Human Nonpancreatic Secreted Phospholipase A_2: Interfacial Parameters, Substrate Specificities, and Competitive Inhibitors†

Timothy Bayburt,‡ Bao-Zhu Yu,§ Hung-Kuei Lin,‡ Jeffrey Browning,∥ Mahendra K. Jain,*,§ and Michael H. Gelb*,‡

Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, and Department of Cell Biology and Immunology, Biogen Inc., Cambridge, Massachusetts 02142

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ABSTRACT: The rate and equilibrium parameters for the interfacial catalysis by recombinant human nonpancreatic secreted phospholipase A_2 were determined. Results show that the enzyme binds to anionic interfaces with considerably higher affinity than to zwitterionic interfaces. The extent of hydrolysis per enzyme on anionic vesicles in the processive scooting mode shows that the enzyme is fully catalytically active as a monomer. Among several secreted phospholipases A_2 tested, the human nonpancreatic secreted enzyme is unique in its ability to undergo slow intervesicle exchange either by dissociation from the interface followed by binding to a different vesicle or by promoting the fusion of vesicles. The equilibrium dissociation constants for calcium, substrate analogs, reaction products, and several competitive inhibitors bound to the enzyme at the interface were determined by monitoring the ligand-conferred protection of the active site histidine residue from alkylation by phenacyl bromide. The interfacial Michaelis–Menten parameters were determined from the analysis of the entire reaction progress curve and also by monitoring the effect of competitive inhibitors on the initial rate of hydrolysis in the scooting mode. The interfacial Michaelis constant (K_M*) for the substrate 1,2-dimyristoylglycero-*sn*-3-phosphomethanol was determined to be considerably above the maximal attainable mole fraction of unity for the substrate in the bilayer. Substrate specificity studies show that the enzyme does not significantly discriminate between phospholipids that differ in the type of polar head group or in the degree of unsaturation of the fatty acyl chains. Competitive inhibitors are described that display a high degree of selectivity for binding to the nonpancreatic versus pancreatic phospholipase A_2. The kinetic properties of the human nonpancreatic secreted phospholipase A_2 suggest that the enzyme has evolved to hydrolyze substrates at anionic interfaces and at high calcium concentrations.

Secreted phospholipases A_2 are ubiquitous in nature, and they are mobilized in response to a wide variety of signals. The role of the pancreatic phospholipase A_2 in the degradation of dietary phospholipids is strongly supported by its kinetic and biochemical properties (Verheij et al., 1981; Waite, 1987). The snake venom phospholipases A_2 are structurally related to the pancreatic enzyme, and their primary role is probably related to local tissue damage (necrosis), although some of the venom enzymes are neurotoxic and cardiotoxic (Harris, 1986; Kini & Evans, 1982). Phospholipases A_2 secreted by animal tissues may also play a role in processes such as inflammation and asthma (Wong & Dennis, 1990). In 1980, Vadas and co-workers reported that a soluble phospholipase A_2 was secreted from aggregated platelets and stimulated macrophages (Vadas & Hay, 1980). Levels of circulating phospholipase A_2 have been shown to increase in patients suffering from septic shock (Pruzanski et al., 1985; Vadas et al., 1988). In addition, significant levels of extracellular phospholipase A_2 are found in inflammatory exudates including synovial and peritoneal fluids from patients with arthritis and peritonitis (Vadas & Pruzanski, 1983). Injection of phospholipase A_2 purified from synovial fluid or snake venom into knee joints of experimental animals induces an inflammatory response in the synovium (Bomalaski et al., 1991; Vadas et al., 1989).

Both the cDNA and the genomic DNA for the phospholipase A_2 found in human synovial fluid have been isolated and sequenced (Kramer et al., 1989; Seilhamer et al., 1989). In addition, the sequence of a gene encoding a phospholipase A_2 secreted from human platelets revealed that this enzyme is identical to the one isolated from synovial fluid (Kramer et al., 1989). The predicted amino acid sequence of this enzyme as well as that derived from the genomic DNA sequence of the analogous enzyme from rat (Komada et al., 1990) demonstrates that this enzyme is homologous to type II phospholipases A_2 (from rattlesnake and pit viper venoms) rather than to type I enzymes (pancreatic and elapid venoms) (van den Bergh et al., 1989). This human nonpancreatic secreted phospholipase A_2 (hnps-PLA2)[1] has also been found in other tissues including human spleen (Kanda et al., 1989), peritoneal cells (Seilhamer et al., 1989), placenta (Crowl et al., 1990; Lai & Wada, 1988), and human adult cartilage (Recklies & White, 1991). The same enzyme is secreted from cells in response to interleukins, suggesting that the hnps-PLA2 is involved in the acute inflammatory response (Crowl et al., 1991; Vadas & Pruzanski, 1991; Vadas et al., 1991). Perhaps the best defined role of hnps-PLA2 is in the degradation of bacterial membranes by polymorphonuclear leukocytes in conjunction with bacterial/permeability-increasing protein (Weiss et al., 1991).

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* To whom correspondence should be addressed.
‡ University of Washington.
§ University of Delaware.
∥ Biogen Inc., 14 Cambridge Center, Cambridge, MA 02142.

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The X-ray crystal structures of hnps-PLA2, with and without a bound inhibitor, have been recently determined (Scott et al., 1991; Wery et al., 1991). This work shows that the structural features of the active site are similar to those reported for the phospholipases A_2 from pancreas (Thunnissen et al., 1990), cobra venom (White et al., 1990), and bee venom (Scott et al., 1990a,b), and all are likely to share a common catalytic mechanism in which calcium ion serves as an essential cofactor. Kinetic properties of these enzymes cannot be derived from structural data alone. It is of importance to understand the subtle functional differences between the hnps-PLA2 and other secreted phospholipases A_2. Such information will be useful in deciphering the physiological role of the hnps-PLA2 as well as in developing strategies for the design and evaluation of effective tight binding competitive inhibitors. Thus, in this paper, the kinetic characterization of hnps-PLA2 acting on phospholipid vesicles under conditions in which the enzyme is tightly bound to the interface [scooting mode kinetics (Jain et al., 1986)] is described. In addition, the values of the equilibrium constants for the interaction of the enzyme with the bilayer as well as with the calcium cofactor, substrate analogs, reaction products, and competitive inhibitors are determined. Also included is a quantitative analysis of the substrate specificity of hnps-PLA2.

MATERIALS AND METHODS

Materials. Syntheses of DMPM (Jain & Gelb, 1991), DTPM and DTPC (Jain et al., 1986), HDNS (Jain & Vaz, 1987), and the competitive inhibitors MJ33-96 (Jain et al., 1991c) and MG14 (Yuan et al., 1990) have been previously described. DMPG, DMPS, DPPC, DOPA, OPPC, PAPC, and SAPC were obtained as lyophilized powders from Avanti Polar Lipids. RM2 was a generous gift from Dr. R. Magolda (DuPont Experimental Station, Wilmington, DE). The syntheses of HK30-61 will be described elsewhere. Ellagic acid was from Sigma. (*R*)- and (*S*)-*N*-octadecyl-1-amino-2-propanol were synthesized as described (Funck et al., 1982) from (*R*)- and (*S*)-1-amino-2-propanol (Aldrich). The sources of the radiolabeled phospholipids are as follows: [^{14}C]DPPC (55 mCi/mmol, Amersham); [^{14}C]PAPC (52 mCi/mmol, NEN); [^{3}H]DPPC (50 Ci/mmol, NEN); [^{14}C]SAPI (50 mCi/mmol, NEN); [^{14}C]PAPC (50 mCi/mmol, NEN); [^{3}H]DPPA [5 mCi/mmol, (Ghomashchi et al., 1991)]; [^{3}H]DPPE [10 mCi/mmol (Ghomashchi et al., 1991)]. HPC was a gift from Dr. H. Eibl (Max Planck Institute, Gottingen, FRG). Compound 3 of Oinuma et al. (1991) was a gift from Dr. M. Gresser (Merck Frosst, Montreal, Canada). *all-trans*-Retinoic acid and *all-trans*-retinol were from Sigma. Recombinant hnps-PLA2 was purified to homogeneity [200–300 μmol/(min·mg) on radiolabeled *Escherichia coli* substrate, homogeneous based on reverse-phase HPLC and SDS gel analyses) from the conditioned medium from Chinese hamster ovary cells stably transfected with the hnps-PLA2 gene as previously described (Bomalaski et al., 1991). Concentrations of enzyme were determined from the OD_{280} using $A_{1\%}$ of 9 cm^{-1} (Franken et al., 1992).

Kinetic Studies of Vesicle Hydrolysis. Most experimental protocols to monitor the kinetics of hydrolysis in the scooting mode used in this study have been established previously. Specific experimental conditions are given in the figure legends. The reaction progress curves for the hydrolysis of vesicles was monitored using the pH-stat titration method (Apitz-Castro et al., 1982; Berg et al., 1991). Kinetic studies of the hydrolysis of DMPM vesicles were carried out as described (Berg et al., 1991; Jain & Gelb, 1991; Jain et al., 1986). The reaction progress curves for the hydrolysis of DMPM in the presence of low calcium concentrations (0.6 mM) were fitted to the integrated Michaelis–Menten equation as described previously (Berg et al., 1991) to obtain the value of the initial reaction velocity per enzyme at unity mole fraction of substrate, v_0, and the parameter $N_s k_i$ (defined below). Values of v_0, in the presence and absence of competitive inhibitors, were measured in the presence of 5 μg/mL polymyxin B sulfate (Sigma) as described previously (Jain et al., 1991b). Polymyxin B sulfate induces rapid intervesicle exchange of DMPM so that the initial mole fraction of substrate of unity is maintained for a prolonged reaction time. The reaction progress curves on DMPC vesicles (Apitz-Castro et al., 1982; Upreti & Jain, 1978) and on covesicles of OPPC/DOPA (Ghomashchi et al., 1991) were monitored as described.

Substrate Specificity Studies. The substrate specificity of hnps-PLA2 was examined using the double-radiolabel approach described previously (Ghomashchi et al., 1991). Vesicles containing radiolabeled lipids were prepared by dissolving 5.4 mg of OPPC, 0.6 mg of DOPA, approximately 3.6 μCi of ^{3}H-substrate, and approximately 1–2 μCi of ^{14}C-substrate in $CHCl_3$/MeOH (1:1). The solvent was evaporated under a stream of N_2 and then in vacuo for 2 h. To the lipid film was added 0.6 mL of deionized water, and the suspension was sonicated as described (Jain & Gelb, 1991). The hydrolysis of these mixed-lipid vesicles in the scooting mode was followed by the pH-stat method. Each reaction mixture contained 0.6 mg of total phospholipid in 4 mL of 1 mM NaCl and 2.6 mM $CaCl_2$, pH 8.0, 21 °C. The reactions were quenched by addition of 1 mL of 0.5 M EDTA at times corresponding to approximately 50% of the maximal reaction extent or several minutes after the reaction progress had ceased. Samples were extracted and analyzed as described previously to obtain the relative k_{cat}/K_M^* values (Ghomashchi et al., 1991).

The specificity studies with DMPS and DMPG were done using a gas chromatographic method. The mixed phospholipid vesicles were prepared as described above for the double-radiolabeled experiments. Each enzymatic reaction contained 4 mg of total phospholipid (0.4 mg DOPA, 3.6 mg OPPC, 77 μg DMPS or DMPG, and 87 μg SAPC), in 4 mL of 1 mM NaCl and 2.6 mM $CaCl_2$ at pH 8.0 and 21 °C. Reactions were quenched at about 20–30% of the full reaction extent using the same protocol described for the double-radiolabel experiments except that an internal standard was added (8 μg of heptadecanoic acid). After the elution of the fatty acid from the silica column, the sample was dried down with a stream of nitrogen in a screw cap vial with a Teflon lined cap, and 14% BF_3 in methanol (Sigma) was added. The sample was heated in a boiling water bath for 2 min, then water (1 mL) and pentane (1 mL) were added, and the organic extract was dried down to about 20 μL with a stream of nitrogen. The

[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; DOPA, 1,2-dioleoyl-*sn*-glycerol 3-phosphate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; HDNS, *N*-dansylhexadecylphosphoethanolamine; hnps-PLA2, recombinant human nonpancreatic phospholipase A_2; HPC, hexadecylphosphocholine; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; SAPC, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; SAPI, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphoinositol; SAPC, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine. For all radiolabeled substrates, the *sn*-2 fatty acid contains either ^{14}C (C_1 position) or ^{3}H (C_9, C_{10} positions).

sample was analyzed by gas chromatography using a 30-m Supelcowax 10 column (Supelco). The ratio of (k_{cat}/K_M*) values for the two competing substrates was estimated by dividing the mole ratio of fatty acid products at partial reaction completion by the mole ratio of the two phospholipid substrates present in the vesicle at time zero.

Binding of hnps-PLA2 to Vesicles. Binding of hnps-PLA2 to DTPM or DTPC vesicles containing 2.5 mol % HDNS was characterized by monitoring the increase in fluorescence emission intensity of the dansyl fluorophore as a function of the hnps-PLA2 concentration. Fluorescence measurements were made on an SLM 4800S instrument with excitation at 345 nm and emission at 490 nm with both slit widths set at 4 nm (Jain & Vaz, 1987). The binding of hnps-PLA2 to DMPC vesicles containing 18% reaction products was examined by measuring v_0 as a function of the bulk concentration of vesicles (Jain & Jahagirdar, 1985a).

Protection from Alkylation Studies. The half-time for alkylation of the active site histidine residue of hnps-PLA2 by phenacyl bromide (Aldrich, recrystallized) both in the presence and in the absence of a ligand was carried out as described previously (Jain et al., 1991d) but with the following modifications. HPC (5 mM) was used as a neutral diluent, and the alkylation reactions were carried out in buffer containing 50 mM sodium cacodylate (pH 7.3), 50 mM NaCl, 2 mM $CaCl_2$, 1 mM phenacyl bromide, and 1 mg/mL human γ-globulin (Sigma). The γ-globulin is needed to prevent the nonspecific absorption of the hnps-PLA2 to the glass walls of the reaction vessel.

RESULTS

Interfacial Equilibrium Dissociation Constants. As reported previously (Jain et al., 1991d), the equilibrium constant for the dissociation of a ligand from the active site of the phospholipase A_2 bound to the interface of a neutral diluent can be determined by monitoring the rate of alkylation of the active site histidine residue both in the presence and in the absence of the ligand. A neutral diluent is defined as an amphiphile that forms an aggregate to which phospholipase A_2 can bind, and molecules of neutral diluent have no measurable affinity for the active site of the enzyme so that the active site is occupied only by solvent molecules. With these conditions satisfied, the dissociation constant for the enzyme–ligand complex at the interface is given by the mole fraction of ligand in the interface that increases the half-time for the inactivation by a factor of 2.

For the present studies with hnps-PLA2, HPC was selected as a neutral diluent. It has already been shown the hnps-PLA2 binds to micelles of HPC with a dissociation constant in terms of HPC molecules of 2 mM (Franken et al., 1992). In the present studies it was found that the half-time for alkylation of hnps-PLA2 in the aqueous phase by phenacyl bromide (4.9 min) was similar to that for the enzyme bound to the neutral diluent micelles (3.0 min). From the relative half-times for alkylation of the enzyme bound to HPC micelles in the absence (t_0) and in the presence (t_L) of an active site ligand present in the interface at a mole fraction X_L, it is possible to obtain the value of the equilibrium dissociation constant for the ligand–enzyme interaction (Jain et al., 1991d).

Equilibrium dissociation constants for calcium (K_{Ca}*), the substrate analogs DTPM and DTPC [K_S*(DTPM) and K_S*(DTPC)], and the reaction products of DMPM and DMPC hydrolysis [K_P*(DMPM) and K_P*(DMPC)] are given in Table I. For these constants, the asterisks designate that the enzyme–ligand complex is in the interface. The significance of these equilibrium parameters is evaluated in terms of the kinetic results discussed below. The value of K_{Ca}* reported in Table I is similar to that reported for the recombinant hnps-PLA2 in the absence of an interface (Franken et al., 1992) or when acting on detergent–phospholipid mixed micelles (Reynolds et al., 1992).

Reaction Kinetics for the Action of hnps-PLA2 on DMPM Vesicles in the Scooting Mode. As previously described in detail, the analysis of phospholipase A_2 in the scooting mode allows for the experimental determination of the kinetic constants for the enzyme in the interface (Berg et al., 1991). In the scooting mode, the enzyme remains tightly bound to the vesicle surface and the lipolysis occurs in a processive fashion in which all of the substrate in the outer layer of the vesicles is hydrolyzed without desorption of the enzyme from the interface. During the reaction, the vesicle remains intact and the phospholipids in the inner layer do not flip to the outer layer (Jain et al., 1986). This type of behavior has been observed for several secreted phospholipases A_2 isolated from pancreatic tissues as well as insect and snake venoms acting on negatively charged DMPM vesicles (Jain et al., 1991a) or on covesicles of OPPC and DOPA (Ghomashchi et al., 1991). On small sonicated DMPM vesicles, a first-order reaction progress curve is observed in which the velocity, or slope at each time point in the reaction progress, decreases with time. This is because, in small vesicles, the mole fraction of substrate in the interface is decreasing rapidly (Berg et al., 1991). The reaction progress ceases when all of the substrate in the outer layer of the enzyme-containing vesicles has become hydrolyzed. A first-order curve is observed with calcium concentrations less than 1 mM where the fusion of DMPM vesicles does not occur to any significant extent during the time period of the reaction progress (Berg et al., 1991).

The reaction progress curve for the action of the hnps-PLA2 on small sonicated vesicles of DMPM in the presence of 0.6 mM $CaCl_2$ is at variance with these previous studies (Jain et al., 1991a) in that a slow steady-state rate of hydrolysis persists at the end of the initial rapid phase of hydrolysis (Figure 1, curve a). During the initial phase of the progress curve, the contribution from the slow phase to the reaction velocity is less than 5%. Although the original definition of scooting mode hydrolysis is that the enzyme hydrolyzes all of the available substrate without desorption, this term will be used in the present study with the hnps-PLA2 since the degree of processivity for this enzyme is high. As shown in Figure 1 (curve b), by subtracting the contribution from the slow phase (Jain & Berg, 1989), the overall progress curve could be fitted to the integrated Michaelis–Menten equation (Berg et al., 1991) to obtain the initial velocity per enzyme, v_0, and $N_s k_i$ (Berg et al., 1991), which are listed in Table I. Here, N_s is the total number of substrates hydrolyzed per enzyme molecule for the entire reaction progress curve under the condition that the number of vesicles exceeds the number of enzymes so that the vesicles contain at most 1 bound enzyme. N_s is also equal to the number of DMPM molecules in the outer layer of a vesicle (Jain et al., 1991a). The parameter k_i is the exponential relaxation constant for the first-order reaction progress curve.

Obtaining the value of v_0 for the hnps-PLA2 from the initial portion of the first-order reaction progress curve deserves further comment. With other phospholipases A_2, such as the pig pancreatic enzyme, that have a K_{Ca}* of about 0.2 mM, it is difficult to obtain the true v_0 from the initial portion of the first-order reaction progress curve. This is because the time period in which the mole fraction of DMPM is 1 is only

Table I: Rate and Equilibrium Parameters for the Hydrolysis of DMPM and DMPC Vesicles by hnps-PLA2[a]

parameters	definition	value	$[CaCl_2]$, mM	method
K_{Ca}*	equilibrium constant for dissociation of Ca^{2+} from enzyme in the interface	1.3 mM (0.16)[b]		protection
K_{Ca}*	same as above	0.95 mM (0.17)[c]		intial slope
K_S*(DTPM)	equilibrium constant for dissociation of the DTPM substrate analogue from enzyme in the interface	>1 mole fraction (0.017)[b]	2	protection
K_S*(DTPC)	equilibrium constant for dissociation of the DTPC substrate analogue from enzyme in the interface	0.42 mole fraction (0.067)[b]	2	protection
K_P*(DMPM)	equilibrium constant for dissociation of products of DMPM hydrolysis from enzyme in the interface	0.12 mole fraction (0.025)[b]	2	protection
K_P*(DMPC)	equilibrium constant for dissociation of products of DMPC hydrolysis from enzyme in the interface	>1 mole fraction	2	protection
v_0	initial velocity at unity mole fraction substrate	45 s^{-1}	0.6	eq 12[c]
v_0	same as above	100 s^{-1} (320)[c]	6	initial slope
$N_s k_i$	apparent second-order rate constant $[k_{cat} \times [K_M^*(1 + 1/K_P^*)]^{-1}]$ for the reaction in the presence of product inhibition; N_s is the number of phospholipids in the outer layer of a vesicle and k_i is the exponential relaxation constant for the first-order reaction progress curve	6 s^{-1} (35)[c]	0.6	eq 12[c]
K_M*(DMPM)	Michaelis constant for DMPM interacting with enzyme in the inteface	4 mole fraction (0.3)[c]	0.6	eq 2
K_M*(DMPM)	same as above	1–2.5 mole fraction (0.25–0.68)[b]	6	eq 3[d]
K_M*(DMPM)	same as above	3.5 mole fraction (0.3)[c]	0.6	eq 3[e]
K_I*(MJ33)	equilibrium constant for dissociation of inhibitor from enzyme in the interface	0.14 mole fraction (0.0008)[f]	6	protection
K_I*(MJ45-16)	same as above	0.01 mole fraction (0.0023)[f]	6	protection
K_I*(MJ78)	same as above	0.016 mole fraction (0.0004)[f]	6	protection
K_I*(MG14)	same as above	0.03 mole fraction (0.0011)[b]	6	protection
K_I(HK40)	same as above	0.0045 mole fraction	6	protection
k_{cat}	velocity on DMPM vesicles calculated from the Michaelis–Menten equation using the experimental values of v_0 and K_M*	>200 s^{-1} (400)[c]	6	eq 1
K_d*(DMPC)	equilibrium constant for dissociation of the enzyme from the interface of DMPC vesicles	>35 mM	1	g
K_d*(DMPC/ 18% products)	equilibrium constant for dissociation of the enzyme from the interface of DMPC vesicles containing 18 mol % products of DMPC hydrolysis	0.03 mM (0.26)[h]	6	h

[a] The numbers in parentheses are for the pig pancreatic PLA2 (in the same units). [b] Jain et al., 1991d. [c] Berg et al., 1991. [d] By adding competitive inhibitors to DMPM. [e] By adding HPC to DMPM. [f] Jain et al., 1991d. [g] By monitoring v_0 as a function of the bulk concentration of DMPC. [h] Jain & Jahagirdar, 1985a.

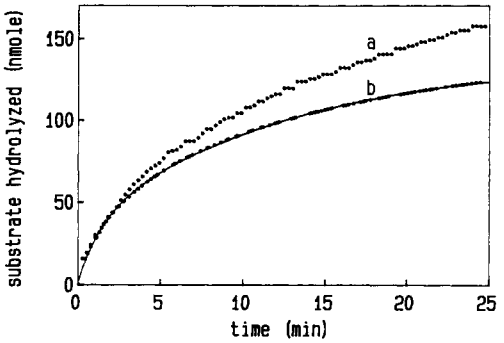


FIGURE 1: (a) Reaction progress curve for the action of hnps-PLA2 on small sonicated vesicles of DMPM. The reaction mixture at pH 8.0 and 21 °C contained 4 mL of 0.6 mM $CaCl_2$, 1 mM NaCl, 0.2 mM DMPM, and 0.4 μg of hnps-PLA2. (b) The computed curve after subtraction of the product formed during the slow-hopping phase seen at longer reaction times. The superimposed line is the fit of the data to the integrated Michaelis–Menten equation adapted for interfacial catalysis [eq 12 of Berg et al. (1991)].

a few seconds (Berg et al., 1991). Since the K_{Ca}* for the hnps-PLA2 is greater than 0.6 mM (Table I), the turnover number of the enzyme in the presence of 0.6 mM $CaCl_2$ is slow enough to permit the estimation of v_0 (Table I) from the initial portion of the reaction progress curve (Figure 1, curve b).

Addition of polymyxin B sulfate to the reaction mixture during the slow phase leads to an immediate reinitiation of the fast phase (not shown). Since polymyxin B sulfate catalyzes intervesicle phospholipid exchange (Jain et al., 1991b), the apparent activation by this agent is most likely due to the replenishment of substrate in the enzyme-containing vesicles. This results also establishes that the slowing of the reaction progress is not due to enzyme inactivation. Addition of a second portion of enzyme during the slow phase leads to a doubling of the slow-phase reaction velocity, and a new fast phase is not seen (not shown). This result suggests that the second portion of hnps-PLA2 binds preferentially to the product-containing vesicles. The presence of 50 mM NaCl in the reaction mixture leads to a 3-fold increase in the amplitude of the fast phase and a 1.5-fold increase in the slope of the slow phase (not shown). This "activating" effect of salt has been previously observed in the action of the pig pancreatic enzyme acting on DMPM vesicles, and it likely arises from

the ability of salt to promote the desorption of the enzyme from the interface (Jain et al., 1986).

The value of K_{Ca}* can also be estimated from the dependence of v_0 on the calcium concentration. The value of v_0 is given by the Michaelis–Menten equation for the action of the enzyme at the interface (Berg et al., 1991):

$$v_0 = \frac{k_{cat}}{1 + K_M^*} \quad (1)$$

Here, k_{cat} and K_M* are the interfacial turnover number and Michaelis constant, respectively. The observed v_0 for the hydrolysis of DMPM vesicles shows a hyperbolic dependence on the calcium concentration (not shown), and a value of K_{Ca}* = 0.95 mM was obtained from this analysis. The maximum value of v_0 measured in the presence of 6 mM $CaCl_2$ is approximately 100 s^{-1}. This value agrees well with the value of K_{Ca}* = 1.3 mM determined by the protection method (Table I). These results suggest that the K_{Ca}* determined from the kinetic data reflects the conversion of the inactive, calcium-free enzyme bound to the interface to the active form containing bound calcium; however, other more complex explanations involving the calcium dependency of K_M* or k_{cat}, or both, cannot be ruled out. The results also suggest that calcium is not required for the binding of the enzyme to the interface.

The ratio of the kinetic parameters obtained from Figure 1 (curve b) is related to K_M* and K_P* by the equation (Berg et al., 1991):

$$\frac{v_0}{N_s k_i} = \frac{1 + 1/K_P^*}{1 + 1/K_M^*} \quad (2)$$

Equation 2 can be used together with the kinetic parameters from Figure 1 and the value of K_P* determined from the protection method (Table I) to obtain a value of K_M* = 4 mole fraction at a calcium concentration of 0.6 mM.

An independent measurement of K_M* comes from the analysis of v_0 in the presence of a competitive inhibitor according to the equation (Jain et al., 1991d):

$$\frac{(v_0)^0}{(v_o)^I} = 1 + \frac{(1 + 1/K_I^*)}{(1 + 1/K_M^*)} \frac{X_I}{(1 - X_I)} \quad (3)$$

Here, $(v_0)^0$ and $(v_0)^I$ are the initial velocities per enzyme in the absence or presence of the competitive inhibitor, respectively. K_I* is the equilibrium dissociation constant for the enzyme–inhibitor complex at the interface in units of mole fraction, and X_I is the mole fraction of the inhibitor in the interface. Using the values of K_I* for four different inhibitors (MJ45-16, MJ78, MG14, HK40) determined using the protection method (Table I) together with the values of $(v_0)^0$ and $(v_0)^I$ determined by adding the inhibitors to vesicles of DMPM, the values of K_M* = 1–2.5 mole fraction were obtained in the presence of 6 mM $CaCl_2$.

The third method for an independent estimate of the value of K_M* is to measure the change in v_0 caused by the addition of the neutral diluent HPC to vesicles of DMPM. Since HPC has no measurable affinity for the active site of hnps-PLA2, the addition of HPC to DMPM vesicles necessarily leads to a decrease in the mole fraction of DMPM in the interface. The consequence of this surface dilution of DMPM substrate is shown in Figure 2. With values of the HPC mole fraction less than 0.13, v_0 decreases slightly. This effect can be used to estimate the value of K_M* according to eq 3. This is because eq 3 includes both the effects of an additive on v_0 due to binding of the additive to the enzyme (K_I*) and due to surface

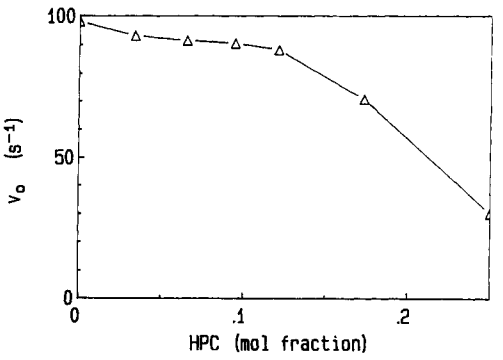


FIGURE 2: The value of v_0 for the action of hnps-PLA2 on small sonicated vesicles of DMPM containing the indicated mole fraction of HPC. The reactions were carried out in 4 mL of 1 mM NaCl, 0.6 mM $CaCl_2$, and 0.6 mg of DMPM, pH 8.0 at 23 °C.

dilution that occurs when the additive is included in the vesicle ($1 - X_I$). Thus, for HPC with $K_I^* \gg 1$, a value of K_m^* = 3.5 mole fraction at 0.6 mM $CaCl_2$ is obtained from the data in Figure 2 and eq 3.

It may also be noted that at higher mole fractions of HPC (>0.13), the initial rate of hydrolysis drops sharply (Figure 2). This is also observed for the action of the pig pancreatic phospholipase A_2 in the presence of 2-hexadecyl-*sn*-glycero-3-phosphocholine as a neutral diluent (Jain et al., 1991d). As elaborated elsewhere, this sharp decrease in the velocity is due to a decrease in the size of the substrate particles as mixed micelles of HPC and DMPM are formed (Jain et al., 1991b). This results in the enzyme being bound to aggregates containing a very small number of DMPM substrates (compared to the relatively large number of DMPM molecules present in vesicles), and the rate of catalytic turnover may now be limited by the replenishment of substrate as it is being rapidly depleted in small aggregates (Jain et al., 1991b).

Thus, by three independent methods, the value of K_M* for the action of hnps-PLA2 on DMPM vesicles is shown to be significantly above 1 mole fraction. The values of K_M* and K_{Ca}* are considerably higher than those for the pig pancreatic enzyme (Berg et al., 1991) (Table I). A similar trend is seen in the binding of the substrate analogs (DTPM and DTPC) and the reaction products (Table I).

The maximal turnover number of the hnps-PLA2 in the presence of saturating amounts of calcium and DMPM substrate, k_{cat}, is determined from eq 1 to be >200 s^{-1} (Table I). This is only a lower estimate since the maximal attainable substrate concentration of unity mole fraction is below the K_M* for the hnps-PLA2.

Reaction Kinetics for the Action of hnps-PLA2 on Covesicles of OPPC/DOPA in the Scooting Mode. The action of secreted phospholipases A_2 in the scooting mode is not restricted to vesicles of DMPM in that similar behavior is seen with zwitterionic OPPC vesicles containing small amounts of negatively charged phospholipids such as DOPA (Ghomashchi et al., 1991). Figure 3 shows that the reaction progress curve for the action of both the pig pancreatic phospholipase A_2 and the hnps-PLA2 acting on OPPC vesicles containing 15 mol % DOPA. Scooting mode hydrolysis is observed for both enzymes in that the reaction ceases after a limited amount of substrate has been hydrolyzed and no slow steady-state rate is observed at long reaction times (in contrast to the results in Figure 1). These curves were obtained with a vesicle to enzyme ratio of about 6 so that the vesicles contain at most 1 bound enzyme. Under such conditions it has been previously shown that the total product formed is equal to the moles of catalytic enzyme species multiplied by the number of substrate

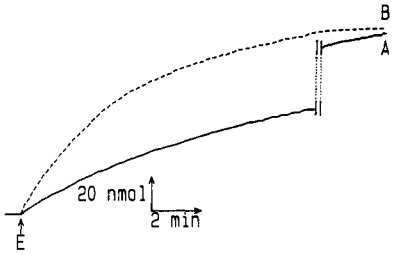


FIGURE 3: Reaction progress curves for the action of 30 pmol of hnps-PLA2 (solid line, A) and 30 pmol of the pig pancreatic enzyme (dashed line, B) on covesicles of 15 mol % DOPA in OPPC. The reactions contained 0.6 mg of total phospholipid in 4 mL of 1 mM NaCl and 1 mM $CaCl_2$, pH 8.0 at 21 °C. A 20-min break is indicated in curve A.

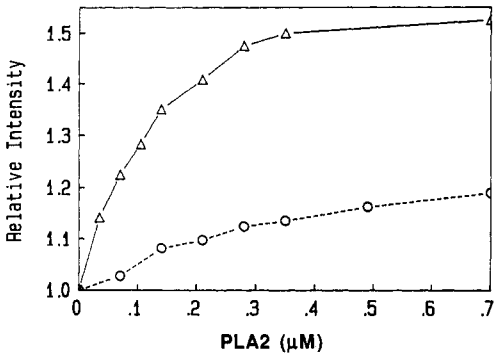


FIGURE 4: Relative fluorescence emission intensity at 490 nm as a function of the amount of hnps-PLA2 added to the vesicles of DTPM (triangles) (similar results were obtained with DTPG vesicles) or DTPC vesicles containing 15 mol % products (myristate and lyso-PC) (circles). The mixtures contained 1.5 mL of 10 mM Tris-HCl, 0.5 mM $CaCl_2$, and 0.065 mM total phospholipid containing 2.5 mol % HDNS at 23 °C.

molecules in the outer layer of the vesicles (Berg et al., 1991). This relationship has been used to demonstrate that the pig pancreatic enzyme and several other secreted phospholipases A_2 are fully active as monomers (as opposed to a higher order aggregate) (Jain et al., 1991a). Thus, the similar total product yields seen with both the pig pancreatic phospholipase A_2 and the hnps-PLA2 (Figure 3) demonstrate that the hnps-PLA2 is catalytically active as a monomer. Furthermore, the results indicate that the hnps-PLA2 preparation is devoid of significant levels of catalytically inactive protein. The curves in Figure 3 show that the time needed for the reaction to cease is significantly longer for the hnps-PLA2 compared to the pig pancreatic enzyme. This is at least partly due to the fact that the K_{Ca}* and the K_M* for the hnps-PLA2 are much larger than that for the pig enzyme and the experiments in Figure 3 were carried out in the presence of 1 mM $CaCl_2$.

Binding of hnps-PLA2 to DTPM and DTPC Vesicles. Binding of hnps-PLA2 to vesicles of the anionic and nonhydrolyzable DTPM containing 2.5 mol % of the fluorescent probe HDNS causes an increase in the fluorescence emission from the dansyl chromophore (Jain & Vaz, 1987). As shown in Figure 4, the increase in the fluorescence emission at 490 nm reaches a maximum with increasing concentrations of enzyme added to the preformed DTPM vesicles. The corresponding increase in the fluorescence intensity with zwitterionic DTPC vesicles containing 2.5 mol % HDNS was negligible (not shown), indicating that the hnps-PLA2 binds very weakly, if at all, to zwitterionic phospholipids. However, if reaction products (1:1 mixture of myristic acid and 1-myristoyl-*sn*-glycero-3-phosphocholine) are present in the DTPC vesicles at 15 mol %, the enzyme binding is detected (Figure 4). Thus, the binding of hnps-PLA2 to anionic vesicles

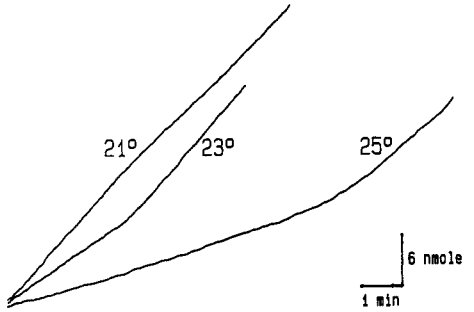


FIGURE 5: Reaction progress curves for the hydrolysis of DMPC vesicles at 21, 23, or 25 °C. The 4-mL reaction mixture at pH 8.0 contained 1 mM $CaCl_2$, 0.15 mM DMPC, and 0.3 μg of hnps-PLA2.

is of considerably higher affinity than to zwitterionic vesicles. This conclusion is also supported by kinetic experiments. Addition of hnps-PLA2 to DTPM vesicles followed by the addition of substrate vesicles (DMPM) does not lead to measurable product formation. This result shows that the enzyme is irreversibly bound to the DTPM vesicles over the time period of the experiment (1 h). In contrast, if the enzyme is first added to DTPC vesicles followed by DMPM, the reaction progress is the same as that seen with DMPM vesicles alone.

In the case of the pig pancreatic enzyme binding to DTPM vesicles, a linear initial rise in the fluorescence emission was observed which suggests that the dissociation constant is below 0.5 μM (Jain & Vaz, 1987). In contrast, the curvature in the initial portion of the binding curve (Figure 4) indicates that hnps-PLA2 binds to DTPM vesicles with lower affinity compared to the pig pancreatic enzyme.

Hydrolysis of DMPC Vesicles. The low-affinity binding of hnps-PLA2 to vesicles of zwitterionic phospholipids was also observed in kinetic experiments. As is the case with the pig pancreatic (Apitz-Castro et al., 1982) and the bee venom (Upreti & Jain, 1978) phospholipases A_2 but not with the enzyme from *Naja melanolueca* venom (Jain et al., 1982), the reaction progress curve for the hydrolysis of DMPC vesicles by hnps-PLA2 shows a latency period (Figure 5). A long latency period is observed at temperatures above the gel–fluid transition temperature, and the latency period has a minimal value at the phase transition temperature, 22 °C in this case. The onset of the steady-state hydrolysis at the end of the latency period is related to a critical mole fraction of reaction products formed (Apitz-Castro et al., 1982; Jain & Berg, 1989; Jain et al., 1982; Jain et al., 1989a). For example, the latency period is not observed with DMPC vesicles containing 15 mol % reaction products (1:1 mixture of myristic acid and 1-myristoyl-*sn*-glycero-3-phosphocholine) (not shown). This suggests that the presence of products increases the fraction of hnps-PLA2 that is bound to vesicles, and this is consistent with the direct binding studies described in the previous section. Addition of myristic acid alone to DMPC vesicles was much less effective in promoting hydrolysis by the hnps-PLA2 compared to the addition of both products. When 10 or 22 mol % myristic acid was added to DMPC vesicles, the initial rate of hydrolysis increased by 5% or 30%, respectively, of that measured when 15 mol % of a 1:1 mixture of myristic acid and 1-myristoyl-*sn*-glycero-3-phosphocholine was added to the DMPC vesicles. Similar trends have been reported for the porcine pancreatic PLA2 (Jain et al., 1982; Jain & Jahagirdar, 1985a; Jain & Yu, 1989).

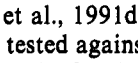
By monitoring the initial rate of hydrolysis of vesicles at 25 °C as a function of the bulk substrate concentration, the apparent dissociation constants (K_d*) for the enzyme bound

to the vesicles were estimated. In the case of pure DMPC vesicles and in the presence of 1 mM $CaCl_2$, v_0 increased linearly as the concentration of DMPC was increased from 0 to 35 mM (not shown). This indicates that $K_d^* > 35$ mM for pure DMPC vesicles. This value for the hnps-PLA2 is considerably higher than the value of $K_d^* = 10$ mM for the binding of the pig pancreatic phospholipase A_2 to vesicles of the nonhydrolyzable phospholipid DTPC (determined by fluorescence methods, (Jain et al., 1982). Binding of the hnps-PLA2 to covesicles of DMPC and 18 mol % reaction products is significantly tighter ($K_d^* = 0.03$ mM) than that measured for the pig pancreatic enzyme ($K_d^* = 0.26$ mM) (Table I). These results suggest that the presence of anionic charge in zwitterionic vesicles is a more critical requirement for hnps-PLA2 binding compared to that for the pig pancreatic enzyme.

Competitive Inhibition Studies. A number of previously reported inhibitors of phospholipases A_2 (Hope et al., 1990; Jain et al., 1991d; Oinuma et al., 1991; Yuan et al., 1990) were tested against the hnps-PLA2 by monitoring v_0 for the hydrolysis of DMPM vesicles in the scooting mode as a function of the mole fraction of inhibitor present in the vesicles. In these studies, polymyxin B sulfate was included in the reaction mixture to keep the mole fraction of DMPM close to unity. The mole fraction of inhibitor in the DMPM vesicles that causes a 50% reduction in v_0 is designated $X_I(50)$, and these are listed in Table II. It may also be noted that the values of K_I^* for four different inhibitors determined by the protection from alkylation method (Table I) are similar in magnitude to the corresponding $X_I(50)$ values. This equality is expected according to eq 3 if $K_M^* > 1$, and this result provides a fourth independent estimation of the value of this parameter.

Substrate Specificity. The ability of hnps-PLA2 to hydrolyze substrates containing different polar head groups and acyl chains was examined in a competitive fashion in which the enzyme is tightly bound to the surface of covesicles of OPPC/DOPA (Ghomashchi et al., 1991). These studies are carried out with OPPC/DOPA vesicles that are doped with small amounts of two competiting substrates. From the amounts of competing substrates in the vesicle at the beginning of the reaction and the amounts of products formed from each of the two substrate after partial hydrolysis, the ratio of k_{cat}/K_M^* values for the two competing substrates is obtained. The results are listed in Table III.

Table II: Competitive Inhibition of hnps-PLA2

inhibitor (structure)[a]	$X_I(50)$, mole fraction[b]
MJ33 ($R_1 = C_{16}H_{33}$, $R_2 = CH_2CF_3$)	0.19 (0.006)[c]
MJ45-8 ($R_1 = C_8H_{17}$, $R_2 = CH_2CH{=}CH_2$)	0.25
MJ45-10 ($R_1 = C_{10}H_{21}$, $R_2 = CH_2CH{=}CH_2$)	0.15
MJ45-12 ($R_1 = C_{12}H_{25}$, $R_2 = CH_2CH{=}CH_2$)	0.06
MJ45-14 ($R_1 = C_{14}H_{29}$, $R_2 = OCH_2CH{=}CH_2$)	0.05
MJ45-16 ($R_1 = C_{16}H_{33}$, $R_2 = CH_2CH{=}CH_2$)	0.02 (0.0023)
MJ45-18 ($R_1 = C_{18}H_{37}$, $R_2 = CH_2CH{=}CH_2$)	0.025
MJ46 ($R_1 = C_{16}H_{33}$, $R_2 = CH{=}CHCH_3$)	0.016 (0.14)
MJ48 ($R_1 = C_{16}H_{33}$, $R_2 =$ )	0.035 (0.011)
MJ78 (R_1 = 2-ethylhexyl, $R_2 = C_8H_{17}$)	0.016 (0.0004)
MJ89	0.024 (0.2)
MJ95	0.01 (0.016)
MJ96	0.13 (0.0005)
RM2	0.024 (0.0094)
MG14	0.052 (0.0034)
HK30 ($X = OCH_2CH_2NH_3^+$)	0.028
HK32 ($X = OCH_2CH_2OH$)	0.006
HK34 ($X = OCH_2CH_2CH_3$)	0.006
HK38 ($X = OCH_3$)	0.0034
HK40 ($X = OCH_2CH_3$)	0.006
HK42 ($X = CH_3$)	0.0042
HK44 ($X = CH_2CH_2CH_2CH_3$)	0.0032
HK61	0.044
all-trans-retinoic acid	no inhibition at $X_I = 0.1$
all-trans-retinol	no inhibition at $X_I = 0.1$
ellagic acid	no inhibition at $X_I = 0.05$
(*R*)-*N*-octadecyl-1-amino-2-propanol	no inhibition at $X_I = 0.05$
(*S*)-*N*-octadecyl-1-amino-2-propanol	no inhibition at $X_I = 0.05$
compound 3 of Oinuma et al. (1991)	no inhibition at $X_I = 0.05$

[a] See Figure 6 for the parent structures. [b] Since the inhibitors are added to preformed DMPM vesicles, the X_I (50)s are calculated as total moles of inhibitor divided by moles of DMPM in the outer leaflet of the vesicles. All measurements were carried out at 21 °C and pH 8.0 in 4 mL of 1 mM NaCl, 2.5 mM $CaCl_2$, 5 μg/mL polymyxin B sulfate, and 0.6 mg of DMPM. [c] The numbers in parentheses are the K_I^* values for the pig pancreatic enzyme (Jain et al., 1991c). $X_I(50)$'s for racemates are calculated based on moles of racemic inhibitor present in the vesicles.

DISCUSSION

Kinetic Properties of hnps-PLA2 on Anionic Vesicles. Numerous secreted PLA2s operate on DMPM vesicles in the scooting mode in which the enzyme never leaves the interface and the reaction ceases when all of the substrate in the outer layer of the enzyme-containing vesicle is hydrolyzed (Jain et al., 1991a). In contrast, the reaction progress curve for the hydrolysis of DMPM vesicles by hnps-PLA2 shows a slow but measurable steady-state rate (Figure 1). Such an effect could arise from the fusion of DMPM vesicles which would replenish the substrate in the enzyme-containing vesicle; however, it has already been demonstrated that fusion of DMPM vesicles does not occur with calcium concentrations less than 1 mM (Jain et al., 1986; Jain & Vaz, 1987). Induction of vesicle fusion by the addition of high calcium, 2.5 mM, or of intervesicle phospholipid exchange by addition of polymyxin B sulfate during the steady-state portion of the reaction progress curve leads to a reinitiation of the fast initial rate. These results together with the observation that the slow steady-state phase continues until all of the enzyme-accessible substrate is hydrolyzed (about 60% of the total DMPM, not shown) demonstrate that the decrease in rate is not due to enzyme inactivation or severe competitive inhibition by the reaction products. Since the rate of exchange of enzyme during the slow phase is much smaller than the time required for a single catalytic turnover (Figure 1, Table I), the enzyme displays processive behavior on DMPM vesicles.

The weaker binding of the hnps-PLA2 to anionic vesicles compared to the pig pancreatic enzyme (Figure 4) is surprising in light of the X-ray crystal structures of the two enzymes (Scott et al., 1991; Thunnissen et al., 1990; Wery et al., 1991). Compared to the pig pancreatic enzyme, the hnps-PLA2 has a much larger number of lysine and arginine residues on the surface that is thought to be in direct contact with the bilayer. In addition, the hnps-PLA2 is unique in that numerous lysine and arginine residues contribute to a dominant positive surface potential on the entire surface of the molecule. Thus, it is possible that a molecule of hnps-PLA2 bound to a DMPM vesicle in a catalytically productive manner could also bind a second DMPM vesicle to form a ternary complex. This complex may provide a pathway for the enzyme to hop to a new vesicle without the need for the enzyme to leave the vesicle

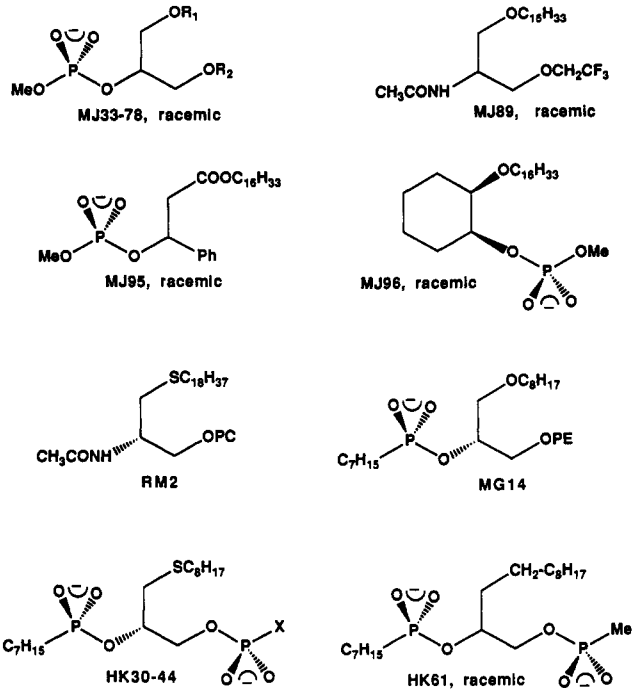


FIGURE 6: Parent structures of the competitive inhibitors listed in Table II.

Table III: Substrate Specificity of hnps-PLA2[a]

substrate	relative k_{cat}/K_M*
[^{3}H]DPPC vs [^{14}C]DPPC	1.01 ± 0.01
[^{3}H]DPPA vs [^{14}C]DPPC	1.49 ± 0.19
[^{3}H]DPPE vs [^{14}C]DPPC	1.09 ± 0.15
[^{14}C]PAPC vs [^{3}H]DPPC	0.53 ± 0.02
[^{14}C]SAPI vs [^{3}H]DPPC	0.26 ± 0.1
[^{3}H]SAPC vs [^{14}C]DPPC	0.6 ± 0.1
DMPC vs SAPC	1.6 ± 0.9
DMPS vs SAPC	3.6 ± 1.6
DMPG vs SAPC	2.7 ± 1.2

[a] The numbers represent the mean ± standard deviation from 3–5 independent trials.

and enter the aqueous phase. It is also possible that the enzyme could promote the fusion of vesicles via this ternary complex, and this could be responsible for the slow steady-state phase in the reaction progress. However, based on steric constraints, it is difficult to imagine that the enzyme could bring two vesicles in direct contact with each other. Although the results of this study show that the hnps-PLA2, like numerous other secreted PLA2s (Jain et al., 1991a), is catalytically active as a monomeric enzyme, it cannot be ruled out that a small amount of dimeric enzyme is present and this could provide a pathway for vesicle fusion. Other possible explanations for the slow intervesicle exchange are given below.

The hnps-PLA2 and numerous other secreted PLA2s hydrolyze DMPM vesicles without displaying any anomalous kinetic effects, for example, a latency period [see Berg et al. (1991), Jain and Berg (1989), Jain et al. (1991a), Jain and Vaz (1987), and Figure 1]. The maximum turnover number for the hnps-PLA2 acting on DMPM vesicles at saturating calcium and substrate is estimated to be >200 s^{-1}. This value is similar to that for the pig pancreatic enzyme (400 s^{-1}) acting on the same substrate under saturating conditions (Berg et al., 1991). The turnover number of hnps-PLA2 acting on DMPM vesicles with unity mole fraction of substrate and at saturating calcium (100 s^{-1}) is similar to the maximum values reported for the same enzyme acting on various substrates. For example, a value of 70 s^{-1} was measured for the purified hnps-PLA2 acting on radiolabeled *E. coli* membranes (Bomalaski et al., 1991; Kramer et al., 1989), and a value of 49 s^{-1} was reported for recombinant hnps-PLA2 acting on vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (Franken et al., 1992). All of these assays utilize negatively charged phospholipids.

Kinetic Properties of hnps-PLA2 on Zwitterionic Vesicles with or without Anionic Phospholipids. With DMPC vesicles, the turnover numbers of hnps-PLA2 are considerably smaller than for DMPM vesicles (Figures 1 and 5). A relatively slow turnover on phosphatidylcholine vesicles has also been reported previously (Franken et al., 1992; Hayakawa et al., 1988). Additional results described in this paper demonstrate that the slow turnover on phosphatidylcholine vesicles is due to the poor binding of the enzyme to the interface and that the anionic reaction products promote the interfacial binding (Figure 4).

On the basis of these results, it is incorrect to conclude that the hnps-PLA2 has an intrinsic preference for anionic phospholipids. The results of Table III demonstrate that the enzyme does not significantly discriminate between zwitterionic and anionic phospholipid substrates under conditions in which the enzyme is fully bound to the vesicle. These substrate specificity studies were carried out with the hnps-PLA2 operating in the scooting mode. It has already been reported that the scooting mode analysis can be used to obtain the relative k_{cat}/K_M* values for competing substrates (Ghomashchi et al., 1991). Furthermore, the data in Table III show that phospholipids containing most of the naturally-occurring polar head groups are all hydrolyzed with similar relative k_{cat}/K_M* values. Thus, the higher activity of the hnps-PLA2 toward phosphatidylethanolamine vs phosphatidylcholine vesicles (Franken et al., 1992; Hayakawa et al., 1988; Kramer et al., 1989) is most likely due to different fractions of enzyme bound to the interface. Indeed, the preference for phosphatidylethanolamine over phosphatidylcholine becomes insignificant when the hnps-PLA2 acts on covesicles of these two substrates (Schalkwijk et al., 1989). On the basis of these results, it is incorrect to classify the hnps-PLA2 as a phosphatidylethanolamine-specific enzyme.

Comparison of the Equilibrium Parameters of hnps-PLA2 to Other Secreted PLA2s. There are significant quantitative differences between the properties of hnps-PLA2 and other secretory enzymes. The affinities of the hnps-PLA2 for calcium ions (K_{Ca}*), substrate (K_M*), substrate analogs (K_S*), and reaction products (K_P*) are low compared to other secreted phospholipases A_2 [Table I, and see, for example, Berg et al. (1991), Dennis (1983), Dupureur et al. (1992), and Verheij et al. (1981)].

Phospholipases A_2 bound to the interface are present mainly in three forms, E*, E*S, and E*P. It is likely that the E* to E*S step requires calcium. This is based on the suggestion that the substrate interacts directly with the active site calcium (Scott et al., 1990b; Thunnissen et al., 1990). The calcium dependence of the substrate–enzyme interaction is also suggested by the observation that the apparent K_M* becomes smaller as the calcium concentration is increased (Table I). The overall equilibrium that describes the binding of the enzyme from the aqueous phase to the phospholipid vesicle is not only composed of the equilibrium constant for the E to E* step (K_d*) but also depends on the equilibrium constants that determine the relative amounts of the different interfacial enzyme species (K_M* and K_P*). Thus, the slow intervesicle exchange of the hnps-PLA2 may arise from the fact that the

K_M* is significantly higher than that for the pig pancreatic phospholipase A_2 (Table I).

The K_{Ca}* for the hnps-PLA2 is also high in comparison to other phospholipases A_2 [Table I and Franken et al. (1992)]. Because of vesicle fusion, it is only possible to examine the issue of intervesicle exchange using small sonicated vesicles of DMPM while keeping the calcium concentration below the K_{Ca}* for the hnps-PLA2. Thus the intervesicle exchange may be due, in part, to the likely possibility that the E* to E*S step is calcium dependent.

Interestingly, addition of a second aliquot of hnps-PLA2 to the reaction vessel during the slow steady-state phase leads only to a doubling of the reaction rate rather than to a new round of rapid initial hydrolysis. This results strongly suggests that the newly added enzyme preferentially binds to vesicles that have become substantially hydrolyzed by the first aliquot of enzyme. This may in part be due to the fact that the products of DMPM hydrolysis bind significantly tighter to the enzyme than the substrate (>10-fold, Table I) and thus the shift from the species E*S to E*P as the product accumulates may help to hold the enzyme on the interface. Such behavior is not seen with the pig pancreatic PLA2 (Jain et al., 1986).

Since the X-ray structures of the hnps-PLA2 and the pig PLA2 complexed with phospholipid analogues are known (Scott et al., 1991; Thunnissen et al., 1990), it is possible to examine the amino acid residues that contact the bound phospholipid analog in an attempt to understand why ligands bind relatively weak to the hnps-PLA2. The identity and position of many of the residues that contact the alkyl chains of the bound phospholipid are the same in both enzymes. There are two exceptions. Leucine-19 of the pig enzyme contacts the methyl group of the phospholipid analogue at the end of the *sn*-1 chain, whereas the hnps-PLA2 has an alanine at this position which does not contact that bound ligand. The β-CH_2 and γ-CH of asparagine-23 of the pig enzyme contacts the middle of the *sn*-1 chain of the bound analogue, whereas the hnps-PLA2 has a glycine at this position. These two additional hydrophobic interactions may be the reason why phospholipid analogs bind relatively tightly to the pig PLA2. The only other difference is that tyrosine-69 of the pig enzyme donates a hydrogen bond to one of the nonbridging oxygens of the *sn*-3 phosphate of the phospholipid analogue, and this structural role is fulfilled by a lysine residue in the hnps-PLA2. The influence of this difference on the ligand binding affinities is difficult to predict. The relatively weak binding of calcium to the hnps-PLA2 may be due to the fact that this enzyme has an exceptionally large number of positively charged amino acids, and this may weaken calcium binding by electrostatic repulsion.

Possible Physiological Relevance of the Kinetic and Equilibrium Parameters for the hnps-PLA2. The kinetic and equilibrium properties of hnps-PLA2 suggest that this enzyme is best able to operate on negatively charged interfaces such as bacterial membranes or on activated platelets that have a high concentration of phosphatidylserine on their extracellular membrane faces. In addition, the affinity of the hnps-PLA2 for phosphatidylcholine vesicles or HPC micelles is significantly weaker than that for the pig pancreatic enzyme [Table I and Franken et al. (1992)]. These results provide a basis for the observations that the enzyme from macrophages, apparently identical to the hnps-PLA2, can be preferentially assayed with autoclaved *E. coli* membranes (Weiss et al., 1991). These results also support the hypothesis that the hnps-PLA2 may play a role in the degradation of foreign bacteria or in the production of free arachidonate for the biosynthesis of eicosanoids in cells such as activated platelets. It may be surmised that secretion of this enzyme by inflammatory cells is the first line of defense. However, if hnps-PLA2 is secreted in larger quantities, the enzyme could also damage the membranes in the host environment.

Competitive Inhibitors of the hnps-PLA2. A number of previously reported competitive inhibitors of secreted phospholipases A_2's were also found to inhibit the action of the hnps-PLA2 on DMPM vesicles in the scooting mode (Table II). This result, together with the fact that the inhibitors protect the active site of the enzyme from alkylation by phenacyl bromide (Table I), establishes that these compounds are true competitive inhibitors of the enzyme (Jain et al., 1989b).

Retinoids, which have been previously shown to inhibit the hnps-PLA2-catalyzed hydrolysis of radiolabeled *E. coli* membranes and to display antiinflammatory activity (Hope et al., 1990), do not significantly inhibit the enzyme under conditions in which it is tightly bound to the interface (Table II). Furthermore, the retinoids do not protect the active site of the enzyme from alkylation (Table II). These results suggest that the reported inhibition by retinoids may be due to the effect of these compounds on the physical state of the phospholipid interface, which may lead to a desorption of the enzyme from the interface (Jain & Jahagirdar, 1985b; Jain et al., 1989b; Jain et al., 1991d). It should be noted that, in the previous work (Hope et al., 1990), the retinoid *all-trans*-retinoic acid was found to produce a 50% decrease in the enzymatic velocity at a concentration of 10 μM, a value that is not much lower than the concentration of substrate phospholipid used in the assay (18 μM). On the basis of the results of the present studies, it is highly unlikely that the antiinflammatory activities of the retinoids are due to the inhibition of the hnps-PLA2. Similar conclusions (Table I) are found for ellagic acid and (*R*)- and (*S*)-*N*-octadecyl-1-amino-2-propanol—both compounds have been previously reported to inhibit secreted PLA2s (Glaser et al., 1991; Davis et al., 1988; Dieter & Fitzke, 1991). A benzenesulfonamide, which has been reported to inhibit the phospholipase A_2 isolated from rabbit heart membranes (Oinuma et al., 1991), did not inhibit the action of the hnps-PLA2 in the scooting mode (Table II). The interpretation of this result is not yet possible since the phospholipase A_2 present in the rabbit heart membranes has not yet been characterized.

Significant differences exist in the potency of the competitive inhibitors listed in Table II depending on the source of the enzyme. For example, the K_I* for MJ33 acting on the pig pancreatic enzyme is 32-fold lower than that for the hnps-PLA2 (Table I) whereas others show a reversal in selectivity (i.e., MJ46 and MJ89). Thus, it should be possible to use these inhibitors to ascertain whether the hnps-PLA2 plays a role in complex biochemical processes. The inhibitors MG14, HK38, and HK44 are among the most potent known inhibitors of the hnps-PLA2, producing inhibition under conditions of one inhibitor per several hundred substrates in the vesicle. Under these conditions, there is little concern that the inhibitors will lead to nonspecific effects arising from the perturbation of the membrane structure. Furthermore, many of the inhibitors in Table II are completely resistant to hydrolytic breakdown by all types of hydrolyases, and this is important in the context of using these compounds as research tools.

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