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Dynamics of Formation of Lysoforms on Enzymatic Hydrolysis of Phosphatidylalkanols

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Abstract—Hydrolysis of 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol (DOPM), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol (DOPEt), 1,2-dioleoyl-*sn*-glycero-3-phosphoethyleneglycol (DOPEG), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) catalyzed by phospholipase A_2 (PLA_2) from porcine pancreas was studied in single-component and binary model bilayer membranes (liposomes). The presence of anionic phosphatidylalkanols increases the rate of hydrolysis of zwitterionic DOPC in liposomes by the action of PLA_2. The rate of formation of lysoforms of anionic ("acidic") lipids at the initial reaction stage in single-component liposomes increased in the following sequence: DOPEG < DOPM < DOPEt (compared with that for the zwitterionic DOPC). In binary liposomes formation of lyso-DOPC increased in the presence of acidic lipids in the following sequence: DOPM < DOPEt < DOPEG. This indicates that the size of polar fragment of the second substrate and the presence of free hydroxy groups in the "head" of DOPEG may possibly activate DOPC hydrolysis by the action of PLA_2 in the presence of anionic phospholipids including cardiolipin; the studied phospholipids model fragments of the latter.

Key words: pancreatic phospholipase A_2, phosphatidylethanol, phosphatidylcholine, phosphatidylmethanol, phosphatidylethyleneglycol, cardiolipin

Phospholipase A_2 (PLA_2, EC 3.1.1.4) hydrolyzing the ester bond at the second position of phosphoglyceride molecules [1] causes formation of bioactive lysophospholipids and fatty acids including arachidonic acid, which then is converted into prostaglandins, leukotrienes, and thromboxanes, mediators of biochemical processes. The efficiency of enzymatic hydrolysis is defined by the composition and structure of bilayer lipid membrane, namely packing density of carbohydrate chains in the polar region [2-5]. Phospholipases and their products participate in the sequence of biochemical reactions resulting in such diseases as heart ischemia and myocardial infarction, which are accompanied by accumulation of lysophospholipids. Pancreatic PLA_2 is known to form an enzyme–substrate complex in the presence of the products of reaction, lysophospholipid and the cleaved fatty acid [6]. Binding to the surface of lipid supramolecular structures occurs instantaneously over a wide temperature range, while on incubation of the enzyme with the substrate in the absence of lysoproducts, lipolysis does not occur at all. Earlier we demonstrated a significant increase in formation of lyso-DOPC by the action of PLA_2 in the presence of DOPEt [7]. Addition of small amounts of DOPM (to 1 mole %) resulted in a 40-fold increase in DOPC lipolysis [8].

The goal of the present work was to study the properties of formation of lysoforms depending on the structure of the "head" of the cosubstrate on lipolysis of phosphatidylcholine by pancreatic PLA_2; for this, synthetic phosphatidylalkanols (DOPM, DOPEt, and DOPEG) modeling the fragments of the native cardiolipin were used.

The structure of these compounds is presented below:

Abbreviations: PLA_2) phospholipase A_2; PC) 1,2-diacyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine); PM) 1,2-diacyl-*sn*-glycero-3-phosphomethanol (phosphatidylmethanol); PEt) 1,2-diacyl-*sn*-glycero-3-phosphoethanol (phosphatidylethanol); PPL) 1,2-diacyl-*sn*-glycero-3-phosphopropanol (phosphatidylpropanol); PBL) 1,2-diacyl-*sn*-glycero-3-phosphobutanol (phosphatidylbutanol); DOPC) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; lyso-DOPC) 1-oleoyl,2-lyso-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine); DOPM) 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol; DOPEt) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol; DOPEG) 1,2-dioleoyl-*sn*-glycero-3-phosphoethyleneglycol; cardiolipin) bis(1,2-diacyl-sn-glycero-3-phospho)-1′,3′-*sn*-glycerol (diphosphatidylglycerol).

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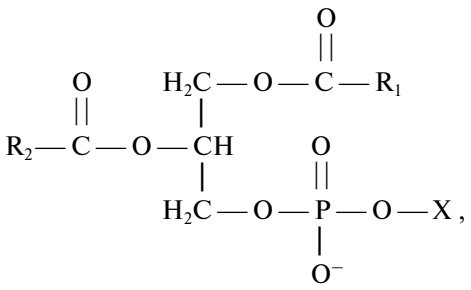
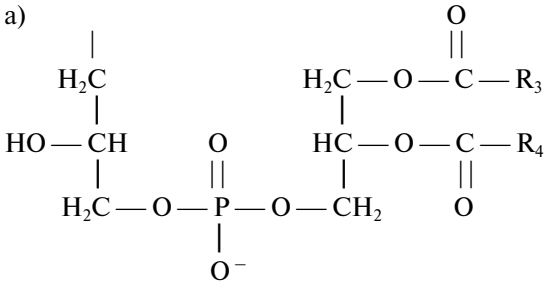


where X =

a)



Diphosphatidylglycerol (cardiolipin)

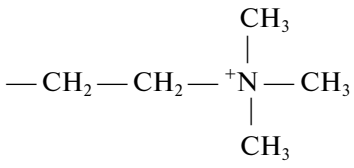
b) $-CH_3$

Phosphatidylmethanol

c) $-CH_2-CH_3$

Phosphatidylethanol

d)



Phosphatidylcholine

e) $-CH_2-CH_2-OH$

Phosphatidylethyleneglycol

MATERIALS AND METHODS

In this work we used Tris, EDTA, and PLA_2 from porcine pancreas from Sigma (USA). DOPC was obtained by acylation of the cadmium complex of *sn*-glycero-3-phosphocholine by imidazolide of oleic acid as described earlier [9]. DOPEt, DOPM, and DOPEG were obtained from DOPC by a transphosphatidylation reaction using phospholipase D from common head cabbage [10]. Using TLC on silica gel in the system chloroform–methanol–water (65 : 25 : 4 v/v), we showed that DOPC, DOPEt, DOPM, and DOPEG appear on chromatograms as the only spot with R_f 0.35, 0.71, 0.65, and 0.71, respectively.

To obtain the liposomes, chloroform solutions containing 2.5 μmol DOPC and 2.7 μmol DOPEt or the mixture of 2.0 μmol DOPC and 0.7 μmol DOPEt (DOPM, DOPEG) were evaporated in vacuum and then 1.6 ml of 0.05 M Tris-HCl buffer, pH 7.4, was added to a phospholipid film. The mixture was intensively shaken at room temperature to complete dispersion of the phospholipids. The resulting dispersions were sonicated with cooling using a UZDN-2T ultrasonic disintegrator (Russia) (frequency 22 kHz, 30 sec, 5 times with 1 min intervals).

To the liposomes incubated at 37°C, 0.4 ml of 0.01 M $CaCl_2$ and PLA_2 (0.07 unit/ml) were added and aliquots of the incubation medium were taken after 2, 5, and 10 min, terminating the reaction by addition of 0.1 M EDTA. Lipids were extracted with chloroform–methanol mixture (2 : 1 v/v) according to Folch, the degree of extraction of lysoproducts being not less than 85%. The chloroform layer was separated, the solvent was evaporated in vacuum, and the reaction products were analyzed by TLC on silica gel in the system chloroform–methanol–25% ammonia (13 : 5 : 1 v/v). The compounds on the plates were visualized as described in [11]; the spots corresponding to the initial phospholipids and their lysoderivatives were scraped out, mineralized with $HClO_4$ for 15 min at 250°C, and the content of lipid phosphorus was determined with molybdate as described in [11]. Silica gel from the zones lacking phospholipids was used as the control. Amount of the enzyme catalyzing formation of 1 μmol of the product per 1 min at 37°C was taken as the PLA_2 activity unit.

The degree of hydrolysis is given either in percent (table) as the ratio of the reaction product (lysophospholipid) and the total amount of lysophospholipid and nonhydrolyzed phospholipid determined as the content of lipid phosphorus, or in arbitrary units (figure). The rate of hydrolysis was estimated by formation of lysoproduct (μmol/min per mg protein). The average data of from two to four experiments in the initial reaction stage (to 2 min), each being performed using two parallel samples, are presented in the table and figure.

RESULTS

The initial rate and the degree of enzymatic hydrolysis of DOPC and DOPEt, DOPM, and DOPEG in single-component and binary liposomes (DOPC + DOPEt, DOPC + DOPM, DOPC + DOPEG) are presented in the table.

In single-component liposomes, the rate of DOPEt hydrolysis is 7.2, 6.9, and 5.5 times higher than the rates of DOPC, DOPM, and DOPEG hydrolysis, respectively

Characteristics of hydrolysis of phosphatidylalkanols in single-component and binary liposomes by pancreatic PLA_2

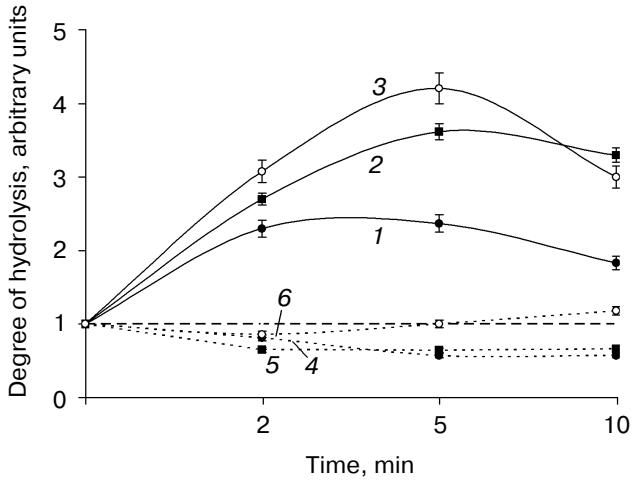
	Rate of hydrolysis, μmol/min per mg protein (% hydrolysis)			
	DOPC	DOPM	DOPEt	DOPEG
	Single-component liposomes			
	3.1 ± 0.1 (10.8 ± 0.3)	18.9 ± 0.2 (65.2 ± 0.7)	22.3 ± 1.1 (76.8 ± 3.6)	17.1 ± 0.5 (58.9 ± 1.7)
	Binary liposomes (3 : 1)			
DOPC + DOPM	7.2 ± 0.5 (24.6 ± 1.6)	15.3 ± 0.5 (51.8 ± 4.9)		
DOPC + DOPEt	8.2 ± 0.5 (27.8 ± 1.6)		15.1 ± 0.8 (50.9 ± 1.7)	
DOPC + DOPEG	9.5 ± 0.5 (32.3 ± 1.5)			14.2 ± 0.5 (47.9 ± 1.6)

Note: Time of hydrolysis 2 min, pH 7.4, 37°C.

(table). Liposomes from DOPEt are almost completely hydrolyzed by PLA_2 in 10 min (85.1 ± 0.1%), while liposomes from DOPC only 12.7 ± 0.5%.

In binary liposomes, the rates of hydrolysis of DOPEt, DOPM, and DOPEG decrease compared with the process in single-component liposomes (figure, curves *4-6*), whereas the relative rate of DOPC hydrolysis in binary liposomes increases almost twice compared with the rate of its hydrolysis in single-component liposomes (figure, curves *1-3*).

In the presence of DOPEt, DOPM, and DOPEG the total hydrolysis of DOPC on saturation of the enzyme with the substrate reaches 45-46% in 10 min. The presence of DOPC in binary liposomes to some extent decreases the total hydrolysis of the second component—DOPEt, DOPM, and DOPEG (55.1 ± 2.3, 46.5 ± 5.0, and 45.14 ± 2.8, respectively).



Enzymatic hydrolysis of phospholipids ($\bar{x} \pm S_x$): DOPC in the composition of binary liposomes with DOPM (*1*), DOPEt (*2*), and DOPEG (*3*) in relation to DOPC hydrolysis in single-component liposomes and hydrolysis of DOPM (*4*), DOPEt (*5*), and DOPEG (*6*) in the composition of binary liposomes with DOPC in relation to hydrolysis in single-component liposomes

DISCUSSION

In cell membranes of animals the ratio of the neutral and anionic (acidic) phospholipids is 3 : 1 [12], so in our model experiments we used the same proportion for the mixture DOPC–"acidic" lipids. Analogously to bilayer biological membranes, under the experimental conditions (37°C) the lipid bilayer of DOPC or its mixtures with DOPEt, DOPM, and DOPEG is in the liquid–crystalline state, and the DOPC hydrolysis catalyzed by PLA_2 is similar to the native PC hydrolysis in kinetic parameters [13].

The maximal increase in the pancreatic PLA_2 activity with phosphatidylmethanol is found when using a number of dioleoyl phosphatidylalkanols as the substrates [8]. The rate of hydrolysis of substrates decreases in the sequence PM > PEt > PPL > PBL. Other scientists also observed an intensive enzymatic hydrolysis of phosphatidylmethanol [3]. Earlier we demonstrated that the degree of hydrolysis of cardiolipin by pancreatic PLA_2 is seven times larger than that of phosphatidylcholine [14].

Under our experimental conditions (pH 7.4, 37°C), in single-component liposomes in the initial reaction stage (to 2 min) the rate of formation of lysoforms of anionic ("acidic") lipids increases in the series DOPEG < DOPM < DOPEt (table), that is, DOPEt is hydrolyzed by PLA_2 better than other phosphatidylalkanols.

Hydrolysis of the zwitterionic DOPC increases in the mixture with DOPEt, DOPM, and DOPEG. The maximal rate of formation of lyso-DOPC is observed in the presence of DOPEG (figure, curve *3*); this seems to indicate that the role of the hydroxy group of this substrate in activation of DOPC hydrolysis in bilayer liposomes is more important, because increase in the carbo-

hydrate chain length of the "head" of phospholipid in the series PM, PEt, PPL, PBL in the absence of polar groups decreases hydrolytic sensitivity of the substrate [8].

The vesicles obtained using the negatively charged phospholipids adopt "looser" packing of carbohydrate chains in the polar region than the vesicles from the neutral phospholipids [5]. It is known that lysophosphatidylcholine and fatty acids formed during the phospholipase catalyzed hydrolysis of phosphatidylcholine also cause loosing of vesicles, accumulating in the membrane, but do not disrupt their structural integrity in the presence of the enzyme [6, 15-19]. Accumulation of lyso-DOPEG, lyso-DOPM, and lyso-DOPEt among the products of phospholipase reaction probably stimulates loosing of bilayer and provides formation of defects which favor binding of the enzyme to the zwitterionic DOPC. It cannot be excluded that increase in the concentration of lyso-components results in flip-flop increase and consequently, in transmission of some additional amount of DOPC earlier inaccessible to the enzyme from the inner to the outer side of the vesicles. As we found earlier, formation of clusters of DOPEt, DOPEG, and DOPM with non-bilayer structure is also possible in the presence of Ca^{2+} [7].

So, the results demonstrate that DOPEt, DOPM, and DOPEG are hydrolyzed by pancreatic PLA_2 with higher rates than DOPC. The presence of DOPEt, DOPM, and DOPEG in the mixture initiates and enhances hydrolysis of the zwitterionic DOPC: increase in the lyso-DOPC formation is observed in the presence of acidic lipids in the sequence DOPM < DOPEt < DOPEG (figure, curves *1-3*); this indicates a possible role of the size of the polar fragment of the substrate in the activation of DOPC hydrolysis in bilayer structures.

The increased content of lysophospholipids in various tissues of a living organism as a consequence of activation of phospholipases [20-24] is known to be one of the main features of stenocardia and myocardial infarction. The heart tissues have anionic phospholipid cardiolipin in the content of cell membranes [25], which stimulates cytochrome *c*-oxidase [26]; this indicates its important effector role in biochemical processes. In liver cells cardiolipin composes up to 10 mole % of the total phospholipids [27].

Stimulation of hydrolysis of phosphatidylcholine in the binary liposomes in the presence of anionic phospholipids including cardiolipin [28] indicates that their role in the process of destruction of the native structure of lipid membrane is essentially negative. This results in the enhanced accumulation of lysoforms which are produced on the enzymatic hydrolysis of phospholipids. The model fragments of the substrate molecule are convenient for clarifying the role of the certain functional groups in the process of phospholipolysis [29, 30]. Since phosphatidylethyleneglycol is a model of the cardiolipin fragment structurally corresponding to a half of its molecule, we can suggest that along with the ability of cardiolipin to form a non-bilayer hexagonal phase [28], the presence of free hydroxy group of the glycerol skeleton at the distance of two methylene units from any phosphate residue in its molecule also contributes to the effect of cardiolipin stimulation of phosphatidylcholine hydrolysis.

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