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The Hydrolysis of Phosphatidyl-Alcohols by Phospholipases A_2

EFFECT OF HEAD GROUP SIZE AND POLARITY

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ABSTRACT. The ability of a variety of secretory phospholipases A_2 (sPLA$_2$: EC 3.1.1.4) to bind to and hydrolyse a series of phosphatidyl-alcohol substrates, in the absence of detergent, was explored by both fluorescence-based kinetic and interfacial binding assays. The enzymes used were sPLA$_2$ from porcine pancreas, *Naja naja* venom and a recombinant human non-pancreatic enzyme. Four dioleoyl phosphatidyl-alcohols were used with different headgroups, methanol, ethanol, propanol and butanol. Comparative kinetic analyses with dioleoyl phosphatidyl-choline, dioleoyl phosphatidyl-glycerol and wheat germ phosphatidyl-inositol are also described. With the phosphatidyl-alcohol series, as the headgroup acyl-chain length increased the susceptibility to hydrolysis decreased. This effect was much more pronounced with the human non-pancreatic and the *Naja naja* venom enzymes than with the pancreatic enzyme. Maximum activity in this assay system was observed with porcine pancreatic sPLA$_2$ and dioleoyl phosphatidyl-methanol (1440 ± 167 μmol/min/mg). We demonstrate that the slow rate of hydrolysis of dioleoyl phosphatidyl-propanol by the human non-pancreatic secretory enzyme (4.56 ± 0.90 μmol/min/mg) is not due to a lack of interfacial binding. The hydrolysis of mixtures of dioleoyl phosphatidyl-choline and dioleoyl phosphatidyl-propanol in various molar proportions by *Naja naja* sPLA$_2$ suggests good mixing of the two phospholipids with minimal phospholipid domain formation under these assay conditions. We present strong evidence for a stimulation of hydrolysis of phosphatidyl-choline by human non-pancreatic sPLA$_2$ in the presence of as little as 1 mol% phosphatidyl-methanol (<40 fold total rate enhancement). Overall, the results demonstrate that the rates of hydrolysis of anionic phospholipids by sPLA$_2$ vary considerably with the different enzymes from this close structurally related family. The tight binding of the human enzyme to poorly hydrolysable anionic phospholipid vesicles provides a novel mechanism of enzyme inhibition by interfacial sequestration. BIOCHEM PHARMACOL **54**;12:1331–1339, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. phospholipase A_2, phosphatidyl-alcohol, interfacial binding, interfacial catalysis, enzyme inhibition

Phospholipases have attracted a good deal of attention not only because of their role in signal transduction pathways but also because they demonstrate the phenomenon of interfacial catalysis as a result of the enzyme first binding to the surface of the aggregated phospholipid substrate. Enzymes acting at this interface do not conform to classical Michaelis Menten kinetics. The phenomenon of interfacial catalysis involves a series of steps (see Scheme 1). The association of the enzyme with the interface may be complex involving both binding and activation steps and this overall process is given the notation E → E*. Once the E* state has been achieved, phospholipid substrate molecules transfer to the active site of the enzyme via an active site channel and hydrolysis will occur with the release of products. The extent of binding of a secretory phospholipase A_2 to the phospholipid interface, the ratio of E to E*, is therefore a critical factor in determining the overall rate of hydrolysis that can be achieved. At its most simple, if the enzyme does not bind to the interface, then hydrolysis cannot proceed. The principles of interfacial catalysis were first recognised by Verger and de Haas in the 1970s and have recently been reviewed [1].

$$\mathbf{E + S \rightarrow E^* + S \rightarrow E^*S \rightarrow E^*P \rightarrow E^* + P}$$

The anionic phosphatidyl-alcohol, phosphatidyl-methanol, has been reported as forming interfaces that are particularly susceptible to hydrolysis by porcine pancreatic and human non-pancreatic secrtory phospholipases A_2 (sPLA$_2$; EC 3.1.1.4)† These mammalian enzymes have

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† *Abbreviations:* DAUDA, 11-(Dansylamino)undecanoic acid; dansyl-DHPE, N-(dansyl)-dihexadecanoyl phosphatidyl-ethanolamine; DOPButanol, dioleoyl phosphatidyl-butanol; DOPC, dioleoyl phosphatidyl-choline; DOPEthanol, dioleoyl phosphatidyl-ethanol; DOPG, dioleoyl phosphatidyl-glycerol; DOPMethanol, dioleoyl phosphatidyl-methanol; DOPPropanol, dioleoyl phosphatidyl-propanol; EDTA, ethylenediaminetetraacetic acid; FABP, fatty acid binding protein; PA, phosphatidic acid; PC, phosphatidyl-choline; hnpsPLA$_2$, human non-pancreatic secretory phospholipase A_2; PG, phosphatidyl-glycerol; PI, phosphatidyl-inositol; PLA$_2$, phospholipase A_2; SUV, small unilamellar vesicle.

been shown to bind with very high affinity to these interfaces [2, 3]. Phosphatidyl-alcohols are not physiologically abundant but may be formed in the presence of low concentrations of free alcohol as a result of phospholipase D-catalysed transphosphatidylation of phosphatidyl-choline. The formation of significant amounts of phosphatidyl-ethanol is seen in alcoholism.

This study explores the effect of increasing the alcohol headgroup acyl chain length and also polarity on PLA_2 activity. Because these substrates are not available in. radioactive form, we have utilised a novel continuous fluorescence displacement assay for phospholipase A_2 which uses natural phospholipids [4] and monitors the release of long chain fatty acids. In this study we compare the relative ability of snake venom, mammalian Group I and mammalian Group II phospholipases A_2 to hydrolyse these phosphatidyl-alcohols. The contributions of both interfacial binding as well as specific active site interactions with the headgroup acyl chains have been assessed for the human non-pancreatic $sPLA_2$. The effect of acyl headgroups on the crucial $E^* + S \rightarrow E^*S$ step of interfacial catalysis is highlighted and suggests a potential role for vesicles of long chain phosphatidyl-alcohols as inhibitors of the human inflammatory enzyme as a result of interfacial sequestration.

MATERIALS AND METHODS

Dioleoyl phosphatidyl-methanol, dioleoyl phosphatidyl-ethanol, dioleoyl phosphatidyl-propanol and dioleoyl phosphatidyl-butanol were obtained as solids from Avanti Polar Lipids. Dioleoyl phosphatidyl-glycerol and *Naja naja* venom PLA_2 were purchased from Sigma. Porcine pancreatic PLA_2 was purchased from Boehringer Ingleheim. Recombinant human non-pancreatic $sPLA_2$ with a N1A mutation was prepared as previously described [5] and protein concentrations were determined by means of Bradford protein assay. DAUDA was purchased from Molecular Probes. Recombinant FABP was prepared and assays were performed as previously described [6, 7]. Solutions of phospholipids (10 mg/ml) were freshly prepared using methanol except for phosphatidyl-ethanol which was dissolved in ethanol. This eliminates any chance of methanolysis of this substrate, propanol and butanol are not efficient leaving groups and significant methanolysis was not anticipated. The alcoholic solutions were directly injected into assay buffer to form small unilamellar vesicles [8]. No difference has been detected between vesicles formed from methanol and ethanol stock solutions [6]. All final phospholipid concentrations were 50 μg/ml in assay cocktail, except where otherwise stated. Activity assays were performed in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.0 at 37°, except where otherwise stated.

Wheat germ phosphatidyl-inositol was purchased from Lipid Products. The sn-1/sn-2 fatty acyl chain composition of wheat germ PI was not specified and differs from the dioleoyl-phospholipids in the study in this regard. Electrospray mass spectroscopy revealed principal constituents with molecular masses of 833, 835 and 857, these masses would correspond to 16:1, 16:1; 16:0, 16:1 and 16:0, 18:2 or 16:1, 18:1 respectively (results not shown). However, the effect of the fatty acyl chain composition on overall activity is far more subtle than that of the headgroup and significantly the same trends in specific activity apply to all enzymes [6]. There is no significant difference in the relative DAUDA-displacing ability of the fatty acids released by hydrolysis of wheat germ PI compared to oleic acid (results not shown). Calibrations were therefore performed using oleic acid as with the other substrates.

Specific Activity Assays

The FABP-linked fluorescence displacement assay for the release of long chain fatty acids has already been described [4] and was used to determine specific activities in the usual manner. An immediate linear initial rate is seen in all assays that was dependant on enzyme concentration. The period of initial rate measurement (up to 60 sec) corresponds to <2% substrate hydrolysis ensuring minimal change in the physical properties of the substrate aggregate. Calibrations were carried out for every cocktail prepared by addition of oleic acid (0.2 mM in methanol). Data points for calibration were recorded once equilibrium partition had been achieved. The calibration curves obtained with this series of phosphatidyl-alcohols varied with the headgroup under investigation. The percentage decrease in fluorescence for 1.0 nmole of oleic acid present was 27.5% for DOPMethanol, 20.2% for DOPEthanol, 17.5% for DOPPropanol and 10.8% for DOPButanol. These data probably reflect the increasing affinity of phospholipid vesicles for oleate with increasingly hydrophobic headgroups making the overall assay less sensitive to released fatty acid. This partitioning phenomenon does not affect the kinetic measurements as all initial rates of phospholipid hydrolysis were recorded without an apparent lag-phase. The assay has been shown to be a reproducible and reliable mechanism for the determination of specific activities of a variety of enzymes releasing long chain fatty acids [4, 6, 7, 9–13].

Preincubation with DOPPropanol and Subsequent Hydrolysis of DOPG

The effect of preincubation of $hnpsPLA_2$ with DOPPropanol on the rate of hydrolysis of subsequently added DOPG was examined to determine the extent of the $E \rightarrow E^*$ transition at the DOPPropanol interface. Preincubation assays were carried out in buffer containing 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl and 0.25 mM $CaCl_2$ final concentrations with 1 nmole DAUDA and 12 μg FABP. The lower calcium concentration was used to ensure the maintenance of discrete vesicles [14]. To a cuvette containing this buffer, 0 to 25 μg DOPPropanol was added to give a final volume of 900 μl. Hnps PLA_2 (20 ng) was then added. The rate of

decrease of fluorescence was recorded in the usual manner. This background decrease in fluorescence is due to the slow hydrolysis of the DOPPropanol. The amount of enzyme free in solution, the E form, was then assessed by addition of 100 μl of DOPG (500 μg/ml) as preformed SUVs in the same buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.25 mM $CaCl_2$, pH 8.0). The enhanced rate of hydrolysis (decrease of fluorescence) following addition of the second substrate is attributable to the hydrolysis of DOPG.

Binding of hnpsPLA$_2$ to dansyl-DHPE Containing Vesicles

This technique has been described elsewhere [3, 5, 15]. It was employed here to confirm the binding of hnpsPLA$_2$ to otherwise pure DOPMethanol or DOPPropanol vesicles as well as mixtures of DOPC and phosphatidyl-alcohols. DOPC/dansyl-DHPE vesicles were used as a control. The production of SUVs was performed using standard methods. Briefly, 1.5 μmoles of the phospholipid, containing 60 nmoles (4 mol%) dansyl-DHPE, were dried down under a stream of nitrogen. The residue was resuspended in 1 ml of cocktail containing 10 mM Tris HCl, 1 mM EDTA, pH 8.0 by rotamixing and sonicated with a Heat Systems XL2020 probe sonicator fitted with a microprobe. A 100-fold dilution of the sonicate was made using 10 mM Tris HCl, 1 mM EDTA, pH 8.0. HnpsPLA$_2$ (590 μg/ml) was then titrated into 1 ml of this preparation, containing 15 nmoles of phospholipid. Fluorescence emission spectra were recorded using an Hitachi F4500 fluorescence spectrophotometer over 400 to 600 nm with the excitation wavelength set at 335 nm. Slit widths were 10 nm for both excitation and emission. The scan speed was 240 nm/min.

RESULTS

Hydrolysis of Phosphatidyl-Alcohols by Phospholipases A$_2$ Using Single Substrate Vesicles

The interfacial binding step (E → E* in Scheme 1) in the hydrolysis of a phospholipid vesicle is often crucial in determining overall catalytic activity, an effect that is often described in terms of the quality of the interface. Because of this problem, the comparison of different enzymes on the same substrate were always carried out using the same preparation of phospholipid vesicles. Similarly, in order to allow a comparison of phospholipids varying only in the headgroup, the dioleoyl-molecular species of each phospholipid was used except for the case of phosphatidyl-inositol which was from wheat germ. It is anticipated that all phospholipids tested, being unsaturated, were above their critical phase transition temperature. Moreover, any major differences in catalytic activity due to the different interfacial properties of different substrates would affect all enzymes to a similar extent. Hence major differences between enzymes in terms of substrate preference should reflect active site specificities of the enzymes.

The results of the comparison of different substrates using porcine pancreatic PLA_2, hnpsPLA$_2$ and *Naja naja* PLA_2 are shown in Table 1. The porcine pancreatic PLA_2 demonstrated the highest specific activities with all phosphatidyl-alcohols that were assayed. Moreover, very high levels of activity were maintained for the series phosphatidyl-methanol to phosphatidyl-butanol with DOPButanol still having about 35% of the activity of that seen with phosphatidyl-methanol under these assay conditions and confirms a previous comparison of phosphatidyl methanol and butanol using this enzyme [16]. This result is in contrast with the human and *N. naja* enzymes where there is a dramatic fall in expressed activity, particularly going from DOPMethanol to DOPEthanol. Both DOPPropanol and DOPButanol are poor substrates for these enzymes.

TABLE 1. Specific activities of sPLA$_2$'s with single substrate vesicles

Substrate	Pancreatic PLA_2	hnpsPLA$_2$	*Naja naja* PLA_2
DOPMethanol	1440 ± 167	82.8 ± 23.3	78.8 ± 15.9
DOPEthanol	1140 ± 109	24.2 ± 11.8	16.2 ± 4.2
DOPPropanol	746 ± 43	4.56 ± 0.90	4.86 ± 0.44
DOPButanol	501 ± 86	5.66 ± 0.34	2.16 ± 0.02
DOPG	198 ± 28	75.3 ± 7.6	51.8 ± 1.5
Wheat germ PI	122 ± 12	3.40 ± 1.4	16.7 ± 5.2
DOPC	73.2 ± 13.4	0.065 ± 0.02	495 ± 91

For a particular substrate, the same phospholipid vesicle preparation was used to assay each enzyme. Specific activities ± standard deviations are based on triplicate assays, data for DOPC are based on triplicate assays of each of two cocktails performed on different days. Calibrations were performed with oleic acid throughout. All assays and calibrations were at 37° and in the presence of 5 mM $CaCl_2$. Specific activities are expressed as μmol/min/mg under these assay conditions.

Two more prevalent naturally occurring phosphatidyl-alcohols have been included for comparison. DOPG has been shown to be an excellent substrate for mammalian phospholipases A_2 and is particularly suited for the purposes of this investigation because the three carbon headgroup allows comparison with phosphatidyl-propanol in terms of chain length, however the two hydroxyl moieties of PG greatly enhance the resultant hydrophilicity of the headgroup. Although the inositol headgroup of PI is of similar hydrophilicity, it is a poorer substrate for all enzymes assayed. It is anticipated that the slower rates probably reflect primarily steric effects due to the enhanced size of this headgroup. The human enzyme is only able to express low activity with this phospholipid. It should be noted that because PI is from a biological source, it contains a mixture of fatty acyl chains which were identified by electrospray mass spectrometry (see "Materials and Methods") however the 14-kDa family of PLA_2 shows minimal acyl chain specificity [6, 17].

Overall the data reveal two principal effects. Firstly, larger headgroups are hydrolysed less rapidly than smaller ones. This holds for all enzymes with the phosphatidyl-alcohol series and DOPG versus PI. The pancreatic enzyme is most tolerant to head group size as seen by the fact that the PI/PG rate ratio is 0.61 for porcine pancreatic PLA_2 whereas rate ratios of 0.32 and 0.045 are observed for *N.*

naja and hnpsPLA$_2$ respectively. Secondly, there is an effect which is probably due to the hydrophobicity of the headgroup, and is seen by comparing DOPPropanol with DOPG. The ratio of specific activities of DOPPropanol: DOPG for porcine pancreatic, *N. naja* and hnpsPLA$_2$ are 3.77, 0.094 and 0.061 respectively under these conditions. In this regard the hnpsPLA$_2$ is more like the *N. naja* enzyme than the other mammalian PLA$_2$.

All the above phospholipids are anionic and therefore provide the preferred interface for those sPLA$_2$ enzymes that have been examined for interfacial binding [1–3]. In contrast, PC provides a neutral zwitterionic interface that discriminates between types of PLA$_2$ due to differences in interfacial binding and activation. Results obtained using DOPC highlight the ability of the highly penetrating *N. naja* venom enzyme to interact with this condensed interface. This contrasts with the pancreatic enzyme and, more dramatically with the human enzyme which exhibits negligible activity with this substrate.

The Effect of Preincubation of Human PLA$_2$ with DOPPropanol on the Rate of Hydrolysis of Subsequently Added DOPG

In the case of interfacial enzymes, the inability of a pure phospholipid to act as a good substrate may be due to an interfacial effect or an effect on classical Michaelis–Menten binding and catalysis. In the present study, the lack of activity of the human enzyme on DOPPropanol might have been attributed to reduced interfacial binding. Therefore, the potential ability of anionic phospholipid vesicles to trap enzyme was utilised as a method of demonstrating interfacial binding. If this human enzyme is able to bind tightly, albeit non-productively, to DOPPropanol vesicles it will not be available to hydrolyse a good substrate, DOPG, if this is subsequently added to the assay. Preincubation of human enzyme with increasing concentrations of DOPPropanol gave decreasing rates of hydrolysis of subsequently added DOPG. No significant difference was recorded for the calibration in the presence of DOPPropanol compared to that in the absence of the phosphatidyl-alcohol. The decrease in the subsequent rate of hydrolysis of DOPG is clearly demonstrated in Fig. 1. At concentrations of DOPPropanol above 1 nmol/mL in the assay, there is essentially no increase in the rate of hydrolysis on subsequent addition of DOPG. Because DOPG is a good substrate for the enzyme, this result suggests that under these assay conditions in the presence of DOPPropanol, the human enzyme is tightly bound to the DOPPropanol interface and is not available to hydrolyse the DOPG. This approach has been used previously to demonstrate interfacial binding to non-hydrolysable phospholipids [18]. From Fig. 1 it can be seen that 50% inhibition of the rate without preincubation can be achieved with 0.03 nmol/mL DOPPropanol present. Assuming this equates to 50% of the total enzyme present being bound to DOPPropanol interfaces, the molar ratio of bound enzyme to DOPPropanol is 1:42.

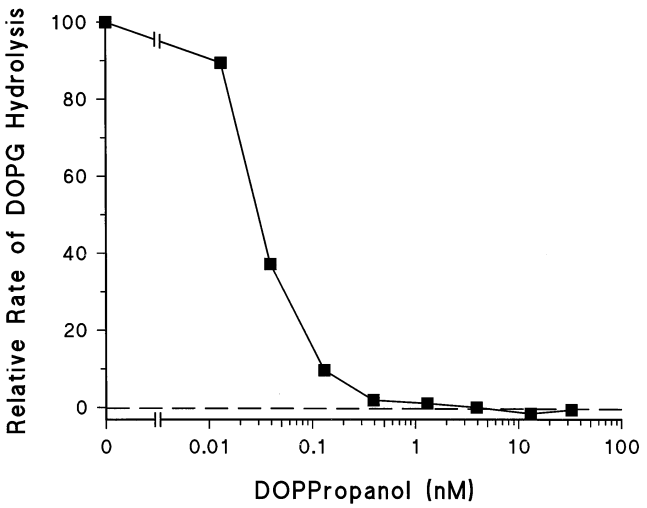

FIG. 1. Trapping of hnpsPLA$_2$ on vesicles of DOPPropanol. The hnpsPLA$_2$ (20 ng) was preincubated with DOPPropanol vesicles and subsequently DOPG vesicles were added as substrate to determine the amount of free enzyme. Preincubations were performed in 900 μL standard assay buffer. The rate due to the slow hydrolysis of DOPPropanol was recorded and subtracted from the rate of hydrolysis achieved upon subsequent addition of preformed DOPG vesicles in 100 μL of assay buffer.

Given that about 70% of phospholipid molecules in a small unilamellar vesicle are exposed and therefore available to bind enzyme the ratio of bound enzyme molecule to exposed phospholipid molecules is about 1:30. This ratio is similar to that observed when an sPLA$_2$ coats the surface of an anionic vesicle [18] and therefore must represent tight binding of hnpsPLA$_2$ to DOPPropanol vesicles.

Binding of hnpsPLA$_2$ to DOPPropanol, DOPMethanol and DOPC Vesicles Containing 4 mol% dansyl-DHPE

The binding of pancreatic and human PLA$_2$ to anionic phospholipid vesicles can be monitored as a result of the change in environment at the phospholipid interface as this becomes coated with added enzyme [3, 16, 19]. This binding has been monitored by means of tryptophan-3 of the porcine pancreatic enzyme [16] and most conveniently by measuring the fluorescence change of a dansylated phospholipid probe such as dansyl-DHPE present in low molar proportions with respect to the bulk phospholipid. Desolvation of the interface causes a large increase in fluorescence intensity of the polarity-sensitive dansyl-reporter group. Such measurements would normally require a non-hydrolysable phospholipid however the interfacial binding step does not require the presence of calcium [5, 20]. Hence interfacial binding studies may be performed with normal phospholipids in the presence of EDTA. Catalysis is prevented by the absence of calcium which is essential for phospholipid binding at the active and is part of the catalytic mechanism of these 14-kDa enzymes.

The effect of increasing hnpsPLA$_2$ concentration on the fluorescence of vesicles comprised of either DOPMethanol

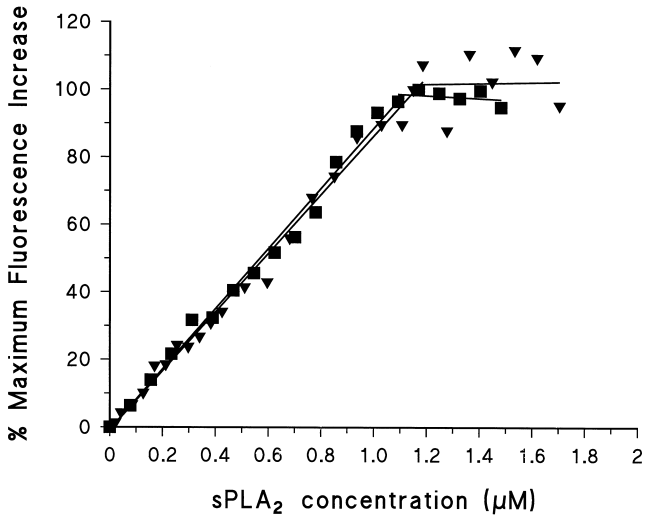

FIG. 2. Interfacial binding of $hnpsPLA_2$ to DOPMethanol and DOPPropanol. Interfacial binding assays were performed using vesicles of DOPMethanol (▼) or DOPPropanol (■) each containing 4 mol% dansyl-DHPE. Assays were performed in 1 mL 10 mM Tris pH 8.0, 1 mM EDTA in the presence of 15 nmol phospholipid. The enzyme was added as a 590 μg/mL solution in 0.6 M KCl, 20 mM Tris-HCl, pH 8.0. Fluorescence emission spectra were recorded and the fluorescence at 500 nm plotted as a percentage of the fluorescence measured in the absence of protein against enzyme concentration.

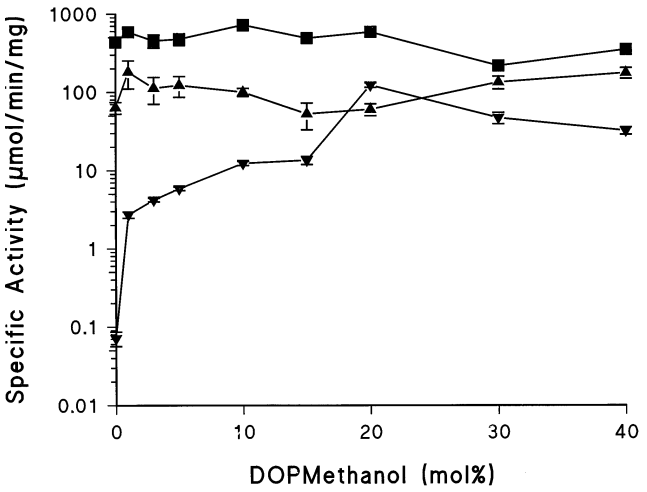

FIG. 3. Effect of varying the molar ratios of DOPMethanol and DOPC on the rate of hydrolysis by PLA_2's. The enzymes used were from *N. naja* venom (■), porcine pancreas (▲) and $hnpsPLA_2$ (▼). Phospholipid vesicles in the assay mixtures were prepared by injection of the appropriate molar ratio of phospholipids in methanol. Total phospholipid present was maintained as 63.6 nmoles per assay. Assays were carried out at 37°C in the usual manner using the same stock cocktail for all enzymes. Calibrations were performed for every assay mixture by addition of oleic acid (0.2 mM in methanol). Specific activities thus calculated are plotted as mean ± standard deviation based on triplicate assays.

or DOPPropanol together with 4 mol% dansyl-DHPE is shown in Fig. 2. The fluorescence of the dansylated phospholipid in the presence of either phosphatidyl-alcohol is enhanced with increasing concentrations of enzyme consistent with high affinity interfacial binding and desolvation ($E \rightarrow E^*$). The results confirm that the $E \rightarrow E^*$ transition for $hnpsPLA_2$ does not require calcium for either of the phosphatidyl-alcohols tested. Although the two titration curves are superimposable, it does not necessarily mean that the enzyme binds to both interfaces with the same affinity. The K_d for such interfaces is extremely low ($K_d \ll 10^{-6}$ M) [1] and as a result this technique is unable to discriminate between differences in very high affinity binding and what is seen is a titration of enzyme onto the vesicle surface. The stoichiometry of this titration corresponds to an end point of about 1 nmol of enzyme per 15 nmol of phospholipid which will approximate to 10 nmol of phospholipid in the outer monolayer of a small unilamellar vesicle. This value is lower than the value of about 1:30 reported in other studies, using ditetradecyl phosphatidylmethanol, and indicated in preincubation assays described above. It would be expected that the dioleoyl phospholipids used here might occupy a larger interfacial area than phospholipids with saturated acyl chains used elsewhere. Any overestimate of the final concentration of enzyme in the binding assay will also result in a reduced binding stoichiometry. The important conclusion from this experiment is that the same enzyme sample binds with high affinity to both DOPMethanol and DOPPropanol vesicles and hence the low catalytic activity seen with DOPPropanol and the human enzyme cannot be attributed to reduced interfacial binding as compared to DOPMethanol.

In contrast to anionic phospholipids, no increase in fluorescence was observed when using DOPC vesicles containing 4 mol% dansyl-DHPE, even with $hnpsPLA_2$ concentrations exceeding 10 μg/ml. This result confirms the lack of productive binding of this enzyme to this type of interface and is consistent with the observation that the human enzyme expresses negligible catalytic activity with DOPC and egg yolk PC vesicles [7]. We suggest that this lack of activity is primarily due to minimal productive interaction ($E \rightarrow E^*$) of the human enzyme with this type of interface under these assay conditions and not because the enzyme is unable to accommodate the substrate at the active site.

Specific Activity Assays with Mixed Substrate Vesicles Containing DOPC and Phosphatidyl-Alcohol

The presence of an increasing molar proportion of DOPMethanol in DOPC vesicles was shown to cause enhanced hydrolysis of the mixed substrate with $hnpsPLA_2$ and porcine pancreatic PLA_2 but not with *N. naja* PLA_2 (see Fig. 3). It was of interest that enhanced rates of $hnpsPLA_2$ mediated hydrolysis were observed even in the presence of molar ratios of phosphatidyl-methanol as low as 1 mol%. This enhancement effect contrasts with incorporation of other amphiphilic anions, such as cholesterol-3-sulphate, into DOPC vesicles. We have previously demonstrated that such compounds induce significantly enhanced susceptibil-

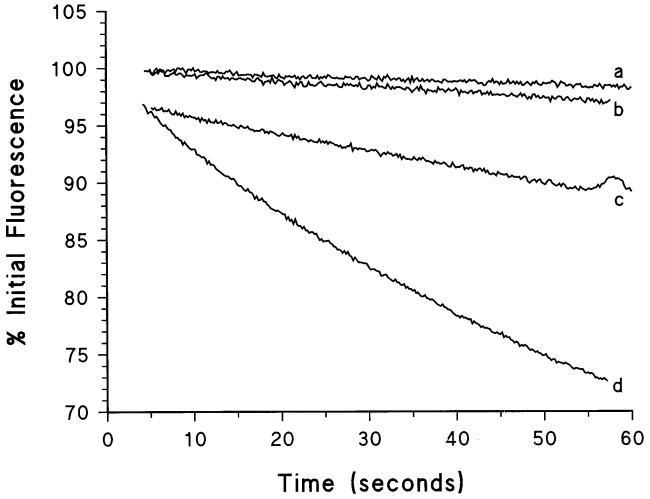

FIG. 4. Fluorescence displacement assay traces for the hydrolysis of DOPC vesicles containing 1 mol% of phosphatidyl-alcohol. Fluorescence values are plotted as a percentage of the fluorescence recorded immediately prior to the addition of the enzyme. Trace a relates to the assay of 1.18 μg of hnpsPLA$_2$ with 63.6 nmol DOPC as the only substrate. Traces b, c, and d were achieved by addition of 1.18 μg hnpsPLA$_2$ to DOPC vesicles containing 1 mol% DOPPropanol, DOPEthanol and DOPMethanol respectively. Total phospholipid present was maintained as 63.6 nmol per assay.

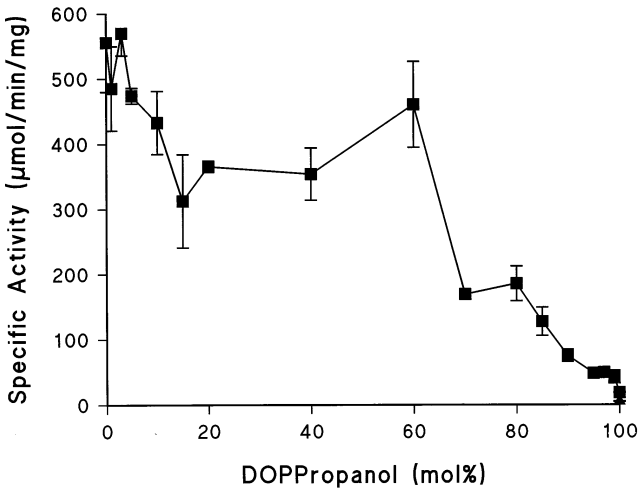

FIG. 5. Effect of varying the molar ratios of DOPPropanol and DOPC on the rate of hydrolysis by N. *naja* sPLA$_2$. Conditions used were as described for Fig. 3 except that in this experiment the molar ratios of DOPPropanol and DOPC were varied between 0–100%.

ity to hnpsPLA$_2$ mediated hydrolysis but only when present at mole fractions exceeding 5 to 10 mol% [7, 21]. At 1 mol% DOPMethanol the amount of total hydrolysis observed extended beyond 0.64 nmol, the total amount of DOPMethanol present under these conditions. This demonstrates that the enhanced activity involves the hydrolysis of DOPC and cannot be attributed solely to hydrolysis of the small amount of DOPMethanol present and is indicative of an effect on interfacial binding rather than an interaction at the active site of the enzyme. The ability of very low molar proportions of DOPMethanol to stimulate hnpsPLA$_2$ suggest that there is not a critical electrostatic charge density required over the interface for activation of this enzyme and that a specific interaction of the enzyme with individual molecules of DOPMethanol may be involved. The results of representative traces of hydrolysis of vesicles containing 1 mol% of various phosphatidyl-alcohols by hnpsPLA$_2$ are shown in Fig. 4.

The effect of the incorporation of different ratios of DOPMethanol:DOPC in mixed vesicles on the porcine pancreatic enzyme (see Fig. 3) is much less dramatic in percentage terms than that seen with the human enzyme. This is due to the relatively high rate seen with porcine pancreatic PLA$_2$ and pure DOPC. A significant stimulation is seen at the low (1 mol%) ratio of DOPMethanol which, again, must be due to enhanced DOPC hydrolysis. There is little effect on the rate of *N. naja* PLA$_2$ mediated hydrolysis caused by the incorporation of DOPMethanol other than an expected gradual decline in activity as DOPC is replaced in the interface by the poorer substrate, DOPMethanol.

It was of particular interest to observe the effect of a changing ratio of DOPPropanol:DOPC on catalysis by *N. naja* PLA$_2$. The enzyme will bind very tightly to the anionic interface that would be produced by a domain of DOPPropanol but DOPPropanol is a very poor substrate compared to DOPC (Table 1). The results are shown in Fig. 5 and overall the effect was of a continual decline in activity as a good substrate is replaced by a poor one. However, the shape of the curve is of interest because at 60 mol% DOPPropanol the activity has only declined by about 30% whereas with pure DOPPropanol the activity is about 1% of that seen on pure DOPC. As already stated, this type of venom enzyme, in common with other sPLA$_2$'s tested, has a very high affinity for anionic vesicles [22] while binding to PC is reported to be of the order of micromolar [1]. Therefore, if domains of DOPPropanol were present, the venom enzyme would be expected to bind to these domains with a resultant low expression of hydrolytic activity. This appears not to be the case and suggests good mixing of the two components. Ideal mixing has previously been reported for PC/PA mixtures [23] and the same is assumed to be true for mixtures of other anionic phospholipids with PC [24]. Moreover, this venom enzyme must have reduced ability to cause selective clustering of anionic lipids within its interfacial binding region as this would be inhibitory. Such clustering of anionic phospholipids has been reported in the case of cardiotoxin II [24] and other membrane binding proteins [23, 25, 26].

DISCUSSION

The interfacial binding to and subsequent hydrolysis of phospholipid aggregates by enzymes is of increasing biomedical interest and has recently been reviewed [1]. The practical importance of interfacial binding is seen most acutely in understanding the role of the hnpsPLA$_2$ in a

variety of inflammatory disorders. Under these conditions the plasma membranes of cells are usually exposed to potentially pathological levels of this phospholipid-degrading enzyme yet the cell membrane is highly refractory to hydrolysis by this particular enzyme [13] and Kinkaid, Voysey and Wilton (paper in preparation). In contrast, plasma membranes may be readily hydrolysed by the structurally related venom enzymes such as that from *N. naja*. There is an argument that the human enzyme is able to hydrolyse the membrane of activated cells possibly resulting from alteration or perturbation of the membrane [27] such as exposure of anionic phospholipids and that under these conditions the human enzyme can exacerbate an inflammatory state. Therefore, a complete understanding of the catalytic properties of this enzyme at the interface and the structural requirement for potential inhibitors of $hnpsPLA_2$ is of continued interest.

The results described in this paper have used a fluorescence assay to monitor the hydrolysis of phospholipid substrates that are not available in radioactive form. The results using this assay demonstrate the ability of porcine pancreatic $sPLA_2$ to hydrolyse the phosphatidyl-alcohols with longer headgroup acyl-chains. In contrast *N. naja* venom PLA_2 and a recombinant $hnpsPLA_2$ expressed only modest hydrolytic activity with propanol and butanol headgroups when compared with phosphatidyl-methanol and phosphatidyl-glycerol (see Table 1). The slightly higher activity recorded for $hnpsPLA_2$ mediated hydrolysis of DOPButanol compared to DOPPropanol may not be significant ($P = 0.12$, Student's *t*-test) but has been observed consistently.

Examination of enzyme crystal structures has revealed that the channel that allows substrate access to the active site is narrower for the human non-pancreatic enzyme than for the pancreatic enzyme [28] although the rigidity of this structural feature has been questioned elsewhere [29]. However, steric reasons for the ability of the pancreatic enzyme to hydrolyse the larger phosphatidyl-alcohols at high rates, as compared to the human enzyme, are not supported by the comparison with the hydrolysis rates of DOPG. DOPG is a preferred substrate for the human enzyme whereas only low rates are seen with this enzyme on the sterically smaller DOPPropanol (Table 1).

In contrast to our results, it has been reported that phospholipid analogues with methanol, ethanol and propanol-derived head groups inhibit both the porcine pancreatic and human enzymes to a similar extent [30]. This result suggests that there are minimal steric effects with different alcohol-derived head groups when binding such ligands at the active of the human enzyme. Since we have confirmed that the human enzyme is able to bind with high affinity to the interface of DOPPropanol vesicles, (Figs. 1 and 2), the reason for DOPPropanol being a poor substrate for the human enzyme will require further consideration and an explanation consistent with the data and experimental conditions reported by Gelb's laboratory [30].

An explanation involving substrate hydrolysis or product release to explain the low catalytic activity of the human enzyme with DOPPropanol would be consistent with the different data. Normally, the chemical step is rate limiting during hydrolysis in scooting mode when the enzyme is bound to anionic vesicles [1] while the kinetics of the hydrolysis step should not be altered by the presence of the various phosphatidyl-alcohols, as supported by the results with the pancreatic enzyme. However it is possible that the human enzyme is able to bind the DOPPropanol in a high affinity but catalytically non-productive orientation. Another explanation is that lysophospholipid product release becomes rate limiting with these higher molecular weight phosphatidyl-alcohols. It should be noted that the hydrolysis of dimyristoyl phosphatidyl-methanol by pancreatic PLA_2 in oxygen-18 water gave a single oxygen-18 in the fatty acid which was interpreted as establishing that the product release step was very rapid as compared to chemical hydrolysis and is unlikely to be limiting [31]. However, this may not be the case with the human enzyme and higher molecular weight phosphatidyl-alcohols.

The effect of increasing the molar concentration of phosphatidyl-alcohol in DOPC vesicles produced some interesting results. In the case of $hnpsPLA_2$ because the rate of hydrolysis of pure DOPC is almost zero, dramatic effects were achieved by addition of small amounts of anionic phospholipid. Thus the inclusion of 1 mol% of DOPMethanol resulted in a considerable increase in activity and the extent of this enhanced rate was too large to be attributable to DOPMethanol hydrolysis alone. It must be due in part to increased DOPC hydrolysis. This supports observations in a previous study where cholesterol sulphate, which cannot be a substrate, caused a dramatically enhanced rate of DOPC hydrolysis [7]. Because there is normally ideal mixing of monoanionic phospholipids with phosphatidyl-choline [24], the sensitivity of this system to 1 mol% DOPMethanol requires further considerations. It has been shown previously that one molecule of $sPLA_2$ interacts with about 30 phospholipid molecules at the interface [1], therefore the predicted number of DOPMethanol molecules within this enzyme footprint is considerably less than unity at a 1 mol% concentration. As a consequence a significant enhancement of binding and subsequent hydrolysis may be achieved by the specific interaction with one molecule of DOPMethanol. It is possible that, as a result of lateral diffusion, enrichment of DOPMethanol occurs at the interface of this highly cationic enzyme allowing hydrolysis of both DOPMethanol and DOPC. There are many precedents for this phenomenon with proteins that bind with high affinity to anionic lipids [24–26].

In the case of hydrolysis of mixtures of DOPC and DOPPropanol by the *N. naja* enzyme the results are of particular interest. In this case DOPC is an excellent substrate while pure DOPPropanol is hydrolysed at about 1% the rate of pure DOPC. Mixtures of DOPC and DOPPropanol only show a dramatic fall in rate at high molar proportions of DOPPropanol. Since it is predicted that the *N. naja* enzyme would bind with very high affinity

to domains of pure DOPPropanol [22] and therefore show greatly reduced activity, this result would imply that there is good phospholipid mixing under these assay conditions allowing a high rate of DOPC hydrolysis to be maintained even when the concentration of DOPC was below 50 mol% (Fig. 5).

The ability of DOPPropanol vesicles to trap the human enzyme is indicative of the high affinity of this enzyme for DOPPropanol and agrees with numerous examples of $sPLA_2$ having a high affinity for anionic vesicles that allows scooting mode kinetics to be observed [22]. The stoichiometry of this effect was consistent with the enzyme being able to coat such vesicles. The results were confirmed by direct binding assays to vesicles where high affinity binding to both DOPMethanol and DOPPropanol could be observed with this enzyme. Interfacial sequestration may provide a future novel method of inhibition of this inflammatory human enzyme in addition to classical active site-directed inhibitors of this enzyme [32, 33]. Such sequestration is already inherent to studies of Jain and others using non-hydrolysable ditetradecyl phosphatidyl-methanol [1, 18], however DOPPropanol is a more natural acyl-containing phospholipid with potentially lower toxicity while the propanol headgroup should metabolise to propionic acid.

Overall the results clearly highlight both the similarities and differences of three different $sPLA_2$ in terms of their ability to hydrolyse a series of monoanionic phospholipids with different headgroups. The sensitivity of the human enzyme to trace (1 mol%) amounts of such phospholipids in PC provide a possible mechanism for allowing cell membrane hydrolysis by this enzyme with potential inflammatory consequences for the tissue.

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