Phospholipase A₂ and Cobra Venom Cytotoxin V₅
Interactions and Membrane Structure

S. E. GASANOV¹, N. E. GASANOV² and E. D. RAEL¹

¹Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968, USA;
²Institute of Bioorganic Chemistry Uzbekistan Academy of Sciences, 83 Kh. Abdullaeva Ave., Tashkent 700143, Uzbekistan

Abstract. The hydrolytic activity and interaction of acidic and neutral phospholipase A₂ (PLA₂) with large unilamellar liposomes treated with cobra venom cytotoxin V₅ (CT V₅) were studied to more fully understand the modulating effects of cationic membrane-active peptides on PLA₂. Studies were done by fluorescence displacement, EPR spin probes, and ³¹P-NMR. The results showed that CT V₅ inhibits PLA₂ activity on phosphatidylcholine liposomes. Enzymatic activity of both acidic and neutral PLA₂s were enhanced on liposomes containing cardiolipin and pretreated with cytotoxin. The cytotoxin, however, inhibited enzyme lipid hydrolysis if these same liposomes were first treated with acidic PLA₂. The highest enzymatic activity was found on substrates with nonbilayer lipid packing. Using EPR of spin labeled enzymes, it was shown that CT V₅ inhibited binding of acidic PLA₂ to liposomes and caused displacement of acidic PLA₂ from liposomes. No direct interaction was found between CT V₅ and neutral PLA₂. It is suggested that cytotoxin perturbs packing of lipid molecules in liposomes containing cardiolipin and is responsible for increased catalysis, whereas direct interaction between CT V₅ and acidic PLA₂ inhibits enzyme activity. It is concluded that variability in substrate composition and the chemical nature of both PLA₂ and cationic peptide determine whether enzyme activity is affected by substrate packing or by direct enzyme-peptide interaction. Models of interactions of PLA₂ with CT V₅ and phospholipid membranes are presented.

Key words: Snake venom PLA₂ — Cytotoxin V₅ — Phospholipid membranes — Fluorescence displacement — EPR — ³¹P-NMR
Introduction

Phospholipase A₂, an enzyme that catalyzes the hydrolysis of the 2-acyl ester bond in phosphoglycerides, has been detected in virtually every tissue and cell type examined (Dennis 1983). This enzyme, found in both intracellular and extracellular forms, exhibits a number of physiological actions and is responsible for the release of fatty acids (Davidson and Dennis 1990). Fatty acids provide the substrates required for the synthesis of eicosanoids, which are involved in pathophysiological processes such as inflammation, platelet aggregation, and acute hypersensitivity reactions (Dennis 1987; Lister et al. 1989). Thus, modulation of PLA₂ activity is a current pharmacological goal, and there is much interest in controlling the consequences of PLA₂ action in chronic inflammatory conditions, such as rheumatoid arthritis and asthma.

Regulation of PLA₂ activity \textit{in vivo} appears to be mediated by a group of glucocorticoid controlled proteins called lipocortins (Crompton et al. 1988; Klee 1988). It has been reported that small cationic peptide sequences derived from the lipocortin family are potent modulators of PLA₂ activity \textit{in vitro} and, in parallel, show anti-inflammatory potential \textit{in vivo} (Miele et al. 1988). The precise mechanism of action of the lipocortins remains controversial, and centers around whether the peptides act by interacting directly with the enzyme or, alternatively, by binding and sequestering the phospholipid substrate (Davidson et al. 1987; Haigler et al. 1987). In contrast to these studies, two synthetic anti-inflammatory peptides having a high amino acid sequence homology with lipocortins did not show binding to pancreatic PLA₂ and additionally did not inhibit enzyme activity \textit{in vitro} (Van Binsbergen et al. 1989). In other studies, an endogenous cationic peptide isolated from murine smooth muscle, bovine endothelial cells and human arthritic synovial fluid (Clark et al. 1987; Bomalaski et al. 1989) has been proposed to regulate PLA₂ activity through direct interaction with enzyme. The peptide bears structural similarity to melittin, the bee venom PLA₂-stimulating peptide, and is recognized by anti-melittin antibodies (Clark et al. 1987). In more recent studies, another distinct PLA₂-stimulating peptide has been identified in murine embryonic genital tracts (Gupta and Braun 1990).

Snake venom PLA₂s show a high degree of structural homology with mammalian extracellular PLA₂ and these PLA₂s have similar physiological activities (Davidson and Dennis 1990; Kelley et al. 1992). Membrane-active cationic peptides, such as snake venom cardotoxin, bee venom melittin and \textit{Pyrularia} thionin, influence the physiological and esterase activities of both snake venom PLA₂ and endogenous PLA₂ in intact cells (Shier 1979; Harvey 1985; Fletcher and Lizzo 1987; Vernon and Bell 1992). In the course of our studies on snake venom PLA₂, we have found that cardotoxin from \textit{Naja naja kaouthia}, cytotoxin from \textit{Naja naja oxiama} and thionin from \textit{Pyrularia pubera} are capable of either enhancing, inhibiting or
showing no effects on activities of cobra and rattlesnake venom PLA$_2$ (Gasanov and Rael 1992; Gasanov et al. 1991, 1994). These effects on PLA$_2$ depend on the sequence in which the enzyme and the toxin are added to the liposome samples and on the phospholipid composition of the liposomes (Gasanov et al. 1991, 1994).

The present paper extends previous investigations on the relationships between membrane-active peptides and PLA$_2$ in their ability to influence the structural state of phospholipid membranes. To better understand the mechanism of PLA$_2$ modulation by membrane-active peptides, we investigated the interactions between PLA$_2$ and cobra venom cytotoxin V$_c$5 in aqueous solutions in the presence and absence of phospholipid liposomes. The effect of cytotoxin V$_c$5 on PLA$_2$ binding to membranes of liposomes was also studied.

**Materials and Methods**

**Reagents**

Venom from *Crotalus molossus molossus* (Northern blacktailed rattlesnake) was purchased from Sigma Chemical Co. (St. Louis, MO). Venom from *Naja naja oxiana* (Central Asian cobra) was obtained from the Institute of Biochemistry (Tashkent, Uzbekistan). Egg yolk L-α-phosphatidylyceroline (PC), cardiolipin from *E. coli* (CL), 5-doxylstearic acid (5-DSA), 4-(2-iodoacetamido)-TEMPO, and oleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 11-(dansylamino)undecanoic acid (DAUDA) was purchased from Molecular Probes (Junction City, OR).

**Enzymes and peptides**

Two PLA$_2$s, one acidic (M1) and the other neutral (M2), were isolated from *C. m. molossus* venom as we previously described (Gasanov et al. 1994). Basic cytotoxin V$_c$5 (CT V$_c$5) was purified from *N. n. oxiana* venom according to procedure (Grishin et al. 1974), and the acidic PLA$_2$ (AEnz) from the same venom was purified as described (Gasanov et al. 1991). All proteins were homogeneous by isoelectric focusing and SDS-polyacrylamide gel electrophoresis. CT V$_c$5 was treated with p-bromophenacyl bromide to reduce lipase activity from trace PLA$_2$ contamination (Fletcher et al. 1991a). Rat liver fatty-acid-binding protein (FABP) was prepared as described by Wilton (1989).

**Phospholipase A$_2$ assay**

Phospholipase A$_2$ activity was determined using a continuous fluorescence displacement assay (Wilton 1990). Large unilamellar liposomes prepared by the ether evaporation method (Deamer and Bangham 1976) were used as substrates for PLA$_2$. Aqueous solution of liposomes consisted of 2 mmol/l lipid, 10 mmol/l Tris-HCl (pH 7.8), 0.1 mol/l NaCl and 0.02 mmol/l CaCl$_2$. Liposomes were composed of PC or PC + 20 mol% CL. PLA$_2$s (10$^{-5}$ mol/l) were incubated with liposomes for 30 min at 37°C. Control samples were incubated in the absence of PLA$_2$. In some assays liposomes were pre- or post-treated with 1.5 x 10$^{-5}$ mol/l of CT V$_c$5. Liposomes were initially treated with either M1, M2 or AEnz with vigorous mixing for 5 min, followed by addition of CT V$_c$5 and an additional 25 min incubation. In a second experiment, liposomes were initially treated with CT V$_c$5 (5 min vigorous mixing) and then either M1, M2 or AEnz added to the reaction mixture.
and incubated for an additional 30 min. Lipid hydrolysis was terminated by addition of EDTA to a final concentration of 20 mmol/l. The liposomes were then dissolved by addition of Triton X-100 to a final concentration of 5 mmol/l followed by vigorous mixing with a Vortex shaker. Dissolved liposomes (1 ml) were mixed with 1 ml of 10 mmol/l Tris-HCl buffer (pH 7.8) containing 25 µg of rat liver FABP. This mixture was added to a 4 ml plastic fluorimeter cell containing 20 µl of 0.1 mol/l DAUDA in methanol. The solution was excited at 350 nm and fluorescence of DAUDA measured at 500 nm with a Perkin-Elmer LS3B fluorescence spectrometer. The excited state lifetime of DAUDA that reflects an equilibrium between DAUDA and free fatty acids in competition for binding to rat liver FABP was estimated from the time dependence of attenuation of the probe glow using semilogarithmic coordinates. A standard curve of the DAUDA excited state lifetime as a function of free fatty acid concentration was prepared using defined concentrations of oleic acid. PLA₂ activity was expressed as mmoles fatty acid released per mg of enzyme. Each data point is the mean of three separate experiments with a standard deviation within ±5% of means.

**EPR spin probes and ³¹P-NMR studies**

The structure of membranes modified by PLA₂ and CT V₅ was studied by EPR oriented multibilayers and ³¹P-NMR (Aripov et al. 1986; Gasanov et al. 1993). Large unilamellar liposomes were treated with PLA₂ and toxin as described above. Lipid hydrolysis was terminated by EDTA. These liposomes were not treated with Triton X-100. Concentrations of 2 × 10⁻² mol/l lipid, 10⁻⁸ mol/l PLA₂, and 1.5 × 10⁻⁴ mol/l CT V₅ were used in samples for EPR studies. Oriented multibilayer films were prepared by squeezing 50 µl of liposome samples between two glass plates as described (Aripov et al. 1986). The lipid/5-DSA molar ratio in oriented lipid films was 200:1. Orientation of lipid films in the applied magnetic field was done with the resonator accessory (equipment GV33).

EPR spectra of 5-DSA 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. EPR spectral analysis was done in terms of the ratio B/C, and the order parameter S (Berliner 1979). B is the intensity of the low-field component and C is the intensity of the central component of spectra taken with the magnetic field perpendicular to the bilayer normal. The parameter S was calculated from the formula

$$S = \frac{T_{II} - T_{\perp}}{T_{xx} - 1/2 (T_{xx} + T_{yy})} \cdot \frac{a}{a'},$$

where $T_{II}$ and $T_{\perp}$ are values of tensors of the hyperfine structure at parallel and perpendicular orientations respectively of the long molecular axis of the spin probe in the applied magnetic field. The values for parameter $a'$ were calculated from the formula $a' = 1/3 (T_{II} + T_{\perp})$, and for parameter a from the formula $a = 1/3 (T_{xx} + T_{yy} + T_{zz})$. The values of $T_{xx}$, $T_{yy}$ and $T_{zz}$ were from (Berliner 1979). Each sample for the EPR assay was prepared and tested at least in triplicate and the means of these measurements were used as experimental data points. The standard deviation was always within ±3% of means. The aqueous solution of large unilamellar liposomes prepared for ³¹P-NMR studies contained 30% by volume of D₂O. The concentrations of lipid, PLA₂ and CT V₅ in these samples were 0.2 mol/l, 10⁻⁷ mol/l, and 1.5 mmol/l respectively. ³¹P-NMR spectra of liposomes were recorded with a Varian XL-200 spectrometer at an operating frequency of 80.99 MHz under conditions of proton decoupling. The width of the 90° pulse was 12 µs, the sweep width was 20 kHz, and the distance between pulses was 0.8 s. To enhance the
signal to noise ratio, the free induction decay was multiplied by an exponential function resulting in a 50-Hz line broadening. 10,000 acquisitions were accumulated for each spectrum. After the spectrum recordings were completed, each sample was kept in an NMR tube for 1 h, then the $^{31}$P-NMR spectrum was recorded again. No visible changes were noted in the spectrum for each sample.

**EPR of spin labeled enzymes**

PLA$_2$ binding to the surfaces of liposomes was studied by EPR of spin labeled enzyme (Gasanov et al. 1991). The PLA$_2$'s were covalently labeled with 4-(2-iodoacetamido)-TEMPO as we previously described (Gasanov et al. 1991). Spin labeling decreased the hydrolytic activity of M1 and AEnz by 5% and M2 by 7%. Different concentrations of large unilamellar liposomes composed of PC + 20 mol% CL were incubated with $10^{-5}$ mol/l of spin labeled enzymes for 30 min at 37°C. In some assays liposomes were post-treated with $5 \times 10^{-4}$ mol/l of CT V$_5$ for 5 min. In other assays liposomes were pretreated with $5 \times 10^{-4}$ mol/l of CT V$_5$ for 5 min and then post-treated with $10^{-5}$ mol/l of spin labeled enzymes for 30 min. CT V$_5$ ($5 \times 10^{-4}$ mol/l) was also incubated with $10^{-5}$ mol/l of spin labeled enzymes in buffer (10 mmol/l Tris-HCl, pH 7.8, 0.1 mol/l NaCl, 0.02 mmol/l CaCl$_2$ without liposomes for 30 min. EPR spectra of the spin labeled PLA$_2$'s were recorded at the same conditions as described above for multibilayer films.

![Figure 1](image)

**Figure 1.** PLA$_2$ activity of AEnz ($A$ and $B$), M1 ($C$ and $D$), and M2 ($E$ and $F$) on untreated liposomes (black bars), CT V$_5$ post-treated liposomes (checked bars), or CT V$_5$ pretreated liposomes (striped bars). PC liposomes are represented by $A$, $C$, and $E$, and PC + 20 mol% CL liposomes are represented by $B$, $D$, and $F$. The molar ratio of enzyme to toxin to lipid was 1 to $1.5 \times 10^4$ to $2 \times 10^6$. 
Results

The hydrolytic activity of the PLA\(^2\)s on liposomes in the presence and absence of CT\(V_\text{c}5\) is presented in Fig 1. In the absence of CT\(V_\text{c}5\), the PLA\(^2\)s were more active on phosphatidylcholine (PC) liposomes than on those composed of PC and cardiolipin (CL). M2 was markedly better at liberating fatty acids from liposomes than either M1 or AEnz. CT\(V_\text{c}5\) affected the activity of all three PLA\(^2\)s, and the effect depended on the order in which the two reagents were added to the liposomes. However, regardless of addition order, CT\(V_\text{c}5\) reduced the liberation of fatty acids by the acidic enzymes (AEnz and M1) when the liposomes were composed of PC. With M2, a significantly reduced liberation of fatty acids was seen when toxin was added first and then followed by addition of the enzyme.

In liposomes containing 20 mol\% CL, CT\(V_\text{c}5\) reduced fatty acid liberation by AEnz and M1 when liposomes were first treated with enzyme and then post-treated with toxin. However, a pronounced increase in activities of AEnz and M1 was observed when PC + 20 mol\% CL liposomes were first treated with toxin and then with enzyme. CT\(V_\text{c}5\) also increased the activity of M2 on PC +

![Figure 2. EPR spectra of 5 DSA in oriented lipid films of PC + 20 mol\% CL. Lipid films were prepared from liposomes that were (a) untreated, (b) treated with CT\(V_\text{c}5\), (c) treated with AEnz, (d) pretreated with AEnz and post treated with CT\(V_\text{c}5\), (e) pretreated with CT\(V_\text{c}5\) and post treated with AEnz, (f) treated with M2, (g) pretreated with M2 and post treated with CT\(V_\text{c}5\), and (h) pretreated with CT\(V_\text{c}5\) and post treated with M2. The molar ratio of spin probe to lipid was 1 to 200. EPR spectra taken with the magnetic field parallel (solid line) and perpendicular (broken line) to the bilayer normal. The molar ratio of enzyme to toxin to lipid was 1 to 1.5 \times 10^4 to 2 \times 10^6.](image)
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20 mol% CL liposomes and this effect was most pronounced when toxin was added first (Fig. 1).

The effects of PLA$_2$ and CT V$_{c5}$ on the EPR spectra of 5-DSA in multibilayers from PC + 20 mol% CL are shown in Fig. 2. Liposomes incubated without enzymes and toxin formed highly ordered multibilayer lipid films. This is reflected by the pronounced anisotropy of the EPR spectra taken at different orientations of the bilayer normal in the magnetic field (Fig. 2a). CT V$_{c5}$ did not visibly change the angular dependence of the EPR spectra (Fig. 2b). The broadening of the hyperfine splitting in the spectrum taken with the field parallel to the bilayer normal reflects the limitation of the molecular mobility of spin probes. This effect was also noted in other lipid films containing CT V$_{c5}$ (Fig. 2d, e, g, h). Liposomes treated with AEnz, or pretreated with AEnz and then post-treated with CT V$_{c5}$, showed a strong spectral anisotropy of 5-DSA (Fig. 2c, d). Treatment of liposomes initially with CT V$_{c5}$ then with AEnz resulted in the appearance of spectral components with resonances that coincide at different orientations of the lipid films in the applied field (Fig. 2e). The same was noted for liposomes treated with M2 (Fig. 2f). A further reduced spectral anisotropy of 5-DSA resulted with M2 and CT V$_{c5}$ regardless of the sequence of reagent addition (Fig. 2g, h). The effects of M1 alone or together with CT V$_{c5}$ on EPR spectra of 5-DSA closely resembled those of AEnz.

EPR spectral analysis was performed in terms of $B/C$ and the order parameter $S$. The $B/C$ ratio is very sensitive to macroscopic disordering (Aracava et al. 1981) and the presence of a nonbilayer phase (Aripov et al. 1986; Gasanov et al. 1990a). The higher value of the $B/C$ ratio reflects the higher ordering of bilayer lipid packing. The order parameter $S$ mainly reflects the rate of spin label rotational movement (Gasanov et al. 1990b). As is seen in Fig. 3, CT V$_{c5}$, AEnz and M1 caused a slight decrease in the $B/C$ ratio. The pronounced decrease in the $B/C$ ratio was observed when membranes were pretreated with CT V$_{c5}$ and post-treated with either AEnz or M1. M2 alone induced a marked drop in the value of the $B/C$ ratio. This effect was even greater when membranes were treated both with M2 and CT V$_{c5}$. Neither AEnz nor M1 appreciably affected parameter $S$, while M2 induced a decrease in the value of parameter $S$ (Fig. 4). CT V$_{c5}$ profoundly restricted mobility of spin probes in membranes in trials both with and without the PLA$_2$s.

Polymorphic behavior of the phospholipid phase of membranes that were treated with the PLA$_2$s and with CT V$_{c5}$ was examined with $^{31}$P-NMR spectroscopy. The $^{31}$P-NMR spectrum of large PC + 20% CL liposomes had a curve typical for lamellar phospholipid systems (Fig. 5a). The applied concentrations of CT V$_{c5}$, AEnz and M1 did not appreciably affect the $^{31}$P-NMR spectra of liposomes (Fig. 5b, c, f). Pretreatment of liposomes with AEnz of M1 followed by treatment with CT V$_{c5}$ also did not change the $^{31}$P-NMR spectra of liposomes.
Figure 3. $B/C$ ratio of the EPR spectra of 5-DSA in oriented lipid films. Lipid films were prepared from (A) untreated liposomes (checked bar) or liposomes treated with CT V, 5 (black bar), (B) liposomes treated with AEnz (checked bar), liposomes pretreated with AEnz and post-treated with CT V, 5 (black bar), or liposomes pretreated with CT V, 5 and post-treated with AEnz (striped bar), (C) liposomes treated with M1 (checked bar), liposomes pretreated with M1 and post-treated with CT V, 5 (black bar), or liposomes pretreated with CT V, 5 and post-treated with M1 (striped bar), (D) liposomes treated with M2 (checked bar), liposomes pretreated with M2 and post-treated with CT V, 5 (black bar) or liposomes pretreated with CT V, 5 and post-treated with M2 (striped bar).

(Fig. 5d, g). When liposomes were initially treated with CT V, 5 and then with AEnz or M1, a narrow resonance from isotropic nonbilayer phospholipid structures was observed at 0 ppm (Fig. 5e, h). This effect was more pronounced for M1. Liposomes modified with M2 also contained nonbilayer structures (Fig. 5i). The action of M2 and CT V, 5 induced a considerable transformation of the lamellar phase to the phospholipid phase with rapid (in the NMR time scale) isotropic molecular mobility (Fig. 5j, k).

To study the effect of CT V, 5 on the capacity of PLA$_2$ to bind to liposomes membranes, the PLA$_2$'s were labeled with 4-(2-iodoacetamido)-TEMPO. The EPR spectrum of spin labeled AEnz in buffer is shown in Fig. 6a. This spectrum with narrow triplet peaks of relatively same intensity implies a slight restricted mobility of spin labels conjugated to enzyme that isotropically rotates in the water phase. The addition of liposomes into the buffer containing spin labeled PLA$_2$ resulted in the appearance of a broad hyperfine splitting (Fig. 6b). This indicates that PLA$_2$'s bind to liposomes which restrict enzyme mobility. The narrow resonance superimposed
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Figure 4. Order parameter $S$ of the EPR spectra of 5-DSA in oriented lipid films. Lipid films were prepared from (A) untreated liposomes (checked bar) or liposomes treated with CT V\textsubscript{c} 5 (black bar), (B) liposomes treated with AEnz (checked bar), liposomes pretreated with AEnz and post-treated with CT V\textsubscript{c} 5 (black bar), or liposomes pretreated with CT V\textsubscript{c} 5 and post-treated with AEnz (striped bar), (C) liposomes treated with M1 (checked bar), liposomes pretreated with M1 and post-treated with CT V\textsubscript{c} 5 (black bar), or liposomes pretreated with CT V\textsubscript{c} 5 and post-treated with M1 (striped bar), (D) liposomes treated with M2 (checked bar), liposomes pretreated with M2 and post-treated with CT V\textsubscript{c} 5 (black bar) or liposomes pretreated with CT V\textsubscript{c} 5 and post-treated with M2 (striped bar).

on the broad resonance is due to enzymes that did not bind to liposomes. With an increase in liposome concentration the intensity of the narrow resonance decreased (Fig. 6c). At a lipid concentration of $8 \times 10^{-2}$ mol/l all enzymes were bound to liposomes (Fig. 6d). The addition of CT V\textsubscript{c} 5 into the sample of liposomes bearing AEnz induced the appearance of a narrow signal superimposed on the broad signal from PLA\textsubscript{2} bound to liposomes (Fig. 6e). Splitting of the outer lines of this narrow signal coincided with that of spin labeled enzymes that were incubated with CT V\textsubscript{c} 5 in buffer without liposomes (Fig. 6f). This implies that the narrow signal in Fig. 6e is from enzymes that were displaced from liposomes by CT V\textsubscript{c} 5. Hyperfine splitting of the outer lines of the spectrum in Fig. 6f was 6.52 G greater than that of the spectrum in Fig. 6a. This indicates that CT V\textsubscript{c} 5 interacts with AEnz in the water phase to form a complex with a more restricted and axis-symmetrical mobility. The EPR spectrum of AEnz incubated with CT V\textsubscript{c} 5 (Fig. 6f) did not change after addition of liposomes at lipid concentration of $8 \times 10^{-2}$ mol/l and
Enzyme to Toxin to Lipid molar ratio
1: 1.5 x 10^4: 2 x 10^6

Figure 5. $^3$P-NMR spectra of large unilamellar liposomes from PC + 20 mol % CL. Liposomes were (a) untreated, (b) treated with CT V, 5, (c) or with AEnz, (d) pretreated with AEnz and post-treated with CT V, 5, (e) pretreated with CT V, 5 and post-treated with AEnz, (f) treated with M1, (g) pretreated with M1 and post-treated with CT V, 5, (h) pretreated with CT V, 5 and post-treated with M1, (i) treated with M2, (j) pretreated with M2 and post-treated with CT V, 5, and (k) pretreated with CT V, 5 and post-treated with M2.

Figure 6. EPR spectra of spin labeled AEnz ($10^{-5}$ mol/l) in (a) Tris-HCl buffer, and in PC + 20 mol% CL liposomes at lipid concentrations of (b) 2 x $10^{-2}$ mol/l, (c) 4 x $10^{-2}$ mol/l, and (d) 8 x $10^{-2}$ mol/l. In (e) the liposomes (8 x $10^{-2}$ mol/l lipid) were pretreated with spin labeled AEnz and post-treated with 5 x $10^{-4}$ mol/l of CT V, 5, in (f) spin labeled AEnz in Tris-HCl buffer incubated with CT V, 5, and in (g) liposomes (8 x $10^{-2}$ mol/l lipid) were pre-treated with CT V, 5 and post-treated with spin labeled AEnz.
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higher suggesting that the AEnz-CT V\textsubscript{c5} complex does not bind to liposomes. The broad “restricted” EPR signal was recorded after spin labeled enzymes were added to liposomes pretreated with CT V\textsubscript{c5} (Fig. 6g). This indicates that pretreatment of membranes with toxin did not interfere with binding of AEnz to liposomes. The same experiments performed with spin labeled M1 and M2 showed that CT V\textsubscript{c5} influenced the binding behavior of M1, but not M2, in the same manner as with AEnz. Addition of CT V\textsubscript{c5} to PC + 20 mol\% CL liposome pretreated with M2 (10\textsuperscript{-5} mol/l enzyme, 5 × 10\textsuperscript{-4} mol/l toxin and 8 × 10\textsuperscript{-2} mol/l lipid) resulted in the appearance of a “restricted” EPR signal of membrane-bound enzyme and no narrow signal. Addition of M2 into the liposomes pretreated with CT V\textsubscript{c5} resulted in the same broad EPR spectrum of spin labeled enzyme bound to liposomes. CT V\textsubscript{c5} did not affect the EPR spectra of M2 dissolved in the water phase.

Discussion

A considerable amount of work has been done on the synergistic effects of cationic peptides, such as cobra venom cardiotoxin and bee venom melittin, and the action of PLA\textsubscript{2} on synthetic lipid bilayers and cellular membranes (Shier 1979; Harvey 1985; Jiang et al. 1989; Fletcher et al. 1991a; Vernon and Bell 1992). There is much interest in the regulation of the activity of PLA\textsubscript{2}, particularly since it appears that the body is able to stimulate the activity of this enzyme via small cationic peptides with structural similarity to exogenous peptides such as melittin (Clark et al. 1987; Bomalaski et al. 1989). Other endogenous peptides as yet undiscovered may also regulate PLA\textsubscript{2} activity. PLA\textsubscript{2} might be inhibited by specific membrane-binding peptides called lipocortins and calpactins (Davidson et al. 1987; Miele et al. 1988). There is controversy, however, in how inhibition is mediated (Davidson et al. 1987; Haigler et al. 1987). There is data that suggests that lipocortins have no PLA\textsubscript{2} inhibitory activity (Van Binsbergen et al. 1989).

In our previous studies we have shown that cardiotoxin and cytotoxin V\textsubscript{c5} from cobra venom are capable of either enhancing or inhibiting PLA\textsubscript{2} activity on lipid liposomes and on human and mouse lymphocytes (Gasanov and Rael 1992; Gasanov et al. 1991, 1994). We suggested that increases in PLA\textsubscript{2} catalysis resulted from toxin-induced formation of nonbilayer lipid structures, and inhibition of PLA\textsubscript{2} activity from an increased substrate density due to tight lipid packing (Gasanov et al. 1994). In the present paper it was shown that CT V\textsubscript{c5} inhibits esterase activity of the three enzymes studied on pure PC liposomes. This relates well with the ability of CT V\textsubscript{c5} to increase the packing density of PC as determined previously (Aripov et al. 1987). In liposomes composed of PC + 20 mol\% CL, a pronounced increase in M2 activity occurred in the presence of CT V\textsubscript{c5}. These same liposomes if pretreated with CT V\textsubscript{c5} were also more susceptible to attack by both M1 and AEnz. However, CT V\textsubscript{c5} decreased lipid hydrolysis in liposomes that were ini-
tially treated with M1 or AEnz. In an attempt to explain this phenomenon we
examined the structural organization of lipid membranes treated with CT V, 5 and
PLA2s to relate the substrate organization with the rate of lipid hydrolysis. The
results indicate that the highest enzymatic activity was detected in samples having
nonbilayer lipid structures. The applied concentration of CT V, 5 did not induce
bilayer-nonbilayer transitions albeit it did perturb lipid packing causing decreases
in the B/C ratio. CT V, 5 is capable of inducing phase segregation of acidic phos-
pholipids (Aripov 1987, 1989), and PC is a preferred substrate for snake venom
PLA2 (Salgo et al. 1992). Therefore liberation of pure PC zones on the membrane
surface as a result of toxin-induced CL segregation should enhance enzymatic ac-
activity. Facilitated catalytic production of free fatty acids further destabilize bilayers
and speed up both enzyme activity and membrane degradation.

The suggested mechanism (of modified packing of substrate molecules) for
enhanced PLA2 activity does not explain how CT V, 5 inhibits enzyme activity.
however, when the cytotoxin is added to liposomes pretreated with M1 or AEnz.
One possible explanation is that liposomes pretreated with PLA2 are not suscepti-
ble to CT V, 5 induced disturbances of the bilayer and the consequent enhancement
of enzymatic activity. However, our EPR spin probe results do not support this
explanation. The parameter S value (which is sensitive to CT V, 5) increased re-
gardless of the addition sequence of toxin and enzyme. This suggests that the
liposome surface is accessible to CT V, 5 regardless of whether liposomes are pre-
or post-treated with PLA2. Another explanation is that acidic enzyme on the li-
posome surface and/or in the water phase interacts directly with basic CT V, 5.
Electrostatic interaction between acidic enzyme and basic toxin may affect the con-
formation of the enzyme thereby inhibiting enzyme activity and/or enzyme binding
to substrate. There is no data available at present to support toxin-induced confo-
nentional changes in enzymes. Nevertheless, the EPR of the spin labeled enzymes
strongly demonstrates that basic toxin affects the interaction of acidic enzymes with
liposomes. Acidic AEnz and M1 incubated with CT V, 5 do not bind to liposomes
and CT V, 5 causes displacement of acidic PLA2 from the surface of liposomes. A
plausible explanation for the inhibitory action of CT V, 5 is that acidic PLA2 and
toxin interact directly preventing the enzyme from binding to liposomes, and addi-
tionally may cause enzyme displacement from the substrate. Membrane-bound CT
V, 5, however, does not interact with acidic PLA2 or at least does not prevent M1
and AEnz from interacting with liposomes containing CL. It is likely that acidic CL
molecules surrounding basic toxin interfere with electrostatic attraction of acidic
PLA2 to CT V, 5 (Aripov et al. 1989; Gasanov et al. 1990b).

Interaction between CT V, 5 and neutral M2 in the water phase has not been
demonstrated. Basic toxin is also incapable of displacing M2 from liposomes and
does not interfere with the binding of M2 to liposomes regardless of the sequence
of reagent addition. It is obvious that inhibition (in PC liposomes) and stimulation
(in PC + CL liposomes) of M2 activity is mediated by the mode in which toxin induces alterations in the packing of substrate (increased density of lipid packing or formation of nonbilayer lipid particles), and not by direct toxin-enzyme interaction.

Figure 7. Models for the interactions of PLA₂ with phospholipid membranes and cytotoxin. The shape, size and penetration depth of cytotoxin (CT) into the bilayer of PC membranes (model A) are illustrated according to Oimatov et al. (1986). Model B depicts CT-induced displacement of PLA₂ from the membrane surface. Localization of a CT molecule on the surface of a membrane containing cardiolipin (model C) and in the core of nonbilayer structure (model D) is illustrated according to (Gasanov et al. 1990a,b, 1993). Cardiolipin is illustrated as a phospholipid with four hydrocarbon chains.

In Fig. 7 we illustrate the events in the relationships between cationic peptide and PLA₂ that can be drawn from the observations described above. Model A illustrates inhibition of PLA₂ activity from toxin-induced tight lipid packing. This illustration applies to neutral PLA₂ acting on PC liposomes. For acidic PLA₂ on PC liposomes, the most likely explanation is that there is direct interaction (on the membrane surface and/or in the water phase) between enzyme and toxin followed by inhibition of PLA₂ activity.

Model B depicts the displacement of acidic PLA₂ from the membrane surface by CT V₅. The displacement of acidic PLA₂ probably occurs both with PC and PC+CL liposomes that were pre-treated with the acidic enzyme. Model C describes toxin-induced phase segregation of acidic phospholipids (Gasanov et al. 1990a,b) which results in stimulation of PLA₂ activity on pure PC zones. Another
mechanism of PLA$_2$ activity enhancement is shown in model $D$. Here the formation of a nonbilayer structure as the result of toxin-induced intermembrane contact (Aripov et al. 1989; Gasanov et al. 1990a,b, 1993) brings transient polymorphic irregularities in lipid packing which makes the phospholipid substrate more susceptible to attack by PLA$_2$. Other polymorphic nonbilayer transitions induced by cationic peptides have been described (Gasanov et al. 1988, 1990b, Gasanov and Gasanov 1994). These transient membrane states may also result in an interface configuration more conducive to higher PLA$_2$ activity (Bell and Biltonen 1992).

The events described in models $C$ and $D$ are accurate for membranes containing acidic phospholipids, such as CL and phosphatidic acid, and most likely work for both acidic and neutral PLA$_2$s.

Our hypothetical models describing relationships between phospholipid substrate packing and cationic peptide-PLA$_2$ interactions are not comprehensive and there is still little data with which to elucidate a detailed mechanism (or mechanisms) of PLA$_2$ regulation. However, our concept proposing variations in cationic peptide behavior on enzyme activity explains the existing controversy concerning regulatory action of lipocortins and calpactins on PLA$_2$ activity. Transient changes in organization and composition of living cell membranes provide a variety of substrate interfaces that likely underlie what may be a flexible physiological regulation of PLA$_2$ activity.

Thus, the results of this study allow us to conclude that there is no single universal mechanism for regulation of PLA$_2$ activity by membrane-active peptides. Variability of substrate composition, and the chemical nature of both PLA$_2$ and membrane-active peptides appear to determine whether enzyme activity is affected by substrate packing or by direct enzyme-peptide interaction. Lipid composition specifies the mode of substrate modification by peptide and the consequent effects (stimulation or inhibition) on PLA$_2$ activity. The overall protein charge could be one of the parameters that determines whether enzyme-peptide interaction occurs, such as in our study where PLA$_2$ activity was inhibited.

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