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Competitive Inhibition of Phospholipase A_2 in Vesicles†

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ABSTRACT: Kinetic studies with phospholipase A_2 are complicated by the fact that binding of the enzyme to the interface precedes catalytic turnover. This difficulty can be overcome by monitoring interfacial catalysis in the scooting mode where the enzyme does not leave the interface. The kinetics of inhibition by transition-state analogues shows that specific competitive inhibition is the result of competition between inhibitor and substrate for the binding to the active site of the enzyme in the interface. Several lipophilic compounds, including alkanols, substituted butyrophenones, aristolochic acid, and mepacrine apparently reduce the rate of lipolysis by promoting the desorption of phospholipase A_2 from the interface.

The products of hydrolysis of phospholipids in biomembranes by phospholipase A_2 (PLA) are biosynthetic precursors of several regulatory molecules in a wide range of tissues under a variety of physiological and pathological conditions. For example, arachidonic acid is the precursor for eicosanoids (Irvine, 1982) and lysophosphatidylcholine for platelet activating factor (Snyder, 1985). It is believed that inhibitors of PLA could exhibit a range of desirable pharmacological effects (Dennis, 1987). Interfacial catalysis by PLA is quantitatively described in terms of Figure 1 (Verger et al., 1973; Jain et al., 1986a; Jain & Berg, 1989). The enzyme in the water layer

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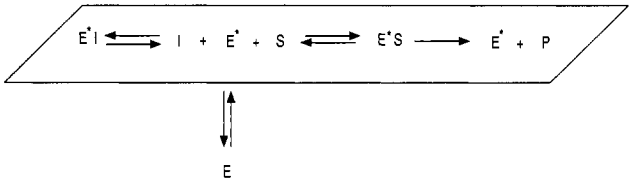


FIGURE 1: Interfacial catalysis by PLA. The enclosed box represents the membrane bilayer. Symbols: E, enzyme in the aqueous phase; E*, enzyme bound to the bilayer; S, substrate in the bilayer; E*S, enzyme–substrate complex in the bilayer; I, inhibitor in the bilayer; E*I, enzyme–inhibitor complex in the bilayer.

(E) binds to the bilayer (E*). Once in the interface, the enzyme can bind the substrate at the catalytic site to give the E*S complex which goes on to products. E* is recycled either within the bilayer (scooting) or through the aqueous phase (hopping). Under optimal conditions for catalysis, scooting predominates and hopping tends to slow down the catalytic turnover due to the slow nature of the desorption and resorption of enzyme intrinsic in the E to E* step (Jain et al., 1988). Thus attachment of the enzyme to the interface is a step distinct from catalytic turnover. This is also implicit in the hypothesis that the interfacial recognition site on the enzyme is distinct from the catalytic site (Verger et al., 1973).

Many compounds have been reported that inhibit the action of PLA including lipocortin (Flower et al., 1984), alkanols (Jain, 1982), cationic amphiphiles (Goldhammer et al., 1975), and substituted butyrophenones (Wallach & Brown, 1981). Most, if not all, of the problems associated with previous inhibition studies on PLA can be attributed to nonspecific effects in which the additive promotes the desorption of bound enzyme (Jain, 1982; Jain & Jahagirdar, 1985; Jain et al., 1984; Davidson et al., 1987). Activation is also possible if the additive shifts the E to E* equilibrium in favor of E*. It is likely that compounds which lower the activity of PLA by influencing the E to E* equilibrium will not be sufficiently potent and selective for inhibition in vivo because they will have to be present in sufficient quantity to change the organization of the interface and they could perturb other membrane-bound enzymes. On the other hand, useful inhibition could be achieved with compounds that bind tightly to the active site of the enzyme and compete with substrate for the binding to E*. With these concerns in mind, we set out to develop a general procedure for testing inhibitors of interfacial catalysis that will unambiguously identify those compounds that function as specific tight-binding inhibitors of PLA.

Materials and Methods

Materials. 1,2-Dimyristoylphosphatidylmethanol and 1,2-ditetradecylphosphatidylmethanol were prepared as described (Jain et al., 1986a). The phosphonate-containing inhibitors were prepared as described (Yuan et al., 1989) and shown to be pure by thin-layer chromatographic and combustion analyses. The structures were verified by accurate mass–liquid secondary ion mass spectrometry and high-resolution ^1H and ^{31}P NMR. Aristolochic acid and mepacrine were purchased from Sigma and Aldrich, respectively. PLA's from *Naja naja naja* and *Agkristodon halys blomhoffii* (basic) venoms were purified as described (Hazlett & Dennis, 1985; Hanahan et al., 1980). Porcine and human pancreatic PLA's were generous gifts from Dr. H. M. Verheij (State University of Utrecht).

Kinetic Studies. Small unilamellar vesicles of dimyristoylphosphatidylmethanol were prepared as follows: The desired amount of solid phospholipid was suspended in pure water by briefly vortexing. The cloudy suspension was sonicated in a bath sonicator (Lab Supplies Model G112SPIT) until the solution became clear (typically 2–3 min). The sonicated vesicles were stored at room temperature for up to several hours. Prior to a kinetic run, the desired amount of vesicles in water was mixed with a solution of $CaCl_2$ in water to give the desired final phospholipid and $CaCl_2$ concentrations. The solution of $CaCl_2$ was preequilibrated to pH 8.0 in a pH stat (Radiometer ETS822 system) prior to the addition of vesicles. The reaction was initiated by the addition of enzyme (typically 0.05 μg). The reaction was maintained at pH 8.0 by continuous pH-stat titration with 3 mM NaOH. All reactions were carried out in a thermostated vessel at 21 °C.

Vesicles containing inhibitor were prepared by delivering the desired amounts of substrate and inhibitor stock solutions to tubes, followed by solvent removal in vacuo and sonication as described above. Reaction volumes and concentrations of phospholipids and calcium are given in Table I and the figure legends.

Fluorescent Binding Studies. Binding of porcine pancreatic PLA to sonicated vesicles of the nonhydrolyzable phospholipid analogue ditetradecylphosphatidylmethanol was studied by using fluorescence titration as described previously (Jain et al., 1986b). Briefly, experiments were carried out by titrating a solution of porcine pancreatic PLA (typically 3 μM) with phospholipid (0–500 μM) in 20 mM Tris and 0.6 mM $CaCl_2$, pH 8.0, with sonicated vesicles. The fluorescence emission was monitored in a SLM 4800S spectrofluorometer with emission at 333 nm, excitation at 285 nm, and slit widths of 4 nm.

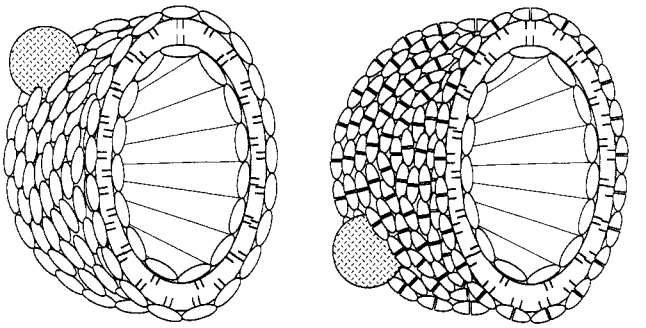


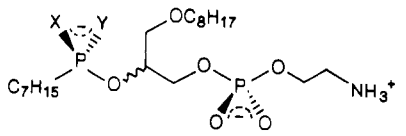
FIGURE 2: Schematic representation of interfacial catalysis by PLA in the scooting mode. The enzyme (small hatched sphere) binds tightly to the vesicle and hydrolyzes all of the substrate in the outermonolayer. After the completion of the reaction, the enzyme still remains in the vesicle. The vesicles maintain their physical structure even after all of the substrate is hydrolyzed. The vesicles have been sliced in half to show their inner and outer surfaces. The glycerol backbone and polar head groups of the phospholipid are drawn as ellipses and the fatty acid chains as lines.

Results

Interfacial catalysis in the scooting mode eliminates the contribution of the E to E* step in the steady-state turnover. This is accomplished by working under conditions in which the enzyme never leaves the interface. We have previously shown that PLA binds irreversibly to vesicles of 1,2-dimyristoylphosphatidylmethanol and undergoes several thousand catalytic turnovers without leaving the interface (Jain et al., 1986a; Jain & Berg, 1989). This processive reaction continues until all of the substrate in the outer monolayer of enzyme-containing vesicles is hydrolyzed. Throughout the entire reaction course, the vesicles retain their overall physical structure (trapped aqueous medium) and the enzyme remains bound (Jain et al., 1986a). This is illustrated schematically in Figure 2.

The inhibitors described in the present study are phospholipid analogues in which the enzyme-susceptible ester has been replaced with a phosphonate group (Table I). These com-

Table I: Mole Fraction of Inhibitor in Vesicles for 50% Inhibition of PLA[a]



compd	structure	confign at glycerol	$X_i(50)$
1	X = Y = O	*sn*-3	0.003
2	X = Y = O	*sn*-1	>0.2
3	X = O; Y = S	*sn*-3	0.005
4	X = S; Y = O	*sn*-3	0.135

[a] 1,2-Dimyristoylphosphatidylmethanol (0.8 mg) vesicles containing various amounts of inhibitor were added to 4 mL of $CaCl_2$ (6 mM) in water. The reaction was followed in a pH stat as described under Materials and Methods. The reactions were started by the addition of enzyme (0.05 μg).

pounds are predicted to bind tightly to the catalytic site of PLA because they mimic the geometrical features of the putative tetrahedral intermediate in the esterolysis reaction. Vesicles of 1,2-myristoylphosphatidylmethanol containing various amounts of inhibitor were prepared as described under Materials and Methods and in Table I. In the absence of inhibitor, an initial linear reaction velocity is seen that persists for several minutes. The presence of compound **1** in the vesicles caused a decrease in the initial reaction velocity without changing the total extent of hydrolysis. Table I gives the mole fraction of inhibitor in the substrate required to reduce the initial velocity by 50%.

Several observations suggest that the enzyme is operating in a scooting mode of catalysis both in the presence and in the absence of inhibitor. The initial rate of the enzymatic reaction does not change even when the concentrations of substrate and inhibitor are lowered over 10-fold while the relative amounts are kept the same. This is shown in Figure 3 both in the presence and in the absence of inhibitor **3**. Thus the percent inhibition is a function of the mole fraction of inhibitor in the substrate rather than the bulk concentration (molarity) of inhibitor in the solution. This would be expected if and only if the enzyme and inhibitor were tightly bound to the substrate interface and if these species where not present in the aqueous phase to any significant extent. The effect of **1** on the binding of enzyme to vesicles was also studied. Porcine pancreatic PLA contains a single tryptophan (Trp 3) in the interfacial recognition region, which is about 8 Å away from the catalytic site. Trp 3 can be used as an intrinsic fluorescence probe to monitor the binding of enzyme to vesicles (Jain et al., 1986b). The binding of enzyme to vesicles of the nonhydrolyzable substrate analogue 1,2-ditetradecylphosphatidylmethanol was measured as described under Materials and Methods. The binding was not noticeably altered (reduced or increased) by the presence of **1** in the bilayer when tested at a mole fraction of 0.05 where the rate of hydrolysis is 95% inhibited. This provides direct evidence that the presence of **1** in vesicles does not promote the desorption of bound enzyme as a means of inhibition.

Since the enzyme retains its affinity for the interface, the observed inhibition must be due to an interaction of the inhibitor with E* (Figure 1). Compound **1** is an effective inhibitor of porcine pancreatic PLA, producing 50% inhibition when present in vesicles at a mole fraction of 0.003 (Table I). The mole fraction of **1** required for 50% inhibition of PLA from human pancreas is 0.0013, corresponding to only one inhibitor molecule for every 770 substrate molecules in the vesicle. Compound **1** also inhibited the enzymes from the snake venoms of *A. halys blomhoffii* (basic) and *N. naja naja*

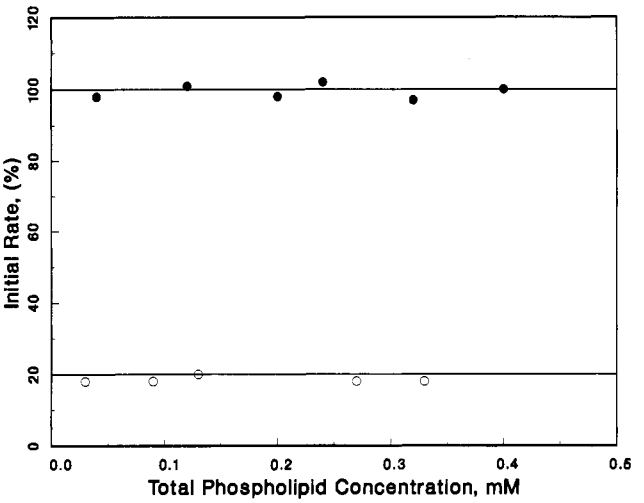


FIGURE 3: Initial enzymatic velocity plotted as a function of the bulk concentration of total phospholipid (substrate plus inhibitor) in the assay. The mole fraction of inhibitor in the vesicles was kept at a constant value of 0.024 by changing the amount of substrate and inhibitor together. Assays were conducted in 4 mL of 6 mM $CaCl_2$ in a pH stat at pH 8.0 and 21 °C. Porcine pancreatic PLA (0.05 μg) was added to start the reaction. (●) Without inhibitor; (○) with inhibitor **3** at 0.024 mole fraction.

with $X_i(50)$ values of 0.082 and 0.0027, respectively.

There are additional criteria that were developed to ascertain the nature of the inhibition by **1**. For competitive inhibition of interfacial catalysis, inhibitor and substrate molecules compete for the binding to E*. In deriving a velocity equation for competitive inhibition in vesicles, the following assumptions are made: (1) The inhibitor is similar to the substrate in surface properties so that it contributes to the surface area in proportion to its mole fraction. (2) The inhibitor, substrate, and product always mix perfectly on the surface of the vesicle. Assumption 1 allows the surface concentration of inhibitor to be expressed as its mole fraction and should be approximately valid for all inhibitors, regardless of surface area, as long as the inhibitor mole fraction values are small (true in the present study). Equation 1 relates the initial enzymatic velocities to

$$\frac{V_0}{V_i} = 1 + \left(\frac{X_i}{1 - X_i}\right)\left(\frac{K_m}{1 + K_m}\right)\left(\frac{1 + K_i}{K_i}\right) \qquad (1)$$

the mole fraction of inhibitor present in the vesicles. Equation 1 is analogous to the standard equation for competitive inhibition except that the concentrations of inhibitor and substrate are expressed in terms of their mole fractions (X_i and $1 - X_i$) rather than molarities. V_i and V_0 are the initial velocities in the presence and absence of inhibitor, respectively. K_m is the interfacial Michaelis constant (mole fraction units), and K_i is the inhibitor dissociation constant (mole fraction units). A plot of V_0/V_i versus $X_i/(1 - X_i)$ should be linear. As shown in Figure 4, a linear plot is indeed observed. It is not possible to extract the absolute value of K_i from the data in Figure 4. This is because the value of K_m is not yet known due to the fact that the usual experiments to determine K_m involve measuring the reaction velocity as a function of the mole fraction of substrate. This is difficult with vesicles where substrate mole fraction is always near unity. However, it is possible to say that K_m cannot be much greater than 1 mole fraction. If K_m were much larger than 1, the mole fraction of substrate would always be much less than K_m and the reaction progress curve would be purely first order. This is inconsistent with the observation of a linear reaction velocity that persists for several minutes (pseudo-zero-order kinetics). If we assume that K_m is 1, then a K_i value of 0.0015 for **1** can

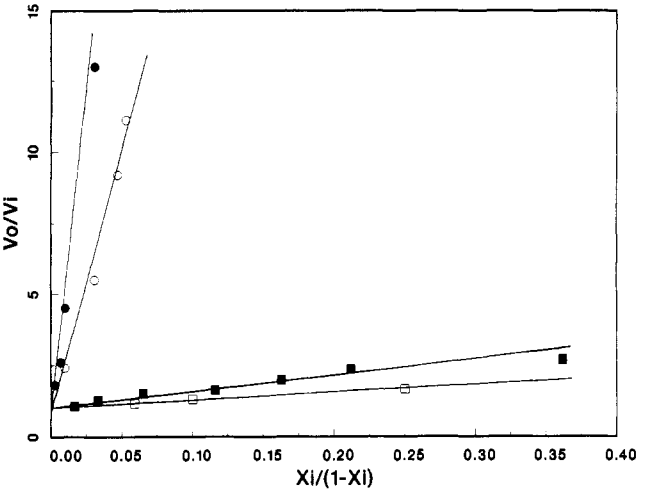


FIGURE 4: Ratio of the initial enzymatic velocity in the absence of inhibitor (V_0) to the initial velocity in the presence of inhibitor (V_i) plotted as a function of $X_i/(1 - X_i)$, where X_i is the mole fraction of inhibitor in the vesicle. Assay conditions are identical with those described in Table I. (●) Inhibitor **1**; (□) inhibitor **2**; (○) inhibitor **3**; (■) inhibitor **4**.

be calculated as an upper limit from the data in Figure 4 and eq 1.

It can also be ruled out that **1** inhibits the PLA-catalyzed hydrolysis of vesicles by somehow altering the physical nature of the substrate that renders it less susceptible to attack by the enzyme. This is based on the fact that inhibition is achieved with extremely small mole fractions of **1** in the vesicle and the fact that the data are quantitatively consistent with competitive inhibition as expressed by eq 1. To further test the idea that **1** is binding tightly to the catalytic site of PLA, we also studied the inhibition by the enantiomer of **1** (compound **2**, Table I). Since PLA hydrolyzes only *sn*-3 phospholipids, it is expected that **2**, which is an analogue of an *sn*-1 phospholipid, would be a poor inhibitor. In order to achieve 50% inhibition, an estimated 0.5 mole fraction of **2** is required (Table I, Figure 4). Even more subtle structural modifications of **1** were made by replacing one of the nonbridging phosphonate oxygens with sulfur. This creates a chiral center at phosphorus, and both diastereomers **3** and **4** were prepared in pure form (Table I). The absolute stereochemistry of **3** and **4** has not yet been determined. The results in Figure 4 and Table I show that one of the thiophosphonates is 45-fold less potent than **1** whereas the other isomer is only about 2-fold less potent. The 170-fold difference in potencies between **1** and **2** and the 27-fold difference in potencies between **3** and **4** strongly suggest that the inhibition by these compounds is the result of a specific interaction between inhibitor and the active site of the enzyme. If the inhibition was due to an E to E* effect, it is likely that all of the compounds would inhibit to similar extents.

A number of previously reported inhibitors of PLA were examined in 1,2-dimyristoylphosphatidylmethanol vesicles. Primary alkanols (C_4–C_{14}), aristolochic acid, mepacrine, and indomethacin all had no effect on the enzymatic reaction when present in vesicles at concentrations up to a mole fraction of 0.1 (data not shown).

Discussion

It has been well documented that PLA binds to the membrane bilayer in a step that it distinct from the binding of substrate to the catalytic site (Verger et al., 1973; Jain & Berg, 1989). With this fact in mind, three modes of reversible inhibition of interfacial catalysis can be envisioned. Mode 1: The inhibitor exists predominantly in the bilayer where it interacts directly with the enzyme in the bilayer (E*) and competes with the substrate for the binding to the catalytic site. Mode 2: The inhibitor is also mainly bound in the bilayer but it inhibits the reaction not by binding directly to the enzyme but by changing the physical organization of the bilayer in a way that promotes the desorption of bound enzyme (E to E* effect). Mode 3: The inhibitor is a water-soluble compound that binds directly to either the active site or the interfacial binding site(s) of the PLA and prevents the enzyme from binding to the interface (E to E* effect).

The results in the present study clearly show that the phosphonate-containing phospholipid analogues inhibit PLA in the scooting mode. The independence of the degree of inhibition on the bulk concentration of inhibitor, the inability of the inhibitor to displace the enzyme from the interface, the competitive nature of the inhibition, and the structure–activity data for the inhibitors all support a specific interaction between the enzyme and the inhibitor in the interface (mode 1).

A number of previously reported inhibitors of PLA are probably functioning via mode 2. These include lipophilic compounds such as alkanols (Jain, 1982), substituted butyrophenones (Wallack & Brown, 1981), aristolochic acid (Vishwanath et al., 1987), indomethacin (Franson et al., 1984), and mepacrine (Vadas, 1986). All of these compounds partition essentially completely into the membrane bilayer and are therefore not likely to inhibit via mode 3. In the present study, these compounds had no inhibitory effect on PLA in the scooting mode when present in 1,2-dimyristoylphosphatidylmethanol vesicles at concentrations up to a mole fraction of 0.1. Thus it is clear that these compounds do not bind directly to E* (mode 1). For these lipophilic compounds, previous inhibition studies were carried out with substrates such as phosphatidylcholine. PLA binds much more weakly to zwitterionic phospholipids (Jain et al., 1986a), and the E to E* equilibrium is therefore sensitive to the presence of additives in the bilayer. Indeed, fluorescence studies that measure the direct binding of PLA to zwitterionic vesicles have shown that these agents promote the desorption of the vesicle-bound enzyme (Jain & Jahagirdar, 1985). Furthermore, these inhibitors only function when present at levels comparable to the amount of substrate in the assay. At these levels, the inhibitors could have a significant effect on the organization of the substrate in the interface. Lipocortins (Flower et al., 1984; Davidson et al., 1987) probably also inhibit PLA via mode 2. In this case, the lipocortin binds tightly to phospholipid vesicles in the presence of calcium and prevents the association of PLA to the interface. Compounds that function in this manner will probably not be useful inhibitors of PLA in vivo.

Inhibitors that bind reversibly to the enzyme in the water layer and prevent the binding of the enzyme to the interface (mode 3) have not yet been adequately described. Such a compound might inhibit the action of PLA on zwitterionic vesicles but not on anionic vesicles in the scooting mode. This is because the enzyme binds extremely tightly to vesicles of 1,2-dimyristoylphosphatidylmethanol. Thus it is possible that anionic vesicles may effectively compete with the water-soluble inhibitor for the interactions with the water-soluble enzyme. The dissociation constant for PLA binding to these anionic vesicles has been estimated to be less than 10^{-9} M (Jain et al., 1986a; Jain & Berg, 1989). Various experiments can be envisioned to establish whether the inhibition is via mode 3. This type of inhibition demands that the enzyme and inhibitor have a strong affinity for each other in the water phase. This could be measured by direct binding studies. In addition,

inhibitors that operate via mode 3 must be soluble in water at concentrations in the range consistent with the inhibition. Finally, the degree of inhibition should depend on the bulk concentration of inhibitor.

Finally, irreversible inhibitors of PLA have been reported. Manoalide (de Silva & Scheuer, 1980; Jacobs et al., 1985; Reynolds et al., 1988) inhibits PLA by covalent modification of lysine residues on the surface of the enzyme. Manoalide produces only partial inhibition of PLA, suggesting that the enzyme can still bind to the interface and that the catalytic site is not completely blocked. The alkylating agent *p*-bromophenacyl bromide modifies a catalytic histidine residue and abolishes the enzymatic activity. The derivatized enzyme is still able to bind to the interface (Volwerk et al., 1974).

The above arguments are generally applicable to inhibition studies of all enzymes that operate at interfaces. Only when the reversible association of the enzyme to the interface has been eliminated can the nature of the inhibition be fully characterized.

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