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TRANSVERSE ASYMMETRY OF PHOSPHOLIPIDS IN SUBCELLULAR MEMBRANES OF RAT LIVER

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Summary

Subcellular membranes isolated from rat liver in a form impermeable to macromolecules were treated with phospholipase A_2 from *Naja naja* venom. The phosphatidylserine, phosphatidylethanolamine and about half of the phosphatidylcholine of microsomes, Golgi membranes, inner mitochondrial membranes, lysosomes and nuclear membranes were hydrolyzed. It is proposed that these phospholipids are localized in the outer surface of the membrane bilayer, which represents the cytoplasmic side in the living cell, while the remaining phosphatidylcholine and most of the phosphatidylinositol, sphingomyelin and cardiolipin may be assigned to the inner side of the bilayer.

Investigations during recent years have established that in many biological membranes, such as plasma membranes [1], inner mitochondrial membranes [2], microsomes [3] and erythrocytes [4], a number of enzyme proteins are asymmetrically distributed in the transverse plane. Similar findings have also been described for the glycoproteins of plasma membranes [5], microsomes [6], Golgi membranes [7], erythrocytes [8] and reticulocytes [9].

Other investigations using specific phospholipases have led to the conclusion that the phospholipids of the erythrocytes are also asymmetrically distributed in the transverse plane. It has been proposed that sphingomyelin and the majority of phosphatidylcholine are situated in the outer half of the erythrocyte membrane bilayer while phosphatidylserine, a large part of the phosphatidylethanolamine and a part of the phosphatidylcholine are located in the inner half [10—12]. The use of non-penetrating probes reacting with phosphatidylethanolamine and -serine gave results which are consistent with this conclusion [13,14].

We have recently shown that phospholipase A_2 from *Naja naja* venom hydrolyzes all of the phosphatidylethanolamine and -serine and about half of the phosphatidylcholine in intact microsomal vesicles from rat liver [3, 15]. In this investigation the same approach is employed to study the phospholipid distribution in various cytoplasmic membranes which were shown by control experiments to be impermeable to macromolecules.

In all experiments starved rats were injected intraperitoneally with a solution of carrier-free isotonic ^{32}P-labelled sodium phosphate from the Radiochemical Centre, Amersham, U.K. (1.2 mCi per 100 g body weight) 16 h before decapitation. Total microsomes and nuclei were prepared as described previously [16,17]. For isolation of Golgi membranes starved rats were given 1.2 g 50% ethanol per 100 g body weight by stomach tube 90 min before decapitation [18]. The total Golgi fraction floating on top of a 1.15 M sucrose layer was used. For the preparation of inner mitochondrial membranes the procedure described by Chan et al. [19] was employed. For the preparation of lysosomes rats were injected intraperitoneally with 1 ml Triton WR-1339 (364 mg per ml) four days before being killed [20].

Phospholipase A_2 from *N. naja* cobra venom was purified as described by Cremona and Kearny [21] with the modification of Verkleij et al. [11] 0.5 units of the purified enzyme per 10 mg of membrane protein was used and incubation carried out in the presence of 50 mM Tris·HCl, pH 7.5, and 0.25 M sucrose. The incubation medium also contained 50 mg defatted bovine serum albumin per ml. Calcium ions, which are known to be essential for the activation of the phospholipase A_2, were not added to the incubation medium, since the subcellular fractions used by us contained amounts of this ion sufficient to achieve activity. Addition of calcium caused aggregation of several types of particles which is very disadvantageous in this type of experiment. Incubations were performed in an ice-water bath for 20 min (if not otherwise indicated). This low temperature was used because incubation at 30°C followed by cooling during centrifugation may cause irreversible changes in membrane structure [22,23]. Maximal effect could be obtained even after 10 min incubation at this low temperature. The reaction was stopped by the addition of EDTA to give a final concentration of 5 mM.

Phospholipid extraction and partition were performed as described previously [24]. Phospholipids were separated by thin-layer chromatography on silica gel H according to Parker and Peterson [25]. The spots were scraped off, phospholipid extracted, radioactivity measured using a toluol scintillator, and the amount of phosphorus assayed according to Marinetti [26]. Since phosphatidylserine and-inositol move as a single spot in the chromatographic system used here, these two lipids were extracted and separated on silica gel-loaded paper [27]. Protein was determined by the procedure of Lowry et al. [28]. The phospholipid content of various subcellular fractions was analyzed as described previously [24].

Phospholipase A_2 treatment of membranes liberates both fatty acids and lysophospholipids, compounds which exert a detergent-like effect on most subcellular membranes. This fact is illustrated in Table I.

TABLE I

EFFECT OF PHOSPHOLIPASE TREATMENT ON THE PERMEABILITY OF SUBCELLULAR MEMBRANES

The water space which is not accessible to dextran (70 000 mol. wt.) is considered to represent the intravesicular water. Estimation of the intravesicular space was performed by the ultracentrifugation procedure [22]. Incubations with phospholipase were carried out in the presence or absence of 50 mg defatted bovine serum albumin per ml incubation medium. The values given are means ± S.E.M. (n=6).

Fraction	Total H_2O	Intraparticular H_2O (μl per mg dry wt.)		
		Control	Phospholipase treatment	Phospholipase treatment in the presence of albumin
Microsomes	2.8 ± 0.3	1.19 ± 0.09	0.48 ± 0.08	1.14 ± 0.12
Golgi membranes	2.3 ± 0.4	1.05 ± 0.11	0.46 ± 0.07	0.98 ± 0.14
Inner mitochondrial membranes	2.4 ± 0.3	1.30 ± 0.09	0.39 ± 0.07	1.19 ± 0.10
Lysosomes	2.5 ± 0.5	1.13 ± 0.11	0.30 ± 0.12	1.01 ± 0.17
Nuclear membranes	2.5 ± 0.4	0.90 ± 0.08	0.21 ± 0.06	0.80 ± 0.05

Phospholipase treatment of the intact particles in the absence of albumin renders them permeable to 70 000 mol. wt. dextran. However, in the presence of albumin the lysophospholipids and fatty acids are bound up and the particles remain impermeable to macromolecules.

Phospholipase A_2 treatment of Golgi membranes in the absence of albumin results in an almost complete hydrolysis of all the phospholipids, with the exception of sphingomyelin, during the first 10 min of incubation (Fig. 1A). Phosphatidylinositol is also hydrolyzed by the enzyme used. We did not attempt to find out whether hydrolysis of this lipid is caused by phospholipase A_2 or some other phospholipase still present as contaminant in our purified preparation. In the presence of albumin some of the phospholipids are not hydrolyzed, some of them are hydrolyzed to a certain extent

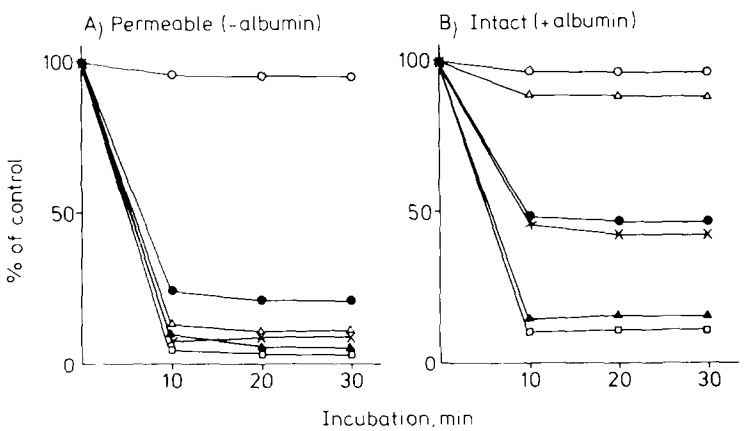

Fig. 1. Effect of phospholipase treatment on Golgi membrane phospholipids. Golgi membranes were prepared from rats injected 16 h before decapitation with carrier-free ^{32}P intraperitoneally (1.2 mCi/ 100 g body weight). Incubations were performed in the absence (A) or in the presence (B) of albumin (50 mg/ml). The values are given as per cent of the total radioactivity which is recovered in the pellet after treatment relative to the control. The values are the means of 3 (A) or 5 (B) experiments. ○—○, sphingomyelin; △—△, phosphatidylinositol; X—X, phosphatidylcholine; ▲—▲, phosphatidylserine; □—□, phosphatidylethanolamine; ●—●, total phospholipid.

and some are completely hydrolyzed during the first 10 min of incubation (Fig. 1B). This pattern is not changed by extending the incubation period. These experiments gave similar results with all of the subfractions used in this investigation. The cardiolipin of the inner mitochondrial membrane was also hydrolyzed completely by incubation in the absence of albumin. Thus, phospholipase A_2 is capable of hydrolyzing all of the membrane phospholipids except sphingomyelin. In addition, the differential breakdown of phospholipids in the presence of albumin cannot be explained by a difference in the rate of hydrolysis, since the phospholipids not hydrolyzed during the initial 10 min of incubation are not available for hydrolysis during prolonged incubation.

In our experiments the extent of hydrolysis was followed by measuring radioactivity in the individual spots from a thin-layer chromatogram. This approach is valid only if the phospholipids which are hydrolyzed do not represent a non-representative compartment into which the precursor is incorporated at a special rate. Table II demonstrates that the specific radioactivities of the individual phospholipids of Golgi membranes do not change during phospholipase treatment, a finding which validates the use of incorporated $^{32}P_i$ as a measure of phospholipid amount. Again, these experiments were repeated with all the subcellular membranes with results which were the same as those obtained with Golgi membranes.

TABLE II

EFFECT OF HYDROLYSIS ON THE SPECIFIC RADIOACTIVITY OF GOLGI MEMBRANE PHOSPHOLIPIDS

Golgi membranes were prepared as described in the legend to Fig. 1 and subjected to phospholipase treatment in the presence of albumin. After thin-layer chromatography of both control and treated Golgi membrane phospholipids, the amount of phospholipid and radioactivity was determined in the individual spots. The values shown are means of 3 experiments.

Phospholipid	Control		Phospholipase A_2-treated	
	mg phospholipid per g liver	cpm per mg phospholipid	mg phospholipid per g liver	cpm per mg phospholipid
Sphingomyelin	0.06	22 100	0.05	22 000
Phosphatidylcholine	0.22	233 000	0.10	221 000
Phosphatidylserine + phosphatidylinositol	0.07	72 300	0.04	70 500
Phosphatidylethanolamine	0.09	390 000	0.01	372 000

With all of the subcellular membranes tested phospholipase A_2 hydrolyzed 50—55% of the total phospholipid (Table III). In the case of nuclear membranes this value is somewhat higher. Phospholipase A_2 does not hydrolyze sphingomyelin. Sphingomyelinase C (kindly provided by Dr. R.F.A. Zwaal, Utrecht University) does not hydrolyze sphingomyelin in the intact particles but effectively hydrolyzes the sphingomyelin of permeable vesicles or sphingomyelin isolated from the various subcellular fractions and suspended in water in micelle form by sonication. Phosphatidylcholine behaves as does total phospholipid: 50—60% is removed from the various membranes by phospholipase A_2 treatment. Phosphatidylserine is present in microsomes,

TABLE III

EFFECT OF PHOSPHOLIPASE A_2 TREATMENT ON THE PHOSPHOLIPID CONTENT OF SUBCELLULAR MEMBRANES

Fractions labeled in vivo were prepared as described in the text and were treated with phospholipase A_2 in the presence of 50 mg albumin/ml. The values given are the means (7 experiments) of the % of the control phospholipid recovered in the pellet after phospholipase A_2 treatment.

Phospholipid	Phospholipid content (% of control)				
	Microsomes	Golgi membranes	Inner mitochondrial membranes	Lysosomes	Nuclear membranes
Total phospholipid	46	47	44	49	40
Sphingomyelin	91	94	—	92	87
Phosphatidylcholine	46	42	46	48	40
Phosphatidylserine	14	15	—	—	17
Phosphatidylethanolamine	9	11	9	21	12
Phosphatidylinositol	87	87	85	80	81
Cardiolipin	—	—	82	—	—

Golgi and nuclear membranes and almost all of this phospholipid is hydrolyzed by short incubation with phospholipase A_2. The behavior of phosphatidylethanolamine, a relatively abundant phospholipid with a high rate of turnover in all membranes, is similar to that of phosphatidylserine: complete or almost complete hydrolysis occurred upon incubation. On the other hand, 80–90% of the phosphatidylinositol of all particles as well as 82% of the mitochondrial inner membrane cardiolipin is non-accessible to phospholipase A_2 in intact particles. The results obtained with the nuclei fraction are probably unreliable, since the cytoplasmic part of the double membrane may be damaged during the isolation and/or the incubation procedure.

The conclusion suggested by these experiments is that phospholipids are asymmetrically distributed in the transverse plane of all cytoplasmic membranes. However, assignment of different phospholipids to the outer and inner halves of the membrane bilayer is not unequivocal, since the reason why phospholipase A_2 only hydrolyzes some of the phospholipids in intact particles has not been elucidated. As with erythrocytes [12], shielding of membrane lipids by proteins does not seem to occur, since pretreatment with proteases was not able to increase the amount of phospholipids hydrolyzed. Lateral surface pressure may be an important factor in determining the efficiency of phospholipase action [29] but at present no information is available about the lipid packing in subcellular membranes. On the basis of experiments performed in the absence of albumin it seems unlikely that substrate specificity would explain the incomplete hydrolysis.

It appears that several cytoplasmic membranes are more sensitive to lysocompounds that are the membranes of human erythrocytes [8]. The addition of high concentrations of albumin during incubation is therefore essential to maintain membrane impermeability to the phospholipase A_2. It cannot be excluded, however, that small amounts of lysocompounds not bound up by albumin or that removal of part of the membrane phospholipid results in perturbation and partial reorganization of the membrane.

This point will require further investigation in the future.

In spite of the fact that phospholipase treatment does not give direct information, it is reasonable to propose a tentative distribution of phospholipids in cytoplasmic membranes on the basis of the present experiments. We propose that phosphatidylcholine is present both in the outer and inner halves of the membrane bilayer, phosphatidylethanolamine and -serine are localized mainly at the outer cytoplasmic side, while phosphatidylinositol and sphingomyelin and cardiolipin (where they are present) are located at the inner surface.

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