Phospholipase A_2 and Cobra Venom Cytotoxin V_c5 Interactions and Membrane Structure

S. E. GASANOV[1], N. E. GASANOV[2] and E. D. RAEL[1]

1 *Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968, USA;*
2 *Institute 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 A_2 (PLA_2) with large unilamellar liposomes treated with cobra venom cytotoxin V_c5 (CT V_c5) were studied to more fully understand the modulating effects of cationic membrane-active peptides on PLA_2. Studies were done by fluorescence displacement, EPR spin probes, and ^{31}P-NMR. The results showed that CT V_c5 inhibits PLA_2 activity on phosphatidylcholine liposomes. Enzymatic activity of both acidic and neutral PLA_2's 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 PLA_2. The highest enzymatic activity was found on substrates with nonbilayer lipid packing. Using EPR of spin labeled enzymes, it was shown that CT V_c5 inhibited binding of acidic PLA_2 to liposomes and caused displacement of acidic PLA_2 from liposomes. No direct interaction was found between CT V_c5 and neutral PLA_2. 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 V_c5 and acidic PLA_2 inhibits enzyme activity. It is concluded that variability in substrate composition and the chemical nature of both PLA_2 and cationic peptide determine whether enzyme activity is affected by substrate packing or by direct enzyme-peptide interaction. Models of interactions of PLA_2 with CT V_c5 and phospholipid membranes are presented.

Key words: Snake venom PLA_2 — Cytotoxin V_c5 — Phospholipid membranes — Fluorescence displacement — EPR — ^{31}P-NMR

Correspondence to: Professor Eppie D. Rael, Department of Biological Sciences, University of Texas at El Paso, El Paso, TX 79968-0519, USA

Introduction

Phospholipase A_2, 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 PLA_2 activity is a current pharmacological goal, and there is much interest in controlling the consequences of PLA_2 action in chronic inflammatory conditions, such as rheumatoid arthritis and asthma.

Regulation of PLA_2 activity *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 PLA_2 activity *in vitro* and, in parallel, show anti-inflammatory potential *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 PLA_2 and additionally did not inhibit enzyme activity *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 PLA_2 activity through direct interaction with enzyme. The peptide bears structural similarity to melittin, the bee venom PLA_2-stimulating peptide, and is recognized by anti-melittin antibodies (Clark et al. 1987). In more recent studies, another distinct PLA_2-stimulating peptide has been identified in murine embryonic genital tracts (Gupta and Braun 1990).

Snake venom PLA_2's show a high degree of structural homology with mammalian extracellular PLA_2 and these PLA_2's have similar physiological activities (Davidson and Dennis 1990; Kelley et al. 1992). Membrane-active cationic peptides, such as snake venom cardiotoxin, bee venom melittin and *Pyrularia* thionin, influence the physiological and esterase activities of both snake venom PLA_2 and endogenous PLA_2 in intact cells (Shier 1979; Harvey 1985; Fletcher and Lizzo 1987; Vernon and Bell 1992). In the course of our studies on snake venom PLA_2, we have found that cardiotoxin from *Naja naja kaothia*, cytotoxin from *Naja naja oxiana* and thionin from *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_c5 in aqueous solutions in the presence and absence of phospholipid liposomes. The effect of cytotoxin V_c5 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-phosphatidylcholine (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_2s, 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_c5 (CT V_c5) 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_c5 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_2s (10^{-9} 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 1.5×10^{-5} mol/l of CT V_c5. Liposomes were initially treated with either M1, M2 or AEnz with vigorous mixing for 5 min, followed by addition of CT V_c5 and an additional 25 min incubation. In a second experiment, liposomes were initially treated with CT V_c5 (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. PLA_2 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 $\pm 5\%$ of means.

EPR spin probes and ^{31}P-NMR studies

The structure of membranes modified by PLA_2 and CT V_c5 was studied by EPR oriented multibilayers and ^{31}P-NMR (Aripov et al. 1986; Gasanov et al. 1993). Large unilamellar liposomes were treated with PLA_2 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 PLA_2, and 1.5×10^{-4} mol/l CT V_c5 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

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

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 $\pm 3\%$ of means. The aqueous solution of large unilamellar liposomes prepared for ^{31}P-NMR studies contained 30% by volume of D_2O. The concentrations of lipid, PLA_2 and CT V_c5 in these samples were 0.2 mol/l, 10^{-7} mol/l, and 1.5 mmol/l respectively. ^{31}P-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 5×10^{-4} mol/l of CT V_c5 for 5 min. In other assays liposomes were pretreated with 5×10^{-4} mol/l of CT V_c5 for 5 min and then post-treated with 10^{-5} mol/l of spin labeled enzymes for 30 min. CT V_c5 (5×10^{-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.

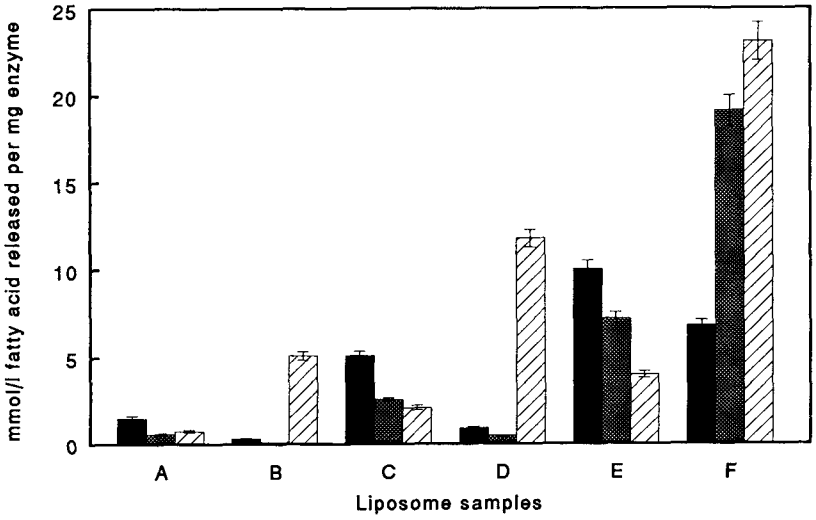

Figure 1. PLA_2 activity of AEnz (*A* and *B*), M1 (*C* and *D*), and M2 (*E* and *F*) on untreated liposomes (black bars), CT V_c5 post-treated liposomes (checked bars), or CT V_c5 pretreated liposomes (striped bars). PC liposomes are represented by *A*, *C*, and *E*, and PC + 20 mol% CL liposomes are represented by *B*, *D*, and *F*. The molar ratio of enzyme to toxin to lipid was 1 to 1.5×10^4 to 2×10^6.

Results

The hydrolytic activity of the PLA_2's on liposomes in the presence and absence of CT V_c5 is presented in Fig 1 In the absence of CT V_c5, 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_c5 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_c5 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_c5 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 5 also effected an increase in the activity of M2 on PC +

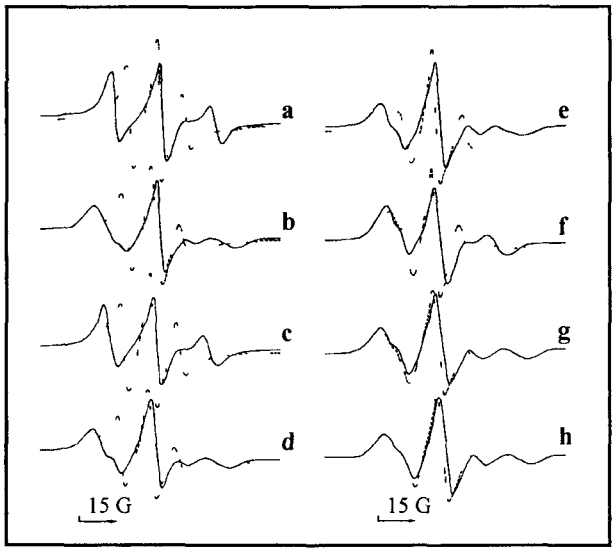

Figure 2. EPR spectra of 5 DSA in oriented lipid films of PC + 20 mol% CL Lipid films were prepared from liposomes that were (*a*) untreated (*b*) treated with CT V_c5 (*c*) treated with AEnz, (*d*) pretreated with AEnz and post treated with CT V_c5 (*e*) pretreated with CT V 5 and post treated with AEnz (*f*) treated with M2 (*g*) pretreated with M2 and post treated with CT V_c5, and (*h*) pretreated with CT V_c5 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 × 10^4 to 2 × 10^6

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. 2*a*). CT V_c5 did not visibly change the angular dependence of the EPR spectra (Fig. 2*b*). 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. 2*d*, *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. 2*c*, *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. 2*e*). 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. 2*g*, *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. 5*a*). The applied concentrations of CT V_c5, AEnz and M1 did not appreciably affect the ^{31}P-NMR spectra of liposomes (Fig. 5*b*, *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

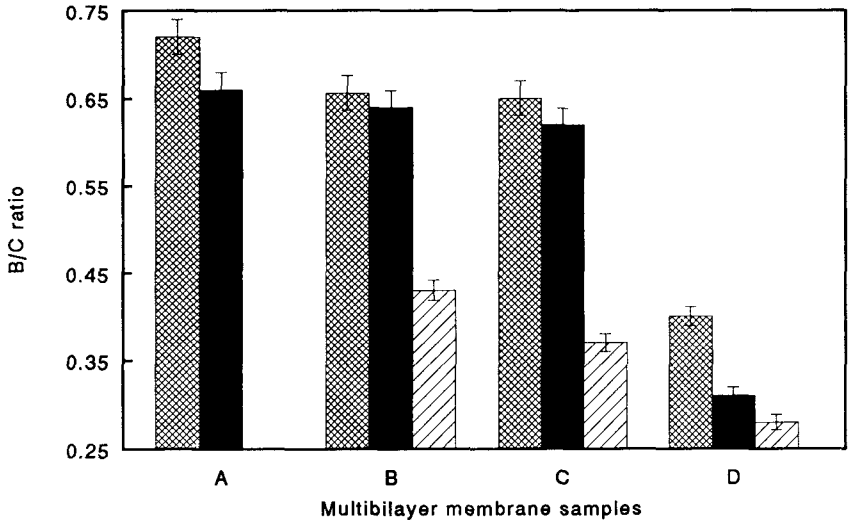

Figure 3. B/C ratio of the EPR spectra of 5-DSA in oriented lipid films. Lipid films were prepared from (A) untreated liposomes (checked bar) or liposomes treated with CT V_c5 (black bar), (B) liposomes treated with AEnz (checked bar), liposomes pretreated with AEnz and post-treated with CT V_c5 (black bar), or liposomes pretreated with CT V_c5 and post-treated with AEnz (striped bar), (C) liposomes treated with M1 (checked bar), liposomes pretreated with M1 and post-treated with CT V_c5 (black bar), or liposomes preteated with CT V_c5 and post-treated with M1 (striped bar), (D) liposomes treated with M2 (checked bar), liposomes pretreated with M2 and post-treated with CT V_c5 (black bar) or liposomes pretreated with CT V_c5 and post-treated with M2 (striped bar).

(Fig. 5d, g). When liposomes were initially treated with CT V_c5 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_c5 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_c5 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 isotropicly 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

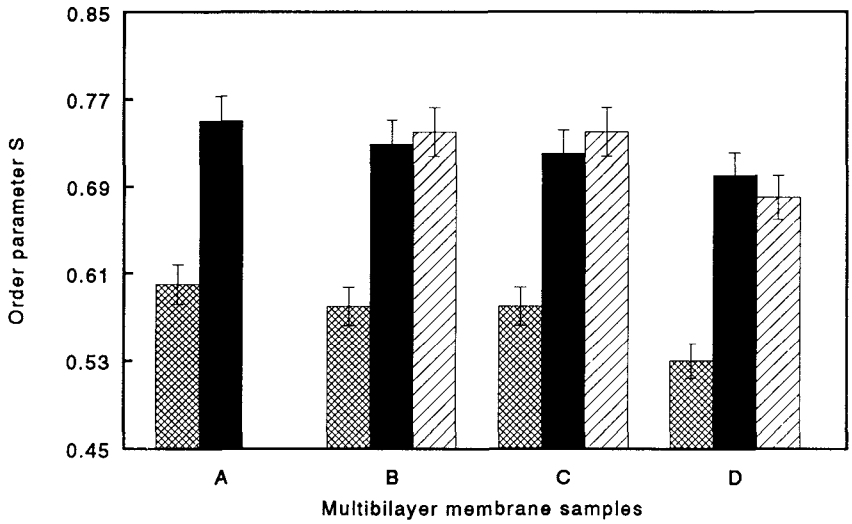

Figure 4. Order parameter S of the EPR spectra of 5-DSA in oriented lipid films. Lipid films were prepared from (*A*) untreated liposomes (checked bar) or liposomes treated with CT V_c5 (black bar), (*B*) liposomes treated with AEnz (checked bar), liposomes pretreated with AEnz and post-treated with CT V_c5 (black bar), or liposomes pretreated with CT V_c5 and post-treated with AEnz (striped bar), (*C*) liposomes treated with M1 (checked bar), liposomes pretreated with M1 and post-treated with CT V_c5 (black bar), or liposomes pretreated with CT V_c5 and post-treated with M1 (striped bar), (*D*) liposomes treated with M2 (checked bar), liposomes pretreated with M2 and post-treated with CT V_c5 (black bar) or liposomes pretreated with CT V_c5 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. 6*c*). At a lipid concentration of 8×10^{-2} mol/l all enzymes were bound to liposomes (Fig. 6*d*). The addition of CT V_c5 into the sample of liposomes bearing AEnz induced the appearance of a narrow signal superimposed on the broad signal from PLA_2 bound to liposomes (Fig. 6*e*). Splitting of the outer lines of this narrow signal coincided with that of spin labeled enzymes that were incubated with CT V_c5 in buffer without liposomes (Fig. 6*f*). This implies that the narrow signal in Fig. 6*e* is from enzymes that were displaced from liposomes by CT V_c5. Hyperfine splitting of the outer lines of the spectrum in Fig. 6*f* was 6.52 G greater than that of the spectrum in Fig. 6*a*. This indicates that CT V_c5 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_c5 (Fig. 6*f*) did not change after addition of liposomes at lipid concentration of 8×10^{-2} mol/l and

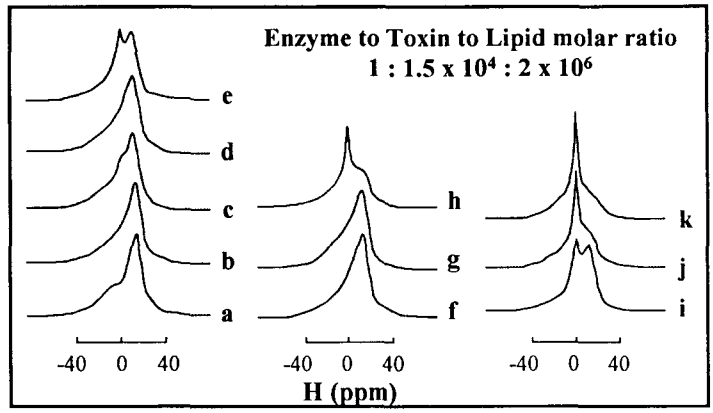

Figure 5. ^{31}P-NMR spectra of large unilamellar liposomes from PC + 20 mol % CL. Liposomes were (*a*) untreated, (*b*) treated with CT V_c5, (*c*) or with AEnz, (*d*) pretreated with AEnz and post-treated with CT V_c5, (*e*) pretreated with CT V_c5 and post-treated with AEnz, (*f*) treated with M1, (*g*) pretreated with M1 and post-treated with CT V_c5, (*h*) pretreated with CT V_c5 and post-treated with M1, (*i*) treated with M2, (*j*) pretreated with M2 and post-treated with CT V_c5, and (*k*) pretreated with CT V_c5 and post-treated with M2.

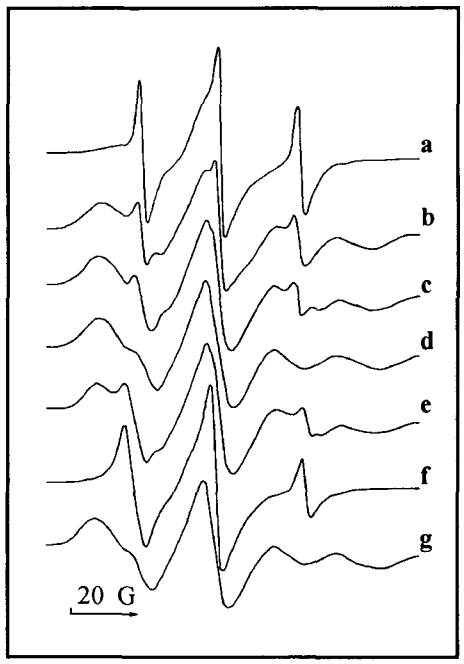

Figure 6. EPR spectra of spin labeled AEnz (10^{-5} mol/l) in (*a*) Tris-HCl buffer, and in PC + 20 mol% CL liposomes at lipid concentrations of (*b*) 2×10^{-2} mol/l, (*c*) 4×10^{-2} mol/l, and (*d*) 8×10^{-2} mol/l. In (*e*) the liposomes (8×10^{-2} mol/l lipid) were pretreated with spin labeled AEnz and post-treated with 5×10^{-4} mol/l of CT V_c5, in (*f*) spin labeled AEnz in Tris-HCl buffer incubated with CT V_c5, and in (*g*) liposomes (8×10^{-2} mol/l lipid) were pretrated with CT V_c5 and post-treated with spin labeled AEnz.

higher suggesting that the AEnz-CT V_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_c5 (Fig. 6*g*). 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_c5 influenced the binding behavior of M1, but not M2, in the same manner as with AEnz. Addition of CT V_c5 to PC + 20 mol% CL liposome pretreated with M2 (10^{-5} mol/l enzyme, 5×10^{-4} mol/l toxin and 8×10^{-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_c5 resulted in the same broad EPR spectrum of spin labeled enzyme bound to liposomes. CT V_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_2 on synthetic lipid bilayers and cellular membranes (Shier 1979; Harvey 1985; Jiang et al. 1989; Fletcher et al. 1991b; Vernon and Bell 1992). There is much interest in the regulation of the activity of PLA_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_2 activity. PLA_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_2 inhibitory activity (Van Binsbergen et al. 1989).

In our previous studies we have shown that cardiotoxin and cytotoxin V_c5 from cobra venom are capable of either enhancing or inhibiting PLA_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_2 catalysis resulted from toxin-induced formation of nonbilayer lipid structures, and inhibition of PLA_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_c5 inhibits esterase activity of the three enzymes studied on pure PC liposomes. This relates well with the ability of CT V_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_c5. These same liposomes if pretreated with CT V_c5 were also more susceptible to attack by both M1 and AEnz. However, CT V_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_c5 and $PLA_2's$ 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_c5 did not induce bilayer-nonbilayer transitions albeit it did perturb lipid packing causing decreases in the B/C ratio. CT V_c5 is capable of inducing phase segregation of acidic phospholipids (Aripov 1987, 1989), and PC is a preferred substrate for snake venom PLA_2 (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 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 PLA_2 activity does not explain how CT V_c5 inhibits enzyme activity, however, when the cytotoxin is added to liposomes pretreated with M1 or AEnz. One possible explanation is that liposomes pretreated with PLA_2 are not susceptible to CT V_c5 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_c5) increased regardless of the addition sequence of toxin and enzyme. This suggests that the liposome surface is accessible to CT V_c5 regardless of whether liposomes are pre- or post-treated with PLA_2. Another explanation is that acidic enzyme on the liposome surface and/or in the water phase interacts directly with basic CT V_c5. Electrostatic interaction between acidic enzyme and basic toxin may affect the conformation of the enzyme thereby inhibiting enzyme activity and/or enzyme binding to substrate. There is no data available at present to support toxin-induced conformational 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_c5 do not bind to liposomes and CT V_c5 causes displacement of acidic PLA_2 from the surface of liposomes. A plausible explanation for the inhibitory action of CT V_c5 is that acidic PLA_2 and toxin interact directly preventing the enzyme from binding to liposomes, and additionally may cause enzyme displacement from the substrate. Membrane-bound CT V_c5, however, does not interact with acidic PLA_2 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 PLA_2 to CT V_c5 (Aripov et al. 1989; Gasanov et al. 1990b).

Interaction between CT V_c5 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.

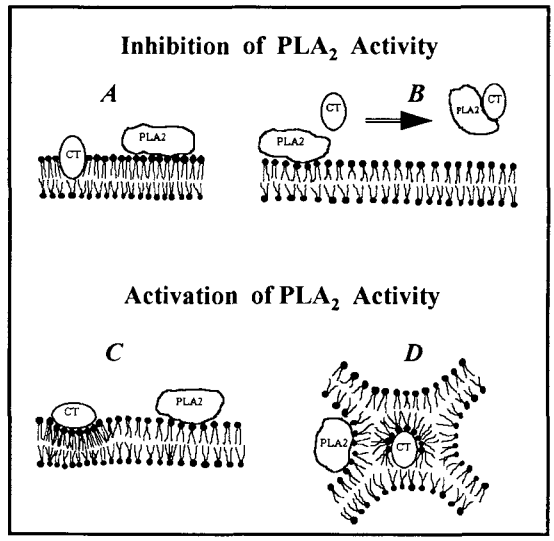

Figure 7. Models for the interactions of PLA_2 with phospholipid membranes and cytotoxin. The shape, size and penetration depth of cytotoxin (CT) into the bilayer of PC membranes (model *A*) are illustrated according to Oimatov et al. (1986). Model *B* depicts CT-induced displacement of PLA_2 from the 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 PLA_2 that can be drawn from the observations described above. Model *A* illustrates inhibition of PLA_2 activity from toxin-induced tight lipid packing. This illustration applies to neutral PLA_2 acting on PC liposomes. For acidic PLA_2 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 PLA_2 activity.

Model *B* depicts the displacement of acidic PLA_2 from the membrane surface by CT V_c5. The displacement of acidic PLA_2 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 PLA_2 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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