Inhibition of Type I and Type II Phospholipase A_2 by Phosphatidyl-Ethanolamine Linked to Polymeric Carriers†

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ABSTRACT: We have previously shown that cell surface proteoglycans protect the cell membrane from the action of extracellular phospholipase A_2 (PLA_2) enzymes [Dan, P., Nitzan, D. W., Dagan, A., Ginsburg, I., and Yedgar, S. (1996) *FEBS Lett. 383*, 75−78]. Cell-impermeable PLA_2 inhibitors (ExPLIs) were prepared by linking phosphatidylethanolamine (PE) to polymeric carriers, specifically, carboxymethylcellulose, heparin, or hyaluronic acid. The structure of these inhibitors enables the incorporation of their PE moiety into the membrane while the polymer remains at the membrane surface. In the present study, we show that the ExPLIs are effective inhibitors of the hydrolysis of different phospholipids in biological (*Escherichia coli*) and model (phospholipid vesicle) membranes, by diverse types of PLA_2 enzymes, specifically human recombinant synovial fluid and *C. atrox* (type II), as well as *Naja mocambique* and porcine pancreatic (type I) PLA_2. It is proposed that the external polymers of the ExPLIs, which are anchored to the membrane by the PE, mimic the naturally occurring cell surface proteoglycans and similarly protect membranes from the action of exogenous PLA_2.

Phospholipase A_2 (PLA_2) is a family of enzymes that hydrolyze phospholipids at the sn2 position, releasing a fatty acid and a lysophospholipid. The PLA_2s are divided into several groups by their size, structure, charge, requirement for calcium, and their behavior in a reducing environment (*1*, *2*). A major group of these enzymes is the low molecular weight secretory PLA_2s, investigated in the present study. They are found in snake venoms, mammalian pancreas, platelets, synovial fluid, and other sites (*1*, *3*). Hydrolysis of phospholipids that are esterified with arachidonic acid at the sn2 position by PLA_2 produces two biologically active products. One, arachidonic acid, is the precursor of the eicosanoids, which are mediators of diverse inflammatory and allergic processes (*3*), and involved in signal transduction (*1*, *5*). The other product, lysophospholipid, can induce cell activation and exocytosis, such as histamine release by mast cells (*6*). In addition, extracellular PLA_2, such as that secreted into body fluids during inflammation, pancreatitis, and sepsis, can directly attack the cell membrane to induce cell lysis and subsequent tissue damage associated with these pathological states (*3*, *7*).

Altogether, the products of phospholipid hydrolysis by PLA_2 and secreted PLA_2s themselves play a pivotal role in the pathophysiology of diverse inflammatory and allergic diseases (*7*, *8*). Accordingly, inhibition of PLA_2 activity has long been proposed for the control of the activity of these enzymes and the treatment of related diseases, and a number of compounds have been proposed for this purpose. However, small inhibiting molecules, which enter the cell, may interfere with the vital phospholipid metabolism in the cell. Since the major cause of tissue damage in the pathophysiology of inflammation is the action of the secreted PLA_2 on the cell membrane (*7*), it has long been suggested that adequate treatment requires cell-impermeable PLA_2 inhibitors which affect the enzyme activity at the cell surface but do not enter the cell (*9*). In accord with this, we recently found that cell-surface proteoglycans protect the cell membrane from the action of exogenous PLA_2 (*10*). To obtain selective inhibition of PLA_2 activity at the cell membrane, we have designed and synthesized cell-impermeable PLA_2 inhibitors (ExPLIs), composed of PLA_2 inhibiting molecules linked to a polymeric carrier which prevents their internalization into the cell (*11*−*13*). One group of these inhibitors is based on N-derivatized phosphatidylethanolamine (PE) linked via the polar headgroup to a polymer, in a way which enables the PE to incorporate into the cell surface membrane, but its internalization is prevented by the polymeric carrier. These ExPLIs have been shown to be effective in inhibiting PLA_2 activity in cell cultures and ameliorating related pathological states in animal models (*13*). In the present study, we have explored the effect of the ExPLIs on the hydrolysis of phospholipids in model membranes (liposomes) by different types of exogenous PLA_2 and the inhibitors' mode of action. It was found that, similar to cell surface proteoglycans (*10*),

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[1] Abbreviations: PLA_2, phospholipase A_2; $NMPLA_2$, *N. mocambique* phospholipase A_2; $CAPLA_2$, *C. atrox* phospholipase A_2; $PPPLA_2$, porcine pancreas phospholipase A_2; $HRsPLA_2$, human recombinant synovial fluid phospholipase A_2; ExPLI, Cell-impermeable PLA_2 inhibitor; PE, phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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the polymer at the membrane surface protects the membrane from the action of diverse types of PLA_2.

MATERIALS AND METHODS

PLA_2 enzymes. *Naja mocambique* (type I), *Crotalus atrox* (type II), and *Porcine pancreas* (type I) were purchased from Sigma (St. Louis, MO). Human recombinant synovial fluid PLA_2 was provided by W. Pruzanski and P. Vadas (*14*).

PLA_2 Substrates. Dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), Sephadex G-50, and Sepharose CL-2B were purchased from Sigma (St. Louis). 1-Oleoyl-2-hydroxyphosphatidylcholine (lysoPC) and 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproylphosphatidylcholine (C_6-NBD-PC) were purchased from Avanti Biochemicals (Alabaster, AL). 1-Palmitoyl-[^{14}C]-2-palmitoyl-PC ([^{14}C]DPPC) was purchased from Amersham (Aylesbury, England).

[^{3}H]Oleic-acid-labeled *Escherichia coli* was prepared according to a published procedure (*15*).

Cell-Impermeable PLA_2 Inhibitors (ExPLIs). ExPLIs were synthesized in the laboratory of S. Yedgar by linking phosphatidylethanolamine (PE) to one of the following polymers: carboxymethyl cellulose of molecular weight 50−100 kDa, hyaluronic acid (molecular weight 30 kDa) and heparin (molecular weight 30 kDa). The ExPLIs were designated, respectively, CMPE, HyalPE, and HepaPE.

Determination of PLA_2 Activity. PLA_2 activity was determined with different enzymes and substrates as described below. In all assays, the reaction conditions (enzyme and substrate concentrations, and the reaction duration) were chosen to yield hydrolysis of 5−10% of the phospholipid substrates in the absence of inhibitors.

1. Using 1-Palmitoyl-2-[^{14}C]-palmitoyl PC/lysoPC Liposomes as Substrate. Liposomal vesicles containing [^{14}C]-DPPC (25 nCi), DPPC (34 nmol), and lysoPC (24 nmol) were prepared by evaporating the mixture of lipids in organic solvents under nitrogen, resuspending in 50 μL of Ca^{2+}-free Tris buffer, and vortexing thoroughly. The reaction with *N. mocambique* PLA_2 or *C. atrox* PLA_2 (at concentrations indicated in the respective experiments) was carried out in Tris buffer (100 mM, pH = 8.0) supplemented with 10 mM Ca^{2+} (total volume 200 μL) for 40 min, terminated by the addition of 1.25 mL of 2-propanol:heptane:1 N H_2SO_4/200:50:5, 1.0 mL of heptane and 0.75 mL of H_2O, and subjected to phase separation by centrifugation (*16*). An aliquot of the upper phase (0.8 mL) was transferred into a tube containing 1 mL of heptane and 20 mg of silica gel and was thoroughly vortexed and centrifuged. The radioactivity in 1 mL of the supernatant was determined in a scintillation counter.

2. Using [^{3}H]oleic Acid Labeled E. coli Membranes as Substrate. *E. coli* membranes were labeled with [^{3}H]oleic acid according to a published procedure (*14*). Labeled membranes, containing 12 000 DPM were reacted with 10 ng of human recombinant synovial fluid PLA_2 in a final volume of 400 μL, for 40 min at 37 °C. The reaction was terminated by the addition of 100 μL 2 N HCl. Bovine serum albumin (100 μg) was added, the tubes were centrifuged, and aliquots of the supernatant were taken for scintillation counting.

3. Using C_6-NBD-PC as Substrate. Mixed liposomes of DOPC and C_6-NBD-PC (3:2 molar ratio) were formed by mixing the lipids (dissolved in a chloroform/methanol solution) and evaporating the solvent under a stream of nitrogen. The lipids were then resuspended in Ca^{2+}-free Tris buffer (100 mM, pH = 8.0) and filtered through polycarbonate Millipore filters of 0.4 and 0.2 μM. The reaction was carried out with 0.014 units/mL for the *N. mocambique* PLA_2 in Tris buffer supplemented with 10 mM Ca^{2+} (final volume 800 μL) for 40 min, and terminated with 3 mL of chloroform:methanol:2 N HCl/166:133:5. The lipids were then extracted into organic phase, dried down under a stream of nitrogen, resuspended in 40 μL of chloroform:methanol, and chromatographed on silica thin layer plates (Whatman, LK6) in chloroform:methanol:water/65:35:5 (*17*). The band corresponding to the NBD-caproic acid (C_6-NBD-FA) was scraped, the C_6-NBD-FA was extracted into chloroform:methanol/1:1, and its fluorescence intensity was measured by excitation at 470 nm and emission at 535 nm.

In all the enzymatic assays for inhibition of PLA_2 activity by the ExPLIs, the inhibitors were incubated with the lipid membranes for 10−15 min prior to introduction of the enzymes.

Interaction of ExPLIs with N. mocambique PLA_2 and Crotalus atrox PLA_2. To examine possible interaction between the ExPLIs and PLA_2s, mixtures of inhibitors and enzymes were subjected to gel filtration on a Sephadex G-50 (coarse) column, for the *N. mocambique* PLA_2, or a Sephadex G-100 column for the dimeric *C. atrox* PLA_2, using Tris buffer or Tris-1.5 M NaCl buffer as the eluant. CMPE was determined in the fractions using anthrone staining (*18*), and PLA_2 was identified by its activity using the continuous fluorescence assay for monitoring the hydrolysis of C_6-NBD-PC (*19*).

Interaction of ExPLI with Lipid Membranes. To examine the interaction between the CMPE and the lipid membranes, mixtures of liposomes and CMPE were subjected to gel filtration on a Sepharose CL-2B column, using Tris buffer (100 mM, pH = 8.0) as the eluant. CMPE was determined as above, and the lipids quantitated by determining their phosphate content (*20*).

Each experiment presented in the Results is representative of four reproducible experiments.

The statistical significance was analyzed by the *t*-test.

RESULTS

The inhibition capacity of the ExPLIs was examined by determining their effect on the hydrolysis of different phospholipid substrates by diverse types of PLA_2.

The ExPLIs used here, as noted in the Materials and Methods, are CMPE, carboxymethycellulose-linked PE; Hyal-PE, hyaluronic-acid-linked PE; Hepa-PE, heparin-linked PE.

We first examined the ExPLIs' effect on the activity of the human recombinant synovial fluid PLA_2, which is of particular interest when considering inflammatory processes. For this we determined the hydrolysis of *E. coli* membranes by $HRsPLA_2$ in the presence of increasing concentrations of CMPE. Figure 1, showing a dose-dependent inhibition of $HRsPLA_2$ activity by CMPE, demonstrates that the activity

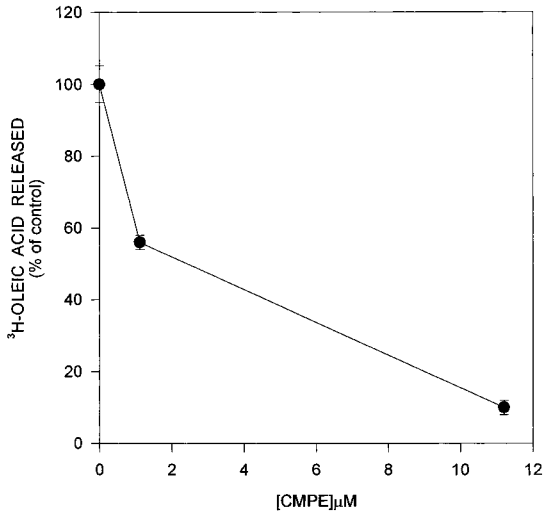

FIGURE 1: Inhibition of human recombinant synovial fluid PLA_2 activity by CMPE: ^{3}H-labeled *E. coli* membranes were interacted with $HRsPLA_2$ for 40 min in the presence of increasing concentrations of CMPE, and the release of [^{3}H]oleic acid was determined as described in Materials and Methods. Each datum is mean ± SD for three replications. CMPE = carboxymethylcellulose-linked PE.

Table 1: Effect of CMPE on the Activity of Diverse PLA_2s[a]

enzyme	IC_{50} for CMPE (μM)
$NMPLA_2$	1.00 ± 0.06
$CAPLA_2$	0.80 ± 0.16
$HRsPLA_2$	8.52 ± 0.62
$PPPLA_2$	1.83 ± 0.14

[a] Liposomes consisting of [^{14}C]DPPC (25 nCi), DPPC (34 nmol), and lysophosphatidylcholine (24 nmol) were subjected to hydrolysis by various PLA_2s in the presence of CMPE for 40 min. The release of [^{14}C]palmitic acid was determined as described in the Materials and Methods. Each datum is ±SD for three replications. $NMPLA_2$ = *N. mocambique* PLA_2; $CAPLA_2$ = *C. atrox* PLA_2; $HRsPLA_2$ = human recombinant synovial fluid PLA_2; $PPPLA_2$ = porcine pancreas PLA_2; CMPE = carboxymethylcellulose-linked PE.

of this enzyme toward a natural membrane can be practically blocked by inhibitors of this kind.

In the following, we focused mainly on the *C. atrox* PLA_2, which is of the same type as the human recombinant synovial fluid PLA_2 (type II), and on *N. mocambique* PLA_2 (type I), both commercially available. *C. atrox* PLA_2 in solution is a negatively charged dimer, while the *N. mocambique* PLA_2 is a positively charged monomer (*21*). Thus, these two enzymes may be taken as an appropriate representation of the diverse types of PLA_2 enzymes.

To examine the scope of inhibition by the ExPLIs, we determined the effect of CMPE on the activity of diverse types of PLA_2, the effect of CMPE on the hydrolysis of different phospholipid substrates by *C. atrox* PLA_2, and the effect of different ExPLIs on the activity of *C. atrox* PLA_2.

Table 1 shows that CMPE inhibits the activity of all enzymes studied, which included *N. mocambique* and *P. pancreas* (type I) PLA_2, as well as human recombinant (synovial fluid) and *C. atrox* (type II) PLA_2s.

Figures 1 and 2 depict the effect of CMPE on the hydrolysis of *E. coli* membranes (Figure 1) and of phospholipid liposomes with radioactive or fluorescent label (Figure 2). These figures show that the hydrolysis of all substrates is effectively inhibited by CMPE.

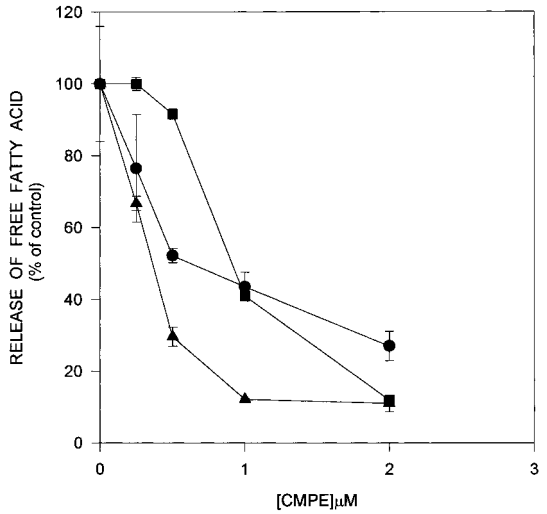

FIGURE 2: Inhibition of *C. atrox* PLA_2 action on C_6-NBD-PC/DOPC liposomes and [^{14}C]DPPC liposomes by CMPE. $CAPLA_2$ was interacted with either C_6-NBD-PC/DOPC liposomes (●) or [^{14}C]DPPC liposomes (▲, 85 nmol/mL lipid substrate; ■, 170 nmol/mL lipid substrate) for 40 min in the presence of increasing concentrations of CMPE. Release of C_6-NBD-FA or [^{14}C]palmitic acid was determined as described in Materials and Methods. Each datum is mean ± SD for three replications. C_6-NBD-PC = 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproylphosphatidylcholine; CMPE = carboxymethylcellulose-linked PE.

Table 2: IC_{50} for Various ExPLIs[a]

ExPLI	[ExPLI] (μM)	[PE] (μM)[b]
CMPE	0.83 ± 0.07	4.01 ± 0.26
Hepa-PE	8.09 ± 0.72	16.03 ± 0.88
Hyal-PE	14.14 ± 1.26	21.03 ± 1.68

[a] Liposomes consisting of [^{14}C]DPPC (25 nCi), DPPC (170 μM), and lysophosphatidylcholine (120 μM) were subjected to hydrolysis by *C. atrox* PLA_2 in the presence of the various ExPLIs for 40 min. The release of [^{14}C]palmitic acid was determined as described in the Materials and Methods. The results are representative of four reproducible experiments. Each datum is mean ±SD for three replications. [b] This column depicts the IC_{50} expressed in terms of the molar concentration of the PE linked to the polymeric carrier in the ExPLIs. Thus, the ratio between the values in the PE column to those in the ExPLI column reflect the molar ratio between the PE and the polymer in the ExPLI tested. CMPE = carboxymethylcellulose-linked PE; Hyal-PE = hyaluronic acid-linked PE; Hepa-PE = heparin-linked PE.

The ExPLIs examined here differ in the nature and size of the polymer linked to the PE, which included carboxymethylcellulose, heparin, and hyaluronic acid. As shown in Table 2, the activity of *C. atrox* PLA_2 was inhibited by all the ExPLIs examined to different degrees. Similar results were obtained also with the *N. mocambique* PLA_2 (not shown). It should be noted that the number of PE molecules bound to each polymer molecule differs for each ExPLI. The CMPE had an average of 5 PE molecules/carboxymethylcellulose molecule, the Hepa-PE had 2 PE molecules/heparin molecule, and Hyal-PE had 2.5 PE molecules/molecule of hyaluronic acid. As shown in the Table 2 (right column), the differences in the IC_{50} between the ExPLIs is smaller when calculated for the PE content, suggesting that the inhibition capacity depends on both the polymer type and its PE content. Thus, the ExPLIs may be considered as general inhibitors of the action of extracellular PLA_2 on membrane phospholipids.

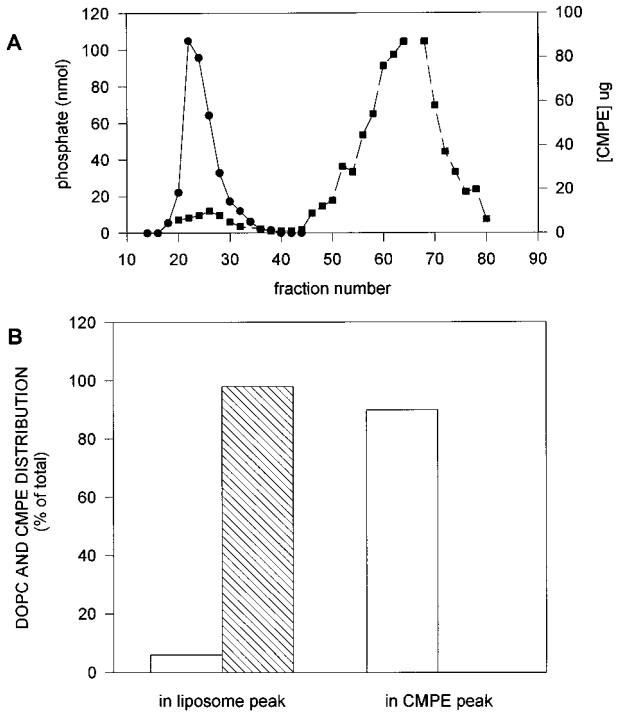

FIGURE 3: Elution profile (A) and content of CMPE and phospholipids in column fractions (B) following separation by gel filtration. A total of 2 mg of CMPE was mixed with DOPC liposomes (total lipid 2 μmol) in 800 μL of buffer and loaded onto a 30 mL Sepharose CL-2B column. The fractions (400 μL each) were eluted with Tris buffer. The corresponding fractions (shown in panel A) were pooled and subjected to determination of phospholipid (hatched bars) and CMPE (empty bars) content, shown in panel B. CMPE = carboxymethylcellulose-linked PE.

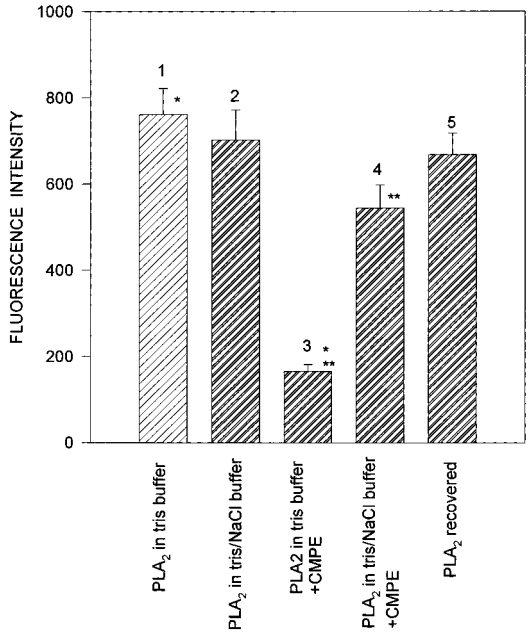

FIGURE 4: Effect of high salt concentration on the binding of CMPE to *N. mocambique* PLA_2. A total of 10 μg of $NMPLA_2$ in the presence or absence of 1 mg of CMPE was chromatographed on a Sephadex G-50 column. The fractions were analyzed for PLA_2 activity as described in Materials and Methods. When eluted separately, the CMPE is eluted in the void volume (fractions 6−12) and the PLA_2 is eluted well after it, in fractions 30−34. These fractions were collected and subjected to determination of PLA_2 activity. Columns 1 and 2 depict the activity of PLA_2 chromatographed in the absence of CMPE in Tris buffer or 1.5 M NaCl, respectively. Column 3 and 4 depict the PLA_2 activity in fractions 30−34 when the enzyme was mixed with CMPE and eluted in either Tris buffer or 1.5 M NaCl, respectively. Column 5 depicts the PLA_2 activity recovered from CMPE/PLA_2 complex which had been first eluted in Tris buffer. These fractions (6−12) were re-eluted in 1.5 M NaCl which yielded full recovery of the PLA_2 activity. (*) $p < 0.001$. (**) $p < 0.001$. CMPE = carboxymethylcellulose-linked PE.

It should be noted that, in all of the above assays, equivalent concentrations of the carriers alone (the polymers to which the PE was linked) did not affect the activity of any of the enzymes tested.

When considering the mode of action of the ExPLIs as PLA_2 inhibitors, we referred to previous findings reporting that cell surface glycosaminoglycans (GAG) protect the cell membrane from the action of exogenous PLA_2 (*10*) and that heparin at the cell surface can bind PLA_2 (*22*) and inhibit its activity (*23*). On these grounds, we examined possible association of the ExPLI with the lipid membranes and/or the enzymes.

To examine the association of the inhibitors with the lipid membranes, mixtures of DOPC liposomes with CMPE were subjected to chromatography on a Sepharose CL-2B gel filtration column. The liposome fractions were collected, concentrated by lyophilization, and subjected to CMPE determination, whereas the CMPE fractions were assayed for phosphate content. As shown in Figure 3, CMPE was found in the liposome fraction, while no lipid phosphate was detected in the CMPE fraction, demonstrating that CMPE associates with the liposomes. This association was further examined by measuring the effect of CMPE on the fluorescence of C_6-NBD-PC [1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproylphosphatidylcholine] in DOPC/C_6-NBD-PC liposomes at self-quenching ratios (60:40 and 90:40). The addition of CMPE to these liposomes increased the NBD fluorescence as a function of the CMPE concentration (not shown), corresponding to dilution of the C_6-NBD-PC and subsequent reduction of its self-quenching. This suggests that the PE moiety of CMPE incorporates into the liposomes.

To examine the possibility that the CMPE might interact with PLA_2 and this interaction takes part in the inhibition of its activity, mixtures of *N. mocambique* PLA_2 and excess CMPE were chromatographed on a gel filtration column. When chromatographed alone, the PLA_2 was eluted well after the void volume (fractions 30−34), while in the presence of CMPE, the enzyme is eluted together with the inhibitor in the void volume (fractions 6−12). By contrast, when the elution of the CMPE/PLA_2 mixture was done in high salt (1.5 N NaCl), which is known to dissociate proteins from cell surfaces, the enzyme was again eluted as the free enzyme. Correspondingly, as shown in Figure 4, the enzyme activity was inhibited when associated with the CMPE fraction (column 2) and resumed when the fractionation was done in high salt (column 4). Similarly, when the CMPE/PLA_2 fraction was rechromatographed in high salt, the enzyme activity was obtained (column 5). This suggests that the free inhibitor can bind the *N. mocambique* PLA_2 and inhibit its activity. However, this was not the case with the *C. atrox* PLA_2. When the same experiments were conducted with the *C. atrox* PLA_2 (using a Sephadex G-100 column, which is suitable for the dimeric enzyme), no binding of this

Table 3: Relative Inhibition of PLA_2 Activity by the Membrane-Associated CMPE vs the Bulk CMPE[a]

	% inhibition	% inhibition per μg of CMPE
Bulk CMPE 400.00 μg	90.0 ± 4.5	0.22
Membrane associated CMPE 28.00 μg	68.0 ± 3.4	2.43

[a] A total of 2 mg of CMPE and DOPC/C_6-NBD-PC liposomes (total lipid 2 μmol) were mixed and separated on a Sepharose CL-2B gel filtration column. The fractions containing the liposomes (into which 5% of the CMPE was incorporated) were collected and interacted with *N. mocambique* PLA_2 in Tris buffer. Identical liposomes, without CMPE were also chromatographed, and then mixed with 400 μg of CMPE, and interacted with the enzyme. The free C_6NBD-FA was determined as described in the Materials and Methods. Each datum is mean ± SD for three replications. CMPE = carboxymethylcellulose-linked PE; C_6-NBD-PC = 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)aminocaproylphosphatidylcholine.

enzyme to the free CMPE was detected. This selectivity is consistent with the fact that *N. mocambique* PLA_2 is positively charged and thus can bind to the anionic ExPLIs, whereas the positively charged *C. atrox* PLA_2 cannot. Yet, this enzyme's activity is inhibited as well by the CMPE.

Table 3 shows that in the experiment of Figure 3 only about 7% of the CMPE is associated with the liposomes. Similar proportion was found at different liposome/CMPE ratios. Yet, this was sufficient to maintain about 70% of the inhibition capacity, showing that the inhibition capacity of the ExPLI at the membrane surface is an order of magnitude greater than that of the free ExPLI in the solution. This is consistent with the finding that the *C. atrox* PLA_2 does not bind to the free CMPE, yet its action on the liposomes is inhibited as well. It was also found that the inhibition of the PLA_2 activity is increased upon increasing the molar ratio of the ExPLI to the substrate (Figure 2). Altogether, these findings suggest that inhibition of the phospholipid hydrolysis by the ExPLIs is exerted mainly by the membrane-associated inhibitor. In accord with this, we found that the inhibition capacity depends mainly on the ratio between the ExPLI and the phospholipid substrate (and not the enzyme concentration). Kinetically, the inhibition was found to be competitive, and accordingly, the apparent K_i for the CMPE (derived using the Lineweaver−Burk presentation) was $0.5 \pm 0.2 \times 10^{-6}$ M. It should be noted, however, that the conventional kinetic constants (by the Michaelis−Menten model) are defined and formulated for the interaction between the enzyme and the inhibitor (and not for substrate−inhibitor interaction). In the present case, in principle, the values of IC_{50} are more meaningful than K_i.

DISCUSSION

In this study, we examined the inhibition of exogenous PLA_2 activity by novel PLA_2 inhibitors selected from a large group of compounds composed of PE covalently linked to polymeric carriers. The data presented here demonstrate that these compounds are potent inhibitors of phospholipid hydrolysis in diverse membranes (pure liposomes or bacterial membranes) catalyzed by various types of PLA_2 enzymes. This may present the ExPLIs as universal inhibitors of the action of exogenous PLA_2s on membrane phospholipids.

The ExPLIs used here, (containing carboxymethylcellulose, heparin, or hyaluronic acid as carriers) are negatively charged, and thus can bind to the positively charged *N. mocambique* PLA_2 in solution. However, this does not explain the inhibitory effect, since the inhibitory capacity of the free ExPLI is markedly lower than that of the membrane-associated inhibitor (Table 3). Furthermore, the ExPLIs do not bind the negatively charged *C. atrox* PLA_2, yet inhibit its activity as well. This, in addition to the fact that the carriers alone, at comparable concentrations, do not inhibit the activity of the enzymes studied, suggests that the inhibition is primarily due to the ExPLI at the membrane surface. This conclusion is consistent with experiments with cultured cells, in which the cells were exposed to ExPLIs then washed prior to introduction of PLA_2. Although this procedure removed the inhibitors from the culture medium (thus leaving that interacted with the cell membrane), the inhibition of PLA_2 activity remained practically the same (unpublished results). In this respect, the ExPLIs are similar to the cell surface proteoglycans which block the accessibility of extracellular PLA_2 to the cell membrane and prevent the hydrolysis of its phospholipids (*10*).

The activity of PLA_2 at the membrane surface is known to be susceptible to diverse physical and chemical propeties of the lipid bilayer (*24*). The mechanism of the inhibition of PLA_2 activity by the ExPLIs could involve effects of the polymer at the membrane surface and/or of the PE incorporated into the membrane. Since the ExPLIs at the membrane surface inhibit both negatively charged (type II) and positively charged (type I) PLA_2, simple electrostatic interaction between the enzymes and the inhibitors does not provide a sufficient explanation for the mode of inhibition. In search of the mechanism of this phenomenon, we are considering the following possibilities: PLA_2 activity is known to be affected by the hydration of the phospholipid headgroup (*25*), and this has been recently shown to be modified by poly(ethylene glycol) (*26*). It is of course likely that a similar effect is exerted by the ExPLIs' polymer component. Another possibility is the masking of the substrate by the polymer covering the bilayer ("substrate depletion"), similar to that proposed for annexins (*27*). A recent study has shown that the inhibition by annexins cannot be explained by simple steric hindrance (as 100% inhibition is achieved already at 50% coverage) and suggested a reduction of substrate lateral mobility as well (*27*). This could be even more so for the ExPLIs, due to the membrane-incorporated PE, and thus both membrane coverage and lateral mobility could be relevant to the inhibition mechanism. Last, PLA_2 activity has long been shown to be sensitive to the phospholipid packing density and membrane fluidity (*24*), which is obviously likely to be affected by the incorporation of the PE component of the ExPLIs into the membrane. These issues are the subject of a separate comprehensive study. The present study together with results obtained in cell cultures (*13*) suggests that the ExPLIs, containing a polymer which is anchored to the membrane by the PE, seem to mimic the naturally occurring cell surface proteoglycans and similarly protect the membrane from the action of extracellular PLA_2.

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