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EFFECT OF THE IONOPHORE A23187, THAPSIGARGIN, CAFFEINE AND HEPARIN ON PHOSPHATIDYLSERINE SYNTHESIS IN RAT LIVER MICROSOMAL FRACTION

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Summary: It has been shown that the incorporation of [^{14}C]serine into phosphatidylserine in rat liver microsomal fractions is distinctly stimulated by ATP and Mg^{2+} at low (10 μM) Ca^{2+} concentration. This stimulation occurred in untreated microsomes, but did not occur in fully disrupted microsomal vesicles. The stimulatory effect of ATP and Mg^{2+} on phosphatidylserine synthesis was reduced by calcium ionophore A23187 and by thapsigargin, which is a specific blocker of Ca^{2+}-ATPase. It was also diminished by caffeine and enhanced by heparin, agents known to modulate Ca^{2+} release by receptor channels. It is therefore postulated that the synthesis of phosphatidylserine occurs on the luminal side of the endoplasmic reticulum and can be regulated by events responsible for Ca^{2+} release and entry into these structures.

In animal tissues phosphatidylserine (PS) is believed to be solely synthesized by the base exchange reaction that occurs mainly in the endoplasmic reticulum and needs high (mM) concentrations of Ca^{2+} for its activity [1,2]. Our previous study [3] on rat liver microsomes showed that at low (μM) Ca^{2+} concentrations serine incorporation into PS was strongly stimulated by ATP and Mg^{2+}, and this stimulatory effect was reduced by calcium ionophore A23187. It was therefore suggested that ATP in this process is utilized for Ca^{2+} accumulation inside the microsomal vesicles by Ca^{2+}-dependent, Mg^{2+}-stimulated ATPase. These data [3] led us to the conclusion that the serine base-exchange reaction occurs at the luminal leaflet of the endoplasmic reticulum and could be regulated by Ca^{2+}-ATPase, which maintains high (mM) Ca^{2+} levels in the lumen of the endoplasmic reticulum, compared to low (nM) external Ca^{2+} concentrations in the cytosol.

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Abbreviations: PS - phosphatidylserine; $InsP_3$ - inositol 1,4,5-trisphophate.

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However, the calcium sequestration in the endoplasmic reticulum reflects the balance between Ca^{2+} uptake and the release of Ca^{2+} through Ca^{2+} channels [4,5]. Therefore, the present investigation was designed to check whether the PS synthesis could be influenced not only by calcium ionophore, but also by the disruption of microsomal membranes, by thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase [6], and by heparin and caffeine, known to modulate Ca^{2+} release by receptor channels.

Materials and Methods

Chemicals: ATP, EGTA, HEPES, imidazole, dithiothreitol, caffeine, heparin, oligomycin and the ionophore A23187 were obtained from Sigma Chemical Co.,ST.Louis, MO, USA, and thapsigargin was obtained from L.C.Service Corporation, Woburn, USA. Fura-2 free acid was purchased from Molecular Probes,Inc., Eugene, OR, USA. L-[^{14}C]serine was obtained from Prague, Czecho-Slovakia.

Microsomal preparation: Liver of Wistar male rats was homogenized in 10 volumes of medium containing 225 mM mannitol, 125 mM sucrose, 3 mM HEPES (pH 7.4) and 0.5 mM EGTA. The homogenate was centrifuged at 1500 x g for 10 min, and the supernatant was further centrifuged at 15000 x g for 15 min. This supernatant was centrifuged at 100000 x g for 90 min in a Beckman L8-80M ultracentrifuge. The microsomal pellet was suspended in the same medium but without EGTA to a final concentration of 20-30 mg of protein/ml. Microsomes were stored at -70 °C. The disruption of microsomal membranes was performed by a freeze-thawing and sonication procedure. The microsomal vesicles were suspended in 3 volumes of 250 mM sucrose and sonicated thoroughly five times for 20 s periods at 0 °C. The suspension was rapidly frozen at -70 °C and thawed at room temperature. The sonication and the freezing-thawing procedure were repeated 3 times. The suspension was sonicated just before using.

The serine base-exchange assay: The assay of L-serine incorporation was a modification of the method of Bjerve [7]. The incubation medium contained 1-1.2 mg per ml microsomal protein, 60 mM imidazole-HCl buffer (pH 7.5), 20 mM sucrose, 1 mM dithiothreitol, 10 μM $CaCl_2$, 3 μg/ml oligomycin, 10 μM L-[U-^{14}C]serine (spec. act. 200000 dpm/nmol). The medium did or did not contain 7 mM $MgCl_2$ and 6 mM ATP/KOH; other additions are as described in the text. The final volume was 0.25 ml. The incorporation was started by the addition of microsomal protein. All incubations were carried out at 37 °C for 20 min in a water bath shaker. The reaction was terminated by the addition of methanol:chloroform (2:1, v/v). Phospholipids were extracted according to Bligh and Dyer [8]. The washed chloroform phase was transferred to a scintillation vial, evaporated to dryness and measured for radioactivity. Radioactivity incorporated in the control zero time samples was subtracted from other samples in each experiment. Phospholipids were analyzed by two dimensional TLC as described elsewhere [9].

Calcium measurements in rat liver microsomes: Microsomal vesicles (1.2 mg protein/ml) were added to a cuvette with 3 ml of incubation medium containing 60 mM imidazole-HCl buffer (pH 7.5), 20 mM sucrose, 1 mM dithiotreitol, 10 μM L-serine, 10 μM $CaCl_2$ and 3 μg/ml oligomycin. The uptake of Ca^{2+} into microsomal vesicles was studied by an addition of 7 mM $MgCl_2$ and 6 mM ATP/KOH. The changes in the free calcium concentration were monitored using the fluorescent calcium indicator Fura-2 free acid (1.5 μM) in a Perkin-Elmer LS-5B fluorescence spectrophotometer. The data were collected using an excitation wavelength of 340 nm (slit width, 5 nm) and emission wavelength of 510 nm (slit width, 10 nm).

Determination of protein was performed according to the method of Lowry et al. [10].

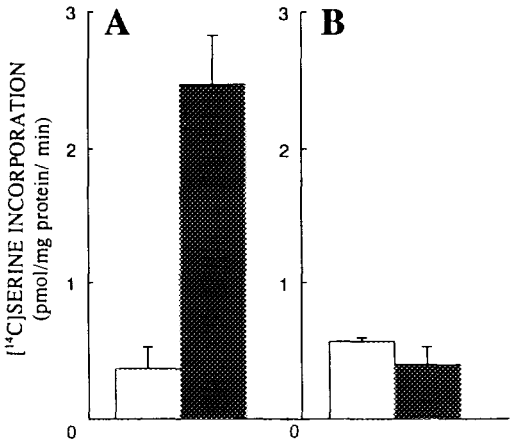

Fig.1. Effect of ATP and Mg^{2+} on the incorporation of serine into total phospholipids in untreated (A) and fully disrupted (B) rat liver microsomes.
A. Microsomal vesicles were incubated (1 - 1.2 mg of protein per ml) in a medium containing 60 mM imidazole buffer (pH 7.5), 20 mM sucrose, 1 mM dithiotreitol, 10 μM $CaCl_2$, 3 μg/ml oligomycin, 10 μM L-[U-^{14}C]serine (spec. act. 200000 dpm/nmol), and with or without 7 mM $MgCl_2$ and 6 mM ATP, as indicated.
B. Microsomal membranes were fully disrupted by a freeze-thawing and sonication procedure as described under Materials and Methods and incubated (1.2 mg of protein per ml) in the same medium as used for A. Other experimental details are given in the Materials and Methods section.
The results are the means ± SD for 6 experiments (A) and 3 experiments (B), using different microsomal preparations.
□ , without ATP and Mg^{2+}; ■ , with ATP and Mg^{2+}.

Results

Our previous investigation [3] indicated that the stimulatory effect of ATP and Mg^{2+} on PS formation occurred only at low (μM), and did not occur at high (mM), Ca^{2+} concentrations. For this reason, in the present study only a low (10μM) Ca^{2+} concentration was used.

Fig. 1 shows that in untreated microsomes ATP and Mg^{2+} distinctly stimulated (about 8 fold) serine incorporation into total phospholipids (Fig. 1A). When fully disrupted microsomal vesicles were assayed, ATP and Mg^{2+} had no such stimulatory influence (Fig. 1B). The results of thin-layer chromatographic analysis, which demonstrated that PS is the main phospholipid synthesized in the microsomal fraction (above 90%, not shown), pointed out that the incorporation of [^{14}C]serine into total phospholipids can be regarded as a measure of PS formation.

In order to examine if ATP indeed induces the uptake of Ca^{2+} into microsomal vesicles under the experimental conditions used (i.e. without ATP-regenerating system), the effect of ATP was measured with Fura-2 free acid. Fig. 2A illustrates that the addition of ATP and

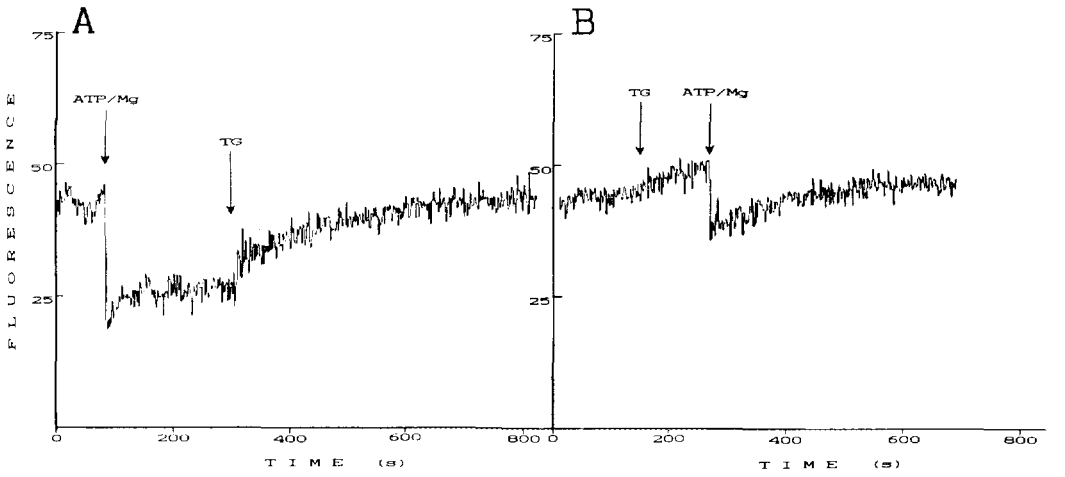

Fig. 2. Effect of thapsigargin (TG) on ATP-induced Ca^{2+} uptake into rat liver microsomal vesicles.
Extramicrosomal calcium changes were monitored by measuring Fura-2 free acid fluorescence. Ca^{2+}-ATPase activity was stimulated by the addition of ATP (6 mM) and $MgCl_2$ (7 mM) to the microsomal preparation. Incubation mixture contains 3 μg/ml oligomycin to prevent mitochondrial ATPase. Other experimental details are given in the Materials and Methods section. **A.** ATP inducing Ca^{2+} uptake into the microsomal vesicles reduces the extramicrosomal calcium concentration and decreases fluorescence. The addition of thapsigargin (100 nM) increases again extramicrosomal Ca^{2+}. **B.** Thapsigargin (100 nM) added before ATP treatment prevents the ATP-induced Ca^{2+} uptake.

Mg^{2+} caused the ATP-induced Ca^{2+} uptake. Thapsigargin added to these vesicles loaded with Ca^{2+} caused a net loss of these ions, as shown by the increase in the ambient calcium level. In contrast, ATP failed to induce the uptake of Ca^{2+} if thapsigargin was added before ATP to the microsomal preparation (Fig. 2B).

Table I demonstrates the effect of calcium ionophore A23187 and thapsigargin on PS synthesis. As is apparent, the action of the ionophore and thapsigargin was dependent on the presence or absence of ATP and Mg^{2+} in the assay mixtures. In their absence, microsomal vesicles were not loaded with Ca^{2} and Ca^{2+}-ATPase was not active, so both A23187 and thapsigargin were without any effect on serine incorporation. In the presence of ATP and Mg^{2+}, thapsigargin and the ionophore strongly inhibited PS formation (Table I). This inhibition could be interpreted as a result of depletion of microsomal intravesicular Ca^{2+} stores from Ca^{2+}. In the mixture containing Mg^{2+} alone without ATP, thapsigargin and A23187 did not influence serine incorporation (not shown).

Table II shows that in Ca^{2+}-loaded microsomal vesicles the addition of caffeine reduced the stimulatory effect of ATP and Mg^{2+} on PS synthesis. On the other hand, heparin distinctly enhanced this process in a concentration dependent manner.

Table I
Effect of calcium ionophore A23187 and thapsigargin on serine incorporation into microsomal phospholipids

Additions	L-[U-^{14}C]serine incorporation (pmol/mg protein/min)	
	ATP and Mg^{2+} omitted	ATP and Mg^{2+} added
none	0.32 ± 0.14	2.42 ± 0.38
+ A23187 (10 μM)	0.35 ± 0.04	0.11 ± 0.07
+ thapsigargin (100 nM)	0.43 ± 0.04	0.24 ± 0.09
+ EtOH	N.d.	2.40 ± 0.30
+ DMSO	N.d.	2.76 ± 0.20

Assays were conducted in the medium as described in Fig. 1., with or without the addition of ATP (6 mM) and $MgCl_2$ (7 mM), and A23187 or thapsigargin as indicated. All values are means ± SD of 4 separate experiments. EtOH and DMSO were added to the medium in the same proportion as that present in samples with A23187 and thapsigargin, respectively. N.d., not determined.

Discussion

The present study shows that the stimulatory effect of ATP and Mg^{2+} on PS formation is reduced not only by the calcium ionophore A23187, but also by the disruption of the microsomal membranes, and by the plant derived sesquiterpene lactone thapsigargin. The ionophore A23187 completely permeabilizes the vesicular membranes for Ca^{2+}, and due to that

Table II
Effect of caffeine and heparin on serine incorporation into microsomal phospholipids in the presence of ATP and Mg^{2+}

Additions	L-[U-^{14}C]serine incorporation (pmol/mg protein/min)	
none	2.73 ± 0.08	(100%)
+ caffeine		
(5 mM)	1.98 ± 0.12	(73%)
(10 mM)	2.05 ± 0.06	(75%)
+ heparin		
(50 μg/ml)	3.75 ± 0.34	(137%)
(100 μg/ml)	4.93 ± 0.59	(181%)
(200 μg/ml)	5.90 ± 0.75	(216%)

Rat liver microsomes were incubated in the presence of ATP (6 mM) and $MgCl_2$ (7 mM) and with or without caffeine and heparin as indicated. Other experimental details were as described in Fig. 1 and under Materials and Methods. The data presented are means ± SD of 4 experiments using different microsomal preparations. The numbers in parentheses indicate % of control.

no ATP-dependent Ca^{2+} accumulation can be achieved [11]. In fully disrupted microsomal vesicles the membrane permeability barrier is also eliminated, whereas thapsigargin is a specific blocker of the endoplasmic reticulum Ca^{2+}-ATPase [6]. The presence of this enzyme in the liver endoplasmic reticulum has been recently documented and its properties and functions characterized [11,12]. According to Inesi and Sagara [13], thapsigargin acts on Ca^{2+}-ATPase by an inhibition both Ca^{2+}- and ATP- binding, and prevents reuptake of calcium into the lumen of the endoplasmic reticulum. The action of thapsigargin on Ca^{2+} uptake by the microsomal vesicles is also demonstrated in this study (Fig. 2).

Thus, all of these observations provide additional evidence to support our previous suggestion [3], that ATP-dependent PS formation is in fact a base-exchange reaction stimulated by endogenous Ca^{2+} accumulated inside the microsomal vesicles by Ca^{2+}-ATPase. These results are compatible with those obtained by us on PS synthesis in glioma C6 cells [9]. We showed that the inhibition of PS synthesis in these cells is caused by Ca^{2+} depletion from the endoplasmic reticulum and suggested that it occurs on the luminal leaflet of these structures. Similar results and similar conclusions for PS synthesis in Jurkat T lymphocytes were recently reported by Pelassy et al. [14].

The present study demonstrates that PS synthesis was also influenced by the modulators of Ca^{2+} release receptor channels. The modulation of these channels can be caused by a variety of substances [4]. For example, caffeine is known as an agent which causes calcium release through ryanodine receptor channel, whereas heparin inhibits channel opening by competing at the $InsP_3$ binding sites [4,5]. More recently, Kraus-Friedmann and coworkers [11,15,16] demonstrated the presence of $InsP_3$- and ryanodine- binding sites in rat liver microsomes, and showed that the binding of ryanodine is inhibited by caffeine and binding of $InsP_3$ by heparin.

To our knowledge, the data presented here are the first to show that caffeine and heparin, the agents capable of modulating Ca^{2+} release receptor channels, can also affect the phospholipid synthesis. Since PS formation in Ca^{2+}-loaded microsomal vesicles was enhanced by heparin and diminished by caffeine, these results provide also a proof for the luminal localization of serine base-exchange reaction in rat liver endoplasmic reticulum.

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