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Respiratory State and Phosphatidylserine Import in Brain Mitochondria In Vitro

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Abstract. The mechanism of phosphatidylserine (PS) movement from donor membranes into rat brain mitochondria was investigated. Mitochondria were incubated with liposomes and subjected to density gradient centrifugation. The energized state was monitored by flow cytometry measuring the fluorescence of membrane-potential-sensitive rhodamine-123 dye. Mitochondria density decreased upon increase of the respiratory rate, as a consequence of their association with liposomes. After interaction of mitochondria with ^{14}C-PS containing liposomes, ^{14}C-PS became a substrate of PS decarboxylase, as monitored by the formation of ^{14}C-phosphatidylethanolamine (PE), indicating translocation of ^{14}C-PS to the inner membrane. The kinetics of ^{14}C-PE formation showed a high rate upon addition of ADP, malate and pyruvate (state 3) compared to control (state 1). In state 3, ^{14}C-PE formation decreased in the presence of NaN_3. Mitochondria-associated membranes (MAM) are the major site of PS synthesis. However, their role in the translocation of PS to mitochondria has not been completely elucidated. A crude mitochondrial fraction (P_2) containing MAM, synaptosomes and myelin was prelabeled with ^{14}C-PS and incubated in different respiratory states. At a high respiratory rate, low-density labeled mitochondria, whose band overlaps that of synaptosomes, were obtained by centrifugation. A parallel decrease of both radioactivity and protein in MAM fraction was observed, indicating that the association of MAM and mitochondria had occurred. Synthesis and translocation of ^{14}C-PS in P_2 membranes were also studied by incubating P_2 with ^{14}C-serine. In the resting state ^{14}C-PS accumulated in MAM, indicating that the transfer to mitochondria was a limiting step. In state 3 both the transfer rate of ^{14}C-PS and its conversion to ^{14}C-PE increased. Respiratory mitochondrial activity modulated the association of MAM and mitochondria, triggering a mechanism that allowed the transport of PS across the outer mitochondrial membrane.

Key words: Phosphatidylserine translocation — Phosphatidylserine decarboxylase — Mitochondria and phosphatidylserine import

Introduction

Fluorescent lipid analogues and radiolabeled phospholipid precursors have been used to illustrate an intense intracellular phospholipid movement between membranes. This trafficking is a consequence of the biosynthetic origin of phospholipids which are mainly synthesized at the endoplasmic reticulum level, thus compelling newly formed molecules to move towards membranes for assembly or phospholipid remodeling.

Several models have been proposed in which lipids are translocated intracellularly between membranes (Trotter & Voelker, 1994): (i) transport through phospholipid exchange/transfer proteins, (ii) vesicle-mediated transfer whereby vesicles bud from the donor membrane and fuse with the acceptor membrane, (iii) flow of phospholipid through direct contact between two juxtaposed membranes.

Mitochondria do not have the capacity to synthesize all their phospholipids and therefore an active import of these molecules is necessary (Daum & Vance, 1997). Enzymatic decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE) by mitochondrial PS decarboxylase has been used as a tool for studying lipid movement between the endoplasmic reticulum and mitochondria. The main features of PS translocation can be summarized as follows: (i) a restricted pool of PS (i.e., the newly formed rather than pre-existing PS) is prefer-

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entially translocated to mitochondria (Bjerve, 1985; Vance, 1991; Corazzi et al., 1993), (ii) cytosolic factors, such as phospholipid transfer proteins, are not required (Voelker, 1989*a*, 1990; Van Heusden et al., 1990), (iii) a supply of ATP is necessary in intact cells (Voelker, 1985) but not in cell-free systems (Voelker, 1989*b*, Corazzi et al., 1993). In a study on PS synthesis in the endoplasmic reticulum and its transport in the Golgi/vacuole, a mutant of the yeast *Saccharomyces cerevisiae* that accumulates PS and diminishes PE formation despite normal PS decarboxylase 2 activity has been isolated. The lesion in PS metabolism is consistent with a defect in interorganelle lipid transport (Trotter et al., 1998).

Mitochondria-associated membranes (MAM) are candidates in the movement of newly synthesized PS to mitochondria (Vance, 1990). They should be considered as bridges where PS molecules are *en route* from the endoplasmic reticulum to mitochondria (Shiao, Lupo & Vance, 1995). In contrast, in the mitochondrial import of PE, MAM are not intermediate membranes for PE movement but appear to play a role in PE synthesis and transfer to mitochondria (Camici & Corazzi, 1995). Thus, membrane contact of MAM with the outer mitochondrial membrane could provide a mechanism for the transfer of phospholipid. In the yeast *Saccharomyces cerevisiae*, a protein localized on the MAM surface and on mitochondria appears to stimulate membrane association and translocation of PS towards the inner mitochondrial membrane (Tuller et al., 1998). A mitochondrial protein has also been claimed to be involved in the transfer of PS to liver mitochondria (Shiao, Balcerzak & Vance, 1998). Recently, a protein promoting the fusion of liposomes with brain mitochondria at acidic pH and belonging to the outer mitochondrial membrane has been purified in our laboratory (Camici & Corazzi, 1997). The finding that a mitochondrial factor is possibly involved in the import of phospholipids in a pH-dependent manner prompted us to hypothesize that the driving force for the association of MAM with mitochondria may reside in the mitochondrion itself.

Our data demonstrate that the amount of PS transferred from MAM to mitochondria correlates with the energized state of mitochondria and indicate that during respiration the association of MAM with mitochondria occurs, thus promoting a flow of PS to mitochondria.

Materials and Methods

Chemicals

L-[3-^{14}C] serine (specific radioactivity, 54 mCi/mmol) was supplied by Amersham Italia Srl. HEPES, NADPH, cytochrome c, ADP (K^+ salt), malic and pyruvic acid were purchased from Boehringer Biochemie. Prior to use, solutions of pyruvic and malic acid were brought to pH 7.0 with KOH. Ficoll 400 and Percoll were obtained from Pharmacia Biotech (Uppsala, Sweden). Rhodamine-123 was a Fluka Chemie product. Other chemicals, all of analytical grade, were purchased from Carlo Erba (Italy).

Preparation of Subcellular Fractions

Brains from CD rats (Charles River, approximately 200 g) were homogenized in 9 volumes of 0.32 M sucrose plus 2 mM HEPES (pH 7.0, S/H buffer) in a teflon-glass homogenizer. The homogenate was centrifuged twice at 1,500 × *g* for 10 min and the pellets were discarded. The supernatant was used as the homogenate or centrifuged at 8,000 × *g* for 30 min to prepare the P_2 pellet (crude mitochondrial fraction). The P_2 pellet was fractionated by centrifugation (75,000 × *g* for 90 min) on a discontinuous sucrose gradient formed in 5 ml bucket tubes (SW50.1 Beckman rotor) by successively layering 0.8 ml each of 1.6, 1.4, 1.2, 1.0 and 0.8 M sucrose. After centrifugation, highly purified 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 designated as MAM. Myelin membranes were found on top of the gradient whereas synaptosomes were localized at the 1.0/1.2 M sucrose interface. The 1.4/1.6 M sucrose interface was always free of mitoplasts, indicating that the integrity of the outer mitochondrial membrane was preserved during purification. Sucrose excess was removed by diluting subfractions with a solution of 2 mM HEPES (pH 7.0) and centrifuging for 20 min (72,000 × *g*, MAM; 30,000 × *g*, synaptosomes; 8,000 × *g*, mitochondria). The final pellets were resuspended in a proper amount of S/H buffer. Subfractions were also purified using Percoll (Sims, 1990) or Ficoll-400 (Lai & Rex Sheu, 1985) as gradient medium. Microsomes were prepared by centrifuging the supernatant of the P_2 pellet (Corazzi et al., 1986). Subcellular fractions were assayed for the following enzymes: lactate dehydrogenase (LDH) (Bradford, 1969), cytochrome c oxidase and NADPH: cytochrome c reductase (Corazzi et al., 1986), serine base-exchange enzyme and PS decarboxylase (Corazzi et al., 1993).

Preparation of Liposomes

^{14}C-PS labeled liposomes were prepared by incubating brain microsomes (15 mg protein) for 30 min at 37°C with [3-^{14}C]serine (4 μCi, specific radioactivity, 54 mCi/mmol), 2.5 mM $CaCl_2$ and 40 mM HEPES (pH 8.0). After incubation, ^{14}C-PS containing phospholipids were extracted and an aliquot was analyzed by TLC as described in analytical procedures. Only the spot corresponding to PS was labeled; no label was found in PE. The phospholipid extract was dried under nitrogen and resuspended in approximately 2 ml of S/H buffer. The suspension was sonicated with a MSE tip sonicator and aggregated material was pelleted by centrifugation at 100,000 × *g* for 20 min. Unlabeled liposomes were prepared as described, omitting incubation with ^{14}C-serine. Phospholipid composition was the following: 41% phosphatidylcholine; 30% phosphatidylethanolamine; 12% phosphatidylserine; 12% phosphatidylinositol + sphingomyelin; 5% others.

Interaction of Liposomes with Energized Mitochondria

(i) Purified and metabolically active mitochondria (0.3 ml, 0.4 mg protein) were mixed with liposomes (unlabeled, 0.3 ml, about 2 μmol lipid P) in the presence of 1 mM K-phosphate (pH 7.0). Respiratory activity of mitochondria was modulated with ADP (0.8 mM, state 2) or with pyruvate and malate (1.5 and 3.0 mM, respectively, state 4) or

ADP, pyruvate and malate (0.8, 1.5 and 3.0 mM, respectively, state 3, energized mitochondria). In the resting state (state 1) substrates were omitted. In selected experiments 3.0 mM NaN_3 or 0.5 mM KCN were added. The mixtures were incubated for 20 min at 37°C, centrifuged on a sucrose density gradient as described above and the mitochondria-containing band was recovered.

(ii) ^{14}C-PS labeled liposomes (2 μmol lipid P, 13 nCi) were mixed with mitochondria (1 mg protein) in the presence of 1 mM K-phosphate (pH 7.0, final volume 0.5 ml) and incubated for 30 min at 37°C at different respiratory rates (*see above*). Mixtures were incubated in PS decarboxylase conditions by adding 0.25 ml solution of 0.45 M Na-phosphate, 9 mM EDTA (pH 7.0). Aliquots of the incubation mixtures were taken at fixed times for the extraction of phospholipids and determination of labeled PE.

Incubation of P_2 in Different Respiratory States

(i) P_2 was first labeled with ^{14}C-PS by incubating homogenate (about 25 mg protein) with 4 μCi of [3-^{14}C] serine (specific radioactivity, 54 mCi/mmol) in a buffered solution containing 0.24 M sucrose, 40 mM HEPES (pH 8.0), (final volume 6 ml). The reaction was carried out at 37°C for 30 min, stopped by adding 20 ml of cold S/H buffer and the mixture was centrifuged at 8,000 × *g* for 30 min. Aliquots of labeled P_2 pellet in S/H buffer (about 3.5 mg protein, 5300 dpm) were incubated in medium containing 1 mM K-phosphate (pH 7.0) in the different respiratory states, as described above (final volume 0.8 ml). In some experiments ^{14}C-PS-loaded mitochondria, purified by centrifuging P_2 aliquots on sucrose density gradient, were incubated in PS decarboxylase conditions in the presence of respiratory substrates.

(ii) P_2 (about 3 mg protein) was incubated with 0.5 μCi of [3-^{14}C]serine (specific radioactivity, 54 mCi/mmol) in a solution containing 1 mM K-phosphate (pH 7.0) in the presence of respiratory substrates (*see above*) in a final volume of 0.8 ml. To avoid mitochondrial membrane potential dissipation (Gunter & Pfeiffer, 1990), in both incubations (i) and (ii) the synthesis of labeled PS was performed omitting calcium. After incubation for 30 min at 37°C in the presence of respiratory substrates, subfractions were separated on sucrose density gradient as described. The bands were recovered and protein content and PS radioactivity determined. In all experiments protein and ^{14}C-PS radioactivity recoveries were always higher than 95%.

Flow Cytometry Analysis and Oxygen Uptake

In flow cytometry studies, P_2 fraction, mitochondria or synaptosomes (about 0.3 mg protein) were incubated for 20 min at 37°C in the presence of 1 mM K-phosphate (pH 7.0), respiratory substrates and rhodamine-123 (1 μM). Samples were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a focused argon laser (power 400 mW). For excitation a 450–490 nm bandpass filter was used. To capture the green fluorescence of rhodamine a 510 nm dichroic mirror and a 520 nm longpass filter were used. Autofluorescence of the samples was tested without the dye and its variation, observed under different metabolic conditions, was negligible. The flow rate was set at 12 μl/min. Data were analyzed and stored with the use of a data management system (LYSYS software). The fluorescence was plotted on a logarithmic scale *vs.* the frequency of events. The mean value of the integral of green fluorescence (IGFL) was also evaluated (Petit et al., 1990).

Oxygen uptake by freshly prepared mitochondrial preparations was measured using a Clark-type oxygen electrode as described (Lai, Rex Sheu & Carlson, 1985). O_2 consumption during respiration (state 4) was measured by addition of pyruvate and malate, whereas phosphorylation (state 3) was measured after the addition of ADP.

Analytical Procedures

Lipids were extracted as described (Folch, Lees & Sloane Stanley, 1957). For the preparation of liposomes, neutral lipids, glycolipids and proteins were removed by column chromatography (Camici & Corazzi, 1995). Phospholipid composition was determined after separation of each lipid class 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 ^{14}C-PE from ^{14}C-PS. Spots corresponding to lipids were visualized by exposure to I_2 vapors and identified by pure reference standards. Radioactivity was determined using a liquid scintillation counter (model TriCarb 1600CA; Packard, Chicago, IL) and Emulsifier Safe Packard as the scintillation mixture. Protein was quantified as described (Bradford, 1976). Phospholipid phosphorus was assayed as previously reported (Camici & Corazzi, 1995).

Abbreviations

MAM, mitochondria-associated membranes; PS, phosphatidylserine; PE, phosphatidylethanolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; S/H buffer, sucrose-HEPES buffer (0.32 M sucrose + 2 mM HEPES, pH 7.0).

Results

Characterization of Subfractions

Biochemical parameters of subfractions are reported in Table 1. A feature distinguishing MAM of brain tissue from the bulk of endoplasmic reticulum was that NADPH:cyt c reductase activity in MAM was much lower than in microsomes, whereas PS base-exchange activity was significantly higher than in microsomes. These results are in agreement with those reported for liver MAM (Vance, 1990). The marker protein proposed for liver MAM, PE N-methyltransferase-2 (Cui et al., 1993), is not present in brain MAM. Among P_2 subfractions, myelin possessed the highest phospholipid/protein ratio and the marker myelin basic protein (Karthigasan et al., 1994). Synaptosomes showed the highest value of LDH (Bradford, 1969) whereas mitochondria were characterized by the highest cytochrome c oxidase activity.

Respiratory properties of mitochondria were studied in the presence of pyruvate and malate. Mitochondrial preparations were metabolically active with a respiratory control ratio (ratio of state 3 to state 4) in the range 5–6. This value is in agreement with data reported in the literature (Lai et al., 1985) and was not significantly influenced by the type of medium utilized in mitochondria purification.

Table 1. Biochemical characterization of subcellular fractions

	Microsomes	Myelin	MAM	Synaptosomes	Mitochondria
Lipid phosphorus[a]	720 ± 10	960 ± 15	570 ± 12	500 ± 11	400 ± 12
Cytochrome oxidase[b]	*n.d.*	*n.d.*	55 ± 12	290 ± 10	1350 ± 25
Lactate dehydrogenase[c]	238 ± 7	151 ± 10	170 ± 15	510 ± 11	197 ± 5
NADPH-cytochrome c reductase[d]	12.9 ± 1.5	*n.d.*	2.5 ± 0.9	*n.d.*	*n.d.*
PS synthase[e] (exchange enzyme)	1.7 ± 0.4	0.3 ± 0.1	2.6 ± 0.2	0.2 ± 0.1	0.13 ± 0.05
Myelin basic protein	*n.d.*	+	*n.d.*	*n.d.*	*n.d.*

Values represent the mean ± SD of at least four independent experiments. In units of: [a]nmol/mg protein, [b]nmol of oxidized cytochrome c/min/mg protein, [c]nmol of reduced NAD formed /min/mg protein, [d]nmol of reduced cytochrome c formed/min/mg protein, [e]nmol of PS synthesized/hour/mg protein; *n.d.*, not detectable

Changes in Fluorescence of Purified Mitochondria After Energization

Single-parameter fluorescence histograms of purified mitochondria stained with the membrane-potential-sensitive rhodamine-123 (Johnson, Walsh & Bo Chen, 1980) are shown in Fig. 1. Under these experimental conditions, autofluorescence of unstained mitochondria was very low. Fluorescence intensity was plotted on the *x* axis as a function of the relative particle number having that intensity on the *y* axis. Upon energization of mitochondria (state 3), a shift towards a higher fluorescence intensity was observed indicating an increase of membrane potential. The mean fluorescence elicited by mitochondria under different metabolic conditions is reported in Table 2. Energization by respiratory substrates such as pyruvate and malate resulted in a significant increase of the mean fluorescence of the dye bound to the organelles. A further increase in fluorescence was observed when ADP was added to the pyruvate/malate mixture. On the contrary, sodium azide and potassium cyanide decreased the membrane potential, as expected. When P_2 was analyzed, results similar to purified mitochondria were obtained. No significant changes in fluorescence were found in synaptosomes under the same experimental conditions.

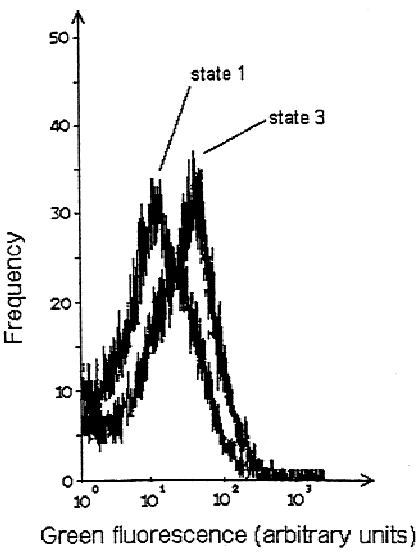

Fig. 1. Flow cytometry analysis of rhodamine-123 stained mitochondria: frequency histograms. Mitochondria (0.3 mg protein) were loaded with rhodamine-123 and incubated for 20 min at 37°C in different respiratory conditions (*see* Materials and Methods). The fluorescence increase in state 3 compared to state 1 is reported.

Interaction of Liposomes with Energized Mitochondria

In a first set of experiments, metabolically active mitochondria, whose energized state was previously monitored by flow-cytometry, were mixed with liposomes. After incubation, mixtures were subjected to density gradient centrifugation. A decrease in mitochondria density was observed that depended on the respiratory state. Mitochondria were recovered at 1.0/1.2 M or 0.8/1.0 M sucrose interface (states 2 and 4, respectively) or on top of the gradient (state 3, energized). Mitochondria incubated in a condition of high respiratory rate, but in the presence of sodium azide, were recovered at 1.0/1.2 M interface. Control mitochondria, incubated with or without liposomes, were recovered at 1.2/1.4 M sucrose interface in both states 1 (resting) and 3 (energized). The observed shift in density for the various conditions was quantitative. Respiratory properties of mitochondria were checked by measuring O_2 consumption. Upon incubation of mitochondria with liposomes the energy state of mitochondria was preserved. These results suggest that liposomes combine with the outer mitochondrial membranes, depending on mitochondrial metabolic activity.

To demonstrate that liposomes-mitochondria interaction was effective in the transfer of PS to the mitochondria, ^{14}C-PS containing liposomes were used in a

Table 2. Flow cytometry analysis of rhodamine-123 stained mitochondria. Changes in fluorescence under different metabolic conditions

Respiratory state	IGFL (AU)
1 (Resting)	20.6 ± 0.8
1 + NaN_3	15.1 ± 0.5
1 + CN^-	15.9 ± 0.4
2 (ADP)	24.2 ± 1.0
4 (Malate and pyruvate)	37.9 ± 1.5
3 (ADP, malate and pyruvate)	44.5 ± 1.8
3 + NaN_3	20.5 ± 0.6
3 + CN^-	28.6 ± 0.7

Mitochondria (0.3 mg protein) were loaded with rhodamine-123 (1 μM) and analyzed by flow cytometry after incubation for 20 min at 37°C in different respiratory states (*see* Materials and Methods). IGFL is the mean value of the integral of fluorescence. Data are expressed in fluorescence arbitrary units (AU). Values are the mean ± SD of three independent experiments.

second set of experiments. Mitochondria were incubated for 30 min with labeled liposomes in energized (state 3), resting (state 1) or deenergized (state 3 plus sodium azide) conditions. The mixtures were then supplemented with a suitable buffer for PS decarboxylase activity and ^{14}C-PE synthesis followed at different incubation times (Fig. 2). Since PS decarboxylase activity is segregated in the inner mitochondrial membrane, ^{14}C-PE formation is a chemical signal for the translocation of ^{14}C-PS towards the inner membrane. The kinetics of ^{14}C-PE formation showed a high rate upon addition of ADP, pyruvate and malate (state 3) compared to resting mitochondria (Fig. 2). In state 3, ^{14}C-PE formation rate decreased noticeably in the presence of sodium azide. PS decarboxylase, assayed by incubating ^{14}C-PS labeled liposomes and mitochondria in the presence of Triton X-100 (Butler & Morell, 1983), was not significantly affected by the addition of respiratory substrates or sodium azide (*results not shown*).

Interaction of MAM with Mitochondria Depends on the Respiratory Rate

The homogenate was incubated with ^{14}C-serine and then P_2 fraction labeled with ^{14}C-PS was prepared as described. P_2 was incubated in several respiratory conditions and subcellular fractions were thereafter separated by centrifugation on a sucrose gradient. A progressive increase of protein content in the synaptosomal band was measured by enhancing the respiratory rate from 1 (resting) to 2 (only ADP), to 4 (only pyruvate and malate) and to 3 (energized). At the same time a significant decrease of protein was observed in mitochondria, myelin and MAM. Table 3 reports data for resting, energized and deenergized mitochondria. The values of total cytochrome c oxidase activity recovered in mitochondrial

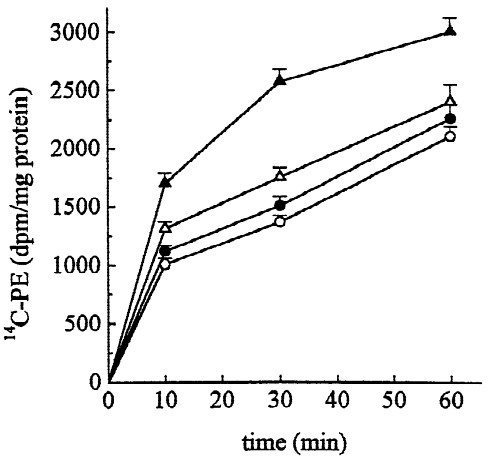

Fig. 2. Kinetics of ^{14}C-PE formation by PS decarboxylase activity. Energized (state 3) or resting (state 1) or deenergized (NaN_3) mitochondria (1 mg protein) were mixed with liposomes labeled with ^{14}C-PS (2 μmol lipid P, 13 nCi) in 1 mM K-phosphate (pH 7.0) in the presence of respiratory substrates and incubated for 30 min at 37°C. Mixtures were then supplemented with a suitable buffer for PS decarboxylase activity (*see* Materials and Methods). At fixed times, aliquots were taken for ^{14}C-PE radioactivity determination. (●----●, state 1; ○----○, state 1 + NaN_3; ▲----▲, state 3; △----△, state 3 + NaN_3). Values are the mean ± SD of four independent experiments.

Table 3. Protein distribution among P_2 subfractions (mg)

	Respiratory state		
	1 (Resting)	3 (Energized)	3 + NaN_3 (Deenergized)
Myelin	0.80 ± 0.02	0.52 ± 0.02	0.64 ± 0.02
MAM	0.56 ± 0.01	0.41 ± 0.01	0.55 ± 0.02
Synaptosomal band	1.08 ± 0.03	1.91 ± 0.06	1.53 ± 0.05
Mitochondria	1.05 ± 0.03	0.65 ± 0.02	0.78 ± 0.03

Aliquots of ^{14}C-PS prelabeled P_2 (3.5 mg protein, 5300 dpm) were incubated in the presence of 1 mM K-phosphate (pH 7.0) in different respiratory states for 30 min at 37°C. P_2 subfractions were then separated by centrifugation on sucrose density gradient (*see* Materials and Methods). Values are the mean ± SD of three independent experiments.

and synaptosomal bands demonstrate that a gradual shift of mitochondria from 1.2/1.4 M sucrose interface to 1.0/1.2 M sucrose interface had occurred by increasing the respiratory rate. At the same time the cytochrome c oxidase specific activity did not change in mitochondria but increased in the synaptosomal band (Table 4). Moreover, in parallel experiments, incubating unlabeled P_2 at high respiratory rate, the specific activity of PS synthase in the synaptosomal band almost doubled compared to nonenergized P_2. Therefore, low-density mitochondria, whose band overlaps that of synaptosomes, must be formed as a consequence of MAM-mitochondria asso-

Table 4. Cytochrome c oxidase activity in mitochondria and synaptosomal bands

Respiratory state	Mitochondria		Synaptosomal band	
	Specific activity	Total activity	Specific activity	Total activity
1 (Resting)	1350 ± 40	1323 ± 15	290 ± 10	293 ± 6
2 (ADP)	1280 ± 35	1242 ± 12	309 ± 12	423 ± 9
4 (Malate and pyruvate)	1350 ± 42	931 ± 10	432 ± 15	648 ± 10
3 (ADP, malate and pyruvate, energized)	1410 ± 31	846 ± 10	626 ± 30	1252 ± 12

Aliquots of ^{14}C-PS prelabeled P_2 (3.5 mg protein, 5300 dpm) were incubated in the presence of 1 mM K-phosphate (pH 7.0) in different respiratory states for 30 min at 37°C. P_2 subfractions were then separated by centrifugation on sucrose density gradient. Specific activity and total activity are expressed in units of nmol/min/mg protein and nmol/min of oxidized cytochrome c, respectively. Values are the mean ± SD of four independent determinations.

ciation. Distribution of ^{14}C-PS radioactivity among P_2 subfractions confirmed a substantial enhancement in radioactivity of the synaptosomal band when the respiratory rate increased (Fig. 3). Meanwhile, the radioactivity decreased in MAM and mitochondria bands. In state 3 about 38% of mitochondria (protein or ^{14}C-PS radioactivity, Table 3 and Fig. 3) were transferred to a lower-density synaptosomal band. At the same time 26% of proteins and 60% of PS radioactivity were lost from MAM. Table 5 reports data of ^{14}C-PS labelling in P_2 subfractions expressed as dpm/mg protein. In the resting state MAM presented the highest value; however, the value decreased noticeably when the respiratory rate was enhanced. This indicated a better transfer of ^{14}C-PS compared to the protein fraction of MAM. In state 3 ^{14}C-PS transfer decreased when the mitochondrial membrane potential was disturbed by adding NaN_3. The intactness of mitochondria following MAM-mitochondria association was evaluated by measuring cytochrome c oxidase activity. In all tested conditions the latency of cytochrome c oxidase was higher than 95%.

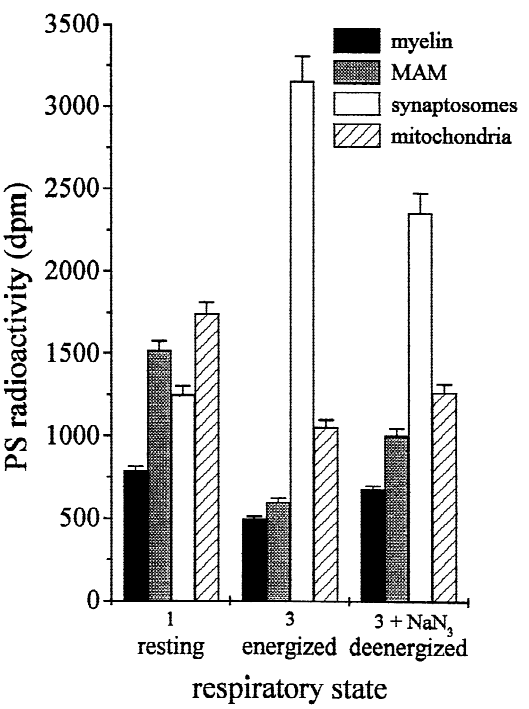

Fig. 3. ^{14}C-PS distribution among P_2 subfractions. Aliquots of ^{14}C-PS prelabeled P_2 (3.5 mg protein, 5300 dpm) were incubated in the presence of 1 mM K-phosphate (pH 7.0) in different respiratory states for 30 min at 37°C. P_2 subfractions were then separated by centrifugation on sucrose density gradient (*see* Materials and Methods). Values are the mean ± SD of four independent experiments.

PS Transfer, Not Synthesis, is a Rate-Limiting Step During Mitochondrial PS Import

Mitochondrial PS import can be divided into two main steps: first, the synthesis of PS and second, the movement of the newly synthesized phospholipid towards mitochondria. The two steps were studied by incubating P_2 with ^{14}C-serine in different respiratory states. PS synthesis was not influenced by respiratory substrates. ^{14}C-PS labelling was 1267 ± 60 and 1202 ± 55 dpm/mg protein in the nonenergized state (1) and energized state (3), respectively (values of three independent experiments), and was linear up to 30 min. In the resting state ^{14}C-PS accumulated in the synthesizing membrane (i.e., MAM) whose label was about 2700 dpm/mg protein and 2.0, 3.2 and 3.5 times higher than mitochondria, myelin and synaptosomes, respectively. This indicated that, in this condition, ^{14}C-PS transfer was a limiting step of the overall process. In state 3 the flow of ^{14}C-PS occurred at a higher rate, as shown by the decrease of PS labeling in MAM (about 1300 dpm/mg protein) and by the increase in the synaptosomal band (about 1020 dpm/mg protein). Taken together these results indicate that ^{14}C-PS synthesis and transport are not linked and the latter process is slower than the former. This finding is in agreement

with PS synthesis and translocation in CHO-K1 permeabilized cells (Voelker, 1990).

To study whether ^{14}C-PS became a substrate of PS decarboxylase, following MAM-mitochondria interaction, aliquots of the synaptosomal band, obtained as reported above and containing MAM-mitochondria structures, were incubated in PS decarboxylase conditions for different times. In both states 1 and 3 ^{14}C-PS became a substrate of PS decarboxylase. At all incubation times the labeling of ^{14}C-PE was higher in state 3 than in state 1 (*data not shown*) and paralleled the difference in specific radioactivity of ^{14}C-PS precursor, indicating that the relative amount of ^{14}C-PE formed in either state was similar.

Table 5. PS labeling in P_2 subfractions (dpm/mg protein)

	Respiratory state		
	1 (Resting)	3 (Energized)	3 + NaN_3 (Deenergized)
Myelin	981	954	1064
MAM	2728	1460	1825
Synaptosomal band	1152	1650	1534
Mitochondria	1658	1619	1620

The reported values were calculated from the data of ^{14}C-PS radioactivity (Fig. 3) and protein content (Table 3) of each fraction.

Discussion

The import of PS into mitochondria has been investigated in vitro, using a reconstituted system in which PS was translocated from donor membranes to mitochondria.

Mitochondria were prepared by centrifuging a P_2 pellet on sucrose density gradient. Although Ficoll-400 or Percoll are recommended as medium for mitochondria purification, we found sucrose a more suitable medium for gradients, since it permitted both mitochondria purification and MAM separation from myelin and synaptosomes.

Data reported in the literature indicate that the fusion of liposomes to biological membranes can be triggered either by divalent cations (Ekerdt, Dahl & Gratzl, 1981), acidic medium (Chernomordik et al., 1997) or fusogenic proteins (Arts et al., 1997). In this paper we report that mitochondria can capture liposomes without the addition of triggering agents, provided that mitochondrial respiration is permitted. The importance of an electrochemical gradient across the inner mitochondrial membrane for the capture of liposomes was demonstrated when the gradient collapsed with NaN_3.

Liposomal ^{14}C-PS can become a substrate of PS decarboxylase for the production of ^{14}C-PE. This means that ^{14}C-PS flows from the outer to the inner mitochondrial membranes through the internal contact sites (Ardail et al., 1990, 1993; Jasinska, Zborowski & Somerharju, 1993) suggesting that not a simple association between liposomes and mitochondria but mixing of lipidic phases must have occurred. The activity of PS decarboxylase is strictly dependent on the amount of substrate flowing towards the inner mitochondrial membrane (Voelker, 1989*b;* Ardail, Lerme & Louisot, 1991; Hovius et al., 1992). Since in metabolically active mitochondria a higher frequency of interactions between the inner and outer membranes occurs (Knoll & Brdiczka, 1983), the increase of ^{14}C-PE synthesis appears to be consistent with an increase in intramitochondrial transport of ^{14}C-PS.

Vance (1990) was the first to isolate MAM, membranous structures organized in the vicinity of mitochondria and possessing enzymes for the synthesis of phospholipids (Rusiñol et al., 1994). The localization of MAM around mitochondria, together with morphological evidence, led us to hypothesize that MAM could interact with mitochondria, depending on mitochondrial metabolic activity.

The role of cellular energetic charge on the intracellular movement of phospholipids has been extensively investigated in recent years. Poisoning of nonpermeabilized CHO-K1 or BHK-21 cells with fluoride, cyanide or sodium azide causes the accumulation of PS in the microsomal fraction (Voelker, 1985) or in MAM (Shiao et al., 1995). In permeabilized CHO-K1 cells, whose energetic state was modified by addition of exogenous ATP, interorganelle translocation of PS required ATP and was largely independent of soluble cytosolic proteins (Voelker, 1989*a*, 1990). Since the addition of ATP was not needed in a reconstituted system of rat liver mitochondria and microsomes (Voelker, 1989*b*), the conclusion was drawn that the ATP requirement in the mitochondrial import of PS precedes the events that are reconstructed with isolated organelles (Voelker, 1989*a*). However, incubation conditions were sometimes critical for the presence of Ca^{+2} and K^+ that, in the presence of extracellular ATP, are involved in dissipation of the mitochondrial membrane potential and cell death (Gunter & Pfeiffer, 1990; Zoeteweij et al., 1992).

It has been reported that the translocation of PS to mitochondria is a process restricted to autologous organelles, i.e., an endoplasmic reticulum-like fraction structurally linked to mitochondria (Voelker, 1993). P_2 contains autologous mitochondria and MAM, whose interaction is consistent with the respiratory state of mitochondria, as inferred by the amount of mitochondria and MAM shifted to the synaptosomal band (Table 3). In agreement with Voelker (1993), no interactions were observed when purified MAM and mitochondria were mixed (*results not shown*). This could be due to loss of specific contact points between MAM and mitochondria during purification. Although liposomes and MAM be-

have differently, in both cases a mitochondrial electrochemical gradient is necessary. MAM-mitochondria interaction promotes the preferential translocation of ^{14}C-PS to mitochondria (Table 5). This result is in agreement with data indicating that, although bulk PS is confined to the inner leaflet of membranes (Dominski et al., 1983), its synthesis should occur on the cytoplasmatic surface where it is easily exported (Vance, 1991; Corazzi et al., 1993). Also in baby hamster kidney cells, hydrophilic PS species are more readily transported to mitochondria than hydrophobic species (Heikinheimo & Somerharju, 1998).

PS synthesis takes place in MAM where transfer of the newly synthesized phospholipid to mitochondria occurs. When synthesis and transfer of PS were studied simultaneously, the respiratory rate influenced the transfer of PS from MAM to mitochondria but not PS synthesis. At high respiratory rate the nature of MAM-mitochondria interaction was such that MAM could not be separated from mitochondria by centrifugation on a sucrose gradient medium. The result that the mitochondrial outer membrane retains its impermeability to cytochrome c indicates membrane interaction which allows the flux of lipids to mitochondria.

In this work we demonstrated that the energy status of mitochondria might affect the amount of PS that translocates into mitochondria. However, the nature of the contact between MAM and the outer mitochondrial membrane and the identification of factor(s) involved in PS translocation are unsolved. Energy requirement for PS translocation may be due to the need of ATP for transbilayer movement of PS across the outer mitochondrial membrane. ATP-dependent P-glycoprotein membrane transporters of the ABC protein family have been described (Toti et al., 1997). However, mitochondria could provide energy not only through ATP but directly through the protonic gradient developed across the inner membrane during respiration. A mitochondrial membrane protein mediating the translocation of PS from liposomes to mitochondria by a fusion process was purified in our laboratory (Camici & Corazzi, 1997). Since fusogenic activity is triggered at acidic pH, the biological relevance of this protein could be emphasized only if an acidic medium is produced around it. We speculate that the driving force for the acquisition of lipids by mitochondria could reside in the mitochondrion itself, through a protein whose activity could be modulated by the acidic environment produced in the intermembrane space of the mitochondria during respiratory activity.

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