydroxamate by the method of Fehrentz and Castro.[29] Coupling of the protected

amino acid aldehyde by reductive alkylation was performed according to Coy *et al.*[30]

N-Methylation of *t*-Boc-L-Trp and *t*-Boc-Thr(OBzl) was carried out by methylation with CH_3I–NaH in tetrahydrofuran as described by Cheung and Benoiton.[31]

The purity of the final products was controlled by analytical HPLC [Nucleosil 120-3 C_{18} column; buffer A, water–0.1% TFA (v/v); buffer B, acetonitrile–0.1% TFA (v/v)] to contain less than 1% of the unmethylated *t*-Boc amino acid.

Synthesis of t-*Boc-2,3,4,5-tetrahydro-β-carboline-4-COOH (t-Boc-Tcc).* The cyclic Trp analog, Tcc (Fig. 1) was synthesized according to Harvey *et al.*[23] Briefly, 3.7 g of L-Trp (18.1 mmol) were dissolved in 250 ml of water (pH 6.5) and 10 ml of formaldehyde (37%) (v/v) were added. The solution was stirred overnight at room temperature. The white precipitate was filtered and dried to yield 3.84 g (17.7 mmol, 98%). The amino acid was protected with the *t*-Boc-group according to the procedure described by Stewart and Young.[32] Analytical HPLC [Nucleosil 120-3 C_{18} column; buffer A, water–0.1% TFA (v/v); buffer B, acetonitrile–0.1% TFA (v/v)] showed that the product contained less than 1% of impurities. As a control, Boc-Trp and Boc-Tcc were mixed (in ratio 2:1) and separated by analytical HPLC.

Preparation of galanin fragments. All peptides were synthesized manually on a phenylacetamidomethyl resin (Pam resin; 1 mmol g^{-1}).[33] The side-chain protective groups for *t*-Boc amino acids were Ser(OBzl), Thr(OBzl), Tyr(2-Br Z), His(Bom) and Trp(For). Tyr, His, Tcc, N-MeTrp and N-MeThr were coupled as hydroxybenzotriazole esters. All other amino acids were activated in a 1:1 ratio with *N,N'*-diisopropylcarbodiimide. The detailed protocol for solid-phase peptide synthesis has been published before.[34]

Removal of the formyl, benzyloxycarbonyl and benzyl ester groups were carried out by the 'low TFMSA' method of Tam[35] in a mixture of TFMSA, TFA, DMS, *p*-cresol and ethanedithiol [10:50:30:8:2, (v/v)]. The resin was washed and dried followed by the final removal of the Bom-protective group and the cleavage of the peptide from the resin with hydrogen fluoride containing 10% *p*-cresol at 0°C over 30 min.

Purification of the cleaved peptides was carried out by HPLC on a C_{16} reversed-phase column (Diasorb 130 C_{16} T, 14 μm) to more than 95% as determined by analytical HPLC. The molecular weight of the synthesized ligands was determined with a BIO-ION 20 (Applied Biosystems, USA) time-of-flight plasma desorption mass spectrometer using ^{252}Californium as the source of fission fragments. The observed molecular weights of the peptides were within the 0.1% error range of the instrument when compared with the calculated values.

Membrane preparation. For all studies Sprague–Dawley adult male rats (200 g) were used. Animals were decapitated, brains rapidly removed, hypothalami dissected and homogenized in 10% (w/v) sucrose–HEPES (20 mM) buffer (pH 7.4). Membrane preparations (10000 g, 45 min, P2) were obtained as previously reported[28] except that the final homogenization was performed in HEPES-buffered (20 mM) Krebs–Ringer solution containing bacitracin (1 mg ml^{-1}).

Ligand binding studies. The affinity of galanin analogs as ligands to the hypothalamic galanin receptor was determined in displacement experiments which made use of the filtration technique described earlier by Land *et al.*[28] using [^{125}I]galanin at sub-K_D concentration (0.2–0.3 nM) as the radioligand. Specific binding was defined as that of [^{125}I]galanin displaceable by different concentrations of the ligands studied.

The fitting of the experimental data to the mass-action equation was carried out on Macintosh II using a non-linear least-squares method of the 'Kaleidagraph' software package. The K_D values of the synthetic ligands were calculated from the IC_{50} values using the Cheng–Prusoff correction.[36]

Acknowledgements. This study was supported by the Swedish Board for Technical Development (STU/NUTEK), *Wenner–Gren Stiftelse* and *Ivar Bendixsons Stiftelse*. K. K. is the recipient of the scholarship from the Swedish Institute.

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Received October 5, 1993.