Accelerated Publications

Total Synthesis and Further Characterization of the γ-Carboxyglutamate-Containing "Sleeper" Peptide from *Conus geographus* Venom†

Jean Rivier,‡ Robert Galyean,‡ Lajos Simon,‡,§ Lourdes J. Cruz,∥ Baldomero M. Olivera,*,∥ and William R. Gray∥

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, California 92037, and Department of Biology, The University of Utah, Salt Lake City, Utah 84112

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ABSTRACT: The total synthesis of the Gla-containing "sleeper" peptide (Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn-NH_2) from *Conus geographus* is described.[1] A new strategy for the synthesis of acid-sensitive peptide amides was developed, which allowed complete deprotection and cleavage of the L-γ-carboxyglutamate-containing peptide from the 2,4-dimethoxybenzhydrylamine resin. Synthetic sleeper peptide, after preparative high-performance liquid chromatography (HPLC) purification, was shown to be identical with the native peptide by all criteria (coelution experiments on HPLC, sequence analysis, and biological activity). In addition, a developmental switch in the behavioral symptoms induced by the peptide after intracerebral administration in mice was documented. At low doses of the peptide (4–30 pmol/g), a sleeplike state was induced in mice under 2 weeks old; in contrast, older mice became markedly hyperactive. It is proposed that, in the presence of Ca^{2+}, the sleeper peptide assumes an α-helical configuration in which all the γ-carboxyglutamate residues are located on the same side of the α-helix.

The fish-hunting cone snail (*Conus geographus*) uses its venom to paralyze prey, and the venom is sufficiently toxic to be lethal to man. This venom has already been shown to contain three classes of paralytic toxins (Olivera et al., 1985): α-conotoxins that block nicotinic acetylcholine receptors (Gray et al., 1981), μ-conotoxins that directly block muscle sodium channels (Cruz et al., 1985), and ω-conotoxins that block presynaptic voltage-sensitive Ca channels (Olivera et al., 1984). Several homologous forms of each of these paralytic toxins have been found, and at least one of each class has been synthesized (Olivera et al., 1985a). All are directly paralytic to vertebrates. In addition to the paralytic toxins, which have been extensively characterized (Olivera et al., 1985a), the venom contains a number of nonparalytic biological activities.

The "sleeper" peptide (also called conotoxin GV) was isolated from *C. geographus* venom on the basis of its ability to induce a sleeplike state in mice after intracerebral injection (Olivera et al., 1985b). Its primary structure was determined to be Gly-Glu-Gla-Gla-Leu-Gln-Gla-Asn-Gln-Gla-Leu-Ile-Arg-Gla-Lys-Ser-Asn-NH_2[1] (McIntosh et al., 1984). The most striking features were the presence of five Gla residues and a C-terminal asparagine amide.

γ-Carboxyglutamate was originally identified in the vitamin K dependent blood-clotting factors including prothrombin (Stenflo & Suttie, 1977). It was subsequently found in several other vertebrate proteins such as osteocalcin (Hauschka et al., 1975) and in ribosomal proteins (van Buskirk & Kirsch, 1978). Its occurrence in a neuroactive peptide was quite unexpected, but we have since found it in other peptides from the venom of *C. geographus* and other related snails. The latter are currently being characterized, and it is clear that they have diverse biological activities.

The target of the sleeper peptide in the mammalian central nervous system is not known. In this paper, we describe two types of studies that should help to elucidate the mechanism by which the sleeper peptide evokes its biological effects. First, we undertook the synthesis of this heptadecapeptide in the hope of obtaining enough material to carry out in-depth physiological and biochemical studies. The strategy that led to the successful synthesis of this molecule should make possible the relatively routine synthesis of most Gla-containing peptides which are of interest in other areas of biochemistry. Second, we demonstrate that with increasing age of the mice there is a dramatic switch in the response elicited by the peptide.

Experimental Procedures

Synthesis of Sleeper Peptide (Conotoxin GV). Fmoc amino acids except that of glycine were of the L configuration. Most were purchased from Bachem, Torrance, CA, but Fmoc-γ,-γ′-di-*t*-Bu-γ-carboxyglutamic acid and Fmoc-isoasparagine were synthesized in our laboratory (see below). Fmoc amino acids included Asn, Gln, Ser(O-*t*-Bu), Lys(ε-Boc), Arg(MTR), and Glu(O-*t*-Bu).

Fmoc-iso-Asn(β-O-t-Bu) was prepared by the reaction of Fmoc-L-Asp(β-O-*t*-Bu)-OH with the ammonium salt of

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* Author to whom correspondence should be addressed.

‡ The Salk Institute.

§ Visiting Scientist, Institute of Pharmaceutical Chemistry, University Medical School, Szeged, Hungary.

∥ The University of Utah.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; Fmoc, fluorenylmethyloxycarbonyl; MTR, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; HOBT, hydroxybenzotriazole; DMBHA, 2,4-dimethoxybenzhydrylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; TFA, trifluoroacetic acid; DCM, dichloromethane; DMSO, dimethyl sulfoxide; TEAP, triethylammonium phosphate; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment. All other amino acid abbreviations are those recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984).

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Table I: Characterization of Gla Derivatives

Gla derivative	this work mp (°C)	this work $[\alpha]^{22}_D$ (deg)	lit. data mp (°C)	lit. data $[\alpha]^{2}_D$ (deg)	ref
Z-DL-Gla(O-*t*-Bu)$_2$OMe	60–64	0	oil	0	*a*
			oil	0	*b*
Z-DL-Gla(O-*t*-Bu)$_2$OH	oil	0	oil	0	*a*
Z-D-Gla(O-*t*-Bu)$_2$OH quinine salt	141–143	−55.5[c]	132–133	−76.8[c]	*a*
			139.5–140	−71.9[c]	*b*
Z-L-Gla(O-*t*-Bu)$_2$OH (1*S*,2*R*)-(−)-ephedrine salt	121–123	−7.9[c]	122–124	−6.7[c]	*a*
Z-L-Gla(O-*t*-Bu)$_2$OH	85–87	−10.8[d]	87–89	−10.9 ± 0.3[d]	*a*
		+2.05[c]		+12.3 ± 0.3[c]	*a*
			87–89	−11.2[d]	*b*
Z-D-Gla(O-*t*-Bu)$_2$OH	87–89	+10.60[d]	86–88	−11.3 ± 0.3[c]	*a*
		−2.2[c]	87–88	+11.4[d]	*b*
L-Gla(O-*t*-Bu)$_2$OH[e]	173–175	+5.95[d]	170–171	+5.6 ± 0.3[d]	*a*
D-Gla(O-*t*-Bu)$_2$OH	167–171	−6.03[d]	165.5–167.5	−5.6 ± 0.3[d]	*a*
			165–167.5	−5.7[d]	*b*
Fmoc-L-Gla(O-*t*-Bu)$_2$OH[f]	121–123	−7.9[d]			
Fmoc-D-Gla(O-*t*-Bu)$_2$OH[g]	120–121	+7.65[d]			

[a] Marki et al., 1977. [b] Boggs et al., 1979. [c] $CHCl_3$, *c* 1. [d] MeOH, *c* 1. [e] 98% L, 2% D, by analysis; see Buck and Krummen (1984). [f] 99% L, 1% D, by analysis; see Buck and Krummen (1984). [g] 99% D, 1% L, by analysis; see Buck and Krummen (1984).

HOBT and DCC in DMF–CH_2Cl_2 solution. After removal of the crystalline dicyclohexylurea, the solvent was evaporated under vacuum, and the desired product was crystallized from methanol. Removal of the *tert*-butyl ester was accomplished in 50% TFA–CH_2Cl_2 at room temperature to yield Fmoc-iso-Asn-OH (mp 183–185 °C).

Fmoc-L-Gla(O-t-Bu)$_2$-OH. Condensation of Z-L-Ser-(Tos)-OMe with di-*tert*-butyl malonate to give Z-DL-Gla(O-*t*-Bu)$_2$-OMe was carried out by a modification of the procedure of Cerovsky and Jost (1984). Sodium hydride was rinsed twice with pentane, suspended in absolute benzene, and then added to the benzene solution of di-*tert*-butyl malonate. The reaction was allowed to proceed to completion within 10 min of reflux. The resulting suspension was cooled in an ice bath, and the Z-L-Ser(Tos)-OMe dissolved in benzene–tetrahydrofuran was added under an argon atmosphere with vigorous stirring and continued cooling at 0 °C for 2 h. Stirring was maintained for additional 48 h at room temperature. At this time, the suspension was cooled and washed successively with ice water, 1 N HCl, and water. After rotary evaporation at room temperature, the oil was dissolved in benzene and pentane was added to initiate crystallization. The yield was 40–60% for a preparation of 0.5 mol. The methyl ester was hydrolyzed by dissolving in alcohol and adding 1.2 equiv of KOH dissolved in water–ethanol. The solution was allowed to remain at room temperature for several days; the reaction was monitored by HPLC using a C_{18} 5-μm column, with 0.1% TFA–acetonitrile as the solvent. When the reaction was complete, the solution was evaporated at room temperature and the product extracted with ethyl acetate after the addition of $NaHSO_4$. The ethyl acetate was dried over Na_2SO_4 and evaporated under reduced pressure. The yield was 80–90%.

The D and L isomers were resolved by crystallization of the quinine salt of the D isomer. The Z-DL-di-*t*-Bu-Gla-OH in ethyl acetate was reacted with an equivalent amount of quinine. The crystals were separated from the mother liquid, and the Z-D-di-*t*-Bu-Gla-OH was recrystallized from ethyl acetate. The quinine salt was suspended in ether, and quinine was removed by the addition of a 20% citric acid solution at 0 °C; the same process was used to remove quinine from the liquid phase. The L isomer was precipitated in the form of its ephedrine salt from ethyl acetate–pentane and recrystallized (Marki et al., 1977). Elimination of ephedrine by acid extraction, hydrogenation of the Z group, and introduction of the Fmoc are all standard laboratory procedures. Optical purity of the L and D isomers of Fmoc-Gla(O-*t*-Bu)$_2$-OH was assessed by the method of Buck and Krummen (1984) after hydrolysis to Glu (6 N HCl, 110 °C, 20 h). Each was approximately 99% pure. Analytical data of derivatives are given in Table I.

DMBHA resin was obtained by reaction of 50 g of 2,4-dimethoxybenzoyl chloride with 100 g of Bio-Beads SX-1 (200–400 mesh) from Bio-Rad in the presence of $AlCl_3$ (50 g) in nitrobenzene for 30 min at room temperature. The resin was successively washed with nitrobenzene, 2-propanol, 2-propanol–water (50/50), water, MeOH, CH_2Cl_2, and MeOH and dried. Weight gain was 20 g/100 g of resin. Leuckart's reductive ammonolysis was performed in liquid ammonium formate at 165 °C for 48 h. Hydrolysis of the formate was achieved in 6 N HCl in EtOH under reflux. The substitution level was 0.25 mequiv of NH_2/g of resin, according to the Gisin test (Gisin, 1972).

Coupling of Fmoc-iso-Asn-OH to the DMBHA resin was accomplished in DMF by using dicyclohexylcarbodiimide (DCC). Stepwise buildup of the peptide on 1 g of the 2,4-dimethoxybenzhydrylamine resin was done manually to facilitate a rapid synthesis (1.0–2.0 mmol of amino acid/g) and to ensure a complete coupling/deblocking for each cycle. Monitoring was done by using the Kaiser test (Kaiser et al., 1970). Removal of the Fmoc group was effected by a 20% solution (v/v) of freshly distilled piperidine in dimethylformamide (DMF) for 10 min. Thorough resin washing was accomplished by repeated application of DMF, methanol, or dichloromethane (DCM). Couplings were mediated by DCC in either DCM, DMF, or mixtures thereof, depending upon the solubility of the particular amino acid derivative. Fmoc-Asn and -Gln were incorporated into the peptide with unprotected side chains, in the presence of 2 equiv of HOBT, and were coupled in DMSO–DMF or DMSO–DCM. The peptide was released from 1 g of the peptide resin as the C-terminal amide by treatment with a freshly prepared mixture of TFA, thioanisole, H_2O, and DCM (40/10/1/49) (10 mL) at 37 °C for 6 h. Trial cleavages on small amounts (10 mg of peptide resin) had demonstrated to our satisfaction that the peptide would be freed and all side-chain protection including the difficult MTR would be removed, while the Gla would remain intact. The peptide was precipitated from the cleavage solution and washed by the addition of a 10-fold volume excess of methyl *tert*-butyl ether (3 times). The peptide was then dissolved in distilled water, the pH of the resulting solution was adjusted to approximately 6 with dilute NaOH, the resin was separated by filtration, and the supernatant was

Table II: Characterization of Sleeper Peptide Conotoxin GV

	HPLC analysis[b]					
	solvent system					
$[\alpha]_D$[a]	A	B	flow rate (mL/min)	gradient	vol (mL)	% purity
−39.7	TEAP, pH 2.25	40% CH_3CN in A	2	25% B (20 min), 45% B	25.7	≥98

[a] *c* 0.5; 1% HOAc, 20 °C. [b] UV monitoring at 210 nm, 0.1 absorbance unit at full scale. Column was Vydac (0.46 × 25 cm) packed with C_{18}, 5-μm particles, 300-Å pore size.

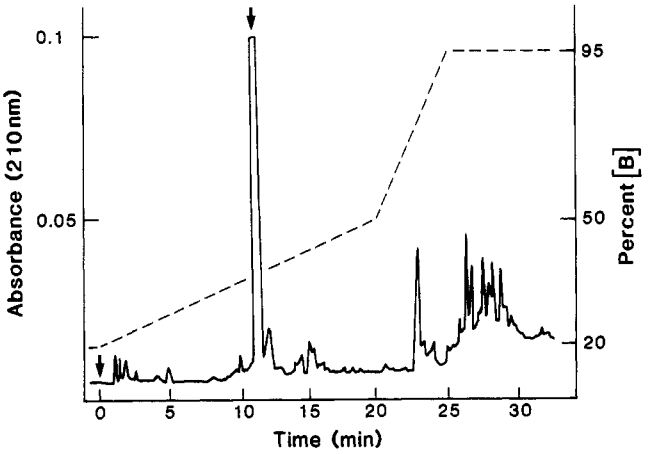

FIGURE 1: HPLC profile of crude synthetic sleeper peptide. Conditions: Vydac C_{18} silica 5-μm (0.46 × 25 cm) column; flow rate 2 mL/min at 2400 psi; solvent A = TEAP, pH 2.25; solvent B = 50% CH_3CN in solvent A. The gradient is shown by a dashed line. The arrow indicates the biologically active material (approximately 5 μg).

lyophilized. The crude peptide (yield 400 mg) was purified by preparative HPLC as previously described (Rivier et al., 1984). The gradient of acetonitrile applied to the preparative cartridge (15–20-μm, 300-A Vydac C_{18}) in TEAP, pH 2.25, was 3–18% in 1 h, with a flow rate of 100 mL/min. Analysis of the generated fractions was achieved under isocratic conditions (14% acetonitrile in TEAP, pH 2.25) on a 5-μm Vydac C_{18} column. Desalting was carried out with an acetonitrile gradient from 0% to 28% in 0.1% TFA in 40 min. Highly purified fractions were pooled and lyophilized, yielding sleeper peptide (conotoxin GV) as the TFA salt (yield 130 mg). Results of the HPLC analysis of this material are given in Table II along with the optical rotation in 1% acetic acid. Amino acid analysis gave the following ratios with expected values in parentheses: Asp (2), 1.90; Ser (1), 0.80; Glu (8) including presence of five Gla residues, 7.69; Gly (1), 1.00; Ile (1), 0.76; Leu (2), 1.96; Lys (1), 0.89; Arg (1), 0.92. FAB mass spectrometry was performed on the peptide, and the spectrum showed a protonated molecular ion (MH^+) at m/z = 2263.86, corresponding to the calculated monoisotopic peptide amide of 2263.95. A chromatogram of the crude GV preparation after TFA cleavage and deprotection is shown in Figure 1 and illustrates that the major product is particularly pure and that a relatively small amount of hydrophobic impurities are present. Sequencer analysis gave the expected residues at each cycle, except for blanks with Gla residues.

Biological Assays. The biological assays on mice were carried out by ic injection as described by McIntosh et al. (1984).

RESULTS

Total Synthesis of Sleeper Peptides. Chemical synthesis of peptides containing Gla has been handicapped by the relative instability of this amino acid to acid conditions, which results in loss of one of the γ-carboxyls with production of glutamic acid. Some success has been obtained with liquid-phase approaches (ten Kortenaar et al., 1980), but these would have been particularly tedious for the conotoxin. In attempting this synthesis, therefore, we tried to devise conditions that would be generally applicable for solid-phase synthesis of Gla-containing peptides.

The presence of five Gla residues in this 17-peptide amide dictated a synthetic strategy using base deprotection of the α-amino function of the residues added during peptide buildup on a solid support. Side-chain protecting groups were such as to be stable under the basic conditions of repeated α-deprotection but labile under acidic conditions that would not lead to decarboxylation of the Gla residues. Finally, because we feared that ammonolysis of the Asn C-terminal linkage to the resin might not be free of racemization or aspartimide formation, we used our recently developed 2,4-dimethoxybenzhydrylamine resin that yields peptide amides under mild acidic cleavage (Penke & Rivier, 1987). We had shown previously that a Gla-containing hexapeptide amide could be obtained by this approach, using Fmoc protection for the α-amino groups and *tert*-butyl side-chain protection (Penke et al., 1987).

Several special concerns were addressed. First, the sleeper peptide contains an Arg residue whose favored side-chain protection by MTR is notoriously resistant to removal by acid. We did use this protection, however, as our experience in sequencing the native peptide suggested that the Gla residues were relatively unaffected by a cumulative exposure of about 1 h to heptafluorobutyric acid at 55 °C. In fact, we experienced no problem with decarboxylation in the final deprotection and cleavage of GV at 37 °C for 6 h in 40% TFA. Second, in another synthesis with C-terminal Asn, cleavage from the DMBHA resin was extremely slow, suggesting possible problems with aspartimide formation during the repeated base treatments for α-deprotection. We therefore coupled Fmoc-isoasparagine to the resin through the β-carboxyl. Final cleavage from the resin was trouble-free.

A final hurdle was that of unavailability of large quantities of the desired starting material, Fmoc-L-Gla(O-*t*-Bu)$_2$-OH. Our earlier synthesis of a Gla-containing peptide was made with the more readily available Fmoc-DL-Gla(O-*t*-Bu)$_2$-OH and separation of the two peptide isomers by preparative HPLC. The presence of five Gla residues in the current target peptide made this approach unworkable. We therefore investigated several synthetic routes for the large-scale synthesis of L-Gla appropriately derivatized for solid-phase synthesis. This was finally achieved by condensation of Z-Ser(Tos)-OMe with di-*tert*-butyl malonate (Cerovsky & Jost, 1984), followed by saponification of the α-methyl ester and resolution of the Z-DL-Gla(O-*t*-Bu)$_2$-OH using quinine and ephedrine (Marki et al., 1977). Physical constants for the synthetic intermediates and final derivatives are given in Table I. It should be noted that a few of our results do not match those of earlier investigators; optical purity of our final Fmoc derivatives of D- and L-Gla(O-*t*-Bu)$_2$ was 99%, as analyzed after HCl hydrolysis. We have no explanation for the minor discrepancies at this time. The synthesis of the DMBHA resin proceeded smoothly and is described under Experimental Procedures. We found

Table III: Biological Effects of Synthetic Sleeper Peptide

dose (pmol/g body wt)	observed effects		
	10–11-day-old mice	18-day-old mice	26–32-day-old mice
<2.5	no effect	no effect	no effect
4.0–6.0	drowsy in <30 min; slept at least 70 min	hyperactive	slightly hyperactive
20–30	slept for more than 9 h	alternate periods of drowsiness and hyperactivity	hyperactive for several hours
110–130	drowsy in <10 min; still affected after 13 h	alternate periods of drowsiness and hyperactivity	"sleepy climber"[a] symptoms for 30–60 min; then alternate drowiness and hyperactivity
400–650	drowsy in <6 min; sleeping at >15 h; short bursts of hyperactivity at 23 h	initially flat on belly for ~30 min; long alternating periods of drowsiness and hyperactivity; "climber"[a] symptoms	"sleepy climber"[a] symptoms for 4 h

[a] "Climber" symptoms are a marked tendency to climb wall cages during periods of hyperactivity. "Sleepy climber" symptoms are an initial period of weakness (mice are flat on their bellies), followed by dragging themselves against the wall of the cage in an apparent effort to climb, but remaining immobile in a climbing posture against the cage wall. Two to four mice were used for each age–dose observation.

that those Fmoc amino acids poorly soluble in DMF or CH_2Cl_2, such as Fmoc-Asn and -Gln, could be coupled in DMSO in the presence of HOBT. Cleavage of the final products from the resin and of all side-chain protecting groups was carried out at 37 °C in CH_2Cl_2–TFA–thioanisole–water (49/40/10/1 by volume) and monitored by HPLC. After 6 h of cleavage the complex pattern of partially deprotected peptides had simplified into one having a single major peak (Figure 1). Extending the cleavage to 24 h made no further changes, suggesting that Gla may be significantly more stable to TFA exposure than originally suspected. By preparative HPLC (Rivier et al., 1984), we obtained 130 mg of apparently homogeneous peptide, which was indistinguishable from the natural material by amino acid analysis, HPLC, sequence analysis, mass spectrometry, and biological activity. Coelution of native and synthetic GV is shown in Figure 2.

Comparison of Biological Activity of Native and Synthetic Toxin: Change in Biological Activity as a Function of Development. Preliminary work in our laboratories (D. Johnson and A. Azimi-Zonooz, unpublished results) indicated that the activity elicited by sleeper peptide in mice depended on the age of the injected animal. Sleep was induced only in relatively young animals, while in older mice a distinctive hyperactive syndrome with "climber" symptoms was found. This remarkable switch with development was therefore used as a diagnostic biological test to compare native and synthetic material.

Natural and synthetic peptides were compared by ic injection into mice of different ages. The biological activity obtained with low doses of the native peptide was dependent on the age of the injected animal, going from pure sleeper syndrome (for mice <14 days old), to a mixture of sleeper and hyperactive (14–21-day-old mice), and finally to a pure hyperactive state (>21-day-old mice). The diagnostic reversal with age of whether the peptide tranquilizes or excites was found with both native and synthetic peptide. In addition, native and synthetic peptides were equally potent; thus in 11-day-old mice, symptoms are elicited first at a dose of ~5 pmol/g. The biological activities of native and synthetic peptide are thus indistinguishable from each other. This biological test, coupled with the identity of the two peptides by various physical and chemical criteria, confirms that the synthesis of the *C. geographus* sleeper peptide has in fact been achieved.

Because of the availability of relatively large amounts of synthetic peptide, we were able to characterize the developmental transition more thoroughly. The results are presented in Table III. A surprising feature of the data is that although sleeper symptoms are dominant in young mice, sporadic hyperactivity is seen at very high doses; correspondingly, although

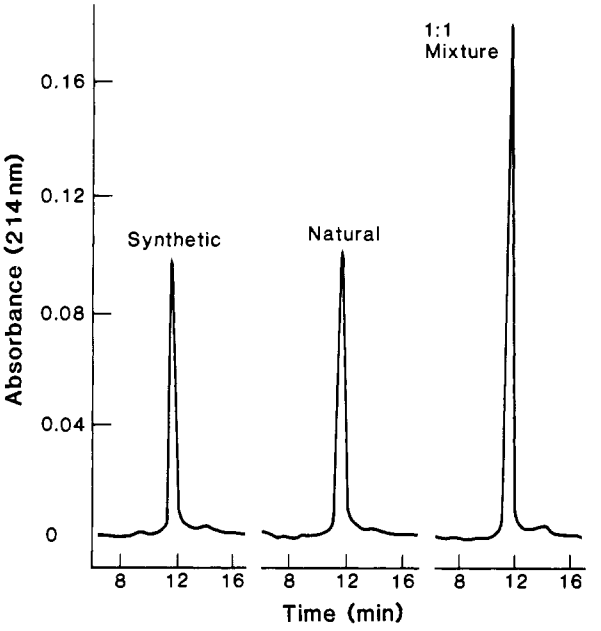

FIGURE 2: HPLC comparison of natural and synthetic material. The natural purified sleeper peptide, the synthetic peptide, and a mixture of natural and synthetic peptide were analyzed by HPLC. The TFA–acetonitrile system [solvent A, 0.1% TFA; solvent B, 0.1% TFA in 60% (v/v) acetonitrile] was used to elute the peptide from a Vydac C_{18} column. Peptides were eluted with linear gradients, expressed as percent solvent B achieved at times given in parentheses (minutes): 25 (0)/25 (2)/40 (17). Approximately 2 nmol of natural, 2 nmol of synthetic, and a mixture of the two are shown in the figure. The slight shoulders eluting later than the major peaks in all cases are probably due to a different form of the peptide produced under these conditions; when this material is rerun, it regenerates mostly the major peak.

hyperactivity is dominant in older mice, they become "sleepy climbers" when high doses are injected. This mixing of excitatory and tranquilizing symptoms may be indicative of at least two types of targets for the sleeper peptide in the central nervous system, with the biological effects of one being dominant in young mice and of the other in older mice.

Discussion

The sleeper peptide is one of many biologically active constituents of *C. geographys* venom. Although the target of the sleeper peptide has not been identified, it is remarkable in two respects. First, the biological effects are most unusual, particularly the developmental switch in symptomatology between sleep and hyperactivity described above. The symptoms induced by the peptide in 18-day-old mice are particularly striking: injected animals continuously cycle between periods of drowsiness and rapid running and climbing. Since younger

Table IV: Helix-Forming Values of Amino Acids in Sleeper Peptide

position	amino acid	P_α[a]	position	amino acid	P_α[a]
1	Gly	0.56	10	Gla	*b*
2	Glu	1.44	11	Leu	1.30
3	Gla	*b*	12	Ile	0.97
4	Gla	*b*	13	Arg	0.96
5	Leu	1.30	14	Gla	*b*
6	Gln	1.27	15	Lys	1.23
7	Gla	*b*	16	Ser	0.82
8	Asn	0.90	17	Asn	0.90
9	Gln	1.27			

[a] The P_α values are taken from Chou and Fasman (1978). [b] The P_α for Gla is unknown; we presume that the presence or absence of Ca^{2+} could significantly change the stabilizing influence of Gla on α-helix formation.

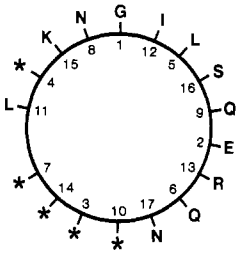

FIGURE 3: Proposed α-helical structure of the sleeper peptide from *C. geographus*. "Helical wheel" projection (Schiffer & Edmundson, 1968) of the sleeper peptide sequence showing the clustering of Gla residues (*) on one side.

mice only show periods of drowsiness, such symptoms suggest the onset of feedback neural circuits at a certain point in development. The sleeper peptide provides a molecular probe for dissecting developmental changes in such circuitry.

Second, an unusual feature of the sleeper peptide is the presence of five residues of the uncommon γ-carboxyglutamate (Gla). The presence of this acid-labile amino acid required that normal strategies of solid-phase peptide synthesis be modified. The successful synthesis required a number of technical innovations: large-scale synthesis of the appropriate L-Gla derivative, a judicious choice of side-chain protecting groups, deprotection of α-amino functions under basic conditions, and coupling to a new resin that permitted release of the peptide amide under mild acidic conditions (which did not cause significant loss of γ-carboxyglutamate groups).

The sequence of the sleeper peptide suggests a possible conformation. As shown in Table IV, residues 2–15 (excluding Gla for which no figure is available) show a strong tendency to α-helix formation (average P_α = 1.18; Chou & Fasman, 1978). In such a conformation, all Gla residues would be located on the same side of the helix, as shown in the "helical wheel" diagram (Figure 3).

In all cases where γ-carboxyglutamate has previously been found in a protein, an interaction of Gla with Ca^{2+} is central to biological activity, suggesting that the sleeper peptide may interact with Ca^{2+} to cause sleep or hyperactivity. The helical structure suggested above would surely be destabilized by the enormous negative charge density, but Ca^{2+} binding could overcome this. When Ca^{2+} is present, Gla may well be a helix-stabilizing amino acid, as is Glu. CD spectra of the sleeper peptide have been taken to assess secondary structure; however, in the relatively concentrated solutions required for such measurements, it was found that the peptide slowly equilibrates between multiple (possibly oligomeric) molecular forms (J. Haack, unpublished results), making the reproducibility and straightforward interpretation of CD spectra problematic. However, shifts between various molecular forms (perhaps depending on Ca^{2+} or the presence of membranes) could very well be central to the biological activity of this peptide. We are presently attempting to establish conditions for stabilizing and purifying each of the forms to better assess the significance of the different molecular species of the sleeper peptide.

With the successful synthesis of the sleeper peptide, a much greater variety of studies to characterize how this peptide acts become possible and are under way in our laboratories. Gla-containing peptides are of interest in several other areas of biochemistry. The synthetic strategy developed for the rather exotic sleeper peptide should be generally applicable to all such peptides.

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REFERENCES

Boggs, N. T., Goldsmith, B., Gawley, R. E., Koehler, K. A., & Hiskey, R. G. (1979) *J. Org. Chem. 44*, 2262–2269.

Buck, R. H., & Krummen, K. (1984) *J. Chromatogr. 315*, 279–285.

Cerovsky, V., & Jost, K. (1984) *Collect. Czech. Chem. Commun. 49*, 2562–2565.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Cruz, L. J., Olivera, B. M., Kerr, L., Yoshikami, D., Zeikus, R. D., Gray, W. R., & Moczydlowski, E. G. (1985) *J. Biol. Chem. 260*, 9280–9288.

Gisin, B. F. (1972) *Anal. Chim. Acta 58*, 248–249.

Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., & Cruz, L. J. (1981) *J. Biol. Chem. 256*, 4734–4740.

Hauschka, P. V., Lian, J. B., & Gallop, P. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3925–3929.

IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984) *Eur. J. Biochem. 138*, 9–37.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.

Marki, W., Oppliger, M., Thanei, P., & Schwyzer, R. (1977) *Helv. Chim. Acta 60*, 798–806.

McIntosh, J. M., Olivera, B. M., Cruz, L. J., & Gray, W. R. (1984) *J. Biol. Chem. 259*, 14343–14346.

Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., & Gray, W. R. (1984) *Biochemistry 23*, 5087–5090.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., de Santos, V., & Cruz, L. J. (1985a) *Science (Washington, D.C.) 230*, 1338–1343.

Olivera, B. M., McIntosh, J. M., Clark, C., Middlemas, D., Gray, W. R., & Cruz, L. J. (1985b) *Toxicon 23*, 277–282.

Penke, B., & Rivier, J. (1987) *J. Org. Chem. 52*, 1197–1200.

Penke, B., Gray, W. R., Johnson, D., Hoeger, C., & Rivier, J. (1987) Tenth American Peptide Symposium, St. Louis, MO, Abstr., p 71.

Rivier, J., McClintock, R., Galyean, R., & Anderson, H. (1984) *J. Chromatogr. 288*, 303–328.

Schiffer, M., & Edmundson, A. B. (1968) *Biophys. J. 7*, 121–135.

Stenflo, J., & Suttie, J. W. (1977) *Annu. Rev. Biochem. 46*, 157–172.

ten Kortenaar, P. B. W., Wilkerson, M. W., Boggs, N. T., Madar, D. A., Koehler, K. A., & Hiskey, R. G. (1980) *Int. J. Pept. Protein Res. 16*, 440–449.

van Buskirk, J. J., & Kirsch, W. M. (1978) *Biochem. Biophys. Res. Commun. 82*, 1329–1331.