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The alkaline phospholipase A_1 of rat liver cytosol

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1. Rat liver cytosol contains a heat-sensitive phospholipase A_1 active against phosphatidylethanolamine, 1-acylglycerophosphoethanolamine and, to a very much lesser extent, phosphatidylcholine and phosphatidylinositol. 2. Activity towards a pure phosphatidylethanolamine substrate is invoked by the presence of water-soluble cations that do not precipitate at the pH optimum of the enzyme (9.5). In this activation bivalent cations, e.g. Mg^{2+}, Ca^{2+}, Mn^{2+}, Sr^{2+} and Ba^{2+}, are effective at much lower concentrations (2.5–5 mM) than univalent cations K^+, Na^+ and NH_4^+ (100 mM). 3. In the absence of such cations the enzyme can be activated by cationic amphiphiles containing quaternary nitrogen or by basic proteins. 4. It is concluded that these agents activate the enzyme by reducing the negative zeta potential on the substrate at the high pH optimum (9.5) and allow interaction with the enzyme whose isoelectric point is at 7.15. 5. The activated enzyme is markedly inhibited by mixing the phosphatidylethanolamine substrate with many other phospholipids that exist in cell membranes, e.g. phosphatidylcholine, phosphatidylinositol. On the other hand, both phosphatidylcholine and phosphatidylinositol can be hydrolysed much more readily if they are mixed with an excess of phosphatidylethanolamine. 6. Such results on the inhibition and substrate specificity of the enzyme, coupled with birefringence measurements, allow the tentative conclusion that phospholipid substrates are only attacked when they exist in a hexagonal or non-bilayer structure and not in the bilayer (lamellar) form.

Although soluble phospholipase activity has previously been detected in the cytosolic (supernatant) fraction of rat liver homogenate, the initial site of attack on the phosphatidylethanolamine substrate was not determined owing to heavy contamination of the enzyme preparation with phospholipase B (Waite & van Deenen, 1967). When the latter enzyme was inhibited with deoxycholate both 1-acyl- and 2-acyl-glycerophosphoethanolamine were produced at neutral pH, indicating the presence of phospholipase A_1 and A_2 activities. More recently, a cytosolic phospholipase A_1 has been described in rat liver that was detergent-activated and had a pH optimum of 3.6; the investigators believe that this is different from the soluble phospholipase A_1 of lysosomes (Suzuki & Matsumoto, 1978).

Recently we obtained evidence that the supernatant fraction obtained from sucrose homogenates of rat liver contained an active phospholipase deacylating phosphatidylethanolamine at an alkaline pH (Dawson *et al.*, 1982). In the present paper the properties of the partially purified enzyme are described.

Methods

Labelled substrates

^{32}P-labelled phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol were purified from *Saccharomyces cerevisiae* grown in limiting [^{32}P]P_i (Irvine *et al.*, 1978). The fatty acid composition of the products as determined by g.l.c. showed almost exclusively C_{16} and C_{18} acids, principally palmitoleate and oleate (Table 1). Phosphatidylethanolamine, the preferred substrate, contained 92% of these monoenoic acids, so the principal molecular moieties present must be dimonoenoic esters of glycerophosphoethanolamine.

Glycerol tri[^{14}C]oleate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. 1-Acylglycero[^{32}P]phosphoethanolamine was prepared by incubating [^{32}P]phosphatidylethanolamine (4 μM) in 6 ml of 33 mM-NaOH/maleate buffer, pH 6.8, containing 3.3 mM-$CaCl_2$. To this substrate was added 4 mg of cobra (*Naja naja*) venom as a source of phospholipase A_2 and 0.8 ml of diethyl ether was then added to saturate the medium. After incubation for 90 min at 37°C the substrate had

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Table 1. *C_{16} and C_{18} fatty acid content of phospholipid substrates*
The substrates were hydrolysed with alkali and the liberated fatty acids were determined by g.l.c.

	Fatty acid composition (%)			
	Palmitate	Palmitoleate	Stearate	Oleate
Phosphatidylethanolamine	5.5	54.8	0.1	37.5
Phosphatidylcholine	5.6	59.8	2.7	30.6
Phosphatidylinositol	23.1	32.5	7.3	32.8

been quantitively hydrolysed. The lysophosphatidylethanolamine was twice extracted from the incubation medium with 6ml of butanol, and after adding 4vol. of ethanol the butanol extract was taken to dryness. The lipoidal products were applied as a strip to a t.l.c. plate (silica gel 60F-254 from Merck) and separated by the solvent chloroform/methanol/water/acetic acid (60:50:4:1, by vol.). The 1-acylglycero[^{32}P]phosphoethanolamine was located by autoradiography and eluted [chloroform/methanol (1:1, v/v)]. The lysophosphatidylethanolamine (2-acyl) formed by the rat liver phospholipase was isolated in a similar manner.

Preparation of enzyme

Rat liver (12g) was cooled on ice and homogenized (Potter–Elvehjem homogenizer) with 48ml of ice-cold 0.25M-sucrose. It was centrifuged at 100000 *g* for 60min, and the supernatant was collected. To 25ml of the supernatant at 0°C was gradually added 4.85g of solid $(NH_4)_2SO_4$ with stirring. At 10min after it had completely dissolved, the mixture was centrifuged (5min at 10000 *g*), the supernatant rejected and the pellet dissolved in 2.5ml of water. This was mixed with 0.25ml of 0.2M-2-amino-2-methylpropane-1,3-diol/HCl buffer, pH8.6, and put on to a Sephadex G-150 column (1cm diameter × 110cm long) poured and equilibrated with 0.02M-2-amino-2-methylpropane-1,3-diol/HCl buffer, pH8.6. The phospholipase A_1 was excluded from the column and eluted with the void volume. The fractions (5ml) containing significant enzymic activity were collected. Purification was 8–10-fold on a protein basis, and the preparation, when tested under the conditions specified below, released mainly lysophosphatidylethanolamine and little glycerophosphoethanolamine. Although whole rat blood contained low enzymic activity of a similar nature, it would contribute only a few per cent of the total phospholipase A_1 activity observed in whole liver.

Determination of enzymic activity

[^{32}P]Phosphatidylethanolamine and inactive yeast phosphatidylethanolamine were mixed in chloroform solution to give 0.34μmol and (4–8) × 10^4 d.p.m. The solvent was removed at low pressure in a 10ml stoppered incubation tube and the phospholipid was dispersed in 5mM-glycine/glycylglycine/NaOH buffer, pH9.5, containing 5mM-$MgCl_2$ or other activators if required. Enzyme (0.05ml; 7.5μg of protein) was added, making a total volume of 1ml and the mixture incubated for 30min at 37°C. The reaction was stopped by immersion of the tube for 2min in a boiling-water bath and, after cooling, lysophosphatidylethanolamine and unchanged substrate were extracted into n-butanol (2 × 0.75ml). The butanolic solution was mixed with 4vol. of ethanol and taken to dryness *in vacuo*. The lipid residue was quantitatively spotted on to a t.l.c. plate (Merck silica gel 60F-254) with an internal marker of lysophosphatidylethanolamine (1μg of phosphorus) and the plate was developed in chloroform/methanol/water/acetic acid (65:50:4:1, by vol.). Phosphatidylethanolamine and lysophosphatidylethanolamine were located by spraying with ninhydrin (0.25% in acetone) and warming to 70°C, and the appropriate areas scraped into scintillation vials and counted for radioactivity. In some experiments the butanol-extracted aqueous phase was extracted twice with an equal volume of diethyl ether and a 0.5ml portion of the aqueous phase was counted by scintillation counting.

When [^{32}P]phosphatidylinositol and [^{32}P]phosphatidylcholine were used as substrates, the same procedure was adopted, except with the latter substrate chloroform/methanol/diethylamine/water (110:50:8:5, by vol.) was used as developing solvent for t.l.c. to give a more adequate separation of the substrate and its lyso-derivative.

1-Acyl-2-[^{14}C]linoleoylglycerophosphoethanolamine and 2-acyl-1-[^{3}H]palmitoylglycerophosphoethanolamine were biosynthesized by the procedure of Smith *et al.* (1973) and the liberation of lysophosphatidylethanolamine and fatty acid was followed by t.l.c. (as described above).

In experiments testing the release of [^{14}C]oleate from triacylglycerol, the hydrolysis was terminated as above and, after cooling, the incubation mixture (1ml) was treated with 1.5ml of methanol and 3ml of chloroform. After shaking and separating the lipid-containing chloroform layer by centrifugation, the lipids were resolved by t.l.c. on silica-gel plates in diethyl ether/light petroleum (b.p. 60–80°C)/acetic

acid (50:50:1, by vol.) and any fatty acid liberated examined for by autoradiography or counting the radioactivity in carrier spots (oleate) located with I_2 vapour.

Isoelectric focusing

The enzyme solution was concentrated (6×) by dialysis against polyethyleneglycol 4000 solid (1h). Isoelectric focusing was carried out in 5% gel [mixture of acrylamide and methylenebisacrylamide (323:10, w/v) containing 5% (v/v) ampholyte (LKB, Bromma, Sweden; mixture of pH interval range 2.5–11.0), 0.25% (v/v) *NNN'N'*-tetramethylethylenediamine (BDH) and 1.8×10^{-4}% (w/v) riboflavin]. The gels were prefocused at 50V for 15min and the concentrated enzyme was loaded on thick filter paper 0.3cm inside the catholyte. The gel was focused for 2h at 10W and then 5mm sections were sliced. The proteins in the slices were eluted with 0.25ml of 0.1M-glycine/NaOH buffer, pH 9.5, at 4°C overnight and 0.15ml of the eluate was assayed for phospholipase A_1.

Electrophoretic mobilities

Mobilities of substrate particles were measured as described previously (Dawson *et al.*, 1976).

Birefringence

The structure of the substrate particles was studied optically by using a Reichert microscope. Crossed polaroids were used with a first order red compensator so that lamellar structures showing various degrees of birefringence could be differentiated from non-birefringent structures.

Results

General properties of liver alkaline phospholipase A_1

When examined under the conditions specified so that the total substrate degradation was no greater than 15%, activity was approximately linear with respect to time (up to 30min) and enzyme concentration (up to 10μg of protein). The pH optimum, when examined in a series of glycine/glycylglycine buffers, was 9.5. The isoelectric point of the enzyme determined by gel isoelectric focusing was 7.15. Compared with many phospholipases the enzyme was heat-sensitive; when it was kept at 55°C for 5min in glycine/glycylglycine buffer at pH 9.5 84% of the activity was lost and at 60°C 96% of the activity was lost.

Substrate specificity

Phosphatidylethanolamine was very much the preferred substrate for deacylation compared with phosphatidylinositol and phosphatidylcholine (Table 2). Phosphatidylcholine was attacked at less than 5% of the rate of deacylation of phosphatidylethanolamine with either $MgCl_2$ or EDTA as activator (see below). Phosphatidylinositol was hydrolysed at about a quarter the rate of phosphatidylethanolamine with EDTA as activator, and hardly at all when $MgCl_2$ was used as activator. This latter result presumably is due to a coagulation of the substrate as an insoluble Mg^{2+} complex. Surprisingly, the hydrolysis of both the latter substrates was appreciably stimulated when they were presented to the enzyme admixed with high concentrations of phosphatidylethanolamine in spite of the dilution of the ^{32}P-labelled substrate with non-radioactive phosphatidylethanolamine, which would presumably also be attacked by the enzyme. Thus, under conditions when some 12% of a pure phosphatidylethanolamine substrate was deacylated in 30min about 3% of either phosphatidylinositol or phosphatidylcholine was deacylated when these substrates were mixed with six times their molar ratio of phosphatidylethanolamine (Fig. 1). However, the hydrolysis of phosphatidylcholine was not stimulated by various concentrations of Triton X-100 and dodecyl sulphate or by reducing the pH of the incubation medium.

On hydrolysing 1-acyl-2-[^{14}C]linoleoylglycerophosphoethanolamine with the enzyme, radioactivity was largely liberated (95%) as 2-[^{14}C]-linoleoylglycerophosphoethanolamine, suggesting that phospholipase A_1 is the predominant activity. With 2-acyl-1-[^{3}H]palmitoylglycerophosphoethanolamine as substrate, only ^{3}H-labelled fatty acid was formed, confirming this specificity. Both these substrates gave the reverse result when treated with a known phospholipase A_2 (*Naja naja* venom), i.e. ^{14}C in fatty acid and ^{3}H in lysophosphatidylethanolamine. 1-Acylglycero[^{32}P]phosphoethanolamine was hydrolysed at about 40% of the rate of phosphatidylethanolamine with Mg^{2+} (7.5mM) as activator. Under the usual conditions

Table 2. *Substrate specificity of alkaline phospholipase A_1*

Activator	Amount of substrate deacylated (μg of phosphorus): Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylinositol
$MgCl_2$ (7.5mM)	1.63	0.08	0.05
EDTA (7.5mM)	1.30	0.05	0.33

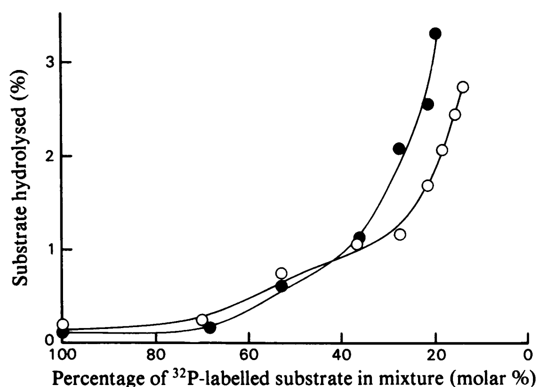

Fig. 1. *Hydrolysis of other ^{32}P-labelled phospholipids stimulated by admixture with phosphatidylethanolamine*
[^{32}P]Phosphatidylcholine (0.11 μmol; ○) or [^{32}P]-phosphatidylinositol (0.054 μmol; ●) was mixed with various amounts of phosphatidylethanolamine in chloroform solution, the solvent removed and the mixed phospholipids incubated as stated in the Methods section using 0.08 M-KCl + 1.5 mM-$MgCl_2$ as activator. The ^{32}P-labelled lysophospholipids produced were separated by t.l.c. and their radioactivity was determined.

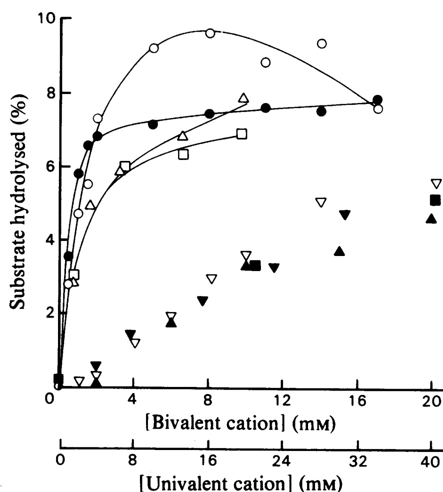

Fig. 2. *Activation of phospholipase A_1 produced by metallic cations*
The enzyme was incubated with [^{32}P]phosphatidylethanolamine at pH 9.5 as indicated in the Methods section without added activator. A series of metallic salt solutions adjusted to pH 9.5 were added to the system and the ^{32}P-labelled lysophosphatidylethanolamine produced was separated by t.l.c. and the radioactivity determined. In one instance EDTA adjusted to pH 9.5 with NaOH was included in the incubation and the results presented as a plot of activity versus mM-Na^+ added. Symbols: ○, $CaCl_2$; ●, $MgCl_2$; △, $SrCl_2$; □, $BaCl_2$; ▲, NaCl; ■, KCl; ▽, Na_2SO_4; ▼, sodium EDTA.

of incubation negligible quantities of glycerophosphoethanolamine were liberated from phosphatidylethanolamine. If the lysophosphatidylethanolamine liberated by the enzyme preparation (presumably 2-acylglycerophosphoethanolamine) was re-isolated and incubated with the enzyme some hydrolysis occurred (29%) compared with phosphatidylethanolamine. However, it is not possible to conclude that this represents a direct attack at the 2-acyl position. The liberation could be caused by lysophospholipase in the preparation or alternatively by non-enzymic acyl migration to the 1-position at the high alkalinity used for enzyme assay (pH 9.5).

The enzyme preparation did not liberate fatty acid from glycerol tri[^{14}C]oleate either alone or in the presence of deoxycholate (0.5 and 1 mg/ml) both with and without $CaCl_2$ (5 mM), so there is no evidence that a triacylglycerol lipase is responsible for the phospholipase activity. On the other hand, there was limited breakdown of added diacylglycerol (egg) visible on t.l.c. of the lipids extracted from the incubation medium and appreciable hydrolysis of 1-oleoylglycerol. When the latter monoacylglycerol was added to [^{32}P]phosphatidylethanolamine in equimolar porportions there was a reduction in the formation of [^{32}P]lysophosphatidylethanolamine on incubation to 41% of the control value. However, it is impossible to judge whether this results from true competition for a single active centre on the enzyme or from a change in substrate structure (see below).

Action of bivalent metal cations on phospholipase A_1 activity

The activity of the enzyme was barely detectable when it was incubated with phosphatidylethanolamine alone. The chloride salts of all bivalent metal cations that did not precipitate as insoluble hydroxides at pH 9.5 acted as good activators of the reaction. This included Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+}; about 50% of the full activation was achieved at 1 mM and maximum activation at about 5 mM (Fig. 2). $MnCl_2$ produced a small variable activation, which was undoubtedly influenced by the partial precipitation of manganous hydroxide.

Action of univalent cations on phospholipase A_1 activity

NaCl and KCl produced some activation of the reaction at concentrations above 2 mM (Fig. 2) but quite insufficient for the activation produced by the bivalent cation chlorides described above to be ascribed to the halide ion component. Sodium sulphate also produced an activation and when this was plotted on a scale relating the normality of the sodium concentration to activity, the activation curves were quite similar (Fig. 2). At first sight

EDTA appeared to be an excellent activator of the reaction. However, when the amount of NaOH needed to bring the pH to 9.5 was measured (equivalent to 3.84 Na atoms/molecule of EDTA) and the results plotted as above, the activation it brings about can be ascribed to the univalent cation content (Fig. 2). Using KCl concentrations equivalent to those pertaining inside the liver cell (80 mM) the reaction was activated to the same level as achieved with bivalent cations (above) and the addition of $MgCl_2$ produced no further activation. In addition the reaction could be fully activated by using NH_4^+ (0.1 M) as a source of univalent cations.

Activation by cationic detergents

Cationic detergents containing a quaternary nitrogen group, which would retain its positive charge at the high pH optimum of the enzyme (9.5), produced substantial activation of the enzyme acting on a phosphatidylethanolamine substrate (Fig. 3). However, when these agents such as cetyltrimethylammonium hydroxide or cetylpyridinium hydroxide were added in excess the activation was totally lost.

Activation by basic proteins

The basic proteins histone and cytochrome *c* produced substantial activation of the phospholipase A_1 acting against a phosphatidylethanolamine substrate (Fig. 4).

Action of agents that perturb hydrated phospholipid structures

All of the anionic or non-ionic detergents tested in graded concentrations produced no activation of the phospholipase and inhibited the reaction strongly with the cation-activated system (80 mM-KCl + 1.5 mM-$MgCl_2$). Triton X-100 and sodium dodecyl sulphate produced 100% inhibition at 0.01% concentration, and deoxycholate was almost as effective (92% inhibition at 0.5 mg/ml, 98% at 1.5 mg/ml). Ultrasonication of phosphatidylethanolamine produced no activation of the hydrolysis in the absence of cations.

Saturating the incubation medium with diethyl ether, which stimulates many phospholipases, caused strong inhibition (89% of the system activated with 7.5 mM-$MgCl_2$). The fluidizing solvent benzyl alcohol (Colley & Metcalfe, 1972; Gordon *et al.*, 1980) caused 86% inhibition at 30 mM, whereas hexanol, an activator of many phospholipases (Jain & Cordes, 1973), produced only a minor inhibition (16% at 28 mM).

Electrophoretic mobility of phosphatidylethanolamine substrate particles

Phosphatidylethanolamine particles at pH 9.5 are highly negatively charged owing to deprotonization of the amino group (Dawson, 1963) and this results

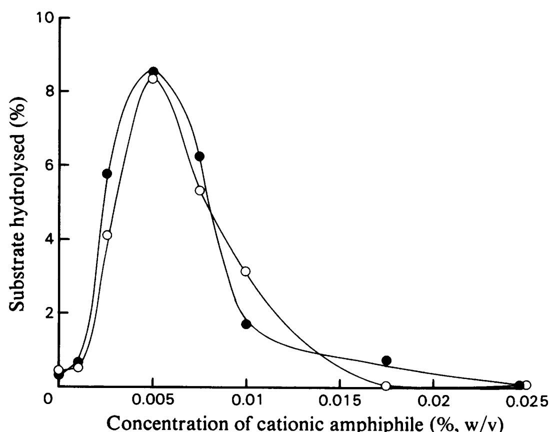

Fig. 3. *Effect of quaternary N-containing amphiphiles on phospholipase A_1 activity*

The phospholipase A_1 activity with [^{32}P]phosphatidylethanolamine as substrate was determined as described in the Methods section using no metallic activator. Cetyltrimethyl ammonium bromide (○) or cetylpyridinium bromide (●) in aqueous solution and adjusted to pH 9.5 were added to the system.

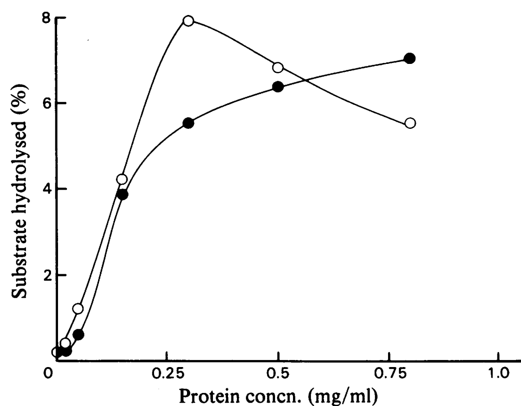

Fig. 4. *Effect of basic proteins on phospholipase A_1 activity*

The phospholipase A_1 activity with [^{32}P]phosphatidylethanolamine as substrate was determined as described in the Methods section using no metallic activator. Cytochrome *c* (●) or histone (○) adjusted to pH 9.5 were added to the system.

in a dispersion of the substrate due to polar-head-group repulsion between adjacent phosphatidylethanolamine molecules. Consequently, the very small size of the particles rendered them difficult to detect with the limited magnification available for observing particle electrophoresis. Measurement of the cathodic mobility of the yeast phosphatidylethanolamine gave a mean value of $-6.02\,\mu$m/s per

cm per V in buffer alone (pH 9.5). Previously a value of −7.4 μm/s per cm per V for egg phosphatidylethanolamine (probably subjected to some autoxidation because of its high polyunsaturated fatty acid content) had been obtained at pH 9.3 (Dawson, 1963).

The addition of $CaCl_2$ or $MgCl_2$ to the suspension of yeast phosphatidylethanolamine caused a distinct cloudiness to develop, presumably because counter-ion binding to the negative sites on the substrate particles reduce head-group repulsion, so that some aggregation could occur. At 3 mM, $CaCl_2$ and $MgCl_2$ reduced the cathodic mobility of the substrate particles to −2.03 and −2.14 μm/s per cm per V respectively. NaCl at 7.5 mM and 11.25 mM reduce the mobility to −5.41 and −4.88. Addition of larger amounts of Na^+ with the consequent higher current passing on application of the voltage made observation unsatisfactory because of convection in the electrophoresis tube.

Inhibitory action of other lipids

The activity of the enzyme was strongly inhibited when the substrate was admixed with small amounts of other lipids. This was true when the activator was either 5 mM-$CaCl_2$ or 80 mM-KCl + 1.5 mM-$MgCl_2$, the latter being used to mimic the internal ionic environment of the cell. All the phospholipids possessing a phosphocholine-containing polar head-group were particularly effective as inhibitors, e.g. phosphatidylcholine, lysophosphatidylcholine and sphingomyelin (Fig. 5). One molecule of these phospholipids with two molecules of substrate produced an inhibition amounting to 86–92%. Phosphatidylinositol was equally as effective as an inhibitor, whereas phosphatidylserine and phosphatidic acid were much less so (Fig. 5). When a series of saturated phosphatidylcholines with acyl groups of differing chain length were tested, (dihexanoyl) phosphatidylcholine was ineffective as in inhibitor, yet (dioctanoyl) phosphatidylcholine and longer chain lengths were very active in this respect (Fig. 6).

Experiments were conducted to test whether the inhibition could be due to phosphatidylcholine and other phospholipids reducing the dispersion of the substrate. However, when phosphatidylethanolamine containing various quantities of phosphatidylcholine was sonicated for 7.5 min in an ultra-

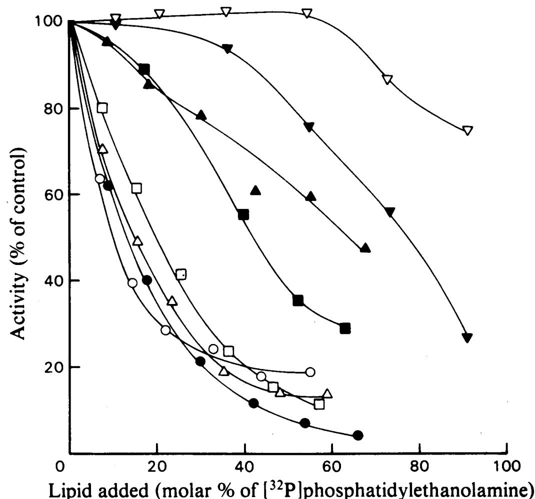

Fig. 5. *Effect of other phospholipids and fatty acids on the activity of phospholipids A_1 towards [^{32}P]phosphatidylethanolamine*

The other lipids were mixed with [^{32}P]phosphatidylethanolamine in organic solvent solution, and after removal of solvent the phospholipase A_1 activity was determined as stated in the Methods section using 80 mM-KCl plus 1.5 mM-$MgCl_2$ as activator. Symbols: ●, phosphatidylcholine; ○, sphingomyelin; △, lysophosphatidylcholine; □, phosphatidylinositol; ▲, phosphatidylserine; ■, phosphatidate; ▽, palmitate; ▼, palmitolate.

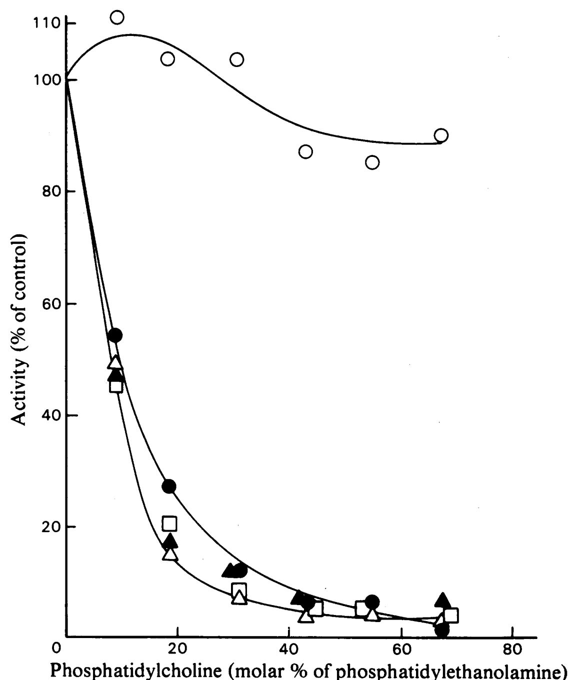

Fig. 6. *Effect of saturated phosphatidylcholines of different chain length on phospholipase A_1 activity towards [^{32}P]phosphatidylethanolamine*

Phospholipase A_1 was assayed as described in the legend to Fig. 5. Symbols represent results for phosphatidylcholines with the following acyl-group composition: ○, di-$C_{6:0}$; ●, di-$C_{8:0}$; △, di-$C_{10:0}$; ▲, di-$C_{12:0}$; □, di-$C_{14:0}$.

sonic bath (Decon F.S. 100; 200 W output), then the inhibition curve produced was exactly equivalent to the non-sonicated control, even though this sonication had produced a visible clearing of the substrate suspension.

Fatty acids, one of the lipoidal products of the enzymic hydrolysis, only produced inhibition of the system at much higher concentrations than the inhibitory phospholids (Fig. 5). Palmitate was much less effective than palmitoleate (Fig. 5), which g.l.c. analysis showed was the main product of the enzymic hydrolysis of the substrate used in the present investigation. The activity of the enzyme was tested against [^{32}P]phosphatidylethanolamine contained in liposomes, which approximated in composition to the internal lipid lamellae of the liver cell plasma membrane (Higgins & Evans, 1978; Dawson *et al.*, 1980). In spite of these containing 24% of their phospholipids as phosphatidylethanolamine the hydrolysis of this component in the mixed liposome was negligible.

Birefringence

Under the polarizing microscope the substrate particles (phosphatidylethanolamine) gave little evidence of lamellar structure both without and with activator added (80mM-KCl + 1.5mM-$MgCl_2$). When sufficient ovolecithin (equimolar) was added to the substrate to produce substantial inhibition of the reaction (>95%) the mixed substrate particles clearly became lamellar with distinct birefringence. A similar result was obtained with equimolar phosphatidylinositol (inhibition >95%), although the birefringence was less marked, probably indicating a wider spacing between the bilayers.

Discussion

A cytosolic phospholipase A_1 active at an alkaline pH has not previously been described in rat liver. In its properties, e.g. pH optimum, preferred substrate, heat stability, the enzyme closely resembles the alkaline phospholipase A_1 originally described by Rooke & Webster (1976) in extracts of acetone-dried human brain tissue, and recently identified as a cytostolic enzyme in rat brain tissue by Doherty & Rowe (1980). Both groups of workers reported that the brain phospholipase A_1 was activated by Ca^{2+}, which we have confirmed for the liver enzyme. However, the present studies make it clear that the calcium is not acting as an obligatory cofactor as it does for many of the other deacylating phospholipases present in mammalian cells and secretions. Such enzymes are completely inhibited by EDTA, whereas the present enzyme is apparently stimulated by the addition of this chelating agent.

It would appear instead that the function of the Ca^{2+} is a general one shared by many metallic cations. Thus, Mg^{2+}, Sr^{2+} and Ba^{2+} are equally effective and activation can also be produced by both Na^+ and NH_4^+, although higher concentrations are needed to produce the equivalent activation. It would seem that any cation that is not precipitated as its insoluble hydroxide at the high pH optimum of the enzyme is effective as an activator. The stimulation produced by EDTA can be entirely ascribed to the univalent cation (Na^+) required to adjust its pH to 9.5 before adding it to the incubation medium.

There seems to be a strong possibility that the activation of the enzyme by cations is due to their ability to act as counter ions, reducing the negative zeta potential near the surface of the substrate. The phosphatidylethanolamine substrate would be negatively charged at pH 9.5 owing to deprotonization of the amino groups (Dawson, 1963) and this has been confirmed by direct particle micro-ionophoresis of the present yeast phosphatidylethanolamine. The negative zeta potential of the substrate particles is substantially reduced by activating concentrations of Ca^{2+} and Mg^{2+}. Similar observations on adding Na^+ and K^+ confirm the theoretical expectation that much more is required to produce an equivalent reduction in the zeta potential, which is in line with their less effective activation of the phospholipase A_1, except at higher concentrations. The activation produced by the introduction of long-chain quaternary cations into the substrate and the addition of basic proteins to the system could be explained by a similar phenomenon.

It is now well known that the electrostatic conditions pertaining at a phospholipid/water interface can have a pronounced effect on the initiation and rate of phospholipase attack (Bangham & Dawson, 1959; Dawson, 1965; Dawson *et al.*, 1976). Depending on the nature of the enzyme, either a negative or positive zeta potential on the substrate is required for successful hydrolysis, although a constant feature is that a too highly charged interface results in inhibition of the enzyme. In the present instance the enzyme with an isoelectric point of 7.15 would possess a net negative charge so that if the substrate is also negatively charged, the electrostatic repulsion might be of sufficient magnitude to prevent successful collision complexes between the enzyme and substrate. Alternatively, the enzyme molecule might be denatured by adsorption on the highly charged surface and rendered inoperative as a catalyst.

The marked inhibition of phosphatidylethanolamine hydrolysis by admixture with other phospholipids does not seem to correlate in any way with the anticipated effect that these would have on the zeta potential. Thus, phosphatidylcholine, being zwitterionic, would reduce the negative zeta potential by surface dilution, whereas phosphatidylinositol, by

virtue of the negative charge on its head group, is unlikely to produce a significant change. From X-ray diffraction and ^{32}P-n.m.r. measurement, it is now believed that naturally-occurring hydrated phosphatidylethanolamine particles suspended in excess of aqueous phase exist in a non-bilayer form and most likely in the hexagonal-phase (Cullis & de Kruijff, 1978*a*,*b*). Addition of phosphatidylcholine causes a conversion from this form through transitional states into the bilayer lamellar structure (Cullis & de Kruijff, 1978*a*; Noordam *et al.*, 1980; Hui *et al.*, 1981). The inhibition by phosphatidylcholine could be accounted for by assuming that the enzyme can only readily attack phosphatidylethanolamine when it exists in a non-bilayer configuration. In fact, the concentration of phosphatidylcholine required to produce almost total inhibition of the system correlates well with the phase diagrams of hexagonal-bilayer structure conversions drawn-up for phosphatidylethanolamine/phosphatidylcholine/water structures by Hui *et al.* (1981). Recent evidence would suggest that phosphatidylinositol admixed with phosphatidylethanolamine can also cause a reversion of the phosphatidylethanolamine to be bilayer system (Nayer *et al.*, 1982).

This suggestion that the present substrate is only attacked when in the non-bilayer form is supported by our birefringence observations. Pure fully hydrated phosphatidylethanolamine shows no birefringence. Both phosphatidylcholine and phosphatidylinositol produce birefringent structures indicative of bilayer lamellae when added to phosphatidylethanolamine in quantities equivalent to those producing almost total inhibition of the enzyme. In addition the stimulation of both [^{32}P]phosphatidylcholine and [^{32}P]phosphatidylinositol hydrolysis produced by adding excess phosphatidylethanolamine is consistent with the same theory, i.e. they can only be readily attacked when forced into a non-bilayer structure. This assumed property of the enzyme could also explain the stability of phospholipids in liver cell membranes, which presumably exist mainly in a bilayer form and are in contact with a cytoplasm containing an active phospholipase A_1, which could potentially degrade them very rapidly.

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