The properties of a cloned human high-molecular-mass cytosolic phospholipase A₂ investigated using a continuous fluorescence displacement assay: evidence for enzyme clustering on phospholipid vesicles

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The 85 kDa human cytosolic phospholipase A₂ has been cloned and expressed in insect Sf21 cells. The pure enzyme has been investigated using a fluorescence displacement assay that provides a continuous record of phospholipid hydrolysis [Wilton (1990) *Biochem. J.* **266**, 435–439]. The unusual kinetic properties of this enzyme, previously described using radioactive assays, were readily demonstrated using the continuous fluorescence assay and were examined in detail. It is proposed that the enzyme clusters on the surface of a fixed number of substrate vesicles during the initial stages of catalysis and that the characteristic burst phase of hydrolysis represents the hydrolysis of these vesicles. This clustering produced a molar ratio of total phospholipid substrate to enzyme of about 450:1 at vesicle saturation with enzyme. Under limiting substrate conditions, the lower secondary rate that is observed results eventually in almost

complete hydrolysis of the phospholipid; this was confirmed using radioactive substrate. Evidence is presented that during the initial burst phase, equivalent to hydrolysis of the outer monolayer of the vesicle, the enzyme remains tightly bound but is released as the reaction proceeds towards complete hydrolysis of the phospholipid substrate. In the presence of excess substrate, about 370 mol of fatty acid are released per mol of enzyme during the burst phase and it is calculated that this value also approximates to hydrolysis of the outer monolayer of the vesicle. It is proposed that the formation of a stable enzyme-vesicle complex during the burst phase of phospholipid hydrolysis may be due, at least in part, to protein-protein interactions between adjacent enzyme molecules in order to account for the clustering phenomenon.

INTRODUCTION

Human cytosolic phospholipase A₂ (cPLA₂) is an 85-kDa enzyme that displays a preference for arachidonic acid-containing phospholipids (Sharp et al., 1991; Clark et al., 1991) and was first detected in human neutrophils and platelets (Kramer et al., 1986; Alonso et al., 1986). The enzyme shows a calcium-dependent translocation to membranes (Channon and Leslie, 1990), appears to be hormonally regulated (Gronich et al., 1988) and is modulated by phosphorylation (Lin et al., 1992, 1993; Nemenoff et al., 1993). Therefore, the enzyme may play a primary role in arachidonic acid release and subsequent eicosanoid biosynthesis during the inflammatory response by cells.

Detailed kinetic studies of the pure enzyme using radioactive arachidonyl-containing phosphatidylcholine have highlighted the complex kinetics of this system (Leslie, 1991; Ghomashchi et al., 1992; Reynolds et al., 1993), the lysophospholipase (Leslie, 1991) and transacylase (Reynolds et al., 1993) activity of the enzyme and the fact that calcium is not required as part of the catalytic mechanism (Ghomashchi et al., 1992; Reynolds et al., 1993).

We have examined the kinetic properties of pure human recombinant cPLA₂ expressed in insect Sf21 cells. The enzyme activity has been measured using a fluorescence displacement assay involving normal substrates which allows a continuous record of reaction progress under a variety of conditions (Wilton, 1990; Kinkaid and Wilton, 1991). The particular advantage of

the fluorescence assay with this enzyme is that the assay allows a rapid and detailed demonstration of the unusual kinetic properties of the enzyme. We have been able to confirm the results of previous work that used radioactive substrates and to define further the properties of this important and unusual enzyme under conditions that have not involved the addition of other lipids or detergents. We would propose that under these assay conditions a phenomenon of essentially irreversible co-operative enzyme clustering occurs that covers the surface of the phospholipid vesicles. This stable cooperative clustering is the basis of the burst kinetics, which corresponds to the rapid and complete hydrolysis of the outer monolayer of these small vesicles. The enzyme is subsequently released as phospholipid hydrolysis proceeds to completion.

MATERIALS AND METHODS

11-(Dansylamino)undecanoic acid (DAUDA) was obtained from Molecular Probes. Rat liver fatty acid-binding protein (FABP) was purified from a synthetic gene expressed in *Escherichia coli* following established procedures (Worrall et al., 1991). All phospholipids and other chemicals were obtained from Sigma. 1-Stearoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine was obtained from Amersham Life Sciences.

Abbreviations used: DAUDA, 11-(dansylamino)undecanoic acid; FABP, fatty acid-binding protein; SAPC, 1-stearoyl-2-arachidonyl-phosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; DLPC, dilinoleoyl-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secreted phospholipase A₂.

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Table 1 The ability of cPLA₂ to hydrolyse different types of vesicles prepared from 1-stearoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine

Small unilamellar vesicles (SUVs) were prepared by solvent (methanol) injection. Multilamellar vesicles (MLVs) were prepared by rehydration and resuspension of solvent-evaporated lipids. The MLV preparation was also subjected to two periods of probe sonication involving (a) three cycles of 15 s or (b) three cycles of 30 s. In all cases the SAPC was at a concentration of 12.3 μ M. The determination of the % total hydrolysis during the burst phase used the fluorescence assay. Sedimentation of vesicles was determined by ultracentrifugation followed by the measurement of radioactivity in the supernatant (see the Materials and methods section).

Vesicle preparation	Hydrolysis in burst phase (%)	Sedimentation of vesicles (%)
SUVs (from MeOH injection)	7.89 $\pm$ 1.27	8.81 $\pm$ 2.34
MLVs (unsonicated)	3.57 $\pm$ 1.1	85.38 $\pm$ 5.87
MLV + 3 $\times$ 15 s sonication	9.65 $\pm$ 0.56	23.51 $\pm$ 3.96
MLV + 3 $\times$ 30 s sonication	10.20 $\pm$ 3.32	3.26 $\pm$ 3.34

Expression of cPLA₂

A full-length cPLA₂ cDNA was isolated using PCR amplifiers from cDNA derived from U937 cell RNA. The 2000 bp fragment obtained was sequenced and subcloned into the insect cell expression vector pacYM1 to generate the expression construct, pYM1/cPLA₂. This expression construct was co-transfected into *Spodoptera frugiperda* (Sf21) cells using the 'Baculogold' system (AMS Biotechnology). A 20 l insect cell fermentation was infected (at a cell density of 0.6×10^6 cell/ml) with virus at a multiplicity of infection of 0.1. A fermentation time course in which cPLA₂ activity in cells was measured using a cPLA₂ assay (Takayama et al., 1991) showed that cell-associated cPLA₂ activity rises to a maximum 48–72 h post-infection. All fermentations were harvested at or beyond this 72 h point.

Purification of cPLA₂

Infected cells were lysed using a Polytron homogenizer in the presence of an EDTA-containing buffer and then centrifuged at high speed. The supernatant was subsequently applied to a Fractogel TSK DEAE-650 column (E. Merck) washed and eluted with a buffered gradient of 0–1 M KCl. The enzyme was monitored throughout the purification using Western blotting employing a cPLA₂-specific anti-peptide serum (Sharp et al., 1991) and enzyme assays (Takayama et al., 1991). The pooled fractions from the DEAE step were applied to a hexyl-agarose 4XL hydrophobic interaction column (Affinity Chromatography Ltd., Ballasalla, I.O.M., U.K.). After washing, the enzyme was eluted from the column using a gradient of 1.0–0 M KCl. The fractions from the hexyl-agarose step were pooled and applied to a Fractogel TSK HW-55 gel-filtration column (E. Merck). Two protein peaks were obtained from the TSK HW-55 column, the second of which was positive in the enzyme assay. The overall yield of the whole purification, based on mass of protein, was 7.8%. The purified protein preparation from the final step was analysed by SDS/PAGE and was found to run as a doublet at 100 kDa and was observed to be > 95% pure.

Fluorescence assay of PLA₂ activity

The fluorescence displacement assay has been described previously (Wilton, 1990, 1991; Kinkaid and Wilton, 1991). All enzyme assays were performed in a total volume of 1 ml in plastic

fluorimeter cuvettes using an Hitachi F2000 fluorimeter coupled to a microcomputer for data recording. A basic assay cocktail consisted of 0.1 M Tris/HCl, pH 9, containing 0.1 M NaCl, 1 μ M DAUDA and recombinant rat liver FABP (10 μ g/ml) together with phospholipid substrate. Initial fluorescence experiments were performed in the presence of 5 mM Ca²⁺. However, this was subsequently reduced to 0.3 mM as indicated. The normal substrate concentration was 12.3 μ M and was obtained by injecting stock substrate prepared in methanol into the assay buffer such that the final methanol concentration did not exceed 1% (v/v).

The dilute small unilamellar vesicle preparations produced as a result of this solvent injection method are not amenable to detailed analysis. The detailed studies of Kremer et al. (1977) indicate that only the phospholipid concentration in the injected solvent had a significant effect on the size of the vesicles and a diameter between 25 and 50 nm would be anticipated. No significant difference was obtained using vesicles prepared by injection of phospholipid concentrations in methanol of 1 mg/ml or 10 mg/ml.

The small size and hence high curvature of these vesicles was confirmed by ultracentrifugation.

Over 90% of vesicles prepared by the injection method using 1-stearoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine ([¹⁴C]SAPC) were not sedimented by ultracentrifugation at 500 000 g for 10 min in a Beckman TLX ultracentrifuge. In comparison, liposomes (multilamellar vesicles) prepared by the standard 'handshaking' method (New, 1990) were mostly sedimented under these conditions (Table 1).

Vesicles produced by methanol injection were stable over a 4 h period after preparation, as judged by sedimentation, while there was no significant change in the kinetics of hydrolysis by cPLA₂ over this period. All assays with a preparation of vesicles were performed within this time period.

The standard amount of enzyme that was used was 0.88 μ g (10.4 pmol). The fall in fluorescence at 500 nm was recorded with time after addition of enzyme to the assay. All reactions curves have been normalized so that the fluorescence value immediately after addition of the enzyme is 100%. All fluorescence traces that are shown are representative of normally three experiments. Any variation from standard conditions is described in the text. In particular, the pH of the assay was later reduced to 8 and also 7.4 without major effects on the kinetic properties of the enzyme.

Calibration of the assay was achieved by adding up to 5 nmol of arachidonic acid as a 1 mM solution in methanol to the complete assay in the absence of enzyme. The assay will lose sensitivity at high substrate concentrations due to significant partitioning of the DAUDA into the vesicles with a resultant increase in non-FABP-bound fluorescence.

Radioactive assay of PLA₂ activity

All assays used 1.23 nmol of [¹⁴C]SAPC unless otherwise stated. The fluorescence assay was first performed under standard conditions using this radiolabelled SAPC. Assays were then stopped at various time points by addition to chloroform/methanol (2 ml; 1:2, v/v) and extracted. The chloroform layer was dried using N₂, resuspended in methanol and applied to a t.l.c. plate that had been previously run with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). The plate was then run in the same solvent and the resulting separated lipids detected using both autoradiography and a radiochromatogram scanner. Areas of radioactivity were removed from the plate and quantified using a scintillation counter. Only two peaks of

radioactivity were observed in all reactions, corresponding to substrate and arachidonic acid.

RESULTS

Assay of cPLA₂ activity

The kinetics of hydrolysis of 2-arachidonyl-phosphatidylcholine by cPLA₂ are dominated by a burst phase of product release followed by a slow second phase of hydrolysis when using sonicated vesicle preparations; this loss of activity has been attributed to the trapping of the enzyme on product-containing vesicles (Ghomashchi et al., 1992). The characteristic reaction profile is clearly illustrated using the continuous fluorescence assay while the extent of the burst phase in the presence of excess substrate is a function of enzyme concentration whether presented as a single addition of enzyme or successive additions after cessation of the burst phase (Figure 1). This result clearly confirms that the burst phase is not due to depletion of substrate but due to loss of enzyme activity, although the enzyme has not been irreversibly inhibited (Ghomashchi et al., 1992). Also, in the presence of excess substrate, the initial rate of the burst phase of the reaction is linearly related to the enzyme concentration, using up to 5 µg/ml (results not shown).

When non-sonicated multilamellar vesicles were used at the same phospholipid concentration, activity was greatly reduced and the burst phase was much less apparent. This reduced activity may reflect the lower curvature of these vesicles. Sonication resulted in activation and the emphasis of the characteristic burst kinetics (Table 1) seen with vesicles prepared by methanol injection, an observation that confirms the studies of Wijkander and Sundler (1991) using mouse macrophage membranes.

These results establish that the indirect fluorescence assay is a faithful measure of phospholipid hydrolysis catalysed by this enzyme. The enzyme activity measurements described in this paper were performed using conditions that involved assays at pH 9 as this was the reported pH optimum of the enzyme (Kramer et al., 1987). Subsequent studies, performed at more

Figure 1 The effect of sequential additions of cPLA₂ on the reaction rate

Assays were carried out at pH 9 and with 5 mM Ca²⁺ using successive amounts of cPLA₂ under standard conditions in the presence of excess substrate (12.3 µM). The cPLA₂ (0.88 µg) was added at time 0, 60 and 150 s and the burst reaction was recorded.

Figure 2 The effect of using different substrates (SAPC, DOPC and DLPC) under standard assay conditions

(a) Blank rate is the change in fluorescence of an assay containing SAPC in the absence of enzyme; (b) rate in the presence of 12.3 µM DOPC; (c) rate in the presence of 12.3 µM DLPC; (d) rate in the presence of 12.3 µM SAPC. Assays were in the presence of 0.88 µg of enzyme.

physiological pH values (8 and 7.4), show that the kinetic properties of the enzyme were not significantly affected. Likewise, Ca²⁺ in the assay was initially fixed at 5 mM but subsequently reduced to 0.3 mM and again no difference was noted under the standard assay conditions reported in this paper.

Substrate specificity of cPLA₂

An important characteristic of cPLA₂ is the preference of the enzyme for arachidonic acid at the *sn*-2 position of the phospholipid and this has recently been investigated in detail (Hanel et al., 1993). A particular advantage of the fluorescence assay is that it uses normal substrates and, therefore, it was possible to compare activity on a range of substrates. In practice, this approach is difficult with interfacial enzymes because of the need to discriminate between interfacial effects, i.e. interfacial binding and activation, as opposed to the intrinsic substrate preference of the enzyme (Ghomashchi et al., 1991). However, comparison of simple enzyme activity measurements would be more significant within a single class of phospholipids such as the phosphatidylcholines where different interfacial effects produced by the head group are minimized.

When three different phosphatidylcholines, namely SAPC, dioleoyl-phosphatidylcholine (DOPC) and dilinoleoyl-phosphatidylcholine (DLPC), were compared (Figure 2) the results clearly highlight the preference of this enzyme for arachidonyl-containing phospholipids. No burst phase was noted within the timescale of the reactions involving DOPC and DLPC. These results provide further confirmation of the validity of the fluorescence assay for measuring the activity of this enzyme.

Effect of substrate and enzyme concentration on cPLA₂ activity and the extent of the burst reaction

As discussed above, cPLA₂ activity on arachidonyl-containing phosphatidylcholine is characterized by a rapid burst reaction followed by a slow second phase of enzyme activity when small vesicles are used. These characteristic kinetic properties of this enzyme may be interpreted in terms of the inactivation of the enzyme because it becomes irreversibly bound to product-

Figure 3 The effect of excess enzyme on the extent of the burst reaction

The cPLA₂ added was varied using a limiting substrate concentration (2.5 nmol) and the product released (nmol) during initial burst and also the percentage phospholipid hydrolysis was plotted against nmol of cPLA₂ added. Assays were performed in 0.3 mM Ca²⁺. The minimum molar ratio of phospholipid to enzyme that gave maximum product release was determined by extrapolation of the initial part of the curve to the point of maximum product release. The amount of enzyme (nmol) was calculated at this point and was determined to be approximately 0.004 nmol.

containing vesicles (Ghomashchi et al., 1992). This explanation was consistent with the known properties of the system and, in particular, conditions that removed products of the reaction or promoted intervesicular exchange enhanced the burst phase of the reaction.

Of particular interest to us was a quantitative measure of the entrapment of enzyme on product-containing vesicles and the percentage of product present in the vesicle before enzyme activity became minimal following the burst phase. Moreover, the maximum number of enzyme molecules that could be trapped on these vesicles (molar ratio of phospholipid to enzyme) would provide valuable information on the overall steric constraints of the process.

When the effect of enzyme concentration on enzyme activity was examined by incubating excess substrate with various concentrations of enzyme both the initial rate and the size of the burst reaction was related to enzyme concentration (results not shown). However, at lower substrate concentrations a limiting value for the extent of the burst reaction was achieved with increasing amounts of enzyme as shown in Figure 3. Moreover, the addition of excess free enzyme under these conditions failed to produce further catalysis unless there was a further addition of substrate to these substrate-limited assays (Figure 4).

The effect of enzyme and substrate concentration on the extent of the burst reaction under normal assay conditions can be explained if the enzyme shows a phenomenon of cooperative clustering on the surface of vesicles and the extent of the burst reaction simply reflects the number of substrate vesicles that are coated with enzyme and are available for hydrolysis. These vesicles undergo hydrolysis to an end-point which is dictated by substrate availability within the vesicles while at this end-point of the burst phase the enzyme is essentially irreversibly trapped on this product-containing vesicle as suggested by others (Ghomashchi et al., 1992). In other words, a certain number of enzyme molecules are able to titrate out (by tight binding and

Figure 4 The effect of further enzyme and substrate additions at the end of the burst phase under limiting substrate conditions

Using limiting substrate, cPLA₂ was added and a burst of reaction resulted. Further addition of cPLA₂ did not produce a subsequent burst while addition of SAPC resulted in a further round of hydrolysis. The assay was performed in 0.3 mM Ca²⁺.

hydrolysis) a certain number of vesicles under the burst phase conditions of these assays.

The stoichiometry of the phenomenon suggested above can be determined from the data described in Figure 3. When increasing amounts of enzyme are added to a constant limiting substrate concentration then the minimum molar ratio of substrate to enzyme to give maximal burst hydrolysis is about 600 mol of phospholipid/mol of enzyme. When higher concentrations of enzyme are present there is excess enzyme in free solution that is available to hydrolyse additional added substrate (results not shown but see Figure 4). Thus, it is possible to titrate out a low fixed concentration of phospholipid with enzyme.

As a result of combining several experiments of this type and plotting the percentage of the maximum initial hydrolysis for each experiment against the molar ratio of cPLA₂:SAPC (Figure 5) a more accurate molar ratio at saturation was achieved and approximated to a value of 450 mol of phospholipid/mol of enzyme with an estimated error of ± 100 .

The topology of binding of cPLA₂ to phospholipid vesicles

The experiments described above (and see below) allowed an approximate quantification of enzyme binding to the surface of the vesicle. Using the value of 450:1 derived from Figure 5, this value should represent the maximum occupancy of enzyme on the phospholipid surface and has to be adjusted to account for the percentage of phospholipid in the outer monolayer. Although the precise dimensions of these unsonicated vesicles are not known they are of small size (see the Materials and methods section). Therefore we will use values of 70% or 60%, which approximate for those expected for the outer monolayer of highly curved sonicated vesicles of 25 or 50 nm diameter respectively. This will give a ratio for outer monolayer of phospholipid to enzyme of 315:1 for a 25-nm-diam. vesicle and 270:1 for a 50-nm-diam. vesicle. These values assume that all phospholipid

Figure 5 The effect of the molar ratio of cPLA₂:SAPC on the extent of the burst reaction

The data from a combination of several graphs of the type shown in Figure 3 were combined and plotted as percentage maximum initial hydrolysis for each experiment against the molar ratio of cPLA₂:SAPC.

is present in the form of small unilamellar vesicles and as such is a maximal value. Assuming the planar dimension of a single phospholipid molecule is 65 Å² (Mohwald, 1990) and the projected surface area of the 85 kDa cPLA₂ is 3200 Å² [based on a radius of 32 Å (Hanel et al., 1993)] then a minimum footprint of 50 mol of phospholipid/mol of enzyme would be predicted assuming a monolayer array of interacting protein molecules on the vesicle surface. This theoretical value of 50 assumes a spherical protein molecule and does not take into account the phospholipid molecules that must exist in the outer monolayer between the protein molecules and any distortions at the protein/phospholipid interface that would increase the footprint size; hence the value of 50 must be a minimal value and can be compared with a value of 35–40 obtained experimentally for the much smaller (14 kDa) pancreatic enzyme (Ramirez and Jain, 1991).

Stoichiometry of hydrolysis during the burst phase

It should be noted that in the case of secreted phospholipase A₂ (sPLA₂), e.g. from porcine pancreas, hydrolysis in the scooting mode as a result of essentially irreversible binding to the anionic vesicle produced a molar ratio of product to enzyme of 4400 in the presence of excess substrate and this was consistent with only one bound enzyme molecule per substrate vesicle (Ramirez and Jain, 1991).

In order to determine the value for cPLA₂ in the presence of excess substrate, the stoichiometry of hydrolysis was determined over a range of enzyme concentrations using a fixed concentration of substrate, and the data was plotted in terms of mol of product/mol of cPLA₂ as a function of [SAPC]/[cPLA₂]. The results are shown in Figure 6(a) using a fixed substrate concentration of 3 μM and a value for the stoichiometry of hydrolysis of 368 ± 50 mol of product per mol of enzyme was obtained at saturating substrate concentrations (Figure 6b). Similar results were obtained using substrate concentrations of 6.15 μM and 2.46 μM. This value of 368 is about 10-fold lower than the value

Figure 6 The relationship between the molar ratio of substrate to enzyme and the molar ratio of product to enzyme released during the burst phase of hydrolysis

(a) The number of mol of fatty acid released during the burst phase of hydrolysis per mol of enzyme was determined under conditions in which various concentrations of enzyme were assayed with a fixed concentration of SAPC (3 μM) to produce a range of molar ratios of substrate to enzyme (see Figure 7). (b) The data is plotted as reciprocals in order to determine the value of product release in the presence of excess substrate (368 ± 50 mol of product per mol of enzyme).

(4400) for sPLA₂, indicating multiple protein molecules may be bound per vesicle and the possible significance of this figure is discussed below.

A direct demonstration of the capacity of phospholipid vesicles to trap added cPLA₂

The experiments described above provide indirect kinetic evidence for the cooperative entrapment of enzyme on a limiting number of substrate vesicles. In order to provide an alternative method for demonstrating this phenomenon, a fixed concentration of SAPC vesicles were incubated with increasing amounts of cPLA₂ for 90 s to allow the enzyme to become tightly bound to the vesicles during burst hydrolysis. After this time a small amount of radioactive SAPC was added. Initial hydrolysis of this substrate to radioactive products will give a measure of the

Figure 7 The relationship between the ratio of phospholipid to enzyme and the concentration of free enzyme

Increasing amounts of cPLA₂ were added to assays containing a fixed concentration of non-radioactive SAPC (3 μ M) and reaction was allowed to proceed for 90 s during which time hydrolysis (expressed as a percentage of total substrate) was determined using the fluorescence assay. After this time, [1-¹⁴C]SAPC (1.23 μ M) was added and free enzyme activity was assayed by measuring radioactive fatty acid released after 90 s. The results are expressed as a percentage of total radioactive substrate, assuming radioactive and non-radioactive substrates do not mix, over the short time scale of this experiment. ■, % hydrolysis of SAPC using fluorescence assay; ▲, % hydrolysis of radioactive SAPC determined by solvent extraction and t.l.c.

amount of enzyme in the assay that is not bound to the original non-radioactive SAPC vesicles. It is thus possible to perform a titration of the capacity of a fixed amount of this original non-radioactive SAPC to bind enzyme. The results are shown in Figure 7 and it is apparent that there is a clear break point at about 6 nM cPLA₂ above which there is a rapid increase in the amount of free enzyme. This break point corresponds to a total phospholipid:enzyme ratio of about 500:1 and is in good agreement with the value of 450:1 determined from Figure 5.

Stoichiometry of hydrolysis in the burst phase using excess enzyme

In the presence of excess enzyme the maximum percentage phospholipid hydrolysis during the burst phase corresponds to approximately 50% of the total phospholipid present in the assay. Since hydrolysis is assessed indirectly as a fall in fluorescence, which was calibrated by addition of free arachidonic acid to the complete system, this approx. 50% figure was confirmed using radioactive SAPC. The products of the reaction were extracted and separated by t.l.c. and the radioactivity in SAPC and arachidonic acid was measured. The hydrolysis values by this method were $59 \pm 16\%$ and $69 \pm 21\%$ for 0.5 and 1 min incubations respectively.

The limiting hydrolysis that was observed approximates to the anticipated value if essentially complete hydrolysis of the outer monolayer of the bilayer is being observed in the burst phase. In order to confirm that the partial hydrolysis did not reflect a situation in which the remaining phospholipid was refractory to phospholipase A₂ hydrolysis, due to some unappreciated physico-chemical property of the vesicle, the 'highly penetrating' (Van der Wiele et al., 1988) 14 kDa *Naja naja* phospholipase A₂ was

added after completion of the burst phase of the reaction catalysed by the cPLA₂. An additional hydrolysis was observed, as denoted by a further fall in fluorescence (results not shown). In comparison, no additional hydrolysis was observed after further addition of cPLA₂ under these substrate-limiting conditions (Figure 4).

It is of interest that this product vesicle, which must still have bound cPLA₂ after the burst phase, was hydrolysed by the 14 kDa secreted phospholipase A₂. However, this rate of hydrolysis was less than 1% of the rate observed when this *N. naja* enzyme was added to an assay containing the equivalent quantity of unhydrolysed vesicles in the absence of the cPLA₂ (results not shown). Therefore, whatever the structure of the product vesicle-enzyme complex, the rate of hydrolysis of the residual SAPC by the 14 kDa *N. naja* enzyme is inhibited by over 99%.

It was noteworthy that even when the burst reaction in the presence of excess enzyme reflected approx. 50% of total substrate hydrolysis, a significant rate of fall in fluorescence was observed in the second phase. Since the only substrate left should be within the inner monolayer of the vesicles then this additional rate should result from vesicle restructuring and possible flipping of phospholipid molecules from the inner to the outer monolayer. Alternatively it might reflect lysophospholipase activity that is demonstrated by this enzyme. To clarify this point, the fluorescence assay was measured using the radioactive substrate, [1-¹⁴C]SAPC (1.23 μ M) and the products were analysed at various time intervals by t.l.c. The results showed that the percentage phospholipid hydrolysis at 0.5, 1, 5 and 30 min was $59 \pm 16\%$, $69 \pm 21\%$, $80 \pm 20\%$ and $85 \pm 6\%$ respectively. An additional radioactive experiment for 5 h gave > 95% hydrolysis. Therefore the slow second phase of the reaction observed under these substrate-limiting conditions by the fluorescence assay clearly represents continued hydrolysis of the phospholipid substrate with a resultant release of bound enzyme.

Thus, depending on the precise incubations that are employed, particularly in terms of enzyme-substrate stoichiometry and duration of the assay, the enzyme will appear to be more or less irreversibly bound to product-containing vesicles.

DISCUSSION

Since its original independent identification by several groups, the arachidonic acid-specific high-molecular-mass phospholipase A₂, now called cPLA₂, has attracted much attention as a possible regulator in the production of pro-inflammatory mediators derived from arachidonic acid. With the availability of highly purified forms of the enzyme, the unusual kinetics that were suggested by earlier work were confirmed and were the subject of detailed studies (Ghomashchi et al., 1992). Since these burst kinetics required more than initial rate measurements, a continuous rather than a radioactivity-based discontinuous assay had obvious attractions. The classical pH stat-based continuous assays used with great effect to study the 14 kDa sPLA₂ are about 1000-fold less sensitive than the fluorescence assay and are not, at present, viable with the cPLA₂.

In this paper we have used a continuous fluorescence displacement assay to investigate the properties of this enzyme. This assay measures the fall in fluorescence as the fluorescent probe, DAUDA, is competitively displaced from rat liver FABP by released long-chain fatty acids. Using this assay, we have been able to confirm all the basic properties of the enzyme previously elucidated by following the release of radioactive fatty acids. These results establish that the information derived from the fluorescence assay is a true reflection of data obtained using radioactive measurement. In addition, we have been able to

extend these data to reveal some unusual characteristics of this enzyme when assayed *in vitro* using small vesicles. The effect of the molar ratio of phospholipid to enzyme on the extent of the burst reaction has been analysed in detail.

Calculation of the phospholipid to protein stoichiometry of the burst reaction at the point when substrate is saturated produced a limiting ratio of total phospholipid to enzyme of about 450:1. This limiting ratio at saturation, which can be obtained in the presence of either limiting enzyme or substrate, suggests that the enzyme aggregates or clusters on the surface of the vesicle during the initial phase of hydrolysis. As a consequence of this aggregation phenomenon, a certain amount of enzyme will initially bind to and hydrolyse a certain number of vesicles to produce the burst kinetics with quantal release of products. Moreover, using excess enzyme an initial burst of hydrolysis of about 50% suggests that an amount of phospholipid corresponding to the outer monolayer is available to the enzyme and that vesicle integrity is maintained during hydrolysis, possibly as a result of a stabilizing effect by the enzyme.

It is possible to calculate an approximate figure for the molar stoichiometry of enzyme to vesicles at saturation. If the substrate vesicles are 25 nm diameter then, assuming a phospholipid headgroup area of 0.65 nm² (Mohwald, 1990) and a bilayer thickness of 4 nm (Cullis and Hope, 1991), each vesicle will contain 4400 phospholipid molecules with about 3000 being in the outer monolayer. The corresponding values for a 50-nm-diam. vesicle are 20600 and 12100 phospholipid molecules. [Jain et al. (1991) use a value of 0.45–0.50 nm² for the area of a phospholipid containing two saturated acyl chains. Using 0.45 nm² the corresponding figures for 25-nm-diam. vesicles would be about 6400 for total phospholipid with about 4400 molecules in the outer monolayer. The values for a 50-nm-diam. vesicle are about 29800 total phospholipid molecules and with 17500 in the outer monolayer.]

The experimental value of 450:1 for the ratio of total phospholipid to enzyme at equivalence would mean that on average there would be about 10 and 46 enzyme molecules bound to 25- and 50-nm-diam. vesicles respectively. (14 or 66 molecules using a phospholipid area of 0.45 nm².) If these 10 or 46 molecules of enzyme were completely hydrolysing the outer monolayer of the appropriate vesicle in the burst phase then they should release 300 or 263 mol of fatty acid respectively. The value of 368 ± 50 determined experimentally is close to these theoretical values, suggesting that the burst phase may indeed approximate to the hydrolysis of the outer monolayer of a fixed number of vesicles on which the enzyme has clustered.

The results with cPLA₂ have some parallels with the elegant studies with porcine pancreatic sPLA₂ operating under scooting conditions with anionic vesicles (Ramirez and Jain, 1991). In the case of the pancreatic enzyme a limiting amount of product formation was also achieved in the presence of excess substrate but this much higher value (4400 mol of product per mol of enzyme) equated to one enzyme molecule per vesicle hydrolysing the outer monolayer for vesicles of 23 nm diam. and larger values for product formation were obtained with larger vesicles (Berg et al., 1991). Thus the critical difference is that the data for the pancreatic enzyme is readily interpreted by monomeric behaviour of the enzyme (Jain et al., 1991), while the burst kinetics of cPLA₂ are best explained by enzyme clustering on a smaller number of vesicles with a resulting hydrolysis of these vesicles. Moreover, the cPLA₂ showed a marked preference for smaller vesicles and, unlike the pancreatic enzyme (Berg et al., 1991), larger vesicles produced a smaller burst reaction (Table 1).

It should be noted that the enzyme molecules must be organized in a loose array since the number of bound protein molecules per

vesicle that has been calculated is considerably less than the maximum number of molecules that might be anticipated assuming a protein footprint for a spherical molecule equivalent to about 50 phospholipid molecules (see the Results section). These numbers would be 60 or 240 for 25- or 50-nm-diam. vesicles respectively. One possible model which would enhance the surface area of the phospholipid-binding surface of the protein would be for the protein to have a more flattened structure with a concave interfacial surface. An interesting consequence of such a structural model is that it should prefer to bind to curved phospholipid vesicles and membranes.

The concept of protein aggregation/clustering on the phospholipid surface finds support from studies using other Ca²⁺- and phospholipid-binding proteins. One protein that has been studied in considerable detail is annexin V (35 kDa) and binding studies have produced values for the binding stoichiometry ranging from 42 to about 250 mol of monolayer phospholipid/mol of annexin depending on the technique used (Tait et al., 1989; Andree et al., 1990, 1992; Meers et al., 1991) that would indicate protein clustering.

The physiological significance of these *in vitro* observations on cPLA₂ remains to be clarified. The concept of protein clustering on a planar phospholipid surface has been proposed to explain the anti-coagulation properties of the phospholipid-binding protein annexin V. In this case, a sheet of protein was observed attached to phospholipid vesicles using electron microscopy (Andree et al., 1992). Moreover, a two-dimensional array of annexin V on phosphatidylserine-containing liposomes has been proposed, which is stabilized by both protein–lipid and protein–protein interactions (Pigault et al., 1994). In another example, the aggregation of the lung surfactant protein SP-C on phospholipid vesicles containing dipalmitoyl-phosphatidylcholine has been observed using fluorescence energy transfer experiments and may be linked to the physiological role of this protein (Horowitz et al., 1993).

There is an apparent requirement for small, highly curved, substrate vesicles or membranes (Wijkander and Sundler, 1991) *in vitro* in order to demonstrate the enhanced activity and unusual burst kinetics of cPLA₂ with quantal release of arachidonic acid. It remains to be established if clustering of this enzyme could occur *in vivo* during membrane hydrolysis. It is noteworthy that, for example, such curved membrane structures are produced *in vivo* as a result of membrane budding in a GTP-binding-protein-linked process during intracellular protein transport (Rothman, 1994). The potential effect of phosphorylation and/or changes in intracellular free [Ca²⁺] on the kinetics of this enzyme is also of particular interest.

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