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Mechanism of the ATP-dependent phosphatidylserine synthesis in liver subcellular fractions

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It has been shown that the ATP-dependent incorporation of [^{14}C]serine into phosphatidylserine in rat liver mitochondrial and microsomal fractions is prevented by EGTA. On the other hand, at low (μM) Ca^{2+} concentrations, serine incorporation is strongly stimulated by ATP and Mg^{2+}. This stimulatory effect is reduced by calcium ionophore A23187. It is therefore suggested that the ATP-dependent process is that of serine base-exchange reaction, stimulated by endogenous Ca^{2+} accumulated inside the microsomal vesicles by Ca^{2+},Mg^{2+}-ATPase. The mitochondrial activity can be accounted for by contamination by the endoplasmic reticulum.

Phosphatidylserine; Base-exchange reaction; ATPase, Ca^{2+},Mg^{2+}-; (Endoplasmic reticulum)

1. INTRODUCTION

The synthesis of phosphatidylserine in animal tissues appears to occur solely by the base-exchange reaction. It requires Ca^{2+} and proceeds in the absence of any source of metabolic energy. A part of this reaction, ATP-dependent and Mg^{2+}-activated system for phosphatidylserine formation in animal cells has been described [1]. This process, first reported 30 years ago in rat liver mitochondria by Hübscher et al. [2,3], was later corroborated by other authors and found to occur in mitochondrial [4–6] and in microsomal [7,8] fractions. The requirement for ATP suggested that this might be a different process than that of serine base-exchange. The mechanism of this synthesis was, however, obscure. The previous investigation [6] led to the conclusion that none of the known pathways for phospholipid biosynthesis described so far could be applied in this case.

The present study was undertaken to elucidate this process. It appears that it occurs through the same mechanism as that of serine base-exchange and is stimulated by endogenous calcium ions accumulated inside the microsomal vesicles by Ca^{2+},Mg^{2+}-ATPase. Mitochondrial activity can be accounted for by contamination by the endoplasmic reticulum.

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2. MATERIALS AND METHODS

Liver of Wistar rats was homogenized in 9 vols of standard medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl (pH 7.4) and 0.5, 1.0 or 5.0 mM EGTA as indicated. Mitochondria and microsomes were isolated by a conventional procedure [9].

The base-exchange reaction was assayed according to Bjerve [10] by incubating the particles (1 mg protein) in the medium containing 60 mM imidazole buffer (pH 7.4), 20 mM sucrose, 4 mM $CaCl_2$, 1 mM dithiothreitol and L-[U-^{14}C]serine in a final volume of 0.25 ml (medium A).

ATP-dependent incorporation of L-[U-^{14}C]serine into phospholipids was assayed as described by Hübscher et al. [2,3], with slight modifications. The particles (1 mg protein) were incubated in the medium containing 60 mM KCl, 10 mM Tris-HCl (pH 7.4), 7 mM ATP, 6 mM $MgCl_2$, 2 mM CMP, 2 mM NaN_3, 1 mM *sn*-glycerol 3-phosphate, 0.3 mM sodium palmitate, 0.2 mM CoA-SH and L-[U-^{14}C]serine in a final volume of 0.25 ml (medium B).

All incubations were carried out at 37°C for 60 min. Incubations were terminated by the addition of methanol/chloroform

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(2:1, v/v). Lipids were extracted as described elsewhere [11]. Phospholipids were separated and identified by thin-layer chromatography on silica gel H in the system of chloroform/methanol/acetic acid/water (65:25:8:4, v/v). Spots were visualized using iodine vapour, ninhydrin and autoradiography.

Radioactivity was measured with a liquid scintillation spectrometer. Radioactivity incorporated in the control samples at zero time was subtracted from other samples in each experiment.

NADPH-cytochrome *c* reductase (EC 1.6.2.4) was used as a marker for the microsomal fraction and was measured as described by Sottocasa et al. [12].

Protein was determined by the biuret method [13].

L-[U-^{14}C]Serine was from Prague, Czechoslovakia.

3. RESULTS

Table 1 shows that in the absence of added Ca^{2+} a higher incorporation of L-[U-^{14}C]serine into rat liver microsomal phospholipids occurred at lower concentration of EGTA in the homogenization medium used for preparing the particles. Addition of EGTA into the incubation mixture not containing exogenous Ca^{2+} further decreased serine incorporation.

Table 1 also shows that not only the base-exchange reaction in microsomes but also the ATP-dependent process in mitochondria was prevented by inclusion of EGTA in the assay mixture. When mitochondria were prepared with 5 mM EGTA (expt 2), EGTA added to the incubation mixture strongly diminished serine incorporation into phospholipids. In the microsomal fraction of rat liver the incorporation of serine was also dependent on ATP (table 2). Addition of 1 or 5 mM EGTA into the incubation mixture containing ATP reduced the incorporation by 80 and 90% (not shown). These observations led to the conclusion that ATP-dependent serine incorporation proceeded, in fact, by the base-exchange mechanism in which endogenous Ca^{2+} was required. Mitochondrial activities could be correlated with the contamination of this fraction by the endoplasmic reticulum which amounted to 5–10% as determined by NADPH-cytochrome *c* reductase (not shown).

In order to examine the role of ATP in serine incorporation, microsomes were incubated with L-[U-^{14}C]serine and with or without ATP and other cofactors, as indicated (table 2). As it is seen, the incorporation was strongly diminished in the absence of ATP. The omission of *sn*-glycerol 3-phosphate and CMP had a much lower effect, and palmitoyl-CoA was unable to replace ATP, CoA-SH and palmitate. The same requirement concerning ATP (table 1), *sn*-glycerol 3-phosphate, palmitoyl-CoA and CMP (not shown) were found for mitochondrial serine incorporation. Thus, the requirement for ATP does not seem to be related to the activation of fatty acids and subsequently to the synthesis of phosphatidic acid.

It has to be noted that the L-serine concentration in the experiments shown in tables 2–4 was 10 times lower than that in table 1. Hence, the absolute amounts of serine incorporation per mg protein are about one order of magnitude lower, in line with a rather high K_m of the serine-exchange enzyme [14].

The exchange of L-[U-^{14}C]serine with microsomal phospholipids was performed either in high (KCl) or in low (sucrose) ionic strength medium, with or without ATP and Mg^{2+}, and with

Table 1

Effect of EGTA on the incorporation of serine into microsomal and mitochondrial phospholipids by the base-exchange and ATP-dependent process

Incubation system	Serine incorporation (pmol/mg protein)	
	Expt 1	Expt 2
Microsomes (medium A)		
complete system	1075	1205
$CaCl_2$ omitted	201	10
$CaCl_2$ omitted, 1 mM EGTA added	14	8
$CaCl_2$ omitted, 5 mM EGTA added	3	2
Mitochondria (medium A)		
complete system	66	78
Mitochondria (medium B)		
complete system	48	80
ATP omitted	8	10
1 mM EGTA added	23	8
5 mM EGTA added	17	2

The particles were prepared by homogenization in the medium containing 0.5 mM (expt 1) and 5 mM EGTA (expt 2). The microsomal and mitochondrial pellets were suspended without EGTA and incubated in medium A for the base-exchange reaction and in medium B for the ATP-dependent process, as described in section 2, with 50 μM L-[U-^{14}C]serine (spec. act. 20000 dpm/mg protein). Other indications were as indicated. Mean values of duplicate (expt 1) and triplicate (expt 2) samples are presented

Table 2

Cofactors requirements for the incorporation of serine into microsomal phospholipids by the ATP-dependent process

Incubation system	Serine incorporation (pmol/mg protein)	
	Expt 1	Expt 2
Complete system	10.4	13.8
ATP omitted	2.7	0.9
sn-Glycerol 3-phosphate omitted	8.1	9.7
ATP, CoA-SH, palmitate omitted, 0.2 mM palmitoyl-CoA added	2.3	0.7
CMP omitted	8.7	11.5

Microsomal membranes obtained in the presence of 1 mM EGTA were incubated in medium B (see section 2) with 5 μM L-[U-^{14}C]serine (spec. act. 200000 dpm/nmol)

varying amounts of $CaCl_2$. The microsomal system appeared to be sensitive to the ionic strength (table 3). A similar sensitivity of the rat brain serine-exchange enzyme was observed [15]. In both media, low concentrations of Ca^{2+} (μM), ATP and Mg^{2+} distinctly stimulated serine incorporation, whereas at higher Ca^{2+} concentrations (mM), ATP and Mg^{2+} had no influence on this process. Mg^{2+} alone was without effect on the incorporation of serine (not shown) but together with ATP significantly enhanced this process.

The data of table 4 show that addition of calcium ionophore A23187 reduced the stimulatory effect of ATP and Mg^{2+}. Again, this phenomenon occurred only at low Ca^{2+} concentrations and did not occur in the presence of 1 or 4 mM $CaCl_2$. In expt 2, the effect of calcium ionophore on the incorporation of serine into total microsomal phospholipids (a) and into phosphatidylserine (b) is compared. As is seen, the range of incorporation was similar and the effect of ATP, Mg^{2+} and ionophore was in both cases the same. This finding and the results of thin-layer chromatographic analysis, which showed that phosphatidylserine is the main phospholipid syn-

Table 3

Effect of varying $CaCl_2$ concentrations on the incorporation of serine into phospholipids of liver microsomes in the absence and presence of ATP and Mg^{2+}

$CaCl_2$	Serine incorporation (pmol/mg protein)			
	High ionic strength medium		Low ionic strength medium	
	Without ATP and Mg^{2+}	With ATP and Mg^{2+}	Without ATP and Mg^{2+}	With ATP and Mg^{2+}
Single experiment				
0	0.9	7.5	1.0	17.5
5 μM	0.8	6.8	1.3	23.8
10 μM	1.3	7.4	1.6	29.7
50 μM	2.2	10.7	5.2	37.9
100 μM	2.3	11.4	16.7	51.2
1 mM	20.9	17.8	51.9	81.4
4 mM	41.3	37.2	91.0	105.2
Mean for 5 experiments ± SD				
10 μM			1.1 ± 0.2	20.5 ± 5.0
100 μM			13.9 ± 2.7	39.4 ± 6.0
4 mM			123.0 ± 23.0	102.2 ± 22.0

Microsomes were prepared in the medium containing 1 mM EGTA. Microsomal membranes (1 mg protein) were incubated in the high ionic strength medium containing 60 mM KCl, 20 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 1 μg oligomycin, 5 μM L-[U-^{14}C]serine (spec. act. 200000 dpm/nmol) and with or without 7 mM ATP and 6 mM $MgCl_2$ in the final volume of 0.25 ml. Low ionic strength medium contained 60 mM imidazole buffer (pH 7.4) instead of 60 mM KCl and 10 mM Tris-HCl. The concentration of $CaCl_2$ was varied as shown

Table 4

Effect of calcium ionophore A23187 on the incorporation of serine into total microsomal phospholipids and phosphatidylserine

$CaCl_2$	Serine incorporation (pmol/mg protein)		
	No addition	ATP and Mg^{2+} added	ATP, Mg^{2+} and A23187 added
Expt 1			
0	0.8	8.9	0.9
5 μM	0.8	12.0	1.1
10 μM	0.9	20.7	1.5
50 μM	4.1	23.9	3.1
100 μM	10.0	36.0	10.6
1 mM	92.8	95.8	97.7
4 mM	123.5	112.3	122.5
Expt 2			
10 μM (a)	1.1	20.9	2.8
(b)	1.0	15.6	0.9
100 μM (a)	16.4	38.9	18.4
(b)	15.0	34.9	11.8
4 mM (a)	157.0	140.5	126.8
(b)	120.6	112.6	110.1

Microsomes were prepared and incubated in low ionic strength medium as described in table 3. 7 mM ATP, 6 mM $MgCl_2$ and 4 μg ionophore A23187/mg protein were added as indicated. The concentration of $CaCl_2$ was varied as shown. After incubation lipids were extracted and counted for radioactivity (expt 1). In expt 2, aliquots of the lipid extracts were counted for radioactivity (a) and separated by thin-layer chromatography. The spots of phosphatidylserine were visualized, scraped and counted for radioactivity (b)

thesized from serine in the endoplasmic reticulum (about 80%), demonstrated that the incorporation of serine into total phospholipids can be regarded as a measure of phosphatidylserine formation. In mitochondria L-[U-^{14}C]serine was incorporated mainly into phosphatidylserine and the product of its decarboxylation, phosphatidylethanolamine (not shown).

The stimulatory effect of ATP and Mg^{2+} on serine incorporation into mitochondrial phospholipids was also prevented by ionophore A23187. The particles were incubated with 100 μM $CaCl_2$ under conditions similar to those shown in table 4, except that 10 μM ruthenium red, an inhibitor of Ca^{2+} uptake into mitochondrial matrix, was added. In the presence of ATP and Mg^{2+}, the incorporation amounted to about 3 and 0.2 pmol/mg protein per h in the absence and presence of A23187, respectively. In the absence of ATP and Mg^{2+} the incorporation was 0.6 pmol/mg protein per h.

4. DISCUSSION

The present study clarifies the long puzzling problem of the so-called energy (ATP)-dependent phosphatidylserine synthesis in animal mitochondria. It shows that ATP and Mg^{2+} do not act as cofactors in some unknown phosphatidylserine biosynthetic route but points to the participation of the microsomal Ca^{2+},Mg^{2+}-ATPase in this process. The presence in the liver endoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase which can actively take up Ca^{2+} has been documented [16–18]. The system of this transport is similar to that in the sarcoplasmic reticulum and is characterized by the absolute requirement for ATP and Mg^{2+} [19]. It is therefore suggested that the ATP-dependent serine incorporation into phosphatidylserine is the serine base-exchange reaction. ATP in this process is utilized for Ca^{2+} accumulation inside the microsomal vesicles by Ca^{2+},Mg^{2+}-ATPase. Due to that, at low (μM) external Ca^{2+} concentrations the internal one reaches the level high enough to enable the base-exchange reaction. The fact that the serine base-exchange enzyme needs high (mM) concentration of Ca^{2+} for its activity is generally agreed upon [20].

The mitochondrial serine incorporation occurs in the presence of azide (inhibitor of mitochondrial ATPase, see table 1) and ruthenium red (inhibitor of mitochondrial Ca^{2+} influx). This indicates that this process observed in the mitochondrial fraction is virtually a microsomal one.

Besides ATP and Mg^{2+}, CoA-SH, *sn*-glycerol 3-phosphate and CMP are required for the optimal incorporation of serine [2–8]. These substances are known as cofactors or substrates (with the exception of CMP) for the synthesis of phosphatidic acid. In the light of the present data it can be suggested that phosphatidic acid may function as Ca^{2+} ionophore, facilitating Ca^{2+} uptake into the vesicles. Phosphatidate-mediated Ca^{2+} transport has been reported to proceed in liposomes [21], and has been suggested to occur in the plasma membrane [22]. The participation of CMP is more difficult to explain. One might speculate that CMP protects phosphatidic acid from its further metabolic conversion, mainly to

phosphatidylinositol, by stimulation of the reverse reaction [23].

The results of the present work allow to speculate that the serine base-exchange enzyme can react at the inner leaflet of the right-side oriented liver microsomes, although they do not exclude its availability to Ca^{2+} from the external leaflet. At low external Ca^{2+} concentrations, and in the presence of ATP and Mg^{2+}, only the active sites in the inner leaflet are saturated with Ca^{2+} and reactive, whereas at high external concentrations those on the cytoplasmic surface of the vesicles are also included. Topology experiments of Ballas and Bell [24] and Vance and Vance [14] suggest that important domains of the serine base-exchange enzyme are exposed to the cytoplasmic surface of hepatic microsomal vesicles. However, Ballas and Bell [24] found that serine base-exchange activity was not affected by mercury-dextran and pronase. Moreover, Vance and Vance [14] reported that after 10 min incubation with trypsin only about half (59%) of the enzyme activity was lost. In rat brain microsomes, serine base-exchange enzyme is claimed [25,26] to be located in their inner leaflet. Besides, Corazzi et al. [26] suggested that the enzyme activity may be present in both luminal and cytoplasmic compartments. This is what can be concluded from the present results.

Therefore, it can be supposed that in vivo serine base-exchange reaction occurs at the luminal leaflet of the endoplasmic reticulum and is regulated by Ca^{2+},Mg^{2+}-ATPase, since the cytosolic Ca^{2+} concentration is 1–2 μM [27] only, and the microsomal intravesicular steady-state concentration of 7–8 mM can be achieved by operation of Ca^{2+},Mg^{2+}-ATPase [16].

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