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Phospholipid asymmetry of the outer membrane of rat liver mitochondria

Evidence for the presence of cardiolipin on the outside of the outer membrane

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The phospholipid topology of the outer membrane of intact rat liver mitochondria and derived outer membrane vesicles was investigated by determining the accessible pool of the various phospholipid classes towards phospholipase A_2, a phosphatidylcholine-specific transfer protein and by chemical labeling using trinitrobenzene sulfonic acid. The outer membrane vesicles are sealed and have a right-side-out topology with a proposed localization of 55%, 77%, 100%, and at least 30% of the phosphatidylcholine, phosphatidylethanolamine, cardiolipin, and phosphatidylinositol plus phosphatidylserine in the outer leaflet, respectively. The outer membrane in intact mitochondria appears to have a similar phospholipid distribution.

Rat liver; Mitochondrion; Outer membrane; Phospholipid asymmetry; Cardiolipin; Phospholipase A_2; Phosphatidylcholine transfer protein

1. INTRODUCTION

The outer membrane of mitochondria separates the organelle from the cytosol and acts as the initial barrier to the import of the majority of the organelle's lipids and proteins [1,2]. It has been proposed that the negatively charged lipids present in this membrane play a role in the import of mitochondrial precursor proteins [3–5]. Cardiolipin, a unique mitochondrial lipid which occurs in both mitochondrial membranes [6], may play a special role because of the reported specific interactions with presequence-containing precursor proteins in model systems [3,4,7].

For an understanding of the mechanisms of protein and lipid import into mitochondria and of the interactions of the outer membrane with its surrounding environment, it is essential to know the topological arrangement of the phospholipids in this membrane. The only information available so far is a study on the asymmetry of phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE) and phosphatidylinositol (PI) in isolated yeast outer membrane vesicles of an assumed right-side out topology [8]. In this study we report on the topology of all major phospholipid classes in the outer membrane of rat liver mitochondria and in derived outer membrane vesicles which in addition are analyzed for their intactness and sidedness.

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2. MATERIALS AND METHODS

2.1. *Materials and analytical methods*

Trypsin, soy bean trypsin inhibitor, leupeptin, phenylmethylsulfonyl fluoride, benzamidine, bovine serum albumin (essentially fatty acid free) and bee venom phospholipase A_2 (bee venom PLA) were obtained from Sigma (USA); deoxycholate, thin-layer chromatography plates from Merck (Germany); dextran T 40 (average molecular weight 40 kDa) from Roth (Germany); 1,2-di-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DOPC) (105 Ci/mol) and [1α,2α-^{3}H]cholesteryloleoyl ether (57.7 mCi/mmol) from Amersham (UK); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), -3-phosphoethanolamine (DOPE), and -3-phosphoserine (DOPS) were synthesized as described in [9,10]. PC-specific transfer protein (PCTP) was purified from bovine liver and stored as described previously [11]. Microsomal glutathion transferase (GT), partially trypsinized microsomal GT and the anti-GT anti-serum raised in rabbits were donated by Dr. R. Morgenstern (Stockholm, Sweden). Pig pancreatic phospholipase A_2 (pig pancreatic PLA), was purified as in [2]. Protein was determined by the BCA method (Pierce, USA) supplemented with 0.1% sodium dodecylsulfate and using bovine serum albumin as standard.

2.2. *Isolation of mitochondria and outer membrane vesicles*

Mitochondria were isolated from male Wistar rats as described in [6], combining differential and Percoll gradient centrifugation. Outer membrane vesicles derived from these mitochondria were isolated employing a swell–shrink–sonicate method as described in [6]. For the last wash step and for suspending the final pellets, a buffer was used to meet the various experimental requirements. The purity of every batch of mitochondria and of outer membrane vesicles used was checked via marker enzyme analysis as described in [6] and yielded comparable results as described in [6]. In short, the specific activities of the marker enzymes monoamine oxidase (mitochondrial outer

membrane), succinate cytochrome *c* reductase (mitochondrial inner membrane), glucose-6-phosphatase (microsomes), acid phosphatase (lysosomes) and 5'-nucleotidase (plasma membrane), respectively, relative to the homogenate, were 2.6, 5.6, 0.18, 0.7 and 0.33 in the mitochondrial preparations and 36.4, 1.3, 0.59, 5.81, and 3.1 in the outer membrane preparations, respectively [6]. The intactness of the outer membrane in intact mitochondria was found to be 90–95%, based on the ability of cytochrome *c* oxidase to oxidize externally added reduced cytochrome *c*, analyzed as described in [6]. 85–100% of activity of adenylate kinase (AK; [6]), enclosed in outer membrane vesicles, was protected against externally added trypsin (see below).

For experiments on the topology of outer membrane proteins, all media used for the isolation of mitochondria and outer membrane vesicles, except those used for suspending the final pellets, were supplemented with the protease inhibitor cocktail: 0.2 mM benzamidine, 0.1 mM phenylmethyl sulfonylfluoride, 50 μg/ml soy bean trypsin inhibitor and 1 μg/ml leupeptin.

2.3. *PLA treatment*

Mitochondria or outer membrane vesicles (3 or 0.6 mg protein per ml, respectively) in 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.1 mM $CaCl_2$ (pH 7.4) were incubated for the indicated periods at room temperature with 0.4 U PLA per ml. Higher PLA concentrations did not result in an increased lipolysis. PLA activity was inhibited by the addition of 0.75 mM EGTA. For phospholipid analysis, mitochondria or outer membrane vesicles were reisolated from the incubation mixtures by centrifugation for 10 min at 14,000 rpm in an Eppendorf Centrifuge 5415 or 15 min at 60,000 rpm in a Beckman TLA 100.2 rotor, at 4°C, respectively. The pellets from the PLA incubations were suspended in 50–100 μl of 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.75 mM EGTA (pH 7.4) and extracted [12] The individual lipid classes were separated, visualized and quantified as described in [6]. Phosphatidylserine (PS) and PI were not always completely separated and were therefore treated as one lipid class denoted PS/PI Determination of the extent of digestion of a phospholipid class by the decrease of the parent phospholipid class or by the increase of the corresponding lysophospholipid yielded identical results. Digestion of CL resulted in the appearance of both mono and dilyso-CL. No hydrolysis of phospholipids was detected in control experiments without PLA.

2.4. *Trypsin treatment of mitochondria and outer membrane vesicles*

Mitochondria or outer membrane vesicles (both 1.25 mg protein per ml) in 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES (pH 7.4) were incubated for 20 min on ice with 0–2 mg trypsin per mg protein. Then a 2.5 times weight excess of soy bean trypsin inhibitor was added and the mixture was left on ice for 5 min before further analysis.

2.5. *Immunodetection of glutathione transferase (GT)*

Mitochondria or outer membrane vesicles were prepared for electrophoresis according to Lugtenberg et al. [13] and proteins (9 or 2.5 μg per sample, respectively) were separated in 15% sodium dodecylsulfate polyacrylamide gel according to Laemmli [14], transferred to nitrocellulose sheets by Western blotting and immunodecorated with an anti-GT anti-serum. Immunopositive bands were stained using immuno decoration kit 170-6509 from BioRad (USA). The anti-serum specifically identified GT on Western blots of total mitochondrial protein (not shown).

2.6. *PCTP-catalyzed exchange of mitochondrial PC*

Sonicated vesicles composed of 50, 30 and 20 mol%, respectively, of DOPC (0.5–2 μCi [^{14}C]DOPC), DOPE and DOPS, and containing 2×10^{-4} mol% cholesteryloleoyl ether as non-exchangeable marker, were prepared as described in [15].

Mitochondria (3 mg protein per ml) were incubated with donor vesicles (60 μM lipid) and PCTP (16 μg per ml) in 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.05% (w/w) bovine serum albumin (pH 7.4) at 30°C. The exchange between donor vesicles and mitochondria, the intramitochondrial localization and the metabolism of [^{14}C]PC were quantified as in [15] Imported [^{14}C]DOPC was found to be not metabolized. The import of labelled PC into mitochondria was corrected using the ^{3}H-radioactivity for unspecific association and/or fusion of the donor vesicles with the mitochondria. This non-specific association proceeded 50–70 times slower than the PCTP catalyzed transfer of PC and amounted to maximally 4–6% of the specifically transferred PC. The radiolabelled PC was localized in the mitochondrial membranes using digitonin to separate the outer from the inner membrane [6]

2.7 *Intactness of outer membrane and outer membrane vesicles during the various incubations*

Of the mitochondrial incubations, 50 μl was centrifuged for 5 min at 14,000 rpm in an Eppendorf Centrifuge 5415 and the fraction of AK activity [6] present in the pellet was taken as a measure for the intactness of the outer membrane. At the start of the experiment, the mitochondria showed very high latency of cytochrome *c* oxidase (see above). AK activity was quantitatively recovered in pellet and supernatant Outer membrane vesicles were treated with 1.7 mg trypsin per mg sample protein (see section 2.4) and the remaining fraction of AK activity was taken as a measure for the intactness of the outer membrane vesicles.

The intactness of the outer membrane in mitochondria and outer membrane vesicles was not effected by incubation with trypsin or PCTP.

3. RESULTS

3.1. *Phospholipid topology studies in intact mitochondria*

PLAs are the most versatile tools for topological studies of membrane phospholipids [16]. However, incubation of rat liver mitochondria with PLA caused outer membrane lysis because it resulted in a rapid and complete release of the intermembrane space marker AK as is shown for the pancreas enzyme in Fig. 1. Inclusion of dextran in the medium retarded lysis for at least 10 min (Fig. 1) which opened up a time window for the topology studies. In the experiment shown in Fig. 2 constant levels of degradation of CL, PE, and PC are observed after only 3 min. Only when outer membrane lysis occurred, increased amounts of these lipids were degraded. No digestion of PS/PI could be detected.

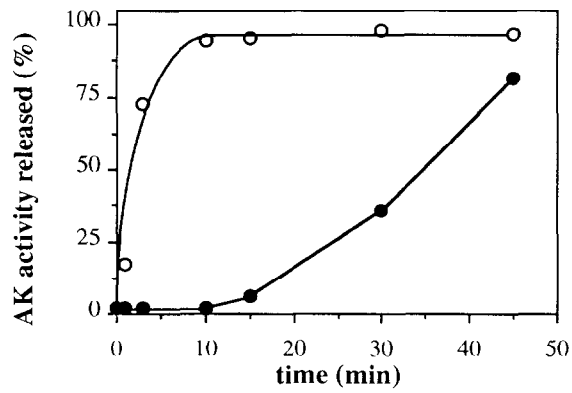

Fig. 1 Protection of the outer membrane by dextran. Mitochondria (3 mg protein per ml) were incubated with 0.4 U ppPLA per ml in the absence (○) or presence (●) of 10% dextran T40. At the indicated time points the intactness of the outer membrane was determined by measuring the amount of AK released from the mitochondria.

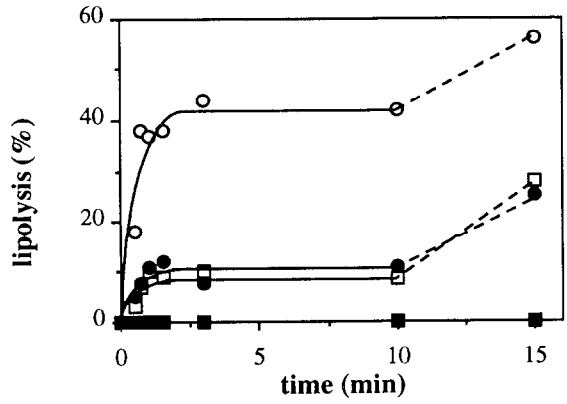

Fig. 2. Digestion of mitochondrial phospholipids by ppPLA. Mitochondria (3 mg protein per ml) were incubated with 0.4 U ppPLA per ml in the presence of 10% (w/v) dextran T40. Samples were drawn at the indicated time points and analyzed for lipolysis of the individual classes: (●), PE; (□), PC; (○), CL; (■), PS/PI. Data of a representative experiment are shown.

The results of several experiments are quantified in Table I and show that a defined, and in case of CL, a remarkably high fraction of the mitochondrial CL, PE and PC is accessible to pancreatic PLA. It is unlikely that the enzyme (molecular weight 19.5 kDa) can pass the outer membrane which is impermeable to cytochrome *c* (12 kDa) [17]. The stronger membrane penetrating bee venom enzyme could not be used because of its very lytic behavior towards intact mitochondria which could not be inhibited by dextran (data not shown).

Next, we used PCTP to specifically study the localization of PC in the mitochondrial outer membrane. Incubation of mitochondria with [^{14}C]DOPC containing donor lipid vesicles resulted in a rapid decrease in the specific radioactivity of the PC in the donor vesicles from 3,600 ± 40 to a stable level of 1,750 ± 10 dpm/nmol (not shown) which is paralleled by an increase in the specific radioactivity of the PC in the mitochondria to a stable level of 155 dpm/nmol (Fig. 3). The radiolabelled PC is exclusively localized in the outer membrane where it reaches at equilibrium a specific radioactivity of 293 ± 8 dpm/nmol. In agreement with previous studies [18,19], we established that 60% of the vesicle PC was accessible to PCTP (not shown). It can then be calculated that after 30 min the specific radioactivity of PC in the outer leaflet of the vesicles will be 520 ± 5 dpm/nmol. This implies that approximately 30% of the mitochondrial and 56 ± 2% of the outer membrane PC is rapidly exchangeable (Table I).

The topology of amino phospholipids can be specifically assessed via labelling with trinitrobenzene sulfonic acid (TNBS) under conditions where membrane permeability of TNBS is negligible. This condition is hard to fulfill for the mitochondrial outer membrane because of the presence of pores. However, blocking these pores with polyanion [20] greatly reduces the passive diffusion of small solutes [21] which we could confirm by incubating the mitochondria (3 mg protein/ml) with 300 μg polyanion/ml and by assessing AK activity. Under these conditions at room temperature a biphasic labelling with 3 mM TNBS of mitochondrial PE was observed (not shown) with a rapid labelling ($t_{1/2}$ = 3 min) of 27% of the mitochondrial PE, suggesting that this fraction, which corresponds to 79 ± 9 of the outer membrane pool, is localized at the outer surface (Table I).

Table I

Rapidly accessible pools of phospholipids in mitochondria and outer membrane vesicles towards phospholipases, PCTP and TNBS

	Rapidly accessible phospholipids (% of each class)				
	PC	PE	CL	PS/PI	*n*
Mitochondria					
Pancreatic PLA					
Total pool	9 ± 3	15 ± 6	42 ± 7	n.d.	3
Outer membrane pool	18 ± 6	44 ± 18	180 ± 40		
PCTP					
Total pool	30 ± 1				2
Outer membrane pool	56 ± 2				
TNBS					
Total pool		27 ± 3			3
Outer membrane pool		79 ± 9			
Outer membrane vesicles					
Bee venom PLA	54 ± 6	72 ± 3	n.d.	n.d.	3
Pancreatic PLA	(a)	(a)	100 ± 1	31 ± 2	2
Bee venom + pancreatic PLA	55 ± 1	77 ± 1	100 ± 1	30 ± 7	3

For mitochondria the table shows data on the total mitochondrial pool of a lipid class and the outer membrane pool as calculated from the distribution of the lipid classes over both membranes [6]. The data are obtained from experiments described in the legends of Figs. 2, 3 and 6 and are presented as the mean ± standard deviation of *n* experiments. n.d. = not detected. (a) = no stable levels reached.

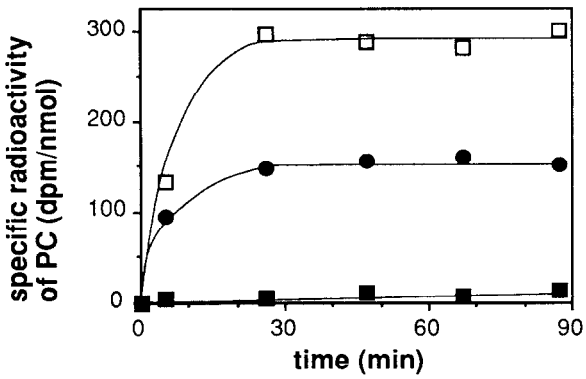

Fig. 3. PCTP-catalyzed introduction of [^{14}C]PC into mitochondria. Mitochondria were incubated with PCTP and donor vesicles containing [^{14}C]PC. At the indicated time points, the specific radioactivity of PC in the mitochondria (●), the inner (■) and the outer membrane (□) were determined

3.2. *Characterization of isolated outer membrane vesicles*

As a second experimental system, we used isolated outer membrane vesicles prepared by the new swell–shrink–sonicate method described in [6]. To be useful for topological studies, these vesicles should be sealed and of known and defined sidedness. The results of Fig. 4 clearly show that the large majority of the AK activity in both mitochondria and derived outer membrane vesicles is protected against trypsin and thus that the enzyme is present behind the barrier of the outer membrane. We assayed the topology of the vesicles using antibodies in conjunction with proteolysis of the outer membrane enzyme GT [22] (Fig. 5). Purified GT migrates as a single immunopositive band at 16 kDa (lane 1). In the presence of protease inhibitors in the isolation of the mitochondria (see experimental section) the 16 kDa band of the intact protein is also observed (lane 2). Addition of excess trypsin digests virtually all GT, resulting in the appearance of a minor band at 8 kDa (lane 3). Despite the presence of protease inhibitors in isolated outer membrane vesicles, the antibodies indicated that approximately half of the GT was proteolytically cleaved (lane 4) to an 8 kDa band which is also observed upon partial trypsinization of GT (not shown). However, subsequent trypsin treatment resulted in full digestion of the 16 kDa protein (lane 5), demonstrating that GT is completely accessible to trypsin in both mitochondria and outer membrane vesicles, suggesting a similar orientation of the protein in the two systems. Accordingly, we observed that: (1) treatment of both mitochondria and outer membrane vesicles with small amounts of trypsin resulted in a similar stimulation of GT activity (assayed as in [23], data not shown); (2) the activity of the outer membrane enzyme rotenone-insensitive NADH cytochrome *c* reductase (assayed as in [24]) is destroyed to a similar extent by trypsin in both mitochondria and outer membrane vesicles (not shown).

3.3. *Phospholipid topology in outer membrane vesicles*

In contrast to mitochondria, the outer membrane in the isolated vesicles remained intact upon prolonged incubation with both bee venom and pancreatic PLA and a combination of the two (data not shown). Incubation of the vesicles with pancreatic PLA results in degradation of all CL present (Fig. 6A), consistent with the results obtained in mitochondria (compare Fig. 2). PS/PI, PC and PE were also degraded but like CL, at a slower rate as in intact mitochondria. No stable end levels were observed for PC and PE. The bee venom enzyme rapidly degrades PE such that a stable level of degradation of 69% is observed (Fig. 6B). PC was initially rapidly degraded by this enzyme after which degradation continued at a much slower rate, possibly as the result of redistribution of PC from the inner to the outer leaflet of the outer membrane. By extrapolation to time zero (dotted line) it can be calculated that 47% of the PC is rapidly degraded. Degradation of CL and PS/PI by the bee venom enzyme was not observed, consistent with its substrate specificity [16]. The combination of both the pancreatic and bee venom enzyme was found to be optimal for stable levels of degradation of all major phospholipids in one experiment (Fig. 6C). These amounted to 100%, 55%, 20%, and 77% for CL, PC, PS/PI, and PE, respectively, for the experiment shown. A prerequisite in the analysis of such data in terms of topology is that the enzyme cocktail is in principle able to degrade all phospholipids. This was confirmed by the observation that these enzymes, added to intact mitochondria, caused degradation of 97% of phospholipids in 30 min, the 3% remaining being pre-

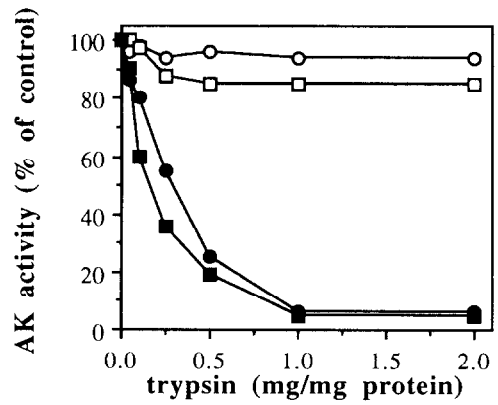

Fig. 4. Trypsin sensitivity of AK activity. Mitochondria and outer membrane vesicles (both 1.25 mg protein per ml) were incubated for 20 min on ice with the indicated amounts of trypsin. After the addition of trypsin inhibitor the AK activity was determined for intact (open symbols) and disrupted (closed symbols) mitochondria (○,●), and outer membrane vesicles (□,■), respectively. Mitochondria were disrupted by two freeze-thaw cycles and the outer membrane vesicles by the addition of 0.4% (w/v) deoxycholate. The latter treatments had no effect on the specific activity of AK which was determined to be 770 ± 70 and 625 ± 70 nmol per minute per mg protein for mitochondria and outer membrane vesicles, respectively. The data presented are the mean of 5 experiments with standard deviations less than 10%.

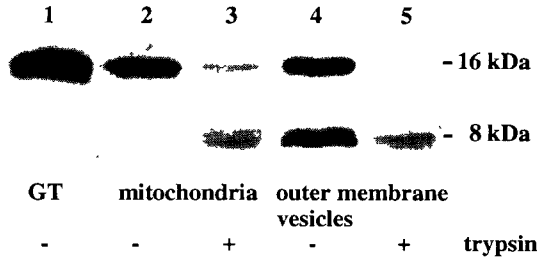

Fig. 5. Immunological analysis of tryptic digestion of GT. Mitochondria and outer membrane vesicles (9 and 2.5 μg protein, respectively) were subjected to electrophoresis, Western blotting, decoration with a specific anti-GT anti-serum and immuno staining: lane 1, purified GT; lane 2, mitochondria; lane 3, trypsin treated mitochondria; lane 4, outer membrane vesicles; lane 5, trypsin treated outer membrane vesicles. Proteolysis was carried out by addition of 0.5 mg trypsin per mg protein.

dominantly PS/PI of which 81% in total was digested. The phospholipase data on the isolated outer membrane vesicles are summarized in Table I.

4. DISCUSSION

Our studies provide for the first time an insight into the topology of all major phospholipid classes in mitochondrial outer membranes. The outer membrane vesicles used as a derived model system are shown to be sealed and right-side out and resistant to lysis by phospholipases. It is proposed that the stable degradation levels obtained reflect the phospholipid topology. The results obtained under different conditions are in good agreement (Table I) and strongly suggest that 55% and 77% of the PC and PE, respectively, are localized in the outer leaflet of the outer membrane in the vesicles and that there is no or only a slow redistribution of the molecules from the inner to the outer layer. The outer membrane contains 9–11 mol% CL (based on lipid phosphorus) as a true component of the membrane [6]. This lipid is very rapidly and completely degraded with kinetics indicating it to be accessible as a single pool. This demonstrates that it either is exclusively localized in the outer leaflet or has a fast transbilayer movement. We favor the first explanation although we cannot rigorously exclude the second possibility. Our arguments are: (1) the transbilayer movement would have to be exceedingly fast because incubation of the vesicles with 0.2 U pancreatic and 0.01 U bee venom PLA resulted in the degradation of 100% of the CL within 30 s (not shown). There are no precedents for such a rapid transbilayer movement of one specific and major phospholipid class in a biomembrane; (2) in a study on the topology of CL in the inner membrane no indications of transbilayer movement of the CL was obtained [25]. At least 30% of the PS/PI fraction is localized in the outer leaflet of the outer membrane. Taking into account the lipid composition of the outer membrane vesicles, it can be calculated that 56% of the total phospholipids are accessible to the PLAs which is close to the value expected for a bilayer considering the small size of the vesicles ranging from 50 to 350 nm in diameter [6]. The data on PC asymmetry in the rat liver outer membrane vesicles are in good agreement with the data reported for similar vesicles from yeast [8].

When the results on phospholipid accessibility in the outer membrane vesicles are compared to the results for intact mitochondria, interesting similarities and differences are detected. The results on PC and PE in mitochondria obtained via the mildest experimental approaches, i.e. PCTP and TNBS are in excellent agree-

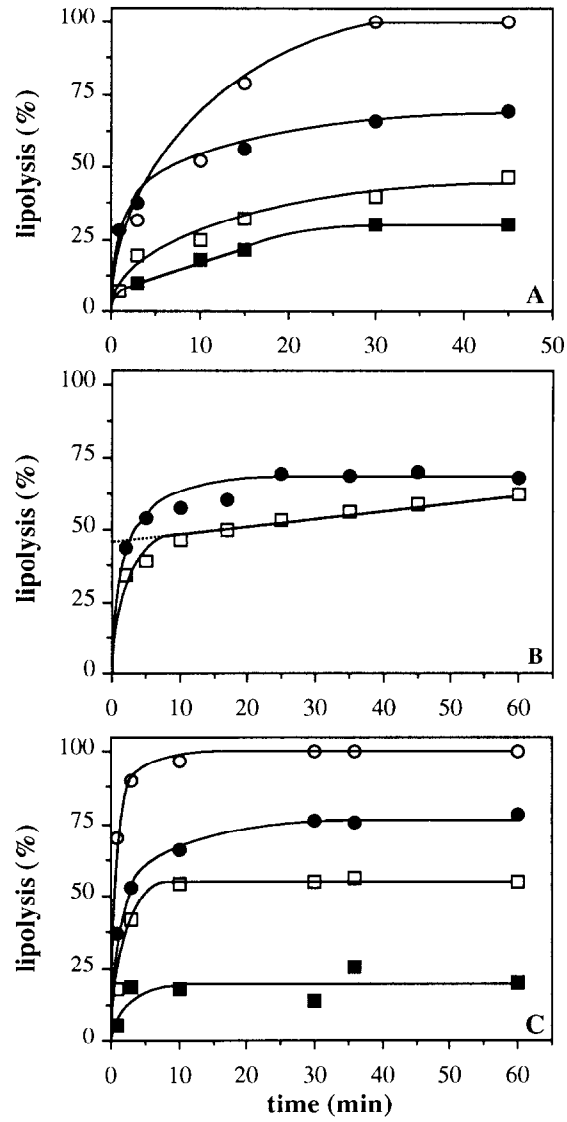

Fig. 6. PLA treatment of outer membrane vesicles. Outer membrane vesicles (0.6 mg protein per ml) were incubated with (A) 0.4 U pancreatic PLA per ml, (B) 0.4 U bee venom PLA per ml, or (C) 0.4 U bee venom and 0.4 U pancreatic PLA per ml. Samples were drawn at the indicated time points and analyzed for lipolysis of the individual classes: (●), PE; (□), PC; (○), CL; (■), PS/PI. Data of representative experiments are shown.

ment with the data on the outer membrane vesicles, justifying this approach and emphasizing the correctness of the sidedness of the outer membrane vesicles. The results of the phospholipases are more difficult to compare in view of the extreme lability of the outer membrane towards phospholipase treatment which only could be partly overcome by the inclusion of a high molecular weight dextran (Fig. 1). This stabilizing effect is probably due to a balancing of the colloid-osmotic pressure in the intermembrane space, originating from the high protein concentration in this compartment [26]. This osmotic stress on the outer membrane is apparently absent in the vesicles because the majority of the intermembrane space proteins are lost during their formation [6]. The observed low extent of lipolysis of PC and PE in intact mitochondria could be due to the preference of the pig pancreatic enzyme for negatively charged phospholipids [31].

The pancreatic PLA degradation levels of PC and PE in the outer membrane in intact mitochondria are lower than in isolated vesicles for which we have no clear explanation. Nevertheless, the phospholipase data on CL in intact mitochondria are in qualitative agreement with the isolated vesicles because in both cases a large fraction of the CL is rapidly degraded by the enzyme. However, the amount of CL digested (42% of the total mitochondrial CL) exceeded the amount of CL present in the outer membrane (23%) as inferred from digitonin extractions [6]. Although 5–10% of the mitochondria have a broken outer membrane and therefore the phospholipase could have access to the inner membrane, this cannot account for this difference. We propose that the difference in accessibility of CL in both systems is due to the presence of small regions of the outer membrane rich in CL which remain attached to the inner membrane in the procedures used to separate both membranes [6]. This explanation is corroborated by the observations that contact sites between the inner and outer membrane are enriched in CL [27,28] and that these sites are relatively resistant to digitonin [29]. Alternatively, the inner membrane CL may be rapidly transferred to the outer membrane to replenish the hydrolyzed outer membrane CL.

Our observations that anionic lipids are present on the outer leaflet of the mitochondrial outer membrane are in good agreement with the proposed roles of these phospholipids in apocytochrome *c* import in the organelle [30]. The proposed localization of CL in the outer leaflet of the outer membrane and the specific interactions with mitochondrial presequences [3,4,7] suggest that CL-presequence interactions are involved in early steps in the general protein import pathway.

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