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An antagonist of ATP-regulated potassium channels, the guanidine derivative U-37883A, stimulates the synthesis of phosphatidylserine in rat liver endoplasmic reticulum membranes

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Abstract The guanidine derivative U-37883A has been found to stimulate in vitro synthesis of phosphatidylserine in endoplasmic reticulum membranes, catalyzed exclusively by a serine-specific base exchange enzyme. The stimulation of the enzyme activity by the drug was concentration-dependent, with EC_{50} of 54 μM, while the biologically inactive analog of U-37883A, U-42069, was without effect. The stimulation caused by U-37883A was enhanced under the conditions when active transport of Ca^{2+} into the lumen of microsomal vesicles was induced, whereas it was inhibited by a calcium ionophore, A23187, and by a specific inhibitor of Ca^{2+}-ATPase, thapsigargin. On the other hand, a potassium ionophore, valinomycin, had no effect on phosphatidylserine synthesis. U-37883A did not affect the K_m of the base exchange enzyme for serine, but greatly reduced the EC_{50} value of the enzyme for calcium. Furthermore, Ca^{2+} uptake by endoplasmic reticulum vesicles has been found to increase in the presence of U-37883A. These observations suggest that U-37883A enhances phosphatidylserine synthesis indirectly by acting on calcium transport, thus affecting calcium concentration within the lumen of endoplasmic reticulum membranes. Alternatively, the effect of the drug could be propagated via the mechanism by which phospholipid flip-flop movement, known to regulate the serine-specific base exchange reaction, is modulated.

Key words: Phospholipid base exchange reaction; Phosphatidylserine; K_{ATP} channel antagonists; Ca^{2+}-ATPase; Endoplasmic reticulum (rat liver)

1. Introduction

The amount of phosphatidylserine (PS) in mammalian cell membranes varies from 1 to 10 mol% of total phospholipids, and is usually tissue specific [1]. PS is asymmetrically distributed within the transversal plane of biological membranes, on the cytoplasmic leaflet of plasma membranes and on the extracytoplasmic side of intracellular ones, for example of endoplasmic reticulum (ER) membranes [2–4]. Such a localization of this phospholipid can be transiently modulated upon metabolic activation of a cell [4] or in various pathologies. PS is solely formed by a unique enzyme catalyzed, bi-substrate phospholipid base exchange (PLBE) reaction [5] in ER membranes of mammalian cells. It has been documented [6] that, in rat liver ER membranes, the specific stearoyl-polyunsaturated molecular species of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are preferentially converted to PS via this reaction. Since, the PLBE reaction is calcium-dependent, the activities of various ER membranes proteins influencing calcium homeostasis within a cell, like Ca^{2+}-ATPase [7] or electron transport systems, cytochrome P450 isoforms and other metalloproteins [8], are potential modulators of the efficiency of PS synthesis. On the other hand, PE formed from the proper molecular species of PS by the ethanolamine-specific PLBE reaction [9], and PS itself, are both main bulk phospholipids which create the membrane environment for the activity of cytochrome P450 isoforms [10]. Recently, a close relationship between PS synthesis, cytochrome P450 and K^+ channel activities has been postulated [11,12], since imidazole antimycotics, inhibitors of cytochrome P450 isoforms, i.e. clotrimazole, econazole, miconazole and α-naphtoflavone [11] at submicromolar or micromolar concentrations, as well as K^+ channel blockers and antiarrhythmic drug, i.e. quinine, quinidine, 4-aminopyridine, tetraethylammonium and clofilium [11–13], were found to enhance the efficiency of PS synthesis in Jurkat T lymphocytes, independently on Ca^{2+}-influx and plasma membrane potential [11]. On the other hand, the inhibition of PC and PE synthesis in these cells by antiarrhythmic drugs has been observed [13].

The ATP-regulated potassium (K_{ATP}) channels, well characterized in plasma membranes of various cells [14], were also identified, by the use of K_{ATP} channel antagonists: antidiabetic sulfonylureas and non-sulfonylureas, or various potassium channel openers, in rat liver mitochondria [15]. It was, therefore, of particular interest to examine the effects of K_{ATP} channel antagonists on PS synthesis in rat liver ER membranes, the main site of phospholipid synthesis in a cell.

2. Materials and methods

2.1. Chemicals

K_{ATP} channel inhibitors were obtained from the following sources: 4-morpholine-carboxamidine-*N*-1-adamantyl-*N'*-cyclohexylhydrochloride (U-37883A), [^{3}H]U-37883A (32.8 Ci/mmol), and 4-morpholinecarboxamidine (U-42069D) were a generous gift from Unpjohn Co. (USA), glibenclamide was obtained from Research Biochemicals Inc. (USA), and glipizide, HB985 and HB699, were kindly supplied by Hoechst (Germany). [3-^{14}C]Serine (51 mCi/mmol) was purchased from Amersham (UK). HEPES, ATP, A23187, and valinomycin were from Sigma (USA). All other chemicals were of the highest purity commercially available.

2.2. Microsomes

Adult male Wistar rats weighing 150–180 g were killed after being

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Abbreviations: PLBE, phospholipid base exchange; ER, endoplasmic reticulum; K_{ATP}, ATP-regulated K^+ channel; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phos-phatidylcholine; U-37883A, 4-morpholinecarboxamidine-*N*-1-adamantyl-*N'*-cyclohexylhydro-chloride; U-42069D, 4-morpholinecarboxamidine

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starved for 16 h. Liver ER membranes were prepared according to Rakowska et al. [16], resuspended at a protein concentration of 10–20 mg/ml in a buffer containing 250 mM sucrose, 40 mM HEPES, pH 7.4, and stored at −72°C.

2.3. Measurements of phospholipid base exchange activity

The reaction mixture for measurements of PLBE activity contained 0.25 mg of microsomal protein (equivalent to 130 ± 12 nmoles of phospholipids) in 0.25 ml of 40 mM K-HEPES, pH 7.4, supplemented with 50 μM $CaCl_2$, 50 μM [3-^{14}C]serine (1.5 mCi/mmol), and when indicated with 1 mM $MgCl_2$+1.2 mM ATP. In some experiments, the concentrations of serine and $CaCl_2$ varied, and the reaction mixture was supplemented with various concentrations of a K_{ATP} antagonist. The reaction was carried out at 37°C in a water bath shaker for 15–20 min, the period when the PLBE activity remained linear. The PLBE reaction was stopped by addition of 2.0 ml of ice-cold chloroform : methanol mixture (1:1, v/v), followed by addition after 15 min of 1 ml of 0.1 N HCl. The organic phase was separated by centrifugation, washed twice with 1.0 ml of 1 mM serine in methanol, and evaporated to dryness. The incorporation of radiolabelled substrate into PS was assessed by scintillation counting in a Beckman LS 6000TA counter (USA). In each assay non-incubated blank samples (immediately after addition of substrate) were used to measure non-specific binding of serine to ER membranes (which did not exceed 1% of total lipid-associated radioactivity), which was subsequently subtracted from the respective experimental data.

2.4. Uptake of calcium by rat liver ER membranes

The uptake of Ca^{2+} by microsomal vesicles (0.7 mg of protein ml^{-1}) was measured in a medium containing 20 mM K-HEPES, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 80 μM $^{45}CaCl_2$ (30 mCi/mmol), and various concentrations of K-EGTA, to obtain the final [Ca^{2+}] as indicated in the abscissa to Fig. 3A or the fixed concentration of calcium estimated to be 3.7 μM when 0.1 mM K-EGTA was present (Fig. 3B). Vesicles were preincubated for 5 min at 37°C, then K-ATP, pH 6.8, was added to reach a final concentration of 1 mM, and incubation was carried out at the same temperature for 1 to 5 min. At various time points, 500-μl aliquots were withdrawn and filtered through Millipore filters (0.45 μm, type HAWP, Millipore Corp.) prewashed with 2×2 ml of 20 mM K-HEPES, pH 7.4, 100 mM KCl. The filters were immediately washed twice with 2 ml of the same ice-cold medium. Filter-associated radioactivity was counted in dioxane scintillator.

2.5. Binding of [^{3}H]U-37883A to endoplasmic reticulum membranes

Binding of radiolabelled U-37883A to ER membranes was assayed as follows. For the equilibrium binding assay, rat liver ER membranes (300–500 μg protein ml^{-1}) were incubated for 60 min at 4°C in 50 mM HEPES-NaOH (pH 7.4), with the required concentrations of [^{3}H]U-37883A (2 nM–20 μM, 32.8 Ci/mmol). The incubation was stopped by rapid filtration through Whatman GF/C filters under reduced pressure. Prior to use, the filters were immersed and incubated in a solution containing 0.5% polyethyleneimine-HEPES, pH 7.4, followed by washing in 30 ml of 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, at 4°C. Subsequently, the filters were incubated for 24 h in 5 ml of Formula 989 (DuPont NEN, Germany) as a scintillation cocktail, and counted for associated radioactivity. Non-specific binding was measured in the presence of 30 μM non-radioactive U-37883A.

2.6. Other procedures

Protein concentration was determined according to Bradford [17] with bovine serum albumin as a standard. Phospholipids were extracted from membranes according to Bligh and Dyer [18], and quantified by inorganic phosphorus measurements, as described by Rouser et al. [19]. Free calcium concentrations were calculated with the aid of a computer program and the log K_a^{EGTA} values of Vianna [20], and verified using a Ca^{2+}-selective electrode (Orion, Res. Inc., USA).

3. Results and discussion

3.1. Influence of U-37883A on phosphatidylserine synthesis via the phospholipid base exchange reaction

The transport activity of K_{ATP} channels of plasma mem-

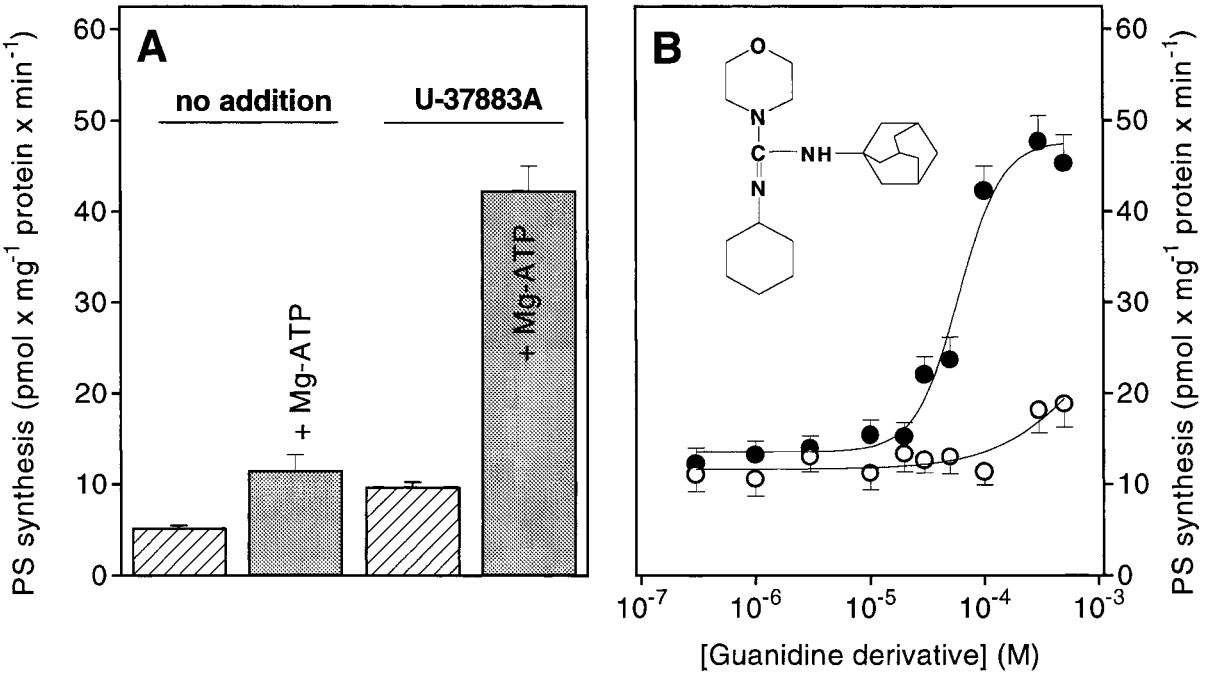

Fig. 1. The effect of non-sulfonylurea K_{ATP} channel inhibitor, guanidine derivative U37883A, on synthesis of phosphatidylserine in rat liver endoplasmic reticulum membranes. (A) The PLBE reaction was performed at protein concentration of 1 mg ml^{-1}, in 40 mM K-HEPES, pH 7.4, 50 μM [3-^{14}C]serine, 50 μM $CaCl_2$, with no further additions, or with 1 mM $MgCl_2$, 1.2 mM ATP, and ± 100 μM U-37883A, for 15 min at 37°C. (B) The concentration dependence of the effect of U-37883A (●) or U-42069D (○) on the serine-specific base exchange reaction in the presence of 1 mM $MgCl_2$, 1.2 mM ATP and 50 μM $CaCl_2$. The determinations of PLBE activity were performed as described in Section 2. The drugs were used at the concentrations indicated on the abscissa. ER membranes were not preincubated with these drugs before the assay of enzyme activity. Mean values ± SD of three experiments are shown. Inset to panel B: chemical formula of U-37883A.

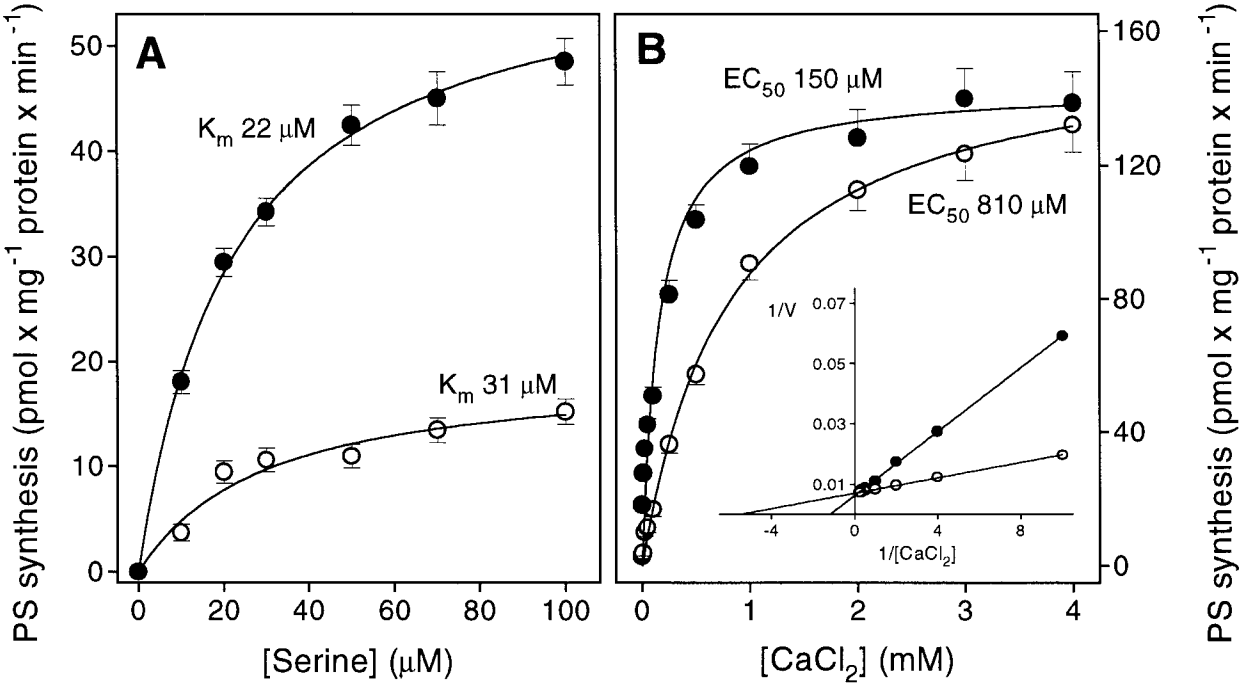

Fig. 2. The influence of 100 μM U-37883A on kinetic parameters of the serine-specific base exchange reaction. (A) The effect of the guanidine derivative U-37883A (●) on K_m of the reaction with serine as a substrate. The control experiment was performed in the absence of guanidine derivative (○). (B) Calcium dependence of PS synthesis in the absence (○) or presence (●) of U-37883A. Other conditions are described in Section 2. ER membranes were not preincubated with U-37883A prior to the assay of PLBE activity. In the inset to B, the double reciprocal plot is shown. The data are combined from four independent experiments.

branes is inhibited by sulfonylureas [21], which serve as potent medicaments in the therapy of diabetes mellitus type II, and by non-sulfonylureas like U-37883A [22]. The K_{ATP} channels are activated by potassium channel openers, which are frequently used in medical treatment of hypertension [23]. Additionally, it has been shown that sulfonylureas are able to enter the cytosol and bind to intracellular membranes, insulin containing granules [24] and liver mitochondria [15]. It has been also found that a non-sulfonylurea blocker of K_{ATP} channels, U-37883A (inset to Fig. 1B), regulating in vivo the function of kidney, i.e. natriuresis/diuresis [25], inhibits K^+ uniport in rat liver mitochondria [26].

We have tested the effect of antidiabetic sulfonylureas and U-37883A on PS synthesis in ER membranes in vitro. All the antidiabetic sulfonylureas applied, glibenclamide, glipizide, HB985, and HB699, even at 100 μM concentration, were unable to affect significantly PS synthesis at 1 mM $CaCl_2$ concentration, equal to EC_{50} value of the PLBE reaction [16]. In contrast, stimulation of PS synthesis by 100 μM U-37883A was observed at 1 mM $CaCl_2$, as well as at 50 μM $CaCl_2$ (Fig. 1A), suggesting a direct interaction of this drug with microsomal membranes. In fact, single class of low-affinity binding sites with K_d of 9 μM was identified in microsomal membranes using [^{3}H]U-37883A. To check whether the U-37883A-stimulated PS synthesis was due to the influence of this drug on K^+ permeability of SR/ER membranes [27] the efficiency of PS synthesis was measured in the presence of valinomycin, a K^+ ionophore. No effect of valinomycin was observed on U-37883A-stimulated PS synthesis, indicating that neither the ER membranes K^+ channel nor changes in membrane potential [16] are involved in the observed phenomena. On the other hand, a K^+ channel modulator, glibenclamide, has been implicated to affect calcium homeostasis in various cells and tissues [28]. Therefore, it seemed of particular interest to check the influence of sulfonylureas (glibenclamide, glipizide, HB985, and HB699) and U-37883A on PS synthesis in ER membranes under the conditions when Ca^{2+}-transport ATPase was activated. We found that only 100 μM U-37883A enhanced PS synthesis via the PLBE reaction when the assay medium was supplemented with 1.2 mM Mg-ATP, and the Ca^{2+} concentration was 19 μM [20]. Stimulation of PS synthesis by the K^+ channel antagonist at 1.2 mM Mg-ATP and 19 μM Ca^{2+} was found to be drug-concentration dependent with EC_{50} value of 54 μM (Fig. 1B). Under the same conditions the analog of U-37883A, designated U-42069, which was found biologically inactive [25,26], did not affect PS synthesis in rat liver ER membranes, although it was used over a broad concentrations range (Fig. 1B). The stimulation of PS synthesis by U-37883A was synergistic with the effect of Mg-ATP at 50 μM $CaCl_2$. The efficiency of PS synthesis in the presence of U-37883A exhibited a linear dependence versus reaction time, up to 15–20 min at 37°C, and was almost by a factor of two enhanced in comparison to control ER membranes (with no drug added) (Fig. 1A). At the same time 1.7-fold stimulation of the PLBE reaction in the presence of 1.2 mM Mg-ATP was observed. Both effectors added together increased the efficiency of PS synthesis by a factor of 3 at 50 μM $CaCl_2$. Preincubation of ER membranes with U-37883A for 15 s to 20 min at 4°C had no significant influence on the reaction (not shown), suggesting indirect effect of the drug on serine-specific PLBE enzyme.

3.2. *Kinetic parameters of serine-specific phospholipid base exchange reaction upon addition of U-37883A*

To study the mechanism by which the applied guanidine derivative may activate in vitro PS synthesis in ER mem-

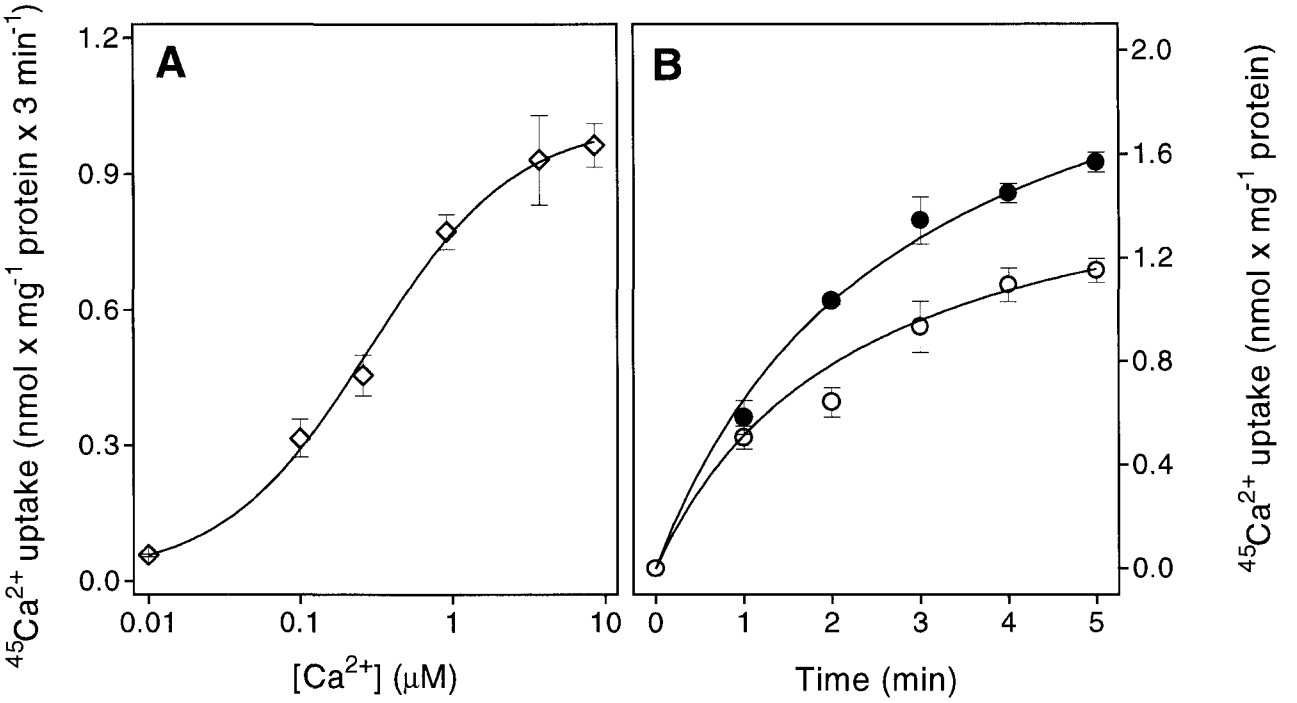

Fig. 3. The effect of U-37883A on calcium uptake by ER membranes. (A) The concentration dependence of $^{45}Ca^{2+}$ uptake by ER membranes not treated with the guanidine derivative was assayed under the conditions specified in Section 2. (B) The time-course of calcium (final [Ca^{2+}] 3.7 μM) uptake by ER membranes in the absence (○) or presence (●) of 100 μM U-37883A. Mean values ± SD for three different membrane preparations are shown.

branes we have examined kinetic parameters of the PLBE reaction. As shown on Fig. 2A, hyperbolic regression curves for serine concentration versus reaction velocity measured in the absence or presence of 100 μM U-37883A, suggest that the serine-specific PLBE obeys in both cases first order kinetics characterized by the Michaelis-Menten law. The K_m value for serine in the presence of 50 μM $CaCl_2$ and 1.2 mM Mg-ATP calculated on the basis of the data obtained for control samples, was found to be 31 ± 8 μM, i.e. similar to that reported by other authors [29,30], and V_{max} of 16 ± 3 pmol per mg protein per min. In comparison, in the presence of U-37883A, K_m of the PLBE reaction for serine was not affected (22 ± 3 μM), while V_{max} value increased more than three-fold (Fig. 2A). This prompted us to conclude once again that the effect of U-37883A on the PLBE enzyme is rather indirect, because it neither does change the affinity of the enzyme for serine nor the permeability of ER membranes to aminoalcohol (not shown). Moreover, the membrane is not destroyed by U-37883A, as phospholipid synthesis via the PLBE reaction was found to be dependent on membrane integrity [16]. Since the V_{max} value of the reaction was significantly enhanced by the drug, it can be speculated that this was due to changes in phospholipid movement across the membrane bilayer or in calcium transport-related events. Since amphiphilic cations and anions have no effect on the K_m value towards L-serine but only upon the V_{max} value of the reaction, it was suggested that their effect was related to interaction of the lipid substrate with the PLBE enzyme [31].

As we reported previously, the EC_{50} value for the serine-specific PLBE reaction with respect to Ca^{2+} concentrations is in the range of 0.6–0.8 mM [16]. In the presence of Mg-ATP, the EC_{50} value of the reaction for calcium was lowered by U-37883A from 810 μM in control ER membranes to 150 μM (Fig. 2B), as confirmed by the double reciprocal plot shown on inset to the figure. This result can be explained by the effect of the drug on permeability of ER membranes to Ca^{2+}.

3.3. *The effect of U-37883A on Ca^{2+} requirement of serine-specific phospholipid base exchange reaction*

PS synthesis in rat ER membranes is inhibited in vitro by calcium antagonists, A23187, caffeine, thapsigargin, and vanadate [32,33], as it was also observed in vivo in Jurkat T cells [13] and glioma C_6 cells [34]. On the other hand, PS synthesis in rabbit platelets [35], human leukocytes [36], and Jurkat T cells [37] was activated by calmodulin antagonists, chlorpromazine, trifluoroperazine, and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide hydrochloride. These cationic amphiphilic drugs acted synergistically to Ca^{2+} since their effect was reversed by the addition of EGTA [37]. Millimolar concentrations of calcium required for maximal activation of the PLBE reaction are reached in a cell only within the lumen of ER membranes [7]. Therefore this reaction either occurs inside microsomes [32,33] or a calcium-responsive domain of the PLBE enzyme is localized on the luminal side of ER membranes [16], where the concentration of this cation is limited in vivo by the presence of Ca^{2+}-binding proteins, such as calmodulin [8], calnexin [38] and calreticulin [39], as well as by negatively charged phospholipids [40]. In hepatocytes, the ER-dependent cellular calcium homeostasis is also regulated by cytochromes P450 [8], by the level of phosphate released from glucose-6-phosphate [41], by the inositol 1,4,5-trisphosphate-dependent pathway, and by Ca^{2+}-mobilizing hormones, vasopressin, glucagon and insulin [42].

Since, depletion of calcium stores inhibits PS synthesis [13,33], $^{45}Ca^{2+}$ uptake measurements were performed in the presence of U-37883A (Fig. 3B) and under the conditions

described as optimal for Ca^{2+}-ATPase activity [43]. The rate of calcium ion accumulation versus $Ca^{2+}{}_{out}$. concentration fitted well the sigmoidal-shaped curve (Fig. 3A) and allowed us to estimate maximal Ca^{2+} accumulation to be 1 nmol per mg protein per 3 min at 37°C. This value is in agreement with those previously reported by other investigators [44]. The uptake was sensitive to calcium ionophores (A23187) and Ca^{2+}-ATPase inhibitors (thapsigargin), as it was also shown by others [33]. U-37883A (100 μM) stimulated calcium uptake by 30% (Fig. 3B), resulting in an increase of Ca^{2+} concentration inside ER membranes by one order of magnitude (taking into account that internal volume of ER vesicles is 2–2.5 μl per mg protein [16]). In addition, the stimulatory effect of U-37883A on PS synthesis was completely reversed in the presence of 250 nM thapsigargin or by 1 μM A23187. Therefore, the influence of the drug studied on calcium transport and/or other calcium-related processes (like redistribution of phospholipids, flip-flop) rather than directly on the protein catalyzing PLBE reaction should be considered as responsible for stimulation of PS synthesis. Changes of PS amount in intracellular membranes may have profound effects in vivo. PS, due to its net negative charge at physiological pH, interacts with amino group of proteins, and with inorganic cations. Calcium interaction with PS headgroup has been found to evoke changes in its configuration, in the molecular area occupied by the phospholipid, and the surface charge density [45], these changes leading to lateral phase separation, to formation of local lipid domains in membranes [40], and affecting localization of PS in the transversal plane of bilayer [2–4,46,47]. Moreover, there are numerous intracellular proteins, among them protein kinase C isoforms, phospholipase A_2 or annexins, which have been found to specifically bind PS in membrane bilayers in the presence of calcium ions, forming ternary calcium/lipid/protein complexes, essential for their biological activity [48].

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