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Minireview

ATP-dependent phosphatidylserine formation in animal cells as a base exchange reaction*

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In living organisms there are two different pathways for the biosynthesis of phosphatidylserine [1]. The first is typical for prokaryotes and was discovered in 1964 by Kanfer & Kennedy [2]. The authors indicated that the synthesis of PS[1] in a soluble fraction from *Escherichia coli* could occur in the presence of CDP-dipalmitin and L-serine with the release of CMP (Fig. 1A). PS formed in this reaction is easily decarboxylated to phosphatidylethanolamine. CTP-dependent PS biosynthesis is the only mechanism known so far that provides a net synthesis of PS.

The second pathway is a base exchange reaction in which serine or other aminoalcohol, such as choline or ethanolamine, is directly incorporated into its corresponding phospholipid (Fig. 1B). In general, this pathway for PS synthesis is typical for eukaryotic organisms. The synthesis of PS in animal tissues appears to occur solely by this reaction [3]. However, this process does not result in net synthesis of phospholipids, and proceeds in the absence of metabolic energy (for review see [1, 3]).

Besides the CTP-dependent bacterial pathway and the base exchange reaction, a third system for PS formation has also been demonstrated in animal cells [4, 5]. In this system, incorporation of L-serine into PS is dependent on the presence of ATP and Mg^{2+}. However, until recently the mechanism of this process was not known [1]. In 1989, we suggested that the ATP-dependent PS formation is in fact the base exchange reaction [6]. Further studies from this laboratory [7, 8] have confirmed this conclusion, and suggested the physiological significance of this process.

The aim of this article is to summarize the data on the ATP-dependent PS synthesis and to propose a possible mechanism for regulation of this process in animal cells.

General properties of the serine base exchange reaction

The base exchange reaction is involved in exchange of serine, choline and ethanolamine with the base moiety of preexisting phospholipids. This reaction does not bring about a *de novo* synthesis of phospholipids but is involved in the modification of their molecular structures [1, 3]. It is characterized by a requirement for Ca^{2+}, and by alkaline pH optimum [3]. The optimal Ca^{2+} concentrations required for the base exchange activities were found to be inversely related to the pH of the incubation mixture [9, 10].

Borkenhagen *et al.* [11] provided evidence that biosynthesis of PS in homogenates of rat liver occurs by the exchange reaction between

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[1]Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine

L-serine and PE (Fig.1B). This reaction proceeds in the absence of metabolic energy and is characterized by requirement for high (2 - 25 mM) Ca^{2+} concentrations.

This enzyme, purified 37 fold, catalysed the reaction between L-serine and PE, which was found to be the most effective substrate. The authors also reported that the enzyme does not

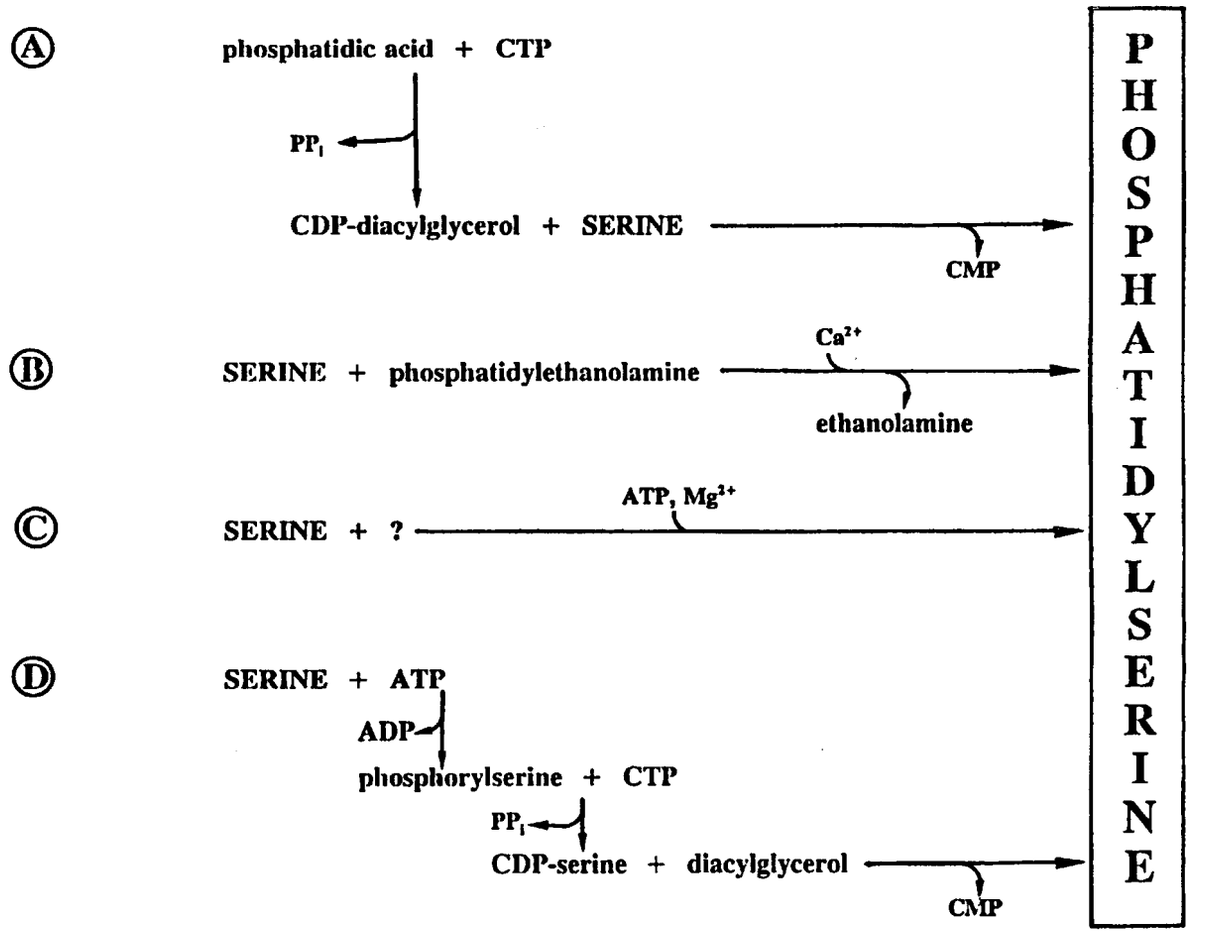

Fig. 1. Possible and speculative pathways for the incorporation of serine into phosphatidylserine (from [25], modified).

On the basis of chase experiments in rat liver microsomes, Bjerve [9] suggested that PC, PE and PS can all act as lipid substrates in the base exchange reactions. Saito & Kanfer [12] reported that the incorporation of radioactive serine into rat brain phospholipids is stimulated by both PE and PC.

From these data a question arose as to whether a single enzyme or separate enzymes are responsible for the exchange of these three bases. Kinetic studies, which demonstrated differences in kinetic constants and in optimal Ca^{2+} concentrations for these bases, have suggested that separate enzymes are involved. Moreover, Taki & Kanfer [13] were able to separate the L-serine exchange enzyme from the ethanolamine and choline exchange activities.

convert PE and PC to phosphatidic acid. This result provided evidence that the base exchange reaction was not associated with phospholipase D activity [13]. Later, Suzuki & Kanfer [14] purified to near homogeneity the base exchange enzyme of rat brain microsomes. This enzyme catalyzes the incorporation of both ethanolamine and serine into their corresponding phospholipids. The pH optimum was the same (7.0) with both substrates, and the optimum Ca^{2+} concentration was 8 mM for serine incorporation.

Studies on the subcellular distribution of the base exchange enzymes have indicated that they are located predominantly in microsomes [9, 15, 16]. In rat brain microsomes, the choline base exchange enzyme is claimed to be lo-

calized on the cytoplasmic side, whereas serine and ethanolamine base exchange enzymes are preferentially located on the luminal surface of the microsomes [17 - 19]. On the other hand, topology experiments on hepatic microsomal vesicles suggested that important domains of the serine base exchange enzyme are exposed on the outer surface of microsomal membranes [16, 20]. However, the authors found that after treatment with trypsin only about half of the enzyme activity was lost [20], and its activity was not affected by mercury-dextran and pronase [16].

ATP-dependent phosphatidylserine formation: historical survey

Thirty five years ago, Hübscher *et al.* [4, 5] presented evidence that serine can be incorporated into rat liver mitochondrial phospholipids by an ATP-dependent and Mg^{2+} activated mechanism. For the optimal incorporation CoA-SH, *sn*-glycerol 3-phosphate and CMP were also required. This evidence was later corroborated by other authors [21 - 25]. It was found to occur in mitochondrial and microsomal fractions of various animal tissues, but its mechanism remained unclear [1] (Fig. 1C).

The requirement for ATP and Mg^{2+} contrasted with the Ca^{2+} dependent incorporation of serine by the base exchange reaction (Fig. 1B) and suggested that this might be a different process. The stimulation by ATP and CMP allowed us to suppose that perhaps the pathway described for bacteria with CDP-diacylglycerol as intermediate (Fig. 1A) is also operating in animal mitochondria [25]. However, we showed that CDP-diacylglycerol added externally had no stimulatory effect on serine incorporation into Ehrlich ascites mitochondrial phospholipids. Also, ^{32}P from [^{32}P]phosphatidic acid was not incorporated into PS in the presence of CTP and L-serine. Similarly, the release of [^{14}C]CMP from [^{14}C]CDP-diacylglycerol was promoted by *sn*-glycerol 3-phosphate but not by L-serine. Therefore, the bacterial pathway for PS formation (Fig. 1A) seemed unlikely [25].

The other possibility was the proposal that serine is incorporated into PS in a way similar to that described for the net synthesis of PC and PE; that is, *via* CDP-serine as an intermediate (Fig. 1D). This possibility was also ruled out since no radioactivity from [β,γ-^{32}P]ATP was recovered in PS [25].

It was therefore concluded that the ATP-dependent process did not correspond to any of the known pathways of PS biosynthesis. It was also suggested that one of the possibilities to be considered may be the stimulation by ATP of the base exchange reaction [25].

To explain this ATP-dependent "energy-requiring" system, Pullarkat *et al.* [26] performed experiments on rat brain microsomal fraction and reported that pyrophosphatidic acid might be an intermediate precursor of PS biosynthesis in an Ni^{2+}- and ATP-requiring pathway. However, this proposal has never been confirmed by other authors.

Another explanation of the ATP-dependent PS formation was proposed by Infante [27]. He postulated that the "storage" form of PS in the membrane reacts with CMP to give CDP-serine. Subsequently, glycerophosphorylserine is formed and is acylated with specific fatty acids. Infante suggested that this pathway explains all the literature data for the incorporation of L-serine into PS stimulated by ATP, Mg^{2+}, *sn*-glycerol 3-phosphate, CoA-SH and CMP. However, even if this pathway could occur [28], it would explain only the requirement for the above reactants, but not the incorporation of labelled serine into phospholipids.

Mechanism of the ATP-dependent phosphatidylserine formation

As presented above, the mechanism of this so called energy(ATP)-dependent PS synthesis was for a long time obscure. The studies from this laboratory [6 - 8] clarified, however, this puzzling problem. Namely, it has been shown [6] that in the absence of added Ca^{2+} the incorporation of L-serine into total phospholipids was prevented by EGTA, both in the case of the base exchange reaction occurring in rat liver microsomes, and in the case of the ATP-dependent process which was examined in mitochondria (Table 1). When the microsomes and mitochondria were prepared with the higher concentration of EGTA (5 mM) in the homogenization medium, a further addition of EGTA to the incubation mixture strongly diminished serine incorporation. In the microsomes the incorporation of serine was also dependent on ATP. Addition of 1 or 5 mM EGTA

Table 1

Effect of EGTA on the incorporation of serine into microsomal and mitochondrial phospholipids by the base exchange reaction and ATP-dependent process (modified from [6])

Incubation system	[^{14}C]Serine incorporation (pmol/mg protein) Homogenized medium contained:	
	0.5 mM EGTA	5 mM EGTA
Medium for the base exchange reaction:		
MICROSOMES		
Complete system (+ $CaCl_2$)	1075	1205
$CaCl_2$ omitted	201	10
$CaCl_2$ + 1 mM EGTA	14	8
$CaCl_2$ + 5 mM EGTA	3	2
MITOCHONDRIA		
Complete system (+ $CaCl_2$)	66	78
Medium for the ATP-dependent process:		
MITOCHONDRIA		
Complete system (+ ATP)	48	80
ATP omitted	8	10
+ ATP + 1 mM EGTA	23	8
+ ATP + 5 mM EGTA	17	2

to the incubation mixture containing ATP reduced the incorporation of serine into microsomal phospholipids by 80 and 90%, respectively (not shown).

This observation led us to the conclusion that the ATP-dependent serine incorporation is in fact the base exchange reaction, in which endogenous calcium ions are required [6]. 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.

When rat liver microsomes were incubated at low (μM) concentrations of Ca^{2+}, ATP and Mg^{2+} distinctly stimulated PS formation, whereas at high (mM) Ca^{2+} concentrations ATP and Mg^{2+} had no influence on this process. This stimulatory effect was completely reduced by calcium ionophore A23187 [6] (Table 2). Moreover, the inhibitory effect of the ionophore on PS synthesis was also manifested in glioma C6 cells [7]. The inhibition of PS synthesis by the ionophore A23187 occurred in Ca^{2+}-free medium and at low calcium concentration (100 μM), whereas at high calcium concentration (10 mM) the ionophore distinctly stimulated this process (Fig. 2). PS synthesis was also inhibited by thapsigargin, a potent and specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, in both the presence and absence of external calcium, and only at 10 mM $CaCl_2$ this effect was not so apparent (Fig. 2).

The ionophore A23187 completely permeabilizes vesicular membranes for Ca^{2+}. Therefore, when the cells are incubated at low $CaCl_2$ concentration (100 μM), the addition of the ionophore increases the level of calcium in the cytosol to the same value, which is a thousand times higher than that in the resting cell [7]. Thus, the inhibition of PS synthesis found in these conditions can be only explained by the Ca^{2+}-depletion of the endoplasmic reticulum, where high (mM) concentrations of Ca^{2+} can be achieved. On the other hand, thapsigargin acts on the endoplasmic reticulum Ca^{2+}-ATPase by inhibition of both Ca^{2+}- and ATP- binding [29], and therefore prevents reuptake of Ca^{2+} into the lumen of these structures [30]. The presence of Ca^{2+}-ATPase in the endoplasmic reticulum

Table 2

Effect of calcium ionophore A23187 on the incorporation of serine into phospholipids of rat liver microsomes at varying Ca^{2+} concentration (modified from [6])

$CaCl_2$	[^{14}C]Serine incorporation (pmol/mg protein)		
	No additions	ATP and Mg^{2+} added	ATP, Mg^{2+} and A23187 added
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

of various tissues has been well documented [31 - 36]. The enzyme is characterized by an absolute requirement for ATP and Mg^{2+}. It binds Ca^{2+} and ATP-Mg from the medium outside the endoplasmic reticulum vesicles, and releases bound Ca^{2+} into the lumen upon utilization of ATP [33]. Recently, we have also found that thapsigargin strongly diminished (up to 90%) PS synthesis in isolated rat liver microsomal fraction [8].

It has therefore been suggested that ATP-dependent PS formation occurs by the serine base exchange reaction stimulated by endogenous Ca^{2+} accumulated inside the microsomal vesicles by Ca^{2+}-ATPase [6 - 8]. In this process, ATP and Mg^{2+} do not act as cofactors of some unknown PS biosynthetic route but, rather, are required for the activity of the Ca^{2+}-dependent, Mg^{2+}-stimulated ATPase. Therefore, at low extravesicular Ca^{2+} concentrations, the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum reaches levels high enough to enable the serine base exchange reaction.

Since in mitochondria incorporation of L-serine occurred in the presence of azide (inhibitor of mitochondrial ATPase) and ruthenium red (inhibitor of mitochondrial Ca^{2+} influx), its activity can be accounted for by contamination by the endoplasmic reticulum [6]. Thus, ATP-dependent PS formation can be considered virtually as a microsomal process.

Serine incorporation was previously described to be stimulated not only by ATP and Mg^{2+}, but also by CoASH, *sn*-glycerol 3-phosphate and CMP [4, 5, 21 - 25]. These compounds are known as cofactors for the synthesis of phosphatidic acid. One might speculate that phosphatidic acid may function as a Ca^{2+} ionophore in facilitating Ca^{2+} uptake into the endoplasmic reticulum. Such a role for this phospholipid in liposomes [37], and plasma membranes [38] has been suggested.

Physiological consequences of the ATP-dependent phosphatidylserine formation as a base exchange reaction and its regulation in the cell

An important consequence of the discovery that the ATP-dependent pathway for PS formation in animal cells is a base exchange reaction, in which ATP is utilized for the accumulation of Ca^{2+} inside the microsomes by Ca^{2+}-ATPase, is that this reaction should proceed inside these structures. In spite of controversial *in vitro* data on the distribution of the serine base exchange enzyme in the endoplasmic reticulum of rat liver [16, 20], and brain [18, 19], the *in vivo* data allow to assume that this enzyme is located within these intracellular Ca^{2+} stores. Specifically, it is well known that the physiological free Ca^{2+} concentration in cytosol of animal cells is very low (from 10^{-7} M in a resting cell to 10^{-6} M in a stimulated cell), whereas Ca^{2+}-ATPase maintains a high (mM) Ca^{2+} level in the lumen of the endoplasmic reticulum [34, 36, 39, 40]. The fact that the serine base exchange enzyme, both in the membrane preparations and in the purified form, needs high (mM) concentration of Ca^{2+} for its activity is generally agreed upon [3, 12, 14].

Such luminal localization of the serine base exchange enzyme was reported for rat brain

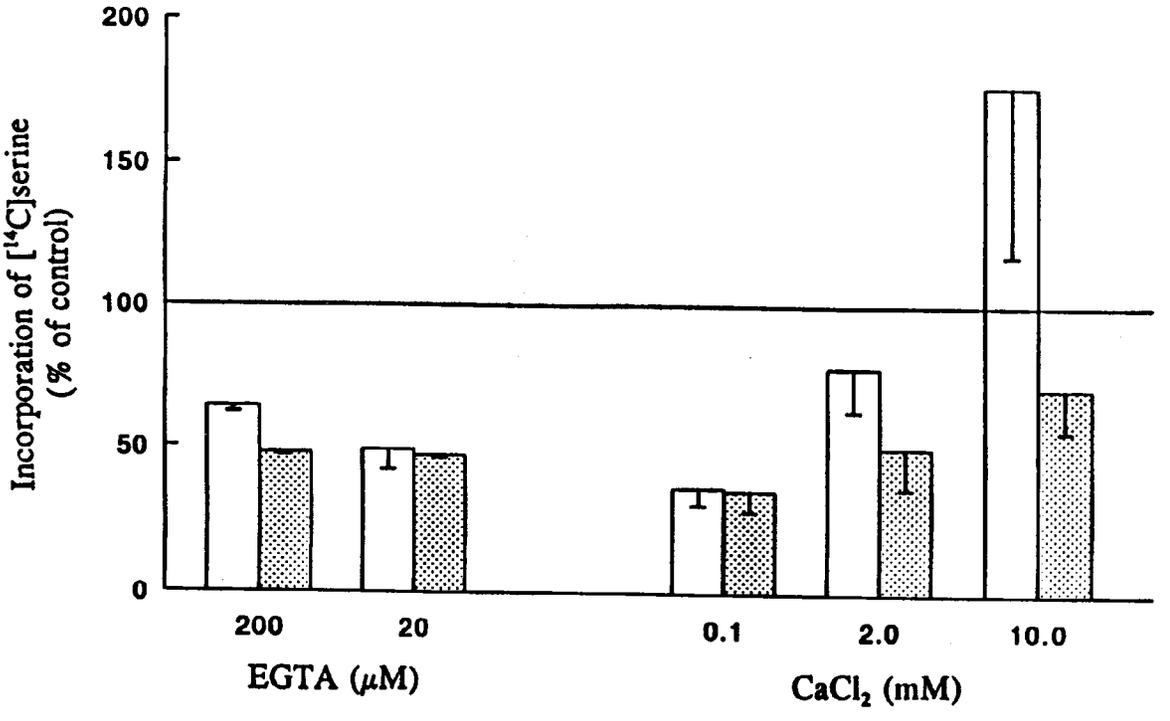

Fig. 2. Effect of calcium ionophore A23187 and thapsigargin on phosphatidylserine formation in glioma C6 cells.

Cells were incubated for 15 min with [^{14}C]serine in the absence (control) or presence of 5 μM A23187 □ or 0.1 μM thapsigargin ▨ and at varying concentration of $CaCl_2$ as indicated. The values obtained when using untreated cells were taken as 100% (basal line, control) (modified from [7]).

microsomes [18, 19] and postulated by us for rat liver microsomes [6, 8]. Moreover, the results on glioma C6 cells led us to the same conclusion [7]. Similar results on Jurkat T lymphocytes, indicating that the inhibition of PS synthesis was caused by Ca^{2+}-depletion of the endoplasmic reticulum by the action of the ionophore and thapsigargin, were recently reported by Pelassy *et al.* [41].

The other physiological consequence of the fact that PS synthesis is dependent on Ca^{2+} and occurs on the luminal side of the endoplasmic reticulum is that this process must be regulated by Ca^{2+}-ATPase. The inhibitory effect of thapsigargin on PS synthesis in glioma C6 cells [7], Jurkat T lymphocytes [41] and isolated rat liver microsomes [8] supports this statement.

The Ca^{2+} sequestration in the endoplasmic reticulum reflects the balance between calcium uptake and Ca^{2+} release through Ca^{2+} channels. Therefore, PS synthesis could be also regulated by the events responsible for Ca^{2+} release from these structures. In glioma C6 cells, neurotransmitters, glutamate and acetylcholine, cause Ca^{2+} release from the endoplasmic reticulum *via* $InsP_3$ and decrease PS synthesis [7]. More recently, we demonstrated that the compounds known to modulate Ca^{2+} receptor channels, such as caffeine and heparin, can also affect the synthesis of PS in rat liver microsomes [8]. As it is known, caffeine causes the release of Ca^{2+} through ryanodine receptor channels, and heparin inhibits Ca^{2+} release by $InsP_3$ receptor channel [40, 42]. The presence of ryanodine- and $InsP_3$- binding sites in rat liver microsomes was recently reported [43, 44]. Since PS formation in microsomal vesicles loaded with Ca^{2+} was enhanced by heparin and diminished by caffeine, these results also pointed to the luminal localization of the serine base exchange reaction [8].

Thus, it seems that PS synthesis can be regulated by intracellular events responsible for calcium fluxes in the cell, and therefore can be under the control of transmembrane signalling systems. It should be noted that the synthesis of this phospholipid can also be regulated by phosphorylation and dephosphorylation pro-

cesses [45], and is one of the earliest cellular responses to mitogenic stimuli [46]. PS dependence of protein kinase C is well established [47]; moreover, PS can also play an essential role in T lymphocyte cells activation, monitored by the measurement of interleukin-2 synthesis [48]. In conclusion, the presented data suggest that the synthesis of PS may be metabolically regulated and that this phospholipid may have a role in mediation of signal transduction in the cell.

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