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ACTION OF PANCREATIC PHOSPHOLIPASE A_2 ON PHOSPHATIDYLCHOLINE BILAYERS IN DIFFERENT PHYSICAL STATES

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SUMMARY

1. Saturated and unsaturated phosphatidylcholines, dispersed as liposomes in water, can be hydrolysed by phospholipase A_2 from pig pancreas. A pure saturated phosphatidylcholine is hydrolysed only near its transition temperature. An unsaturated phosphatidylcholine is hydrolysed preferentially near its transition temperature, but hydrolysis can occur also above the transition temperature, albeit at a much lower rate.

2. An equimolar mixture of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, which shows cocrystallization of the paraffin chains, is hydrolyzed between 25 and 40 °C with a maximum at 32 °C, in agreement with the calorimetric scan of the phase transition.

3. An equimolar mixture of dilauroyl phosphatidylcholine and distearoyl phosphatidylcholine, which shows a monotectic behaviour, is hydrolysed at all temperatures. Hydrolysis is maximal at 0 and 40 °C, at which temperatures dilauroyl phosphatidylcholine and distearoyl phosphatidylcholine undergo their phase transition, respectively.

4. Both in the mixture showing cocrystallization and in the mixture in which phase separation occurs, the phosphatidylcholine species with the shorter fatty acid chains is hydrolysed at a higher rate than the longer chain fatty acid species.

5. Hydrolysis is inhibited by the presence of cholesterol in liposomes prepared of saturated phosphatidylcholine. Inhibition is complete at a cholesterol concentration of 35 mol %. Subsequent addition of filipin and amphotericin B to the mixed cholesterol-phosphatidylcholine liposomes overcomes the inhibitory effect of cholesterol.

INTRODUCTION

Recently we have presented evidence that saturated phosphatidylcholines dispersed as liposomes can be hydrolysed by phospholipase A_2 from pig pancreas only near the transition temperature of the lipids [1]. At this temperature regions of frozen molecules coexist with lipids in the liquid crystalline phase, and it was postulated that

at the border of these regions irregularities in the packing of the lipid molecules might favour ion permeability [2–4] and insertion of enzymes [1, 5] into the interface. An obvious experimental approach to substantiate this hypothesis is to test the enzymatic activity of phospholipase A_2 towards liposomes containing cholesterol. Association between cholesterol and phosphatidylcholine is known to decrease the cooperative phase transition of the phosphatidylcholine molecules [1, 6–8]. At sufficiently high concentrations of cholesterol a liquid condensed packing is reached and the cooperative transition from the liquid crystalline to the gel state is abolished. Therefore it is of interest to test the hypothesis that the introduction of cholesterol into the bilayer will result in an inhibition of phospholipase A_2 activity. Furthermore, the question arises as to whether the characteristic phase transition-dependent action of phospholipase A_2 is restricted to pure saturated phosphatidylcholine. We therefore tested mixtures of saturated phosphatidylcholine and several unsaturated compounds.

EXPERIMENTAL

1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine (diC12 : 0 phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (diC14 : 0 phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (diC16 : 0 phosphatidylcholine), 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (diC18 : 0 phosphatidylcholine), 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (diC18 : 1 *cis* phosphatidylcholine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine (diC18 : 1 *trans* phosphatidylcholine), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (C16 : 0/C18 : 1 *cis* phosphatidylcholine) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (C18 : 0/C18 : 1 *cis* phosphatidylcholine) were synthesized according to the procedures described by Slotboom and Bonsen [9]. Filipin was obtained from the Upjohn Co. (Kalamazoo, Michigan) and dissolved in dimethylformamide. Amphotericin B from Squibb Institute for Medical Research (New Brunswick, N.Y.) was dissolved in dimethylsulfoxide.

Hydrolysis measurements

Handshaken liposomes were prepared in 0.1 M Tris buffer, pH 7.2, containing 1 mM $CaCl_2$ [1]. Single bilayer vesicles were prepared according to the method of Batzri and Korn [10]. Both preparations contained 2 μmol of lipid per ml. 0.2 ml of liposomes or vesicle suspensions were incubated with variable amounts of highly purified phospholipase A_2 from pig pancreas. The reaction was terminated by the addition of 0.5 ml 0.2 M EDTA and 1 ml of methanol. The whole mixture was evaporated to dryness, the lipids were dissolved in chloroform/methanol (2 : 1, v/v) and chromatographed on silica gel H plates in chloroform/methanol/water (65 : 35 : 5, by vol.). The remaining phosphatidylcholine was scraped off the plates and determined quantitatively by a phosphorous assay [11]. In some of the experiments the extent of hydrolysis was measured by gas-liquid chromatography. After termination of the reaction by EDTA and methanol a known amount of phosphatidylcholine was added to the reaction mixture. The phosphatidylcholine which was added as internal standard differed in fatty acid composition from the phosphatidylcholine(s) used in the incubation. Isolation and separation of the lipids was performed as described above. The phosphatidylcholine was hydrolysed and the remaining fatty

acids were converted to the methylesters. Gas-liquid chromatography was carried out as described before [12]. Calorimetric experiments were performed on a Perkin-Elmer DSC-2B apparatus as described by de Kruyff et al. [13].

RESULTS

Unsaturated phosphatidylcholines

The hydrolysis at different temperatures of two mono-unsaturated and two di-unsaturated synthetic phosphatidylcholines has been studied. The results of a typical experiment are shown in Table I. C16 : 0/C18 : 1 *cis* phosphatidylcholine was hydrolysed preferentially around 3 °C, which is the transition temperature of this lipid. Also C18 : 0/C18 : 1 *cis* phosphatidylcholine and diC18 : 1 *trans* phosphatidylcholine are both hydrolysed preferentially near their transition temperatures of 13 °C. However, the unsaturated phosphatidylcholines are also hydrolysed, although at lower rate, at temperatures above their transition temperature. At 40 °C a substantial hydrolysis is obtained with the mono-unsaturated compounds and dioleoyl phosphatidylcholine. It is obvious from these data that the rate of hydrolysis at the higher temperatures is still considerably smaller than the hydrolysis rate at the transition temperature of these lipids.

Mixtures of saturated phosphatidylcholines

Vesicles prepared of equimolar amounts of diC14 : 0 phosphatidylcholine and diC16 : 0 phosphatidylcholine are hydrolysed by pig pancreatic phospholipase A_2 between 25 and 40 °C with a maximum at 32 °C (Fig. 1). Both above and below this temperature range the rate of hydrolysis is greatly decreased. On the other hand, liposomes which are made of pure diC14 : 0 phosphatidylcholine and pure diC16 : 0 phosphatidylcholine are hydrolysed at a lower, respectively, higher temperature than the equimolar mixture of both lipids. A comparison between Figs 1 and 2 shows that there is a striking similarity between the temperature dependence of hydrolysis and

TABLE I

HYDROLYSIS OF UNSATURATED PHOSPHATIDYLCHOLINES BY PHOSPHOLIPASE A_2 OF PIG PANCREAS AT DIFFERENT TEMPERATURES

Samples containing 100 nmol of lipid, dispersed as liposomes were incubated at several temperatures with 10 μl phospholipase A_2 (1 mg/ml) during 20 min. Incubations were terminated as described in Experimental. The extent of hydrolysis was determined by phosphate assay and is expressed as percentage of the initial amount of lipid. n.d., not determined.

	C16 : 0/C18 : 1 *cis*	C18 : 0/C18 : 1 *cis*	diC18 : 1 *cis*	diC18 : 1 *trans*
Transition temperature	3 °C	13 °C	−20 °C	13 °C
Incubation is carried out at:				
Transition temperature	50	85	n.d.	95
20 °C	10	20	10	5
30 °C	20	30	25	5
40 °C	20	30	25	15

the calorimetric scans of both the pure phosphatidylcholine and the cocrystallizing mixtures. It can be concluded that the hydrolysis is only possible in the temperature region of phase transition.

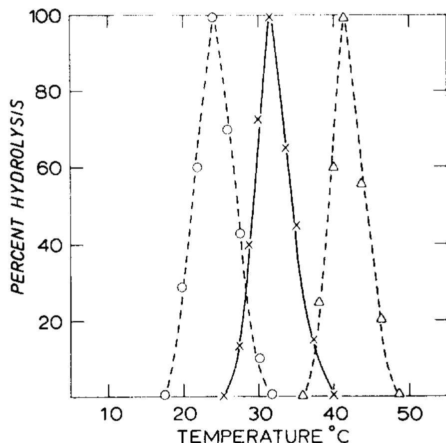

Fig. 1. Temperature-dependent hydrolysis of diC14 : 0 phosphatidylcholine, diC16 : 0 phosphatidylcholine and the equimolar mixture of both lipids. Vesicles were prepared of diC14 : 0 phosphatidylcholine (○), diC16 : 0 phosphatidylcholine (△) and the equimolar mixture of both compounds (×). Incubation with phospholipase A_2 was carried out at several temperatures during 10 min. The residual phosphatidylcholine was determined quantitatively and the results are expressed as percent of the initial amount of lipid which is hydrolysed.

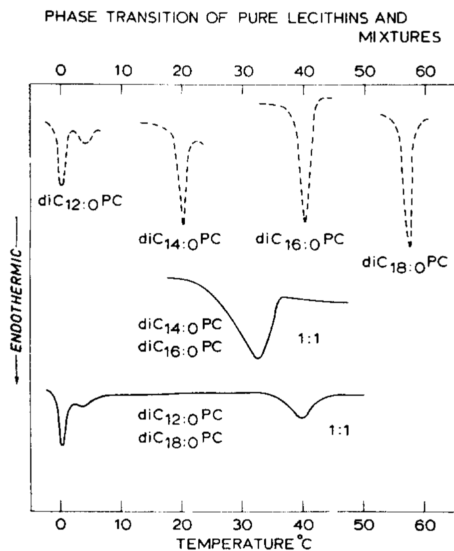

Fig. 2. Differential calorimetric scans of phosphatidylcholines (PC). Pure diC12 : 0 phosphatidylcholine, diC14 : 0 phosphatidylcholine, diC16 : 0 phosphatidylcholine and diC18 : 0 phosphatidylcholine were scanned as described elsewhere [13]. In the lower part of the figure scans are shown of the cocrystallizing mixture of diC14 : 0 phosphatidylcholine and diC16 : 0 phosphatidylcholine and the monotectic mixture of diC12 : 0 phosphatidylcholine and diC18 : 0 phosphatidylcholine.

Somewhat different results have been obtained with a mixture of two phosphatidylcholines which shows a monotectic behaviour. The temperature-dependent hydrolysis profile of the equimolar mixture of diC12 : 0 phosphatidylcholine and the

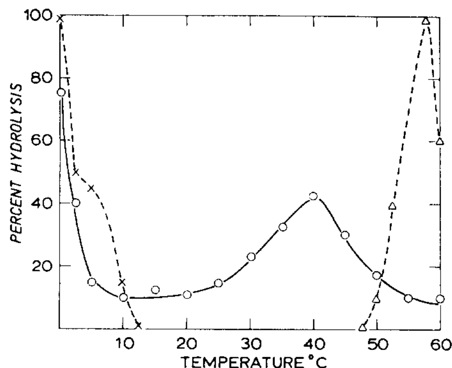

Fig. 3. Temperature-dependent hydrolysis of diC12 : 0 phosphatidylcholine, diC18 : 0 phosphatidylcholine and the equimolar mixture of these lipids. Vesicles were prepared of diC12 : 0 phosphatidylcholine (×), diC18 : 0 phosphatidylcholine (△) and an equimolar mixture of these lipids (○). After 10 min of incubation with phospholipase A_2 the remaining phosphatidylcholine was determined quantitatively. The data are expressed as the percent of the initial amount of lipid which is hydrolysed in 10 min.

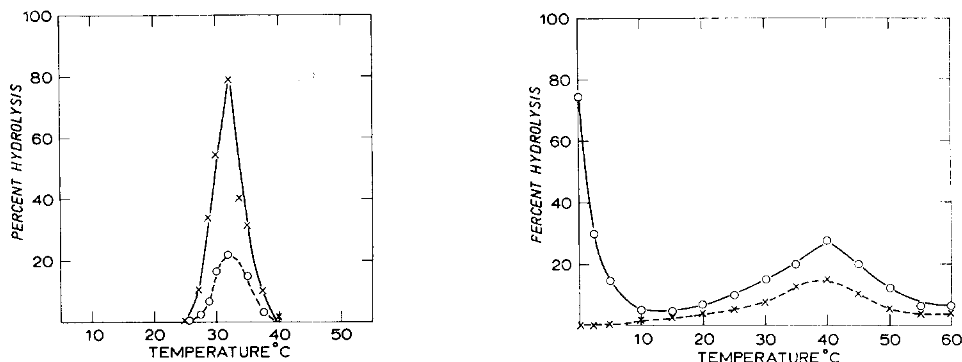

Fig. 4. Preferential hydrolysis of diC14 : 0 phosphatidylcholine in the cocrystallizing mixture of diC14 : 0 phosphatidylcholine and diC16 : 0 phosphatidylcholine. Vesicles prepared from an equimolar mixture of diC14 : 0 phosphatidylcholine and diC16 : 0 phosphatidylcholine were incubated with phospholipase A_2 during 10 min at several temperatures. The residual phosphatidylcholine was assayed for its fatty acid composition as described in Experimental. The amounts of diC14 : 0 phosphatidylcholine (×) and diC16 : 0 phosphatidylcholine (○) which are hydrolysed are expressed as percent of the initial amount of each lipid present.

Fig. 5. Preferential hydrolysis of diC12 : 0 phosphatidylcholine in the monotectic mixture of diC12 : 0 phosphatidylcholine and diC18 : 0 phosphatidylcholine. Vesicles prepared from an equimolar mixture of diC12 : 0 phosphatidylcholine and diC18 : 0 phosphatidylcholine were incubated with phospholipase A_2 during 10 min at several temperatures. The residual phosphatidylcholine was assayed for its fatty acid composition as described in Experimental. The amounts of diC12 : 0 phosphatidylcholine (○) and diC18 : 0 phosphatidylcholine (×) which are hydrolyzed are expressed as percent of the initial amount of each lipid present (average of three independent determinations).

diC18 : 0 phosphatidylcholine is given in Fig. 3. Pure diC18 : 0 phosphatidylcholine is hydrolysed preferentially around 55 °C whereas the diC12 : 0 phosphatidylcholine is hydrolysed very fast at 0 °C. The equimolar mixture is hydrolysed over a wide temperature range. The temperature-dependence curve of hydrolysis (Fig. 3) again coincides with the temperature profile of the calorimeter (Fig. 2). Two optima in the rate of hydrolysis are found, 0 and 40 °C, which temperatures coincide with the phase transition of diC12 : 0 phosphatidylcholine and diC18 : 0 phosphatidylcholine, respectively, in the equimolar mixture. In contrast to the cocrystallizing system, however, hydrolysis proceeds at a reasonable rate at all temperatures tested. This result indicates that not only the cooperative phase transition stimulates hydrolysis but that already the coexistence of two phases in the bilayer allows the action of pig pancreatic phospholipase A_2. In the cocrystallizing mixture and in the monotectic system we also measured the hydrolysis of each of the two phosphatidylcholines. The results are plotted in Figs 4 and 5. In the cocrystallizing system the phosphatidylcholine species with the shorter fatty acid chains is hydrolysed faster than the components containing the longer chain. Also in the monotectic system the more fluid compound is hydrolysed faster over the whole temperature range. It is even possible to hydrolyse all the diC12 : 0 phosphatidylcholine at 0 °C before hydrolysis of the diC18 : 0 phosphatidylcholine starts. In this respect it has to be emphasized that the data which are plotted in Figs 1, 3, 4 and 5 are obtained after relatively short incubation time. Whenever hydrolysis is possible the breakdown of all the components goes to completion, be it at variable rates.

Mixed cholesterol-phosphatidylcholine liposomes

Liposomes and single bilayer vesicles containing cholesterol and diC14 : 0 phosphatidylcholine at different molar ratios have been incubated with pig pancreatic phospholipase A_2 at several temperatures. In the presence of 10 and 20 mol % of cholesterol the hydrolysis proceeds at a slower rate than in the absence of cholesterol (Fig. 6). Maximal hydrolysis still occurs at 23 °C and similar temperature profiles are

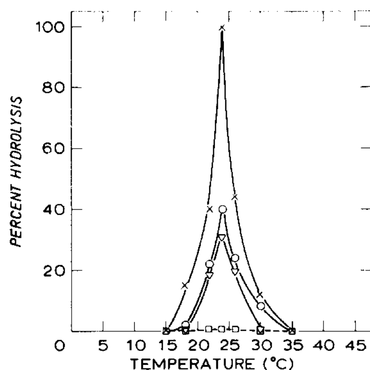

Fig. 6. Hydrolysis of lecithin-cholesterol liposomes by phospholipase A_2 at different temperatures. 1.1 ml of suspensions of liposomes of diC14 : 0 phosphatidylcholine containing 0 (×); 10 (○); 20 (▽) and 35 (□) mol % of cholesterol were incubated with phospholipase A_2 at several temperatures during 10 min. The remaining phosphatidylcholine was measured quantitatively by phosphate assay. The results are expressed as percent of the initial amount of phosphatidylcholine which is hydrolysed.

TABLE II

EFFECT OF FILIPIN AND AMPHOTERICIN B ON THE HYDROLYSIS OF DIC14 : 0 PHOSPHATIDYLCHOLINE BY PHOSPHOLIPASE A_2 IN CHOLESTEROL-CONTAINING LIPOSOMES

Samples of 200 μl liposome suspension, prepared of diC14 : 0 phosphatidylcholine and cholesterol in a molar ratio of 2 : 1, were incubated with 20 μl phospholipase A_2 (1 mg/ml) from pig pancreas during 20 min at the temperature indicated. Filipin and amphotericin were added before the phospholipase in amounts equimolar to the cholesterol present. After the incubation samples were treated as described in Experimental. The extent of hydrolysis was determined by phosphate assay and expressed as percentage of the initial amount of phosphatidylcholine.

Addition	Incubation temperature		
	10 °C	24 °C	37 °C
None	< 1	< 1	< 1
Filipin	15	95	95
Amphotericin B	5	50	40

obtained in the absence and presence of cholesterol. However, in the presence of 35 mol % of cholesterol hydrolysis is completely inhibited at all temperatures tested. Similar results have been obtained with mixtures of cholesterol and diC12 : 0 phosphatidylcholine and cholesterol and diC16 : 0 phosphatidylcholine. In both systems 35 mol % of cholesterol inhibits hydrolysis completely.

It is known that polyene antibiotics like filipin and amphotericin B associate with cholesterol and are able to restore a cooperative phase transition of the phosphatidylcholine molecules [14]. Therefore liposomes of diC14 : 0 phosphatidylcholine containing 35 mol % of cholesterol were preincubated at several temperatures with filipin or amphotericin B. The amounts of these antibiotics used were equimolar to the amount of cholesterol in the incubation mixture. After subsequent addition of pig pancreatic phospholipase A_2 the incubation was continued for 20 min. The results are shown in Table II. Both below, in and above the phase transition temperature the diC14 : 0 phosphatidylcholine appeared to be hydrolysed in the presence of cholesterol and filipin. Also the addition of amphotericin B restores the accessibility of phosphatidylcholine towards phospholipase A_2, although this polyene antibiotic was less effective than filipin.

DISCUSSION

The data presented in this paper and in an earlier report [1] strongly suggest that whenever long chain saturated lecithin molecules are uniformly arranged in a bilayer, hydrolysis of these lecithins by phospholipase A_2 from pig pancreas is negligible. The action of the enzyme is strongly enhanced by irregularities in lipid packing in the bilayer. Such an irregularity is created at the temperature of phase transition of the lecithin when lipids in the liquid crystalline phase and in the gel phase coexist. In this case hydrolysis of the lipid is very fast probably because the penetration of the enzyme into the interface is facilitated at the border of the domains of "frozen" lipid. This explanation is in agreement with results obtained with mixed phosphatidylcholine-cholesterol liposomes. Addition of cholesterol in a concentration

which eliminates a cooperative phase transition also prevents hydrolysis by pig pancreatic phospholipase A_2. Furthermore, the restoration of a phase transition by the addition of filipin and amphotericin B to the cholesterol-phosphatidylcholine liposomes results in a restored susceptibility towards the enzyme. However, the phosphatidylcholine liposomes containing the cholesterol-antibiotic complexes are accessible for the enzyme also below and above the transition temperature. It is likely that in this case the packing of the lipid molecules is disturbed around the cholesterol-antibiotic complex thereby facilitating the penetration of the enzyme. Another type of disturbance of a homogeneous packing is found in the diC12 : 0 phosphatidylcholine/diC18 : 0 phosphatidylcholine mixture. In the temperature region between the two phase transitions of both compounds the diC18 : 0 species is present as aggregates of crystalline material surrounded by the fluid diC12 : 0 species. Also in this case hydrolysis is possible be it at a smaller rate than at the two transition temperatures. Therefore it can be concluded that the coexistence of solid and liquid phases in the bilayer enables the phospholipase A to penetrate into the bilayer but that the possibility of cooperative phase transition of the lipid molecules enhances this penetration.

That at all temperatures diC12 : 0 phosphatidylcholine is hydrolyzed faster than the diC18 : 0 phosphatidylcholine can be readily explained if we assume that phospholipase A_2 is hydrolyzing preferentially those lipids which are in the most fluid environment. In a monotectic system, the concentration of the short chain species in the liquid part of a bilayer is greater than the concentration of the long chain phosphatidylcholine. Indeed our experiments demonstrated that the diC12 : 0 phosphatidylcholine is hydrolyzed preferentially by phospholipase A_2. However, in the case of a cocrystallizing equimolar mixture of phosphatidylcholines a preference for one of two components was not expected. One explanation for our observations that also in this system the short chain compound is hydrolyzed preferentially might be that a preference for the shorter chain containing lipid is an intrinsic property of the pig pancreatic phospholipase A_2. Another possibility is that the lipids in this mixture do not form a complete cocrystallizing system and that within the broad temperature traject in which the phase transition occurs, the diC16 : 0 phosphatidylcholine undergoes its transition mainly at the higher temperature. As a result the fluid phase will contain a higher concentration of the diC14 : 0 phosphatidylcholine.

The results from the experiments with phosphatidylcholine species containing one or more unsaturated fatty acids confirm the conclusion that phospholipase action is enhanced when the lipid system is in a phase transition equilibrium. It is apparent, however, that in this case the packing of the lipid molecules in the bilayer above the transition temperature is not tight enough to prevent the penetration of the enzyme. In this respect it is of interest to note that monolayer studies [15] showed that the introduction of a double bond increases the area occupied by a lipid in a monolayer considerably. Therefore, we ascribe the observed low rate of hydrolysis of unsaturated phosphatidylcholines at temperatures above the phase transition to an increased distance between the lipid molecules which allows penetration of the enzyme. A definite explanation, however, has to await the elucidation of the molecular mechanism of penetration of such an enzyme into a lipid interface.

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