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Cobra Venom Cytotoxin Free of Phospholipase A₂ and Its Effect on Model Membranes and T Leukemia Cells

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Abstract. Membrane-active toxins from snake venom have been used previously to study protein-lipid interactions and to probe the physical and biochemical states of biomembranes. To extend these studies, we have isolated from *Naja naja kaowthia* (cobra) venom a cytotoxin free of detectable phospholipase A₂ (PLA₂). The amino acid composition, pI (10.2), and net charge of the cytotoxin compares well with membrane-active toxins isolated from venoms of other cobras. The cytotoxin, shown by a spin label method, associates with PLA₂ in buffers at pH values between 7.0 and 5.0, but not at pH 4.0. It is suggested that cytotoxin and PLA₂ (pI close to 4.8) associate electrostatically in the native venom. The effect of the cytotoxin on model phospholipid membranes was studied by EPR of spin probes in oriented lipid multilayers and ¹H-NMR of sonicated liposomes. The cytotoxin did not significantly affect the packing of lipids in pure phosphatidylcholine (PC) membranes and in PC membranes containing 10 mol% phosphatidic acid (PA) or cardiolipin (CL). However, the cytotoxin induced an increase in membrane permeability and formation of nonbilayer structures in PC membranes containing 40 mol% of PA or CL. The purified cytotoxin was cytotoxic to Jurkat cells, but had little effect on normal human lymphocytes. However, both Jurkat cells and normal lymphocytes were killed equivalently when treated with 10⁻⁹ M PLA₂ and 10⁻⁵ M cytotoxin in combination. From its effect on model membranes and Jurkat cells, it is suggested that purified cytotoxin preferentially targets and disrupts membranes that are rich in acidic phospholipids on the extracellular side of the plasma membrane.

Key words: Cobra cytotoxin — Cardiotoxin — Phos-

pholipid membranes — Permeability — Nonbilayer structures — T leukemia cells — Phospholipase A₂ — EPR spin probes — ¹H-NMR

Introduction

Snake venom cytotoxins are basic proteins in cobra venoms (*Naja* and *Haemachatus*), where they may represent as much as 40% of the total protein. The cytotoxins that have been studied to date are amphipathic peptides composed of 57–61 amino acid residues in a single chain that is cross-linked by four highly conserved disulfide bonds (Grishin et al., 1974, 1976; Harvey, 1985, 1991; Chaim-Matyas, Borkow & Ovadia, 1991). The coexistence of an exposed hydrophobic surface and a cationic zone is a shared and, most likely, functionally significant characteristic of venom cytotoxins (Kini & Evans, 1989).

Cytotoxins isolated from different sources exhibit various physiological effects, such as modulation of the activity of membrane enzymes, depolarization of excitable membranes, inhibition of platelet aggregation, cardiac arrest, hemolysis and cytotoxicity (Harvey, 1985, 1990, 1991; Teng et al., 1987; Fletcher & Jiang, 1993). The methods used for detection of cytotoxins has resulted in descriptive names such as direct lytic factors, cardiotoxins, cobramines, cytolytins, or membranotoxins (Chien et al., 1991; Rees & Bilwes, 1993). This terminology reflects, for the most part, a superficial understanding of the principal activity of these toxins. It is now generally accepted that cytotoxins bind to cell membranes where they alter membrane organization and function (Vernon & Bell, 1992; Rees & Bilwes, 1993). These toxins, therefore, have now been grouped as membrane-active toxins.

Venom cytotoxins have been widely used as a tool to study protein-lipid interactions, protein-mediated

membrane-membrane interactions, fusion of membranes and membrane permeability (Batenburg et al., 1985; Airpov et al., 1989; Gasanov, Salakhutdinov & Airpov, 1990; Gasanov, Airpov & Salakhutdinov, 1990). Despite the considerable research that has been done, the molecular/physical mode of interaction of cytotoxins with biomembranes has not been completely elucidated. It is unclear whether cytotoxins interact with lipids or with membrane proteins. One of the problems in solving this question has been that most (if not all) venom cytotoxin preparations are contaminated to some degree with venom-derived phospholipase A₂ (PLA₂) since the two copurify (Fletcher et al., 1990; Fletcher & Jiang, 1993). These two venom components synergistically disrupt cell membranes and thus modify the actual mode of action of the cytotoxin itself (Fletcher & Jiang, 1993; Gasanov et al., 1994). Recent studies have shown that the membranotropic properties of cytotoxins can be altered, not only as the result of direct action of PLA₂ on lipid membranes, but also from PLA₂-cytotoxin interactions that may occur either in the water phase or on the membrane surface (Gasanov et al., 1991; Gasanov, Gasanov & Rael, 1995).

The purpose of the present work was to investigate the membranotropic properties of a PLA₂-free venom cytotoxin on model phospholipid membranes and to examine its cytotoxic activity. The purified cytotoxin was found to lack the ability to disturb model membranes composed of either pure phosphatidylcholine (PC) or PC containing small amounts of incorporated acidic phospholipids. However, the cytotoxin caused an increase in membrane permeability and formation of nonbilayer structures in model membranes containing high amounts of acidic phospholipids. The cytotoxin was also found to discriminate, in its cytotoxic activity, between normal and malignant human lymphocytes. It is suggested that the cytotoxin targets malignant lymphocytes via its preferential interaction with acidic phospholipids which are accumulated on the extracellular side of membranes of T leukemia cells (Mirtalipov, 1992).

Materials and methods

REAGENTS

Venom from *Naja naja kaouthia* (Thailand cobra) was purchased in lyophilized form from Captive Born Venoms (Slade, Kentucky). Venom from *Crotalus molossus molossus* (Northern blacktailed rattlesnake) was purchased from Sigma Chemical (St. Louis, MO). Egg yol, L- α -phosphatidylcholine (PC), acardiophospholipin (CL) from *E. coli*, egg yol, L- α -phosphatidic acid (PA), 5-doxylosteic acid (5-DSA), 4-(2-iodoacetamido)-TEMPO, and oleic acid were purchased from Sigma Chemical (St. Louis, MO). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from Miles-Yeda Ltd Research Products (Israel). 11-(dansylamino) undecanoic acid (DAUDA) was purchased from Molecular Probes (Junction City, OR). Rat liver fatty-

acid-binding protein (FABP) was obtained from Dr. E.A. Iagudeeva, Chemistry Department (Moscow State University, Russia).

CHROMATOGRAPHY

Naja naja kaouthia venom (0.5 g) was reconstituted in starting buffer (0.02 M Na₂HPO₄, pH 4.6) and applied to a CM-Sephadex C-50 (Pharmacia LKB, Piscataway, NJ) column equilibrated with starting buffer. A salt gradient was established with eluant buffer (0.02 M Na₂HPO₄, pH 4.6, 1 M NaCl) and monitored at 280 nm. Fractions were collected in 3 ml volumes and each was desalted and lyophilized. The fraction with the highest cytotoxicity on mouse lymphocytes was subfractionated by cation exchange HPLC. For subfractionation, 0.5 ml of a sample (10 mg/ml), dissolved in 0.02 M Na₂HPO₄, pH 4.0, was injected into a SCX 83-C-13-ET1 Hydropore column (Rainin Instrument, Woburn, MA). A linear gradient was established with the same buffer containing 1 M NaCl 10 min after the sample was injected. The column eluate was monitored at 280 nm.

A fraction with high PLA₂ activity, obtained by a CM-Sephadex C-50 fractionation, was also subfractionated by cation exchange HPLC with a SCX 83-C-13-ET1 Hydropore column. The fraction (5 mg), which had been desalted and lyophilized, was reconstituted in 0.5 ml of starting buffer (0.02 M Na₂HPO₄, pH 7.5) and then injected. A salt gradient was established with buffer containing 1 M NaCl and the eluate monitored at 280 nm.

The purity of the fractions was determined by reducing SDS-PAGE and by isoelectric focusing.

M2, a PLA₂ of pI 7.0, was isolated from *Crotalus molossus molossus* venom as previously described (Gasanov et al., 1994).

ANTI-PLA₂ ANTIBODIES

Anti-PLA₂ antibodies were produced by injecting BALB/c mice with 10 μ g/injection of PLA₂ purified from *Naja naja kaouthia*. The Titer-Max (Vaxcel, Norcross, GA) protocol was followed for the immunization schedule. To test for PLA₂, the cytotoxin or venom fractions (100 μ l of a 1 mg/ml solution) were first filtered through a membrane using a slot-blot apparatus and then visualized with the anti-PLA₂ antibodies by a procedure described previously (Anaya et al., 1992). In this procedure, the membranes with the samples were first incubated with blocking solution, washed, then incubated for 2 hr with different dilutions of anti-PLA₂. After washing, the membranes were incubated with a 1/6,000 dilution of secondary antibodies (rabbit anti-mouse IgG conjugated to horseradish peroxidase, from Miles-Yeda Ltd Research Products, Israel), followed by washing, and then development with 4-chloro-1-naphthol in the presence of H₂O₂.

CYTOTOXICITY TEST

Cytotoxicity was determined by trypan blue uptake of treated cells. Lymphocytes from normal human blood and from spleens of adult BALB/c mice were separated by density-gradient centrifugation on lymphocyte separation medium (Bionetics, Kensington, MD). Jurkat cells (clone E6-1, ATCC TIB 152), derived from an acute human T cell leukemia, were maintained in RPMI-1640 medium containing 10% fetal bovine serum and 5 μ g/ml gentamicin at 37°C in a 5% CO₂ humidified atmosphere. After several washes in RPMI-1640 medium, each of the cell samples was adjusted to 10⁷ cells per ml. Venom fractions were tested on mouse lymphocytes by incubating 0.5 ml cells with 6.5 μ g of venom fraction for 30 min at 37°C. Normal human lymphocytes or Jurkat cells (0.5 ml) were incubated for 30 min at 37°C with defined concentrations of cytotoxin in the presence or absence of

10^{-9} M PLA₂. After treatment, the cell samples were centrifuged and resuspended in fresh medium. Cells incubated in the absence of fraction, cytotoxin, or PLA₂ served as controls. Percent cytotoxicity was calculated according to the formula: $(1 - (\text{number of viable cells in the test sample} / \text{number of viable cells in control sample})) \times 100$. Each data point reported is the mean of three experiments, each performed in triplicate, \pm the standard deviation.

PLA₂ ASSAY

PLA₂ activity was determined by a continuous fluorescence displacement assay (Wilton, 1990). In the present study, a stock assay cocktail was prepared by adding 1 ml of 100 mg/ml of phosphatidylcholine in methanol and 0.3 ml of 0.1 M DAUDA in methanol to the 20 ml assay buffer (0.1 M Tris-HCl, pH 7.8, 0.1 M NaCl, 0.1 mM CaCl₂, 0.02 mM Triton X-100). In the test samples, 0.01 mg of each fraction to be tested was added to 2 ml of the assay cocktail and incubated for 30 min at 37°C in a thermostatically regulated fluorimeter cell (Hitachi F200 fluorimeter). Lipid hydrolysis was terminated by addition of 20 mM EDTA, followed by addition of 30 μ g of rat liver FABP. The solution was excited by pulsed laser at 350 nm and fluorescence of DAUDA was measured at 500 nm. The lifetime of the excited state of DAUDA was estimated from the time dependence of the attenuation of the probe glow using semilogarithmic coordinates. A standard curve of the lifetime of the excited state of DAUDA as a function of free fatty acid concentration was prepared using defined concentrations of oleic acid. Each sample was prepared and measured at least in triplicate. The standard deviation was always within $\pm 2.5\%$ of the means.

AMINO ACID ANALYSIS OF CYTOTOXIN

Reduction and carboxymethylation of cytotoxin was carried out according to Grishin et al., 1976. Approximately 50 mmol of protein were reduced with 50 μ l of 20 mM dithiothreitol and subsequently carboxymethylated with 50 μ l of 0.1 M iodoacetic acid. Reduced and carboxymethylated cytotoxin was hydrolyzed in sealed evacuated tubes with 6 N HCl at 105°C for 24 hr. Acid was removed by drying in a Speed-Vac (Savant Instruments). The residue was dissolved in 2 ml of Na citrate buffer, pH 3.2, and its amino acid composition analyzed on a Bio-Cal BC-201 automatic analyzer.

CYTOTOXIN BINDING TO PLA₂

Cytotoxin binding to PLA₂ was studied by electron paramagnetic resonance (EPR) of spin labeled proteins (Gasanov et al., 1991; 1995a). The cytotoxin was covalently labeled with 4-(2-iodoacetamido)-TEMPO according to the protocol provided by Sigma Chemical (St. Louis, MO). Unreacted spin label was removed from the cytotoxin by Sephadex G-100 gel filtration. A detectable EPR signal of the spin labeled cytotoxin (10^{-5} M) with no change in cell toxicity was obtained when a threefold molar excess of spin label over cytotoxin was used. However, at this toxin to spin label ratio, a decrease of 11% in toxicity was observed on cells pretreated with 10^{-9} M PLA₂. Cytotoxin (10^{-5} M) was incubated for 5 min at room temperature in the presence of 5×10^{-4} M PLA₂ in buffers (2 mM Tris-HCl, 0.1 M NaCl, 0.02 mM CaCl₂) having pH values of 7.0, 6.0, 5.0, or 4.0. These same buffers without PLA₂ served as controls. The EPR spectra of spin-labeled cytotoxin were recorded with a Varian E-4 spectrometer at modulation amplitudes not exceeding 2×10^{-4} T and resonator input power not exceeding 20 mW. Each sample was prepared and tested at least two times. The differences in EPR spectra (observed in terms of hyperfine split-

ting and intensity of resonance peaks) of different preparations for each sample were always within 1%.

CYTOTOXIN INTERACTION WITH LIPID MEMBRANES

The interaction of cytotoxin with lipid membranes was examined by the EPR of 5-doxystearic acid (5-DSA) in oriented lipid films and by proton nuclear magnetic resonance (¹H-NMR) of sonicated liposomes. Membranes were composed of phosphatidylcholine (PC), PC + 10 mol% or 40 mol% of phosphatidic acid (PA), or PC + 10 mol% or 40 mol% of cardiolipin (CL). In the EPR studies, lipid (5 mm) was suspended in buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA) containing 10^{-5} M 5-DSA and defined concentrations of cytotoxin. EPR spectra of 5-DSA were recorded at the same conditions as described above for 4-(2-iodoacetamido)-TEMPO. To prepare oriented lipid films, 50 μ l of lipid suspension was applied onto the surface of a microscope cover slip (0.5 cm \times 2 cm) attached to a glass rod. Pre-testing of the coverslip with attached glass rod did not give an EPR signal at the conditions described above. The coverslip with lipid was left at room temperature for 5 min, then an additional 50 μ l of lipid suspension was applied directly to the lipid smear, kept for 2 min, then covered by a second coverslip of equal size. Orientation of the coverslip surface in the applied magnetic field was done with the resonator accessory. EPR spectral analysis was done in terms of the ratio B/C (Aracava, Smith & Schreier, 1981; Gasanov et al., 1990b; Gasanov, Vernon & Airpov, 1993). Each sample for the EPR assay was prepared and tested at least in triplicate and the means of these measurements used as experimental data points. The standard deviation was always within $\pm 3.5\%$ of the means.

In the ¹H-NMR studies, liposomes were prepared in D₂O. Lipid (10^{-2} M) suspended in buffer (2 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.5 mM EDTA) was sonicated (Airpov et al., 1989). Liposomes (1 ml) were mixed with 10 μ l of saturated solution of [Fe(CN)₃]⁻³ in D₂O and incubated with 2×10^{-5} M of cytotoxin for 30 min at room temperature. ¹H-NMR spectra of liposomes were recorded with a Bruker WP 200 wide-bore NMR spectrometer. The measurements were done with an operating frequency of 200 MHz. The width of the 90° pulse was 8.7 μ sec, and the acquisition time for the free induction signal was 1 sec. Each liposome sample was prepared two times and the measurements of the integral intensity of the signals from the N⁺(CH₃)₃ groups of phosphatidylcholine was each made in triplicate. Variation among the triplicates of each sample was less than 1%, and variation among the means of the two preparations for each sample was less than 5%.

Results

Venom was fractionated on a CM-Sephadex C-50 cation exchange column. The major absorbance peaks were pooled into six fractions as shown in Fig. 1. Each fraction was tested for cytotoxicity and of these fractions F₆ was the most cytotoxic for mouse lymphocytes. The fractions were also tested for PLA₂ activity by fluorescence displacement assay. This assay is very sensitive and detects free fatty acid at concentrations down to 0.1 nM. All fractions, except fraction F₁, hydrolyzed phosphatidylcholine, and fraction F₂ had highest PLA₂ activity. Fraction F₂ through F₆ all tested positive with anti-PLA₂ antibodies by slot-blot procedure with the highest visualization at a 1/10 dilution of anti-PLA₂ antibodies.

Fraction F₆ was subfractionated by cation exchange

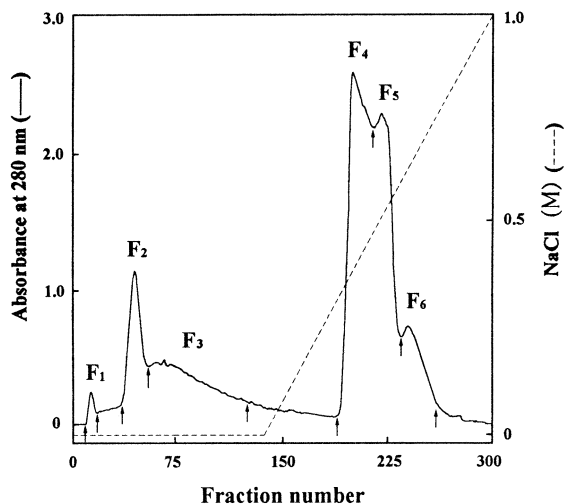


Fig. 1. Fractionation of *naja naja kaouthia* venom by CM-Sephadex C-50 column chromatography. Venom (0.5 g in 0.02 M Na_2HPO_4 , pH 4.6) was applied to the column and a salt gradient established (broken line) with eluant buffer (0.02 M Na_2HPO_4 , pH 4.6, 1 M NaCl) to release the adsorbed proteins. The eluant was monitored at 280 nm and the fractions pooled as indicated by the arrows.

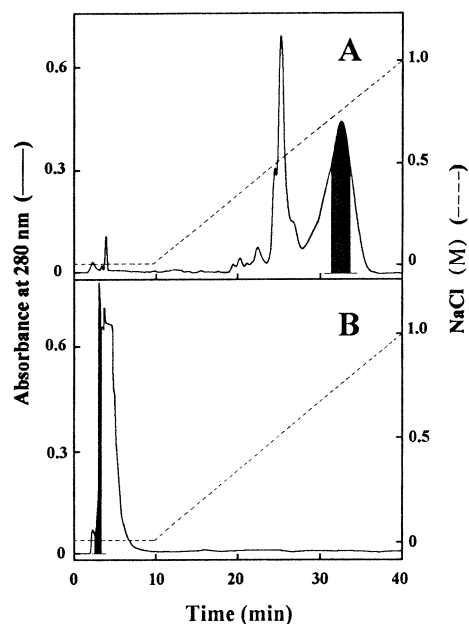


Fig. 2. A Subfractionation of fraction F_6 with an SCX 83-C13-Et1 Hydropore column to purify the cytotoxin (shaded peak). F_6 (10 mg in buffer) was injected into a column that had been equilibrated with 0.02 M Na_2HOP_4 , pH 4.0. A salt gradient in buffer (broken line) was used to elute the adsorbed proteins. (B) Subfractionation of fraction F_2 with an SCX 83-C13-ETI Hydropore column to purify the PLA₂ (shaded peak). F_2 (5 mg in buffer) was injected into a column that had been equilibrated with 0.02 M Na_2HPO_4 , pH 7.5. A salt gradient in buffer (broken line) was used to elute the adsorbed proteins.

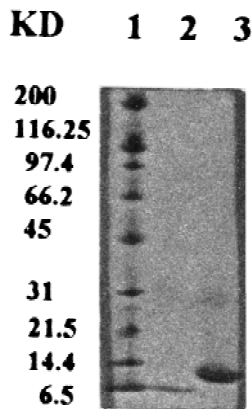


Fig. 3. SDS-PAGE of cytotoxin (lane 2) and PLA₂ (lane 3). Samples for SDS-PAGE were dissolved in buffer composed of 20 mM Tris-HCl, 2 mM EDTA, 5% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue and boiled for 3 min. The samples were resolved in a 8%–25% acrylamide gradient gel. Standard broad-range molecular mass proteins are in lane 1. Molecular masses are indicated in kilodaltons.

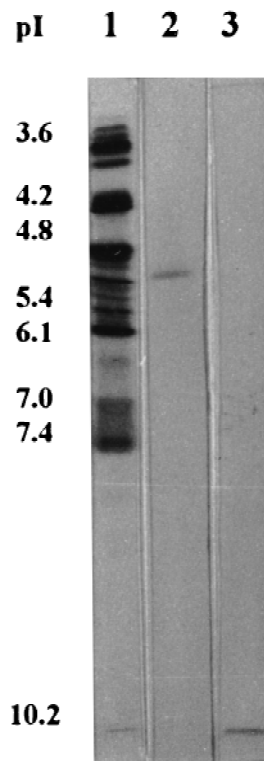


Fig. 4. Isoelectric focusing of cytotoxin (lane 3) and PLA₂ (lane 2). Isoelectric focusing was done in isogel agarose with a pH gradient from 3.0 to 10.0. Standard pI reference proteins are in lane 1.

HPLC, and the fraction shown shaded in Fig. 2A was found to be cytotoxic for mouse lymphocytes. This subfraction was a protein of molecular weight 6.5 kDa (Fig. 3, lane 2) and pI 10.2 (Fig. 4, lane 3). The protein had no

Table 1. Amino acid composition, net charges and pI values of cytotoxin from *Naja n. kaouthia* and related membrane-active toxins from other cobra venoms

Parameter	<i>Naja n. kaouthia</i> cytotoxin	<i>Naja n. oxiana</i> cytotoxins ^{a,b}		<i>Naja n. atra</i> cardiotoxins ^{c,d}				<i>Naja naja</i> cytotoxin II ^e	<i>H. haemachatus</i> direct lytic factor ^f	<i>Naja m.</i> <i>mossambica</i> cardiotoxin IIb ^g
		Vc5	Vc1	CTXII	CTX1	CTX3	CTX4			
Amino acids per molecule										
Basic										
Lys	9	10	8	8	8	9	8	9	10	8
His	0	1	0	0	0	0	0	0	1	0
Arg	2	1	2	2	2	2	3	2	1	2
Acidic										
Asp	2	2	2	2	3	2	2	2	0	0
Glu	0	0	1	0	0	0	0	0	1	1
Nonpolar										
Ala	2	3	3	2	2	2	2	2	1	3
Val	7	7	7	7	4	7	7	7	4	5
Leu	6	6	6	6	6	6	5	6	6	6
Ile	1	1	2	1	4	1	1	1	2	2
Pro	5	5	5	4	4	5	4	5	5	6
Met	2	2	2	2	3	2	2	2	2	2
Phe	2	2	2	2	1	2	2	1	1	1
Trp	0	0	0	0	0	0	0	0	0	1
Polar										
Gly	2	2	2	2	2	2	2	2	2	2
Ser	2	3	3	2	3	2	2	2	3	2
Thr	3	2	2	3	3	3	3	3	3	3
Cys	8	8	8	8	8	8	8	8	8	8
Tyr	3	2	2	3	2	3	3	4	1	2
Asn	4	3	3	6	5	4	6	4	6	6
Total residues	60	60	60	60	60	60	60	60	57	60
Net charge	+9	+10	+7	+8	+7	+9	+9	+9	+11	+9
pI value	10.2	11.0	9.5	10.1	10.04	10.18	10.31	10.3		10.2

Amino acid composition and other properties of membrane-active toxins from cobra venoms were from ^a Grishin et al., 1974, 1976; ^b Yukelson et al., 1974; ^c Bhaskaran et al., 1994; ^d Chiou et al., 1993; ^e Takechi & Hayashi, 1972; ^f Aloof-Hirsch et al., 1968; ^g O'Connell et al., 1993.

PLA₂ activity by fluorescence displacement assay, and it tested negative with anti-PLA₂ antibodies. This protein was designated as the cytotoxin.

Fraction F₂ was also subfractionated by cation exchange HPLC, and the peak that had highest PLA₂ activity was pooled as shown shaded in Fig. 2B. This subfraction, which was pure by SDS-PAGE and isoelectric focusing, was designated as PLA₂. It had a molecular weight close to 14 kDa (Fig. 3, lane 3) that relates well to a molecular weight of other cobra venom PLA₂s (Deems & Dennis, 1975; Reynolds & Dennis, 1991; Reynolds et al., 1995). The pI of PLA₂ was close to 4.8 (Fig. 4, lane 2).

A summary of the amino acid composition, pI value and net charge of the cytotoxin and a comparison to other cobra venom membrane-active toxins described by others are shown in Table 1. The cytotoxin contains no histidine, glutamic acid, or tryptophan, but is rich in cysteine and basic (lysine, arginine) and hydrophobic (valine, leucine, proline) amino acids. This is very similar to data obtained for other cytotoxins.

Since it is very difficult to isolate cytotoxin free of PLA₂, it is reasonable to assume that the two proteins associate strongly by noncovalent bonding. To examine this we studied the association between purified cytotoxin and PLA₂ in buffers of different pH by measuring the EPR spectra of spin-labeled cytotoxin. The EPR spectrum of spin-labeled cytotoxin (Fig. 5A0) appeared as a rather broad triplet signal that one would not expect for a molecule as small as cytotoxin. Such a signal could result from aggregation of spin-labeled cytotoxin. By gel filtration, we have observed a retention time for spin-labeled cytotoxin close to that of proteins of molecular weight of 21.5 kD. This suggests that the toxin aggregated into trimers. Another explanation for resonance broadening could be from dipolar-induced relaxation resulting from interaction between spin-label molecules from neighboring aggregated cytotoxin. Spin-spin interaction within single molecules is not likely to occur, since it was determined previously that the lysines in the cytotoxin are not close enough to induce effective spin-spin relaxation (Gasanov et al., 1990). We recorded the

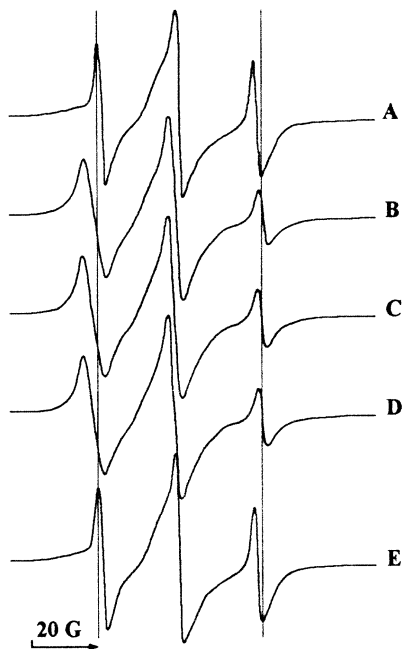


Fig. 5. EPR spectra of spin labeled cytotoxin in Tris-HCl buffers with pH values of 7.0 (A and B), 6.0 (C), 5.0 (D), and 4.0 (E). Cytotoxin (10^{-5} M), labeled with 4-(2-iodoacetamido)-TEMPO, was incubated with PLA₂ (5×10^{-4} M) for 5 min at room temperature, then the EPR spectra recorded. (A) is cytotoxin alone, whereas B, C, D, and E have added PLA₂.

EPR spectra of spin labeled cytotoxin in buffers of pH from 9.0 to 3.0, and no changes in the EPR spectra were found. We have also determined in a previous study, (Gasanov et al., 1991) that acidic cobra venom PLA₂ enzymes maintain the same conformation at pH from 7.0 to 4.0. Addition of PLA₂ to spin-labeled cytotoxin in buffer at pH 7.0 resulted in a noticeable broadening of the resonance signal (Fig. 5B). This broader hyperfine splitting indicates a more restricted and axis-symmetrical mobility, a result of PLA₂ association with the spin-labeled cytotoxin. Similar results were obtained in buffers of pH 6.0 and 5.0 (Fig. 5C and D). At pH 4.0, however, a noticeable change in the resonance of spin-labeled cytotoxin was detected (Fig. 5E). The EPR signal had the same hyperfine splitting and intensity of the triplet peaks as that of the spin-labeled cytotoxin in the absence of PLA₂. This indicates that cytotoxin and PLA₂ had dissociated at pH 4.0 and further suggested that the two proteins associate electrostatically in buffers at pH that is higher than the pI of the enzyme (the pI of *Naja naja kaouthia* PLA₂ is 4.8). Experiments involving M2, the PLA₂ (pI 7.0) from *Crotalus molossus molossus*, showed it had no effect on the EPR spectra of spin-labeled cytotoxin in buffers of pH from 7.0 to 4.0.

The cytotoxic activity of cytotoxin to human cells was examined on normal and cultured lymphocytes (Jurkat cells). Cytotoxin, at the concentrations studied, was

not very active on normal lymphocytes (Table 2). It killed about 6% of normal lymphocytes. At a concentration of 10^{-5} M, the cytotoxin was much more active on Jurkat cells, killing close to 80% of cells. When PLA₂ was added to the samples, the viability of both normal lymphocytes and Jurkat cells incubated with cytotoxin dropped significant, killing 92% of normal lymphocytes and close to 98% of the Jurkat cells at toxin concentration of 10^{-5} M. With PLA₂ alone 95.4% of normal lymphocytes and 98.7% of Jurkat cells were viable (Table 2).

The cytotoxin was further studied to determine its interaction with phospholipid membranes. Its effect on the EPR spectra of 5-DSA in multilayer lipid films was analyzed in terms of the *B/C* parameter. The *B/C* ratio indicates transitions in the macroscopic order of the lipid phase (Aracava et al., 1981; Airpov et al., 1986). Higher values of the *B/C* ratio reflect a higher ordering in the packing of the bilayer, and a decline in *B/C* values indicates a disordering of the lipid bilayer (Berliner, 1979; Aracava et al., 1981). As seen in Fig. 6, the cytotoxin caused a negligible increase in the ordering of lipid bilayer in membranes of pure PC, and, to a lesser extent, in membranes of PC + 10 mol% PA. A slight decline in the *B/C* values was observed in membranes of PC + 10 mol% CL that had been treated with cytotoxin. A pronounced decrease in the *B/C* values was observed in membranes containing 40 mol% of PA or CL with treated with cytotoxin. In these membranes, at high toxin concentration, values for *B/C* were lower than 0.46 reflecting the presence of nonbilayer structures (Berliner, 1979).

The effect of the cytotoxin on the ¹H-NMR spectra of the trimethylammonium groups on PC in liposomes is shown in Fig. 7. In control samples (lower spectrum for each test), the smaller of the two resonance signals is given by the inner monolayer of the liposomes, and the second, larger signal comes from the outer monolayer. The second signal has a higher resonance value as a result of interaction of PC of the outer monolayer with paramagnetic ferricyanide anion [Fe(CN)₆]³⁻. In cytotoxin-treated liposomes, the position of the two resonance signals of the trimethylammonium groups did not change in liposomes of either pure PC or those containing 10 mol% PA or CL (upper spectra). However, addition of cytotoxin to liposomes containing 40 mol% PA or CL resulted in a shift of the signal from the inner monolayer toward the stronger field. This indicates that these liposomes have become permeable to ferricyanide anions. In these liposomes, a new resonance signal also appeared as a high-field shoulder in the outer monolayer signal. This new signal was of higher intensity in liposomes containing 40 mol% of CL than that in liposomes containing 40 mol% PA. This new shift in the resonance frequency reflects the change in the interaction between

Table 2. Toxicity of the cytotoxin (CT) on normal human lymphocytes (NHL) and Jurkat cells (acute human T cell leukemia) treated or untreated with 10^{-9} M PLA_2 . The reported data are percent viable cells after the indicated treatments.

CT concentration (mM)	Cell untreated with PLA_2		Cell treated with PLA_2	
	NHL	Jurkat	NHL	Jurkat
0	100*	100	95.4 ± 2.8	98.7 ± 3.4
10^{-10}	98.1 ± 3.0	91.4 ± 2.5	—	—
10^{-9}	99.3 ± 5.5	86.5 ± 2.0	—	—
10^{-8}	98.3 ± 2.3	74.3 ± 4.5	62.0 ± 5.6	59.5 ± 3.4
10^{-7}	99.0 ± 3.6	46.2 ± 4.9	34.9 ± 5.1	26.5 ± 4.1
10^{-6}	96.7 ± 3.1	35.3 ± 2.0	18.2 ± 3.5	9.1 ± 3.0
10^{-5}	94.0 ± 4.0	21.5 ± 3.1	8.0 ± 3.0	2.6 ± 2.0

* Percent viable cells is based on control cell samples not treated with CT or PLA_2 . The data are based on the means of three preparations \pm the standard deviation.

ferricyanide anions and trimethylammonium groups of the outer monolayer, a result of nonbilayer structure formation in portions of the liposome membrane (Airpov et al., 1989; Gasanov et al., 1993).

Discussion

As a result of considerable research on cobra venom cytotoxins in the past four decades it is now well accepted that the subcellular target of these toxins is the cell membrane. Not clear, however, is whether the specific binding sites for cytotoxins are membrane proteins or lipids since there is experimental evidence supporting interaction with both entities (Harvey, 1991; Dufton & Hider, 1991). Experiments involving radiolabeling with cardiotoxin suggest that cardiotoxin is able to bind specifically to a protein of 59 kDa in cockroach heart membrane (Klowden et al., 1992). Cardiotoxin also has been found to stimulate phosphatidylinositol kinase activity in A431 cell membranes, supposedly by interacting directly with the enzyme (Walker & Pike, 1990). Three cardiotoxins (also called cytotoxins) from Formosan cobra (*naja naja atra*) inhibited protein kinase C activity in phosphatidylserine vesicles presumably by interacting nonspecifically with a hydrophobic regulatory domain of the enzyme and with membrane phospholipids (Chiou et al., 1993).

In our previous studies, we were unable to show experimentally that *naja naja oxiana* cytotoxins V_c5 and V_c1 interact with membrane proteins (Gasanov, Kamaev & Rozenshtein, 1988; Segal et al., 1993). On the contrary, we found that cytotoxin V_c5 modulates the activity of H^+ ATP synthase by inducing structural transitions of lipids in mitochondrial membranes. These lipid phase transitions likely occur from binding of cytotoxin to phospholipids that are tightly bound to F_0 factor of the enzyme. In other studies with model membranes, we

have also shown that both V_c5 and V_c1 are able to dehydrate the membrane surface (Gasanov et al., 1990), aggregate liposomes, increase membrane permeability (Airpov et al., 1989), induce the formation of nonbilayer structures, cause an intermembrane exchange with lipids, and cause membranes to fuse (Gasanov et al., 1990a,b). These effects, caused by toxin-lipid interaction, relate well with the results of others who have shown that cardiotoxins bind to and disrupt lipid membranes and penetrate acidic phospholipid monolayers (Bougis et al., 1981; Dufourcq et al., 1982; Batenburg et al., 1985).

The attraction of cytotoxins to negatively-charged lipids in natural and synthetic membranes is well known (Vincent, Balerna & Lazdunski, 1978). The affinity of cytotoxin for negative lipids is high ($K_d < 10^{-7}$ M), and the stoichiometry is about seven lipid molecules for each toxin molecule. This is slightly less than the number of positive charges available on the toxin molecule (Rees & Bilwes, 1993). The principal argument against a lipid target in vivo is that the cell membranes so far investigated contain only a small amount of negatively charged phospholipids, which are, furthermore, concentrated on the cytoplasmic side of the cell membrane (Rothman & Lenard, 1977; Mirtalipov, 1992). Therefore these phospholipids are not directly accessible to the cytotoxin.

The results of the present study show that cytotoxin from *Naja naja kaouthia*, purified free of PLA_2 , is not effective in perturbing neutral synthetic membranes. The cytotoxin did not significantly affect the parameter B/C of spin probes and ^1H -NMR spectra of liposomes in model membranes of either pure PC or PC containing small amounts of acidic phospholipids. However, with liposomes containing higher amounts of negatively charged phospholipids, the cytotoxin caused noticeable disruptions, such as formation of nonbilayer structures and increasing membrane permeability. This finding supports the hypothesis that acidic lipids are the binding site for cytotoxins. From our studies with cells

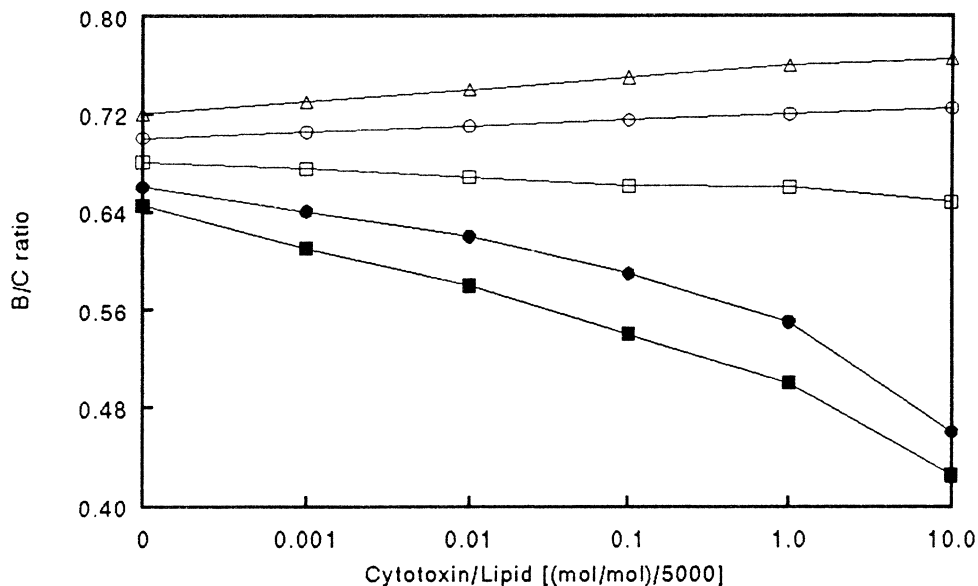


Fig. 6. B/C ratio of the EPR spectra of 5-DSA in oriented lipid films of PC (triangles), PC + 10 mol% PA (open circles), PC + 10 mol% CL (open squares), PC + 40 mol% PA (dark circles), and PC + 40 mol% CL (dark squares) as a function of cytotoxin concentration. Each data point is the mean of three experiments. The standard deviation was always within $\pm 3.5\%$ of the means.

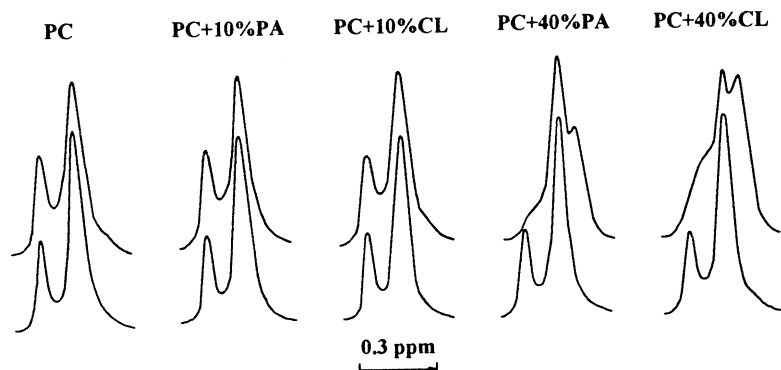


Fig. 7. ^1H -NMR signals of trimethylammonium groups of PC of sonicated liposomes in the presence of potassium ferricyanide. The lower set of signals are from liposomes in the absence of cytotoxin and the upper set of signals are from liposomes in the presence of 2×10^{-5} M cytotoxin. The composition of the liposomes is indicated for each group of cytotoxin-treated and untreated liposomes.

we showed that the cytotoxin was not effective in killing normal human lymphocytes, but was very cytotoxic to Jurkat cells, a cell line derived from a human T cell leukemia. Recent studies on lipid metabolism in blood cells of patients with blood cell malignancies revealed altered activities of endogenous lipases with a resultant preferential accumulation of acidic lipids, such as phosphatidic acid and phosphatidylinositol, on the outer leaflet of membranes of B and T leukemia cells (Mirtalipov, 1992). This allows one to suggest that cytotoxin differentiates between normal and malignant lymphocytes as a result of its preferential binding to membranes of T leukemia cells that contain acidic lipids on the outer leaflet. It seems likely that cytotoxin specifically targets and disrupts cells having membranes rich in negative lipids on the extracellular side.

It is important to be aware that one can obtain dif-

ferent results when very small amounts of PLA_2 , such as may be present in commercial preparations of membrane-active toxins (Fletcher, Michaux & Jiang, 1990), are introduced along with cytotoxin to cell samples. Here, cytotoxin, which works synergistically with PLA_2 , is equally toxic to both normal lymphocytes and Jurkat cells. A mechanism of this synergy has been suggested previously by us in which cytotoxin interacts with PLA_2 to form a complex (Gasanov et al., 1991; 1995a). The nature of the interaction between cytotoxin and PLA_2 appears to be electrostatic, since we found no interaction at pH 4.0 (the pI of cytotoxin is 10.2, and the pI of PLA_2 is 4.8). The cytotoxin- PLA_2 complex, under neutral conditions, attaches to the cell membrane where PLA_2 catalyzes the release of free fatty acids and lysophospholipids (Gasanov et al., 1995a). Cytotoxin in turn becomes surrounded by the produced fatty acids and as a

result gets shuttled away from the enzyme-toxin complex (Gasanov et al., 1991). Both phase segregation of fatty acids and accumulation of lysophospholipids should destabilize lipid bilayers, causing flip-flopping of phospholipids. This activity, which changes the initial asymmetric distribution of membrane lipids, should expose acidic lipids on the outer membrane leaflet, whereby, not only is free cytotoxin attracted to acidic molecules on the extracellular side, but also triggers lipase activity on the cytoplasmic side. Cytotoxin-induced stimulation of endogenous lipase activity is well known (Shier, 1980). The question still remains as to whether the stimulated endogenous enzyme is a PLA₂ or a phospholipase C (Fletcher et al., 1990; Vernon & Bell, 1992).

The ability of highly purified cytotoxin to discriminate between normal and cancer cells is intriguing and has been suggested for specific cell targeting. This property of cytotoxin, however, may not hold true in vivo due to possible synergy between cytotoxin and extracellular lipases. A plausible strategy for using cytotoxins against cancer is to conjugate them to monoclonal antibodies that are specific to epitopes on malignant cells. The cytotoxin-like membrane-active toxin, *Pyrularia* thionin, has been conjugated to an anti-CD5 monoclonal antibody and this conjugate proved to be very effective in specific killing in vitro (Gasanov et al., 1995b). At present, cytotoxin from *Naja naja kaouthia* is being studied in our laboratory as a potential candidate in immunotoxin construction.

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