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Import of Phosphatidylethanolamine for the Assembly of Rat Brain Mitochondrial Membranes

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Abstract. Mitochondria can synthesize phosphatidylethanolamine (PE) through phosphatidylserine decarboxylase (PS decarboxylase) activity or can import this lipid from the endoplasmic reticulum. In this work, we studied the factors influencing the import of PE in brain mitochondria and its utilization for the assembly of mitochondrial membranes. Incubation of rat brain homogenate with [1-^{3}H]ethanolamine resulted in the synthesis and distribution of ^{3}H-PE to subcellular fractions. Twenty-one percent of labeled PE was recovered in purified mitochondria. The import of PE in mitochondria was studied in a reconstituted system made of microsomes (donor particles) and purified mitochondria (acceptor particles). Ca^{+2} and nonspecific lipid transfer protein purified from liver tissue (nsL-TP) enhanced the translocation process. ^{3}H-PE synthesized in membrane associated to mitochondria (MAM) could also translocate to mitochondria in the reconstituted system. Exposure of mitochondria to trinitrobenzensulfonic acid (TNBS) resulted in the reaction of more than 60% of ^{3}H-PE imported from endoplasmic reticulum and of about 25% of ^{14}C-PE produced in mitochondria by decarboxylation of ^{14}C-PS. Moreover, the removal of the outer mitochondrial membrane by digitonin treatment, resulted in the loss of ^{3}H-PE, but not ^{14}C-PE. These results indicate that labeled PE imported in mitochondria is mainly localized in the outer mitochondrial membrane, whereas PE produced by PS decarboxylase activity is confined to the inner mitochondrial membrane. Phospholipase C hydrolyzed 25–30% of both PE radioactivity and mass of the outer mitochondrial membrane indicating an asymmetrical distribution of this lipid across the membrane.

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Key words: Phosphatidylethanolamine — Phospholipid transport — Mitochondria — Phosphatidylserine decarboxylase — Brain

Introduction

Phospholipid synthesis in brain mitochondria is restricted to the formation of phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE). All the other phospholipids are synthesized in the microsomal fraction and from this site distributed to the proper location. Since mitochondrial membranes contain the whole type of cellular phospholipids, a very efficient import of lipidic material from endoplasmic reticulum is necessary (Daum, 1985).

Mitochondrial PE is synthesized by decarboxylation of phosphatidylserine (PS) through an enzymatic activity (PS decarboxylase) localized on the inner mitochondrial membrane (Percy et al., 1983). PS, the substrate for PS decarboxylase, is synthesized in the endoplasmic reticulum and translocated to the inner mitochondrial membrane. Translocation and decarboxylation of PS has been studied in many cellular systems (Voelker, 1990; Hovius et al., 1992; Simbeni et al., 1993). Membranous continuity between inner mitochondrial membrane and endoplasmic reticulum through intermembrane mitochondrial contact sites (Ardail, Lerme & Louisot, 1991) and membranes associated with the outer mitochondrial membrane (Vance, 1990), should be taken into account for the possibility of movement of phospholipids between endoplasmic reticulum and mitochondria. In the liver, the mitochondria-associated membranes, similar in structure and function to the endoplasmic reticulum, contain all the ingredients necessary for the synthesis of PS and for its transfer to mitochondria with subsequent decarboxylation. The newly formed PE is thereafter ex-

pelled from mitochondria via contact sites (Jasinska, Zborowski & Somerharju, 1993) and methylated to phosphatidylcholine (PC) in mitochondria-associated membranes (Vance, 1990). Similarly (Simbeni, Paltauf & Dunn, 1990), PE synthesized through PS decarboxylase activity in yeast mitochondria is easily exported without prior mixing to the pool of PE in the inner membrane. On the contrary, we demonstrated, in a reconstituted system of brain microsomes and mitochondria (Corazzi et al., 1993; Carlini et al., 1993) that labeled newly formed PE obtained by decarboxylation of PS, slowly moves from its site of synthesis to the outer mitochondrial membrane, while a large part of it is internalized in the inner mitochondrial membrane.

PE is also imported into mitochondria from microsomes (Voelker, 1991) where it is formed by the CDP-ethanolamine pathway (Kennedy & Weiss, 1956) or by base-exchange reaction (Corazzi et al., 1986). Movement of PE from endoplasmic reticulum to mitochondria was observed in hepatocytes and kidney cells in culture (Yaffe & Kennedy, 1983). Moreover, experiments in vivo showed that PE formed in brain endoplasmic reticulum is translocated to mitochondria (Butler & Morell, 1983).

In this work, we present some results on the factors influencing the translocation of PE from endoplasmic reticulum to mitochondria and compare the localization of this lipid with that synthesized in the mitochondria through PS decarboxylase activity.

Materials and Methods

Materials

Amersham International (Amersham, UK) supplied L-[3-^{14}C]serine (specific radioactivity, 55 mCi/mmol) and [1-^{3}H]ethanolamine (specific radioactivity, 30 Ci/mmol). Cytidine-5′-diphospho-[1,2-^{14}C]ethanolamine (specific radioactivity, 51 mCi/mmol) was obtained from ICN Radiochemicals (Zoetermeer, The Netherlands). Hepes, NADPH, cytochrome c and ATP were from Boehringer-Biochemie (Mannheim, Germany). Phospholipase C from *B. cereus* (specific activity, 200 units per mg protein), digitonin, standard phospholipids and other reagents used to test the purity of subcellular fractions were from Sigma Chemical (St Louis, MO). TNBS was obtained from Pierce Eurochemie (Rotterdam, The Netherlands). All other chemicals were from Carlo Erba, Milan, Italy.

Subcellular Fractionation

Wistar male rats (weighing 150–200 g) were used to prepare brain subcellular fractions. Brains were homogenized in 0.32 M sucrose plus 2 mM Hepes, pH 7.4 (S/H buffer, 8 ml/g tissue). The homogenized material was centrifuged twice at 1,500 × *g* for 10 min and the pellets were discarded. The supernatant was used as the homogenate or to prepare mitochondrial and microsomal fractions. To prepare mitochondria the supernatant was centrifuged at 8,000 × *g* for 20 min and the pellets (P_1) were considered the crude mitochondrial fraction. Crude mitochondria (7–8 mg protein) were then purified by centrifugation (75,000 × *g* for 90 min) on a discontinuous sucrose gradient formed in 5 ml bucket tubs (SW 50.1, Beckman rotor), by successively layering 0.8 ml each of 1.6 M, 1.4 M, 1.2 M 1.0 M, and 0.8 M sucrose. After centrifugation, pure mitochondria were recovered at the 1.2–1.4 M sucrose interface. The band observed at the 0.8–1.0 M sucrose interface was designed as mitochondria-associated membrane fraction (MAM), following its biochemical characterization. To remove sucrose excess, subfractions were diluted with a solution of 2 mM Hepes, pH 7.4 and recovered by centrifugation (8,000 × *g* for 10 min, mitochondria; 72,000 × *g* for 20 min, MAM). The final pellets were resuspended in a proper amount of S/H buffer. Microsomes were prepared with routine procedure (Corazzi et al., 1986) from the supernatant of the crude mitochondrial pellet.

The nonspecific lipid transfer protein (nsL-TP, specific activity about 800 nmol phospholipid/h per mg protein) was purified from the postmicrosomal fraction of rat liver homogenate as described (Crain & Zilversmit, 1980). The protein suspension was divided into small aliquots which were stored at −20°C and thawed before use.

Characterization of Subcellular Fractions

Subcellular fractions were assayed for the following marker enzymes by standard procedures: cytochrome c oxidoreductase (Vesco & Giuditta, 1966), NADPH: cytochrome c reductase (Sottocasa et al., 1967); Na^+, K^+ - ATPase (Pommier et al., 1977). The intactness of outer mitochondrial membrane, routinely determined as cytochrome c oxidase latency, was 88.4 ± 1.6% (n = 8). Ethanolamine and serine base-exchange and CDPethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase activities were assayed in subcellular fractions as described (Corazzi et al., 1986; Corazzi, Pistolesi & Arienti, 1991; Roberti et al., 1989).

Labeling of Mitochondria with ^{14}C-PE Produced by Decarboxylation of ^{14}C-PS

Mitochondria were first labeled with ^{14}C-PS by incubating homogenate (about 25 mg protein) with 4 μCi of [3^{14}C]serine (specific radioactivity, 55 mCi/mmol) in a buffered solution containing 0.24 M sucrose, 40 mM Hepes (pH 8.0), 2.5 mM $CaCl_2$ (final volume, 4 ml). The reaction was carried out at 37°C for 30 min and stopped by adding 20 ml of cold S/H buffer. Labeled mitochondria were recovered from the homogenate by performing the centrifugation steps as described above. ^{14}C-PS loaded mitochondria (4 mg protein) were then incubated in the conditions for PS decarboxylase activity in presence of 0.1 M phosphate buffer (pH 7.0) and 3 mM EDTA, in a final volume of 2 ml. The reaction was performed at 37°C for 30 min and was stopped by adding hydroxylamine (1 mM final concentration).

Labeling of Mitochondria with ^{3}H-PE Imported from Endoplasmic Reticulum

Mitochondria were loaded with ^{3}H-PE by using homogenate or a mixture of microsomes and mitochondria. Homogenate (69 mg protein) was incubated with [1-^{3}H]ethanolamine (5 μCi, specific radioactivity, 30 Ci/mmol) in presence of 40 mM Hepes (pH 8.0) and 2.5 mM $CaCl_2$ in a final volume of 8 ml. The reaction was carried out for 30 min at 37°C and stopped by adding cold S/H buffer. Subcellular fractions were prepared as described. Alternatively, microsomes (8 mg protein) were incubated for 30 min at 37°C with [1-^{3}H]ethanolamine (5 μCi, specific radioactivity, 30 Ci/mmol) in the same conditions. Labeled

microsomes were recovered by sedimentation at 105,000 × *g* and resuspended in S/H buffer. Aliquots (0.6 mg protein, 22,900 dpm) were mixed with mitochondria (0.3 mg protein) and incubated in different experimental conditions (*see* Results). After the incubation, labeled mitochondria were recovered and purified by sucrose density gradient centrifugation.

Probing of Mitochondria with TNBS

Aliquots of ^{3}H-PE (26,600 dpm) or ^{14}C-PE (3,000 dpm) labeled mitochondria (0.76 mg protein, 0.5 ml) were mixed with 4 ml of 0.6 mM TNBS solution in 40 mM Hepes (pH 8.1), 25 mM KCl, 0.2 M sucrose for various times at 22°C; the reaction was stopped by adding 1 ml of saturated glycine solution (Corazzi et al., 1983).

Treatment of Mitochondria with Digitonin

For subfractionation of mitochondria we used a described procedure (Schnaitman & Greenawalt, 1968). Briefly, suspensions of pure ^{3}H-PE (35,000 dpm) or ^{14}C-PE (4,000 dpm) labeled mitochondria (1 mg protein, 0.3 ml) containing (in mM): 220 mannitol, 70 sucrose, 2 Hepes (pH 7.4) were mixed gently with a solution containing the desired amount of digitonin (0 to 0.4 mg, 0.3 ml). Mixtures were incubated in ice for 20 min with stirring. After incubation, the samples were centrifuged on a discontinuous sucrose gradient as described above. Mitoplasts, formed as a result of solubilization by digitonin of mitochondrial outer membrane, were recovered at 1.4–1.6 M sucrose interface.

Phospholipase C Treatment of Mitochondria Loaded with ^{3}H-PE

Aliquots of ^{3}H-PE-loaded mitochondria (35,000 dpm, 1 mg protein) resuspended in 20 mM Tris-HCl buffer (pH 7.4), 0.85% NaCl and 0.25 mM $CaCl_2$ were incubated with 0.005 units of phospholipase C from *B. cereus* in a final volume of 0.2 ml as described (Dominski et al., 1983). The reaction was stopped by the addition of 3 ml of chloroform/methanol (2:1, v/v) and the lipids were extracted and separated by TLC as described below.

Extraction and Analysis of Lipids

Lipids were extracted as described (Folch et al., 1957). Phospholipids were separated by two-dimensional TLC (6.5 × 6.5 cm; PE SIL G 250 μm, Whatman) with (i) chloroform/methanol/1.6 M ammonia (70:30:5, by volume) and (ii) chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5, by volume). The same chromatographic procedure was used to separate PE from trinitrophenyl-PE (TNPh-PE). Spots corresponding to lipids were visualized by exposure to I_2 vapors and identified with pure reference standards. Radioactivity was determined using a liquid scintillation counter (model TriCarb 1600CA; Packard, Chicago, IL) and Emulsifier Scintillator 299 (Packard) as the scintillation mixture. Protein was quantified as described (Lowry et al., 1951); phospholipid phosphorus was assayed after digestion with 70% perchloric acid (Bartlett, 1959).

Abbreviations

Hepes: 4-2-(2-hydroxyethyl)-1-piperazineethansulfonic acid; Thesit: dodecyl poly(ethylenglycolether)$_9$; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; CL: cardiolipin; TNBS: 2,4,6-trinitrobenzensulfonic acid; nsL-TP: nonspecific lipid transfer protein.

Results

Characterization of Subcellular Fractions

In our preparations, the level and distribution of marker enzyme activities of membranes were similar to those reported (Butler & Morell, 1983). The specific activity of cytochrome c oxidase (mitochondrial marker enzyme) was 5.7 times higher in mitochondria than in the homogenate. NADPH:cytochrome c reductase (microsomal marker enzyme), enriched in microsomal fraction, was found in MAM, but not in mitochondria and mitoplasts. Na^+,K^+-ATPase (marker enzyme for plasma membranes) was mainly found in P_1 pellet, although it was present at low level also in mitochondria, microsomes and MAM.

The highest value of phospholipid to protein ratio was found in microsomes (643.6 nmol/mg protein) and decreased in MAM (542.7), mitochondria (440.2) and mitoplasts (296.7). Cardiolipin (3.7% of total phospholipid) and PG (4.5%) were found in mitochondria, but not in microsomes and MAM. Microsomes and MAM contained more PS (11 and 12%) than mitochondria and mitoplasts (8.8 and 6.5%).

PS decarboxylase activity in mitochondria was 14 and 29 times higher than in microsomes and MAM. Serine and ethanolamine base-exchange in microsomes (specific activity of 8.7 and 12 nmol/h/mg protein) was 29 and 30 times higher than in purified mitochondria. A similar result was found for ethanolaminephosphotransferase (specific activity of 59 nmol/h/mg protein in microsomes). In MAM fraction, serine and ethanolamine base-exchange specific activities were 3.0 and 4.9 nmol/h/mg protein and ethanolaminephosphotransferase specific activity was 19.1 nmol/h/mg protein. These values were lower than in microsomes but 9–12 times higher than in mitochondria.

Synthesis, Translocation and Decarboxylation of PS

In this work, we loaded mitochondria with ^{14}C-PS by incubating homogenate with ^{14}C-serine. Total PS radioactivity in the homogenate was 176,540 dpm. After subfractionation 84,450 dpm were found on crude mitochondria. Only 46% of this radioactivity was recovered in purified mitochondria while a good extent of this extramitochondrial radioactivity was present in MAM. Incubation of purified mitochondria in a suitable medium for decarboxylase activity could transform about 41% of ^{14}C-PS into ^{14}C-PE.

Table 1. Distribution of phosphatidylethanolamine radioactivity after incubation of homogenate with [1-^{3}H]ethanolamine

	Protein content (mg)	Phosphatidylethanolamine (dpm/total fraction)
Homogenate	68.7	230,624
Microsome	8.5	52,619
Microsomal supernatant	29.8	11,060
Crude mitochondria	29.6	143,883
Mitochondria	10.9	48,177 (33%)
MAM	3.5	31,536 (22%)
Synaptosomes + myelin	14.0	56,356 (39%)

Rat brain homogenate (69 mg protein) was incubated with [1-^{3}H]ethanolamine (5 μCi, specific radioactivity, 30 Ci/mmol) for 30 min at 37°C in a buffered solution containing 40 mM Hepes (pH 8.0) and 2.5 mM $CaCl_2$. Subcellular fractions were prepared after stopping the reaction with cold S/H buffer. Lipids were extracted and phosphatidylethaholamine radioactivity was determined in each fraction. Data are expressed as dpm of phosphatidylethanolamine recovered in each fraction and are representative results from three such experiments.

Table 2. Transfer of phosphatidylethanolamine by mixing microsomes and mitochondria

	Phosphatidylethanolamine in mitochondria (dpm/mg protein)
Control	10,906 ± 1,520
2 mM Ca^{+2}	29,580 ± 1,640
Supernatant	12,204 ± 1,115
Supernatant + 2 mM Ca^{+2}	28,190 ± 3,097
nsL-TP	34,889 ± 2,320

Mitochondria (0.3 mg protein) were mixed with [^{3}H]-PE labeled microsomes (0.6 mg protein, 22,900 dpm, *see* Materials and Methods). Incubations were carried out for 30 min at 37°C in a buffered solution containing 40 mM Hepes (pH 7.4) and either nsL-TP (15 μg) or 2 mM Ca^{+2} or supernatant (0.5 mg protein) or Ca^{+2} plus supernatant. After the incubation mitochondria were separated from microsomes by centrifugation on sucrose density gradient. Lipids were extracted and phosphatidylethanolamine radioactivity was determined. Data are expressed as dpm/mg of mitochondrial protein and are means ±SD from three experiments.

In vitro Reconstitution of PE Translocation from Endoplasmic Reticulum to Mitochondria

Table 1 shows the distribution of PE radioactivity after incubation of homogenate with labeled ethanolamine. Crude mitochondrial fraction possesses 62% of total radioactivity of the homogenate. This radioactivity is distributed among mitochondria (33%), MAM (22%), myelin and synaptosomal membranes (39%).

To demonstrate that labeled PE flows from microsomes (donor particles) to mitochondria (acceptor particles), experiments mixing ^{3}H-PE-labeled microsomes and mitochondria were performed. Table 2 shows that PE import into mitochondria occurs mixing ^{3}H-PE-labeled microsomes and mitochondria. The specific activity of mitochondrial ^{3}H-PE increased when Ca^{+2} was added in the incubation medium. Protein from cell supernatant had no effect, whereas the nsL-TP purified from rat liver favored the translocation process. The addition of increasing amounts of MAM to a mixture of ^{3}H-PE-labeled microsomes and mitochondria did not influence the ^{3}H-PE import to mitochondria (Fig. 1, open symbols). On the other hand, the import of ^{3}H-PE to mitochondria was enhanced when experiments were performed mixing unlabeled microsomes, mitochondria and ^{3}H-ethanolamine, (Fig. 1, filled symbols). In control experiments, a flow of ^{3}H-PE was observed by mixing ^{3}H-PE-labeled MAM and mitochondria or MAM and mitochondria in presence of ^{3}H-ethanolamine (*data not shown*). Therefore the increase of ^{3}H-PE import into mitochondria noticed in Fig. 1 should be due to a contribution of ^{3}H-PE synthesized in MAM.

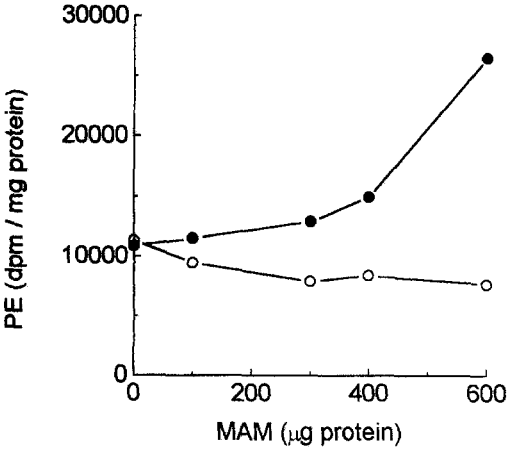

Fig. 1. Transfer of PE from microsomes to mitochondria. Involvement of MAM. In the first experiment mitochondria (0.3 mg protein) were incubated with ^{3}H-PE microsomes (22,900 dpm, 0.6 mg protein, *see* Materials and Methods) in presence of 40 mM Hepes (pH 7.4) and increasing amounts of MAM (○—○). In the second experiment mitochondria (0.3 mg protein) were incubated with unlabeled microsomes (0.6 mg protein) in presence of 40 mM Hepes (pH 8.0), 2.5 mM $CaCl_2$, [1-^{3}H]ethanolamine (0.5 μCi, specific radioactivity, 30 Ci/mmol) and increasing amounts of MAM, (●—●). After incubation for 30 min at 37°C, labeled mitochondria were recovered and analyzed for PE radioactivity.

Treatment of Mitochondria with TNBS

Figure 2 shows the time dependence of the reaction of membrane PE with 0.6 mM TNBS at 22°C. The mass of mitochondrial PE reacting with the probe increased with the time, but after a 50-min reaction time, it reached a

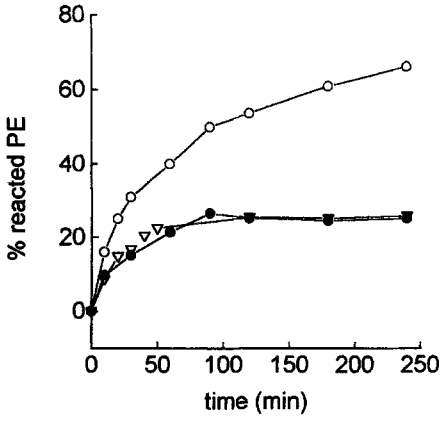

Fig. 2. Time dependence of TNPh-PE formation. Mitochondria were loaded with ^{14}C-PE formed by PS decarboxylase activity or with ^{3}H-PE imported from endoplasmic reticulum in presence of nsL-TP. Aliquots (0.76 mg protein) were incubated for the indicated time intervals at 22°C with 0.6 mM TNBS. Results are expressed as percent of total PE radioactivity (^{14}C-PE, ●—●; ^{3}H-PE, ○—○) or mass (▽—▽) transformed into TNPh-PE. Reacted plus unreacted PE was 3,000 ± 200 dpm of ^{14}C-PE; 26,000 ± 1,500 dpm of ^{3}H-PE and 112 ± 5 nmol in each sample.

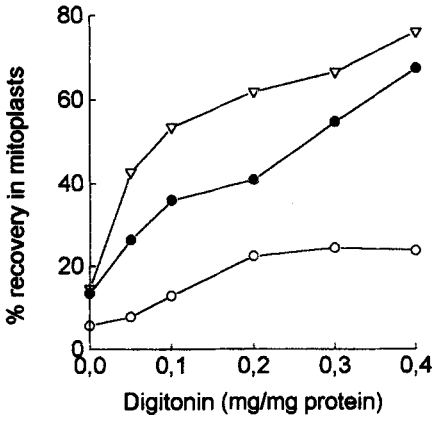

Fig. 3. Treatment of mitochondria with digitonin. Mitochondria were loaded with ^{14}C-PE through PS decarboxylase activity or with ^{3}H-PE imported from the endoplasmic reticulum in presence of nsL-TP. Aliquots (1 mg protein, 4,000 ± 260 dpm of ^{14}C-PE or 35,000 ± 1,900 dpm of ^{3}H-PE) were incubated for 20 min with the indicated amounts of digitonin. Mitoplasts were recovered by centrifugation on a discontinuous sucrose density gradient. Lipids were extracted and the radioactivity of ^{3}H-PE and ^{14}C-PE was determined. Results are expressed as percent of PE radioactivity (^{14}C-PE, ▽—▽; ^{3}H-PE, ○—○) and percent of protein recovered in mitoplasts (●—●).

plateau that never exceeded 25% of the total. On the contrary, ^{3}H-PE which had been previously imported in mitochondria from endoplasmic reticulum, reacted more quickly and more completely than did the bulk of PE present in mitochondrial membrane. Different reactivity was shown by TNBS towards ^{14}C-PE produced in mitochondria by decarboxylation of ^{14}C-PS. Indeed, no more than 25% of labeling was available for the probe, also after a very long reaction time (Fig. 2). The reaction of TNBS with PE mass or radioactivity was however complete after the addition of 0.3% Thesit (*results not shown*).

Subfractionation of Mitochondria by Digitonin Treatment

After treatment of mitochondria with 0.4 mg digitonin/mg mitochondrial protein, 68% of the protein was recovered in the mitoplast fraction (Fig. 3). Taking into account that mitoplasts contain about 85% of total mitochondrial protein (*unpublished results*), we can consider that about 80% of mitochondria were converted to mitoplasts.

When mitochondria loaded with ^{14}C-PE formed through PS decarboxylase activity were submitted to the gradual removal of the outer membrane, we observed an increase of ^{14}C-PE in mitoplasts (Fig. 3). Following the treatment of mitochondria with 0.4 mg digitonin per mg mitochondrial protein, 76% of ^{14}C-PE was recovered in mitoplasts. On the contrary, the digitonin treatment of mitochondria loaded with ^{3}H-PE imported from the endoplasmic reticulum left no more than 20% of ^{3}H-PE in mitoplasts (Fig. 3).

Treatment of Mitochondria with Phospholipase C

Incubation of ^{3}H-PE-loaded mitochondria with phospholipase C led to the hydrolysis of about 30% of labeled PE, but no more than 5% of PE mass in 15 min. Incubation times prolonged to 30 min produced a noticeable hydrolytic effect towards both labeled and unlabeled PE. At the same time, the percent of hydrolysis of total phospholipids increased noticeably and the latency of cytochrome oxidase activity was no longer retained (Fig. 4).

Discussion

The transport of newly synthesized PE from the endoplasmic reticulum to mitochondria has been examined by using labeled ethanolamine precursor and subcellular fractions (Yaffe & Kennedy, 1983). However, the factors influencing the transport phenomenon have not been studied. Ultrastructural studies performed by electron microscopy revealed a continuity between the endoplasmic reticulum and the outer mitochondrial membrane which originates in a cytostructure of a mitochondrial network held together by lamellae of endoplasmic reticulum (Katz et al., 1983). Endoplasmic reticulum associ-

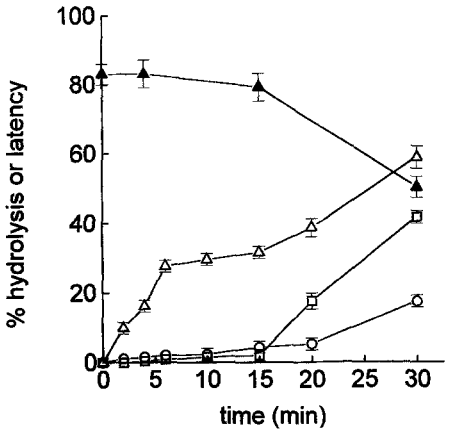

Fig. 4. Hydrolysis of mitochondrial phospholipids with phospholipase C. Mitochondria were loaded with ^{3}H-PE by incubation with ^{3}H-PE microsomes in presence of nsL-TP. Aliquots (1 mg protein, 35,000 ± 1,900 dpm) were treated with 0.005 units of phospholipase C from *B. Cereus* for different time intervals. Lipids were extracted and the radioactivity of ^{3}H-PE was determined. Results are expressed as percent of hydrolysis of PE (mass, ○—○; radioactivity, △—△) and as percent of hydrolysis of total phospholipids (□—□). In parallel experiments, the latency of cytochrome c oxidase of mitochondria during phospholipase C treatment was also determined (▲—▲). Values shown are mean ± SD.

ated with mitochondria has been isolated and characterized (Meier, Spycher & Meyer, 1981). More recently a membranous fraction from rat liver, possessing properties similar to the endoplasmic reticulum and associated with mitochondria, has been examined (Vance, 1990). These membranes seem to be involved in the linked synthesis of PS, PE and PC.

In our laboratory, mitochondria-associated membranes from brain tissue have been prepared. Their biochemical characterization indicates that they are a mixed population of membrane fragments largely originating from endoplasmic reticulum. The quantitative importance of phospholipid synthesizing enzymes in MAM can be deduced assuming the same yield of microsomes and MAM from the homogenate. We obtained 8.5 and 3.5 mg protein/brain of microsomes and MAM, respectively (Table 1). Considering the specific activities of serine and ethanolamine base-exchange and of CDPethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase in MAM and in microsomes (*see* Results), it can be calculated that these activities in MAM represent 12–14% of the total.

Incubation of homogenate with ^{3}H-ethanolamine results in the synthesis and distribution of ^{3}H-PE in different subcellular fractions (Table 1). The ^{3}H-PE labeling found in mitochondria is a consequence of importation from endoplasmic reticulum and MAM, since direct contribution of mitochondria to the synthesis of PE is very low. Import of PE in the mitochondria can occur in a reconstituted system made by mixing microsomes prelabeled with ^{3}H-PE and mitochondria (Table 2). In this experimental model, described for the import of labeled PS in liver mitochondria, translocation of phospholipid results from collision complexes formed between the endoplasmic reticulum and the outer mitochondrial membrane (Voelker, 1989). The import of labeled PE was found to be greatly enhanced by Ca^{+2}, which is known to stimulate aggregation or fusion of membranes (Corazzi, Pistolesi & Arienti, 1991), but was not influenced by the cytosolic fraction of rat brain. Mitochondria may be loaded in vitro with ^{3}H-PE by using a nsL-TP purified from rat liver (Table 2). The efforts to purify a nsL-TP from rat brain in our laboratory have been unsuccessful. However, the transfer of PE from endoplasmic reticulum to mitochondria has been observed in vivo (Butler & Morell, 1983). Since cytosolic cofactors or transport protein have not yet been found in the brain, the possible role of MAM has been examined in this work. In our experiments (Fig. 1), MAM seem not be to an intermediate station for ^{3}H-PE import into mitochondria, but they seem to have a role in the synthesis of ^{3}H-PE and in the direct transfer of the labeled phospholipid to mitochondria.

Import and decarboxylation of PS in mitochondria have been studied in liver (Vance, 1991) and in brain (Corazzi et al., 1993). In this work, the fate of PE imported from endoplasmic reticulum is compared with that of PE produced in mitochondria by decarboxylation of PS. TNBS reacts with a large pool of ^{3}H-PE imported from endoplasmic reticulum and with a restricted pool of ^{14}C-PE produced by decarboxylation of ^{14}C-PS in the inner mitochondrial membrane. At the same time, no more than 25% of PE mass reacts with the probe (Fig. 2). Considering that TNBS reacts poorly with PE of the inner mitochondrial membrane (*unpublished observations*), it is reasonable to argue that TNBS in mild conditions reacts preferentially with PE of the outer membrane (Corazzi et al., 1983). Consequently, we deduce that ^{3}H-PE imported from endoplasmic reticulum is mainly localized on the outer mitochondrial membrane. This indication is confirmed by the experiments performed with digitonin (Fig. 3). Indeed, the gradual removal of the outer mitochondrial membrane is followed by the formation of mitoplasts containing a low amount of labeled PE imported from endoplasmic reticulum and a large amount of labeled PE produced in mitochondria by decarboxylation of PS.

The treatment with phospholipase C of mitochondria loaded with ^{3}H-PE imported from endoplasmic reticulum, hydrolyzed 25–30% of labeled PE and only 4–5% of PE mass, corresponding to about 6–7 nmol/mg protein of total mitochondrial PE (140 ± 12 nmol/mg protein). In the same conditions, mitochondria retained their permeability properties, thus indicating that this enzyme hydrolyzes phospholipid localized on the outer surface of mitochondria (Fig. 4). Since the outer mitochondrial

membrane contains about 28 nmol of PE per mg of total mitochondrial protein (*unpublished results*), we can speculate that during the first period of incubation with phospholipase C about 25–30% of both mass and radioactivity of the outer membrane PE has reacted. This result indicates an asymmetric distribution of PE across the outer mitochondrial membrane, 25–30% of PE being on the outer leaflet. The same distribution was found in studies performed on intact liver mitochondria with fluorescent pyrene-PE species (Jasinska, Zborowski & Somerharju, 1993), whereas the distribution of PE across the outer mitochondrial membrane from liver mitochondria prepared by osmotic shock was found to be the opposite (Hovius et al., 1993).

The data presented in this paper indicate that the mitochondrial utilization of imported PE is mainly restricted to the assembly of the outer mitochondrial membrane, whereas the inner membrane seems to prefer the PE produced *in loco* by decarboxylation of imported PS (Carlini et al., 1993). These results differ from what has been found in the liver, where the PE newly formed from PS by decarboxylation flows directly back to the outer membrane, without mixing with the inner membrane PE (Hovius et al., 1992).

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