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μ-Conotoxin GIIIA, a Peptide Ligand for Muscle Sodium Channels: Chemical Synthesis, Radiolabeling, and Receptor Characterization†

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ABSTRACT: The peptide conotoxin GIIIA from *Conus geographus* L. venom, which specifically blocks sodium channels in muscle, has been synthesized by a solid-phase method. The three disulfide bridges were formed by air oxidation. After HPLC purification, the synthetic product was shown to be identical with the native conotoxin GIIIA from *Conus geographus*. A high specific activity, ^{125}I derivative of μ-conotoxin was prepared and used for binding assays to the Na channel from *Electrophorus* electric organ. Specific binding could be abolished by competition with tetrodotoxin. The radiolabeled toxin was specifically cross-linked to the Na channel. These studies demonstrate that μ-conotoxin GIIIA can be used to define the guanidinium toxin binding site and will be a useful ligand for understanding functionally important differences between Na channel subtypes.

The sodium channel is presently the best understood voltage-sensitive ion channel, both because of the detailed electrophysiology available and because of the wealth of biochemical data including complete sequence information from cloning experiments [for reviews, see Barchi (1988); Salkoff et al. (1987), Strichartz et al. (1987), Catterall (1986), and Agnew (1984)]. This remains the model system for understanding how changes in voltage across a membrane can result in a change in ion permeability. An important remaining goal is to identify the functionally important parts of the molecule. For example, it has long been known that the heterocyclic guanidinium toxins (tetrodotoxin and saxitoxin) bind at a site on the channel and prevent sodium permeation; this well-known toxin binding site has yet to be localized within the

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sodium channel structure. In addition, the molecular basis of functional differences between sodium channel subtypes needs to be understood.

Recently, the μ-conotoxins, a new class of peptide neurotoxins which are promising ligands for addressing the above problems, were characterized from the venom of the piscivorous marine snail *Conus geographus*. They constitute a family of 22-residue peptide amides which block skeletal muscle sodium channels, while having no discernible effect on cardiac muscle, nerve, or brain sodium channels. Seven different homologues have been characterized, the most abundant of which is μ-conotoxin GIIIA,[1] a peptide with three hydroxyproline residues and three disulfide bridges (Cruz et al., 1985; Olivera et al., 1985). This peptide is equivalent to toxin III of Spence et al. (1977) and to geographutoxin I of Nakamura et al. (1983). The μ-conotoxins are only one of three classes of paralytic toxins found in *Conus geographus* venom, the other two being the ω-conotoxins which block voltage-sensitive Ca channels and the α-conotoxins which block the acetylcholine receptor (Olivera et al., 1985).

It has been established that μ-conotoxin GIIIA has strong selectivity for the muscle subtype of sodium channel. The peptide competes with the guanidinium toxins (tetrodotoxin and saxitoxin) for binding to these channels but does not compete with them for binding to the toxin site in neuronal sodium channels (Moczydlowski et al., 1986a,b). Because of the peptidic nature of μ-contotoxin GIIIA, the range of possible modifications that can be made on the toxin without losing biological activity is far greater than can be achieved with the guanidinium toxins.

Natural conotoxin GIIIA is relatively difficult to obtain because *Conus geographus* venom is not commercially available. The chemical synthesis of this peptide has not previously been reported. We describe the complete synthesis of this 22 amino acid peptide; we have also radiolabeled μ-contotoxin GIIIA to yield a biologically active ^{125}I derivative of the peptide and cross-linked the radiolabeled peptide to the sodium channel from the electric fish *Electrophorus*. The availability of synthetic peptide and high specific activity radiolabeled μ-conotoxin GIIIA should make it a much more generally useful probe for understanding sodium channel function.

The structure of μ-conotoxin GIIIA is Arg-Asp-Cys-Cys-Thr-Hyp-Hyp-Lys-Lys-Cys-Lys-Asp-Arg-Gln-Cys-Lys-Hyp-Gln-Arg-Cys-Cys-Ala-NH_2.

Experimental Procedures

Materials. All amino acid derivatives were obtained from Bachem Inc. (Torrance, CA) and were of the L configuration. MBHA-resin with the capacity of 0.35 mequiv/g was prepared from polystyrene cross-linked with 1% divinylbenzene, 200–400 mesh (Biobeads S × 1 from Bio-Rad Laboratories), using the previously published procedure (Rivier et al., 1973). Cation-exchange resin H^+ form was prepared from analytical-grade Na^+-form cation-exchange resin, 100–200 mesh, Bio-Rex 70 from Bio-Rad Laboratories, by washing with 0.5 M HCl. 1,2-Ethanediol (EDT), 1,3-diisopropylcarbodiimide (DIC), and triethylamine (TEA) were obtained from Aldrich Chemical Co. Inc. Trifluoroacetic acid (TFA) was obtained from Halocarbon Products Corp. and redistilled for HPLC use. Acetonitrile for preparative HPLC was obtained from E. M. Science; for semipreparative and analytical HPLC, it was obtained from American Burdick and Jackson. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Sigma Chemical Co. Heptafluorobutyric acid (HFBA) was purchased from Pierce Chemical Co.

Synthesis of Peptide. MBHA-resin (3.0 g) containing ca. 1.0 mmol of amino groups was used for automated solid-phase synthesis on a Beckman Model 990B peptide synthesizer. Previously reported protocols (Marki et al., 1981) were used. Protection of α-amino groups was accomplished throughout synthesis with Boc. Amino acid side chains were protected as follows: Arg(Tos), Asp(OBzl), Cys(MeOBzl), Hyp(Bzl), Lys(2-Cl-Z), and Thr(Bzl). Deblocking steps (20 min each) were performed with TFA/CH_2Cl_2 (60% by volume) containing 2.5% of EDT, and neutralization was with 10% TEA/CH_2Cl_2 (v/v). Couplings in CH_2Cl_2 were performed using 1.0 M DIC/CH_2Cl_2 for 180 min. A 2-fold excess of protected amino acids was used based on the original substitution of MBHA-resin.

Cleavage from the Resin and Formation of Disulfide Bridges. The peptide–resin (4.8 g) was treated with 50 mL of liquid HF at 0 °C for 60 min in the presence of 5 mL of anisole. After cleavage by HF and its removal under vacuum, the product was washed with anhydrous diethyl ether. The peptide was extracted from the resin with 50% aqueous acetic acid and quickly diluted to 2000 mL with distilled water. The pH of the solution was adjusted with ammonium hydroxide to 6.8, and $FeSO_4$ (100 mg) was added. The solution stood under slow stirring in an open beaker at +5 °C for 72 h at which time all sulfhydryl had oxidized when monitored with DTNB (Ellman, 1959). The pH was then adjusted with acetic acid to 5.0, and the solution was passed through a column packed with cation-exchange resin (3 × 12 cm) Bio-Rex 70 (H^+ form). The resin was extensively washed with water and 5% aqueous acetic acid, and the oxidized peptide was removed from the resin with 200 mL of 50% aqueous acetic acid. This fraction was diluted with water and lyophilized; 1.3 g of crude product was obtained.

Purification. Crude product was first partially purified by using a preparative chromatographic system consisting of a Waters Associates Prep LC/System 500A, a Model 450 variable-wavelength UV detector, a Fischer Recordall Model 5000 strip chart recorder, and an Eldex Laboratories Chromat-A-Trol Model II gradient maker. Preparative cartridges were dry-packed in this laboratory (Rivier et al., 1984) with VYDAC C18 silica (15–20-μm particle sizes). Semipreparative purification was performed by using a 1 × 25 cm column filled with 5-μm particle-size VYDAC C18 and a Hitachi Ltd. Model 655A liquid chromatograph with a Model 655-61 processor. Analytical control of individual fractions and coelution experiment with the native conotoxin GIIIA were performed on a Hitachi Ltd. liquid chromatograph using an analytical HPLC (VYDAC C18) column (0.48 × 25 cm) with 5-μm particle size. After each step, purity was determined by carrying out an analytical HPLC run and measuring the fraction of absorbance which traveled at the position of authentic μ-conotoxin GIIIA (native material was run in parallel under identical HPLC conditions). Most impurities are presumably conformational isomers disulfide-bonded in alternative conformations. Details of reverse-phase HPLC purification are given in Table I.

Sequence Analysis. Twenty nanomoles of synthetic peptide was reduced and carboxymethylated in 75 μL of 0.2 M *N*-ethylmorpholine, all incubations being carried out in the dark. The reaction mixture was diluted with 200 μL of 10% acetic

[1] Abbreviations: the symbols of amino acids and peptides are in accordance with 1983 Recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984).

acid and dried down in the spinning cup of a Beckman 890D sequencer, which contained 1 mg of polybrene that had been precycled through several steps of degradation. A 0.25 M Quadrol program was used, the sample being first put through a wash cycle with ethyl acetate. Purity was assessed by measuring "preview" of amino acids, indicative of failure sequences.

Modification of GIIIA. The procedure used for chemical modification of the toxin with *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (SHPP) was based on the method of Bolton and Hunter (1973). μ-Conotoxin GIIIA (20 nmol) was dissolved in 40 μL of 0.125 M KH_2PO_4, pH 7.5, and 5 μL of 0.1 M SHPP in dimethyl sulfoxide was added. The reaction was allowed to proceed for 15 min at 0 °C, with frequent mixing. After incubation, the products were separated from unmodified GIIIA by HPLC (Figure 1).

Unmodified toxin travels at the position indicated by the heavy arrow in Figure 1. Fractions corresponding to the absorbance peaks indicated by arrows in Figure 1 were assayed for biological activity using intraperitoneal injection of the toxin into goldfish and intracerebral injections in young mice. All five fractions tested positive. Material corresponding to the hatched absorbance peak was used for iodination.

Preparation of ^{125}I-Labeled 3-(4-Hydroxyphenyl)-propionyl-GIIIA. The GIIIA derivative (8.6 nmol) indicated by a heavy arrow in Figure 1 was radioiodinated with carrier-free Na^{125}I (1.34 mCi/nmol) by the Iodogen method (Fraker & Speck, 1978) using 0.35 M sodium phosphate, pH 7.2, as buffer. The reaction products were separated on an LKB HPLC system with a Lichrosorb RP C18 analytical column using the TFA/acetonitrile solvent system with a linear gradient of 2% B per minute starting at 3% B (B = 0.1% TFA/60% acetonitrile). The radioactivity of fractions was monitored with a γ counter. Only one peak showed significant amounts of radioactivity. In this system, the unlabeled derivative eluted at 16.5 min and the radioiodinated form at 19.0 min. Approximtely 15% of the derivative was converted to ^{125}I-labeled 3-(4-hydroxyphenyl)propionyl-GIIIA.

Cross-Linking of ^{125}I-GIIIA to Sodium Channels. Membrane fractions from *Electrophorus electricus* were generously provided by Dr. Edward Moczydlowski, Department of Pharmacology, Yale University, and prepared as previously described (Moczydlowski et al., 1986a,b). A reaction mixture containing 168 μg of membrane protein and 0.25 pmol (550 000 cpm) of ^{125}I-labled 3-(4-hydroxyphenyl)propionyl-GIIIA in 200 μL of 20 mM NaHEPES, pH 7.5, was incubated for 30 min at room temperature. As a control, the same amount of membrane protein in buffer was preincubated with 0.1 nmol of unlabeled native GIIIA for 30 min at 0 °C before radioiodinated GIIIA was added.

Toxin-receptor complexes were cross-linked according to the method of Pilch et al. (1980) by the addition of 15 μL of 10 mM disuccinimidyl suberate (DSS). The mixtures were incubated on ice for 15 min; then 3 mL of 50 mM Tris, pH 6.0, was added. After centrifugation of the mixture for 10 min at 15 000 rpm in a Sorvall RC2-B centrifuge (Ss-34 rotor), the radioactivity of pellets was determined in a γ counter. Cross-linked samples were analyzed by SDS-PAGE according to the method of Laemmli (1970) using 4–15% gradient gels. Pellets were dissolved by PAGE sample buffer containing 80 mM DTT just before electrophoresis.

Receptor Binding Assays. Assays of receptors in *Electrophorus electricus* electric organ membranes were carried out in a reaction mixture (0.2 mL) containing 0.32 M sucrose, 5 mM HEPES/Tris, pH 7.4, 45 mM KCl, and ca. 50 μg of electric organ protein. Preincubations with unlabeled μ-conotoxin GIIIA or tetrodotoxin were carried out for 30 min at 0 °C. Incubations with ^{125}I-labeled (4HP)-μ-conotoxin GIIIA (0.21 pmol, 0.48 Ci/μmol) were carried out for 30 min at room temperature. After incubation, samples were filtered on Whatman GF/C titers and washed 3 times with 2 mL of a washing medium (16 mM choline chloride, 1.5 mM $CaCl_2$, 5 mM HEPES/Tris, pH 7.4, and 0.2 mg/mL BSA). The radioactivity of filters was determined by using a Packard γ counter.

Results

Solid-Phase Synthesis of μ-Conotoxin GIIIA. Conotoxin GIIIA was synthesized by the solid-phase procedure on a *p*-methylbenzhydrylamine-resin (MBHA-resin). After the peptide chain had been assembled, the resin was treated with liquid HF at 0 °C in the presence of anisole. The peptide was fully deprotected and concomitantly cleaved from the resin. The disulfide bridges in the 22-peptide amide molecule were formed by an air oxidation procedure in dilute solution in ammonium acetate buffer at pH 6.8 and low temperature. Progress in the formation of the disulfide bridges was monitored by DTNB reagent at pH 8 (Ellman, 1959) and by analytical HPLC. Reaction appeared to be complete by 72 h. After oxidation, the clear solution was passed through a column containing Bio-Rex 70 cation-exchange resin (H^+ form). After extensive washing, the peptide was removed from the resin with 50% aqueous acetic acid. The crude product (1.3 g) was isolated after lyophilization. Analysis by HPLC showed the conotoxin GIIIA to be contaminated with various impurities. One gram of crude product was purified by passing twice through a preparative HPLC column, followed by 25 applications through a semipreparative HPLC column in the buffer system 0.1 M trifluoroacetic acid/acetonitrile. This last step was necessary in view of the unusually hydrophilic character of this peptide and our inability to achieve adequate purification preparatively. Amino acid analysis of the purified synthetic product was consistent with the theoretical values. HPLC coelution experiments with the natural product gave a single sharp symmetrical peak. Sequence analysis confirmed the proposed structure and set an upper limit of 2% for the presence of "deletion peptides". Fast atom bombardment (FAB) mass spectrometry gave the molecular ion at m/z 2607 (MH^+), which is predicted for the fully oxidized peptide amide. The biological activity of the synthetic peptide was quantitatively compared to that of the native toxin. The results indicated that both preparations were equipotent using both intraperitoneal and intracranial injection into mice and injection into goldfish.

Preparation of ^{125}I-Labeled μ-Conotoxin GIIIA. In order to use μ-conotoxin GIIIA as a sensitive probe for sodium channels, it is desirable to radiolabel the toxin with ^{125}I. Although this has been relatively easily achieved with other conotoxins, μ-conotoxin GIIIA presents special problems because it has neither histidine nor tyrosine residues.

Our strategy was to introduce a phenolic ring into μ-conotoxin using the Bolton–Hunter reagent, thereby derivatizing the toxin with a (4-hydroxyphenyl)propionyl (4HP) moiety at one of its amino groups. Since there are four ε-amino groups on lysine residues and a free N-terminus, a variety of derivatized products would be expected if μ-conotoxin GIIIA were coupled to the Bolton–Hunter reagent. As shown in Figure 1, this is indeed the case. The major products of the reaction, indicated by arrows, were tested for biological activity. All of the 4HP derivatives tested proved to be biologically active, as assayed by intracranial injection of mice. The material in

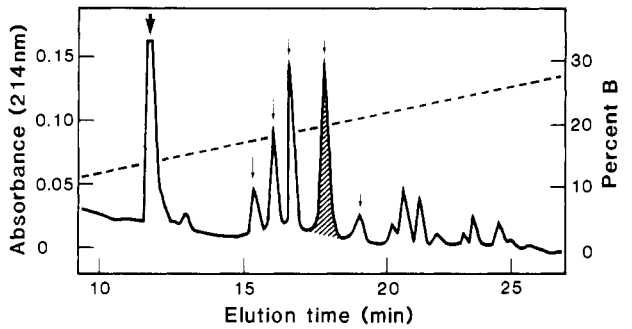

FIGURE 1: Separation of 3-(4-hydroxyphenyl)propionyl derivatives of GIIIA by HPLC. GIIIA was reacted with the Bolton–Hunter reagent as described under Experimental Procedures. The derivatives were separated from unmodified GIIIA by HPLC on a VYDAC C18 analytical column using the LKB system equipped with a spectral detector. A linear gradient of acetonitrile in 0.1% TFA (---) with 0.1% TFA (solvent A) and 60% acetonitrile in 0.1% TFA (solvent B) as limiting buffers was used to elute the peptides. The thick arrow is unmodified toxin; material in peaks indicated by arrows was found to be biologically active (tested intraperitoneally on mice). The hatched peak was then radiolabeled by iodination (see text).

the hatched absorbance peak in Figure 1 was used for iodination experiments. In principle, since the first five modified peaks are all biologically active, any of these could be iodinated. However, in most modification experiments, the amount of material eluting at the position of the hatched peak was greater than any of the other peaks; in addition, the greater separation from other peaks made the collection of homogeneous material not contaminated with other derivatives more facile.

Iodination of the derivatized μ-conotoxin GIIIA was carried out by using the Iodogen procedure as described under Experimental Procedures. With the reaction conditions used, approximately 15% of the derivatized peptide was converted to the ^{125}I-labeled 4HP-μ-conotoxin GIIIA. Carrier-free ^{125}I was used for the derivatization in order to maximize specific activity. By virtue of its greater hydrophobicity and consequent later elution time, the radiolabeled μ-conotoxin GIIIA could be easily separated from the unlabeled, noniodinated 4HP-toxin using HPLC.

Binding and Cross-Linking Studies Using ^{125}I-Labeled μ-Conotoxin GIIIA. The radiolabeled toxin described above was used for binding studies; the receptors assayed were sodium channels present in the *Electrophorus* electric organ membranes. This preparation has previously been shown to be a rich source of sodium channels which are μ-conotoxin targets. It is well established that the sodium channels present in this tissue are also targets for saxitoxin and tetrodotoxin and that there is competition between μ-conotoxin and saxitoxin for binding to electric organ receptors.

As shown in Figure 2, specific binding by μ-conotoxin GIIIA can readily be demonstrated. Specific binding is abolished not only if an excess of unlabeled μ-conotoxin is preincubated with the membrane preparation but also if tetrodotoxin is preincubated with the membrane preparation. However, under these experimental conditions, preincubation with ω-conotoxin GVIA, α-conotoxins MI and GI, or lysozyme (LZ) did not substantially affect specific binding (Figure 2). In displacement assays of ^{125}I-labeled toxin by tetrodotoxin and μ-conotoxin GIIIA, the IC_{50} for tetrodotoxin is 50 nM, and for μ-conotoxin it is 4 nM.

^{125}I-Labeled μ-conotoxin GIIIA is potentially a useful ligand for identifying the common binding site on the Na channel for saxitoxin, tetrodotoxin, and μ-conotoxin. This site has been difficult to define experimentally because heretofore only ^{3}H-labeled toxins have been available. However, the specific

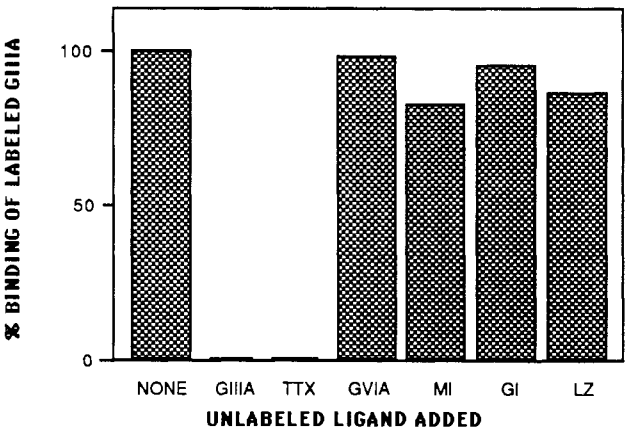

FIGURE 2: Competition assays. Assays were carried out as described under Experimental Procedures. Unlabeled peptides (final concentration 5 μM except lysozyme which was 0.2 mg/mL) were preincubated for 30 min before ^{125}I-labeled (4HP)-μ-conotoxin GIIIA (0.47 Ci/μmol) was added. Abbreviations: GIIIA, μ-conotoxin GIIIA; TTX, tetrodotoxin; GVIA, ω-conotoxin GVIA; MI, α-conotoxin MI; GI, α-conotoxin GI; LZ, lysozyme.

activity of carrier-free ^{125}I-labeled μ-conotoxin GIIIA is much higher than can be achieved with a ^{3}H-labeled toxin. In order to establish the feasibility of such an effort, we performed a preliminary cross-linking experiment.

^{125}I-Labeled μ-conotoxin GIIIA was cross-linked to receptor targets in *Electrophorus* electric organ membrane preparations (see Experimental Procedures) using the bivalent cross-linker disuccinimidyl suberate, which cross-links at free amino groups. In order to analyze receptor targets cross-linked to ^{125}I-labeled μ-conotoxin GIIIA, the preparation was denatured in SDS, disulfide bonds were broken by using dithiothreitol, and the mixture was analyzed by PAGE. The products cross-linked to labeled μ-conotoxin were then identified by autoradiography. Only one diffuse band appears to be radiolabeled specifically with a molecular weight in excess of 200 000. This result is consistent with (but does not prove) specific cross-linking of electric organ sodium channel. These results indicate that ^{125}I-labeled μ-conotoxin GIIIA could be a useful probe for identifying binding sites on the sodium channel.

Discussion

Reports on the chemical synthesis of single-chain polypeptides containing three or more disulfide bridges are relatively few. Zahn and Bullesback (1978), for example, as well as Noburu et al. (1978), independently reported the synthesis of proinsulin, an 84-peptide cross-linked by three disulfide bridges. Both teams used the classical fragment condensation approach in solution followed by air oxidation. An open-chain proinsulin model (a 57-residue peptide), also containing three disulfide bridges, was obtained by coupling in solution two peptide fragments containing one and two preformed disulfide bridges, respectively (Bullesbach, 1982). Using a similar fragment condensation methodology and a new deprotection procedure (trifluoromethanesulfonic acid in trifluoroacetic acid in the presence of thioanisole), Yajima and Fuji (1981) synthesized crystalline ribonuclease A, a 124-residue polypeptide. The four disulfide bridges were obtained by oxidation in the presence of a mixture of oxidized and reduced glutathione. The same oxidation method was recently used by Tam et al. (1984) for the cyclization of transforming growth factor (50-residue peptide cross-linked by three disulfide bridges) and by Kupryszewski et al. (1986, 1987) for the trypsin inhibitors CMTI III and CMTI I (29-residue peptides with three disulfide bridges), all synthesized by the solid-phase approach

Table I: Purification and Characterization of Conotoxin GIIIA by Reversed-Phase HPLC Chromatography

no.	scale of HPLC purification	loaded amount	buffers: system A	buffers: system B	flow rate (mL/min)	monitoring wavelength (nm)	gradient	retention vol (mL)	yield (mg)	% purity
1	preparative	1000 mg	0.1% TFA	50% CH_3CN in A	100	225	0% B, 50 min, 50% B	1900–2200	480	40
2	preparative	480 mg	0.1% TFA	50% CH_3CN in A	100	225	0% B, 30 min, 30% B	1850–2100	150	70
3	semipreparative	6 mg, 25 times	0.1% TFA	60% CH_3CN in A	2.0	210	isocratic, 12% B	26–28	15.6	>95
4	analytical	10 μg	0.1% TFA	60% CH_3CN in A	1.3	210	isocratic, 12% B	6.2		
5	analytical	10 μg	0.05% HFBA		1.3	210	isocratic, 100% A	4.4		

of Merrifield (1963). Other highly cross-linked peptides are found in the venom of some fish-hunting marine snails of the genus *Conus*. Several structures were recently disclosed (Olivera et al., 1985), among which the μ- and ω-conotoxins also contain three disulfide bridges. While Nishiuchi et al. (1986) reported the synthesis in solution of the 27-peptide ω-conotoxin GVIA, we reported (Olivera et al., 1985, 1987) the synthesis of the ω-conotoxin MVIIA by the solid-phase approach and had also succeeded in the total synthesis of ω-conotoxin GVIA (Rivier et al., 1987). Both groups used air oxidation to obtain the desired products.

In this report, we describe the synthesis of the μ-conotoxin GIIIA, which like ω-conotoxin GVIA also contains three L-hydroxyproline residues. This synthesis was a challenge not only because of the 3 disulfide bridges but also because 21 of the 22 amino acids are trifunctional. While random oxidation of 6 cysteine residues will yield 15 possible monomer species and an infinite number of polymeric species, it is apparent that the native toxin has only one defined set of disulfide bridges. A comparison of synthetic to natural peptide confirmed that the major product from cyclization (especially at lower temperature) was indeed the desired product. This suggests that the native conformer for the conotoxin GIIIA is the thermodynamically favored stereoisomer. From the synthetic mixture, several other undesired products could be separated by HPLC, suggesting possible disulfide bridge isomers or imperfect synthesis [i.e., failure sequences, chain termination, and other side reactions from either base or acid treatment (Barany & Merrifield, 1980)]. Final purity achieved after several HPLC steps was at least 98% (Table I).

We also describe the preparation of high specific activity ^{125}I-labeled μ-conotoxin GIIIA. The availability of ^{125}I-labeled toxin derivatives provides a much higher specific activity probe for the guanidinium toxin binding sites on the Na^+ channel; only ^{3}H-labeled ligands have been available to date. It is clear that a variety of biologically active derivatives of μ-conotoxin GIIIa can be made by the strategy for making radiolabeled peptide used here (fluorescent derivatives, biotinylated derivatives). Photoactivatable cross-linking derivatives should be particularly useful. The results in Figure 1 demonstrate that derivatization of several different amino groups on the peptide does not abolish biological activity. The five different amino groups on μ-conotoxin GIIIA would be immediately adjacent to different residues on the Na^+ channel. Thus, placement of photoactivatable cross-linking groups on different amino groups and cross-linking to the Na channel may permit mapping the detailed topography of the μ-conotoxin binding site on the *Electrophorus* Na^+ channel.

Results presented above strongly suggest that the radiolabeled toxin can be used to assay for Na^+ channels; as expected, tetrodotoxin competes with ^{125}I-conotoxin GIIIA for binding to the *Electrophorus* Na^+ channel, but other peptide toxins (ω-conotoxin GVIA, α-conotoxin MI, or proteins like lysozyme) do not. The cross-linked receptor has a denatured molecular weight in excess of 200 000 which is the expected result if cross-linking were occurring at the sodium channel. These experiments indicate the feasibility of using μ-conotoxin GIIIA for defining the guanidinium toxin binding site on the Na^+ channel.

Since μ-conotoxin GIIIA does not appear to bind voltage-sensitive Na^+ channels in motor neuron axons, a comparison of sequence differences in the receptor in the region of the toxin binding site may give insights into the chemical basis for Na^+ channel subtype differentiation. For example, skeletal muscle Na^+ channels bind both saxitoxin and μ-conotoxin with high affinity and neuronal channels bind saxitoxin with high affinity but not μ-conotoxin, while cardiac Na^+ channels do not have a high affinity for either μ-conotoxin or saxitoxin. The molecular basis for such differences in toxin sensitivity is not presently understood, and the availability of ^{125}I-labeled μ-conotoxin GIIIA for defining the toxin binding region in one subtype should make it possible to understand the structural basis for such channel subtype variation. A cautionary note should be introduced at this point. Although it seems reasonable that the modified toxin will show the same discriminatory characteristics between muscle and axonal Na channels as the native toxin, this has not been directly demonstrated. This awaits preparation of more substantial amounts of the modified toxin so that the appropriate electrophysiological experiments can be carried out.

Finally, readily available synthetic toxin is a useful experimental tool for physiological experiments where it is desirable to keep the muscle paralyzed, while not substantially inhibiting axonal or synaptic transmission.

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Registry No. GIIIA, 86394-16-3; Na, 7440-23-5.

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The Growth Inhibitor of African Green Monkey (BSC-1) Cells Is Transforming Growth Factors β1 and β2

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ABSTRACT: The growth inhibitory activity in conditioned medium of African green monkey kidney epithelial (BSC-1) cells that has been shown to arise, at least in part, from transforming growth factor β2 (TGF-β2) [Hanks, S. K., Armour, R., Baldwin, J. H., Maldonado, F., Spiess, J., & Holley, R. W. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 79-82] was tested for growth inhibitory activity prior to and following acidification. Similar to TGF-β1 from human platelets, the inhibitory activity from BSC-1 cells demonstrated an 8-10-fold stimulation following acidification, showing that the activity was secreted from the cells in latent form. Conditioned medium from BSC-1 cells was collected, acidified, and fractionated by procedures that separate TGF-β1 and -2. Biological activity was assayed by using the BSC-1 cell proliferation assay. Two active proteins with properties similar to known TGF-β1 and TGF-β2 were identified. Identity was confirmed by using immunological and amino acid sequencing techniques. These results were consistent with Northern blot analysis of total BSC-1 RNA, using cDNA probes for TGF-β1 and TGF-β2, which demonstrated strong signals for both mRNAs. Metabolic labeling in conjunction with two-dimensional gel electrophoresis revealed that the cells secrete approximately 10% TGF-β1 and 90% TGF-β2.

The multitude of biological activities attributed to transforming growth factor β1 (TGF-β1)[1] and TGF-β2 is remarkable [for reviews, see Sporn et al. (1987), Massague (1987), and Roberts and Sporn (1988)]. The activity was characterized by Roberts et al. (1980, 1981), who showed that in combination with epidermal growth factor it could promote anchorage-independent growth of NRK fibroblasts, hence, the name "transforming growth factor". The factor, now designated TGF-β1, was shown to be present in many normal tissues

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[1] Abbreviations: TGF, transforming growth factor; NRK, normal rat kidney; BSC-1, African green monkey kidney epithelial; GI, growth inhibitor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CM, carboxymethyl; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; ATCC, american type culture collection; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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