331-334, Slovak Academy of Sciences, Bratislava, Czechoslovakia.
Quaas, R., McKeown, Y., Stanssens, P., Frank, R., Blöcker, H., & Hahn, U. (1988) *Eur. J. Biochem. 173*, 617-622.
Ramdas, L., & Nall, B. T. (1986) *Biochemistry 25*, 6959-6964.
Rehage, A., & Schmid, F. X. (1982) *Biochemistry 21*, 1499-1505.
Ridge, J. A., Baldwin, R. L., & Labhardt, A. M. (1981) *Biochemistry 20*, 1622-1630.
Roder, H., Elöve, G. A., & Englander, S. W. (1988) *Nature 335*, 700-704.
Schmid, F. X. (1983) *Biochemistry 22*, 4690-4696.
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764-4768.
Schmid, F. X., & Blaschek, H. (1981) *Eur. J. Biochem. 114*, 111-117.
Schmid, F. X., Grafl, R., Wrba, A., & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 872-876.
Segawa, S.-I., & Sugihara, M. (1984) *Biopolymers 23*, 2473-2488.
Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature 337*, 473-475.
Thomson, J. A., Shirley, B. A., Grimsley, G. R., & Pace, N. C. (1989) *J. Biol. Chem. 264*, 11614-11620.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694-699.
Wood, L. C., White, T. B., Ramdas, L., & Nall, B. T. (1988) *Biochemistry 27*, 8562-8568.

Delineation of the Functional Site of a Snake Venom Cardiotoxin: Preparation, Structure, and Function of Monoacetylated Derivatives†

E. Gatineau,‡ M. Takechi,‡,§ F. Bouet,‡ P. Mansuelle,‖ H. Rochat,‖ A. L. Harvey,⊥ Th. Montenay-Garestier,# and A. Ménez*,‡

Service de Biochimie du Département de Biologie, CEN Saclay, 91191 Gif-sur-Yvette Cédex, France, Unité Associée CNRS 1179 and Unité INSERM 172, Faculté de Médecine Secteur Nord, Boulevard Pierre Dramard, 13326 Marseille Cédex 15, France, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, United Kingdom, and Unité INSERM 201, Muséum d'Histoire Naturelle, 75005 Paris, France

Received June 30, 1989; Revised Manuscript Received February 27, 1990

ABSTRACT: Toxin γ, a cardiotoxin from the venom of the cobra *Naja nigricollis*, was modified with acetic anhydride, and the derivatives were separated by cation-exchange and reverse-phase chromatography. Nine monoacetylated derivatives were obtained, and those modified at positions 1, 2, 12, 23, and 35 were readily identified by automated sequencing. The overall structure of toxin γ, composed of three adjacent loops (I, II, and III) rich in β-sheet, was not affected by monoacetylation as revealed by circular dichroic analysis. Trp-11, Tyr-22, and Tyr-51 fluorescence intensities were not affected by modifications at Lys-12 and Lys-35, whereas Trp-11 fluorescence intensity slightly increased when Lys-1 and Lys-23 were modified. The cytotoxic activity of toxin γ to FL cells in culture was unchanged after modification at positions 1 and 2, whereas it was 3-fold lower after modification at Lys-23 and Lys-35. The derivative modified at Lys-12 was 10-fold less active than native toxin. Using two isotoxins, we found that substitutions at positions 28, 30, 31, and 57 did not change the cytotoxic potency of toxin γ. A good correlation between cytotoxicity, lethality, and, to some extent, depolarizing activity on cultured skeletal muscle cells was found. In particular, the derivative modified at Lys-12 always had the lowest potency. Our data show that the site responsible for cytotoxicity, lethality, and depolarizing activity is not diffuse but is well localized on loop I and perhaps at the base of loop II. This site is topographically different from the AcChoR binding site of the structurally similar snake neurotoxins.

Despite extensive work, a debate still exists as to whether or not snake cardiotoxins have a specific target (Harvey, 1985; Dufton & Hider, 1988) and possess a defined functional site (Louw & Visser, 1977, 1978; Lauterwein & Wüthrich, 1978; Karlsson, 1979; Dufourcq et al., 1982; Hider & Khader, 1982; Batenburg et al., 1985). The debate is complicated by evidence suggesting structural and functional heterogeneity among these small proteins (Botes & Viljoen, 1976; Hider & Khader, 1982; Harvey, 1985; Breckenridge & Dufton, 1987; Grognet et al., 1988). To clarify this situation, we searched for the functional site of toxin γ from *Naja nigricollis*, a well-studied cardiotoxin (Tazieff-Depierre et al., 1969a,b; Boquet, 1970; Tazieff-Depierre & Trethevie, 1975; Lee & Lee, 1979; Grognet et al., 1986, 1988; Gatineau et al., 1987; Roumestand et al., 1989). The overall structure of toxin γ is similar to that of *Naja mossambica mossambica* cardiotoxin $V^{II}4$ (Rees et al., 1987) and of CTX IIb (Steinmetz et al., 1988), which consists of a small core containing four disulfide bonds from which three adjacent loops (I, II, and III) rich in β-pleated sheet protrude (Figure 1).

Previously, we reported that Trp-11 in loop I was implicated in the function of toxin γ (Gatineau et al., 1987). The role

† This work was supported by the Commissariat à l'Energie Atomique, the Institut National de la Santé et de la Recherche Médicale, and the Centre National de la Recherche Scientifique.
* To whom correspondence should be addressed.
‡ CEN Saclay.
§ Present address: Department of Pharmacognosy, Faculty of Pharmacy, Kinki University, Higashi-Osaka 577, Japan.
‖ Faculté de Médecine Secteur Nord.
⊥ University of Strathclyde.
Muséum d'Histoire Naturelle.

0006-2960/90/0429-6480$02.50/0

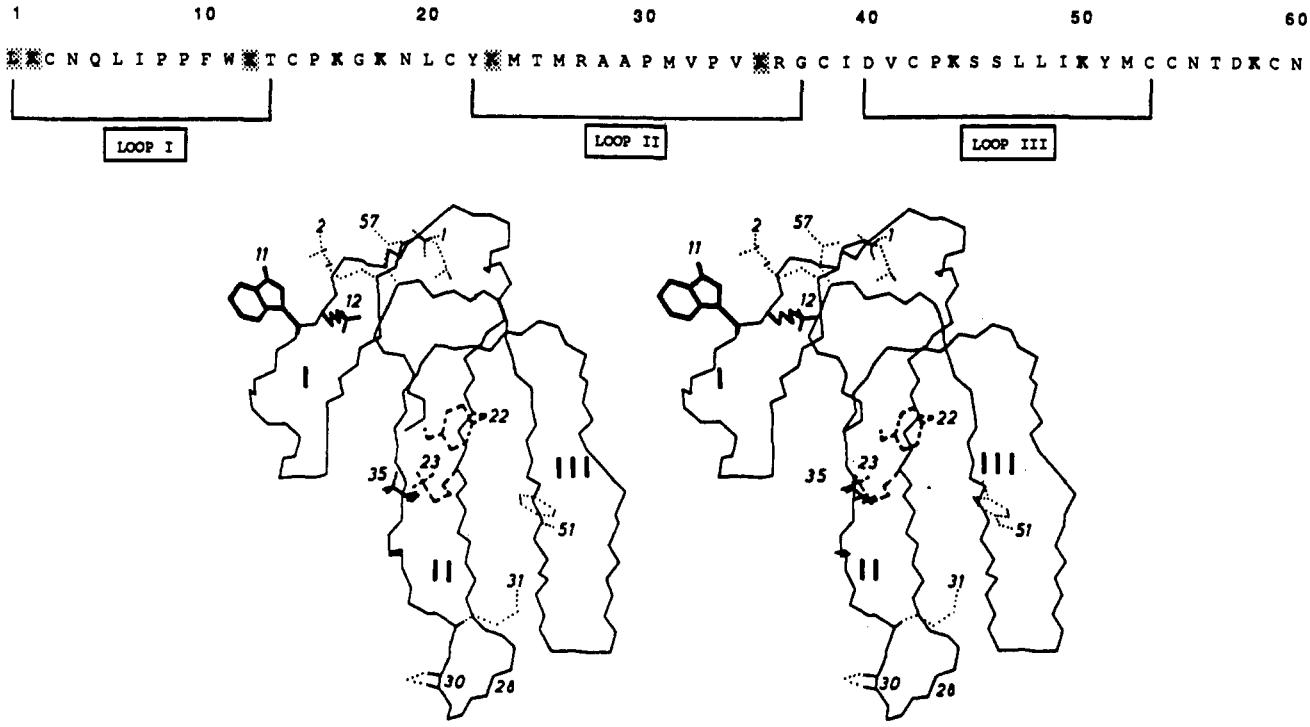

FIGURE 1: (Upper) Amino acid sequence of toxin γ from *N. nigricollis* (Fryklund & Eaker, 1975). All residues possessing an amino group have been emphasized with boldface type. The dotted residues have been individually acetylated in this work. Residues belonging to loops I, II, and III are indicated. (Lower) Stereoview of the backbone of toxin γ folded in a manner similar to that of *N. m. mossambica* cardiotoxin $V^{II}4$ as deduced from X-ray crystallographic data (Rees et al., 1987). The residues are schematically placed on the backbone. The three loops are indicated. Amino acids playing some role in cytotoxicity (—); amino acids having no role in cytotoxicity (···); amino acids playing some role in structure and/or cytotoxicity (- - -).

of this loop was subsequently confirmed (Marchot et al., 1988). Other data based on chemical modifications of Tyr-22 and Tyr-51 further indicated that part of loop II may also play a role in the activity of toxin γ, whereas loop III may be less important (Gatineau et al., 1987). We therefore decided to search for other residues of loop I and loop II that may be involved in the function of toxin γ.

Toxin γ has ten amino groups (Fryklund & Eaker, 1975), and five of them (positions 1, 2, 12, 23, and 35) are in loops I or II (Figure 1). Some of the amino groups are likely to be critical for the cardiotoxin function, since (i) our pilot experiments (not reported) and recent observations (Kini & Evans, 1989a) showed that full acetylation inactivated the molecule, (ii) the cytotoxic activity of cardiotoxins was inhibited by negatively charged phospholipids but not by positively charged phospholipids (Takechi et al., 1986), and (iii) divalent cations reduced the effects of toxin γ and its variants on skeletal and cardiac muscles (Arms & McPheeters, 1975; Tazieff-Depierre & Trethevie, 1975; Harvey et al., 1982). To identify critical amino groups, we acetylated toxin γ, separated the monoacetylated derivatives, and using automated sequencing, identified the five derivatives modified at positions in loop I or II. We investigated the capacity of the derivatives to show cytotoxicity toward FL cells, to kill mice, and to depolarize skeletal muscle fibers. We also examined some structural properties by circular dichroism and fluorescence emission spectroscopy. Our data confirmed our previous conclusions that loop I and possibly loop II are implicated in the biological function of toxin γ and allowed us to delineate a plausible "toxic site".

Materials and Methods

Toxins. Toxin γ was isolated from the venom of *N. nigricollis* (Pasteur Institute) as previously described (Fryklund & Eaker, 1975). CTX IIa and CTX IIb from *N. m. mossambica* venom were generous gifts from Dr. P. Bougis (Marseille, France). Selective monoderivatization of toxin γ aromatic side chains was carried out as described by Gatineau et al. (1987).

Materials. Acetic anhydride was obtained from SDS (Peypin, France). [^{3}H]Acetic anhydride was from Amersham (Buckinghamshire, England). Bio-Gel P2 and Bio-Rex 70 Na^+ (200–400 mesh) were purchased from Bio-Rad (Richmond, CA). The high-performance liquid chromatography (HPLC) C18 μBondapak column was obtained from Waters Associates (Milford, MA), and C4 and C8 bonded-phase columns were from SFCC (Gagny, France). Trifluoroacetic acid was purchased from Fluka Chemie (Buchs, Switzerland) and acetonitrile from BDH (Poole, England), and other analytical-grade reagents were from Prolabo (Paris, France). Purification of derivatives by reverse-phase HPLC (RP-HPLC) was performed on both a Beckman system, including two Model 1108 pumps, a Model 421 gradient controller, a Model 340 injector, and a Model 160 spectrophotometric detector, and a Gilson system, including two Model 303 pumps, a Model 311 dynamic mixer, a Model 802 manometric module, a holochrome spectrophotometer, and an Apple IIe computer.

Acetylation of Toxin γ with ^{3}H-Labeled Acetic Anhydride. The acetylation of amino groups in toxin γ was performed with acetic anhydride (Fraenkel-Conrat et al., 1949; Riordan & Vallee, 1967). ^{3}H-Labeled acetic anhydride (4.35 μmol; 5.75 Ci/mmol or 213 GBq/mmol) in 0.15 mL of toluene was added to 31 mg of toxin γ (4.56 μmol) in 0.1 M sodium phosphate buffer, pH 7 (12 mL). The mixture was left at room temperature in the dark overnight under stirring. The reaction mixture was desalted by filtration through a Bio-Gel P2 column (1 × 10 cm) in 10% acetic acid. The protein fraction was freeze-dried. Then, 0.55 mL of 1 M hydroxylamine hydrochloride, pH 7, was added for 2 h at room temperature and then the reaction mixture was filtered through a Bio-Gel P2

column in 1% acetic acid. After being freeze-dried, the acetylated mixture was chromatographed on a Bio-rex column (1 × 20 cm) equilibrated in 0.1 M ammonium acetate, pH 7, to which was applied a linear gradient from 0.1 to 0.6 M ammonium acetate, pH 7, for 24 h (flow rate = 9 mL/h). The specific radioactivity was determined from the ratio of incorporated radioactivity per mole of protein, estimated from UV absorbance at 278 nm (ϵ_M = 9000). The presence of a single monoacetylated amino group per mole of protein was assessed by automated sequencing.

Acetylation of Toxin γ with Unlabeled Acetic Anhydride. A 560-mg quantity of toxin γ (82.35 μmol) was dissolved in 12 mL of 0.1 M sodium phosphate buffer, pH 7. Acetic anhydride (80 μmol) in 0.8 mL of toluene was added to the toxin solution for 18 h. The desalted reaction mixture was then treated with 10 mL of 1 M hydroxylamine hydrochloride for 2 h. After being desalted and freeze-dried, the acetylated toxin mixture was chromatographed on a Bio-Rex column (1.5 × 29 cm) with a linear gradient from 0.2 to 0.4 M ammonium acetate, pH 7, for 48 h (flow rate = 18 mL/h). Some fractions were further rechromatographed on a Bio-Rex column (1 × 20 cm) with a linear gradient from 0.25 to 0.4 M ammonium acetate, pH 7, for 24 h. In preliminary experiments (not shown), we isolated derivatives acetylated at tyrosine and threonine residues, in addition to those modified on amino groups. Acetylation of tyrosine hydroxyls was easily detected by spectrophotometrical measurement since *O*-acetyltyrosine is characterized by a decrease in absorption at 278 nm, and a derivative modified at Thr-25 was identified by automated sequencing. The presence of these additional derivatives complicated the purification steps; therefore, we deacetylated the phenolic and aliphatic esters by treating the reaction mixture with hydroxylamine at pH 7 (Hestrin, 1949). No side products were detected after this treatment.

Purification of Monoacetylated Derivatives by High-Performance Liquid Chromatography. Each fraction containing monoacetylated derivatives was purified by RP-HPLC on a Nucleosil 300 Å 5-μm C8 column (4.6 × 250 mm) equilibrated in 0.1 M triethylammonium formate, pH 4.2 (solvent A1). Elution was achieved by using a gradient of acetonitrile (solvent B1) in solvent A1. Some fractions were further rechromatographed, either on the same system or on a Nucleosil 300-Å 5-μm C4 column (4.6 × 250 mm) with a gradient of 0.1% TFA in water/0.06% TFA in acetonitrile, 9/1 (solvent A2), to 0.06% TFA in acetonitrile/0.1% TFA in water, 2/1 (solvent B2). Analytical controls were performed either on a μBondapak C18 column with a gradient of solvent B1 in A1 or on a C8 column with a gradient of acetonitrile/0.15 M ammonium formate, pH 2.7, 2/1 (solvent B3), in 0.15 M ammonium formate, pH 2.7/acetonitrile, 9/1 (solvent A3), or on a C4 column with a gradient of solvent B2 in A2. The nature of the alkyl chain (C4, C8, or C18) on the stationary silica phase, the solvents A and B, and the gradient of solvent B in A that were used are indicated in the figure legends.

Amino Acid and Sequence Analysis. To determine amino acid composition and sequence, the purified derivatives (tritiated or not) were reduced and either S-carboxymethylated (Crestfield et al., 1963) or S-pyridylethylated (Tarr et al., 1983). The reduced and S-pyridylethylated derivatives of a monoacetylated toxin γ were further purified by RP-HPLC on a C8 column by using a 0.15 M ammonium formate, pH 2.7/acetonitrile system. All reduced and S-alkylated monoaminoacetylated toxin γ derivatives were submitted to amino acid analysis after hydrolysis by 6 M HCl and PTC derivatization (Heinrikson & Meredith, 1984), using an automatic analyzer (Applied Biosystems). These derivatives (1 nmol of radiolabeled derivative or 3.5–6 nmol of unlabeled derivative) were analyzed by the automated Edman technique. A standard 0.1 M quadrol program (program 349.390 REV C) in a Beckman 890 M.M sequencer was used. Identification of PTH-N^{ϵ}-acetyllysine was obtained either by HPLC and (or) by radioactivity counting (Picofluor 15 and a 460 counter from Packard Instruments). The HPLC set was composed of two Beckman 114 M pumps, a Beckman 165 variable wavelength detector, a Spectraphysics SP 8780 injector, a Kontron anacomp 220 programmer and integrator, and a Beckman ultrasphere ODS column (5 μm, 4.6 × 250 mm). The trifluoroacetate/acetate system recommended by Hawke et al. (1982) was applied with a flow rate of 1 mL/min and a linear gradient from 0 to 100% solvent B in 15 min. In these conditions, a good separation of classic PTH derivatives of amino acids was obtained, and furthermore, PTH-(pyridylethyl)-cysteine and PTH-N^{ϵ}-acetyllysine were eluted, well separated from the other PTH-amino acids, at 10.4 and 12.5 min, respectively (not shown). Quantification of PTH-amino acids was obtained by using a standard mixture from Sigma and assuming similar ϵ_M values at 269 nm for PTH-N^{ϵ}-acetyllysine and PTH-alanine. When the derivative was labeled, the specific radioactivity of PTH-N^{ϵ}-acetyllysine residue was obtained by counting the radioactivity and by estimating the amount of PTH derivative at this step, assuming the same extracting yield for PTH-lysine and PTH-N^{ϵ}-acetyllysine.

Cytotoxicity Assays. Epithelial cells (FL strain) derived from human amniotic membrane (American Type Culture Collection reference number CCL62) were grown at 37 °C in Eagle's medium containing 10% fetal calf serum (Fogh & Lund, 1957). Three- or four-day-old cells were dispersed with 0.1% trypsin for 10 min at 37 °C and suspended in PBS buffer (140 mM NaCl + 8 mM Na_2HPO_4 + 1.5 mM KH_2PO_4 + 2.7 mM KCl, pH 7.2) at a concentration of 3.3 × 10^6 cells/mL. Different concentrations of either toxin γ (15) or its derivatives (5–10 for each derivative) or its homologues in PBS buffer were added to 0.2-mL aliquots of cell suspensions for 30 min at 37 °C. The number of both live and dead cells was then measured by the trypan blue exclusion test in a hemocytometer (Iwaguchi et al., 1985). The cytotoxic activity was expressed as the median effective concentration (EC_{50}) corrected for the blank (4–9%). It is important to notice that lytic activity could also be observed on other types of cells (myotubes, erythrocytes) that did not require pretreatment with trypsin. The data also proved reproducible (not shown).

Toxicity Assays. Various doses of native toxin γ, five of its derivatives, and *N. m. mossambica* (CTX IIb (5–6 for each toxin) in 0.9% NaCl (10 mL/kg of body weight) were injected intravenously into the tail vein of female Balb/c mice (10 mice/dose) weighing 20–22 g. The number of mice that died was recorded, and the percentage of dead mice was calculated for each dose. The median lethal dose (LD_{50}) was determined according to the method of Miller and Tainter (1944).

Depolarization Assays. The depolarizing activities of various concentrations of native toxin γ, its derivatives, and *N. m. mossambica* CTX IIb dissolved in 5 mL of Eagle's minimum essential medium were measured on 5- or 10-day-old skeletal muscle fibers grown in cell cultures (2 cultures/concentration for each compound) as previously described (Harvey et al., 1983). Before toxin addition, control membrane potentials were in the range −25 to −70 mV, but the average control values in individual cultures (16 myotubes/culture) were not significantly different. In the presence of cardiotoxin, the membrane potential of myotubes fell to 0 mV and the time

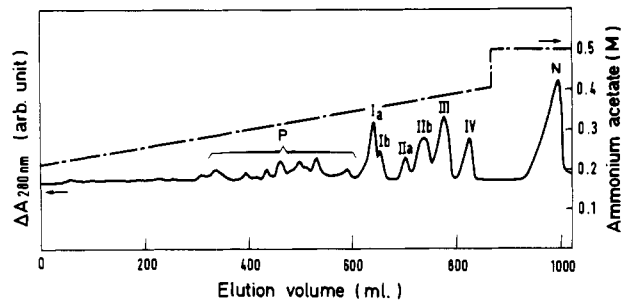

FIGURE 2: Elution on a Bio-Rex 70 column of the desalted reaction mixture after acetylation of 82.35 μmol of toxin γ by 80 μmol of unlabeled acetic anhydride. The compounds were eluted with a linear gradient from 0.2 to 0.4 M ammonium acetate, pH 7, for 48 h and a wash with 0.5 M ammonium acetate, pH 7 (-·-). Fraction P represents mixtures of polyacetylated derivatives. Fractions I–IV correspond to mixtures of monoacetylated compounds, and fraction N corresponds to unreacted toxin.

course of the depolarization was fitted by an exponential function (with correlation coefficients greater than 0.92). Hence, we calculated the time constant (τ) of this process, i.e., the time required for the average membrane potential to reach $1/e$ (≈37%) of the control value. The reciprocal of the time constant ($1/\tau$) was taken as a measure of the rate of depolarization and used to establish concentration–response curves. To compare the differences in potency between toxins, we determined the equieffective concentration (EEC), i.e., the concentration of toxin required to produce a rate of depolarization of 0.002 s^{-1}, which corresponded to 63% depolarization in 500 s.

Correlation between Lethality and either Depolarizing or Cytotoxic Activity. LD_{50}, EC_{50}, and EEC for each compound were determined (see above) or found in the literature (Louw, 1974; Bougis et al., 1983; Gatineau et al., 1987; Hodges et al., 1987). Correlation between EC_{50} and LD_{50} was checked by using a linear independence test on the Pearson correlation coefficient (Zar, 1984).

Circular Dichroism. CD measurements were performed with a Jobin-Yvon dichrograph III equiped with an Olivetti M280 PC/AT computer. The toxins were dissolved in water and the pH was adjusted to 6.7 ± 0.1 with HCl or NH_4OH. The protein concentration was determined on the basis of the optical density at 278 nm measured with a Beckman DU-7 spectrophotometer, by using a molar absorbancy (ϵ_M) equal to 9000. All the spectra were recorded at 20 °C with toxin concentrations close to 10^{-5} M. The path length of the quartz cell was 0.05 cm. For each sample, the spectrum (260–185-nm region) was measured 16 times and the results were averaged and corrected for the base line. Molar ellipticities ($[\theta]$) were expressed as degrees centimeter squared per decimole.

Emission Fluorescence. The emission fluorescence spectra were recorded five times at 20 °C with a Fluorolog 2-FIT 11 SPEX apparatus. The toxins were dissolved in 0.1 M NaCl + 10 mM sodium cacodylate buffer, pH 7, at concentrations close to 5×10^{-6} M. The path length of the quartz cell was 1 cm. The excitation and emission slits were adjusted to 3.6 and 1.8 nm, respectively. The wavelength increment was 0.5 nm, and the integration time was 1 s. The spectra (480–280 nm) were measured for an excitation wavelength equal to 275 or 295 nm. All spectra were corrected for the wavelength dependence of lamp intensity, monochromator transmission, and photomultiplier response.

Results

(1) Isolation of Monoacetylated Derivatives. Figure 2 shows a typical ion-exchange chromatography on Bio-Rex 70 resin of a desalted mixture obtained after acetylation of 560 mg (82.35 μmol) of toxin γ with 80 μmol of unlabeled acetic anhydride. The last and major fraction, N, eluted like native toxin γ, had the same CD spectrum, and had the same cytotoxic and lethal activities as native toxin γ. Clearly, peak N corresponded to unreacted toxin γ. Minor peaks called P and six fractions called Ia, Ib, IIa, IIb, III, and IV eluted before fraction N and corresponded to acetylated toxin derivatives. To determine the number of incorporated acetyl group(s), we repeated the experiment using ^3H-labeled acetic anhydride as a modifying reagent. As expected, only fraction N (see fraction numbering in Figure 2) was not radioactive. The minor fractions P* had a specific radioactivity higher than 3 Ci/mmol, whereas derivatives in fractions Ia*, Ib*, IIa*, IIb*, III*, and IV* had a specific radioactivity around 2 Ci/mmol. Fractions I*–IV* were further purified by RP-HPLC on a C8 bonded-phase column (Figure 3). Fraction Ia* yielded a well-resolved fraction, Ia2*, that eluted at 40 min (Figure 3A). Fraction Ib* led to a major and a minor peak at 35.5 and 37 min, respectively (Figure 3B); fraction Ib1* was rechromatographed to homogeneity. Fractions IIa* and IIb* gave rise to major peaks at 56 min (IIa4*) (Figure 3C) and 43 min (IIb*) (Figure 3D), respectively. Fraction III contained five components (III1*–III5*) eluting at 24, 25.5, 32.5, 34, and 44 min (Figure 3E); the main fractions III3* and III4* were rechromatographed to homogeneity. Fraction Ia2* and III5* were submitted to automated sequencing (see below), and in both cases, about 95–97% of the total radioactivity injected into the microsequencer was located on a single residue (see Table I). Both monoacetylated derivatives had a specific radioactivity equal to 2 Ci/mmol (73.3 GBq/mmol). The other fractions Ib1*, IIa4*, IIb*, III3*, III4*, and IV2* had the same specific radioactivity, implying that they also contained monoacetylated derivatives. Purification of the nonradioactive fractions I–IV led to profiles identical with those shown in Figure 3. Each cold-purified fraction coeluted with its corresponding tritiated fraction on a C8 bonded-phase column. Furthermore, unlabeled and corresponding labeled fractions had the same cytotoxic activity to FL cells (not shown). The cold derivatives I–IV were therefore identical with the radioactive derivatives I*–IV*. The fractions were then submitted to the following RP-HPLC analytical systems: a C4 column with a gradient of acetonitrile in TFA for fractions Ia2, Ib1, and IV2; a C8 column with a gradient of acetonitrile in ammonium formate for fractions Ia2, reduced and S-pyridylethylated Ia2, Ib1, and IV2; and a C18 column under an isocratic condition of acetonitrile in triethylammonium formate for the fractions Ia2, Ib1, IIa4, IIb, III3, III4, III5, and IV2. A single symmetrical peak was always

Table I: Yield of N^{ϵ}-Acetyllysine As Obtained by Sequencing of the Monoacetylated Derivatives[a]

fraction identification[b]	yield of N^{ϵ}-acetyllysine after n sequencing cycles (%)						
	1	2	12	16	18	23	35
Ia2	0	0	92	0	0	0	0
Ib1	0	0	15	0	70	nd[c]	nd
IIa4	0	0	0	0	0	0	0
IIb1′	0	90	0	10	0	0	0
III3	99	nd	nd	nd	nd	nd	nd
III4	0	0	0	0	0	0	0
IV2	0	0	0	0	0	86	nd
Ia2*	0	3	97	0	0	0	0
III5*	0	4	0	0	0	0	95

[a] Only the first seven positions of amino groups in the toxin sequence were thus determined (see text). [b] For the numbering of the fractions, see Figure 5. [c] nd, not determined.

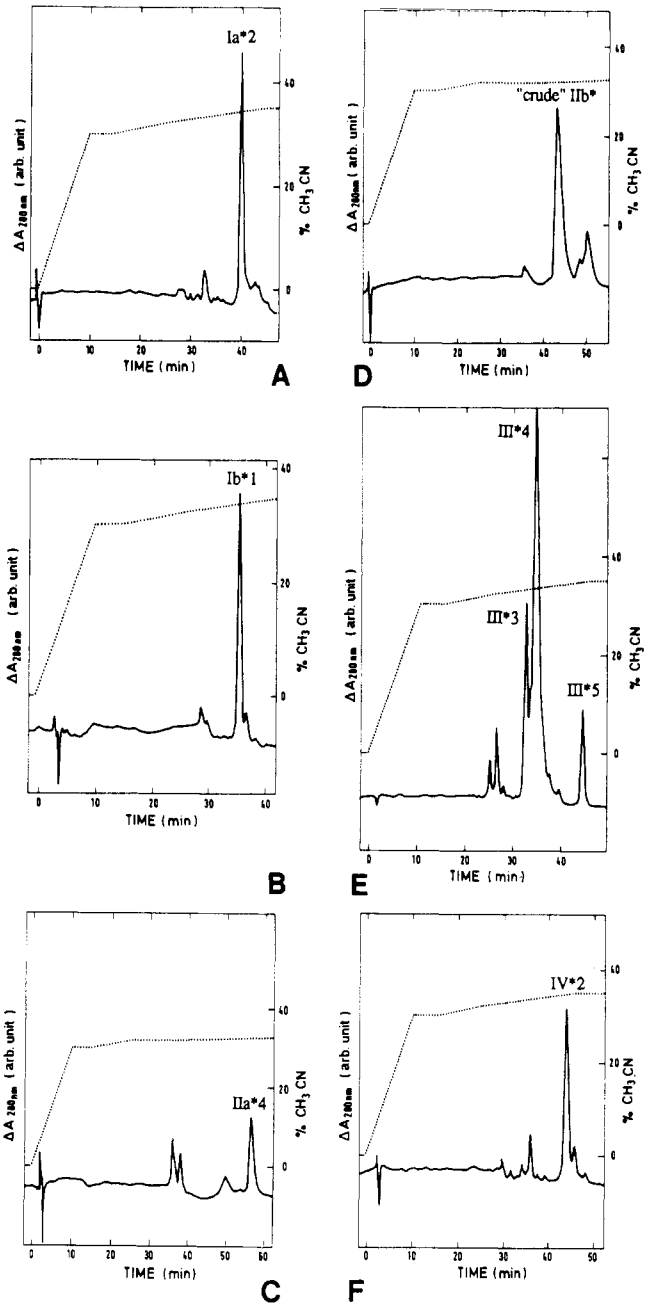

FIGURE 3: Elution profiles on a C8-Nucleosil column of labeled and acetylated fractions Ia* (A), Ib* (B), IIa* (C), IIb* (D), III* (E), and IV* (F) obtained after ion-exchange chromatography on a Bio-Rex 70 column. A binary gradient of acetonitrile (solvent B1) in 0.1 M triethylammonium formate, pH 4.2 (solvent A1), was used (···).

observed; however, data based on automated sequencing (see below) indicated that fractions Ib1 and IIb were not pure. Fraction IIb was rechromatographed on a C4 bonded-phase column, leading to two fractions, IIb1 (65 min) and IIb1′ (67 min) (Figure 4). Fraction Ib1, however, could not be further resolved, irrespective of the system used.

(*2*) *Location of the Acetyl Groups.* We submitted each reduced and blocked derivative to automated sequencing. Only 1% of leucine was cleaved at cycle 1 of the sequence run of fraction III3, indicating that its N-terminus was modified. Other derivatives were sequenced up to steps 19 for fraction Ib1, 25 for fraction IV2, and 36 for fractions Ia2, IIa4, IIb1′, and III4. A tentative quantification of PTH-N^{ϵ}-acetyllysine is shown in Table I. Approximately 90% of N^{ϵ}-acetyllysine was found at positions 2, 12, and 23 in IIb1′, Ia2, and IV2, respectively. In a pilot sequencing experiment (not shown),

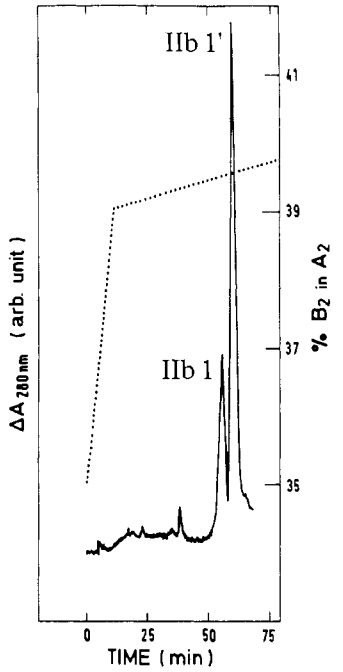

FIGURE 4: Elution profile on a C4-Nucleosil column of acetylated component "crude" IIb obtained after RP-HPLC on a C8-Nucleosil column. A binary gradient, 0.1% TFA in water/0.06% TFA in acetonitrile, 9/1 (solvent A2), to 0.06% TFA in acetonitrile/0.1% TFA in water, 2/1 (solvent B2), was used (···).

we found that fraction IIb (Figure 3D) was labeled at both positions 2 (65%) and 16 (27%). This was due to the presence of two components, IIb1′ and IIb1, which were separated by RP-HPLC in the proportion of 69% and 31%, respectively (Figure 4). Due to the lack of IIb1, we only sequenced fraction IIb1′, which essentially corresponded to (N^{ϵ}-acetyllysine-2)-toxin γ (Table I). By difference, fraction IIb1 probably corresponded to (N^{ϵ}-acetyllysine-16)-toxin γ. Fractions IIa4 and III4 were modified at a residue after position 35. Fraction Ib1 mostly corresponded to a derivative with N^{ϵ}-acetyllysine at position 18 (70%), contaminated by 15% of the derivative modified at position 12, which closely eluted on Biorex chromatography. To assess the validity of our quantification in N^{ϵ}-acetyllysine, we sequenced fraction Ia2* and monitored the amount of radioactivity incorporated up to position 41. Ninety-seven percent of the total radioactivity was located on Lys-12 (Table I), in agreement with direct quantification of N^{ϵ}-acetyllysine, which indicated a value of 92%. We felt safe, therefore, in concluding that fractions Ia2, IIb1′, III3, and IV2, which have approximately 90% N^{ϵ}-acetyllysine at a single position, i.e., 12, 2, 1, and 23, respectively, were essentially pure or highly purified monoacetylated derivatives. In fraction III5*, the radioactive label was located at Lys-35 (Table I). An overview of the procedure has been summarized in Figure 5. It enabled us to obtain, in the 5–27-mg range, five pure derivatives modified at a single amino group located in loop I or loop II of toxin γ.

(*3*) *Effect of Monoacetylation on Cytotoxic Activity.* The dose-dependent lytic effect of toxin γ toward FL cells in culture was highly reproducible (Figure 6A), occurring in a small range of concentrations. The EC_{50} value for native toxin γ was 17 mg/L (2.5 μM). The seven tested monoacetylated toxin γ derivatives also acted in a concentration-dependent manner, the dose–response curves being always parallel to that of native toxin (Figure 6B). According to their EC_{50} values (Table II), the derivatives were classified into three categories. The first group contained derivatives modified at Leu-1 and Lys-2, which were virtually as lytic as native toxin. The second

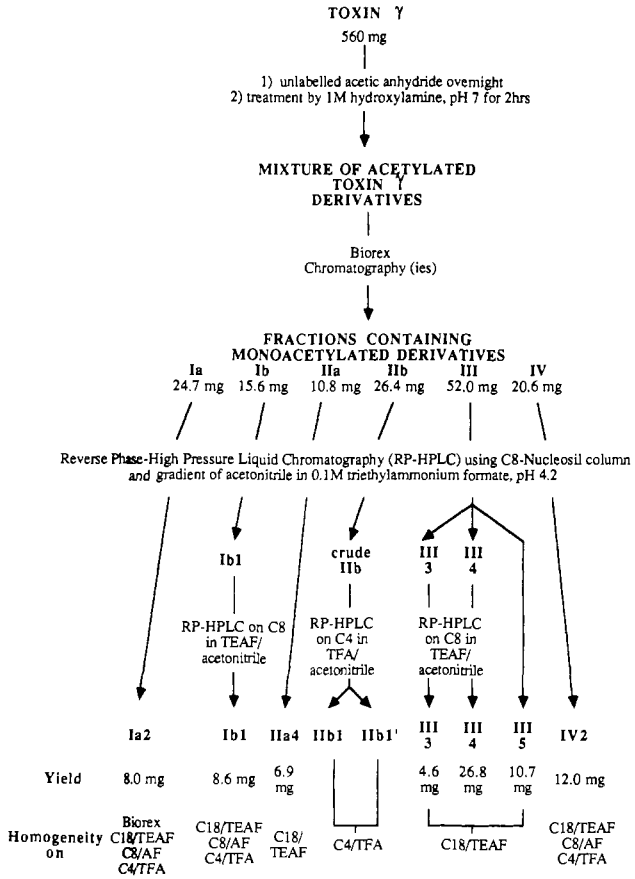

FIGURE 5: Procedure for acetylation of toxin γ from *N. nigricollis* venom and purification of monoacetylated derivatives. Note that fractions Ia, Ib, IIb, and III were chromatographed twice on the Bio-Rex column. AF = 0.15 M ammonium formate, pH 2.7; RP-HPLC = reverse-phase high-performance liquid chromatography; TEAF = 0.10 M triethylammonium formate, pH 4.2; TFA = 0.1% trifluoroacetic acid. See text for further details.

Table II: Cytotoxicity of Native Toxin γ and Its Seven Monomodified Derivatives at a Single Amino Group[a]

toxin	location of substitution	EC_{50} (mg/L)	EC_{50}/EC_{50} of toxin γ
native toxin γ		17	1
(N^{α}-acetylleucine-1)-toxin γ	loop I	23	1.3
(N^{ϵ}-acetyllysine-2)-toxin γ	loop I	17	1
(N^{ϵ}-acetyllysine-12)-toxin γ	loop I	170	10
(N^{ϵ}-acetyllysine-23)-toxin γ	loop II	55	3.2
(N^{ϵ}-acetyllysine-35)-toxin γ	loop II	55	3.2
(N^{ϵ}-acetyllysine-16)-toxin γ[b]		50	2.9
(N^{ϵ}-acetyllysine-18)-toxin γ[b]		50	2.9

[a] The compounds in phosphate saline buffer were added to 6.6×10^5 epithelial cells from cultured human amnion (FL strain). After a 30-min incubation, cytotoxicity was measured by the trypan blue exclusion test. Measurements were repeated at least three times. Cytotoxic activity is given as EC_{50}. [b] Crude fraction.

group contained derivatives modified at Lys-23 and Lys-35 and the crude fractions modified at Lys-16 and Lys-18, which were about 3 times less cytotoxic than native toxin γ. The third category contained only the derivative modified at Lys-12, which was 10 times less potent than native toxin γ. Note also that two other unidentified derivatives (fractions IIa4 and III4) modified after position 35 did not exhibit a decrease in cytotoxic potency higher than a factor of 2–3.

(4) Correlation between Lethal Activity and Cytotoxicity. Although cytotoxicity appears to be a highly reproducible and sensitive procedure, it has not been shown, as yet, if this property reflects directly or indirectly the lethal potency of a cardiotoxin. We therefore tentatively correlated toxicity and

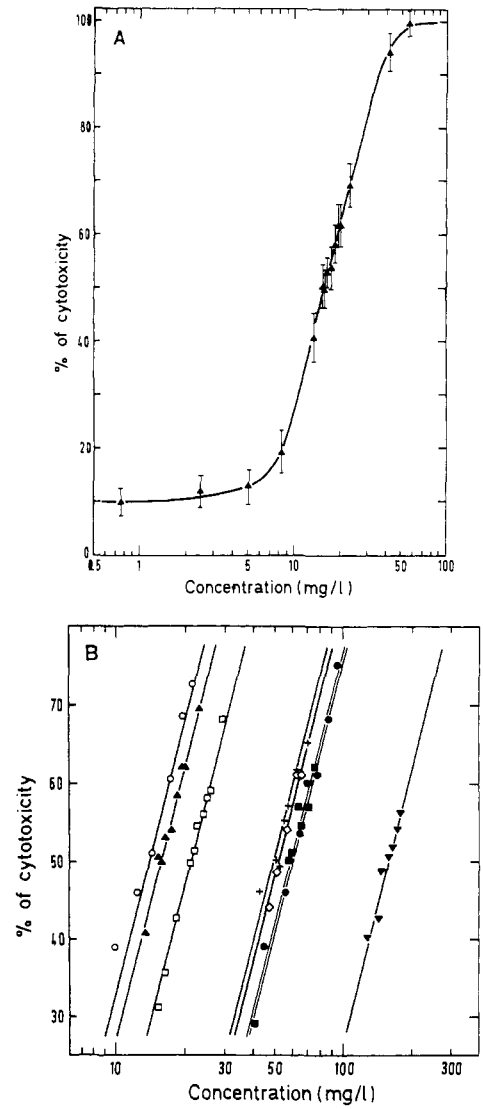

FIGURE 6: Concentration–response curve for cytotoxicity on epithelial cells (cultured human amnion) by native toxin γ (panel A) and derivatives at Leu-1 (□), Lys-2 (○), Lys-12 (▼), Lys-23 (◇), and Lys-35 (+) as well as by the crude fractions Lys-16 (●), Lys-18 (■), and native toxin γ (▲) (panel B). Points represent the means of at least three experiments; SE bars are shown in panel A.

lytic activity for a set of five monomodified derivatives and two analogous cardiotoxins, all chosen because their lytic potency was observed to increase gradually in the range of 14–170 mg/L (see Table III). The five derivatives included three toxins substituted at a hydrophobic residue [Trp-11, Tyr-22, and Tyr-51 (Gatineau et al., 1987)] and two toxins monoacetylated at Lys-12 and Lys-23. The two similar cardiotoxins are *N. m. mossambica* CTX IIa and CTX IIb mentioned above. Figure 7 and Table III showed that cytotoxicity toward FL cells, expressed as EC_{50}, correlated well with the lethal activity in mice, expressed as LD_{50} ($R = 0.93$, $p < 0.001$). The discovery of a wider range of potencies in vitro compared to in vivo is not surprising, as the effect in vivo must be influenced by a large number of factors (such as absorption, distribution, and protein binding) that are not encountered in vitro where the interaction of the toxin with its target membrane is studied more directly. Indeed, similar discrepancies between in vivo toxicity and action on target tissue in vitro (nerve–muscle preparation or purified acetylcholine receptor preparation) have been described for some weakly active curaremimetic toxins from cobra venoms (Ishikawa et al., 1977; Harvey et al., 1984). Also, it was argued

Table III: Cytotoxicity, Lethal Activity, and Depolarizing Potency of Native and Modified Toxin γ as Well as *N. m. mossambica* CTX IIa and CTX IIb[a]

toxin	location of substitution	EC_{50} (mg/L)[b]	LD_{50} (mg/kg)[f]	EEC (mg/L)[b]
native toxin γ		17 (1.0)	1.06 (1.0)[c]	5 (1.0)
(N^{ϵ}-acetyllysine-12)-toxin γ	loop I	170 (10)	3.20 (3.0)[b]	170 (34)
(N^{ϵ}-acetyllysine-23)-toxin γ	loop II	55 (3.2)	2.00 (1.9)[b]	nd
(NPS-tryptophan-11)-toxin γ	loop I	32 (1.9)	2.15 (2.1)[c]	24 (4.8)
(3-nitrotyrosine-22)-toxin γ	loop II	27 (1.6)	1.52 (1.4)[c]	75 (15)
(3-nitrotyrosine-51)-toxin γ	loop III	14 (0.8)	1.00 (0.9)[c]	17 (3.4)
N. m. mossambica CTX IIa	tip of loop II	19 (1.1)	1.17 (1.1)[d]	nd
N. m. mossambica CTX IIb [(deaminated asparagine-57)-toxin γ]	C-terminal region	21 (1.2)	1.09 (1.0)[e]	8 (1.6)

[a] The assays were performed as described in the text. Numbers in parentheses correspond to the ratio of the activity of the compound to the activity of native toxin γ. [b] This work. [c] Gatineau et al., 1987. [d] Mean of values from Louw (1974) and Bougis et al. (1983). [e] Mean of values taken from Louw (1974), Bougis et al. (1983), and our laboratory (this work). [f] In spite of the diversity of the origins of the LD_{50}'s, for the same compound, the values taken from the different laboratories are very close.

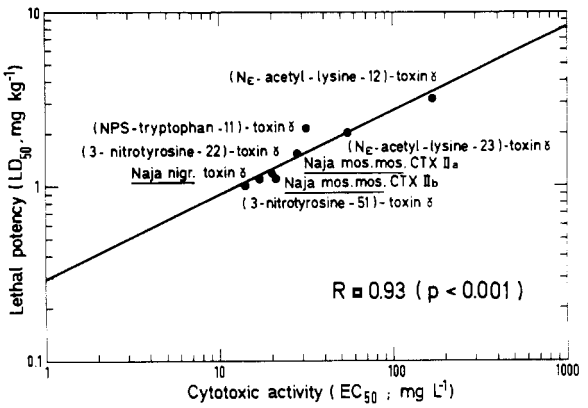

FIGURE 7: Correlation of lethal potency with cytotoxic activity. The inset in the curve indicates the correlation coefficient (R) and the risk associated with the statistic (p).

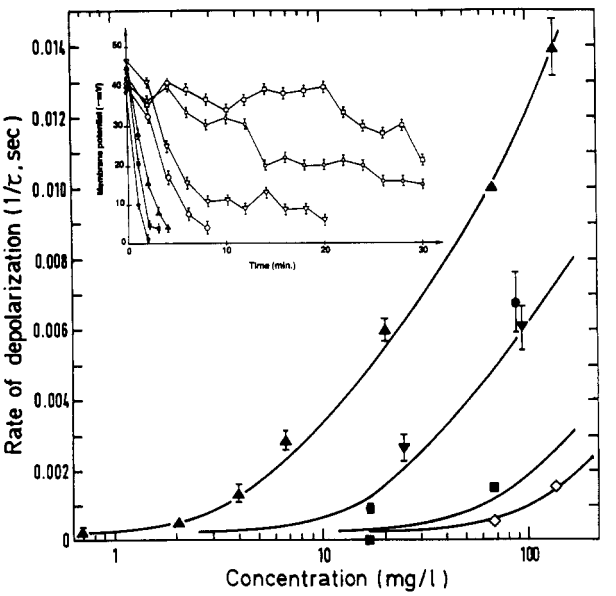

FIGURE 8: Concentration–response curve for depolarization of cultured skeletal muscle fibers by native toxin γ (▲) and its derivatives at Trp-11 (●), Lys-12 (◇), Tyr-22 (■), and Tyr-51 (▼). The rate of depolarization is measured as the reciprocal of the time constant of the decay of membrane potential with time, which is well fitted by an exponential. Points represent the means of at least two experiments; SE bars are shown when they are longer than the symbol. The inset shows depolarization of cultured skeletal muscle fibers by different concentrations of native toxin γ: (□) 0.1, (○) 0.3, (▽) 0.6, (◇) 3, (▲) 10, (■) 20, and (●) 47 μM. Each point represents the mean ± SE of measurements (in at least six cells made in at least two cultures).

that lethality assays with potent toxins reflect the minimum amounts of toxin required to occupy a necessary fraction of binding sites in the body; hence, small changes in affinity to their target tissue may be undetected when lethality is measured (Ishikawa et al., 1977).

(5) Relationships between Cytotoxicity and Cell Depolarization. Like other cardiotoxins (Hodges et al., 1987), toxin γ produced a concentration-dependent depolarization of skeletal muscle fibers in culture (see inset in Figure 8). This effect was irreversible and was followed by obvious morphological damage: loss of cross-striations, extrusion of cytoplasm, and detachment from culture dish [see Figure 7 in Harvey et al. (1983)]. We monitored the concentration-dependent rate of depolarization ($1/\tau$) for myotubes induced by four monomodified derivatives (at Trp-11, Lys-12, Tyr-22, and Tyr-51) and one highly similar cardiotoxin (*N. m. mossambica* CTX IIb) (Figure 8). Table III shows that the equieffective concentration (EEC) was markedly different from one compound to another. CTX IIb was nearly as potent as native toxin γ, suggesting that the single substitution occurring in its C-terminal region (position 57) was of no importance for the depolarizing effect. The modifications at Tyr-51, Trp-11, and Tyr-22 decreased the depolarizing potency by factors of 3, 5, and 15, respectively. Modification of Lys-12 induced the most dramatic decrease in depolarization activity by a factor of 34. Hodges et al. (1987) observed an even weaker decrease in depolarizing activity (×26) for *Hemachatus haemachatus* 9B, which differed from toxin γ by as many as 32 substitutions, of which 10 were supposed to be critical for depolarization. In general, depolarization data were in fairly good agreement with cytotoxicity data. One difference, however, was noted concerning the derivative modified at position 51, which was equally as cytotoxic as native toxin γ whereas it was less potent at depolarizing cell membranes. Perhaps, as suggested by Hodges et al. (1987), the third loop is more critical for depolarization activity than for cytotoxicity.

(6) Effect of Monoacetylation on the Secondary Structure of Toxin γ. The far-UV CD spectra of native toxin γ and derivatives modified at position 1, 2, 12, 23, and 35 (Figure 9) disclosed similar features, with a negative trough centered at 215 nm and an intense positive signal at 192 nm. These two bands correspond respectively to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the peptide chromophores (Woody, 1985) and indicate the presence of β-structure with no helicity (Ménez et al., 1978; Grognet et al., 1988), in agreement with observations from diffraction (Rees et al., 1987) and NMR (Steinmetz et al., 1988) studies. The identity of the CD spectra of native and monoderivatized toxins indicated that the overall structure of the polypeptide backbone was not altered by abolition of individual positive charges at positions 1, 2, 12, 23, and 35.

(7) Effect of Monoacetylation on Fluorescence Emission of Trp and Tyr Residues of Toxin γ. Each loop of the toxin

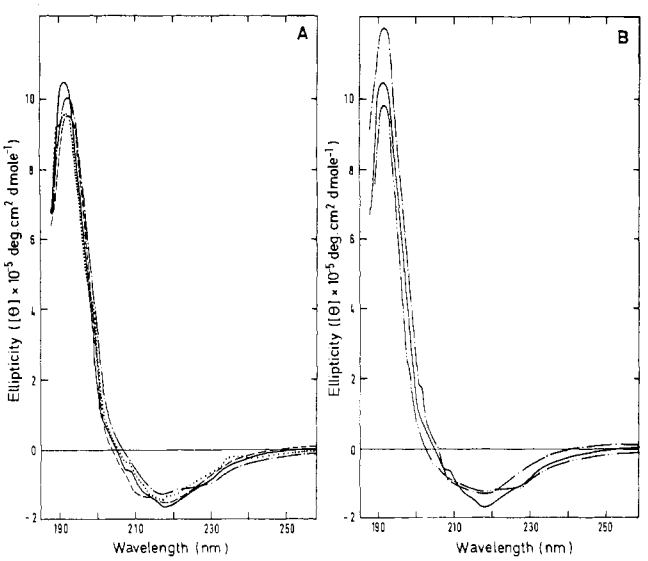

FIGURE 9: Far-UV circular dichroism spectra of native (—) and monomodified toxin γ at positions 1 (---), 2 (···), 12 (-·-) (panel A), 23 (-·-), and 35 (-··-) (panel B). In all cases, the compound was dissolved in water (pH ≈ 6.7) and the protein concentration was approximately 10^{-5} M. The cell path length was 0.05 cm.

has one aromatic residue, which was used to probe locally the structure of the molecule. These residues are Trp-11 in loop I, Tyr-22 at the base of loop II, and Tyr-51 at the base of loop III (Figure 1). This is a particularly fortunate situation, since (i) Trp-11 and Tyr-22 are respectively adjacent to Lys-12 and Lys-23, (ii) both Tyr-22 and Tyr-51 are in spatial proximity to Lys-35, and (iii) both phenolic moieties 22 and 51 are close to each other and sensitive to local structural changes (Gatineau et al., 1987). All fluorescence emission spectra (Figure 10) showed a broad peak centered at 337 nm due to Trp-11 emission, and when excitation was at 275 nm, a shoulder occurred at 305 nm due to emission of both tyrosines (Gatineau et al., 1987). Toxin γ and the derivatives modified at Lys-12 and Lys-35 gave rise to virtually identical emission spectra, indicating that abolition of these positive charges did not affect the local structure of Trp-11 and the two tyrosine residues. The spectra of derivatives modified at Leu-1 and Lys-23 were similar in shape to that of native toxin, though slightly more intense (10%) (Figure 10A) at 337 nm. However, no difference was seen at 305 nm. Only the tryptophan environment appeared to be affected by acetylation at positions 1 and 23. Due to lack of a derivative modified at position 2, we could not monitor its fluorescence characteristics.

Discussion

We have described the preparation and characterization of a set of monoacetylated derivatives of toxin γ, a cardiotoxin from the venom of *N. nigricollis*. The derivatives enabled us to delineate the functional sites of the toxin.

Delineation of the "Cytotoxic Site". By far the largest decrease in cytotoxicity (90%) was associated with modification at Lys-12, a residue located in loop I of toxin γ (Figure 1). As judged from CD and fluorescence data, this decrease was unlikely to be due to global or local conformational change in the molecule. We therefore conclude that the positive charge of Lys-12 is a critical cytotoxic element of toxin γ. Trp-11, another residue of loop I, was also identified as being implicated in cytotoxicity (Gatineau et al., 1987). Strikingly, examination of cardiotoxin sequences (Dufton & Hider, 1988) revealed that positions 11 and 12 are highly conserved, being respectively occupied by an aromatic residue (Trp, 55%, Tyr, 27.5%, and His, 10%) and a lysine (100%). The presence of

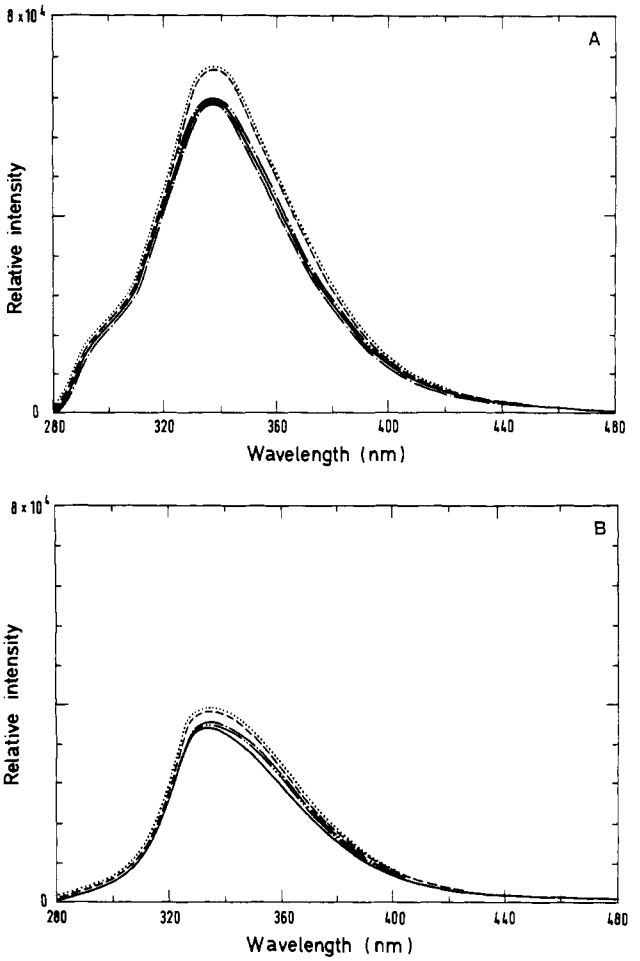

FIGURE 10: Emission fluorescence spectra of native (—) and monomodified toxin γ at positions 1 (---), 12 (-·-), 23 (···), and 35 (-··-) for excitation wavelengths equal to 275 (panel A) and 295 nm (panel B). The compounds were dissolved in 10^{-2} M sodium cacodylate buffer + 10^{-1} M NaCl, pH 7; temperature = 20 °C.

these residues may partly account for the cytotoxic character of some cardiotoxins (Condrea, 1974; Kini & Evans, 1989b). Not all residues of loop I, however, are important for cytotoxicity, since the derivatives modified at Leu-1 and Lys-2 were as potent as the native molecule. The only other modification that induced a decrease in cytotoxicity with no concomitant structural change was at Lys-35. The derivative was 3-fold less potent than native toxin, suggesting, therefore, that the base of loop II was also implicated in cytotoxicity. In contrast, the tip of loop II was of no importance for cytotoxicity, as inferred from the observation that *N. m. mossambica* CTX IIa, whose sequence only differed by three amino acid substitutions, all located at the tip of loop II [Ala-28, Pro-30, and Met-31 in toxin γ are replaced by Gly, Ser, and Lys in CTX IIa (Louw, 1974; Bougis et al., 1986; Otting et al., 1987)], was as potent as toxin γ. We also found that the C-terminal part of the toxin did not seem to be critical for cytotoxicity, since *N. m. mossambica* CTX IIb, which had a single substitution at position 57 [Asp in toxin γ is replaced by Asn in CTX IIb (Bougis et al., 1986; Otting et al., 1987)], was equally as potent as toxin γ. No conclusion could be drawn as to the role of other amino groups; however, none of them, including those modified after position 35 (not shown), was as critical as Lys-12 (Table II). Data from this and previous papers have been summarized in Figure 1. Clearly, the highly basic toxin γ has a well-delineated cytotoxic site, which includes defined residues located on the convex face or on the edge of the molecule, on loop I and perhaps at the base of loop II.

The "Cytotoxic", "Depolarizing", and "Lethal" Sites Are Highly Similar. Change in cytotoxicity correlated well with change in lethal activity and, to a large extent, with change in depolarizing activity. In particular, irrespective of the type of assay, the derivative acetylated at Lys-12 was far less potent. Our data suggest, therefore, that the sites responsible for the different functions of toxin γ are highly similar if not identical. In agreement with our conclusion are the findings of Marchot et al. (1988), who reported that a dodecapeptide mimicking the edge of loop I of *N. m. mossambica* CTX IV was toxic in mice and induced cardiotoxin-like symptoms. Since toxin γ possesses a defined functional site, it is tempting to suggest that there should be a physiological counterpart. This might be a calcium channel (Lin Shiau et al., 1976) or negatively charged phospholipids (Dufourcq & Faucon, 1978; Vincent et al., 1978). The question is still open but our data indicate that it is pertinent to search for it.

The same *N. nigricollis* venom contains another toxin, called toxin α, which differs from toxin γ by approximately 58% substitutions. The two toxins have a similar folded structure, as judged from data on analogous molecules (Dufton & Hider, 1977; Ménez et al., 1978; Low, 1979; Rees et al., 1987), but are functionally unrelated. Toxin α binds to the acetylcholine nicotinic receptor and induces no sign of cytotoxicity. The "toxic" residues of toxin α (Ménez et al., 1984) and toxin γ (this work) are on the same side of the molecule, which is concave in toxin α (Low, 1979) and convex in toxin γ (Rees et al., 1987). However, toxic residues of toxin γ are predominantly located on loop I and the base of loop II, whereas toxic residues of toxin α are mostly located on loops II and III (Low, 1979; Ménez et al., 1984). Therefore, *N. nigricollis* venom contains two and possibly more toxins with similar architecture but topographically distinct sites ensuring fundamentally different functions.

Added in Proof

After this paper was submitted, Kini and Evans reported on the importance of amino groups in determining the lytic activity of the same cardiotoxin, in agreement with our pilot experiments.

Acknowledgments

We are gratefully indebted to Bernard Gilquin for having communicated a schematic view of toxin γ structure and to Pierre Bougis for the gift of *N. m. mossambica* CTX IIa and IIb. We are also gratefully indebted to Pierre Paroutaud for advice in protein sequences. We warmly thank Christian Schneider, who made an interface between a Jobin-Yvon dichrograph III and an Olivetti M280 computer, and Pierre Legay, who wrote Turbo C software for recording and analyzing the CD spectra. We also thank Flavio Toma, Gilles Mourier, and Jean-Marc Grognet for their fruitful suggestions.

References

Arms, K., & MacPheeters, D. (1975) *Toxicon 13*, 333.
Batenburg, A. M., Bougis, P. E., Rochat, H., Verkleij, A. J., & de Kruijff, B. (1985) *Biochemistry 24*, 7101–7110.
Boquet, P. (1970) *C. R. Acad. Sci. Paris, Sér. D 271*, 2422–2425.
Botes, D. P., & Viljoen, C. C. (1976) *Biochim. Biophys. Acta 446*, 1–9.
Bougis, P. E., Marchot, P., & Rochat, H. (1986) *Biochemistry 25*, 7235–7243.
Bougis, P., Tessier, M., Van Rietschoten, J., Rochat, H., Faucon, J. F., & Dufourcq, J. (1983) *Mol. Cell. Biochem. 55*, 49–54.
Breckenridge, R., & Dufton, M. J. (1987) *J. Mol. Evol. 26*, 274–283.
Condrea, E. (1974) *Experientia 30*, 121–129.
Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.
Dufourcq, J., & Faucon, J. F. (1978) *Biochemistry 17*, 1170–1176.
Dufourcq, J., Faucon, J. F., Bernard, E., Pezolet, M., Tessier, M., Bougis, P., Van Rietschoten, J., Delori, P., & Rochat, H. (1982) *Toxicon 20*, 165–174.
Dufton, M. J., & Hider, R. C. (1977) *J. Mol. Biol. 115*, 177–193.
Dufton, M. J., & Hider, R. C. (1988) *Pharmacol. Ther. 36*, 1–40.
Fogh, J., & Lund, R. O. (1957) *Proc. Soc. Exp. Biol. Med. 94*, 532–537.
Fraenkel-Conrat, H., Bean, R. C., & Lineweaver, H. (1949) *J. Biol. Chem. 177*, 365–403.
Fryklund, L., & Eaker, D. (1975) *Biochemistry 14*, 2865–2871.
Gatineau, E., Toma, F., Montenay-Garestier, Th., Takechi, M., Fromageot, P., & Ménez, A. (1987) *Biochemistry 26*, 8046–8055.
Grognet, J.-M., Gatineau, E., Bougis, P., Harvey, A. L., Couderc, J., Fromageot, P., & Ménez, A. (1986) *Mol. Immunol. 23*, 1329–1337.
Grognet, J.-M., Ménez, A., Drake, A., Hayashi, K., Morrison, I. E. C., & Hider, R. C. (1988) *Eur. J. Biochem. 172*, 383–388.
Harvey, A. L. (1985) *J. Toxicol., Toxin Rev. 4*, 41–69.
Harvey, A. L., Marshall, R. J., & Karlsson, E. (1982) *Toxicon 20*, 379–396.
Harvey, A. L., Hider, R. C., & Khader, F. (1983) *Biochim. Biophys. Acta 728*, 215–221.
Harvey, A. L., Hider, R. C. Hodges, S. J., & Joubert, F. J. (1984) *Br. J. Pharmacol. 82*, 709–716.
Hawke, D., Yuan, P.-M., & Shively, J. E. (1982) *Anal. Biochem. 120*, 302–311.
Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65–74.
Hestrin, S. (1949) *J. Biol. Chem. 180*, 249–261.
Hider, R. C., & Khader, F. (1982) *Toxicon 20*, 175–179.
Hodges, S. J., Agbaji, A. S., Harvey, A. L., & Hider, R. C. (1987) *Eur. J. Biochem. 165*, 373–383.
Ishikawa, Y., Ménez, A., Hori, H., Yoshida, H., & Tamiya, N. (1977) *Toxicon 15*, 477–488.
Iwaguchi, T., Takechi, M., & Hayashi, K. (1985) *Biochem. Int. 10*, 343–349.
Karlsson, E. (1979) *Handb. Exp. Pharmacol. 52*, 159–204.
Kini, R. M., & Evans, H. J. (1989a) *Biochemistry 28*, 9209–9215.
Kini, R. M., & Evans, H. J. (1989b) *Int. J. Pept. Protein Res. 34*, 277–286.
Lauterwein, J., & Wüthrich, K. (1978) *FEBS Lett. 93*, 181–184.
Lee, C. Y., & Lee, S. Y. (1979) *Handb. Exp. Pharmacol. 52*, 547–590.
Lin Shiau, S. Y., Huang, M. C., & Lee, C. Y. (1976) *J. Pharmacol. Exp. Ther. 196*, 758–770.
Louw, A. I. (1974) *Biochim. Biophys. Acta 336*, 481–495.
Louw, A. I., & Visser, L. (1977) *Biochim. Biophys. Acta 498*, 143–153.
Louw, A. I., & Visser, L. (1978) *Biochim. Biophys. Acta 512*, 163–171.
Low, B. W. (1979) *Handb. Exp. Pharmacol. 52*, 213–254.

Marchot, P., Bougis, P. E., Céard, B., Van Rietschoten, J., & Rochat, H. (1988) *Biochem. Biophys. Res. Commun. 153*, 642–647.
Mênez, A., Langlet, G., Tamiya, N., & Fromageot, P. (1978) *Biochimie 60*, 505–516.
Mênez, A., Boulain, J.-C., Bouet, F., Couderc, J., Faure, G., Rousselet, A., Trêmeau, O., Gatineau, E., & Fromageot, P. (1984) *J. Physiol. (Paris) 79*, 196–206.
Miller, L. C., & Tainter, M. L. (1944) *Proc. Soc. Exp. Biol. Med. 57*, 261–264.
Otting, G., Steinmetz, W. E., Bougis, P. E., Rochat, H., & Wüthrich, K. (1987) *Eur. J. Biochem. 168*, 609–620.
Rees, B., Samama, J. P., Thierry, J. C., Gillibert, J., Fischer, J., Schweitz, H., Lazdunski, M., & Moras, D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3132–3136.
Riordan, J. F., & Vallee, B. L. (1967) *Methods Enzymol. 11*, 565–570.
Roumestand, C., Gatineau, E., Gilquin, B., Mênez, A., & Toma, F. (1989) 11th American Peptide Symposium, San Diego, CA (in press).
Steinmetz, W. E., Bougis, P. E., Rochat, H., Redwine, O. D., Braun, W., & Wüthrich, K. (1988) *Eur. J. Biochem. 172*, 101–116.
Takechi, M., Tanaka, Y., & Hayashi, K. (1986) *FEBS Lett. 205*, 143–146.
Tarr, G. E., Black, D. S., Fujita, V. S., & Coon, M. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6552–6556.
Tazieff-Depierre, F., & Trethevie, E. R. (1975) *C.R. Acad. Sci. Paris, Sêr. D 280*, 137–140.
Tazieff-Depierre, F., Czajka, M., & Lowagie, C. (1969a) *C. R. Acad. Sci. Paris, Sêr. D 268*, 2511–2514.
Tazieff-Depierre, F., Czajka, M., & Lowagie, C. (1969b) Symposium International:Drugs and Metabolism of Myocardium and Striated Muscles, Nancy, France, Abstract pp 479–485.
Vincent, J. P., Balerna, M., & Lazdunski, M. (1978) *FEBS Lett. 85*, 103–107.
Woody, R. W. (1985) *Peptides (N.Y.) 7*, 15–114.
Zar, J. H. (1984) in *Biostatistical Analysis* (Zar, J. E., Ed.) pp 306–309, Prentice-Hall, Englewood Cliffs, NJ.

Role of GTP Hydrolysis in Microtubule Polymerization: Evidence for a Coupled Hydrolysis Mechanism†

Russell J. Stewart,‡ Kevin W. Farrell, and Leslie Wilson*
Department of Biological Sciences, University of California, Santa Barbara, California 93106
Received January 17, 1990; Revised Manuscript Received April 10, 1990

ABSTRACT: The relationship between GTP hydrolysis and microtubule assembly has been investigated by using a rapid filtration method. Microtubules assembled from phosphocellulose-purified tubulin, double-labeled with [γ-^{32}P]- and [^{3}H]GTP, were trapped and washed free of unbound nucleotide on glass fiber filters. The transient accumulation of microtubule-bound GTP predicted by uncoupled GTP hydrolysis models [Carlier & Pantaloni (1981) *Biochemistry 20*, 1918–1924; Carlier et al. (1987) *Biochemistry 26*, 4428–4437] during the rapid assembly of microtubules was not detectable under our experimental conditions. By calculating hypothetical time courses for the transient accumulation of microtubule-bound GTP, we demonstrate that microtubule-bound GTP would have been detectable even if the first-order rate constant for GTP hydrolysis were 4–5 times greater than the pseudo-first-order rate constant for tubulin subunit addition to microtubules. In a similar manner, we demonstrate that if GTP hydrolysis were uncoupled from microtubule assembly but were limited to the interface between GTP subunits and GDP subunits (uncoupled vectorial hydrolysis), then microtubule-bound GTP would have been detectable if GTP hydrolysis became uncoupled from microtubule assembly at less than 50 μM free tubulin, 5 times the steady-state tubulin concentration of our experimental conditions. In addition, during rapid microtubule assembly, we have not detected any microtubule-bound P_i, which has been proposed to form a stabilizing cap at the ends of microtubules [Carlier et al. (1988) *Biochemistry 27*, 3555–3559]. Also, several conditions that could be expected to increase the degree of potential uncoupling between GTP hydrolysis and microtubule assembly were examined, and no evidence of uncoupling was found. Our results are consistent with models that propose cooperative mechanisms that limit GTP hydrolysis to the terminal ring of tubulin subunits [e.g., O'Brien et al. (1987) *Biochemistry 26*, 4148–4156]. The results are also consistent with the hypothesis that a slow conformational change in tubulin subunits after GTP hydrolysis and P_i release occurs that results in destabilized microtubule ends when such subunits become exposed at the ends.

Microtubules assembled in vitro, in the absence of stabilizing agents such as microtubule-associated proteins (MAPs)[1] or glycerol, can be highly dynamic (Horio & Hotani, 1986; Walker et al., 1988). At steady state under appropriate conditions, a microtubule can be either growing relatively slowly or disassembling rapidly at either end, and the ends may alternate between the growing and shortening phases frequently relative to the lifetime of the microtubule. The sug-

†Supported by USPHS Grants NS13560 (R.J.S. and L.W.) and GM41751 (K.W.F.).
‡Present address: The Biological Laboratories, Harvard University, 16 Divinity St., Cambridge, MA 02138.

[1] Abbreviations: MAP(s), microtubule-associated protein(s); GXP, guanine nucleotide in microtubules, in the form either of GDP or of GTP; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; column buffer, 50 mM Pipes, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8; polymerization buffer, 100 mM Pipes, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.8; stabilizing buffer, 30% glycerol (v/v) and 10% dimethyl sulfoxide (v/v) in polymerization buffer; PBS, phosphate-buffered saline solution; BSA, bovine serum albumin.

0006-2960/90/0429-6489$02.50/0