Comparison of the catalytic properties of phospholipase A₂ from pancreas and venom using a continuous fluorescence displacement assay

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Phospholipases A₂ from pig pancreas and the venoms from bee, *Naja naja* and *Crotalus atrox* have been studied by using a new continuous fluorescence displacement assay that utilizes normal phospholipid substrates [Wilton (1990) *Biochem. J.* 266, 435–439]. With limiting amounts of substrate, the assay demonstrated stoichiometric conversion into products with both pancreatic and venom enzymes, and thus would allow phospholipid determination at concentrations down to about 0.1 μM. The substrate specificity of the enzyme was determined for the four enzymes in terms of both phospholipid head group and fatty acid selectivity. None of the enzymes demonstrated a preference for arachidonic acid-containing phospholipid under the conditions of this assay. No lag was observed with any enzyme with either phosphatidylcholine or phosphatidylglycerol as substrate. With dipalmitoyl-phosphatidylcholine as substrate, the assay clearly highlighted the different membrane-penetrating properties of the pancreatic and *Naja naja* enzymes and demonstrated maximal activity for the pancreatic enzyme in the region of the phase-transition temperature of this substrate, at about 35 °C.

INTRODUCTION

Phospholipase A₂ (PLA₂) catalyses the hydrolysis of the *sn*-2 fatty acyl ester bond of phospholipids to give a long-chain fatty acid and the corresponding lysophospholipid. PLA₂ describes a diverse family of enzymes that are widely distributed in biological systems and are the subject of very considerable interest at present.

Earlier work focused on the digestive enzymes from the pancreas and also found in various venoms and toxins. These enzymes have been used extensively to investigate both membrane structure and the nature of the molecular events that occur at the lipid/water interface (Jain & Berg, 1989). There is now very considerable interest in the much less abundant intracellular enzymes that have important roles in cell function. These roles include phospholipid remodelling of the fatty acyl composition of phospholipids (Samborski *et al.*, 1990), the removal of peroxidized fatty acids from cellular membranes (van Kuijk *et al.*, 1987), and in the formation of the eicosanoids and platelet-activating factor that are used by the cell as intracellular or intracellular messengers. PLA₂ has been implicated as part of a G-protein-regulated receptor-mediated signal-transduction system (Axelrod *et al.*, 1988), and this may involve a recently discovered family of high-molecular-mass PLA₂s that are very sensitive to Ca²⁺ (e.g. Clark *et al.*, 1990; Gronich *et al.*, 1990; Leslie & Channon, 1990).

We have recently described a continuous fluorescence displacement assay for PLA₂ (Wilton, 1990a). This assay involves monitoring the loss of fluorescence when the fatty acid probe 11-(dansylamino)undecanoic acid (DAUDA) is displaced from liver fatty-acid-binding protein (FABP) by long-chain fatty acids released from normal phospholipids as a result of PLA₂ activity. The particular advantages of the assay are its relatively high

sensitivity, coupled with the fact that the assay works apparently with any phospholipid substrate. Moreover, high activity is expressed in the absence of detergents. As a result, comparisons of both substrates and enzymes can be performed under conditions that may approach those that would be experienced within the eukaryotic cell.

Preliminary studies (Wilton, 1990a) concentrated on the enzyme from pig pancreas and evaluated the overall effectiveness of the assay in terms of various phospholipid substrates. In the present paper we describe results in which the pig pancreas is compared with a variety of venom enzymes, using different classes of phospholipids and also different species of phosphatidylcholine that are commercially available containing a variety of long-chain fatty acids. The results confirm the major differences that occur, particularly when comparing the pancreatic and venom enzymes, and highlight the apparently less important contribution of the fatty acyl chain in determining the rate of hydrolysis when comparing phospholipids as substrates. This assay should be useful in evaluating the properties of important intracellular enzymes, such as the human platelet-derived enzyme that is present in rheumatoid-arthritis synovial fluid and which has now been cloned (Kramer *et al.*, 1989; Seilhamer *et al.*, 1989).

EXPERIMENTAL

Materials

Phospholipids were obtained from either Sigma (DOPG, DOPE, SAPC, Sopc, POPC, DLPC) or Lipid Products (DPPC, DOPC). Samples of lipid supplied in methanol, chloroform or chloroform/methanol were dried with N₂ and dissolved in methanol. Solid samples were dissolved directly in methanol without further purification. All PLA₂ enzymes were obtained

Abbreviations used: PLA₂, phospholipase A₂; FABP, fatty-acid-binding protein; DAUDA, 11-(dansylamino)undecanoic acid; DOPC, dioleoyl-phosphatidylcholine; DOPE, dioleoyl-phosphatidylethanolamine; DOPG, dioleoyl-phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; Sopc, 1-stearoyl-2-oleoyl-phosphatidylcholine; SAPC, 1-stearoyl-2-arachidonyl-phosphatidylcholine; DLPC, dilinoleoyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; SUV, small unilamellar vesicle.

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from Sigma, and used without further purification as follows: pig pancreas (P6534), bee venom (P9279), *Naja naja* (P6139) and *Crotalus atrox* (P3770).

Fluorescence displacement assay for PLA₂

The principles and experimental details of this assay have been described previously (Wilton, 1990a). Most of the measurements were carried out on a Hitachi F2000 fluorimeter with a thermostatically regulated cell holder. All assays comparing different phospholipids are the means of at least four initial-rate estimations, of which at least one estimation was performed on the various enzymes with the same assay cocktail. The final concentration of the phospholipid was either 2 or 10 µg/ml as indicated, and was added as a 10 mg/ml solution in methanol.

Preparation of phospholipid vesicles

To 20 ml of standard assay buffer (0.1 M-Tris/HCl, pH 8.0, 0.1 M-NaCl, 5 mM-CaCl₂), phospholipid was added by rapid injection of 20 µl or 100 µl of 10 mg/ml in methanol, followed by vigorous mixing. Then 200 µl of 0.1 mM-DAUDA in methanol was added to produce the assay cocktail. For assays run at 43 °C the cocktail was prepared at 45 °C, whereas 25 °C assays were prepared at 25 °C. Samples (1 ml) were taken for fluorescence measurements, and to this was added an approximately equimolar amount (1 nmol) of FABP.

For comparison, SUVs were prepared by the rapid ethanol-injection procedure of Batzri & Korn (1973) as described by New (1990).

RESULTS

Physical state of the phospholipid substrate

The fluorescence displacement assay (Wilton, 1990a) used a system in which the phospholipid was first dissolved in methanol and then a small volume of methanol solution was added to the buffer, giving a residual methanol concentration at this stage of 0.1 or 0.5 % (v/v). This general method is one of hydration from organic solvent and is, in principle, a standard procedure for generating SUVs (Szoka & Papahadjopoulos, 1980). We will therefore refer to the presentation of phospholipid in methanol as SUVs, although the precise physical structure of the substrate in these assays has not been determined. This limitation of knowing the precise physico-chemical properties of the substrate is a problem with all studies of phospholipid vesicle preparations involving different phospholipids.

A comparison of these vesicles produced by methanol injection with those produced by the ethanol-injection procedure of Batzri & Korn (1973) was performed. With DOPC as substrate, no significant differences in the initial rates of hydrolysis were observed between vesicles produced by the two solvent-injection procedures with the same amounts of enzyme from pig pancreas. Thus initial rates of hydrolysis (expressed as fluorescence change/min, in arbitrary units) of DOPC for vesicles prepared by methanol and ethanol injection were 43.5 (±0.8) and 41.8 (±2.8) respectively.

Where phospholipids show low solubility in methanol (e.g. DOPE), a turbid dispersion of the phospholipid may be produced in the final assay buffer. This is not a major problem, owing to the good spectral characteristics of DAUDA, and, for example, the assay is surprisingly effective in allowing lipase activity to be measured by using dispersions of triacylglycerol (olive oil) in buffer (Wilton, 1991).

Stoichiometry of phospholipid hydrolysis

The fluorescence displacement assay is effective with low concentrations of phospholipids, and therefore it is possible to

study the result of using limiting substrate concentrations on the reaction stoichiometry. This study was performed with DOPC, DOPE and DOPG. When phospholipid concentrations below 1 µM were used in the presence of excess enzyme, a rapid decline in fluorescence to a limiting value was obtained that was proportional to the starting phospholipid concentration. Calibration of each of the assays with added oleic acid allowed this fluorescence end point to be quantified in terms of nmol of oleic acid released (Table 1). The mean value with both enzymes approached the theoretical value of 1 mol of fatty acid released per mol of phospholipid. Thus PLA₂, under the conditions of the assay, is able to hydrolyse completely the *sn*-2 position of DOPC, DOPE and DOPG, and examples of hydrolysis traces for these three phospholipids are shown in Fig. 1 for the enzyme from pancreas or *N. naja*. These results establish that, in principle, the assay could be used to quantify a sample of phospholipid at concentrations down to about 0.1 µM. It has previously been shown that a variation of the assay using triacylglycerol lipase

Table 1. Stoichiometry of phospholipid hydrolysis by PLA₂ from pig pancreas and *N. naja*

All assays (1 ml) were performed with 313, 400 and 625 ng of phospholipid together with 1 µM-DAUDA and 12.5 µg of FABP at 25 °C. The appropriate PLA₂ was added, and completion of hydrolysis was defined as when the residual rate of fall of fluorescence was the same as the rate in the absence of enzyme. For each assay the fall in fluorescence in the absence of enzyme over the period of measurement was subtracted. Each assay was calibrated by adding up to 10 nmol of oleic acid (1 mM in methanol).

Phospholipid substrate	Phospholipase	Fatty acid released (mol/mol of phospholipid)
DOPG	Pig pancreas	0.90 ± 0.01
DOPG	<i>N. naja</i>	0.80 ± 0.18
DOPC	Pig pancreas	0.79 ± 0.05
DOPC	<i>N. naja</i>	1.07 ± 0.16
DOPE	Pig pancreas	0.97 ± 0.11

Fig. 1. Fluorescence displacement traces resulting from hydrolysis of a limiting concentration of dioleoyl-phospholipid by PLA₂ from pig pancreas and *N. naja*

All assays (1 ml) contained 1 µM-DAUDA and 12.5 µg of FABP at 25 °C. The fluorescence displacement traces were: ▲, 313 ng of DOPE with 500 ng of pig pancreas PLA₂; ■, 400 ng of DOPG with 50 ng of pig pancreas PLA₂; ●, 625 ng of DOPG with 25 ng of *N. naja* PLA₂; △, 625 ng of DOPC with 500 ng of *N. naja* PLA₂. The highest blank rate of fall of fluorescence (no enzyme) was shown by 625 ng of DOPG (○).

Fig. 2. First-order plot of the progress curve of substrate utilization

The log % of the total displaceable fluorescence remaining was plotted as a function of time for DOPG, DOPC and DOPE hydrolysis curves. The fall in fluorescence is a linear function of oleic acid released for fluorescence displacement values below 50%. F_t is the fall in fluorescence due to complete phospholipid hydrolysis; F_x is the fall in fluorescence at each time point. The plots are: ●, 625 ng of DOPG and 25 ng of *N. naja* PLA₂; ■, 625 ng of DOPE and 500 ng of pig pancreas PLA₂; ▲, 625 ng of DOPG and 10 ng of pig pancreas PLA₂; ○, 625 ng of DOPC and 500 ng of *N. naja* PLA₂.

can measure di- and tri-acylglycerol levels with a similar sensitivity (Wilton, 1991).

It is assumed that PLA₂ must initially bind to and hydrolyse the outer half of the phospholipid bilayer found in vesicles as a result of interfacial recognition. The hydrolysis was first order with respect to substrates with these low concentrations of DOPG with the enzymes from pancreas and *N. naja* (Fig. 2). This might suggest that the rate of reorganization of the vesicle substrate as hydrolysis proceeded was sufficiently rapid to provide a continuous supply of outer-bilayer structure containing the unhydrolysed phospholipid. However, any conclusions must be very tentative, because the precise physical nature of this system as hydrolysis proceeds is unknown.

Phospholipid specificity of pancreatic and venom PLA₂

There is much information in the literature on the substrate specificity of pancreatic and various venom PLA₂s. However, most of these studies involve presentation of phospholipid in the form of mixed vesicles with neutral or negatively charged detergent. In addition, detailed kinetic analysis has often involved pH-stat measurements requiring relatively high concentrations of enzyme and substrates. The development of the fluorescence assay provided an opportunity to compare the substrate specificity of various enzymes under conditions that would more closely resemble those experienced by phospholipases *in vivo*, in particular with low enzyme concentrations and in the absence of detergents.

The substrate specificity of phospholipases is of crucial importance, for example, in understanding the control of arachidonic acid release in cells as a result of intracellular PLA₂ activity. This type of enzyme catalyses the first step in the synthesis of intra- and inter-cellular messengers in response to appropriate stimuli; however, the precise nature of the enzyme is controversial (see the Discussion section). Although these important intracellular PLA₂s are present in very low concentrations, they are becoming more readily available with the use of cloning technology (Kramer *et al.*, 1989; Seilhamer *et al.*, 1989). Therefore a future potential use of the fluorescence assay

will be for the detailed study of the substrate specificity, mechanism and regulation of these enzymes. As a foundation for this work, we have evaluated the assay by using the pancreatic and venom enzymes, for which crystal structures have been published. This study provides the first detailed description of the substrate specificity of these enzymes assayed under identical assay conditions in the absence of detergents.

Initial rate studies on phospholipid hydrolysis were performed with three different classes of phospholipid, namely phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol, as these are readily available with defined chemical composition. The individual phospholipids used for comparison of head-group specificity were the neutral phospholipids DOPC and DOPE (assumed to be neutral at pH 8) and the negatively charged phospholipid DOPG. The results, which are shown in Fig. 2, are normalized with respect to DOPC for each enzyme studied. In addition, at least one set of results for comparing the four enzymes was obtained by using the same stock assay solution in order to eliminate variation in the physical state of the phospholipid for different preparations. Because subsequent comparisons also involved DPPC, assays were performed at 43 °C to ensure that all substrates were in the fluid phase.

Each type of phospholipid assay was calibrated by addition of the relevant fatty acid to the complete assay in the absence of enzyme, from which the percentage fluorescence displacement for each nanomole of fatty acid released was determined.

As shown in Table 2, major differences in specificity were observed between the four enzymes. The enzyme from pig pancreas had a very pronounced preference for the negatively charged phospholipid DOPG, whereas low rates of hydrolysis were observed with the neutral phospholipid DOPC. This observation is consistent with the literature and may reflect the fact that the normal substrate for this enzyme in the gut is the negatively charged phospholipid/bile salt mixed micelle (Volwerk *et al.*, 1986). On the other hand, the two snake venom enzymes showed a preference for the neutral phospholipid DOPC. The bee venom enzyme appeared to fall into a third category.

The hydrolysis of DOPE highlighted further differences between the four enzymes, with the pancreatic enzyme showing significantly enhanced activity with this substrate as compared with DOPC, whereas the snake venom enzymes both showed very low activity towards DOPE under the conditions of the assay. Again the bee venom showed significant activity, but below that for DOPC. With all the enzymes tested DOPE gave slightly sigmoidal rate curves, with a maximum rate being achieved after about 20 s of reaction. (This phenomenon was not observed when concentrations of DOPE below 1 μM were used for determining hydrolysis stoichiometry.) The potential for phosphatidylethanolamine to demonstrate lipid polymorphism with the formation of a hexagonal H_{II} structure suggests considerable caution in interpreting phosphatidylethanolamine results. However, a major preference of the enzymes from *N. naja* and *C. adamanteus* for phosphatidylcholine over phosphatidylethanolamine has been previously highlighted (Pluckthun & Dennis, 1985).

Effect of fatty acid composition on the hydrolysis of phosphatidylcholine by pancreatic and venom enzymes

The analysis of the initial rates of hydrolysis of a number of commercially available phosphatidylcholines by the four PLA₂s (Table 2) allows several general conclusions to be made.

Firstly, the presence of a saturated fatty acid at the *sn*-1 position significantly decreases the rate of hydrolysis of the phospholipid as compared with DOPC (e.g. compare DOPC with SOPC or POPC), and this trend was shown for all the four

Table 2. Comparison of the activities of various phospholipases with synthetic phospholipids as substrates

The initial rates of hydrolysis of the phospholipids were normalized to that of DOPC for each phospholipase A_2 . All experiments were performed at 43 °C at a phospholipid concentration of 10 $\mu\text{g/ml}$. The values are means $\pm$ s.d. of at least four experiments, and for at least one experiment measurement of all four enzymes was performed with the same assay cocktail preparation.

Enzyme	Substrate	Activity relative to that with DOPC (%)
Pig pancreatic	DOPC	100 $\pm$ 11.4
	DOPE	409 $\pm$ 41.8
	DOPG	3587 $\pm$ 211.4
	DPPC	7.4 $\pm$ 1.7
	POPC	49 $\pm$ 7.6
	SOPC	35 $\pm$ 10.5
	DLPC	114 $\pm$ 14.0
	SAPC	23 $\pm$ 10.9
Bee venom	DOPC	100 $\pm$ 14.7
	DOPE	37 $\pm$ 2.7
	DOPG	259 $\pm$ 26.0
	DPPC	31 $\pm$ 3.5
	POPC	92 $\pm$ 6.6
	SOPC	68 $\pm$ 11.1
	DLPC	98 $\pm$ 21.0
	SAPC	41 $\pm$ 4.8
<i>Naja naja</i> venom	DOPC	100 $\pm$ 2.4
	DOPE	5.2 $\pm$ 0.7
	DOPG	63 $\pm$ 5.1
	DPPC	65 $\pm$ 8.5
	POPC	58 $\pm$ 4.5
	SOPC	62 $\pm$ 11.9
	DLPC	139 $\pm$ 8.7
	SAPC	56 $\pm$ 5.7
<i>C. atrox</i> venom	DOPC	100 $\pm$ 23.9
	DOPE	4.0 $\pm$ 1.7
	DOPG	14 $\pm$ 1.7
	DPPC	17 $\pm$ 1.4
	POPC	86 $\pm$ 0.5
	SOPC	61 $\pm$ 3.2
	DLPC	196 $\pm$ 37.3
	SAPC	42 $\pm$ 3.1

enzymes tested. However, the decrease was small and may have little significance when extrapolated to physiological substrates present in biological membranes.

Secondly, the comparison of SOPC with SAPC indicates that the presence of additional double bonds in the fatty acid to be released has minimal effect on the hydrolysis rate for all four enzymes. There was no evidence of acyl-chain selectivity towards arachidonic acid with the four enzymes tested.

Thirdly, when DPPC was used as substrate a major difference was noted between the enzyme from pig pancreas and the venom enzymes. Whereas high rates of hydrolysis of this substrate were observed with the venom enzymes, low rates of hydrolysis were detected with the pancreatic enzyme under the conditions of this assay, even with extended incubation time of up to 30 min. This result may reflect the difference in what has been termed the penetrating ability of the pancreatic enzyme as opposed to the venom enzymes. The pancreatic enzyme shows the lowest penetrating ability, and the enzyme from *N. naja* the highest penetrating ability (Van der Wiele *et al.*, 1988). Recent discussion of data on the crystal structures of PLA_2 -inhibitor complexes

Fig. 3. Effect of temperature on the rate of hydrolysis of DPPC (■) and DOPC (●) by PLA_2 from pig pancreas

All assays (1 ml) contained 10 μg of phospholipid, 10 μM -DAUDA, 12.5 μg of FABP and 100 ng of enzyme. The initial rates of hydrolysis were determined and expressed as fluorescence change (ΔF) in arbitrary units/min.

(Scott *et al.*, 1990) indicates that the term 'extractability' of the phospholipid from the vesicle may be more appropriate.

The greatly enhanced capacity of the pancreatic enzyme to hydrolyse POPC as compared with DPPC, even though both phospholipids are in the fluid state at 43 °C, highlights the importance of having an unsaturated fatty acid at the *sn*-2 position to allow effective hydrolysis.

PLA_2 activity and the lag phenomenon

The membrane-penetrating ability of PLA_2 s has been linked to the hydrophobicity of the interfacial recognition site and hence the membrane-binding potential of the enzyme. This property of pancreatic PLA_2 has been enhanced by enzyme acylation (Van der Wiele *et al.*, 1988). In addition, the phenomenon of bilayer penetration may be linked to the concept of interfacial activation and a concomitant lag phase or latency. An alternative model to explain the lag phase involves a requirement for enzyme dimerization, and variations of this mechanism involve dimerization of the enzyme either in solution or on the membrane surface (Romero *et al.*, 1987). With the pancreatic enzyme, dimerization may be as a result of enzyme-catalysed enzyme acylation (Tomasselli *et al.*, 1989).

Using the fluorescence displacement assay, we have never seen any lag phase with the pancreatic or venom enzymes and phosphatidylcholine or phosphatidylglycerol substrates. The absence of a lag phase is a surprising result, because the lag or activation phenomenon associated with the pancreatic enzyme appears to be central to the discussion of these enzymes. However, it should be appreciated that the fluorescence assay measures the initial rate of hydrolysis, as detected by fatty acid release. The very nature of the assay does not allow prolonged hydrolysis to be monitored, which would result in the build-up of fatty acids, and it is possible that in many systems involving the pancreatic enzyme this build-up of fatty acids may activate the assay by generating negatively charged vesicles (Pluckthun & Dennis, 1985). The lag phase and its complex relationship to the physico-chemical state of the phospholipid aggregate have been critically discussed (Jain & Berg, 1989).

Effect of temperature on phospholipid hydrolysis

The large differences observed for hydrolysis of liquid-crystalline DPPC, as compared with DOPC, by the pancreatic enzyme may reflect the low membrane-penetrating ability (substrate extraction) of this enzyme. It was therefore decided to

investigate the effect of temperature on the activity of the pancreatic enzyme, with DPPC and also DOPC as substrate in order to determine enzyme activity during the transition of DPPC from gel to fluid state.

The activity increased continuously with increasing temperature when DOPC was used as substrate, as shown in Fig. 3, and this was most apparent above 25 °C. Since the enzyme experiences high temperatures for only a very short period of time, we have no evidence for decreased activity owing to denaturation with assays up to 60 °C. When DPPC was used as substrate, a peak in activity was observed at about 35 °C. This temperature is lower than the reported phase-transition temperature reported for monolayer DPPC (41 °C). However, it is not possible to extrapolate the phase transition temperature from monolayers to other aggregates, and the value for SUVs, for example, is reported to be several degrees lower than that for liposomes (Szoka & Papahadjopoulos, 1980).

The ability of the pancreatic enzyme to hydrolyse DPPC most effectively at the transition temperature has been attributed to the development of fracture patterns at this temperature between regions of gel-phase and liquid-crystalline-phase lipid. Such fracture patterns are presumed to facilitate phospholipid penetration (phospholipid extraction) by the enzyme.

DISCUSSION

A fluorescence displacement assay for lipase activity has been described previously (Wilton, 1990a) and its general features have been discussed. In the present paper we have extended the assay to examine in detail some properties of four PLA₂s that are commercially available, using a variety of substrates in order to demonstrate the effectiveness of the assay for more detailed enzymological studies. The results confirm the usefulness of the assay in being able to analyse very rapidly the different properties of the PLA₂s studied under identical conditions and in the absence of added detergents.

A number of general conclusions may be drawn from this study.

(1) Substrate addition in methanol to generate presumptive SUVs provides a convenient method of phospholipid assay using the low concentrations of phospholipid required in this measurement of PLA₂ activity.

(2) Using DOPC, DOPE and DOPG, the assay allows complete hydrolysis of the *sn*-2 position of the phospholipid to be monitored and can be used to quantify phospholipids in a sample at concentrations down to about 0.1 μM.

(3) The assay was able to confirm and extend various information in the literature concerning the head-group specificity of pancreatic and venom phospholipases. It must be realized that the phenomenon of interfacial binding will make the enzymes sensitive to the precise physical state of the substrate aggregates. Thus apparent substrate specificity will also reflect the effect of substrate structure on the physical state of the aggregate, and as a result conclusions expressed in terms of classical substrate specificity must be treated with caution. This is particularly the case when comparing phosphatidylethanolamine with other substrates.

The simplicity of the assay allowed all enzymes to be measured under identical conditions for the comparison of activity and, in particular, highlighted the known requirement of pancreatic phospholipase for anionic vesicles that may be achieved by using anionic phospholipids. The present work does not attempt to distinguish K_m and K_{cat} effects, as comparisons have been done at a single substrate concentration. For the pancreatic enzyme, the pronounced preference for phosphatidylglycerol over phosphatidylcholine is due to both a K_m and a K_{cat} effect when

assayed in the presence of a neutral detergent (Volwerk *et al.*, 1986).

(4) The use of DPPC as substrate may provide a simple method for demonstrating the different penetrating properties (substrate extractibility) of pancreatic and venom phospholipases and should allow a more detailed investigation of this phenomenon, which has also been linked to enzyme activation. In this context, the assay should be of particular use in protein engineering studies to investigate the interaction of the enzyme with the phospholipid surface.

(5) The results demonstrated minimal fatty acid selectivity under the conditions of the assay, provided that the *sn*-2 fatty acid was unsaturated. This observation has particular relevance to the problem of explaining the specific release of arachidonic acid from membrane phospholipid resulting from activation of intracellular PLA₂(s) by cell mediators, such as in the thrombin stimulation of human platelets.

Recent studies with the 14 kDa platelet PLA₂ failed to provide any evidence for acyl-chain selectivity with phosphatidylcholine substrates (Schalkwijk *et al.*, 1990). However other, high-molecular-mass, forms of PLA₂s have been identified in macrophages (Leslie *et al.*, 1988; Wijkander & Sundler, 1989), monocytes (Clark *et al.*, 1990; Diez & Mong, 1990) and rat kidney (Gronich *et al.*, 1990). These show a preference for arachidonic acid-containing substrates and are sensitive to nanomolar concentrations of Ca²⁺, consistent with a role for Ca²⁺ as a modulator of activity. Therefore these enzymes are a more obvious target in the important cell-signalling pathway to produce arachidonic acid.

The cumulative results presented in this paper provide support for the fluorescence displacement assay as an effective method for evaluating the specificity of important intracellular PLA₂s such as the human platelet PLA₂, which has recently been cloned, and also the high-molecular-mass enzymes as they become available in reasonable quantities as a result of gene cloning. Of particular interest will be an investigation into the role of putative physiological modulators of these enzymes. Such modulators include Ca²⁺, anionic phospholipids (Leslie & Channon, 1990), the lipocortins and other inhibitory proteins (Suwa *et al.*, 1990). Modulators of enzyme activity can be evaluated by using phospholipid vesicles containing other important constituents of plasma membranes, such as cholesterol and sphingomyelin (Lange *et al.*, 1989). The fluorescence assay may be particularly suited to this approach, since it uses natural phospholipids and does not require radioactive substrates. This approach is most relevant at present to the human platelet PLA₂, which is enriched in rheumatoid synovial fluid and has been cloned and sequenced (Kramer *et al.*, 1989; Seilhamer *et al.*, 1989).

The fluorescence displacement assay requires liver FABP and will not work with albumin, although DAUDA binds to this protein (Wilton, 1990b). We have therefore undertaken the total synthesis of a gene coding for rat liver FABP and have over-expressed the active protein in *Escherichia coli* (Worrall *et al.*, 1991) so that it may become more readily available.

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