

NMDA-receptor antagonist requirements in conantokin-G

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Received 29 July 1998

Abstract A series of variants of the neuroactive 17-residue γ -carboxyglutamate (Gla)-containing polypeptide, conantokin-G (con-G), were synthesized with the intention of determining those features that were important for its *N*-methyl-D-aspartate (NMDA) receptor-targeted antagonist activity and for adoption of its divalent cation-dependent α -helical conformation. Employing the binding of [3 H]dizolcipine (MK-801) as an assay for open receptor ion channels in rat brain membranes, which displays inhibition by con-G (IC_{50} = 0.48 μ M), it was found that replacement by an Ala residue of Gla⁴ led to complete inactivation of the peptide, whereas a similar replacement of Gla³ resulted in a 20-fold decreased potency. Ala substitutions for Gla¹⁰ and Gla¹⁴ did not substantially affect [3 H]MK-801 binding. This same substitution at Gla⁷ appeared to slightly enhance binding. Ala replacements of non-Gla residues demonstrated that four of them, viz. Glu², Leu⁵, Gln⁹, and Ile¹², possessed at least 200-fold decreases in inhibitory potency, whereas similar replacements at Gly¹, Leu¹¹, and Arg¹³ resulted in peptides with 8- to 12-fold increases in the IC_{50} values. The remaining amino acid residues tested in the single Ala replacement series showed no significant changes in the inhibitory characteristics of wild-type con-G. Additional studies with carboxyl-terminal truncated peptides revealed that the carboxyl-terminal 4 amino acids were unimportant for this activity. There was no strict correlation of inhibition of [3 H]MK-801 binding with the ability of these peptides to form cation-dependent α -helices. Peptides with notably low α -helical content in the presence of these cations were lacking at least one, or both, of Gla¹⁰ and Gla¹⁴. Con-G[Gla^{3,4,7,10,14}E] and con-G[Gla^{7,10,14}E] were the only peptides that remained in a completely random conformation upon metal ion addition.

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Key words: Conantokin-G; *N*-Methyl-D-aspartate receptor; Ion channel; Polypeptide conformation; Metal binding; γ -Carboxyglutamate

1. Introduction

The conantokins are a group of peptides isolated from the venoms of *Conus* snails. The two most widely investigated members of this class are conantokin-G (con-G) and conantokin-T (con-T), peptides possessing 17 and 21 amino acids, respectively. Contained in these peptides is the amino acid γ -carboxyglutamate (Gla), at levels of 5 and 4 residues, respectively [1,2]. This structural feature allows the peptides to interact with a variety of metal cations [3–5]. The binding of divalent cations, such as Ca²⁺, Mg²⁺, and Zn²⁺, to con-G leads to a conformational change of the peptide, from a random structure to an end-to-end α -helix [3,4,6,7]. Similarly, adoption of a full α -helical conformation is found in con-T

as a result of binding of these cations [8]. However, in this case the change is less dramatic, since apo-con-T already possesses a high α -helical molecular subpopulation [9].

The *N*-methyl-D-aspartate (NMDA) receptor is a subclass of ionotropic glutamate-dependent receptors that is widely distributed in the mammalian CNS. Glutamate and glycine are coagonists of the Ca²⁺-permeable ion channel of this receptor [10,11], with polyamines among the agents displaying allosteric stimulation [12]. Abnormal activation of the ion channel has been associated with a variety of neuropathological states, such as acute neuronal death after ischemic stroke [13], and Ca²⁺-related neurodegeneration in Parkinson's [14] and Alzheimer's [15] diseases.

In the NMDA receptor system con-G and con-T specifically function as non-competitive inhibitors of ion flow through NMDA receptors. These peptides inhibit in a non-competitive manner polyamine-stimulated dizolcipine (MK-801) binding in rat brain membranes [8,16] with an IC_{50} \approx 500 nM, attenuate the NMDA-stimulated rise in neuronal intracellular Ca²⁺ (IC_{50} \approx 2 μ M) [2], decrease the NMDA-stimulated cGMP levels in rat cerebellar granule neurons (IC_{50} = 77–177 nM) [17], and inhibit glutamate-induced neurotoxicity [16]. The mechanisms of the inhibitory effects of the peptides have been attributed to their inhibition of the positive effector roles of spermine [16] and of glutamate and glycine [18,19].

Little is currently known regarding the functions of specific amino acid side chains in elaborating the functional effects of the conantokins. Available data for both con-G and con-T suggest that Gla¹⁰ and Gla¹⁴ are involved in stabilizing binding of the highest affinity metal ion site, and in governing the conformational change induced by multivalent cations [7,8]. The critical nature of Gla³ and Gla⁴ of con-T in inhibition of the spermine-induced potentiation of the binding of MK-801 to open ion channels of the NMDA-receptor was established, as well as the role of Gla⁴ in stabilizing the apo-con-T α -helical conformation [8]. Similarly, with regard to con-G, it was found that substitution at Gla³ and Gla⁴ reduced or eliminated MK-801 binding function, while replacement of Gla⁷, Gla¹⁰, and Gla¹⁴ did not affect NMDA antagonist activity of the resulting peptides [20]. In the light of these results, a systematic investigation of the effects of the structure-function relationships of these peptides is warranted. Thus, we have synthesized an array of synthetic variants of con-G and assessed the role of each amino acid in the NMDA receptor antagonist activity of con-G, as well as in the ability of the peptides to adopt the metal cation-dependent α -helical conformation. The results of this study are reported herein.

2. Materials and methods

2.1. Peptide synthesis, purification, and characterization

Syntheses of con-G (GE γ L₅Q γ NQ γ ₁₀LIR γ K₁₅SN-NH₂)-based peptides on a 0.1-mmol scale were accomplished on PAL resin supports

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(PerSeptive Biosystems, Framingham, MA, USA) using an Applied Biosystems (Foster City, CA, USA) model 433A peptide synthesizer as described previously [3]. The following side chain protecting groups were used: O-tBu for Glu, Glu, and Ser; Trt for Asn and Gln; Pmc for Arg; and Boc for Lys. The derivatized Glu was synthesized as described previously [21]. Peptides were cleaved from the support resin with 88% trifluoroacetic acid in the presence of radical scavengers dithiothreitol and triisopropylsilane. Purifications of the peptides were accomplished by either FPLC chromatography on a Bio-scale DEAE20 (Bio-Rad, Hercules, CA, USA) column or by reverse phase HPLC on a semi-prep Vydac TP218510 (10×250 mm), as previously reported [3]. The target material was lyophilized, and desalted on a Sephadex G-15 column (1.5×100 cm) that was equilibrated and eluted with 0.1% NH₄OH. The peptides were characterized by reverse-phase HPLC and delayed extraction-matrix assisted laser desorption/ionization-time of flight mass spectrometry (DE-MALDI-TOF) as described [3].

The concentrations of the peptides were determined by amino acid analysis and quantitative reverse phase-HPLC.

2.2. [³H]MK-801 binding assays

These assays were performed as previously described [8]. Adult Sprague-Dawley rats were sacrificed by decapitation and forebrains were removed and processed [16]. Inhibition assays were performed in triplicate in a total volume of 500 µl in 5 mM sodium-HEPES, 4.5 mM Tris-Cl, pH 7.4, in the nominal absence of glutamate and glycine, with varying concentrations of peptide. The final concentrations of [³H]MK-801 and spermine were 5 nM and 50 µM, respectively. Binding was initiated by the addition of 300 µl of membrane suspension, containing 100–200 µg protein. Incubations were carried out for 2 h at room temperature. Assays were terminated by rapid filtration over Whatman GF/B filters pretreated with 0.03% polyethyleneimine, using a 24-well cell harvester (Brandel, Gaithersburg, MD, USA). Basal [³H]MK-801 binding was defined as the amount of radioligand bound in the nominal absence of spermine. Observed enhancements in

[³H]MK-801 binding at 50 µM spermine were 60–300% over basal values, depending on the membrane preparation. Non-specific binding was determined in the presence of 50 µM MK-801 and represented 5–10% of total ligand bound in the presence of 50 µM spermine.

2.3. Circular dichroism (CD) spectroscopy

CD spectra of con-G analogues were collected at 25°C in 10 mM sodium borate/100 mM NaCl, pH 6.5, on an Aviv model 62DS spectrometer using a 1-cm pathlength cell. The peptide solutions and buffers used were treated with Chelex-100 resin prior to the experiments to ensure that they contained no multivalent metal ions. CD spectra of the peptides were acquired in 100% aqueous buffer with the above composition, or with the addition of either 20 mM CaCl₂ or 1.5 mM MgCl₂. The peptide concentration was 35 µM. The α-helical content was determined from mean residue ellipticities at 222 nm using the empirical relationship $f\alpha = (-[Q]222 - 2340)/30300$ [22].

3. Results

To test the importance of amino acid side chains in the properties of con-G, a series of con-G-based variant peptides were synthesized. These included peptides with single amino acid replacements to Ala of the first 14 amino-terminal residues, peptides that were truncated at the carboxyl-terminal end at each of the amino acids between 11 and 15, and several peptides in which one or more Glu residues were replaced with Glu, Lys, or Pro, in addition to Ala. For purposes of characterization of the purified peptides, their molecular weights were determined by DE-MALDI-TOF. These values are listed in Table 1.

The NMDA-receptor antagonist activity of each peptide

Table 1
NMDA-Receptor antagonist and conformational properties of con-G analogues

Peptide	Mol. wt. ^a	IC ₅₀ (µM) ^b	% α-helix ^c		
			Apo	Ca ²⁺	Mg ²⁺
Con-G	2264.8 (2265.2)	0.48 ± 0.03	2	50	72
Con-G[G ¹ A]	2278.1 (2278.7)	6.5 ± 1.3	0	32	58
Con-G[E ² A]	2205.9 (2206.6)	> 100 (0)	2	42	68
Con-G[Gla ³ A]	2159.4 (2162.7)	9.6 ± 1.5	5	31	44
Con-G[Gla ⁴ A]	2158.8 (2162.7)	> 100 (0)	0	36	59
Con-G[L ⁵ A]	2224.9 (2222.5)	> 100 (0)	0	35	57
Con-G[Q ⁶ A]	2206.9 (2207.6)	0.51 ± 0.05	0	35	57
Con-G[Gla ⁷ A]	2164.0 (2162.7)	0.12 ± 0.02	5	24	32
Con-G[Gla ⁷ K]	2218.8 (2219.8)	0.22 ± 0.03	10	32	38
Con-G[Gla ⁷ P]	2189.0 (2188.7)	> 100 (0)	1	14	26
Con-G[N ⁸ A]	2223.2 (2221.6)	0.49 ± 0.05	2	44	72
Con-G[Q ⁹ A]	2206.3 (2207.6)	> 100 (38) ^d	0	36	60
Con-G[Gla ¹⁰ A]	2162.0 (2162.7)	2.0 ± 0.3	7	14	31
Con-G[Gla ¹⁰ E]	2219.4 (2220.7)	1.7 ± 0.1	2	10	29
Con-G[L ¹¹ A]	2223.8 (2222.5)	5.9 ± 1.3	0	37	62
Con-G[I ¹² A]	2223.0 (2222.5)	> 100 (15) ^d	0	28	57
Con-G[R ¹³ A]	2181.1 (2179.5)	3.4 ± 0.5	0	15	45
Con-G[Gla ¹⁴ A]	2163.6 (2162.7)	0.23 ± 0.03	11	21	39
Con-G[Gla ^{10,14} A]	2061.4 (2060.8)	0.71 ± 0.11	5	12	24
Con-G[Gla ^{7,10,14} E]	2132.9 (2132.9)	4.7 ± 0.6	2	2	3
Con-G[Gla ^{3,4,7,10,14} E]	2044.4 (2045.1)	> 100 (0)	4	4	6
Con-G[1–15]	2064.3 (2063.5)	1.2 ± 0.08	0	27	48
Con-G[1–14]	1938.1 (1935.3)	5.9 ± 1.5	0	17	51
Con-G[1–13]	1763.6 (1762.3)	2.9 ± 0.3	0	4	18
Con-G[1–12]	1608.4 (1606.1)	> 100 (30)	0	6	16
Con-G[1–11]	1492.6 (1492.9)	> 100 (0)	0	5	17

^aThe observed (and calculated) molecular weights as determined by DE-MALDI-TOF mass spectrometry.

^bValues represent the peptide concentration needed to achieve 50% inhibition of the spermine potentiation of [³H]MK-801 binding to rat brain membranes. Data are mean ± standard error of three experiments. The numbers in parentheses represent the % inhibition of [³H]MK-801 binding at 100 µM peptide.

^cSecondary structure content of con-G analogues in the absence and presence of Ca²⁺ and Mg²⁺. α-Helicity values were determined by CD measurements.

^dThe inhibition reached 100% at suitably high concentrations of peptide.

was measured with the [^3H]MK-801 binding assay. This NMDA receptor specific open channel blocker has been used widely as a radioligand in binding assays to monitor the effects of various modulators of the NMDA receptor [23]. Conantokins have been shown to be capable of inhibiting the spermine-enhanced [^3H]MK-801 binding to rat brain membranes in a dose-dependent manner [8,16]. Representative plots of the inhibition of the spermine-induced phase of [^3H]MK-801 binding by wild-type con-G, con-G[Gla 3 A], con-G[Gla 4 A] and con-G[R 13 A] are shown in Fig. 1. The IC $_{50}$ values obtained from data of this type for each peptide are listed in Table 1.

Two out of the five Gla residues appear to play a significant role in the bioactivity of con-G. The Ala replacement of Gla 3 resulted in a peptide with 20-fold decreased inhibitory activity, whereas the same replacement at Gla 4 resulted in a peptide that lacked inhibitory properties up to a concentration of 100 μM . Ala replacements at Gla 7 , Gla 10 , and Gla 14 effect only small changes in the IC $_{50}$ values.

Examination of single Ala replacements of non-Gla residues demonstrated that four of them, viz., Glu 2 , Leu 5 , Gln 9 , and Ile 12 , led to dramatic decreases of inhibitory potency. These peptides showed less than 50% inhibition at their highest experimental concentrations (100 μM). Therefore, these analogues are at least 200-fold less potent than con-G with respect to inhibition of [^3H]MK-801 binding. Ala replacements of three additional residues, Gly 1 , Leu 11 , and Arg 13 revealed significant contributions to the inhibitory properties of con-G. In these cases, Ala replacements resulted in peptides that displayed 8- to 12-fold increases in the IC $_{50}$ values. The remaining amino acid residues tested in the single Ala replacement series showed no significant changes in the inhibitory characteristics of wild-type con-G. These residues include Gln 6 and Asn 8 . Additional studies with carboxyl-terminal truncations to peptides containing 15, 14, and 13 amino acids from the amino-terminus and onward of con-G resulted in 2-fold, 12-fold, and 6-fold increases in IC $_{50}$ respectively. Further trunca-

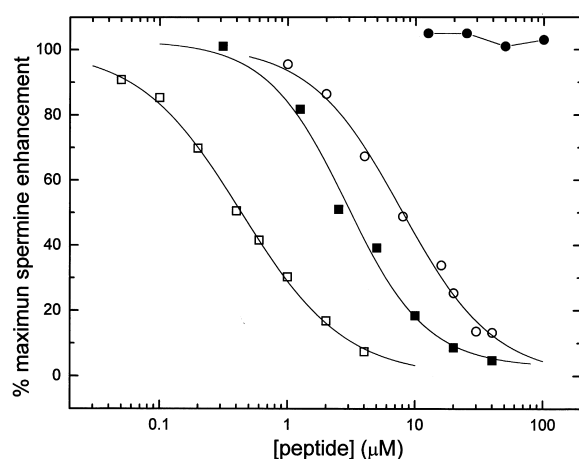


Fig. 1. Inhibition of the polyamine enhanced [^3H]MK-801 binding by con-G analogues. Assay results are presented in a normalized form, 100% being bound ligand in the presence of spermine (50 μM) and 0% being basal binding (no added spermine). Parameter values were fit by non-linear regression using Microcal Origin software. (\square) Con-G; (\circ) con-G[Gla 3 A]; (\bullet) con-G[Gla 4 A]; (\blacksquare) con-G[R 13 A]. These representative experiments were repeated three times with similar results.

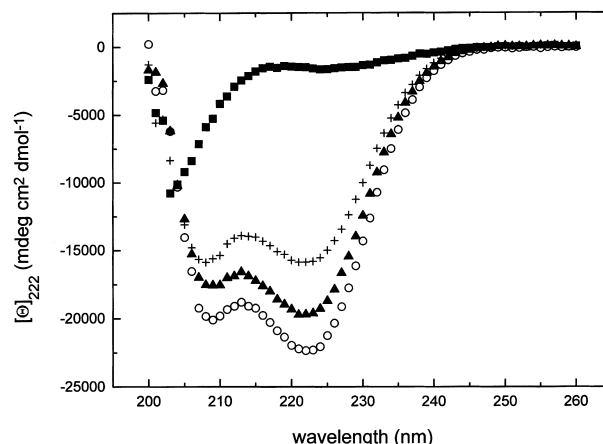


Fig. 2. CD spectra of con-G analogues. Spectra were recorded at 25°C in 10 mM sodium-borate, pH 6.5, 100 mM NaCl, 0 or 1.5 mM MgCl $_2$. (\blacksquare) Con-G, no Mg $^{2+}$; (\circ) con-G+Mg $^{2+}$; (\blacktriangle) con-G[Gla 12 A]+Mg $^{2+}$; (+) con-G[R 13 A]+Mg $^{2+}$.

tions to 12 or 11 residues resulted in at least 200-fold increases in the IC $_{50}$ values.

Two variants with multiple Gla to Glu replacements have largely decreased antagonist activities, as revealed by this same assay. The con-G variant, in which all five Gla residues were altered to Glu, had a higher than detectable ($\gg 100 \mu\text{M}$) IC $_{50}$. A similar analogue, with two Gla residues at positions 3 and 4, and Glu residues at Gla 7 , Gla 10 and Gla 14 , possesses a 10-fold decreased potency as compared to con-G. A Gla to Lys replacement at position 7 is well-tolerated. Moreover, this peptide displays an IC $_{50}$ value that is slightly lower than that of wild-type con-G. This is similar to the behavior of con-G[Gla 7 A]. However, a Pro substitution at Gla 7 leads to a greatly diminished potency.

Since con-G undergoes a large metal cation-induced conformational change, it is also relevant to examine the extent to which this occurs with the synthetic variants. The secondary structure of each variant was evaluated by CD spectroscopy, both in the absence and presence of divalent cations. Fig. 2 illustrates the CD spectra of con-G, con-G[Gla 12 A], and con-G[R 13 A]. Wild-type con-G as well as its analogues show a random conformation in their cation-free apo forms. The CD spectra of cation-bound peptides are characterized by varying degrees of α -helicity, with the wild-type peptide displaying more than 70% α -helix in the Mg $^{2+}$ bound form.

Table 1 summarizes the α -helical contents of con-G variants in the apo, Ca $^{2+}$ -bound and Mg $^{2+}$ -bound states. All apo-peptides possess none-to-very-low ($< 11\%$) α -helicity. Metal cation-bound peptides possess varying degrees of α -helical secondary structure. Peptides with notably low α -helical content in the presence of these cations are lacking at least one, or both, Gla residues at positions 10 and 14. Con-G[Gla 3,4,7,10,14 E] and con-G[Gla 7,10,14 E] are the only peptides that remain in a completely random conformation upon metal ion addition.

4. Discussion

Several amino acids contained in the primary structure of con-G have been identified as contributors to its NMDA receptor inhibitory activity and replacement of these residues

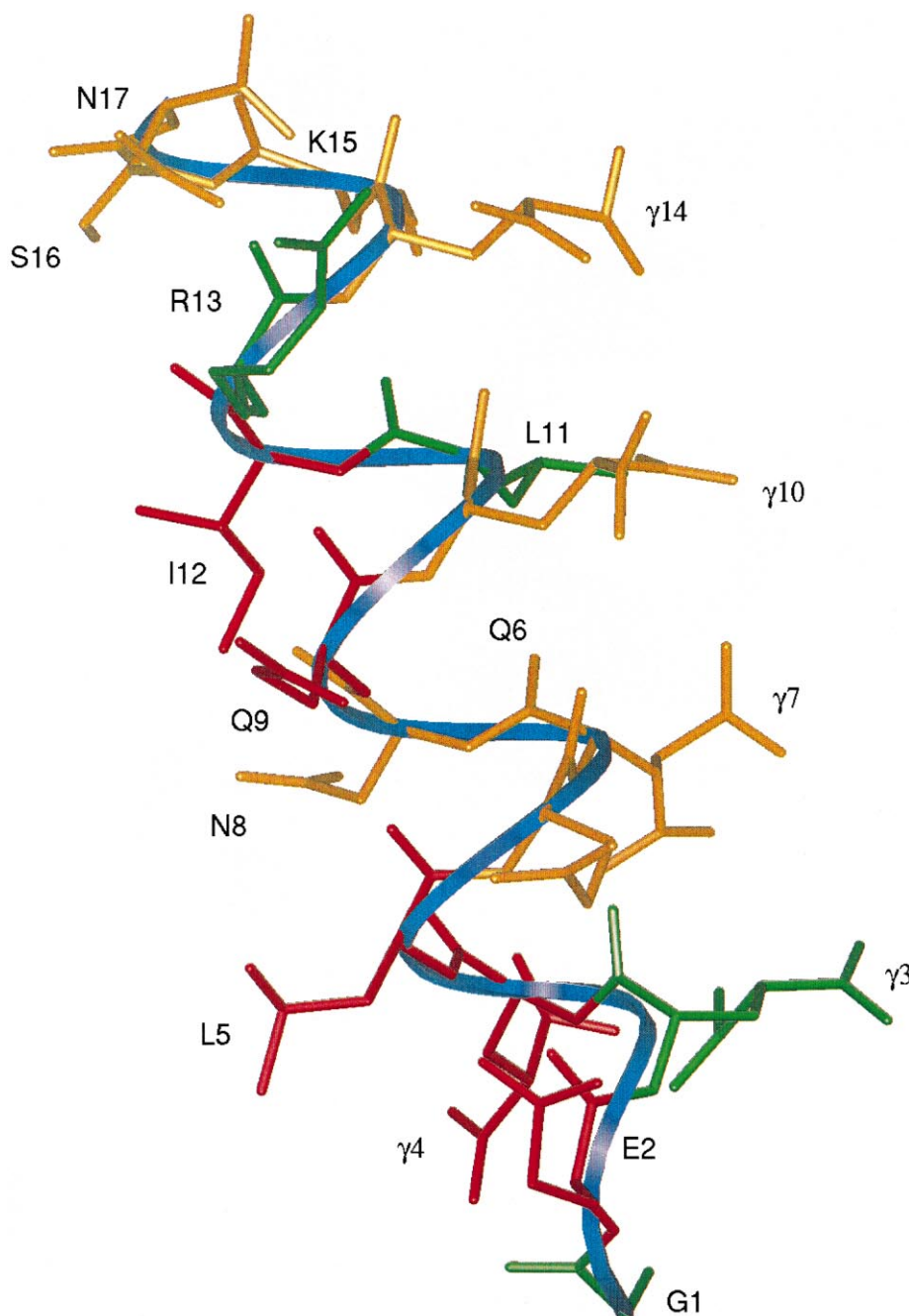


Fig. 3. Role of side chains in the NMDA receptor antagonist action of con-G. This three dimensional structure of con-G was determined in the presence of Mg^{2+} by NMR spectroscopy [7]. Amino acids are color coded according to importance in NMDA receptor binding. Residues in red: essential, when replaced by Ala, potency decreases by at least 200-fold. Residues in green: important, when replaced by Ala, potency decreases by 8–20-fold. Residues in orange: not important, when replaced by Ala, potency does not decrease substantially.

with Ala resulted in a significant reduction in the potency of these variants. As seen from the data of Table 1, replacement of any one of five residues by Ala, that includes Glu², Glu⁴, Leu⁵, Gln⁹, and Ile¹², resulted in an at least 200-fold increase in the IC_{50} value that is associated with the spermine-induced stimulatory phase of [MK-801] binding to open channels of the NMDA receptor. Another group of residues, viz. Gly¹, Glu³, Leu¹¹, and Arg¹³, displayed more minor involvement in function, in that their Ala variants reduced the inhibitory potency of con-G by 8- to 20-fold. The remainder of the residues directly tested, that included Gln⁶, Glu⁷, Asn⁸,

Glu¹⁰, and Glu¹⁴, did not show significant effects on the NMDA receptor inhibitory properties of con-G upon their replacement by Ala as revealed by the spermine-induced [³H]MK-801 binding assay.

These results indicated that amino-terminal residues were heavily involved in this function of con-G, with lesser direct involvement of carboxyl-terminal residues. Thus, a series of carboxyl-terminal truncated variants of con-G were synthesized and tested in this manner. The results (Table 1) showed that there are no essential residues beyond Arg¹³, since con-G containing only the amino-terminal 13 amino acids (con-G[1–

13]) still displayed an IC_{50} that was only 6-fold higher than that of wild-type con-G. Con-G[1–12] and con-G[1–11] proved to be insufficient for inducing the effects of con-G, up to a 100- μ M concentration of these peptides. The latter is consistent with the requirement for Ile¹² that was identified by Ala replacement in a synthetic variant.

Two Gla residues, Gla¹⁰ and Gla¹⁴, that have previously been identified to form the tight metal cation binding site, do not appear to play a role in receptor binding. Ala replacements of each of these residues do not result in significant reductions in bioactivity. Significantly, however, in both of these cases, metal ion binding is compromised substantially [7], and α -helicity is decreased compared to con-G. Further, replacing with Ala both Gla¹⁰ and Gla¹⁴ in the same con-G variant (con-G[Gla^{10,14}A]) causes a slight decrease in bioactivity, although α -helicity is significantly decreased. Also revealing are the results obtained with a variant peptide in which all three Gla residues, at positions 7, 10, and 14, were replaced with Glu (con-G[Gla^{7,10,14}E]). This peptide possessed a 10-fold increased IC_{50} in the [³H]MK-801 binding assay. Interestingly, this same peptide displayed virtually no α -helicity, even in the presence of multivalent cations. The con-G analog in which all five Gla residues were replaced with Glu (con-G[Gla^{3,4,7,10,14}E]) did not retain bioactivity. This result provides additional evidence of the essential nature of Gla⁴, as suggested by the Ala scan results. In this case, even a very conservative variation at this residue severely compromises bioactivity of the peptide.

The amino acid at position 7 is radically different in con-G (Gla) and con-T (Lys), and this residue has been predicted to stabilize the apo α -helix in con-T and destabilize such a conformation in con-G. In con-T, Lys⁷ can interact with both Gla³, Gla⁴, and Gla¹⁰ forming essential (*i, i+3* and (*i, i+4*) interactions, thus stabilizing its α -helical structure. On the other hand, the doubly negatively charged Gla⁷ in con-G contributes to charge repulsion in the apo-form and to metal binding/ α -helix formation in the presence of multivalent cations [7], which is readily observed by CD measurements (Table 1). Correspondingly, both Ala or Lys replacements of Gla⁷ in con-G result in peptides with IC_{50} values slightly lower than that of the wild-type peptide. However, the Gla⁷Pro replacement abolishes the bioactivity of con-G. This peptide also possesses a low α -helical content in the presence of cations (Table 1). The presence of the strong α -helix destabilizing Pro residue in the middle of the sequence not only causes a major disruption in the α -helical backbone, but likely also induces new local conformations in con-G.

A report has been published regarding the NMDA receptor antagonist and conformational properties of a series of con-G derived peptides that contained single amino acid replacements at each of the five Gla residues [20]. These included five Ala peptides that are also included in the present study. The NMDA antagonist activity of these peptides was also determined in this case by measuring the inhibition of the spermine enhanced [³H]MK-801 binding to rat brain membranes. The observed IC_{50} values for the identical molecules are very similar in both of these studies. Our IC_{50} values are consistently about two-fold higher, most likely due to minor differences in assay conditions and/or membrane preparations.

Results of the present study also parallel an earlier report on Gla to Ala replacement variants of con-T [8]. That study

also identified Gla³ and Gla⁴ as the only important bioactivity determinants out of the four Gla residues present in con-T. This correlation, along with the very similar pharmacology, wild-type IC_{50} values, and high chemical homology of the two conantokin peptides, suggests that they have a common mode of action in the inhibition of the NMDA receptor, despite major differences in the conformations adopted by their apo-forms [7,8].

In order to examine the conformational properties of the con-G analogues in greater detail, the secondary structure of each variant was determined by CD spectroscopy both in the absence of multivalent metal ions and in the presence of either Ca²⁺ or Mg²⁺. The Mg²⁺-dependent α -helicity values were determined at 1.5 mM Mg²⁺, a concentration that is comparable to the physiological concentration of this ion. Based on considerations of binding affinities and physiologically available free concentrations of multivalent metal ions, the conantokin exist in Mg²⁺ and/or Zn²⁺ bound states [4,5]. Maximal α -helicities obtainable in the presence of Zn²⁺ were always very close to the Mg²⁺ values, and, therefore, only the latter are reported.

Almost all con-G variants exhibited the cation-dependent conformational change, although maximal helicity values varied. There was no strict correlation observed between α -helicity of the conantokin analogues in solution and their abilities to inhibit MK-801 binding to the NMDA receptor. Several peptides have decreased ability to adopt the α -helical conformation when cations are added, but display wild-type-like IC_{50} values. These variant peptides include con-G[Gla⁷A], con-G[Gla⁷K] and con-G[Gla¹⁴A]. Several peptide variants with substantially reduced potency possess an α -helical secondary structure content that is comparable to the above mentioned fully active molecules. Con-G[Gla^{7,10,14}E], a peptide with a complete lack of secondary structure in the presence of metal cations, possesses an IC_{50} that is only 10-fold higher than its wild-type counterpart.

An illustrative summary of the importance of each con-G residue in its NMDA antagonist activity is provided in Fig. 3. Residues indicated in red lead to a very substantial loss of activity when replaced by Ala. Due to the high extent of their involvement in this property, we propose that these residues directly interact with the binding site for con-G on the NMDA receptor. Considering that both con-G and con-T exist essentially in end-to-end α -helical conformations under physiological conditions of cation concentrations, this model for the interaction interface between con-G and its binding site seems very plausible, as all of the essential residues are clustered on a well defined surface area comprising of the N-terminal turn and one side of the α -helix along Leu⁵, Gln⁹ and Ile¹². All of the synthetic con-G analogues that retained NMDA receptor inhibitory activity possess considerable α -helix forming capabilities. The only exception from this observation seems to be con-G[Gla^{7,10,14}E]. This peptide is in a random conformation at close to physiological solution conditions. Its 10-fold decreased potency can result from receptor-facilitated folding of this peptide, after initial contact by critical amino acid residues. Further support for this consideration is provided by the fact that the introduction of the α -helix breaker Pro at position 7 leads to a complete loss of activity. The β -turn-favoring Pro residue effectively prevents the receptor-induced α -helix formation in the region important for binding, although otherwise this peptide, like

con-G[Gla^{7,10,14}E], possesses every amino acid side chain that was identified as required or important in the NMDA receptor antagonist activity of con-G.

In summary, individual amino acids of con-G have been examined in order to identify those that play a critical role in the NMDA receptor antagonist activity of this natural molecule. Five residues, Glu², Gla⁴, Leu⁵, Gln⁹, and Ile¹², have proven to be required for activity. In addition, four additional amino acids, Gly¹, Gla³, Leu¹¹, and Arg¹³, showed additional contributions, whereas the remainder of the side chains of this 17-amino acid peptide is not important in its inhibition of the NMDA receptor. Overall, we have demonstrated that the α -helix forming capabilities of most of the variant peptides are not compromised significantly with alterations in individual residues. However, exceptions are observed with peptides that are missing one or both of Gla¹⁰ and Gla¹⁴, in that these variants have low maximal metal ion bound α -helicity. These two amino acids have been identified as the contributors of the primary metal ion binding site in both con-G and con-T [7,8]. Based on these bioassay results, it would appear that an intact metal binding site at this locus is not an absolute requirement for the neuroactivity of the conantokins, although such a metal binding site does provide an increased rigidity to these short, otherwise unconstrained peptides. Alternatively, the significance of metal binding and highly α -helical conformations of wild-type conantokins may lie in advantages other than increased receptor affinity, such as increased half-life in both the venom duct and in the body of the prey, solubility and diffusion parameters, or processing subsequent to post-translational modifications.

Acknowledgements: These studies were supported by Grant HL-19982 from the National Institutes of Health (to F.J.C.), the Kleiderer-Pezold family endowed professorship (to F.J.C.), and a predoctoral fellowship from the American Heart Association, Indiana Affiliate (to T.B.).

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