Modulation of Phospholipase A_2 Activity by Membrane-active Peptides on Liposomes of Different Phospholipid Composition

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Abstract. To determine the influence of variations in both lipid species and lipid packing on phospholipase A_2 (PLA_2) hydrolytic activity, the activities of two PLA_2 isolated from *Crotalus molossus molossus* venom, were followed on unilamellar liposomes modified by membrane-active peptides. Enzymatic activity was compared with cytolytic activity on human and mouse lymphocytes. Phosphatidylcholine liposomes were hydrolysed better than liposomes containing acidic phospholipids (phosphatidylserine, phosphatidic acid or cardiolipin) or phosphatidylethanolamine. Both membrane-active peptides, cardiotoxin and thionin, inhibited the PLA_2 activity on phosphatidylcholine liposomes. The activities of the enzymes were profoundly enhanced on thionin-pretreated liposomes containing phosphatidylserine, and on cardiotoxin-pretreated liposomes containing cardiolipin or phosphatidic acid. Both cardiotoxin and thionin facilitated the cytolytic activities of PLA_2 on both human and mouse lymphocytes. Cytolytic activity correlated well with esterase activity. It is proposed that the complex dynamic structure of cell membranes renders a variety of substrate configurations that transiently affect PLA_2 activity.

Key words: Snake venom PLA_2 – Liposomes – Lymphocytes – Cardiotoxin – *Pyrularia* thionin

Introduction

The phospholipid-hydrolysing enzyme, PLA_2, has been studied extensively during the past few decades because of its physiological and pathological importance

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(Rosenberg 1990). PLA_2 plays an important role in maintaining the composition and well being of cell membranes (Sevanian et al. 1988). In addition, the products of the reaction catalyzed by PLA_2 are themselves precursors for hormones, prostaglandins, leukotrienes, platelet-activating factor, as well as possible intracellular second messengers (Dreher and Hanley 1988; Oishi et al. 1988). Pathologically, excess PLA_2 activity has been implicated in a number of disease states including trauma, viral infection, shock, inflammatory disease and pancreatitis (Scheuer 1989).

Snake venom PLA_2 has been widely used to study the mechanism of enzyme activation. It has been found that PLA_2 may be modulated by the organization of the phospholipid substrate that may either facilitate enzyme binding or result in an interface configuration more conducive to higher enzyme activity (Bell and Biltonen 1992; Vernon and Bell 1992). Membrane-active peptides have been found to influence PLA_2 activity by modifying the bilayer structure (Gasanov et al. 1991, Rao 1992). Liposomes pre-treated with cobra cytotoxin, a membrane-active peptide, were far more susceptible to PLA_2 attack than liposomes initially treated with PLA_2 followed by treatment with cytotoxin (Gasanov et al. 1991; 1994).

The present paper is an extension of our study (Gasanov et al. 1991; 1994) on the relationships between membrane-active peptides and PLA_2 activity. The purpose of this work was to investigate the influence of phospholipid liposomes of different composition on PLA_2 activity as ifluenced by the membrane-active peptides, cobra cardiotoxin and *Pyrularia* thionin (Vernon 1992). The hydrolytic activities of PLA_2 on liposomes were compared to their cytolytic activities on human and mouse lymphocytes modified by cardiotoxin or thionin.

Materials and Methods

Reagents

Venom from *Crotalus molossus molossus* (Northern blacktailed rattlesnake) and cardiotoxin (CT) from *Naja naja kaouthia* (Indian cobra) venom were purchased from Sigma Chemical Co. (St. Louis, MO) in lyophilized form. *Pyrularia* thionin (PT) was purified from plant *Pyrularia pubera* according to Vernon et al. (1985). Egg yolk L-α-phosphatidylcholine (PC), bovine brain L-α-phosphatidyl-L-serine (PS), cardiolipin from *E. coli* (CL), bovine brain L-α-phosphatidylethanolamine (PE) and L-α-phosphatidic acid from egg yolk (PA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chromate (^{51}Cr) in sodium chloride solution (sterilized; 250–500 mCi/mg ^{51}Cr) was purchased from Amersham Corporation (Arlinton Heights, IL).

Chromatography

To isolate PLA_2, *C. m. molossus* venom (0.9 g) was reconstituted in starting buffer (0.05 mol/l Tris-HCl, 0.1 mol/l KCl, pH 8.3) and applied to a DEAE-Sephadex A-50 (Pharmacia LKB, Piscaway, NJ) column equilibrated with starting buffer. A linear gradient was established with eluant buffer (0.5 mol/l Tris-HCl, 0.3 mol/l KCl, pH 8.3) and monitored at 280 nm. The first fraction, F1 (Fig. 1A), containing 65% of the PLA_2 activity of

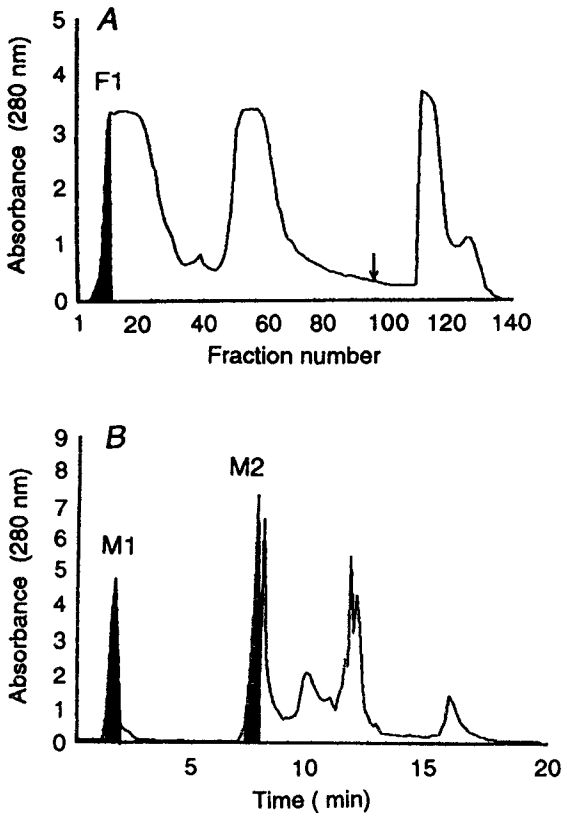

Figure 1. Fractionation of *C. m. molossus* venom and isolation of M1 and M2. The first fractionation (*A*) was by DEAE-Sephadex A-50 column chromatography. The KCl gradient was started where indicated by the arrow. F1 was subfractionated with a SynChrom SynChropak S300 column (*B*) to purify M1 and M2.

the crude venom was dialyzed against water, lyophilized and subfractionated by cation exchange HPLC. A sample (20 mg/ml), dissolved in 0.02 mol/l Bis-Tris, pH 6.5, was injected into a SynChropak S300 column (SynChrom, Inc., Lafayette, IN). A 10 min linear gradient was established with buffer containing 1 mol/l NaCl 5 min after the sample was injected. The column eluate was monitored at 280 nm. The PLA_2 activity was found in the two fractions, M1 and M2 (Fig. 1*B*). Both fractions were homogeneous by isoelectric focusing (Fig. 2) and SDS-PAGE (Fig. 3). The pIs of the enzymes were close to 5.4 for M1 and 7.4 for M2. The molecular weights of both PLA_2 were close to 14.4 kD by SDS-PAGE under reducing conditions.

Phospholipase A_2 assay

PLA_2 activity was determined using the NEFA C Kit (Wako Chemicals USA, Inc., Dallas, TX) according to Kasurinen and Vanha-Perttula (1987). The mode of action of this assay has been described by Yates et al. (1990). Large unilamellar liposomes prepared by the ether evaporation method (Deamer and Bangham 1976) were used as substrates for PLA_2. The aqueous solution of liposomes consisted of 5 mmol/l lipid and 100 mmol/l Tris-HCl (pH 7.8). The phospholipid compositions of the liposomes were 100% PC; 75 mol% PC + 25 mol% PS, PA, PE or CL or 50 mol% PC + 50 mol% PS, PA, PE or CL, respectively. PLA_2 enzymes (2.5×10^{-6} mmol/l) were incubated with liposomes for 20

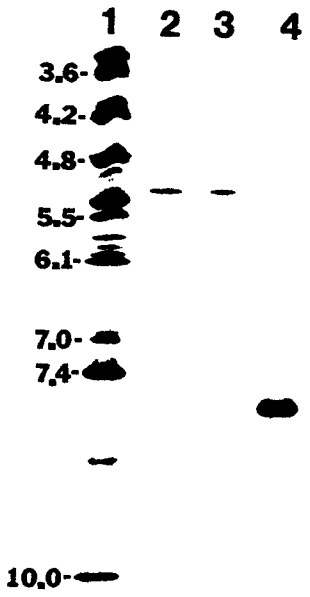

Figure 2. Isoelectric focusing of M1 and M2. Lane 1 contains the pI markers, lanes 2 and 3 have M1, and lane 4 has M2. Isoelectric focusing was done in isogel agarose gels (FMC Corporation, Rockland, ME) with a pH gradient from 3.0 to 10.0. Standard pI markers were from FMC Corporation (Rockland, ME).

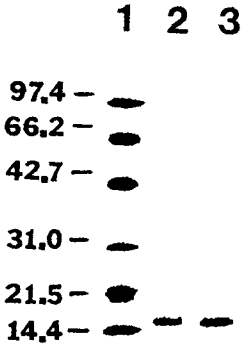

Figure 3. SDS-polyacrylamide gel electrophoresis of M1 and M2. Lane 1 contains the standard low molecular weight markers (BioRad, Richmond, CA), lane 2 has M1, and lane 3 has M2. Molecular weights are given in kilodaltons. The PhastSystem (Pharmacia Fine Chemicals) was used for SDS-PAGE. The samples were dissolved in buffer (20 mmol/l Tris-HCl, 2 mmol/l EDTA, 5% SDS, 10% 2-mercaptoethanol, 0.01% Bromophenol Blue) and boiled for 10 min. A 3 μl portion (1 mg/ml) of each sample was then applied to an 8–25% acrylamide gradient gel.

min. In some assays liposomes were pre- or post-treated with 2.5×10^{-2} mmol/l of CT or PT. Liposomes were then dissolved by addition of Triton X-100 to a final concentration of 10 mmol/l followed by vigorous mixing with a Vortex shaker. Lipid hydrolysis by PLA_2 was terminated by addition of EDTA to a final concentration of 20 mmol/l. All assays were done at 37 °C. Enzymatic activity was expressed as mmoles fatty acid liberated per mg of enzyme during a 20 min incubation. Data are reported as the means of two separate experiments, each performed in triplicate. The reproducibility of data was within ±5%.

^{51}Cr release assay

Lymphocytes from spleens of adult BALB/c mice and human venous blood were separated by density gradient centrifugation on LSM solution (Bionetics, Kensington, MD). Cells suspended in Dulbecco's Minimal Essential Medium (MEM) were labelled with ^{51}Cr

(100 μCi ^{51}Cr per 10^7 cells) by a 1 h incubation at 37 °C. The cells were then washed with MEM (200 × g, 10 min). To reduce nonspecific background radioactivity, cells were incubated in a large volume of MEM (50–100 ml) for 30 min at room temperature. The cells were washed three more times with MEM and adjusted to a concentration of 10^6 cells per ml. The labelled cells, in a total volume of 0.5 ml, were incubated with either membrane-active peptides (0.4×10^{-5} mol/l) or enzymes (1.5×10^{-7} mol/l) for 30 min at 37 °C. In other cases, cells were pre-treated with either PLA_2 or membrane-active peptides for 2 min and then post-treated with membrane-active peptides or PLA_2 for additional 30 min. After incubation, the cells were centrifuged for 2 min in a Micro-centrifuge, then 100 μl of supernatant was removed and mixed with 2 ml of BioSafe II liquid scintillation cocktail (Research Products International Corporation, Mount Prospect, IL). The released radioactivity was measured by liquid scintillation spectroscopy. Cell samples incubated in the absence of PLA_2 and membrane-active peptides were used as controls. The radioactivity of the supernatants from controls was never more than 1000 cpm. All assays were made in two separate experiments, each performed in triplicate. Data were reported as the means of experimental readings. Variation among experimental data points was in every case less than 8%.

Results

The activities of M1 and M2 on pure PC liposomes or PC liposomes containing either acidic (PS, PA or CL) or zwitterionic (PE) phospholipids are presented in

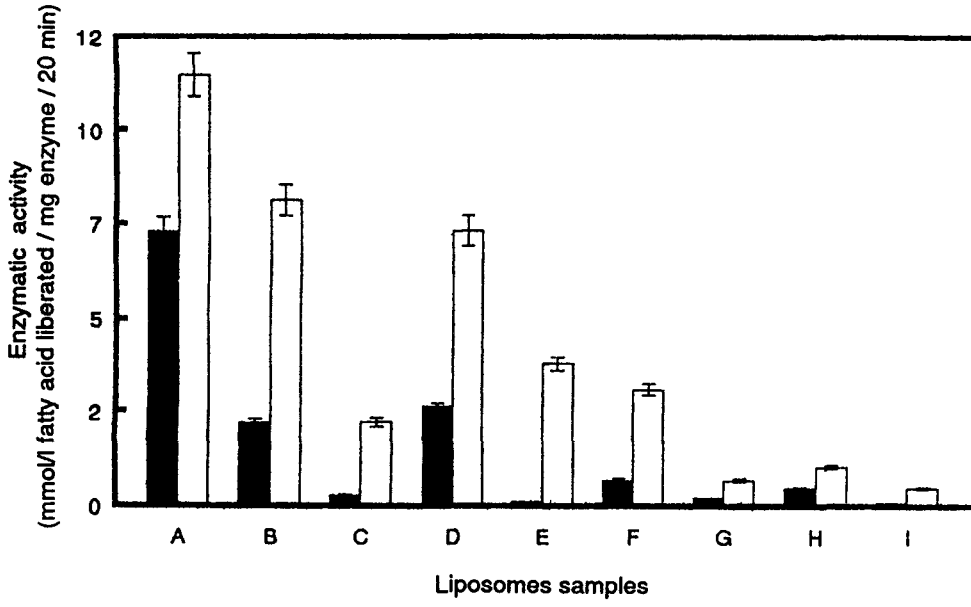

Figure 4. PLA_2 activity of M1 (black bars) and M2 (white bars) on liposomes composed of PC alone (A), PC + 25 mol% PS (B), PC + 50 mol% PS (C), PC + 25 mol% PA (D), PC + 50 mol% PA (E), PC + 25 mol% PE (F), PC + 50 mol% PE (G), PC + 25 mol% CL (H), and PC + 50 mol% CL (I). The molar ratio of lipid : PLA_2 was 2×10^6 : 1.

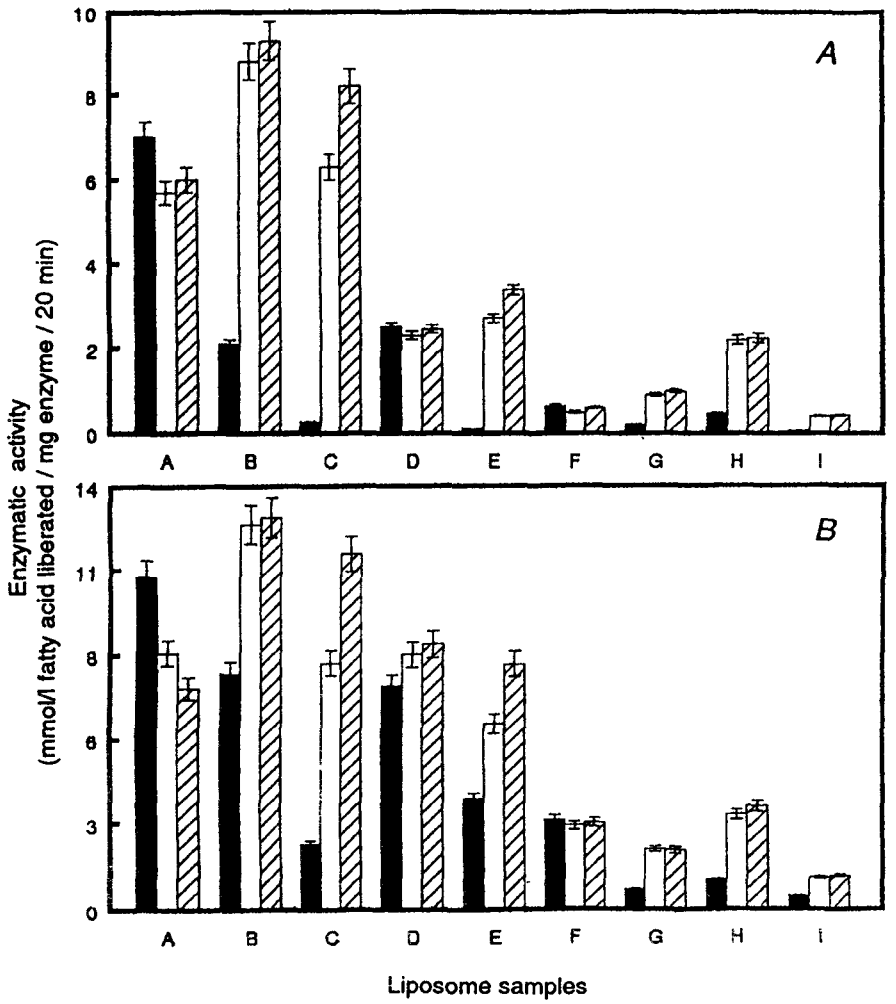

Figure 5. PLA_2 activity of M1 (**A**) and M2 (**B**) on untreated (black bars), post-treated (white bars) or pretreated (striped bars) with PT liposomes. Liposomes were prepared from PC alone (*A*), PC + 25 mol% PS (*B*), PC + 50 mol% PS (*C*), PC + 25 mol% PA (*D*), PC + 50 mol% PA (*E*), PC + 25 mol% PE (*F*), PC + 50 mol% PE (*G*), PC + 25 mol% CL (*H*), and PC + 50 mol% CL (*I*). The molar ratio of lipid : PLA_2 : PT was $2 \times 10^6 : 1 : 10^4$.

Fig. 4. The two enzymes were more active on pure PC liposomes than on those composed of PC and other phospholipids. M2 was markedly better at liberating fatty acids from liposomes than M1 in all cases. With an increase in concentration

of acidic phospholipids in PC liposomes there was a concomitant decrease in fatty acid liberation by both M1 and M2. This effect was markedly pronounced in liposomes containing CL. Liposomes containing PE were also hydrolysed less than pure PC liposomes by both enzymes.

The effect of PT and CT on the hydrolytic activity of the enzymes was examined in two ways. Liposome samples were initially treated with either M1 or M2 with vigorous mixing for 2 min, then one of the two membrane-active peptides was added and the reaction mixtures incubated for additional 18 min, or liposome samples were initially treated with membrane-active peptides (2 min vigorous mixing) and then either M1 or M2 was added to the reaction mixture and incubated for additional 20 min. As shown in Fig. 5, added PT reduced the liberation of fatty acids by both M1 and M2 from pure PC liposomes. The reduced fatty acid liberation did not depend on the addition sequence of M1 and PT, while in the case of M2 the largest inhibition was found when PT was added first. In liposomes containing 25 mol% of PE, PT addition did not change the activity of M1 and M2. The same result was observed in liposomes composed of PC + 25 mol% PA and tested with M1. In all other samples, PT affected an increase in the activity of both enzymes and this effect was most pronounced in liposomes containing PS. In liposomes containing 25 mol% PS or PA the extent of fatty acid liberation did not depend on the addition sequence of enzymes and PT, while in liposomes containing 50 mol% PS or PA the greater enzyme activity occurred when liposomes were first treated with PT. In liposomes containing both 25 mol% and 50 mol% PE or CL the sequence of enzymes and PT addition did not influence the extent of phospholipid hydrolysis.

CT reduced the activities of M1 and M2 in liposomes composed of pure PC (Fig. 6). Unlike the result with PT, the most reduced phopholipid hydrolysis for both M1 and M2 occurred when PC liposomes were first treated with CT. Reduced activity by the two enzymes also occurred with liposomes composed of PC + 25 mol% PE that were initially treated with CT. This same effect also occurred for M2 when liposomes composed of PC + 25 mol% PS and PC + 25 mol% PA were first treated with CT. In all other liposome samples fatty acid liberation was increased with CT treatment regardless of the sequence of reagent addition. In liposomes containing 25 mol% of the negatively charged phospholipids, fatty acid liberation was larger in the samples that were first treated with either M1 or M2 before addition of CT. The converse was observed for liposomes composed of 50 mol% of the negatively charged phospholipids. Virtually no difference was observed in liposomes containing 50 mol% PE, whether they were initially treated by either enzyme, or with CT.

The cytolytic activities of the two PLA_2, in the presence or absence of membrane-active peptides, were examined by the ^{51}Cr release assay. The amount of radioactivity released into cell-free supernatant is directly proportional to the per-

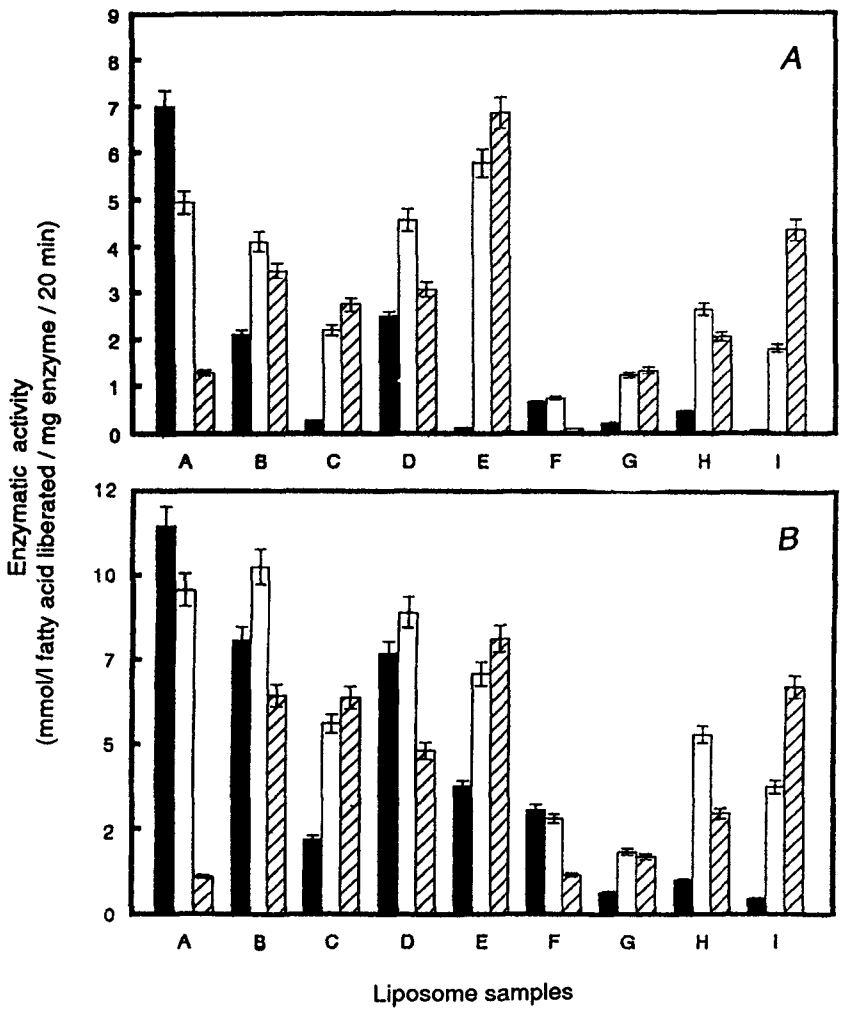

Figure 6. PLA_2 activity of M1 (**A**) and M2 (**B**) on untreated (black bars), post-treated (white bars) or pretreated (striped bars) with CT liposomes. Liposomes were prepared from PC alone (*A*), PC + 25 mol% PS (*B*), PC + 50 mol% PS (*C*), PC + 25 mol% PA (*D*), PC + 50 mol% PA (*E*), PC + 25 mol% PE (*F*), PC + 50 mol% PE (*G*), PC + 25 mol% CL (*H*), and PC + 50 mol% CL (*I*). The molar ratio of lipid : PLA_2 : CT was $2 \times 10^6 : 1 : 10^4$.

centage of target cells killed (Grabstein and Chen 1980). Since both PLA_2 and membrane-active peptides are capable of inducing cell membrane disintegration, we used the lowest reagent concentrations capable of inducing minimal cell lysis in

Table 1. ^{51}Cr release from mouse spleen and human blood lymphocytes treated with PLA_2, M1 and M2, and membrane-active peptides CT and PT*.

Treatment mode (Sequence of reagent addition)	Human lymphocytes (cpm ± SD)	Mouse lymphocytes (cpm ± SD)
Control	925 ± 34	950 ± 29
M1	1074 ± 41	1026 ± 39
M2	1181 ± 43	1035 ± 37
PT	1707 ± 64	1402 ± 51
CT	1584 ± 62	1379 ± 52
M1 + PT	3360 ± 124	2598 ± 87
PT + M1	3465 ± 121	3170 ± 109
M2 + PT	4174 ± 151	3282 ± 112
PT + M2	4217 ± 161	3392 ± 117
M1 + CT	2872 ± 108	2690 ± 92
CT + M1	4082 ± 157	3894 ± 138
M2 + CT	3281 ± 128	3092 ± 120
CT + M2	4390 ± 149	4152 ± 138

*0.5×10^6 cells were treated with 1.5×10^{-7} mol/l PLA_2 and 0.4×10^{-5} mol/l membrane-active peptides as described in Materials and Methods.

order to examine the mutual effects of the enzymes and membrane-active peptides in lymphocyte lysis (Table 1). At given concentrations, the effect of the enzymes on the lymphocytes was lower than that of either PT or CT alone. The release of ^{51}Cr was highest, and more than additive when both PT and enzymes were added to either human or mouse lymphocytes. ^{51}Cr release was not significantly influenced by the sequence of addition of reagents to human lymphocytes. With mouse lymphocytes, the highest ^{51}Cr release occurred when samples were first treated with PT before addition of PLA_2 rather than the converse. With mouse lymphocytes, the synergistic effect was not observed between M1 and PT when the lymphocytes were first treated with M1. For CT, both PLA_2 synergistically enhanced ^{51}Cr release from both human and mouse lymphocytes. Lymphocytes first treated with CT released higher ^{51}Cr amounts after enzyme treatment than when lymphocytes were first treated with enzyme and then with CT.

Discussion

In our earlier study on the relationship between cobra (*Naja naja oxiana*) venom PLA_2 activity and membrane (PC + 20 mol% CL) structure modulated by cobra venom cytotoxin, we found an increase in enzymatic activity (more than 5 times)

on nonbilayer organized membranes that were pretreated with cytotoxin (Gasanov et al. 1991). We hypothesized that the modified structure of the phospholipid bilayer was the result of increased catalysis. In the present study we utilized a variety of membrane phospholipid compositions and obtained a more comprehensive, but complex picture. However, the results obtained present strong evidence to support the hypothesis that PLA_2 is modulated by alterations in membrane structure. It was found earlier that CT is attracted to CL and PA, and the end point of that attraction is the formation of nonbilayer lipid particles (Batenberg et al. 1985; Aripov et al. 1989; Gasanov et al. 1990a, 1990b). CT is also capable of inducing polymorphic transitions in PE membranes (Devaux and Seigneuret 1985). PT in turn induces the formation of nonbilayer structures in PS containing membranes (Gasanov et al. 1993). Our present data are in strong agreement with previous work: CT profoundly facilitated PLA_2 activity in CL, PA or PE containing liposomes, while PT did the same in PS containing liposomes. In pure PC liposomes both CT and PT inhibited enzymatic activity (CT also inhibited M2 activity on liposomes with small amount of PS, PA and PE), which appeared to be the result of more dense packing of substrate molecules. The ability of CT (Aripov et al. 1984, 1987) and PT (our unpublished observation) to increase the lipid packing density of PC bilayers has been noted earlier. There is no information available regarding the relationship between PT and PE membranes. From the present study it appears that PT can slightly perturb the PE containing membranes and stimulate PLA_2 activity in liposomes composed of 50 mol% of PE. Thus, it is obvious that membranes with different phospholipid compositions respond differently to membrane-active peptides. Complex, living cell membranes, therefore, provide a variety of dynamic peptide-lipid interfaces that transiently affect PLA_2 activity. A few endogenous membrane-binding peptides that regulate PLA_2 activity have been recently identified (Bomalaski et al. 1989; Gupta and Braun 1990).

We have examined the cytolytic activities of PLA_2 on lymphocytes treated with membrane-active peptides in an attempt to provide more support to the mechanism of PLA_2 activity modulation by alterations in membrane structure. Since hydrolytic activity of nonpresynaptic PLA_2 enzymes is in direct proportion to their cytolytic activity (Gasanov et al. 1991), we expected to find a correlation between enzyme activity on liposomes and ^{51}Cr release from lymphocytes. The findings noted in Table 1 demonstrate that when lymphocytes are pretreated with membrane-active peptides, the synergism between PLA_2 and membrane-active peptides takes place. These results strengthen the conclusion that PLA_2 activity is modulated by membrane-active peptides through modification of substrate structure.

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