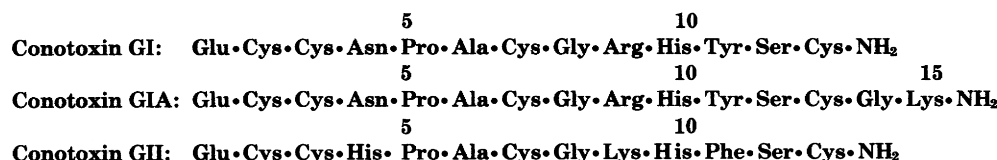
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 10, Issue of May 25, pp. 4734-4740, 1981
Printed in U.S.A.

Peptide Toxins from *Conus geographus* Venom*

(Received for publication, August 1, 1980, and in revised form, December 17, 1980)

William R. Gray, Antonio Luque, and Baldomero M. Olivera
From the Department of Biology, University of Utah, Salt Lake City, Utah 84112
John Barrett
From the Department of Physiology, University of Miami, Miami, Florida 33101
Lourdes J. Cruz
From the Department of Biochemistry, University of the Philippines College of Medicine, Manila, Philippines

Three homologous toxic peptides which cause postsynaptic inhibition at the vertebrate neuromuscular junction have been purified from the venom of the marine snail *Conus geographus*. Their amino acid sequences are:



Conotoxin GI:	Glu•Cys•Cys•Asn•Pro•Ala•Cys•Gly•Arg•His•Tyr•Ser•Cys•NH_2
Conotoxin GIA:	Glu•Cys•Cys•Asn•Pro•Ala•Cys•Gly•Arg•His•Tyr•Ser•Cys•Gly•Lys•NH_2
Conotoxin GII:	Glu•Cys•Cys•His•Pro•Ala•Cys•Gly•Lys•His•Phe•Ser•Cys•NH_2

(Residue positions 5, 10 and 15 are marked above the sequences.)

The biologically active peptides are monomeric, with internal disulfide bonds.

The biochemical characterization of three highly toxic peptides from the venom of the marine snail *Conus geographus* is described in this report. These peptides cause their potent activity by inhibition of the postsynaptic terminus of the vertebrate neuromuscular junction. The relatively small size of the peptides (13-15 amino acids) as well as the polymorphism in their structure makes them potentially attractive probes for synaptic transmission events.

The *Conus* snails are venomous predators that immobilize their prey by a highly specialized venom apparatus (1). The venom is injected into the prey by means of a disposable hollow tooth, which serves both as harpoon and hypodermic needle. The geography cone (*C. geographus L.*, a fish-hunting species from the central Indo-Pacific) is the most venomous of all *Conus*. A number of human fatalities have resulted from the sting of this species (2-6). Although a preliminary purification and characterization of toxins from several *Conus* species has been carried out (1, 7-15), this is the first report of the structure of any toxin from the over 300 species of venomous *Conus*.

EXPERIMENTAL PROCEDURES

Assay for Toxicity

Samples of column fractions and purified peptides were lyophilized, dissolved in water, and tested for activity by intraperitoneal injection into mice, as described previously (14, 16). One unit of activity is defined as the quantity needed to produce death in a 20-g mouse 20 min after injection.

* This research was supported by United States Public Health Service Grant GM-22737 from the National Institutes of General Medical Sciences. Work at the University of the Philippines was supported by Grant G-257 from the International Foundation for Science (Stockholm) and a grant from the Marine Sciences Center, University of the Philippines. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Venom Preparation

Venom was prepared as described previously (14). In some experiments venom from several snails was pooled, while in others material from single animals was used. Venom was lyophilized and stored in the freezer until needed. It was then extracted, and the soluble peptides were subjected to chromatography on phosphocellulose.

Chromatography on Phosphocellulose

Conditions were essentially those described by Cruz *et al.* (14), except that an extended gradient of *N*-ethylmorpholine acetate was used (pH 9; .01-2.0 M) (see Fig. 1). Protein was assayed by the Bio-Rad method (17). Activity was located approximately by pooling small samples from several fractions, and testing as described above. More precise location was made by assaying individual fractions through areas of interest. In certain cases amino acid analysis (see below) was carried out on individual fractions within a peak.

Amino Acid Analysis

Samples were lyophilized, then hydrolyzed *in vacuo* using redistilled constant-boiling HCl (approximately 6 N) at 105 °C for 20 h. After removal of HCl over NaOH *in vacuo*, samples were analyzed on a Beckman model 121C amino acid analyzer (18).

Performic Acid Oxidation

Performic acid oxidation was carried out by the method of Hirs (19), using preformed performic acid, cooled to 0 °C. After reaction for 30 min at 0 °C, samples were diluted 20-fold with distilled water and lyophilized. They were then redissolved in water and lyophilized a second time to remove further traces of acid.

Periodate Oxidation

Periodate oxidation was carried out by a modification of the method of Dixon (20), using 25 µl of 0.01 and 0.05 M $NaIO_4$ in 0.05 M potassium phosphate buffer, pH 7.05, at room temperature for 1 h. Control incubations with Ser·Tyr·NH_2, Thr·Leu, and Glu·Tyr were carried out.

ASBMB
The Journal of Biological Chemistry
jbc
Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

Reduction and Methylation

Methylation of Cys residues was accomplished by selective *S*-methylation of SH groups with methyl *p*-nitrobenzenesulfonate (purchased from Eastman) according to the procedure described by Heinrikson (21).

Preparation of Carboxymethylcysteine-α-methyl Ester and α-Amide

L-Cystine methyl ester was purchased from Sigma. The corresponding amide was prepared by ammonolysis in methanol saturated with NH_3 at 0 °C for 1 h. Samples of the methyl ester and the amide were reduced and carboxymethylated with [^{12}C]iodoacetic acid. They were used as electrophoretic markers without further purification.

Analysis for Free SH

Spectrophotometric titration of thiol groups was carried out by the method of Boyer (22), using *p*-mercuribenzoate.

High Voltage Electrophoresis

A cooled flat-plate apparatus was used. Samples were run on Whatman No. 3MM paper with the following buffers: 0.1 M pyridine acetate, pH 4.4 or 6.4; 0.2 M pyridine acetate, pH 5.4; and 7% formic acid, pH 1.7. For analytical purposes (*e.g.* estimation of peptide charge) samples of approximately 2 nmol were run as small spots. For preparative runs samples were applied as streaks at a loading of approximately 10–20 nmol/cm, and guide strips were cut from the edges for staining. Small amounts of DNS-NH_2[1] and DNS-OH were included as mobility markers, and as guides for cutting strips for subsequent elution of peptides. Elution was carried out chromatographically by allowing 10% acetic acid to permeate along the paper strips and drip into test tubes. Approximately 0.5–1.0 ml was collected/strip.

Stains for Peptide Materials

The most frequently used stains were: ninhydrin-cadmium acetate (23) for amino groups, Pauli stain for His and/or Tyr, Sakaguchi stain for Arg, and Ehrlich's stain for Trp. All these procedures are described by Bennett (24).

NH_2-terminal Analysis

Dansyl chloride procedure (25) was used. Dansyl amino acids were identified by high voltage electrophoresis at pH 4.4 for 2 or 3 h at 54 V/cm, supplemented by electrophoresis at pH 1.7 for 1 to 1.5 h at 45 V/cm.

Enzymatic Digestions

1. *Trypsin and Chymotrypsin*—Samples of peptide (10–100 nmol) were incubated at 37 °C for 4 h in 100 μl of a solution of enzyme (0.2 mg/ml) in 0.2 M NEMAC buffer, pH 8.5.

2. *Aminopeptidase M*—Digestion was carried out on samples of unmodified GI and GII, using 0.1 M Na phosphate buffer, pH 7.0, at 37 °C for 3 h and overnight, respectively. Reaction was terminated by the addition of 0.2 M citrate buffer, pH 2.2, and samples were analyzed for free amino acids as described above.

3. *Carboxypeptidase Y*—Peptide samples were incubated for up to 3 h using 0.5 nmol of carboxypeptidase Y/ml of incubation mixture as described by Hayashi (26). Then, 0.1-ml aliquots were taken at different time intervals (0, 5, 10, 15, 30, 45, 60, 90, 120, and 180 min).

4. *Pronase Digestion*—The COOH-terminal CM dipeptide (2000 cpm) from conotoxins GI and GII was incubated at 37 °C for 12 h in 25 μl of a 1 mg/ml pronase solution in 0.2 M *N*-ethylmorpholine acetate (pH 8), 1 mM $CaCl_2$. The reaction mixture was dried under vacuum, redigested with the same amount of enzyme for an additional 3 h, and dried down again before electrophoresis.

5. *Dipeptidylaminopeptidase I*—Dipeptidylaminopeptidase I (EC 3.4.14.1) was purchased from Sigma. The procedure used for digestion was based on that of Callahan *et al.* (27). Approximately 2–5 nmol of peptide were dried in a small Pyrex tube of the type used for dansylation. They were then dissolved in 25 μl of 0.05 M pyridine acetate buffer (pH 4.5), containing 5 mM EDTA, 15 mM β-mercaptoethanol, and 1% (w/v) NaCl. Digestion was started by the addition of 2.5 μl (0.02 units) of enzyme dissolved in water. After reaction for 0.5–2 h at 37 °C, samples were dried *in vacuo* and dansylated in the usual manner. Controls were run in which enzyme alone was incubated and dansylated; insignificant quantities of dansyl amino acids were produced.

Hydrazinolysis

Samples of peptide were heated for 16 h at 80 °C *in vacuo* in sealed tubes with 0.5 ml of anhydrous hydrazine (28). Tubes were then cooled and opened, and the hydrazine was removed *in vacuo* over sulfuric acid. Samples were then dissolved in 0.5 ml of H_2O and lyophilized over P_2O_5. Amino acid analysis was carried out as described above, without prior separation of amino acid hydrazides.

Sequence Analysis of Peptides

The dansyl-monitored Edman method of Gray (29) was used for most peptides and peptide fragments. Samples of conotoxins GI (performic acid oxidized) and GIA (reduced and methylated) were also analyzed in a Beckman 890B Sequencer (30), using a single-cleavage program with dimethylallylamine buffers. In the case of GIA the analysis was carried out in presence of Polybrene (31). Amino acid phenylthiohydantoins were identified by high performance liquid chromatography on columns of ODS-silica (Dupont); the gradient used for elution was slightly modified from that of Hunkapiller and Hood (32).

Radioactive Labeling of Peptides

For investigation of the carboxyl terminus it was found convenient to label Cys residues using [^{14}C]iodoacetate (Amersham: 54 mCi/mmol). Samples of peptide were reduced using 2.5-fold excess of β-mercaptoethanol in 0.05 M Na borate, pH 7.7, 50 °C for 2 h. Undiluted [^{14}C]iodoacetate was then added and allowed to react for 1 h at room temperature. This was followed by reduction using a further 5-fold excess of mercaptoethanol at 50 °C for 30 min, and finally by addition of a 4-fold excess of [^{12}C]iodoacetate over total SH groups. After 30 min at room temperature, a 4-fold excess of β-mercaptoethanol was added and allowed to react for 30 min. The sample was freeze-dried and redissolved in H_2O. The carboxymethylated peptide was purified by high voltage electrophoresis at pH 1.7 for 1.5 h at 44 V/cm. Throughout the labeling reactions the samples were kept dark by wrapping the tube with aluminum foil. Control experiments with vasopressin were used to establish these conditions as giving high labeling with minimum side-reactions. The buffer used was sodium borate as we found that *N*-ethylmorpholine and pyridine give rise to labeled products, presumably by alkylation of the tertiary nitrogens.

Detection of Labeled Peptides

For electrophoresis fluorescent markers of DNS-OH and DNS-NH_2 were included in all test samples as internal mobility standards. Radioactive materials were located by dividing the paper lengthwise into 1-cm strips and measuring ^{14}C in each strip using a Beckmann LS233 scintillation counter, with a toluene base counting fluid.

Physiology

The cutaneous pectoris muscle of small grass frogs (***Rana pipiens***) was dissected. The muscle was excited either by directly depolarizing the major nerve innervating this muscle using a suction electrode (2 V) or by impaling the muscle with a micropipette filled with 0.5 M K_2SO_4. The transmembrane potential was measured by using another micropipette. For most experiments, normal frog Ringer's solution was used, containing 120 mM NaCl, 2.5 mM KCl, and 1.8 mM $CaCl_2$, using PIPES (1,4-piperazinediethanesulfonic acid) buffer (pH 7.2–7.3) and 10 mM phenol red as a pH indicator. This solution also contained glucose (0.5 g/l). For some experiments a high Mg^{2+}, low Ca^{2+} Ringer's solution was used containing 113 mM NaCl, 2.5 mM KCl, 5 mM Tris-maleate (pH 7.4), 0.5 mM $CaCl_2$, 5 mM $MgCl_2$. All experiments were performed at 15–20 °C. The muscle was observed under the microscope and twitching was monitored by visual inspection.

[1] The abbreviations used are: DNS-NH_2, 1-dimethylaminonaphthalene-5-sulfonamide or dansyl amide; DNS-OH, 1-dimethylaminonaphthalene-5-sulfonic acid or dansyl sulfonic acid; DNS-Glu, dansyl glutamic acid; PTH-Glu, phenylthiohydantoin derivative of glutamic acid; $CySO_3H$, cysteic acid; DNS-$CySO_3H$, dansyl cysteic acid; DNS-Asp, dansyl aspartic acid; CMCys, carboxymethylcysteine; DNS CMCys, dansyl carboxymethylcysteine; Cys-NH_2, cysteine amide; CMCys-NH_2, carboxymethylcysteine amide; CMCys-OMe, carboxymethylcysteine-*o*-methyl ester; DMAA, dimethylallylamine; NEMAC, *N*-ethylmorpholine-acetic acid buffer.

Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

RESULTS

Purification and Bioassay of Conotoxins GI, GII, and GIA

Results of phosphocellulose chromatography of venom from a single snail are shown in Fig. 1. The three biologically active peptides indicated are conotoxins GI, GII, and GIA, respectively. To achieve a separation of conotoxins GI and GII, we subjected the toxins to paper electrophoresis at pH 5.4 as shown in Fig. 2. A clear separation between GI, GII, and GIA (not shown) can be achieved under these conditions. Amino acid analysis (below) shows that GII prepared in this way is essentially free of GI (no detectable Arg or Tyr). GI, however, usually shows traces (<5%) of Lys and Phe, indicative of contamination by GII or a modified form of it.

The quantitative relationship between activity of peptide in the eluted strips (Fig. 2) is very strong confirmatory evidence that the peptides are the active molecules. From the curves the specific activities of GI and GII are 5.2 and 3.7 units/nmol, respectively. We do not have comparable data on GIA, but its activity is roughly equivalent to that of the other toxins on a molar basis.

Amino acid analysis of individual fractions from the composite GI-GII peak (Fig. 1) showed that the peptide eluted from paper accounted well for all the original material, and that GII elutes slightly ahead of GI from phosphocellulose. The recovery from paper was essentially quantitative.

The proportions of the three toxins described in this report vary depending on the venom preparation used. In our earlier work (14), only conotoxin GI was found. However, venom preparations with equal levels of GI and GII, with a predominance of conotoxin GII, and with all three peptide toxins present have since been obtained. Overall, much less GIA has been available for chemical and physiological studies.

Amino Acid Composition and Terminal Residues

The amino acid compositions of the three peptide toxins described above were determined (Table I); the composition for conotoxin GI was previously reported. The three peptides are obviously closely related. There are three differences between GI and GII, and GIA is identical to GI except for the presence of one additional residue of Gly and a residue of Lys.

All three peptides showed DNS-Glu as the only α-labeled amino acid. Aminopeptidase M released Glu as the only free amino acid from an equimolar mixture of GI and GII (0.6 mol/mol of peptide) and no Gln.

The carboxyl termini of GI and GII appeared to be blocked, as evidenced by failure to release significant quantities of amino acids after prolonged incubation with carboxypeptidase Y. Less than 0.05 mol/mol was released from both native and performic acid-oxidized toxins, under conditions in which insulin was extensively degraded. Similarly, hydrazinolysis of native and oxidized GI did not yield significant amounts of free amino acids, while yields of the expected residue from a control protein were about 40% (a λ-type Bence-Jones protein; carboxyl-terminal Ser). Chromatographic and electrophoretic behavior of the peptides was also best interpreted in terms of a blocked carboxyl. More definitive studies are described below.

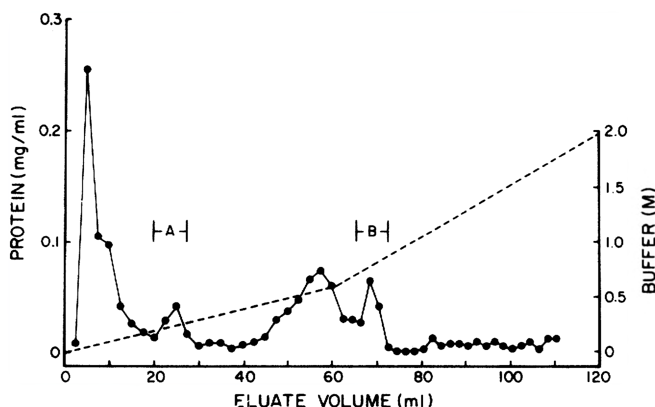


FIG. 1. **Phosphocellulose chromatography of crude venom.** Lyophilized venom from a single snail was extracted with 0.3 ml of 0.01 M NEMAC, pH 9.0. The extract was then applied on a column (10 × 0.5 cm) of phosphocellulose previously equilibrated with the same buffer. Elution was carried out first with a linear gradient consisting of 30 ml of 0.01 M and 30 ml of 0.6 M *N*-ethylmorpholine acetate, pH 9.0, followed by another gradient with 30 ml of 0.6 M and 30 ml of 2.0 M *N*-ethylmorpholine acetate, pH 9.0, as limiting buffers. Conotoxins GI and GII eluted together at around 0.2 M (*Peak A*), and GIA (*Peak B*) eluted at about 0.8 M *N*-ethylmorpholine acetate.

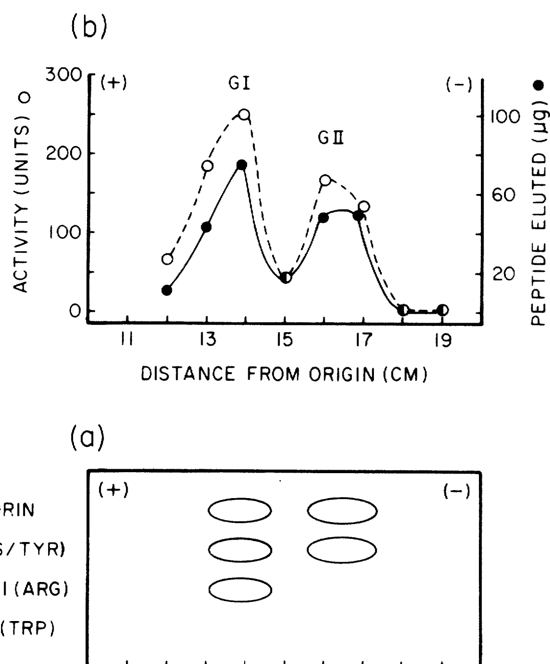
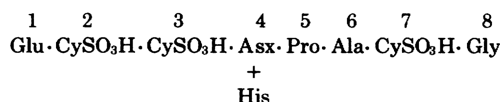


FIG. 2. **Paper electrophoresis for separation of conotoxin GI and GII.** Material from the center region of peak 1 (Fig. 1) was subjected to electrophoresis in 0.2 M pyridine acetate buffer, pH 5.4, for 2 h and 20 min at 27 V/cm. The paper was dried in a hood at room temperature. *a*, Guide strips were cut from the edges of the peptide zones and stained as described in the text. The faster moving peptide stained relatively more intensely with ninhydrin but did not stain positively for Arg or Trp. The slower peptide stained for Arg, but not Trp. *b*, peptides were eluted from the unstained regions of the paper and were tested by bioassay and amino acid analysis.

Sequential Degradation of GI and GII

Sequencer analysis of performic acid-oxidized GI was not successful. Initial yields were very low (approximately 15% of the expected phenylthiohydantoin derivative of glutamic acid), and subsequent yields declined rapidly. Similarly, the yield of DNS-Glu from performic acid-oxidized material was low. Greatly improved yields were obtained from a pool of GI and GII in which the terminal Glu was removed by Edman degradation prior to performic acid oxidation of the peptide. Stepwise application of the dansyl-Edman procedure gave the following sequence:



Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

TABLE I
Amino acid compositions of purified toxins

Amino acid	GI[a]	GII[b]	GIA[c]
Lysine	0.09	0.95 (1)	0.98 (1)
Histidine	0.98 (1)	1.88 (2)	0.96 (1)
Arginine	0.86 (1)	—[d]	0.99 (1)
Aspartic acid	1.01 (1)	0.12	1.17 (1)
Threonine	0.03	—	—
Serine[e]	0.99 (1)	1.06 (1)	0.98 (1)
Glutamic acid	1.04 (1)	1.11 (1)	1.04 (1)
Proline	1.03 (1)	0.97 (1)	0.92 (1)
Cysteine[f]	3.7 (4)	3.8 (4)	3.8 (4)
Glycine[g]	1.55 (1)	1.11 (1)	1.87 (2)
Alanine	0.96 (1)	1.01 (1)	0.99 (1)
Valine	0.04	—	0.16
Methionine	0.07	—	0.26
Isoleucine	0.03	—	0.17
Leucine	0.06	—	0.25
Tyrosine	0.96 (1)	—	0.93 (1)
Phenylalanine	0.09	1.03 (1)	0.19
Tryptophan[h]		—	—

[a] Previous report (14).
[b] After electrophoresis (Fig. 2).
[c] From phosphocellulose (Fig. 1).
[d] —, quantities of the amino acid were below the detection limit.
[e] Uncorrected for hydrolysis losses.
[f] Determined as $CySO_3H$ (GI and GII) and *S*-methylcysteine (GIA).
[g] Subsequent analyses confirm presence of only one Gly residue in GI, the rest being free amino acid.
[h] Determined by staining with Ehrlich's reagent (see Fig. 2).

Tryptic Fragments

Trypsin digestion of a mixture of GI and GII resulted in the appearance of His as the only new end-group revealed by dansylation. The digested material was oxidized with performic acid, and the fragments were separated by high voltage electrophoresis at pH 6.4 (2.5 h at 28 V/cm). Two anodally moving bands were obtained, plus a third band remaining at the neutral point. Elution of the peptides, followed by amino acid analysis and dansyl-Edman degradation yielded the following results.

1. *Faster Anodal band—*

Composition: 1 Arg, 1 Asx, 1 Glx, 1 Pro, 3 $CySO_3H$, 1 Gly, 1 Ala

Sequence: $\underrightarrow{\text{Glx}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{Asx}}\cdot\underrightarrow{\text{Pro}}\cdot\underrightarrow{\text{Ala}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{Gly}}$ (Arg)

The net charge of the peptide was measured by the method of Offord (33), using an extensive set of data for log (molecular weight) *versus* log (mobility relative to DNS-OH). From the measured charge of −3, and the established status of the Glu residue at position 1, we conclude that the residue at position 4 is Asn rather than Asp. This agrees with the results obtained in the poor sequenator run mentioned above, and also with the state of the corresponding residue in GIA (see below).

2. *Slower Anodal band—*

Composition: 1 Lys, 1 His, 1 Glx, 1 Pro, 3 $CySO_3H$, 1 Gly, 1 Ala

Sequence: $\underrightarrow{\text{Glx}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{His}}\cdot\underrightarrow{\text{Pro}}\cdot\underrightarrow{\text{Ala}}\cdot\underrightarrow{\text{CySO}_3\text{H}}\cdot\underrightarrow{\text{Gly}}\cdot\underrightarrow{\text{Lys}}$

The net charge of the peptide was between −2 and −3, compatible with the assignment of Glu as the NH_2-terminal residue.

3. *Neutral Band—*

Composition: 1 His, 1 Ser, 1 $CySO_3H$, 0.5 Phe, 0.3 Tyr

Degradation of this peptide established clearly that it was a mixture of His·Tyr·(Ser, $CySO_3H$) and His·Phe·(Ser, $CySO_3H$). Beyond this point we observed highly anomalous behavior, indicating that chemical rearrangements were occurring. In this particular experiment we obtained a mixture of DNS-$CySO_3H$ and DNS-Ser at the third step, in approximately a 2:1 ratio. Roughly equal amounts of DNS-$CySO_3H$ and DNS-Ser were obtained at the next step. These data suggested the sequence $CySO_3H$·Ser·x for the presumed terminal dipeptide, where x indicates a blocked carboxyl terminus. We repeated the experiments with a preparation of pure GI. Existence of the COOH-terminal block was confirmed by examining the electrophoretic mobility of the tetrapeptide at several pH values and also examining mobility of the tripeptide remaining after one round of degradation. Again Tyr was confirmed as the second residue; at the next step we obtained both DNS-$CySO_3H$ and DNS-Ser, but this time DNS-Ser was the stronger spot. A further round of degradation was carried out to release the presumed Ser·x and/or $CySO_3H$·x. The resulting material was dansylated and submitted to electrophoresis at pH 4.4 without prior hydrolysis; a marker of authentic DNS-Ser-NH_2 was run in parallel, and moved to the expected position towards the cathode. No trace of material ran at that point in the experimental sample, but a strongly fluorescent spot was observed running towards the anode with a mobility slightly greater than that of DNS-Asp, but much less than that of DNS-$CySO_3H$: no marker of DNS-$CySO_3H$-NH_2 was available. The fluorescent material running in this very distinctive position was eluted and hydrolyzed with 6 N HCl for 18 h at 105 °C. To our great surprise we obtained DNS-$CySO_3H$ and DNS-Ser in equal amounts. The material ran as a single spot at pH 6.4 as well as at pH 4.4, so there is little doubt that it is the doubly dansylated form of a rearrangement product of the carboxyl-terminal dipeptide. Since anomalies only showed at steps 3 and 4, we suspect the rearrangement occurs during the trifluoroacetic acid cleavage to remove the Tyr residue. Because of this anomalous behavior we undertook a completely different series of experiments to establish the structure at the carboxyl terminus of GI (see below).

Sequential Degradation of GIA

A sample of reduced and *S*-methylated GIA was analyzed in the sequenator. Results are presented in Table II. The sequence of the first 11 residues was found to be identical with that of the first 11 residues of GI:

$$\underrightarrow{\text{Glu}}\cdot\underrightarrow{\text{Cys}}\cdot\underrightarrow{\text{Cys}}\cdot\underrightarrow{\text{Asn}}\cdot\underrightarrow{\text{Pro}}\cdot\underrightarrow{\text{Ala}}\cdot\underrightarrow{\text{Cys}}\cdot\underrightarrow{\text{Gly}}\cdot\underrightarrow{\text{Arg}}\cdot\underrightarrow{\text{His}}\cdot\underrightarrow{\text{Tyr}}$$

This leaves unassigned four residues Ser, Cys, Gly, and Lys.

Carboxyl-terminal Region of GI

A sample of 25 nmol of GI was alkylated using [^{14}C]iodoacetate, and excess reagents were removed by high voltage paper electrophoresis. Cleavage of the labeled peptide by chymotrypsin was followed by electrophoresis of the fragments in 0.2 M *N*-ethylmorpholine acetate, pH 8.0, at 18 V/cm for 8 h. As expected, two radioactive bands were obtained. Three-fourths of the radioactivity was in a band running towards the anode, presumably the NH_2-terminal undecapeptide containing three residues of [^{14}C]carboxymethylcysteine. The remaining one-fourth was in the neutral band, presumably representing the carboxyl-terminal dipeptide derivative (Ser, CMCys)·x. The material in the neutral band was eluted and subjected to various experiments, with [^{12}C]carboxymethylcysteine added as carrier to minimize oxidative losses.

Experiment 1: Acid Hydrolysis—This was followed by electrophoresis at pH 6.4. All radioactivity migrated with CMCys.

Experiment 2: Dansylation—This was followed by hydrolysis and electrophoresis at pH 1.7. A trace of DNS-Ser was obtained. No radioactivity was associated with DNS-CMCys:

Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

TABLE II

Sequential degradation of conotoxin GIA

Approximately 10 nmol of *S*-methylated peptide were degraded using Polybrene and the volatile buffer program with dimethylallylamine (see text).

Cycle	Residue	
		nmol
1	Glu	6.2
2	Me Cys	5.9
3	Me Cys	7.0
4	Asn	8.8
5	Pro	2.0
6	Ala	6.0
7	Me Cys	2.2
8	Gly	2.5
9	(Arg)[a]	+
10	(His)[a]	+
11	Tyr	1.0
12	—[b]	
13	—	

[a] Detected by weak positive color tests on the aqueous phases.

[b] —, no significant increase of any amino acid was observed at steps 12 or 13.

all migrated with free CMCys.

Experiment 3: Oxidation—Oxidation of the dipeptide by periodate resulted in a shift from a net positive to a zero charge at pH 1.7. Controls on several other small peptides confirmed that only those containing NH_2-terminal Ser or Thr are affected by this treatment (20).

Experiment 4: Pronase Treatment—The [^{14}C]carboxymethylated peptide was treated with pronase (34), and subjected to electrophoresis at pH 6.4 and pH 1.7, with markers of carboxymethylcysteine (CMCys), carboxymethylcysteine-α-methyl ester (CMCys-OMe), carboxymethylcysteine-α-amide (CMCys-NH_2), and undigested dipeptide. At pH 6.4 the original dipeptide, and the pronase-digested material remained at the neutral point, as did the amide and ester derivatives of carboxymethylcysteine: CMCys itself moved towards the anode. At pH 1.7 the pronase-digested material ran rapidly towards the cathode, co-migrating with CMCys-NH_2. The mobility was quite distinctive, greater than that of undigested peptide, or of CMCys, and less than that of CMCys-OMe (Fig. 3). From the mobilities of digested and undigested peptides relative to that of DNS-NH_2 we can set an upper limit of approximately 40–50 daltons for the weight of the blocking group x (in —CO·x). Methyl ester is definitely excluded, while ethyl ester is compatible with the results. By far the most probable blocking group would seem to be amide. Although our data do not constitute rigorous proof, the coincident electrophoretic mobility is highly suggestive, and the amide group is the only common carboxyl-terminal block encountered in natural peptides.

Carboxyl Terminus of GII and GIA

The above results indicate very strongly that the COOH-terminal sequence of GI is -Ser-Cys-NH_2, and we take the same to be true for GII. In the case of GIA the sequence is -Ser-Cys-Gly-Lys-NH_2. Our assignment is based on the following observations.

1. GIA was reduced and carboxymethylated with [^{14}C]iodoacetate. Cleavage of the carboxymethylated peptide by chymotrypsin yielded two radioactive fragments: the first was negatively charged and contained about 3 times as much radioactivity as the second, positively charged fragment. Amino acid analysis of the latter confirmed that it was the tetrapeptide (Ser, CMCys, Gly, Lys). The measured charge of +1.0 indicates that the carboxyl terminus is blocked.

2. Dansylation and hydrolysis of the carboxymethylated tetrapeptide yielded DNS-Ser and ϵ-DNS-Lys, confirming that Ser is amino-terminal; periodate oxidation also resulted in a change of net charge from +2 to +1 at pH 1.7.

3. Dipeptidylaminopeptidase I digestion of the tetrapeptide yielded a radioactive dipeptide which was negatively charged. Dansylation and hydrolysis of this radioactive peptide gave DNS-Ser; periodate oxidation of the dipeptide resulted in a product which was neutral at pH 1.7. Thus, the second residue from the NH_2-terminus is Cys.

4. Dipeptidylaminopeptidase I treatment of tetrapeptide, followed by dansylation and hydrolysis, yielded DNS-Ser, ϵ-DNS-Lys and DNS-Gly. There was no trace of either α- or bis-DNS-Lys.

5. Trypsin digestion of the carboxymethylated tetrapeptide caused all radioactivity to move to the neutral position. Ten nmol of tetrapeptide were trypsin treated and subjected to electrophoresis at pH 6.5. Only the neutral radioactive band showed ninhydrin reactivity; specifically, there was no ninhydrin staining material at the position of glycinamide. Hydrolysis of material eluted from the neutral band showed all four amino acids as major components, but there was sufficient contamination to preclude a definitive test of whether Gly were truly an integral part of the digested peptide.

Together, these results strongly suggest that the sequence of the COOH-terminal tetrapeptide of GIA is Ser-Cys-Gly-Lys-NH_2, although the evidence is not absolutely definitive that the blocking group is an amide. Electrophoretic mobility of the tetrapeptide indicates that the blocking group must be of very low molecular weight, and its ready removal by trypsin is fully compatible with its being amide. The carboxyl-terminal amides, in cases where they have been studied (*e.g.* mellitin (35)), are produced by modification of Gly residues. It is attractive to hypothesize that GIA is converted to GI by processing of the Gly to produce the amide. Presumably then, GIA was itself produced from a larger precursor.

Status of Cys Residues

No free SH (<0.1 mol/mol) was detected in GI or GII. Previous studies (14) indicated M_r = ~1500 by gel filtration. We conclude, therefore, that the molecules exist as monomers containing two internal disulfide bridges. We are attempting separate experiments to elucidate the linkage patterns.

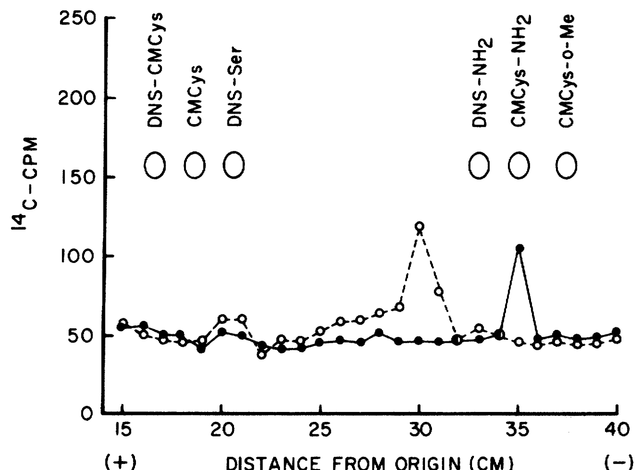


FIG. 3. **Paper electrophoresis of pronase-digested carboxyl terminal dipeptide.** Pronase digestion was carried out as described under "Experimental Procedures." After reaction appropriate aliquots containing DNS-NH_2, CMCys-NH_2, and CMCys-OMe as internal markers and in parallel spots were subjected to paper electrophoresis at pH 1.7 (7% formic acid), 28 V/cm for 3 h. Radioactive spots were located as described in the text. ○- - -○, control; ●——●, pronase-digested.

Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

	4	9	11
GI	Glu·Cys·Cys·Asn·Pro·Ala·Cys·Gly·Arg·His·Tyr·Ser·Cys·NH_2		
GIA	Glu·Cys·Cys·Asn·Pro·Ala·Cys·Gly·Arg·His·Tyr·Ser·Cys·Gly·Lys·NH_2		
GII	Glu·Cys·Cys·His·Pro·Ala·Cys·Gly·Lys·His·Phe·Ser·Cys·NH_2		

FIG. 4.

Physiological Studies

Experiments using the cutaneous pectoris muscle of frogs were performed using purified *C. geographus* toxins at a concentration of 25 μg/ml in Ringer's solution. Purified conotoxin GII, purified conotoxin GI (approximately 90% pure), and mixtures of conotoxins GI and GII were used and gave essentially the same results.

In the presence of conotoxin, the resting potential was in the normal range (80–100 mV) for this muscle, and no measurable depolarization was observed. However, when the end-plate potential was measured, it was found that there was no response to excitation of the nerve in six different experiments. In a seventh experiment, a small evoked response (4 mV) was observed, but this appeared to be from a muscle cell that was beneath the surface and therefore effectively exposed to a lower concentration of toxin. Miniature end plate potentials were completely abolished by the addition of *C. geographus* toxin at these concentrations.

However, muscle action potentials were not detectably inhibited by these concentrations of conotoxins. When a muscle fiber was impaled by two electrodes and a current was passed, a normal muscle action potential was induced. Twitching of the muscle fiber could be observed after direct excitation through an impaled electrode at these concentrations of conotoxin. In addition, nerve action potentials could also be elicited.

Lower concentrations of conotoxins resulted in reduced, but not completely abolished, end-plate potentials. For example, at a concentration of 8 μg/ml, the end-plate potential was in the range of 3–5 mV, although the miniature end plate potentials were no longer detectable. Some experiments were also done at low Ca^{2+} (0.5 mM) and high Mg^{2+} (5 mM) concentrations; under these conditions, the normal end-plate potential was an average of 1.73 ± 0.07 mV. In the presence of a 0.6 μg/ml solution of a 1:1 mixture of GI and GII, the average end-plate potential was reduced to 0.49 ± 0.04 mV. However, the failure rate was 95/189 in the presence of conotoxin, and 0/118 in the control. Mini end-plate potentials, which in the absence of conotoxin were an average of 0.34 mV, were completely abolished in the presence of 0.6 μg/ml conotoxin. Muscles ceased contractions in response to nerve stimulation almost immediately upon addition of toxin; on washing out the conotoxin, the muscles resumed contraction as soon as the solution was changed. Thus, the toxin binding appears to be readily reversible.

DISCUSSION

We have purified three homologous peptides from *C. geographus* venom. The purification and a preliminary biochemical characterization of one of these has been previously described (14). In the venom preparation we had available for those studies, only conotoxin GI was detected. Considerable variation has been observed in toxin composition from one venom preparation to another. The venom preparation shown in Fig. 1 was extracted from a single snail, and all three homologous peptides (conotoxins GI, GIA, and GII) were present. It is not clear whether these differences in toxin composition are genetic, due to changes in snail physiology or due to differences in venom extraction and storage.

The results of our structural studies on these peptides are summarized in Fig. 4.
Clearly they form a very closely related set. Conotoxins GI and GII are both 13-residue peptides identical in ten of their residues. The three substitutions are conservative: Lys for Arg, Phe for Tyr, and His for Asn. This suggests that at these locations the molecule can tolerate some flexibility, but perhaps not a great deal. Both molecules appear to contain two disulfide bridges, as yet unassigned, and to be amidated at their carboxyl terminal. The result is that both peptides are markedly basic, tightly folded molecules. The third peptide is designated GIA since its sequence is identical with that of GI, except for presence of additional residues of Gly and Lys at the carboxyl terminus. Since all three toxins have approximately the same level of activity, there must be a reasonable tolerance for substitution at the carboxyl terminus also; however, all three peptides have a blocked COOH-terminus.

These toxins are all sensitive to agents which break disulfide bonds, such as sodium borohydride or β-mercaptoethanol. Once reduction has occurred, reoxidation does not result in reactivation of toxic activity. These results suggest that the new disulfide bonding pattern is thermodynamically stable, but non-native.

The disulfide reduction/reoxidation results, as well as the presence of GIA and GI in the same venom, suggest that these toxins may be processed from larger precursors. Crude venom contains insoluble birefringent granules, which are largely composed of proteins rich in cystine residues.[2] Preliminary experiments suggest that the protein of the granules may represent an earlier precursor of the active toxins: limited proteolytic digestion with elastase resulted in release of significant amounts of soluble biologically active peptides. A larger molecule may in fact be a necessary precursor for achieving correct folding and disulfide bridge formation.

The physiological data included with this report indicate that conotoxins GI, GII, and GIA all act at the muscle end-plate region; there is no detectable inhibition of either nerve or muscle action potential propagation at the concentrations used. Although some presynaptic effects cannot be rigorously excluded by the data, the postsynaptic effects are sufficient to account for the potent biological activity of these peptides. A much more detailed and quantitative study of the physiological effects of these conotoxins is being carried out.[3] These investigators have used ^{125}I-labeled α-bungarotoxin in competition studies; their initial results indicate that these conotoxins competitively inhibit ^{125}I-α-bungarotoxin binding to the acetylcholine receptor.

The three homologous peptides above are not the only toxins we have detected in *C. geographus* venom. As was first reported by Endean (8), whole *C. geographus* venom inhibits muscle action potential propagation even when the muscle is excited directly. The peptides described in this report do not abolish the muscle action potential; their action appears to be restricted to the muscle end plate region. There is at least one additional peptide in *C. geographus* venom which causes inhibition of the muscle action potential. This toxin, which does not appear to be homologous to conotoxins GI, GIA, and

[2] W. R. Gray, B. M. Olivera, and L. J. Cruz, unpublished results.

[3] O. McManus, J. Musick, and C. Gonzales, submitted for publication.

Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009

GII, has been recovered in very variable amounts from one venom preparation to another. In addition, there are at least two toxins which in vertebrates have effects only on the central nervous system. Recently, Spence and co-workers (13) have reported a purification of three peptide toxins from *C. geographus* venom. It appears that toxins I and II of these workers are homologous (and possibly identical) with the peptides described in this report. A tentative assignment would be that their toxin I is a mixture of our GI and GII, while their toxin II is equivalent to our GIA.

It is clear that conotoxins GI, GIA, and GII are a related family of tightly folded small peptides with common structural features which permit their potent biological effects at the verebrate neuromuscular junction. These peptides are in a size range in which synthesis is feasible and amino acid modification studies are optimal. They may be ideal probes for unraveling some of the molecular details of postsynaptic inhibition.

Acknowledgments—We would like to thank Lorna So, Milagros Bautista, and Craig Clark for their help in purifying the conotoxins used for this study. The contribution of Gloria Corpuz in the preparation of venom from the snails is gratefully acknowledged. Drs. D. Hernandez, A. Bendaña-Brown, and C. Y. Lim-Sylianco made significant early contributions to this work.

REFERENCES

1. Kohn, A. J., Saunders, P. R., and Wiener, S. (1960) *Ann. N. Y. Acad. Sci.* **90,** 706–725
2. Clench, W. J., and Kondo, Y. (1943) *Am. J. Trop. Med. Hyg.* **23,** 105–121
3. Kohn, A. J. (1963) in *Venomous and Poisonous Animals and Noxious Plants of the Pacific Area* (Keegan, H. L., and MacFarlane, W. V., eds) pp. 83–96, Pergamon Press, Oxford
4. Endean, R., and Rudkin, C. (1965) *Toxicon* **2,** 225–248
5. Rice, R. D., and Halstead, B. W. (1968) *Toxicon* **5,** 223–224
6. McMichael, D. F. (1971) in *Venomous Animals and Their Venoms* (Bucherl, W., and Buekley, E., eds) Vol. 3, pp. 373–393, Academic Press, Inc., New York
7. Whysner, J. A., and Saunders, P. R. (1966) *Toxicon* **4,** 177–181
8. Endean, R., Parish, G., and Gyr, P. (1974) *Toxicon* **12,** 131–138
9. Cruz, L. J., Corpuz, G., and Olivera, B. M. (1976) *Veliger* **18,** 302–308
10. Cruz, L. J., Tecson, E. M., and Olivera, B. M. (1976) *Abstracts. Tenth International Congress of Biochemistry*, p. 675
11. Endean, R., Williams, H., Gyr, P., and Surridge, J. (1976) *Toxicon* **14,** 267–274
12. Gray, W. R., Luque, A., Olivera, B. M., and Cruz, L. J. (1977) *Fed. Proc.* **36,** 684
13. Spence, I., Gillessen, D., Gregson, R. P., and Quinn, R. J. (1977) *Life Sci.* **21,** 1759–1770
14. Cruz, L. J., Gray, W. R., and Olivera, B. M. (1978) *Arch. Biochem. Biophys.* **190,** 539–548
15. Elliot, E. J., and Raftery, M. A. (1979) *Toxicon* **17,** 259–268
16. Cruz, L. J. (1977) *Acta Medica Philippina* **13,** 13–16
17. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
18. Spackman, D. H., Stein, W. H., and Moore, S. (1958) *Anal. Chem.* **30,** 1190–1206
19. Hirs, C. W. H. (1967) *Methods Enzymol.* **11,** 197–199
20. Dixon, H. B. F. (1962) *Biochem. J.* **83,** 91–94
21. Heinrikson, R. L. (1971) *J. Biol. Chem.* **246,** 4090–4096
22. Boyer, P. D. (1954) *J. Am. Chem. Soc.* **76,** 4331–4337
23. Dreyer, W. J., and Bynum, E. (1967) *Methods Enzymol.* **11,** 32–39
24. Bennett, J. C. (1967) *Methods Enzymol.* **11,** 330–339
25. Gray, W. R. (1967) *Methods Enzymol.* **11,** 139–151
26. Hayashi, R. (1977) *Methods Enzymol.* **47,** 84–93
27. Callahan, P.X., McDonald, J. K., and Ellis, S. (1972) *Methods Enzymol.* **25,** 282–298
28. Schroeder, W. A. (1972) *Methods Enzymol.* **25,** 138–143
29. Gray, W. R. (1967) *Methods Enzymol.* **11,** 469–475
30. Edman, P., and Begg, G. (1967) *Eur. J. Biochem.* **1,** 80–91
31. Tarr, G. E., Beecher, J. F., Bell, M., and McKean, D. J. (1978) *Anal. Biochem.* **84,** 622–627
32. Hunkapiller, M. W., and Hood, L. E. (1978) *Biochemistry* **17,** 2124–2133
33. Offord, R. E. (1966) *Nature* **211,** 591–593
34. Nomoto, M., Narahashi, Y., and Murakami, M. (1960) *J. Biochem. (Tokyo)* **48,** 593–602
35. Suchanek, G., and Kreil, G. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 975–978

The Journal of Biological Chemistry
ASBMB
jbc ONLINE
Downloaded from www.jbc.org at UNIV OF UTAH, on October 7, 2009