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Kinetic Analysis of the Ca$^{2+}$-dependent, Membrane-bound, Macrophage Phospholipase A$_2$ and the Effects of Arachidonic Acid*

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The kinetics of the Ca$^{2+}$-dependent, alkaline pH optimum, membrane-bound phospholipase A$_2$ from the P388D$_1$ macrophage-like cell line were studied using various phospholipidic choline (PC) and phosphatidyl-ethanolamine (PE) substrates. This enzyme exhibits "surface dilution kinetics" toward PC in Triton X-100 mixed micelles, and the "dual phospholipid model" was found to adequately describe its kinetic behavior. With substrate in the form of sonicated vesicles, the dual phospholipid model should give rise to Michaelis-Menten type kinetics. However, the hydrolysis of dipalmityl-PC, 1-palmitoyl-2-oleoyl-PC, and 1-stearoyl-2-arachidonoyl-PC vesicles exhibited two distinct activities. Below 10 $\mu$m, the data appeared to follow Michaelis-Menten behavior, while at higher concentrations, the data could best be fit to a Hill equation with a Hill coefficient of 2. These PC's had $V_{\text{max}}$ values for the low substrate concentration range of 0.2-0.6 amol min$^{-1}$ mg$^{-1}$ and $K_m$ values of 1-2 $\mu$m. At the high substrate concentration range, the $V_{\text{max}}$ values were between 5 and 7 nmol min$^{-1}$ mg$^{-1}$. PC containing unsaturated fatty acids had an apparent $K_m$, determined from the Hill equation, of about 15 $\mu$m, while the apparent $K_m$ of dipalmityl-PC was 0.6 $\mu$m. When 70% glycerol was included in the assays, a single Michaelis-Menten curve was obtained for both dipalmityl-PC and 1-stearoyl,2-arachidonoyl-PC. Possible explanations for these kinetic results include reconstitution of the membrane-bound phospholipase A$_2$ in the phospholipid vesicle or the enzyme has two distinct phospholipid binding functions. The kinetics for both dipalmityl-PC and dipalmityl-PE hydrolysis in vesicles was very similar, indicating that the enzyme does not greatly prefer one of these head groups over the other. The enzyme also showed no preference for arachidonoyl containing phospholipid. Enzymatic activity toward PC containing saturated fatty acids was linear to about 15% hydrolysis while the hydrolysis of PC containing unsaturated fatty acids was linear to only about 5%. This loss of linearity was due to inhibition by released unsaturated fatty acids. Arachidonic acid was found to be a competitive inhibitor of dipalmityl PC hydrolysis with a $K_i$ of 5 $\mu$m. This tight binding suggests a possible in vivo regulatory role for arachidonic acid. Three compounds of the arachidonic acid cascade, prostaglandin F$_{2\alpha}$, 6-keto-prostaglandin F$_{1\alpha}$, and thromboxane B$_2$, showed no inhibition of enzymatic activity.

The biosynthesis of the prostaglandins, thromboxanes, leukotrienes, and related eicosanoids is dependent on the availability of the precursor arachidonic acid (1, 2). Since this tetraenoic fatty acid is in vivo normally found esterified at the sn-2 position of glycerophosphatidyls, its release from these compounds is thought to be the rate-limiting step in eicosanoid production. From the information currently available about the control of eicosanoid production, the most likely candidate for the putative arachidonic acid releasing enzyme is an alkaline pH optimum, Ca$^{2+}$-dependent, membrane-bound phospholipase A$_2$ (3). In our studies on the phospholipases in the P388D$_1$ macrophage-like cell line (4), we have identified at least four phospholipases as well as a lysophospholipase (5). One of these enzymes, the membrane-bound phospholipase A$_2$, which has an absolute dependence on Ca$^{2+}$ (4) and an alkaline pH optimum, has recently been purified and characterized (6). In contrast to tissue macrophages and other immunologically active cells where only a limited number of homogeneous cells are obtainable, the P388D$_1$ cell line is an attractive source for this enzyme since these cells can be grown in suspension allowing the harvesting of homogeneous cells and the preparation of relatively large amounts of enzyme suitable for kinetic analysis.

While this enzyme is capable of releasing arachidonic acid for the eicosanoid cascade, it could also be involved in general lipid metabolism. Therefore, a detailed kinetic analysis of the solubilized, purified, and reconstituted enzyme is a prerequisite to evaluating its possible role in arachidonic acid release in vivo. Since the extracellular phospholipase A$_2$ from snake venom and mammalian pancreas has been well characterized both structurally and kinetically (for reviews see Refs. 3 and 7), its kinetics was used as a paradigm for the intracellular enzyme. The "dual phospholipid model" (8), which is based on "surface dilution kinetics" (9), allows the kinetic parameters (10-12) and substrate specificities (13, 14) of this enzyme to be determined. This kinetic analysis is complicated by the presence of the lipid/water interface and the dependence of activity on the aggregation state, structure, and surface concentration of the phospholipid substrate (7). Membrane-bound phospholipases pose an extra level of complication over soluble enzymes and have not been subjected to thorough kinetic analysis, although we (15) have applied our kinetic concepts to a membrane-bound phosphatidylserine decarboxylase.

We have now used these kinetic concepts in the evaluation...
of the membrane-bound, Ca\textsuperscript{2+} -dependent phospholipase A\textsubscript{2} from the macrophage-like cell line (6). Kinetic parameters have now been determined which allow the comparison of the enzyme's activity toward substrates containing different fatty acid chains or head groups and allow the analysis of various inhibitor effects. With these data, we can begin to sort out whether the release of arachidonic acid in the cell is controlled by subtle differences in specificities, subcellular localization, or allosteric control of the putative phospholipase. From these studies, we have developed a set of assay conditions that possess distinct advantages for the initial characterization of intracellular and membrane-bound phospholipase A\textsubscript{2}.S. In addition, we have found that unsaturated fatty acids are competitive inhibitors of the macrophage enzyme. A preliminary report of this work has been presented (16).

**EXPERIMENTAL PROCEDURES**

**Materials**—Triton X-100, fatty acid free bovine serum albumin (BSA),\textsuperscript{1} palmitic acid, and arachidonic acid were purchased from Sigma. Linoleic acid, linolenic acid, and eicosapentaenoic acid were obtained from Aldrich. Oleic acid, prostaetadino, F\textsubscript{1}, (PGF\textsubscript{2}e), 6-keto-prostaglandin F\textsubscript{1}, (6-keto-PGF\textsubscript{1})\textsubscript{e}, dipalmitoyl-sn-glycero-3-phosphorylethanolamine (dipalmitoyl-PE), 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (dipalmitoyl-PC), and dipalmitoyl-sniplac-glycerol-3-phosphorylethanolamine (dipalmitoyl-PC) were purchased from Behring Diagnostics (La Jolla, CA). 1,2-Dipalmitoyl-sn-glycero-3-phosphorylcholine (dipalmitoyl-PC), 1-stearoyl-2-arginchoy1-sn-glycero-3-phosphorylcholine (steaoy1, arachidonyl-PC), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (palmitoyl, oleoyl PC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). 1-Palmitoyl-2-[\textsuperscript{14}C]palmitoyl-PC (54 mCi/ mmol) and 1,2-dil-[\textsuperscript{14}C]palmitoyl-PE (110 mCi/mmol) were purchased from Du Pont-New England Nuclear. 1-Palmitoyl-2-[\textsuperscript{3}H]oleoy1-PC (57 mCi/mmol) and 1-stearoy1-2-[\textsuperscript{3}H]arachidonyl-PC (60 mCi/mmol) were purchased from Amersham Corp. Thin layer chromatography plates (250-\mu m layer of Silica Gel G) were purchased from Analtech (Newark, DE). Scintillation fluid (Safety-Solve) was purchased from Research Products International (Mount Prospect, IL). All other reagents were analytical reagent grade or better.

**Phospholipase A\textsubscript{2} Preparation**—The purification of the phospholipase A\textsubscript{2} from the P388D1 macrophage-like cell line is discussed elsewhere (6). The enzyme preparation used for these studies was purified and free of other phospholipases. The enzyme is stored at 4°C over silica gel and is stable for at least 6 months. The lyophilized enzyme was dissolved in 0.01 M Tris buffer, pH 7.5, and kept at -70°C for 1 year. The lyophilized enzyme was used for incubation at 4°C for 90 min. The reaction was stopped, and enzymatic activity was determined by the modified (17) Dole extraction system (18) as previously described (6). This procedure was used instead of the TLC assay system (described above) because of the high content of Triton X-100. Such high levels would have overloaded the TLC plates, interfering with resolution.

**Kinetic Analysis**—Many enzymes that act at lipid-water interfaces exhibit "surface dilution kinetics" (9). Their activities depend not only on the bulk composition of the membrane, but also on its surface concentration. The most dramatic consequence of this phenomenon is that the enzyme's activity can be varied even when the bulk concentration of substrate is held constant. This is accomplished by varying its surface concentration with the addition of inert surface active compounds, such as detergents, that increase the surface area and decrease the substrate's surface concentration. Surface dilution occurs if a kinetically relevant step in the enzymatic reaction occurs while the enzyme is sequestered to the interface. If this occurs, all subsequent substrate binding steps take place on the two-dimensional surface of the interface and the concentration of the reactants must be expressed in terms of their surface concentration. One way this can be achieved is by the "dual phospholipid model" (8) which is represented in Equation 1.

\[
E + S \rightleftharpoons ES \rightleftharpoons ES + P \quad \text{(bulk)} \quad \text{ES} + S \rightleftharpoons ES + P \quad \text{(surface)}
\]

The kinetic equation for such a model has been derived (10) and is given in Equation 2.

\[
\nu = \frac{V_{\text{max}} A B}{K_m A + (K_c + B) A}
\]

Here \( \nu \) is the specific velocity of the reaction (nmol min\textsuperscript{-1} mg\textsuperscript{-1}), \( V_{\text{max}} \) is the maximum specific velocity (nmol min\textsuperscript{-1} mg\textsuperscript{-1}), \( K_c \) is the dissociation constant for the bulk binding step (\mu M), and \( K_m \) is the Michaelis-
Menten constant for the surface binding step (mole fraction), A is the bulk concentration of substrate (mM), and B is the surface concentration of substrate (mole fraction). If pure phospholipid vesicles are used, the surface concentration of substrate is equal to a mole fraction of 1.0 and does not vary under initial rate conditions. Thus, \( K_a + B \) becomes a constant and Equation 2 simplifies to Equation 3:

\[
v = \frac{V_{\text{max}}A}{K_s^* + A}
\]

where \( V_{\text{max}} \) is \( (V_{\text{max}}/(K_a + 1)) \) and \( K_s^* \) is now \( (K_aK_b/(K_a + 1)) \). Overall catalytic efficiency, \( V_{\text{max}}/K_{\text{cat}} \), becomes \( V_{\text{max}}/K_aK_b \) and contains the appropriate relationships of \( V_{\text{max}} \) and the inverse of the binding constants. Since the surface concentration of substrate, \( B \), cannot physically exceed a mole fraction of 1.0, \( V_{\text{max}} \) does in fact represent the maximum rate that could ever actually be achieved, regardless of what the theoretical \( V_{\text{max}} \) might be. Thus, in a sense, \( V_{\text{max}} \) is a true \( V_{\text{max}} \) and the superscript will be omitted in the following discussions. The \( K_b^* \) is a composite of both the bulk binding constant \( K_a \) and the Michaelis-Menten constant, \( K_b \). These two terms cannot be separated unless the surface concentration of the substrate is varied. In the case of pure phospholipid vesicles of a single substrate, this cannot be done and \( K_b^* \) also effectively becomes a true \( K_b \) for vesicles. Again, the superscript will be omitted.

The kinetic data obtained herein were analyzed by either linear least squares or by nonlinear regression analysis using the algorithms presented by Press et al. (19). The equations used to describe the Hill kinetics were those of Segel (20). To study inhibition, simple IC50 values, determined from dose-response curves, were used to screen various fatty acids and prostaglandins for inhibition of the phospholipase A₂. The type of inhibition was determined via a more detailed kinetic analysis of the effects that substrate and inhibitor concentrations had on activity. Double reciprocal plots and replots were carried out as described by Segel (20) for competitive and noncompetitive inhibition.

**RESULTS**

Phospholipase A₂ Time Course—Time courses were carried out for three different PCs and for dipalmitoyl-PE in order to determine the extent of linearity and to assure that all kinetic assays were carried out under conditions that would yield true initial rates. A comparison of the PCs is given in Fig. 1. While dipalmitoyl-PC was linear to about 15% hydrolysis, both palmitoyl, oleoyl-PC and stearoyl, arachidonoyl-PC were linear to only about 5%. As with dipalmitoyl-PC, dipalmitoyl-PE was also linear to about 15% (data not shown). Other studies show that time courses toward dipalmitoyl-PC are linear for at least 180 min as long as the hydrolysis remains below 15%.

**Phospholipase A₂ Activity toward Mixed Micelles**—The enzyme exhibits “surface dilution kinetics” as shown by the dependence of activity on the surface concentration of phospholipid in Fig. 2. These assays were carried out at a bulk substrate concentration of 500 μM and at molar ratios of Triton X-100 to phospholipid above 4 to 1. In calculating the Triton concentration in the mixed micelles, the cmc of Triton was subtracted (21). At these concentrations, the enzyme was already saturated with respect to bulk concentration of substrate so that only the dependence on surface concentration was observed. At lower substrate concentrations in the range below 100 μM, it was not practical to subtract out the Triton X-100 cmc (which is 250 μM) (22) as the cmc value for Triton X-100 would approach its total concentration in the assay.

Also, its actual cmc value in the presence of phospholipid, CaCl₂, buffer, etc., is unclear. Nonetheless, when bulk concentration ratios of Triton:phospholipid of 2:1 were examined, the enzyme was found to be quite active at substrate concentrations below 100 μM and gave simple Michaelis-Menten kinetics (data not shown). The \( V_{\text{max}} \) for this region was 50 nmol min⁻¹ mg⁻¹, and the apparent \( K_m \) was 250 μM when using stearoyl, arachidonoyl PC as substrate. Because the exact form of the substrate is not known in this region, the application of the surface dilution model would not be straightforward, and we have not attempted to analyze these conditions in more detail.

**Phospholipase A₂ Activity toward Vesicles**—Because of the concentration limits imposed by the cmc of Triton X-100 (see “Discussion”), we also investigated the activity toward phospholipids in vesicles. As described under “Experimental Procedures,” the dual phospholipid model simplifies to the Mi-

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**Fig. 1.** Phospholipase A₂ activity toward 1,2-dipalmitoyl-PC (C), 1-palmitoyl,2-oleoyl-PC (D), and 1-stearoyl,2-arachidonoyl-PC (Δ) as a function of time under standard assay conditions.

**Fig. 2.** The dependence of phospholipase A₂ activity on the surface concentration of substrate in mixed micelles with Triton X-100. The bulk 1-stearoyl-2-arachidonoyl-PC concentration was held constant at 0.5 mM while the Triton X-100 concentration was varied over a 5-fold range. Activity is shown as a function of the surface concentration of the substrate expressed as the mole fraction of phospholipid in the micelles, \( [(PC) + (TX)] \). [TX] is the total Triton concentration minus its cmc.
Kinetic Analysis of Phospholipase A$_2$

...chaelis-Menten equation since the surface concentration of substrate is constant and equal to a molar ratio of 1.0 for pure phospholipid vesicles. Thus, one is analyzing only the initial bulk binding step. The activity of the enzyme was measured at substrate concentrations that ranged from 1 to 1000 μM. The velocity versus substrate concentration plot for stearoyl, arachidonoyl PC is shown in Fig. 3, and the double reciprocal plot is shown in Fig. 4. There are two distinct substrate dependence regions; one at concentrations below 10 μM and the other above this concentration. Similar curves were also found for both dipalmitoyl-PC and palmitoyl, oleoyl-PC. Nonlinear regression analysis of these data clearly indicated that neither a single Michaelis-Menten equation nor a single Hill equation could explain the data. The next more complex model would be to assume the presence of two binding events. These two binding events could be either two Michaelis-Menten steps or one Michaelis-Menten and the second a Hill. Nonlinear regression analysis of these two combinations fit the data much better than the single equations. While the data were not sufficiently accurate to unambiguously distinguish between these two models or to confirm that either is definitely applicable, the Michaelis-Menten/Hill combination gave the best fit when a Hill coefficient of 2 was employed.

Table I presents the kinetic parameters obtained from this analysis. The $V_{max}$ for both the Michaelis-Menten and the Hill portions of the functions represent true $V_{max}$ and the $K_m$ for the Michaelis-Menten portion has its standard meaning. The apparent $K_m$ obtained for the Hill portion is composed of the intrinsic dissociation constant, $K_m$, and the interaction factors from the Hill treatment (20). In this case, half-saturation occurs when the substrate concentration is equal to $(K_m)^h$.

Glycerol Effects on Phospholipase A$_2$ Activity—We have previously found that the inclusion of 70% glycerol in the assay activates the enzyme toward dipalmitoyl-PC (6). The vesicle substrate dependence curves were repeated in the presence of glycerol in the hopes that this would shed some light on both this activation and the complex kinetic phenomenon reported above. The substrate dependence of stearoyl, arachidonoyl-PC and dipalmitoyl-PC hydrolysis are presented as a double reciprocal plot in Fig. 5. The incorporation of the glycerol straightened out both plots. The data for both fit a simple Michaelis-Menten equation. The one difference was that the stearoyl, arachidonoyl-PC had a lower $V_{max}$, 1.2 nmol min$^{-1}$ mg$^{-1}$ compared to 6.0 nmol min$^{-1}$ mg$^{-1}$ for the

| Substrate                  | Glycerol (70%) | $V_{max}$ (nmol min$^{-1}$ mg$^{-1}$) | $K_m$ (μM) |
|----------------------------|---------------|-------------------------------------|------------|
| Dipalmitoyl-PC             | -             | 0.44 ± 0.04                         | 1.1 ± 0.3  |
| Dipalmitoyl-PC$^a$        | -             | 5.10 ± 0.14                         | 0.66 ± 0.05|
| Dipalmitoyl-PC$^b$        | +             | 8.60 ± 4                            | 40 ± 20    |
| 1-Palmitoyl, 2-oleoyl-PC$^a$ | -         | 0.20 ± 0.01                         | 0.2 ± 0.2  |
| 1-Palmitoyl, 2-oleoyl-PC$^b$ | -         | 6.30 ± 0.18                         | 18 ± 1     |
| 1-Stearoyl, 2-arachidonoyl-PC$^a$ | -  | 0.57 ± 0.03                         | 2.4 ± 0.3  |
| 1-Stearoyl, 2-arachidonoyl-PC$^b$ | -  | 6.20 ± 0.20                         | 13 ± 1     |
| 1-Stearoyl, 2-arachidonoyl-PC$^c$ | +  | 1.20 ± 0.07                         | 6.1 ± 0.6  |

$^a$ Constants obtained from the Michaelis-Menten portion of the linear regression.

$^b$ Constants obtained from the Hill portion of the linear regression. $K_m$ represents the apparent $K_m$ as defined in the text.

Fig. 3. Phospholipase A$_2$ activity toward vesicles of 1-stearoyl-2-arachidonoyl-PC as function of substrate concentration under standard assay conditions. Inset are data points at low substrate concentrations. The line was drawn from the nonlinear regression analysis of the Michaelis-Menten/Hill combination (see text).

Fig. 4. Double reciprocal plot of phospholipase A$_2$ activity as a function of the concentration of vesicles of 1-stearoyl,2-arachidonoyl-PC. The line was drawn from the nonlinear regression analysis of the Michaelis-Menten/Hill combination (see text).

Fig. 5. Double reciprocal plot of phospholipase A$_2$ activity dependence on the concentration of 1-stearoyl,2-arachidonoyl-PC (●) and dipalmitoyl-PC (□) under standard assay conditions except that 70% glycerol was included in the assay. Linear least squares lines are shown.
assays without glycerol. The dipalmitoyl-PC, on the other hand, had a $V_{\text{max}}$ that was about 2 times higher, 5.0 nmol min$^{-1}$ mg$^{-1}$ without glycerol and 8.6 nmol min$^{-1}$ mg$^{-1}$ with glycerol.

**Head Group and Fatty Acid Chain Specificity of Phospholipase A$_2$**—The substrate concentration dependence of phospholipase A$_2$ toward dipalmitoyl-PC and dipalmitoyl-PE were compared to assess the effect of the change of polar head group on enzymatic activity. Double reciprocal plots for dipalmitoyl-PC and dipalmitoyl-PE hydrolysis are shown in Fig. 6. In order to simplify the analysis, the substrate concentrations were kept above 10 μM. In addition, activities could not be determined for PE concentrations over 100 μM. Above this concentration, Ca$^{2+}$ precipitated the PE. The kinetic constants for dipalmitoyl-PE ($V_{\text{max}} = 4.5 \pm 0.5$ nmol min$^{-1}$ mg$^{-1}$ and $K_m = 37 \pm 6 \mu M$), are nearly within experimental error of those determined for dipalmitoyl-PC ($V_{\text{max}} = 4.3 \pm 0.5$ nmol min$^{-1}$ mg$^{-1}$ and $K_m = 28 \pm 5 \mu M$) under the same substrate concentration range.

Because there was no significant preference for either PE or PC, the fatty acid chain specificity studies were conducted using only PC. Table I lists the results for the saturated dipalmitoyl-PC and two PCs containing unsaturated fatty acids. The $V_{\text{max}}$ and $K_m$ values for substrate concentrations below 10 μM were identical. The $V_{\text{max}}$ values obtained from the Hill analysis of the higher substrate range were also identical. The $K_m$ values, however, were not identical; dipalmitoyl-PC had a $K_m$ that was 20-fold lower than those of the unsaturated PCs. Since these are apparent $K_m$ values derived from the Hill equations, the half-maximum velocity for dipalmitoyl-PC would be about 24 μM and 122 μM for the unsaturated PCs. This is only a 5-fold difference.

![Fig. 6. Double reciprocal plot of phospholipase A$_2$ activity toward vesicles of dipalmitoyl-PE (●) and dipalmitoyl-PC (□) as a function of substrate concentration. Standard assay conditions were employed and linear least square lines are shown.](image)

![Fig. 7. Dose-response curve showing the effects of palmitate (▲), oleate (△), linolenate (■), linoleate (■), arachidonate (●), and eicosapentaenoate (○) on phospholipase A$_2$ activity toward dipalmitoyl-PC under standard assay conditions.](image)

![Fig. 8. A, double reciprocal plot of phospholipase A$_2$ substrate dependence toward dipalmitoyl-PC in the presence of 0 μM (●), 5 μM (○), 20 μM (△), or 50 μM arachidonate (○). B, plot of the slopes as a function of arachidonate concentration.](image)
Fatty Acid Inhibition Studies—In order to survey the inhibitory effects of fatty acids and eicosanoids on phospholipase A₂ activity, dose-response experiments were performed. For these experiments, standard assay conditions for dipalmitoyl-PC were used. The fatty acids and the eicosanoids were each added to assay tubes as ethanol solutions. PC were used. The fatty acids and the eicosanoids were each assayed showed no inhibition and perhaps a slight activation. The other fatty acids showed inhibition with an IC₅₀ for oleate of 400 μM (extrapolated), linoleate and linolenate of 60 μM, and arachidonate and eicosapentaenoate of 20 μM. The eicosanoids surveyed, PGF₂α, 6-keto-PGF₁α, and TxB₂ had no effect over the same range of concentrations used for the fatty acids (data not shown).

More detailed kinetic analysis was carried out on arachidonate, the most potent inhibitor, to determine the nature of the fatty acid inhibition. As with the specificity studies, the substrate concentration was varied from 10 to 500 μM in order to simplify the kinetic analysis. The double reciprocal plot shown in Fig. 8A shows that arachidonate behaves kinetically as a competitive inhibitor. A replot of the slope versus inhibitor concentration (Fig. 8B) gives a Kᵢ for arachidonate of about 5 μM.

In order to confirm that the nonlinearity of the time course of stearoyl, arachidonoyl-PC hydrolysis was due to arachidonate inhibition, BSA was included in an assay to bind and remove the fatty acid produced in the course of the reaction. The BSA did not change the time course when the substrate was in the form of vesicles, presumably because it did not pull out and sequester the fatty acid. However, BSA did increase the linearity when Triton X-100/phospholipid (4:1) mixed micelles were used resulting in a linear time course to at least 10% hydrolysis (data not shown).

A further characterization of the inhibitory effect of arachidonate is shown in Fig. 9. Two time courses, both at standard conditions and both containing 100 μM dipalmitoyl-PC and 100 μM stearoyl, arachidonoyl-PC, were carried out under identical conditions except that one contained radiolabeled dipalmitoyl-PC and the other radiolabeled stearoyl, arachidonoyl-PC. The results show that dipalmitoyl-PC hydrolysis is inhibited to the same extent as stearoyl, arachidonoyl-PC hydrolysis by the arachidonate accumulation.

DISCUSSION

Kinetic Analysis of Intracellular Phospholipase A₂—Because the Ca²⁺-dependent, membrane-bound phospholipase A₂ could play a major role in both eicosanoid production and general lipid metabolism, a detailed kinetic analysis of this enzyme is a prerequisite to evaluating its relative role in either of these major metabolic systems. Despite its importance, the kinetic characterization of the intracellular phospholipases has progressed at a much slower rate than that of their extracellular counterparts. This is due to difficulties in purification, low yields, relative instability, and low enzymatic activities. In this communication, we report initial studies aimed at a detailed kinetic characterization of such an enzyme.

The enzyme clearly exhibited surface dilution kinetics. Thus, at least one step in its mechanism is dependent upon the surface concentration of the phospholipid. The binding constant for bulk phospholipid would have required using Triton X-100 at concentrations below its cmc to achieve reasonable molar ratios of Triton to phospholipid. Therefore, lower bulk phospholipid concentrations could not be used because the mixed micelle structure would not be maintained. Thus, the separation of the two binding terms could not be accomplished. We are currently investigating the use of other detergents with lower cmc values so that lower concentrations of substrate can be used in this system.

Phospholipase A₂, Vesicle Kinetics—The surface dilution equation reduces to a simple Michaelis-Menten model when the substrate is in the form of vesicles. However, the vesicle data exhibited a biphasic nature. The data were best fit by a model containing two activities with one activity following a Michaelis-Menten equation and the second a Hill equation with a Hill coefficient of 2. The data could also be fit to a model containing two Michaelis-Menten steps, but not as well.

There are several possible explanations for this behavior. The first is that there are two different enzymes present in the enzyme preparation employed. We were unable, however, to find any evidence for other phospholipase activities in this preparation. In addition, studies on the highly purified enzyme (6) show that it also exhibits similar complex behavior toward both dipalmitoyl-PC and stearoyl, arachidonoyl-PC vesicles as a function of concentration, yet it gives a linear inverse plot in the presence of 70% glycerol, but with much higher V₅₀ (6). These results show that the complex curves are not due to a second enzyme.

Another possible explanation would be that this effect is due to some change in the substrate aggregate structure. It is well known that extracellular phospholipases exhibit similar kinetic behavior at the cmc when monomeric short chain phospholipids aggregate to form micelles. However, dipalmitoyl-PC has a very low cmc thought to be about 10⁻¹⁰ M (23). No other physical changes in this lipid occur in the 10 μM region arguing that physical changes are not responsible.

The effect could also be due to a reconstitution of the enzyme. We have previously shown (4) that this phospholipase A₂ is associated with the membrane fractions in sucrose density centrifugation. The lipids are removed in the purification process by solubilization in octyl glucoside and extrac-
substrate to use with the Ca\textsuperscript{2+} requiring enzyme (26). This avoids the inherent problems of Ca\textsuperscript{2+}-PE precipitation.

**Fatty Acid Inhibition**—Time courses, routinely performed to determine the extent of linearity, revealed a marked diminution in activity for unsaturated PC above 5% hydrolysis while saturated PC was linear to about 15% hydrolysis. These results suggested the possibility of product inhibition by unsaturated fatty acids. Dose-response curves confirmed this inhibitory effect. Palmitate caused no enzyme inhibition while unsaturated fatty acids did. The inhibition was roughly proportional to the number of double bonds. Arachidonate was one of the most inhibitory fatty acids showing a very strong competitive inhibition with a $K_I$ of about 5 $\mu$M. This inhibition could be overcome by adding BSA to the assay to absorb the arachidonolic acid and thus prevent its binding to the enzyme. However, the linearity of the initial rate could be extended in this fashion only when the substrate was micellar, not when it was in the form of vesicles. This difference is probably due to the differential extraction of the fatty acids by BSA from the two different interfaces. This suggests that BSA should not routinely be used to “linearize” time courses until the nature of this effect has been better characterized.

Other studies have also suggested that unsaturated fatty acids inhibit phospholipase $A_2$. The evidence was either that albumin enhanced activity or that unsaturated fatty acids inhibited, but saturated ones did not. The fact that fatty acid inhibition has been suggested for the enzymes from human platelet (27, 28), polymorphonuclear leukocytes (29), rat liver mitochondria (30, 31), and now a macrophage-like cell line suggests that this inhibition may be a common property of some Ca\textsuperscript{2+}-dependent phospholipase $A_2$.

**Choice of Assay Conditions for Phospholipase $A_2$—**As a result of these studies, we suggest that dipalmitoyl-PC vesicles represents the best substrate for initial enzyme characterization. While the sn-2-unsatuated acyl containing phospholipids especially of the PE variety are the most widely used substrate in the literature, we have shown that this substrate has two factors which complicate kinetic analysis. The first is the precipitation of PE in the presence of Ca\textsuperscript{2+}, which reduces the range of substrate concentrations that are accessible. The second is the competitive inhibition of arachidonic acid (other unsaturated fatty acids) which leads to nonlinear time courses. Since dipalmitoyl-PC lacks these drawbacks, it offers the simplest system to initiate a study. As to the possibility of adding BSA to the assay, previous reports have shown albumin to have varying effects on phospholipase $A_2$ activity in different assays (32) and albumin binding has been shown to be a function of pH, ionic strength, and membrane charge (33). In our own studies, BSA’s efficiency at extending linearity in a time course depended on the physical state of the phospholipid. Based on these findings, BSA would not appear to be an effective general solution to the nonlinearity problem. While the dipalmitoyl-PC vesicle system represents a good choice as a substrate for the initial kinetic studies, the Triton X-100 mixed micelle system offers advantages under certain circumstances. The first is that to completely characterize the enzyme, an investigation of its surface dilution phenomenon is required. This cannot be easily done on vesicles without disrupting the vesicle structure. Thus, these studies are best carried out with Triton X-100/phospholipid micelles where varying the detergent to phospholipid ratio alters the surface concentration without dramatically changing the micellar structure. The second circumstance is that most inhibitors of phospholipase are surface active (34). They could produce varied effects such as the accumulation of inhibitor at the surface, diluting out of the substrate, altering the vesicle

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2. D. Lombardo, R. A. Deems, and E. A. Dennis, unpublished observations.
structure, and, in the extreme, actually causing micelle formation. All of these would affect the apparent activity of the enzyme. The use of mixed micelles at a high detergent to phospholipid mole ratio precludes the later two problems by presenting the substrate in a stable unchanging surface as long as the inhibitor concentrations are kept well below that of the detergent. In summary, we have found that the alkaline pH optimum, Ca2+-dependent phospholipase A2, from a macrophage-like cell line exhibits surface dilution kinetics and that its kinetic behavior can be explained by the dual phospholipid model. These complex kinetics appear to be similar to those found with the cobra venom enzyme. Thus the cobra venom enzyme may require a certain hydrophobic environment to exhibit stable kinetics. This environment can be provided by 70% glycerol or phospholipid vesicles. Furthermore, phospholipase A2 is competitively inhibited for phospholipase A2 and thereby eicosanoid production. The nature of this possible control is unclear and will require further investigation.

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