Biochimica et Biophysica Acta, 556 (1979) 423—433

BBA 78508

EFFECT OF DIFFERENT PHYSICAL STATES OF PHOSPHOLIPID SUBSTRATES ON PARTIALLY PURIFIED PLATELET PHOSPHOLIPASE A_2 ACTIVITY *

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(Received February 20th, 1979)

Key words: Phospholipase A_2; Arachidonic acid; Prostaglandin; Phospholipid; Phase transition

Summary

Partial purification of alkaline phospholipase A_2 (EC 3.1.1.4) from rabbit platelets was carried out and the effect of different physical states of the substrate phosphatidylcholine on the activity was investigated.

(1) The enzyme was purified about 1020-fold by means of Sephadex gel chromatography after extraction from a particulate fraction of rabbit platelets, followed by CM-cellulose chromatography, and had a molecular weight of approx. 12 000 as determined by gel chromatography.

(2) The activity of the purified enzyme was enhanced by the addition of detergents. Sodium deoxycholate and sodium cholate markedly stimulated the activity, and the effect of these substances was observed well below the critical micelle concentrations. Triton X-100 stimulated the activity moderately, and the activation was observed only above the critical micelle concentration.

(3) The addition of negatively charged phospholipids to the substrate egg phosphatidylcholine induced a moderate activation of hydrolysis.

(4) The addition of long-chain cation to the substrate induced an inhibition of the activity, whereas the addition of long-chain anion activated the hydrolysis of egg phosphatidylcholine, but did not activate the hydrolysis of phosphatidylcholine in the total lipid extract of rabbit platelets.

(5) Hydrolysis of dimyristoyl phosphatidylcholine increased in the temperature region of the phase transition of the substrate. Addition of cholesterol at the concentration of 20 mol% diminished the effect of phase transition.

* A preliminary report of these experiments was made at the XVIIth Congress of the International Society of Hematology, Paris, July 25, 1978.

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Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

(6) Release of [1-^{14}C]arachidonic acid from an equimolar mixture of egg phosphatidylcholine with dipalmitoyl or distearoyl phosphatidylcholine was activated at the temperature of 0°C or 20°C, respectively.

From these results, we suggest that platelet phospholipase A_2 can be activated to release fatty acids from the platelet phospholipids at the domains within membranes, where exist the structural irregularities and/or accumulation of negative charge within the bilayers.

Introduction

Prostaglandins and thromboxanes have been implicated with platelet function [1—3]. Since the release of arachidonic acid from membrane phospholipids is necessary for prostaglandin and thromboxane syntheses, phospholipases are assumed to be the triggering enzyme [4]. In preliminary experiments, we observed that rabbit platelets contained phospholipase A_1 activity at pH 4.5, phospholipase A_2 activity at pH 9.5 and lysophospholipase activity at pH 8.5 [5]. Since it was reported that the release of arachidonic acid from incubated human platelet membranes had a peak at pH 9.5 [6], we considered the phospholipase A_2 activity at pH 9.5 to be at least partly responsible to the release of arachidonic acid during platelet activation. Since phospholipases are considered to constitute the rate-limiting step in prostaglandin and thromboxane biosynthesis in platelets, the mechanism of activation of phospholipases has been the subject of the extensive research. There is the hypothesis that phospholipases are stored in platelets in a form of zymogen, and are activated by some endogenous proteases [7]. While such a proposal is attractive since many serine-protease inhibitors do play a role in platelet aggregation and biosynthesis of prostaglandins and thromboxanes [8,9], there are other mechanisms in which the regulation of phospholipases can be achieved through the change in the structural arrangement of phospholipid membranes which constitute the substrate for the enzyme [10]. In view of this, we set out to purify the enzyme and to investigate the effect of different physical states of substrate phosphatidylcholine on the activity.

Materials and Methods

Chemicals. Dimyristoyl, dipalmitoyl and distearoyl phosphatidylcholine (synthetic), phosphatidylcholine (egg), phosphatidylinositol (plant), phosphatidylserine (bovine), phosphatidic acid (egg) and coenzyme A were purchased from P-L Laboratory, Milwaukee, WI, U.S.A. [1-^{14}C]Arachidonic acid were obtained from the Radiochemical Centre, Amersham, U.K. [1-^{14}C]-Dimyristoyl phosphatidylcholine was a gift from Nippon Shoji, Osaka, Japan. The purity of the fatty acids in the synthetic lipids was verified by gas-liquid chromatography of the fatty acid methyl esters. All other chemicals were of reagent grade or the best commercially available.

Preparation of rabbit platelet sonicates. Platelet-rich plasma was prepared by low-speed centrifugation of freshly obtained citrated whole blood from New Zealand white rabbits. Platelets were isolated from the platelet-rich plasma,

washed three times and freed of residual erythrocytes by repeated differential centrifugations. Microscopical examination revealed that red and white cell contamination was negligible. Platelet buttons were finally resuspended in saline ($1—4 \cdot 10^9$ platelets/ml) and sonicated in a vessel surrounded by ice for two periods of 30 s, with 15 s between each period, using a Branson Sonifier, Branson Sonic Power. Co., Plainsview, NY; output control setting 4 (approx. 80 W). Plastic or silicon-coated glassware was used throughout.

Preparation of labelled substrates. The [1-^{14}C]arachidonic acid-labelled egg phosphatidylcholine was prepared by the acylation of 1-acyl-*sn*-glycero-3-phosphorylcholine with [1-^{14}C]arachidonic acid according to the method of Mulder et al. [11], using human erythrocyte lysate as follows: 3 μmol egg lysophosphatidylcholine, 2 mg ATP, 200 μg coenzyme A and 5 μCi [1-^{14}C]arachidonic acid in 1/15 M sodium phosphate buffer, pH 7.4, containing 1 μmol/ml sodium deoxycholate and 10 mM $MgCl_2$, were sonicated. To this preparation, 0.3 ml of human erythrocyte lysate was added and incubation was carried out for 4 h at 37°C. After incubation, the lipids were extracted according to the method of Folch et al. [12], and the labelled phospholipids were separated by silicic acid column chromatography. More than 98% of the radioactivity was found in the second position of glycerol of phospholipid molecules as ascertained by enzymic hydrolysis with *Crotalus Adamanteus* venom. The specific radioactivity of the substrate was adjusted to be $1 \cdot 10^5$ dpm/μmol using corresponding non-isotopic compounds.

Assay of phospholipase A_2 activity. To 200 nmol of phosphatidylcholine in chloroform solution (egg phosphatidylcholine unless otherwise stated), the activating amphipathic substances dissolved in an appropriate solvent, e.g. chloroform was added.

The solvent was removed in vacuo and the lipid mixture was emulsified by vortex mixing over the phase transition temperature after addition of 0.4 ml of 0.1 M glycine/NaOH, buffer, pH 9.0. The reaction was started by the addition of 50 μl of enzyme solution (2 μg protein, 1016-fold purified) and 50 μl of 0.1 M $CaCl_2$. After the incubation with shaking for 60 min at the temperature indicated in each experiment, the reaction was stopped by adding 2.0 ml of chloroform/methanol (2 : 1, v/v), and the lipids were extracted according to the method of Folch et al. [12]. The combined extract was evaporated and the lipids were separated by thin-layer chromatography with a solvent system of chloroform/methanol/water (100 : 40 : 6, v/v/v). The lipids were visualized by exposure to iodine vapour and β-camera (LB 290, Berthold, F.R.G.), and the spots corresponding to the different lipids were scraped off into the counting vials. The radioactivity of the samples was measured using a Nuclear Chicago liquid scintillation counter (Model Mark II). The liquid scintillation solution consisted 4 g PPO, 0.1 g POPOP and 20 g of thixotropic gel powder in 1000 ml toluene. The efficiency ranged between 87 and 90% as ascertained by the external standard method.

Fluorescence polarization measurements. The thermotropic behavior of the substrates was studied using fluorescence depolarization of the fluorophore, 1,6-diphenyl-1,3,5-hexatriene (DPH) according to the procedure described by Shinitzky and Barenholz [13]. For labelling of substrate dispersion, an equal volume of 2 μM DPH dispersion in 0.1 M glycine/NaOH buffer, pH 9.0, was

added to the substrate and incubation was carried out for 60 min at 25°C. The thermotropic behavior of the substrate was expressed as the fluorescence polarization, P, according to the following equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ is the fluorescence intensity parallel to, and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam. $I_{\parallel}$ and $I_{\perp}$ were measured by using Elscint Microviscosimeter MV-1a (Elscint, Israel) and were corrected for light scattering.

Analytical procedures. Protein concentration was routinely determined by the method of Lowry et al. [14], with bovine serum albumin as standard. Lipid phosphorus was estimated according to the modified method of Bartlett [15].

Results

Enzyme purification

The purification procedure was as follows: The pH of platelet sonicate in 0.15 M NaCl was adjusted to 5.0 with 20 mM citrate buffer and heated at 60°C for 10 min. Phospholipase A_2 activity was hardly changed by this procedure, whereas lysophospholipase activity lost about 90% of its original activity. The heat-treated sonicate was centrifuged at 105 000 × g for 60 min at 4°C and the pellet was resuspended in 1.0 M KCl. The extraction was carried out at 4°C for 12 h. About 45% of protein and 60% of enzyme activity were extracted, respectively. Changes in KCl concentration at the range of 0.75—2.0 M produced no appreciable effect on extraction. Solutions of NaCl, KSCN and NaSCN were equally effective. When the extracted enzyme was concentrated and subjected to Sephadex G-100 gel filtration at 4°C in the presence of 1.0 M KCl, a single peak of activity appeared at a molecular weight of around 12 000 (Fig. 1a and b). This value falls within the range of monomers of most phos-

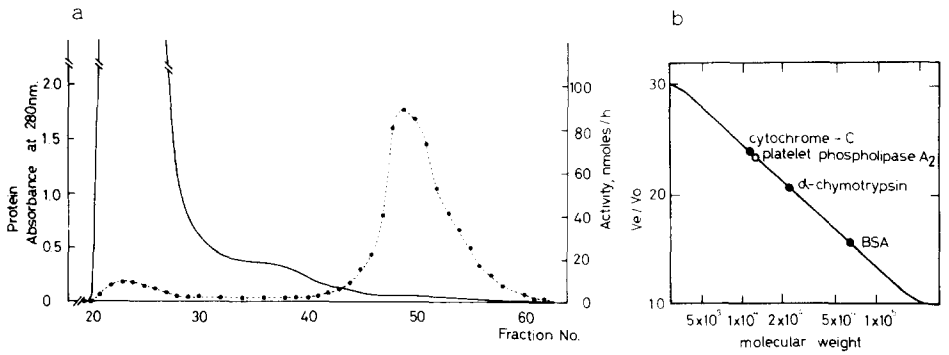

Fig. 1. (a) A typical elution pattern of platelet phospholipase A_2 from Sephadex G-100 column (3.6 cm × 40 cm). ———, protein concentration determined according to absorbance at 280 nm; ●· · · · · ●, phospholipase A_2 activity of 50 µl aliquot of each fraction (nmol fatty acid released/h). Fraction size, 2.5 ml. (b) Molecular weight determination of platelet phospholipase A_2 by gel filtration on Sephadex G-100. Cytochrome *c*, α-chymotrypsin and bovine serum albumin with known molecular weights were used as reference proteins. V_e, elution volume for the protein; V_0, elution volume for Blue Dextran 2000 (Pharmacia).

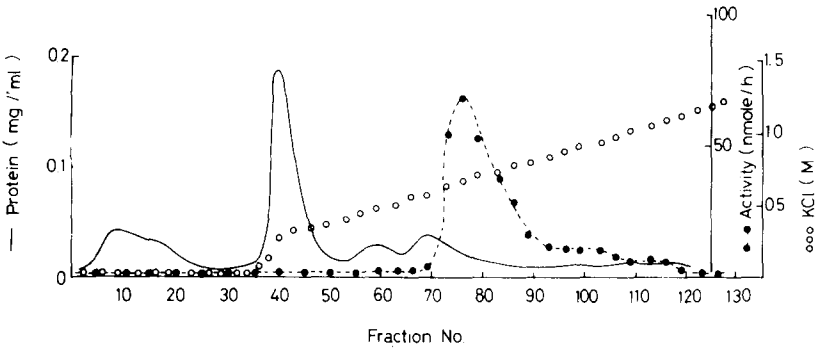

Fig. 2. A typical elution pattern of phospholipase A_2 from a CM-cellulose column (1.0 cm × 35 cm). ———, protein concentration determined according to Lowry et al. [14]. Fraction size, 2.5 ml; ●- - - - -●, phospholipase A_2 activity (nmol fatty acid released/h) by 200 µl aliquot of each fraction; ○○○, KCl concentration.

pholipases so far purified. The phospholipase A_2 was purified 140-fold by subsequent gel filtration through Sephadex G-75. The effluent fractions containing phospholipase A_2 were condensed with Diaflo Membrane DM-5 (Amicon, U.S.A.) and freed of salts by extensive dialysis, first against distilled water and subsequently against 20 mM Tris/malate buffer, pH 5.5. After the CM-cellulose column was equilibrated against 20 mM Tris/malate buffer, pH 5.5, the sample was applied and eluted with the same buffer. When the breakthrough peak was apparent, the remaining proteins were eluted by a linear gradient developed by use of 1.5 M KCl in the same buffer. A typical elution pattern is given in Fig. 2. The enzymically active fractions were combined, dialysed at 4°C against saline and stored at −65°C. Table I shows the yield and the increase in specific activity of the phospholipase A_2 at the various stages during purification. The phospholipase A_2 was purified 1020-fold by the procedures described above without use of detergents. The purified enzyme contained only a negligible amount of phospholipids, i.e., approximately 1.4 nmol

TABLE I

PURIFICATION OF PHOSPHOLIPASE A_2 FROM RABBIT PLATELETS ACHIEVED AT EACH STEP OF PREPARATION

Phospholipase A_2 activity of each fraction was assayed under the condition as described in Materials and Methods. Sodium deoxycholate was added at the final concentration of 0.05% to obtain maximal activity. Enzyme activities were expressed in µmol arachidonic acid released/h at pH 9.0 and 37°C. Specific activities were expressed in µmol arachidonic acid released/h per mg protein under the same assay conditions. Data in parentheses are percent values.

Fraction	Protein (mg)	Activity	Specific activity	Purification (-fold)
Sonicate	499.0	24.4	0.049	—
Heat treatment at pH 5.0	486.0 (97.4)	22.3 (91.4)	0.046	—
105 000 × *g* precipitate	223.6 (44.8)	21.4 (87.7)	0.096	1.96
1.0 M KCl extract	99.8 (20.0)	12.4 (50.8)	0.124	2.54
Sephadex G-100	4.69 (0.94)	10.5 (43.0)	2.24	45.8
Sephadex G-75	1.49 (0.30)	10.2 (41.8)	6.85	140.0
CM-cellulose	0.19 (0.04)	9.4 (38.7)	49.7	1016.4

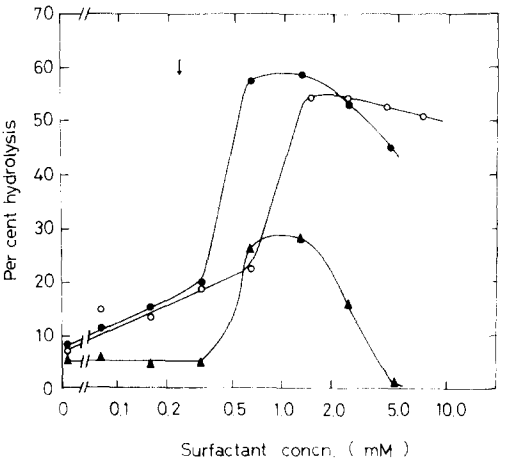

Fig. 3. Effect of two anionic detergents, sodium deoxycholate (●) and sodium cholate (○), and a non-ionic detergent, Triton X-100 (▲) on the activity of platelet phospholipase A_2. Incubation was carried out at 37°C for 60 min. Critical micelle concentration of Triton X-100 (0.24 mM) was taken from the literature [16], and is marked with an arrow.

phospholipids/μg protein, but the final preparation was not a homogenous protein, as determined by polyacrylamide gel electrophoresis.

Effect of detergents on platelet phospholipase A_2 activity

As shown in Fig. 3, addition of detergents to the substrate enhanced the enzymic activity. For anionic detergents, sodium deoxycholate and sodium cholate, two phases of activation were observed. Activation observed at the low concentrations was moderate and related to the concentration of detergents, while a markedly stimulatory effect was observed above a certain concentration of the respective surfactant. This concentration was still lower than critical micelle concentration of the respective surfactant (4—6 mM for sodium deoxycholate and 10—12 mM for sodium cholate) as reported in the literature [16].

In the case of a non-ionic detergent, Triton X-100, no activation was observed below the critical micelle concentration. A moderately stimulatory

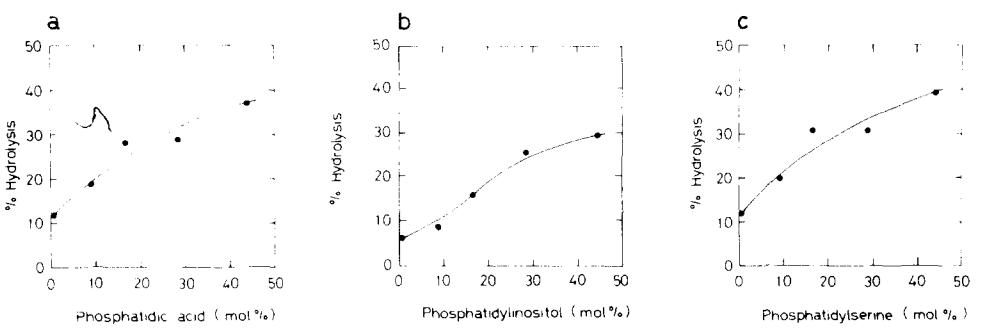

Fig. 4. Effect of highly charged phospholipids; phosphatidic acid (a), phosphatidylinositol (b) and phosphatidylserine (c) on the rate of hydrolysis of egg phosphatidylcholine with platelet phospholipase A_2. Incubations were carried out at 37°C for 120 min.

effect was observed at the concentration above the critical micelle concentration, but the effect was less prominent in comparison with that of anionic detergents. For most detergents, the activating effect was limited to a relatively narrow concentration range, above which inhibition of the activity was observed.

Effect of negatively charged phospholipids

Addition of negatively charged phospholipids, phosphatidic acid, phosphatidylserine and phosphatidylinositol to the substrate activated the hydrolysis of phosphatidylcholine moderately as indicated in Fig. 4. The stimulatory effect of phosphatidylinositol was less pronounced in comparison with the other two compounds.

Effect of long-chain anions and cations on the activity

The addition of stearic acid or dicetylphosphate to the substrate egg phosphatidylcholine enhanced the activity, whereas the addition of long-chain cations, i.e. stearylamine or cetyltrimethylammonium chloride strongly inhibited the activity (Fig. 5). When the total lipid extract of rabbit platelets was utilized as substrate instead of egg phosphatidylcholine and the rate of hydrolysis of the phosphatidylcholine fraction was studied, the activation effect of dicetylphosphate was no longer observed, whereas the addition of stearylamine was still inhibitory. The phospholipid composition of the total lipid extract of rabbit platelets used was as follows: phosphatidylcholine

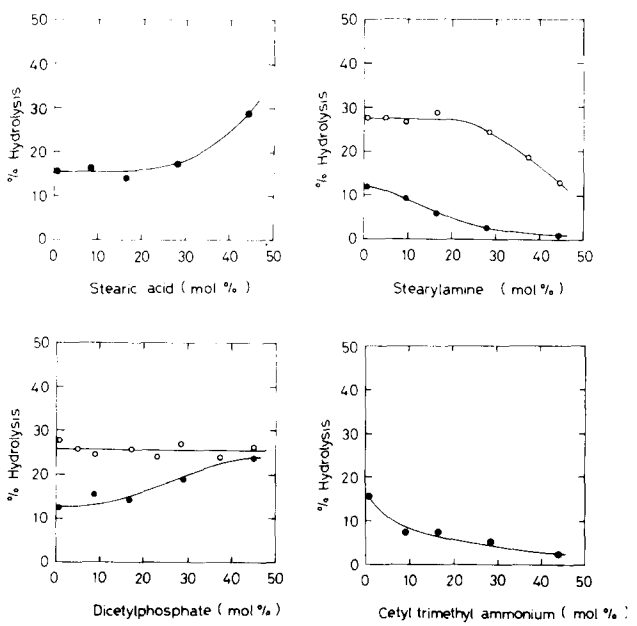

Fig. 5. Effect of long-chain anions or cations on the rate of hydrolysis of egg phosphatidylcholine (●——●) or phosphatidylcholine in total lipid extract of rabbit platelets (○——○) with platelet phospholipase A_2. In the case of total lipid extract as substrate, 200 nmol phospholipid was used as substrate and the hydrolysis of a phosphatidylcholine fraction within the extract was plotted. Incubations were carried out at 37°C for 120 min.

TABLE II

HYDROLYSIS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE AT DIFFERENT TEMPERATURES AND THE EFFECT OF CHOLESTEROL

The extent of hydrolysis of dimyristoyl phosphatidylcholine was expressed as percent radioactivity of the phosphatidylcholine hydrolyzed to the total radioactivity recovered. The initial amount of phosphatidylcholine was 200 nmol ($4 \cdot 10^4$ dpm). Incubations were carried out for 60 min.

Molar ratio cholesterol:dimyristoyl phosphatidylcholine	Incubation temperature (°C)		
	10	23 (T_m)	37
No cholesterol	4.8	24.6	7.1
0.10	3.6	9.4	6.3
0.20	1.8	3.2	3.9

34.1 mol%, phosphatidylethanolamine 28.7 mol%, phosphatidylserine plus phosphatidylinositol 17.3 mol%, sphingomyelin 14.0 mol% and lysophosphatidylcholine 1.1 mol%.

Hydrolysis of dimyristoyl phosphatidylcholine. Effect of cholesterol

Temperature-dependent hydrolysis of unsonicated dimyristoyl phosphatidylcholine dispersions is shown in Table II. Preferential hydrolysis of the substrate was observed at 23°C, the phase transition temperature of the synthetic phospholipids.In the presence of 20 mol% of cholesterol, hydrolysis was inhibited at all temperatures tested, and no preferential hydrolysis at the phase transition temperature was observed.

Release of arachidonic acid from egg phosphatidylcholine. Effect of coexistence of some synthetic lipids

Temperature-dependent release of [1-^{14}C]arachidonic acid from the equi-

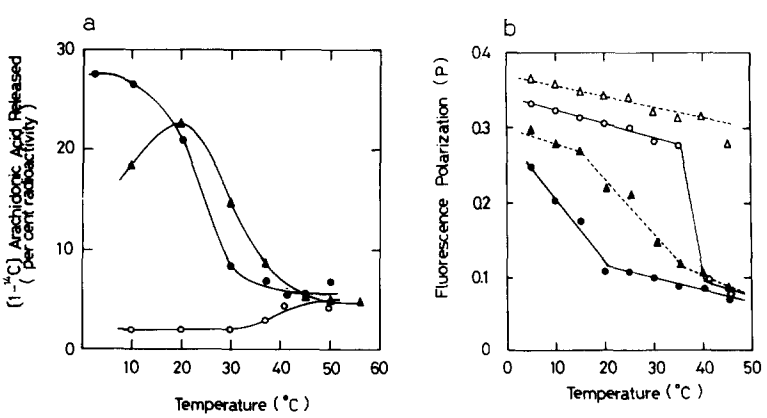

Fig. 6. (a) Temperature-dependent release of [1-^{14}C]arachidonic acid from egg phosphatidylcholine (○) and the effect of dipalmitoyl (●) and distearoyl (▲) phosphatidylcholine on the release of arachidonic acid from egg phosphatidylcholine with platelet phospholipase A_2. In the latter cases, the substrates used were the equimolar mixture of 100 nmol of egg phosphatidylcholine and 100 nmol of each synthetic lipid. Incubations were carried out for 60 min. (b) The thermotropic behavior of the substrates; dipalmitoyl phosphatidylcholine (○), distearoyl phosphatidylcholine (△) and equimolar mixtures of egg phosphatidylcholine with dipalmitoyl (●) or distearoyl (▲) phosphatidylcholine.

molar mixture of egg phosphatidylcholine and other synthetic lipids is shown in Fig. 6a. The release of arachidonic acid from egg phosphatidylcholine increased by a factor of about 2.0 at 50°C compared with that at 20°C. This is a reasonable rate factor for a 30°C temperature change and presumably reflects only the normal temperature effect on the rate of hydrolysis. Maximal release of arachidonic acid was observed at 0°C when egg phosphatidylcholine was mixed with dipalmitoyl phosphatidylcholine, and at 20°C when mixed with distearoyl phosphatidylcholine. The thermotropic behavior of mixed lipid dispersions used as substrates studied using the fluorescence depolarization method was characterized by a broad phase transition at the same temperature range where the enzymic activity showed a maximum (Fig. 6b).

Discussion

This study was concerned with a partial purification of platelet phospholipase A_2 and the effects of different physical states of phospholipid substrates on the enzymic activity were investigated. Since the anionic surfactants were more effective activators, it was assumed that the rabbit platelet phospholipase A_2 may require a net negative charge of the substrate micelles. This tentative conclusion was supported by the observation that the addition of charged phospholipids or long-chain anions accelerated the enzymic breakdown of phosphatidylcholine. An electrostatic effect seems to be operative in the action of platelet phospholipase A_2. The introduction of a negative electrical charge at the surface of the substrate tended to enhance the activity, whereas a positive charge at the surface of the substrate inhibited the activity. These findings are similar to those reported by de Haas and coworkers with pancreatic phospholipase A_2 [17,18]. The fact that the hydrolysis of phosphatidylcholine included in the total lipid extract of platelets was not facilitated by the addition of dicetylphosphate is probably due to the existence of a sufficient amount of charged phospholipids in the extracted lipids. Thus, the activity of the enzyme would not as a whole be affected by subtle changes in the net electrical charge of platelets. However, it does not imply that changes in the electrical charge are not important in specialized regions, if the charged phospholipids are not uniformly distributed within the bilayers.

As to the effect of physical state of the substrate on phospholipase A_2 activity, two factors have to be considered; first, the surface arrangement of the phospholipid bilayers; second, the electrostatic conditions on this surface. Since the introduction of an electrical charge onto the surface of liposomes will also alter the geometric structure of the surface, it is difficult to attribute the observed effect solely to the electrostatic attraction and repulsion of enzyme and lipid bilayers. In view of this, effects of the change in arrangement of lipid bilayers on the enzymic activity other than electrostatic effect were studied, and here an electrically neutral substrate, i.e. phosphatidylcholine, without an added surfactant was utilized. From the experiments using dimyristoyl phosphatidylcholine as substrate, it was demonstrated that platelet phospholipase A_2 preferentially hydrolyzes phospholipids undergoing phase transition. Similar results have already been reported with pancreatic enzyme and venom enzyme [19—22]. These data were compiled from studies on phos-

pholipase A_2 from exocrine glands, and mammalian intracellular phospholipases have apparently not been reported.

As arachidonic acid is quite unsaturated, the bulk thermotropic phase transition of phospholipids containing arachidonyl chains is completed below 0°C, this temperature being far below the physiological range. Therefore, the thermotropic behavior of phosphatidylcholine containing arachidonyl chains was modified by the addition of synthetic lipids, i.e. distearoyl phosphatidylcholine, etc., and the rate of enzymic release of arachidonic acid was investigated. The results obtained showed that the peak of release of arachidonic acid from the modified substrate can be shifted into the physiological temperature range by the addition of synthetic phospholipid. Under these conditions, the maximum release of arachidonic acid was observed at temperatures lower than normal body temperature. It seems that the coexistence of lipids with a higher transition temperature may be required to shift the peak of arachidonic acid release more in line with body temperature. In this context, it is of interest to note van der Bosch and McConnell's [23] suggestion that integral membrane proteins have an annulus of tightly bound phospholipids, the hydrocarbon chains of which are not free to move.

Cholesterol, which eliminates thermotropic phase transition of phospholipids and prevents hydrolytic action of phospholipase A_2 towards substrates, is abundant within platelet membranes. However, Bieri and Wallach [24] reported that, in biomembranes, cholesterol molecules are not distributed uniformly and some cholesterol-poor domains may exist within the bilayers.

It has been reported that the phospholipases A_2 from pig pancreas and *C. adamanteus* venom fail to hydrolyze intact platelet membrane phospholipids [25], and such was the case with the platelet enzyme (Kannagi, R. and Koizumi, K., unpublished observations). It should be noted that the uniformly arranged phosphatidylcholine bilayers were highly resistant to platelet phospholipase A_2 in spite of the presence of a sufficient amount of Ca^{2+}, when surfactant was depleted from the incubation medium. At least some perturbations of phospholipid bilayers are required for the enzyme to release a significant amount of fatty acids. It seems that the platelet phospholipase A_2 can hydrolyze only disarranged phospholipids at the limited domains within platelet membranes, thus avoiding excess hydrolysis and the lysis of whole cells. Although our approach was indirect, nevertheless, our results suggest that platelet phospholipase A_2 can be activated at the limited domains within platelet membranes; either areas rich in charged phospholipids, or cholesterol-poor areas where the cooperative phase transition of the substrate may occur due to the coexistence of other lipids, the hydrocarbon chains of which are not free to move.

Thrombin and other platelet-activating agents presumably set up a series of orderly events, resulting in profound modifications of the content and distribution of membrane phospholipids. Several workers have reported the accumulation of phosphatidic acid during platelet activation [26,27]. This accumulation of charged phospholipid may play a role in phospholipase A_2 activation since the presence of such a negatively charged phospholipid in substrate liposomes enhances the enzymic activity. Furthermore, there are possibilities that the structural arrangement of platelet membrane phospholipids may change

through the interaction of platelet-aggregating agents such as thrombin with receptors at the cell surface, resulting eventually in alterations of the membrane fluidity [28,29] or in appearance of a considerable amount of phosphatidylethanolamine on the cell surface [30]. The physiological significance of these observations is now being investigated.

Acknowledgements

Thanks are due to Drs. T. Murachi, S. Yamamoto, M. Okuma and H. Hidaka for pertinent discussion, K. Hirashima and S. Tanoue for technical assistance, and M. Ohara for assistance with the manuscript.

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