Calcium Binding Properties of Synthetic γ-Carboxyglutamic Acid-Containing Marine Cone Snail "Sleeper" Peptides, Conantokin-G and Conantokin-T†

Mary Prorok, Scott E. Warder, Tamas Blandl, and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

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ABSTRACT: Total chemical synthesis of two Conus-derived peptides, conantokin-G (con-G), a 17-residue polypeptide containing five residues of γ-carboxyglutamic acid (Gla), and conantokin-T (con-T), a 21-residue polypeptide possessing four residues of Gla, was accomplished. Calcium binding isotherms were obtained for each peptide, and these differed considerably from each other. The binding isotherm for con-G was complex and could only be fit to degenerate models involving multiple Ca^{2+} binding sites. The data for Ca^{2+} binding to con-T was uniquely fit to a simple one-site model. In the case of con-G, circular dichroism (CD) studies revealed a polypeptide without observable α-helicity in the absence of Ca^{2+} and a dramatic shift to a high degree of α-helix at saturating Ca^{2+} concentrations. In contrast, apo-con-T possessed significant α-helical structure, and saturation with Ca^{2+} produced a less substantial change in its α-helical content. Titrations with Ca^{2+} of the change in α-helical content of con-T produced a C_{50} value for Ca^{2+} that was essentially the same as its K_d from direct binding studies, demonstrating that occupancy of the single macroscopic binding site resulted in the conformational change. Similar titrations with con-G provided a C_{50} value in concert with the K_d for binding of Ca^{2+} to this peptide. Moreover, in agreement with these particular Ca^{2+}-induced structural changes, gel filtration analyses demonstrated significantly reduced hydrodynamic volumes of both of these polypeptides after saturation of their apo forms with Ca^{2+}, with con-G showing a more pronounced change than con-T. One-dimensional ^{1}H-NMR spectra showed both line broadening and changes in chemical shifts of several peptide amide proton resonances after addition of Ca^{2+} to con-G, again suggestive of a large Ca^{2+}-induced conformational change in this polypeptide. A derivative of con-G was synthesized with all amino acids present in the D-configuration (D-con-G). This variant peptide displayed Ca^{2+} binding isotherms nearly identical to those of con-G and underwent a Ca^{2+}-induced conformational change very similar to that of con-G. Intracranial injections of con-G and con-T in young (<2 weeks) and older (3−4 weeks) mice produced the expected "sleep-like" and hyperactive effects, respectively. The variant, D-con-G, was inactive in these assays. These studies demonstrate that synthetic con-G and con-T possess their expected bioactivities and undergo large and defined conformational alterations in the presence of Ca^{2+}. We propose that binding of Ca^{2+} to these polypeptides contributes to their ability to adopt a defined conformation, and this divalent cation-dependent conformation is necessary for their neuroactivities.

Predator snails of the Conus family possess genetic material that encodes small peptides which are bioactive in the predator−prey relationship. Approximately 500 species of cone sea snails that feed on fish, mollusks, and polychaete worms have been identified. A variety of polypeptides are employed as paralytic toxins by the snails, many of which inhibit communication between motor neurons and skeletal muscle *via* effects on nicotinic ACh[1] receptors (α-conotoxins), muscle sodium channels (μ-conotoxins), and voltage-sensitive neuronal calcium channels (ω-conotoxins). These peptidic conotoxins are injected into the prey by envenomation and result in rapid paralysis, thus facilitating the feeding process for the snails [reviewed in Olivera et al. (1991) and Myers et al. (1993)].

Other types of neuroactive Conus peptides exist, among which are the "sleeper" peptides, con-G and con-T, that are found in the venoms of *Conus geographus* (McIntosh et al., 1984) and *Conus tulipa* (Haack et al., 1990), respectively. The bioactivity of these peptides is characterized by production of a sleep-like state in young mice and a hyperactive state in older mice (Rivier et al., 1987). On a molecular level, these conantokins act on ligand-gated Ca^{2+} channels in the CNS and specifically function as antagonists of glutamate/glycine receptors of the NMDA subclass, a target of modern stroke drug development. These peptides inhibit NMDA-stimulated increases in neuronal intracellular Ca^{2+}

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* Correspondence: Professor Francis J. Castellino, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556. Telephone: (219) 631-6456. Fax: (219) 631-8149. E-mail: castellino.1@nd.edu.

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[1] Abbreviations: ACh, acetylcholine; con-G, conantokin-G with all amino acids in the L-configuration; D-con-G, con-G with all amino acids in the D-configuration; con-T, conantokin-T with all amino acids in the L-configuration; Gla, γ-carboxyglutamic acid; NMDA, *N*-methyl-D-aspartate; DE−MALDI−TOF, delayed extraction−matrix-assisted laser desorption ionization−time of flight mass spectrometry; HPLC, high-performance liquid chromatography; CD, circular dichroism; Fmoc, 9-fluorenylmethyloxycarbonyl; O-tBu, *tert*-butyl ester; O-Bzl, benzyl ester; tBoc, *tert*-butyloxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PAL, 5-[4-[[(9-fluorenylmethyloxycarbonyl)-amino]methyl]-3,5-dimethoxyphenoxy]valeric acid; Trt, trityl.

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(Haack et al., 1990) and cGMP levels (Mena et al., 1990), as well as glutamate-induced neurotoxicity (Skolnick et al., 1994). The mechanism of these antagonist effects has been attributed to attenuation by these peptides of the positive effector roles of polyamines at NMDA receptors (Skolnick et al., 1992; Chandler et al., 1993). The relationships between these molecular events and the *in vivo* activities of the conantokins are not as yet well understood.

A most interesting chemical feature of con-G and con-T is their high relative content of Gla. Specifically, con-G, a 17-residue polypeptide, contains five Gla residues per mole, and con-T, a 21-residue polypeptide, possesses Gla residues in a molar ratio of 4:1. All known Gla-containing proteins and peptides, especially those composed of paired Gla residues, bind Ca^{2+} in a functionally relevant manner. Thus, this characteristic implicates Ca^{2+}, and/or other divalent cations, as an important structural and/or functional element in defining the bioactivities of con-G and con-T. Since small polypeptides normally equilibrate among a spectrum of conformations (Davis et al., 1993; Sevilla et al., 1993; Hill et al., 1996), and since it would be expected that a stable and defined conformation would be needed for very tight and discriminate receptor binding, we believed that Ca^{2+} should be important in stabilizing unique conformations of these peptides. Therefore, we have investigated the Ca^{2+} binding properties of con-G and con-T and the effect of this cation on their conformational states. The results of this study are reported herein.

MATERIALS AND METHODS

Materials. N^{α}-Fmoc(γ,γ'-di-O-tBu)-L-Gla and N^{α}-Fmoc-(γ,γ'-di-O-tBu)-D-Gla-OH were chemically synthesized as previously described (Marki et al., 1977a,b; Colpitts & Castellino, 1993). N^{α}-Fmoc-D-Ile-OH was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). All other N^{α}-Fmoc-L-amino and N^{α}-Fmoc-D-amino acids required to synthesize the peptides were purchased from either the Sigma Chemical Co. (St. Louis, MO) or Nova Biochem (La Jolla, CA). The following amino acids contained the specified side chain protecting groups: Glu (O-tBu), Tyr (O-tBu), Ser (O-tBu), Lys (N^{ϵ}-tBoc), Gln (N^{δ}-Trt), Asn (N^{γ}-Trt), and Arg (N^{G}-Pmc).

Peptide Synthesis. The peptides (0.1 mmol scale) were synthesized by automated solid state methodology on a PAL resin support (PerSeptive Biosystems, Framingham, MA), using an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer. Each residue was double-coupled using the manufacturer's protocols for *FastMoc* chemistry with the exception of the Gla derivatives which were single-coupled for 90 min at a 5-fold molar excess. Deprotection of blocking groups and cleavage from the resin were effected using TFA/H_2O/thioanisole/ethanedithiol/phenol (40:2:2:1:3, v/v/v/v/w) for 2 h at room temperature. The resin was separated from the peptide deprotection cocktail by filtration, and the TFA was removed *in vacuo* by rotary evaporation. Cold diethyl ether was added to the filtrate to precipitate the peptides. Following filtration and several ether washes, the crude peptide products were dissolved in water and lyophilized.

Peptide Purification. Purification of each peptide was accomplished by ion-exchange chromatography on a 1.5 cm $\times$ 20 cm column of DEAE-Sephadex A-25 resin (Pharmacia Biotech, Inc., Piscataway, NJ) equilibrated in 10 mM sodium borate/150 mM NaCl at pH 8.0. A 400 mL linear gradient from 10 mM sodium borate/150 mM NaCl at pH 8.0 (start solvent) to 10 mM sodium borate/500 mM NaCl at pH 8.0 (limit solvent), was applied. Fractions corresponding to the major peak were pooled and lyophilized. Desalting was carried out on a 1.5 cm $\times$ 100 cm bed of Sephadex G-15 (Pharmacia Biotech, Inc.) equilibrated in 0.1% NH_4OH. Pooled peptide fractions were lyophilized to constant weight and dissolved in water at final concentrations of 15−20 mM. These stock solutions were retained at −20° C until needed.

Peptide Characterization. The purity of the peptides was assessed by reverse-phase HPLC using a 5 mm Vydac 218TP column (4.6 mm $\times$ 250 mm) equilibrated in a solution containing 95% of 0.1% TFA in H_2O and 5% 0.1% TFA in CH_3CN at a flow rate of 1.0 mL/min. At a time of 3 min after injection onto the column, a 60 min linear gradient was implemented to a limiting value of 60% of 0.1% TFA in H_2O and 40% of 0.1% TFA in CH_3CN. Absorbance detection was performed at 215 nm.

The synthetic peptides were further characterized by DE−MALDI−TOF on a Voyager-DE spectrometer (PerSeptive Biosystems). Samples were prepared by mixing 0.5 μL of a 10^{-4}−10^{-5} M aqueous solution of peptide and 0.5 μL of 50 mM α-cyano-4-hydroxycinnamic acid in 50% CH_3CN/0.1% TFA on a 100-well sample plate. The 1 μL drops were air-dried and analyzed in the instrument. The samples were irradiated with a nitrogen laser (337 nm, 4 ns pulse time). The instrument was operated in the delayed extraction mode (Vestal et al., 1995). The accelerating voltage was +20 or −20 kV. Signal transients were recorded with a digitizer at a time resolution of 5 ns. Spectra were generated from the sum of 50−100 laser pulses using external calibration. The estimated weight accuracy over the observed weight range was ±0.05%. Linear mode positive and negative ionizations were used.

Gla Analysis. The Gla content of the peptides was established by amino acid analysis using alkaline hydrolysis of the peptide samples (Kuwada & Katayama, 1983). These analyses, using HPLC methodology, were carried out after precolumn derivatization with *o*-phthaldehyde/mercaptoethanol, employing a 2.0 mm (inside diameter) $\times$ 250 mm reverse-phase C8 (5 μm) column at 46 °C. A Hewlett-Packard (Palo Alto, CA) 1090L liquid chromatography system was employed with an absorbance filter detector (340 nm). The gradient used for elution consisted of various combinations of two solvent systems. Solvent A contained 110 mL of 1 M sodium acetate (pH 7.2), 95 mL of methanol, 5 mL of tetrahydrofuran, and 790 mL of H_2O. Solvent B consisted of 100% methanol. The column was equilibrated against 95% A/5% B. After application of the hydrolysate, the first gradient used immediately stepped the solvent to 85% A/15% B (time = 0). From 0−10 min, a linear gradient to a limit of 80% A/20% B was applied, followed by continued elution with 80% A/20% B for another 5 min. Under these conditions, Gla was eluted at 3.53 min with baseline separation from neighboring peaks, Asp at 4.98 min, and Glu at 5.95 min. Remaining materials were then batch eluted from the column with 2% A/98% B for 5 min, after which column start buffer (95% A/5% B) was applied for 5 min. The Gla content was determined using a standard synthetic Gla-containing peptide as described earlier (Zhang & Castellino, 1990).

Calcium Binding. The Ca^{2+}-binding isotherms for each peptide were determined potentiometrically at 25 °C using a Ca^{2+}-selective electrode (Orion Research, Inc., Boston, MA) with a single-junction Ag/AgCl reference electrode. Peptide samples were dissolved in 3 mL of 10 mM sodium borate/100 mM NaCl at pH 8.0 to a final concentration of 2.0 mM. The samples were then treated with Chelex-100 (Bio-Rad Laboratories, Hercules, CA) prior to titration with the appropriate concentration range of $CaCl_2$ in the previously indicated buffer. Electrode calibrations were performed immediately before and after titration of the sample. Slopes of 29.0 ± 0.5 mV per decade of Ca^{2+} concentrations were typically obtained and were subsequently used for the determination of free Ca^{2+} in the sample titrations. Treatment of the data by Scatchard analysis was carried out as previously described (Colpitts & Castellino, 1994).

Circular Dichroism. CD spectra were recorded between 190 and 260 nm on an AVIV model 62DS spectrometer. Peptides were dissolved in 10 mM sodium borate/100 mM NaCl at pH 8.0 to a final concentration of 2.0 mM (con-G and D-con-G) or 1.5 mM (con-T). A 0.01 cm path length cell was employed. Each spectrum represents the average of five scans collected at a 1.0 nm bandwidth at 1.0 nm intervals. Mean residue ellipticities were calculated using a mean residue molecular weight of 133 for con-G and D-con-G and 127 for con-T. The α-helical content was determined from the mean residue ellipticities at 222 nm according to a previously published method (Chen et al., 1972). Titrations of changes in ellipticity at 222 nm as a function of the Ca^{2+} concentration were performed in a 1.0 cm path length cell at peptide concentrations of 35 μM in 10 mM sodium borate/100 mM NaCl at pH 8.0. Titrations of changes in ellipticity at 222 nm as a function of peptide concentration at 40 mM Ca^{2+} (10 mM sodium borate/100 mM NaCl at pH 8.0) were performed using path lengths of 0.01, 0.2, or 1.0 cm, changed as required for signal optimization.

Gel Filtration Chromatography. Size exclusion experiments on the conantokins were carried out on a Superdex 10/30 peptide column (Pharmacia Biotech, Inc.) equilibrated in either Chelex-treated 10 mM sodium borate/100 mM NaCl at pH 8.0 or 10 mM sodium borate/100 mM NaCl/100 mM $CaCl_2$ at pH 8.0. Peptide solutions (20 μM) were injected onto the column through a 0.5 mL injection loop at a flow rate of 0.2 mL/min. Detection was performed at 214 nm.

^{1}H-NMR. The samples for one-dimensional ^{1}H-NMR experiments were prepared by dissolving 10 mg of con-G in 450 μL of an aqueous solution containing 10 mM sodium borate/100 mM NaCl at pH 8.0 with either 0, 20, or 40 mM $CaCl_2$. A volume of 50 μL of ^2H_2O was then added to provide the deuterium lock for the spectrometer. The pH was adjusted to 6.5, using a dilute solution of HCl.

^{1}H-NMR spectra were resolved on a Bruker AMX-500 MHz NMR spectrometer with a proton frequency of 500.13 MHz. The H_2O resonance was suppressed by presaturation. The spectra were acquired at 25 °C without spinning over a 6024 Hz sweep width with 16K data points. A total of 256 scans were collected for samples containing 0, 20, and 40 mM $CaCl_2$.

Bioassays. The purified peptides were administered to mice by intracerebral injection into the right hemisphere of the brain using a 26 gauge (pre-2-week-old mice) or 25 gauge (post-3-week-old mice) 3″/8″ tuberculin needle on a 1 mL syringe. Injections consisted of 10 μg of the desired peptide in 30 μL of buffer (150 mM NaCl/1 mM $CaCl_2$ at pH 7.4). The shield of the needle was trimmed and placed back on the needle to allow for no more than 2 mm of penetration during injection. Control mice received a 30 μL intracerebral injection of buffer. Other injections (subcutaneous and intramuscular) were accomplished in a similar fashion. All solutions were sterilized by passage through 0.2 μm syringe filters prior to introduction into the mice.

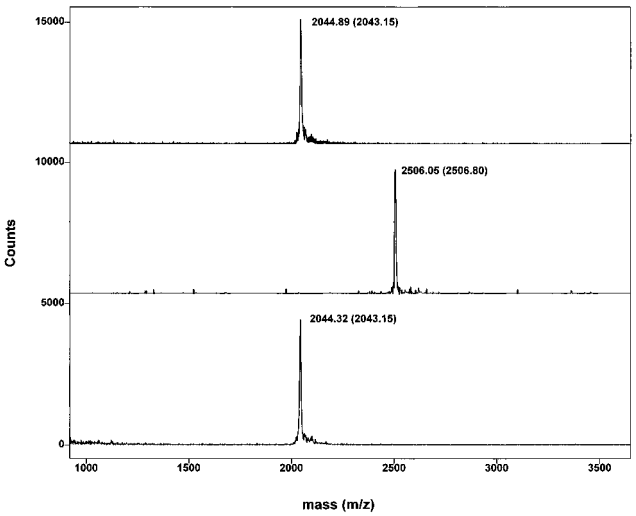

FIGURE 1: Linear mode, negative ion DE−MALDI−TOF spectra of the peptides. Top to bottom: con-G, con-T, and D-con-G. Full γ-decarboxylation of the peptides resulted during these experiments. The molecular weights obtained are listed beside each peak. The calculated values are placed in parentheses.

RESULTS

Two wild-type conantokins, *viz.* con-G and con-T, and a variant of con-G, D-con-G, were chemically synthesized and purified to apparent homogeneity. On a 0.1 mM synthetic scale, the final yields were 50−70%. Their structures are as follows: con-G, NH_2-Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn; and con-T, NH_2-Gly-Glu-Gla-Gla-Tyr-Gln-Lys-Met-Leu-Gla-Asn-Leu-Arg-Gla-Ala-Glu-Val-Lys-Lys-Asn-Ala.

Positive ion DE−MALDI−TOF analyses of the peptides yielded a series of mass peaks for the samples, each differing by 44 Da. This is due to γ-decarboxylation of Gla residues in the laser field and not to the presence of partially γ-decarboxylated material in the peptides, since the relative amounts of these peaks were dependent upon laser power and not predictable from experiment to experiment. When the same mass spectrometric analyses were performed in the negative ion mode (Figure 1), full γ-decarboxylation occurred and single-molecular weight species were observed for each peptide. The molecular weights were 2044.89 for des-γ-carboxyl-con-G (calculated, M − H = 2043.15), 2506.05 for des-γ-carboxyl-con-T (calculated, M − H = 2506.80), and 2044.32 for des-γ-carboxyl-D-con-G (calculated, M − H = 2043.15). These results substantiate the integrity of the peptides. The purity of the intact peptides was also assessed by ion-exchange (Cl^-) chromatography. A single peak containing at least 95% of the total material was obtained in each case, demonstrating that essentially a sole ionic species was present. Reverse-phase HPLC analysis

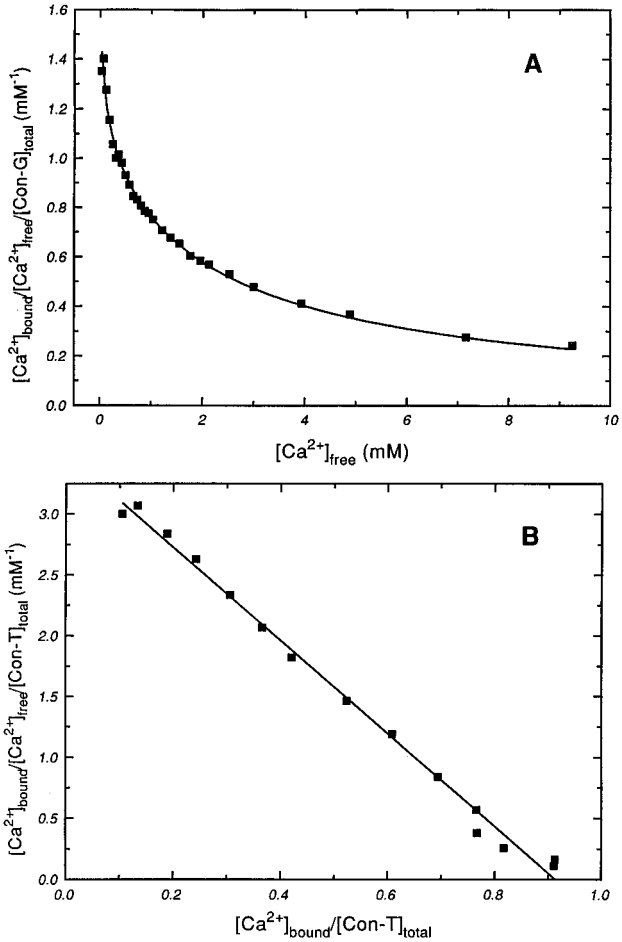

FIGURE 2: Scatchard analysis of the binding of Ca^{2+} to con-G and con-T using the Ca^{2+}-specific electrode. In both cases, the peptide concentrations were 2.0 mM and titrations were performed in a buffer of 10 mM sodium borate/100 mM NaCl at pH 8.0 and 25 °C. (A) Binding isotherm to con-G. The fit shown is for a single class of sites ($n = 2.9$ mol of Ca^{2+} per mole of peptide, $K_d = 2.8$ mM). (B) Binding isotherm to con-T. The data were fit to a single Ca^{2+} binding site ($n = 0.92$ mol of Ca^{2+} per mole of peptide, $K_d = 0.26$ mM). In this case, the format for a typical Scatchard plot was used to clearly show the linearity over the entire titration.

on each peptide also showed the presence of a single peak containing at least 95% of the material, confirming the very high purity of these products. In this latter case, the retention times for con-G (and D-con-G) and con-T were 28.1 and 41.78 min, respectively. Gla analyses of each peptide demonstrated that con-G and D-con-G contained 4.7 ± 0.1 and 5.0 ± 0.1 mol of Gla/(mol of peptide), respectively (calculated, 5.0 mol/mol), and con-T contained 4.1 ± 0.1 mol of Gla/(mol of peptide) (calculated, 4.0 mol/mol). All of these values are very close to the calculated Gla contents.

Calcium binding isotherms of each peptide were determined by Ca^{2+}-specific electrode titrations. Scatchard plots of the titration data are illustrated in Figure 2 for con-G (A) and con-T (B). The binding isotherm for con-G is complex and cannot be defined uniquely. The best models for Ca^{2+} binding to this peptide were a single class of two or three Ca^{2+} binding sites [$n = 2-3$ mol of Ca^{2+}/(mol of peptide), $K_d = 2.8$ mM, $H = 0.89$] and a two-site model [$n_1 = 0.2$ mol of Ca^{2+}/(mol of peptide), $K_{d1} = 0.24$ mM, $n_2 = 2.8$ mol of Ca^{2+}/(mol of peptide), $K_{d2} = 3.6$ mM]. The Ca^{2+} binding isotherm for D-con-G was essentially the same as that for con-G. On the other hand, the data for con-T were

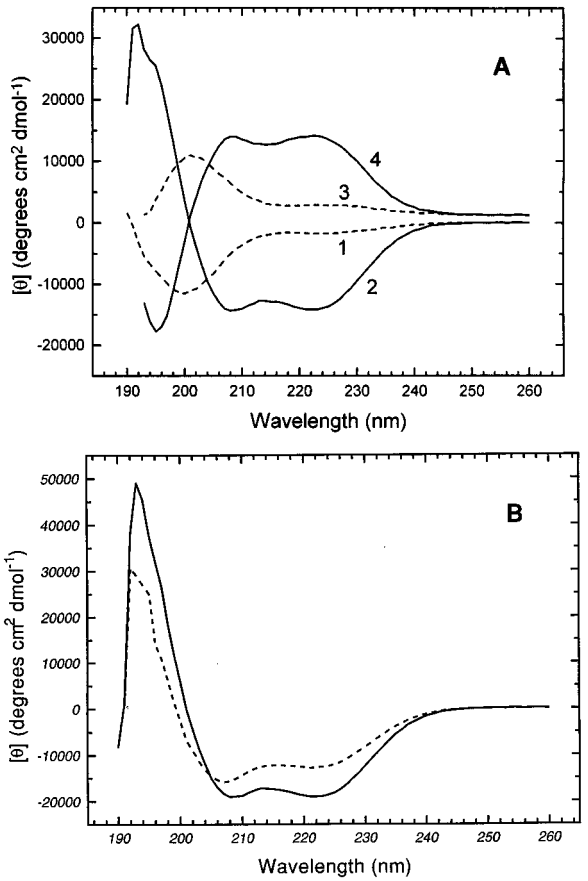

FIGURE 3: Circular dichroism spectra of con-G and con-T. The buffers used were 10 mM sodium borate/100 mM NaCl ± 40 mM $CaCl_2$ at pH 8.0 and 25 °C. (A) Con-G and D-con-G: curve 1, apo-con-G; curve 2, con-G + 40 mM $CaCl_2$; curve 3, apo-D-con-G; and curve 4, D-con-G + 40 mM $CaCl_2$. (B) Apo-con-T (broken line) and con-T (solid line) + 40 mM $CaCl_2$. The molar ellipticities are plotted against the wavelength. Each curve represents the accumulation of five individual scans. The concentration of con-G and D-con-G was 2.0 mM, and that for con-T was 1.5 mM.

fit to a unique single-site model [$n = 0.92$ mol of Ca^{2+}/(mol of peptide), $K_d = 0.26$ mM].

CD analyses were performed to evaluate the effects of Ca^{2+} on the conformations of the conantokins. Figure 3A illustrates the data obtained for con-G and apo-con-G. The plots clearly show that apo forms of con-G possess no observable α-helical structure and, from the minimum at approximately 197 nm, appear to contain a large degree of disordered structure. However, after addition of a Ca^{2+} concentration (40 mM) that saturates its binding sites on con-G, a substantial amount of α-helix is found as shown by the spectral minima at approximately 208 and 222 nm. Regarding D-con-G, the CD data were virtually identical to the data for con-G, except that the values of the molar ellipticities, as expected, were opposite in sign (Figure 3A). An estimate of the α-helical content of Ca^{2+}-loaded con-G and D-con-G is 50%. On the other hand, similar plots of con-T data (Figure 3B) show that its apo form does possess significant α-helix (*ca.* 38%), and this value increased to 55% for the Ca^{2+}-bound form.

Titration with Ca^{2+} of the change in α-helicity of these peptides yielded C_{50} values for the Ca^{2+}-induced conformational alteration. The data obtained are illustrated in Figure 4 for con-G (A) and con-T (B). The C_{50} for Ca^{2+} toward con-G (D-con-G) was 3.6 mM (4.0 mM), which is consistent

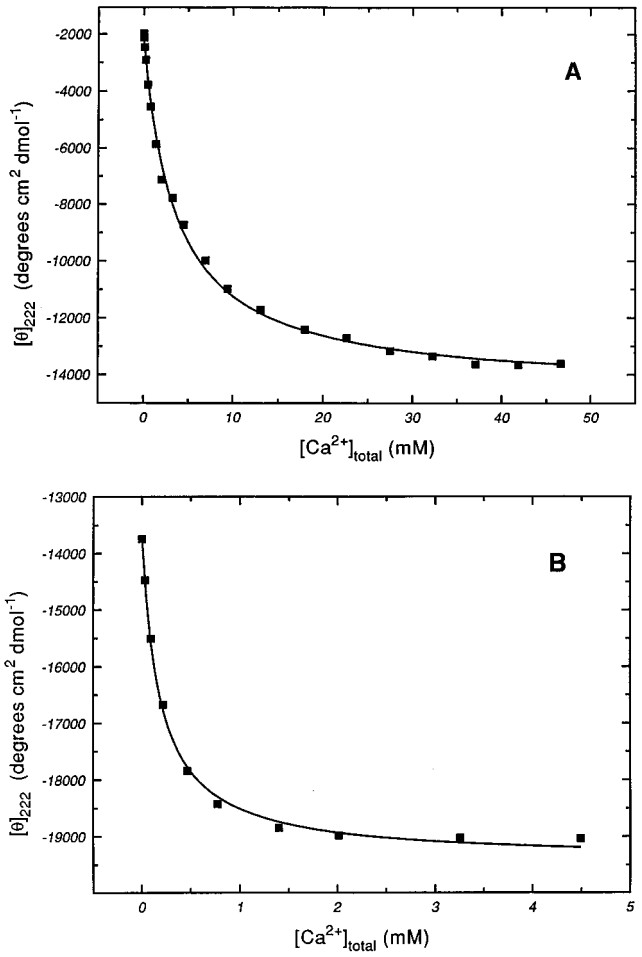

FIGURE 4: Titration with Ca^{2+} of the change in molar ellipticities of con-G and con-T. The starting buffer was 10 mM sodium borate/100 mM NaCl at pH 8.0 and 25 °C. Titrations with Ca^{2+} were accomplished in a 1 cm path length cell at a wavelength of 222 nm: (A) con-G (35 μM) and (B) con-T (35 μM).

with the K_d observed for the single-class site model or the K_d of the weaker Ca^{2+} sites in the two-site model. As revealed by this same CD titration, the C_{50} of Ca^{2+} for the con-T conformational change was 0.24 mM, a value in excellent agreement with the K_d of its lone Ca^{2+} binding site.

In order to determine whether binding of Ca^{2+} led to aggregation of the conantokins, we performed gel filtration experiments on the peptides in the absence and presence of suitable concentrations of Ca^{2+}. The elution profiles are plotted in Figure 5 for con-G (A) and con-T (B). Surprisingly, an increase in elution volumes was observed for both peptides in the presence of Ca^{2+}, demonstrating that significant aggregation likely did not occur in either case under these conditions. The observed decrease in the hydrodynamic volumes of the Ca^{2+}-bound peptides is likely a reflection of a Ca^{2+}-induced conformational change to a more compact structure.

The downfield 500 MHz ^{1}H-NMR spectrum of con-G, in the range of 6.3−9.5 ppm, is shown in Figure 6, along with changes in this region as a result of addition of Ca^{2+}. Resonance shifts in the peptidic amide proton ($7 < \delta < 10$) region of the spectra show clear evidence of a Ca^{2+}-induced conformational alteration in the presence of this cation. Line broadening in some areas is also noted, which could be a result of peptide aggregation in the presence of Ca^{2+}, despite

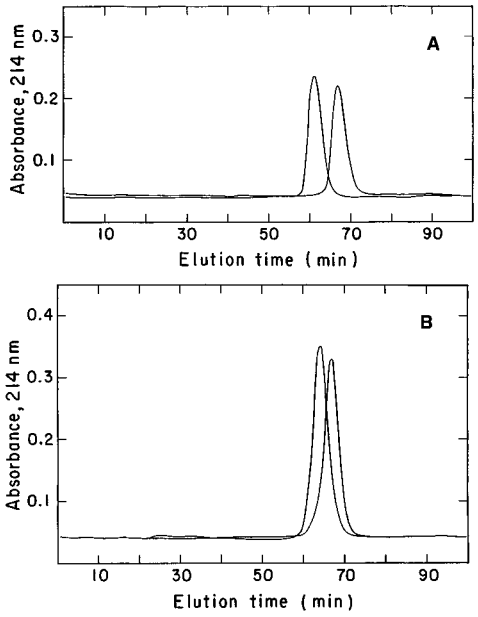

FIGURE 5: Size exclusion analysis of con-G and con-T in the absence and presence of Ca^{2+}. A volume of 0.5 mL of a solution of the relevant peptide (20 μM) was injected onto a Superdex 10/30 peptide column. The column was equilibrated with a Chelex-100-treated buffer containing 10 mM sodium borate/100 mM NaCl at pH 8.0 in the cases of the earlier-eluting fraction or with a buffer of 10 mM sodium borate/100 mM NaCl/100 mM $CaCl_2$ at pH 8.0 in the cases of the later-eluting fractions. The flow rate was 0.2 mL/min at room temperature: (A) con-G and (B) con-T.

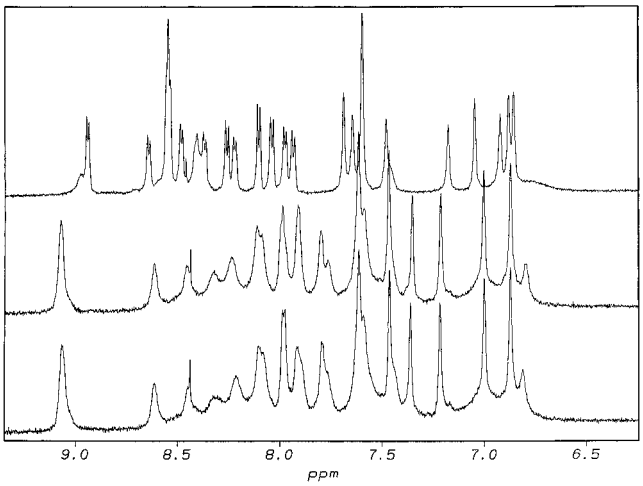

FIGURE 6: Downfield 500 MHz ^{1}H-NMR spectra of con-G in the absence and presence of Ca^{2+} at 25 °C: (top) apo-con-G, (middle) con-G + 20 mM $CaCl_2$, and (bottom) con-G + 40 mM $CaCl_2$. The buffer was 10 mM sodium borate/100 mM NaCl at pH 8.0 and 25 °C.

the fact that there is no evidence for such aggregation *via* other techniques.

Finally, bioassays were conducted to evaluate the efficacies of these synthetic peptides. Intracranial injections of 10 μg of con-G and con-T into mice <2 weeks of age resulted in sleep within 20 min, continuing for approximately 6 h, after which seemingly normal activity occurred. Similar injections in mice between 3 and 4 weeks of age produced mixed effects. In these cases, fitful sleep ensued within 30 min and continued for approximately 2 h. After this time, extreme hyperactivity occurred, characterized by continuous rapid movement around the periphery of the cages, for approximately 12−14 h. After this time, and likely as a

result of metabolic challenges due to the hyperactivity, the con-T mice consumed large amounts of food, while the con-G mice behaved lethargically and were nearly comatose. The former mice recovered, while the latter mice did not and were sacrificed. D-Con-G was inactive in this same assay, as was con-G and con-T when injected subcutaneously or intramuscularly.

DISCUSSION

In this study, two conantokins, *viz.* con-G and con-T, were chemically synthesized, along with a third peptide, D-con-G, in which each of the amino acids in con-G was substituted by D amino acids. With this latter peptide, we were able to test the importance of specific peptide−receptor interactions in dictating the biological activities of these peptides in animals, since D-con-G would be chemically identical to con-G, but the specific interactions with receptors and other effectors of con-G *in vivo* would not be expected to occur with D-con-G.

The chemical compositions of con-G and con-T are unusual not only in the presence of residues of Gla but also in the fact that the Glu at position 2 in each peptide is not γ-carboxylated. In cases of vitamin K-dependent mammalian proteins of blood coagulation and related systems that contain Gla residues in their amino-terminal domains, every residue of Glu within the Gla domain-containing exon (*ca.* 40 amino acid residues) is γ-carboxylated (Magnusson et al., 1975; Fernlund & Stenflo, 1982; Hagen et al., 1986; Park & Tulinsky, 1986; Grinnell et al., 1987; Takeya et al., 1988). In fact, insertions of additional Glu residues within these domains also result in their γ-carboxylation (Zhang et al., 1992). Thus, the existence of Gla residues in these snail-derived small neuroactive peptides, and the refractiveness of Glu[2] to this post-translational modification, at least in these particular cases, makes a further delineation of this discriminate γ-carboxylation system of special interest. Perhaps a different γ-carboxylation system is employed in the cone snail materials, and/or one with a different sensitivity to the exact placement of Glu residues in the peptide, than that employed by the vitamin K-dependent coagulation proteins.

No unusual aspects were present in the workup or purification of these peptides, except that the standard procedures of DE−MALDI−TOF that we employed for characterization of the intact conantokins led to partial (positive ion mode) or complete (negative ion mode) γ-decarboxylation. These same phenomena with other Gla-containing peptides have been observed earlier (Nakamura et al., 1996). Given the increasing use of this spectrometric technique in peptide and protein characterization, it would be of great utility to have conditions in place that eliminated γ-decarboxylation side reactions, so that molecular weights of intact materials would be obtained. In our case, however, we show that the γ-decarboxylated peptides possessed nearly the exact molecular weights expected, demonstrating that full length peptides were synthesized and purified, without any evidence of truncated forms. Further, the intact purified peptides possessed only one chromatographic species, when either anion-exchange or reverse-phase resins were employed. Gla analyses indicated that the correct numbers of Gla residues were contained in each of the peptides, confirming that the synthetic materials used in these investigations were indeed highly purified conantokins.

As with all proteins containing paired Gla residues, these three peptides interacted with Ca^{2+}. Con-G, with five Gla residues, possessed the most complex binding isotherm. One model that fits the data requires that Ca^{2+}-induced aggregation occurs at a K_d for Ca^{2+} of 0.24 mM. We have eliminated this model, on the basis of two lines of evidence. First, gel filtration experiments with con-G, conducted in the presence and absence of a high Ca^{2+} concentration (Figure 5A) or at 0.25 mM Ca^{2+} (data not shown), do not provide evidence for such Ca^{2+}-mediated aggregation. Second, CD experiments with con-G at 40 mM Ca^{2+} demonstrate that the ellipticity values at 222 nm showed a linear dependency on the concentration of con-G (over a concentration range of 5 μM to 5 mM), with the plot extending through the origin. While the line broadening in the ^{1}H-NMR spectrum in the presence of Ca^{2+} (Figure 6) would be consistent with Ca^{2+}-mediated aggregation, the more direct evidence cited above does not support such an interpretation, and a detailed NMR study is currently underway that will provide solution structures of these two conantokins. The issue of Ca^{2+}-mediated aggregation will be finally resolved at that time. At this juncture, however, we employ the single-class multiple ($n = 2-3$, $K_d = 2.8$ mM) binding site model for con-G. On the other hand, the Ca^{2+} binding data for con-T, a peptide with four Gla residues, were uniquely fit to a single-site model. Similar titrations with Ca^{2+} and D-con-G yielded results virtually identical to those of con-G.

The metal ion binding differences in these peptides are of interest. On the basis of analogies with the Gla-containing region of bovine prothrombin fragment 1, the X-ray crystal structure of which has been determined for the Ca^{2+}-loaded form, and where malonate-, unidentate-, and bidentate-type binding have been observed, the tightest Ca^{2+} binding occurs with paired Gla residues and results in a Ca^{2+}-induced, functionally relevant conformational change in this fragment, as well as in the intact protein (Soriano-Garcia et al., 1992). These conantokins contain consecutive Gla residues at amino acid sequence positions 3 and 4, and this situation may be critical for the tighter cation binding sites in the cases herein. With con-G, a dramatic conformational alteration occurs as a result of Ca^{2+} binding, perhaps providing other Ca^{2+} sites. In this regard, Gla[7], a residue not present in con-T, may be important for metal ion coordination and for provision of the additional Ca^{2+} binding sites in con-G. The 10-fold stronger binding of Ca^{2+} to con-T with respect to the binding to con-G is most likely explained by the fact that a significant α-helical conformation is present in con-T, which may provide a more optimal Ca^{2+} binding site than in the case of apo-con-G, which does not present such a structured environment. Since cooperativity does not seem to be present, then the preformed cation site in con-T appears to allow stronger interaction with Ca^{2+} than does that of con-G.

A dramatic change in secondary structure occurs in con-G consequent to Ca^{2+} site occupancy. The type of alteration is from a disordered structure in apo-con-G to a high degree of α-helix in its Ca^{2+}-bound form. The extensive nature of this alteration had not been observed in one study with this peptide (Zhou et al., 1996) but had in another (Myers et al., 1990), and we believe that it is of extreme importance in the adoption of a defined and optimal structure for binding

[2] T. Blandl, F. J. Castellino, manuscript in preparation.

to its neuroreceptor and, most likely, for its neuroactivity. It is likely that the Ca^{2+}-induced conformational alteration in con-G was not observed in this previous work due to the lack of appropriate removal of metal cations from the peptide and the consequent overestimation of the amount of α-helix in apo-con-G. Since other divalent cations, *e.g.*, Mg^{2+}, interact with these conantokins much more tightly than Ca^{2+}, rigorous and repeated chelation is necessary for their complete removal.[2] When this is accomplished for con-G, no α-helix is found in the apo-peptide. A similar, but not as extensive, change occurs in con-T upon Ca^{2+} binding. The reason for the smaller change in secondary structure is because the apo form of con-T already possesses a high degree of α-helix. However, Ca^{2+} still induces a very meaningful increase in the α-helical content of this peptide, a result that decreases the number of available structures for con-T, thereby, as we propose, enhancing its specific binding to neuroreceptors. The involvement of specific receptors in the bioactivity of these molecules is underpinned by the *in vivo* data for D-con-G, which possesses a chemical composition and a Ca^{2+}-mediated conformational change identical to those of con-G but does not display any observable neuroactivity. This indicates that a specific chirality is required for neuroactivity, and such a situation is best explained by interaction with a receptor that recognizes such differences between con-G and D-con-G. High receptor selectivity appears to be essential for the functioning of these peptides since their effects as neuroactive agents are related to the age of the mice, likely suggestive of developmental changes in the concentration distribution of the conantokin receptors in brain cells.

In summary, we have delineated the Ca^{2+} binding properties of two synthetic neuroactive sea snail conantokins, con-T and con-G, and have clearly demonstrated that both peptides undergo large and defined conformational changes upon binding of Ca^{2+}. We propose that Ca^{2+} and likely other divalent cations induce conformations in these peptides that allow discriminate binding to neuroreceptors, an event required for elicitation of their bioactivities.

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