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Influence of Ethanol and Phosphatidylethanol on the Activity of Pancreatic Phospholipase A_2

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Abstract—Hydrolysis of dioleoylphosphatidylethanol (DOPEt) and dioleoylphosphatidylcholine (DOPC) catalyzed by phospholipase A_2 (PLA_2) from porcine pancreas has been studied in single-component and binary liposomes in the absence and in the presence of ethanol. DOPEt (an anionic phospholipid) was found to increase the rate of hydrolysis of zwitterionic DOPC in liposomes under the action of PLA_2.

Key words: pancreatic phospholipase A_2, phosphatidylethanol, phosphatidylcholine, ethanol

Phospholipase A_2 (PLA_2; EC 3.1.1.4) hydrolyzes the ester bond in the second position of phosphoglycerides [1], the efficiency of the enzymatic hydrolysis being dependent on the chemical composition and structure of the bilayer lipid membrane [2-4]. *In vitro* experiments showed that ethanol affects the ordered arrangement of lipids and, as a result, the sensitivity of the lipid bilayer to hydrolysis by PLA_2 [5]. Administration of ethanol into the organism results in the appearance of resistance to the disturbing action of ethanol with respect to membrane phospholipids (so-called membrane tolerance). Membrane tolerance was first found in membranes of brain synaptosomes [6]. It was in synaptic membranes that the greatest activation (44%) of membrane-bound PLA_2 was observed after *in vivo* administration of ethanol [7]. Nevertheless, the data on the effect of ethanol on PLA_2 activity are contradictory: the results obtained by different authors pointed to inhibitory [5, 8-11] or activating [12-14] effects of ethanol or the lack of any effect [14-17]. In response to ethanol administration, phosphatidylethanol (PEt) is formed in cell membranes [18], PEt preventing the membranotropic action of ethanol [19, 20]. In our opinion, inconsistencies in the results are due to distinctions in the content of PEt in the membranes under study, since, as was shown in [18], the accumulation of PEt in the membranes depends on the concentration of ethanol being administered and the duration of action.

Our preliminary data showed that dioleoylphosphatidylethanol (DOPEt) might activate PLA_2 [21]. From the above reasoning, we decided to study the effect of ethanol on hydrolysis of dioleoylphosphatidylcholine (DOPC) catalyzed by PLA_2 in the presence of DOPEt with the intent of elucidating the possible role of PEt in modulation of the membranotropic properties of ethanol.

MATERIALS AND METHODS

Tris, EDTA, and PLA_2 from porcine pancreas were purchased from Sigma (USA). DOPC was prepared by acylation of cadmium complex of *sn*-glycero-3-phosphocholine by the imidazolide of oleic acid as described in [22]. DOPEt was prepared from DOPC by the reaction of transphosphatidylation catalyzed by phospholipase D from white cabbage [23]. The purity of phospholipids obtained was monitored using TLC in the system chloroform–methanol–25% NH_3 (13 : 5 : 1). Both DOPC and DOPEt are seen on chromatographs as a single spot with R_f equal 0.35 and 0.82, respectively.

To obtain liposomes, 2.5 μmoles of DOPC or 2.7 μmoles of DOPEt (or the mixture of 2 μmoles of DOPC and 0.7 μmole of DOPEt) were dissolved in chloroform. The solutions were evaporated under vacuum. After the addition of 1.6 ml of 0.05 M Tris-HCl buffer, pH 7.4, to the phospholipid film, the resulting mixture was intensively shaken at room temperature until the phospholipids were completely dispersed. The resulting dispersions were sonicated using a UZDN-2T ultrasound disintegration apparatus (frequency 22 kHz) for 30 sec with cooling. The procedure was repeated five times at 1-min intervals.

Abbreviations: DOCh) deoxycholate; DOPC) dioleoylphosphatidylcholine; DOPEt) dioleoylphosphatidylethanol; PEt) phosphatidylethanol; PLA_2) phospholipase A_2.

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After incubation of liposomes with $CaCl_2$ (0.01 M) and PLA_2 (0.1 U/ml) at 37°C for a certain length of time (2, 5, and 10 min), the reaction was stopped by the addition of 0.1 M EDTA, then ethanol (3.0, 4.5, and 9.0%, by volume) was added to aliquots withdrawn. Lipids were extracted from the reaction medium with chloroform–methanol mixture (2 : 1) by the Folch method. The chloroform phase was separated, the solvent was evaporated under vacuum, and the reaction products were analyzed using TLC on silica gel in the system of solvents chloroform–methanol–25% NH_3 (13 : 5 : 1). The lipids were detected by spraying with Vaskovsky reagent [24]. Then the spots corresponding to the initial phospholipids and their derivatives were scraped off. Mineralization was carried out in $HClO_4$ for 15 min at 250°C. The content of lipid phosphorus was determined using the molybdate reagent [24]. The amount of the enzyme catalyzing the formation of 1 μmole of the product per min at 37°C was taken as the unit of the PLA_2 activity.

Mixed micelles of phospholipids with surfactants were prepared by addition of solutions of Triton X-100 or sodium deoxycholate (DOCh) to a lipid film containing 1 μmole of DOPC or DOPEt (or their equimolar mixture: 0.5 μmole of DOPC and 0.5 μmole of DOPEt). The molar ratio surfactant/lipid was 4 : 1. The mixture was shaken for 5 min using a Vortex apparatus. The micelles were hydrolyzed as described above. The reaction was started by the addition of PLA_2 (0.01 U/ml) to the sample. The degree of hydrolysis was expressed as a percentage of lysophospholipid to the amount of non-hydrolyzed phospholipid (table). The rate of hydrolysis (in μmoles/min per mg) was calculated from the formation of lysoproduct. Data presented in the figure and table are averages of 2-4 experiments, each experiment involving two parallel measurements.

In calorimetric experiments, liposomes contained 5 mg of phospholipid per ml. The curves of heat absorption were recorded using a DASM-1M differential scanning calorimeter. The rate of scanning was 1 deg/min.

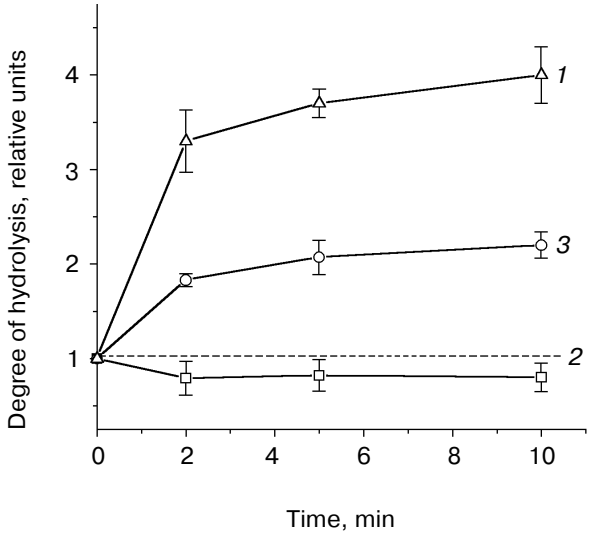
RESULTS

The initial rate and degree of the enzymatic hydrolysis of DOPC and DOPEt in single-component and binary (DOPC + DOPEt) liposomes in the absence and in the

Characteristics of hydrolysis of DOPC and DOPEt in single-component and binary liposomes and micelles with surfactants catalyzed by pancreatic PLA_2 in the absence and in the presence of ethanol

Ethanol, %	Rate of hydrolysis, μmoles/min per mg (degree of hydrolysis, %)			
	Single-component liposomes			
	DOPC		DOPEt	
0	4.8 ± 0.1 (16.5 ± 0.3)		17.4 ± 2.3 (56.1 ± 7.2)	
	Binary liposomes (DOPC + DOPEt, 3 : 1)			
0	9.5 ± 1.0 (31.1 ± 1.9)		17.3 ± 0.5 (55.4 ± 1.7)	
3.0	9.7 ± 1.2 (31.8 ± 4.0)		19.5 ± 1.4 (62.4 ± 4.3)	
4.5	11.0 ± 0.9 (35.9 ± 3.1)		18.6 ± 1.9 (59.7 ± 6.4)	
9.0	13.5 ± 1.4 (44.0 ± 4.5)		22.6 ± 1.8 (72.5 ± 5.8)	
Surfactant	Micelles with surfactant			
	DOPC	DOPEt	DOPC + DOPEt (1 : 1)	
			DOPC	DOPEt
Triton X-100	12.5 ± 1.0 (5.3 ± 0.5)	181.0 ± 16.4 (76.1 ± 6.9)	120.4 ± 20.2 (50.6 ± 8.5)	160.4 ± 30.1 (67.4 ± 15.4)
DOCh	74.3 ± 4.0 (31.5 ± 1.5)	206.7 ± 30.0 (86.8 ± 13.0)	84.5 ± 12.0 (35.5 ± 5.0)	162.8 ± 3.0 (68.4 ± 1.1)

Note: For data representation, see "Materials and Methods" section.



Ratio of degree of enzymatic hydrolysis of DOPEt in single-component liposomes to that for DOPC in single-component liposomes (*1*), ratio of degree of hydrolysis of DOPEt in binary liposomes containing DOPC to that for DOPEt in single-component liposomes (*2*), and ratio of degree of hydrolysis of DOPC in binary liposomes containing DOPEt to that for DOPC in single-component liposomes (*3*).

presence of ethanol are presented in the table. The data on hydrolysis in the micelles containing surfactants (Triton X-100 and DOCh) are also shown.

The rate of hydrolysis of DOPEt in single-component liposomes in the absence of ethanol is three times as great as that for DOPC (curve *1* in the figure). The outer layer of liposomes composed of DOPEt is almost completely hydrolyzed by PLA_2. In the case of liposomes composed of DOPC, the degree of hydrolysis is slightly higher than 25% (table). In binary liposomes, the rate of hydrolysis of DOPEt is less than that for single-component liposomes (curve *2* in figure). At the same time, the relative rate of hydrolysis of DOPC in binary liposomes is twice as large as that for single-component liposomes (curve *3* in figure). In the presence of DOPEt, the degree of hydrolysis of DOPC is as great as 31%, whereas the presence of DOPC in binary liposomes has practically no affect on the degree of hydrolysis of the second component, namely DOPEt (table).

Preliminary experiments showed that the addition of 3.0, 4.5, or 9.0% ethanol to liposomes composed of DOPC does not affect the rate of its hydrolysis by PLA_2 (data not presented). In the presence of ethanol (3-9%), the rate of hydrolysis of DOPC in binary liposomes increases by a factor of 2-3.5 (table).

Comparison of the values of the hydrolysis degree for the individual phospholipids in binary liposomes in the absence and in the presence of ethanol shows that increasing the ethanol concentration to 9% increased the degree of hydrolysis of DOPC and DOPEt by 13 and 17%, respectively (table).

To provide support for the activating effect of DOPEt, hydrolysis of phospholipids was carried out in mixed micelles composed of neutral and anionic surfactants that ensure maximum accessibility of the substrate to PLA_2. As seen from the table, the rate of hydrolysis of DOPC in neutral single-component micelles is rather low, whereas the presence of DOPEt results in a 10-fold increase in the rate of hydrolysis of DOPC. At the same time, the effect of DOPEt in anionic micelles is negligible.

To elucidate the phase state of DOPEt-containing liposomes under the conditions of hydrolysis, we studied the thermotropic properties of single-component liposomes composed of DOPEt by differential scanning calorimetry. In the absence of $CaCl_2$, no heat transitions were detected when the temperature was varied from 5 to 80°C even at rather high concentrations of DOPEt. However, the addition of 0.01 M $CaCl_2$ resulted in the appearance of a low energy (1.8 kJ/mol) endothermic phase transition in the interval from 41 to 45°C.

DISCUSSION

The DOPC/DOPEt ratio (3 : 1) in the liposomes used in our experiments corresponds to the ratio between neutral and anionic (acidic) phospholipids in the cell membranes of animals [25]. Under the conditions of the experiments (37°C), the lipid bilayer of DOPC and its mixture with DOPEt, much like the bilayer of the biological membranes, is in the liquid crystalline state, the kinetic characteristics of hydrolysis of DOPC catalyzed by PLA_2 being very similar to those for hydrolysis of natural phosphatidylcholine [26].

Based on studies of the properties of PEt and the mechanism of its biosynthesis, one can conclude that the phospholipid, apart from synthesis by occasional transphosphatidylation with the participation of ethanol, is purposely synthesized by cells that become tolerant to ethanol [19, 20]. PEt stimulates protein kinase C [27] and has some effect on the phosphoinositide signal transduction system [28], suggesting that the phospholipid has a mediator function in the action of ethanol.

The results show that two factors are essential for the enhancement of the rate of hydrolysis of DOPC: a) the presence of ethanol; b) the presence of anionic phospholipid (figure and table). It should be noted that, when studying the substrate properties of dioleoylphosphatidylalkanols (phosphatidylmethanol, phosphatidylethanol, phosphatidylpropanol, and phosphatidylbutanol), the highest activity of PLA_2 was observed with phosphatidylmethanol [29]. Intensive enzymatic hydrolysis of this phospholipid was also observed by other investigators [3]. The presence of small amounts of phosphatidylmethanol (to 1 mole %) in the mixture with DOPC resulted in a

40-fold increase in the rate of hydrolysis of the latter [29]. This fact was explained by favorable interaction of the enzyme with the structurally ordered lipid surface formed by zwitterionic DOPC in the presence of anionic lipids.

According to our experiments, the incorporation of DOPEt in liposomes also resulted in a substantial increase in the rate of hydrolysis of DOPC (figure). The degree of hydrolysis of liposomes composed of DOPEt alone within 10 min after the initiation of the reaction was as much as 80%, whereas the degree of hydrolysis was only 23% for DOPC in single-component liposomes. There are some reasons for the facilitation of DOPC hydrolysis in binary liposomes. The important role of the charge of the lipid surface in this process was supported by the data on hydrolysis of these phospholipids in liposomes composed of individual phospholipids, liposomes composed of equimolar mixture of phospholipids, and micelles with surfactants having different charge (table).

Only the outer layer of liposomes is accessible for PLA_2. This is evident from the fact that the reaction ceased when two thirds of the substrate was hydrolyzed. The higher degree of hydrolysis of DOPC in the presence of DOPEt (figure) may indicate that additional amounts of DOPC appear on the outer surface of the membrane via rapid phospholipid exchange by a flip-flop mechanism. Such an exchange was demonstrated in PEt-containing liposomes using ^{13}C-NMR [30].

The substantial increase in the rate of hydrolysis of liposomes containing DOPEt may be due to phase segregation or the formation of non-bilayer, in particular, hexagonal structures, due to the addition of the cofactor of the enzymatic reaction, Ca^{2+}, to liposomes. Changes in the liposome structure cause the appearance of "defects" on the liposome surface and in consequence higher accessibility of the substrate to the action of lipolytic enzymes [31]. It was shown in [32] that the incorporation of diglyceride, cardiolipin, or monogalactosylglyceride (these agents induce non-bilayer hexagonal (HII) structures in the membrane) to liposomes composed of phosphatidylcholine is accompanied by increase in the rate of the enzymatic transformation catalyzed by PLA_2. Judging from our differential scanning calorimetry data, the incorporation of DOPEt in liposomes composed of DOPC should result in a decrease in enthalpy and temperature of the phase transition. There is a high probability that the phases (lamellar and hexagonal) exist in liposomes under the conditions of the enzymatic reaction (37°C, 0.01 M $CaCl_2$).

Based on present knowledge, one can formulate three main reasons for the increase in the rate of the enzymatic hydrolysis of liposomal phospholipids in the presence of DOPEt: a) occurrence of negative charge on the interphase (lipid/water) boundary; b) facilitation of flip-flop exchange of phospholipid molecules by DOPEt; c) formation of DOPEt clusters having a non-bilayer structure in the presence of Ca^{2+}.

Thus, the results show that DOPEt is hydrolyzed by pancreatic PLA_2 with higher rate than DOPC. DOPEt initiates and enhances the hydrolysis of zwitterionic DOPC and potentiates the action of ethanol on this process.

It is known that the change in the activity of PLA_2 in acute pancreatitis caused by excessive alcohol consumption may serve as an indicator of the severity of the disease [33]. Since the PLA_2 activity changes are accompanied by the increase in the content of PEt that potentiates the action of ethanol on the enzymatic hydrolysis of phosphatidylcholine, it seems that the formation of PEt plays an important role in the pathogenesis of acute pancreatitis.

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