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Serine Base Exchange Enzyme Activity Is Modulated by Sphingosine and Other Amphiphilic Compounds: Possible Role of Positive Charge in Increasing the Synthesis of Phosphatidylserine

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It has been found that sphingosine and sphingosylphosphorylcholine (amphiphilic cations) have a stimulatory, and cholesterol 3-sulfate (an amphiphilic anion), an inhibitory, effect on [^{14}C]serine incorporation into phosphatidylserine in glioma C6 and rat liver microsomes. In glioma intact cells sphingosine stimulates phosphatidylserine synthesis in a process independent of protein kinase C, but suppressed by thapsigargin. We suggest that the stimulation of the enzyme occurs by the interaction of amphiphilic cations with the membrane cosubstrate phospholipids, leading to a charge redistribution on their phosphate groups, and hence facilitating the enzyme action. A new hypothesis concerning the mechanism of the serine base exchange reaction is discussed.

Phosphatidylserine (PS) synthesis in animal cells occurs solely by the base exchange reaction in which serine is directly exchanged for the ethanolamine moiety of phosphatidylethanolamine (PE), or choline moiety of phosphatidylcholine (PC). This reaction proceeds in the absence of metabolic energy, is characterized by a requirement for Ca^{2+} and occurs mainly in the endoplasmic reticulum [1,2]. Whereas different pathways exist for the synthesis of PE and PC, the base exchange reaction may be the only pathway for PS synthesis. The molecular mechanism of this reaction remains, however, obscure.

It has been reported that compounds belonging to cationic amphiphilic drugs (CADs) increase PS synthesis in various cell lines [3-5]. CADs represent a large number of effective therapeutic compounds characterized by two distinct hydrophobic and hydrophilic domains and by a positively charged aminogroup at physiological pH. Sphingosine and other sphingoid bases (e.g. lysosphingolipid - sphingosylphosphorylcholine) are natural long chain amino bases with a hydrophilic positively charged aminogroup (headgroup) and a hydrophobic long carbon chain (tail), and therefore may be categorized as endogenous cationic amphiphilic molecules [6]. The main interest in possible functions of sphingosine stems from discovery that sphingosine is a potent inhibitor of protein kinase C (PKC) and interferes with biological actions of PKC in multiple cell systems [7]. Among many biological activities of sphingosine, it has been also shown that it generates intracellular Ca^{2+} mobilization by release of Ca^{2+} from internal stores [8]. In a neuronal cell line [4] and rat brain microsomal membranes [9] sphingosine has been shown to stimulate the serine base exchange activity, whereas cholesterol 3-sulphate and other amphiphilic anions have an opposite effect. It was therefore of interest to check whether similar effect of sphingosine, sphingosylphosphorylcholine and cholesterol 3-sulfate could be observed in glioma C6 intact cells and microsomal membranes. To examine whether opposite effects of cationic and anionic amphiphilic compounds might have a more general character, experiments on rat liver microsomes were also performed. The present study shows that amphiphilic cations have a stimulatory, and anions an inhibitory, effect on the serine base exchange activity and provides an additional insight into unclear problem of the mechanism of the base exchange synthesis of PS. A new hypothesis concerning the mechanism of the serine base exchange reaction is proposed.

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Abbreviations: BSA, bovine serum albumin; CADs, cationic amphiphilic drugs; DMSO, dimethylsulfoxide; LDH, lactate dehydrogenase; MEM, Minimum Essential medium; PBS, phosphate buffer saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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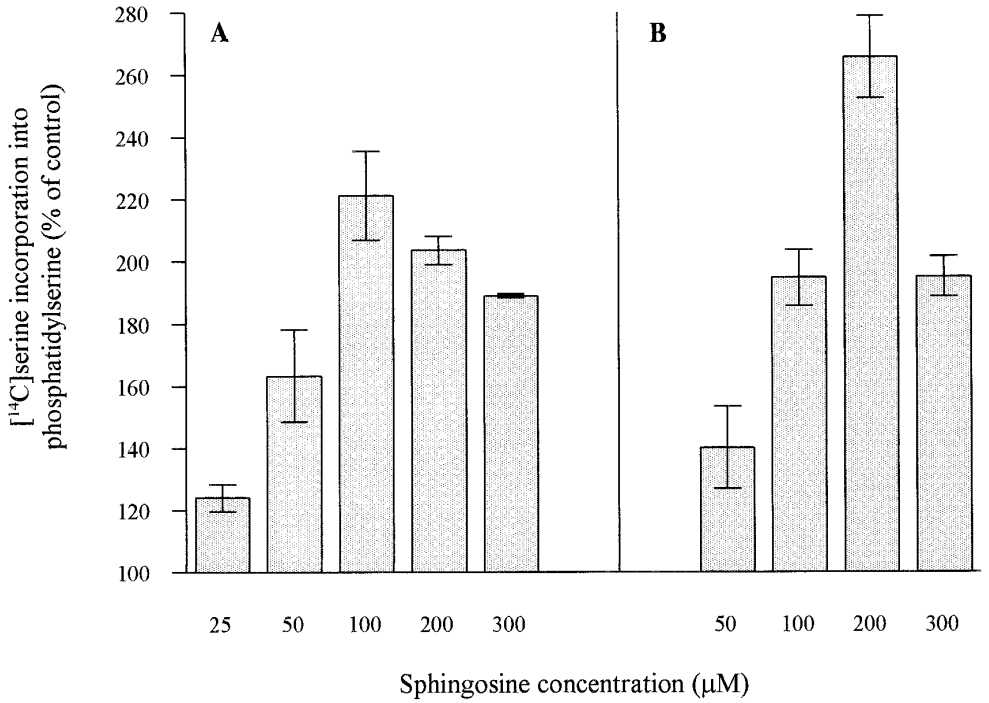

FIG. 1. Effect of various concentrations of sphingosine on [^{14}C]serine incorporation into phosphatidylserine in glioma C6 cells. The cells were incubated for 30 min under conditions as described in Materials and Methods. Results are expressed at % vs. control untreated cells, taken as 100% ([^{14}C]serine incorporation into phosphatidylserine in control cells amounted to 2880 ± 167 dpm/10^6 cells). All values are the mean ± SEM of four (A) and two (B) experiments done in triplicate. Asterisks indicate statistical significance compared to the control group (** $p < 0.01$; *** $p < 0.001$, Student's t-test). (A) Sphingosine added in DMSO (DMSO concentration in the assay medium did not exceed 0.3%). (B) Sphingosine stabilized by a equimolar complex with BSA.

MATERIALS AND METHODS

Chemicals. Minimum Essential Medium (MEM), calf serum, antibiotics and PBS were from Gibco BRL, UK. 12-O-tetradecanoylphorbol-13-acetate (TPA), bovine serum albumin (BSA), EGTA, NADH, D-(+)-erythro-trans-sphingosine, sphingosylphosphorylcholine and cholesterol 3-sulfate were purchased from Sigma Chemical Co., St. Louis, USA. Triton X-100 was from Romil Chemicals, Shepsed, UK and thapsigargin from LC Services Corporation (Woburn, USA). L-[U-^{14}C]serine was purchased from Amersham, UK.

Cell culture. Glioma C6 cells were cultured in MEM supplemented with 10% (v/v) calf serum and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 at 37°C as previously described [10].

Preparation of microsomal membranes from glioma C6 cells and rat liver. Microsomes from glioma C6 cells were prepared as described by Rodriguez et al. [11]. Briefly, the cells were homogenized in a buffer containing 0.32 M sucrose, 1 mM EDTA and 2 mM HEPES (pH 7.4) and centrifuged at 20,000 g for 20 min. Thereafter, the supernatant was centrifuged at 100,000 g for 60 min to provide a pellet, referred to as the microsomal fraction. This pellet was then resuspended in a buffer containing 40 mM HEPES (pH 7.45) and 1 mM $CaCl_2$, protein content measured, and stored at −70°C until use. The microsomes from liver of Wistar male rats were prepared exactly as described previously [12]. The microsomal pellet obtained by differential centrifugations was resuspended in 40 mM HEPES (pH 7.45) and 1 mM $CaCl_2$, protein content measured, and stored at −70°C until use.

Assay for the serine base-exchange activity in glioma C6 cells. Confluent cultures of cells (1.3-1.5 × 10^6 cells per dish) were incubated in the culture medium with L-[U-^{14}C]serine (0.63 μM, 0.1 μCi/ml) in the presence or absence of sphingosine and other agents prepared in a stock solution in DMSO (the final concentration of DMSO never exceeded 0.3%) or in the presence of sphingosine prepared in equimolar concentration of BSA [13]. All incubations were carried out at 37°C. After an incubation, the medium was removed and the cells were washed, scraped off and collected as described [10]. Lipids were extracted using methanol:chloroform (2:1 v/v) according to Bligh and Dyer [14], and phospholipids were analyzed by two dimensional TLC as described [10]. Phospholipid spots were visualized with iodine vapour and ninhydrin and scraped off for radioactivity counting.

Assay for the serine base exchange in glioma C6 and rat liver microsomal membranes. The serine base exchange reaction was carried out according to Hu et al. [15] with minor modifications. Briefly, microsomal membranes (50 μg protein) were incubated for 20 min in 40 mM HEPES (pH 7.45) and 1 mM $CaCl_2$ in the presence of L-[U-^{14}C]serine (31.6 μM, 0.5 μCi) and other additions as described in the text. The final volume was 100 μl and samples were incubated in a shaker bath at 37°C. The reaction was terminated by adding 400 μl of methanol:chloroform (2:1, v/v) and phospholipids were extracted according to Bligh and Dyer [14]. 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 others samples in each experiment. Phospholipids were analyzed by two dimensional TLC as described [10].

Assays of cell viability. Cell viability was assayed by Trypan Blue exclusion and by release of the cytosolic enzyme, lactate dehydrogenase (LDH). The LDH concentration was determined after incubation of the cells without (control) or with sphingosine, and in the cells permeabilized with 1% Triton X-100 (final concentration) for

complete release of cellular LDH. The cells were centrifuged and LDH activity in the supernatants was monitored by following the oxidation of NADH at 340 nm [16]. Trypan Blue exclusion was determined microscopically.

Determination of protein content and statistical analysis. Proteins were quantified according to Bradford [17]. The results are presented as mean values ± SEM. Statistical significances were evaluated by the Student's *t*-test.

RESULTS

Figure 1 shows that the action of sphingosine on the incorporation of radioactive serine into PS in glioma C6 cells was concentration dependent. During 30 min of incubation, sphingosine increased [^{14}C]serine incorporation twofold, with a maximal increase at 100 μM sphingosine added to the assay medium in DMSO (Fig. 1A), or an increase of about threefold at 200 μM sphingosine added as BSA complex (Fig. 1B), used to minimize the cytotoxicity [13]. The decrease in the uptake of radioactivity, evident at higher sphingosine concentration (300 μM, Fig. 1) is most likely due to the known cytotoxic effect of this compound [13]. However, we found that when glioma C6 cells (1.3-1.5 $\times$ 10^6 cells per dish) were incubated for 15 or 30 min in the absence of BSA with 50 and 100 μM sphingosine, there was no effect on cell viability. Trypan Blue exclusion showed 97% viability and the release of LDH in both the absence and the presence of sphingosine was lower than 5 %. Moreover, PS formation was similarly affected by 50 and 100 μM sphingosine added to the medium in form of DMSO solution or stabilized as BSA complex (Fig 1, A and B).

[^{14}C]Serine was incorporated solely into PS and not significantly into PE (not shown). The percentage of PE formed after 30 min of incubation, in the control cells, and in those incubated with sphingosine, amounted to 5 and 3 %, respectively. Since the cell viability was preserved, these data indicate that sphingosine affects PS labelling at the level of PS formation because of enhancement of the serine base exchange activity.

To check whether the stimulatory effect of sphingosine on PS synthesis may be PKC-dependent, we compared the incorporation of [^{14}C]serine into PS in the presence and absence of sphingosine and phorbol ester (TPA), a known PKC activator, in another set of experiments (Table 1). This process was also investigated in the cells exposed to TPA for 18h, the condition used for PKC down-regulation. As we have recently reported [18], both PKC activation and down-regulation caused by TPA treatment (100 nM) diminished PS formation. Table 1 shows that sphingosine was able to neutralize and reverse these effects, indicating that enhanced PS formation is a PKC-independent process. In contrast, stimulatory effect of sphingosine was suppressed by thapsigargin, a selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (Table 1).

TABLE 1

Effect of Sphingosine on [^{14}C]Serine Incorporation into Phosphatidylserine in Control (Untreated), PKC-Activated, PKC-Down-regulated, and Thapsigargin-Treated Glioma C6 Cells

Treatment	[^{14}C]serine incorporation	
	dpm/10^6 cells	%
control cells, no addition	2866 ± 119	100
+ sphingosine	5818 ± 358	203
+ TPA (PKC activated cells)	1232 ± 243	43
+ TPA + sphingosine	5187 ± 114	181
PKC down-regulated cells	1690 ± 106	59
PKC down-regulated cells + sphingosine	4614 ± 343	161
+ thapsigargin	1010 ± 93	35
+ thapsigargin + sphingosine	956 ± 67	33

Note. Cells were incubated for 30 min with [^{14}C]serine as described in Materials and Methods in the absence and presence of 100 μM sphingosine and 100nM TPA, a PKC activator. PKC-down regulated cells were treated for 18 h with 100 nM TPA [18], washed and incubated with or without of 100 μM sphingosine. The effect of thapsigargin was measured in the cells incubated for 30 min in the presence or absence of 100 μM sphingosine and 100 nM thapsigargin. The data are means ± SEM of triplicate determination from three individual experiments.

To further investigate the effect of sphingosine on serine uptake in an *in vitro* assay, the uptake of radioactive serine was examined in microsomal membranes from glioma C6 cells and rat liver. In both these systems thin-layer chromatographic analysis demonstrated that PS was the main phospholipid synthesized in the microsomal fraction (above 90%, not shown) and pointed out that the incorporation of [^{14}C]serine into total phospholipids could be regarded as a measure of PS synthesis.

Figure 2 shows that in glioma C6 microsomal membranes, sphingosine produced a dose-dependent increase in the serine base exchange activity. This increase reached a maximum at 50 μM, followed by a decline below the baseline activity at 300 μM sphingosine (Fig. 2). Another amphiphilic cation, sphingosylphosphorylcholine had a similar effect, although the profile of this increase was different than that observed for sphingosine. On the other hand, an amphiphilic anion, cholesterol 3-sulfate, inhibited the serine base exchange reaction (Fig. 2).

As above observations were done on the membrane fraction composed of microsomes from glioma C6 cells, it was of interest to determine whether such phenomenon could also occur in the microsomes of different origin. Therefore, in the next set of experiments we examined the effect of these amphiphilic

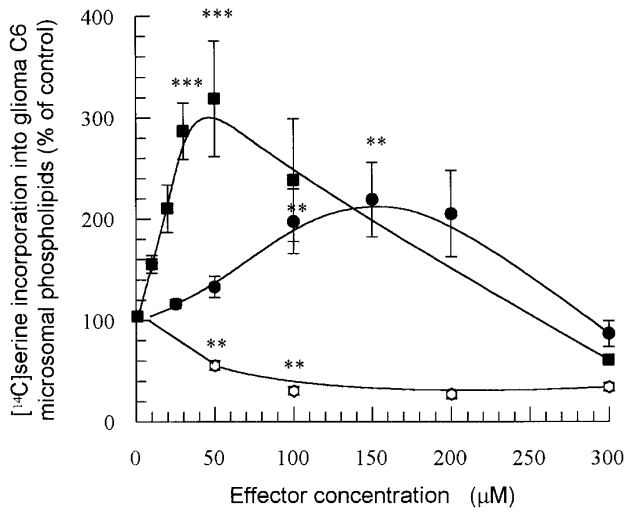

FIG. 2. Effect of sphingosine (■), sphingosylphosphorylcholine (●) and cholesterol 3-sulfate (○) on serine incorporation into glioma C6 microsomal phospholipids. Microsomal membranes were incubated at 37°C for 20 min in the presence of [^{14}C]serine and the indicated concentration of the drugs. Results are the means of ±SEM for 5 experiments (sphingosine and sphingosylphosphorylcholine) and 3 experiments (cholesterol 3-sulfate) using different microsomal preparations and are expressed as percentage of control untreated microsomes taken as 100% ([^{14}C]serine incorporation into phospholipids of control microsomes amounted to 56 pmol/mg protein/min). Statistical significance was assessed by Student's t-test (** $p < 0.01$; *** $p < 0.001$).

substances on PS synthesis in rat liver microsomes. Table 2 shows that in these membranes sphingosine, sphingosylphosphorylcholine and cholesterol 3-sulfate behaved similarly as it was found in glioma microsomal membranes. Sphingosine and sphingosylphosphorylcholine increased PS synthesis with a twofold stimulation of serine base exchange activity by 50 μM and 100 μM, respectively. Cholesterol 3-sulfate, similarly to glioma microsomes, had the opposite effect. There was 40% and about 70% inhibition of the enzyme activity at 50 and 100 μM cholesterol 3-sulfate, respectively (Table 2).

DISCUSSION

It has been demonstrated that a number of drugs belonging to CADs stimulate the incorporation of labelled serine into PS in various cell lines: intact rabbit platelets [3], human neuroblastoma cell line LA-N-2 [4] and human Jurkat T cells [5]). The same phenomenon occurred in rabbit platelet microsomal membranes, where the effect of calmoduline antagonists, chlorpromazine and trifluoperazine was tested [19]. In rat brain microsomal membrane fraction, PS synthesis was enhanced by sphingosine and other amphiphilic cations, whereas amphiphilic anions, cholesterol 3-sulfate and bis(2-ethylhexyl)hydrogen phosphate diminished this process [9]. The same relationships for sphingosine in intact glioma C6 cells, and sphingosine, sphingosylphosphorylcholine and cholesterol 3-sulfate in glioma C6 and rat liver microsomal membranes were presented in this study. Thus, from the examples described here, it can be generalized that with the serine base exchange activity the amphiphilic cations are stimulatory and amphiphilic anions are inhibitory.

Emptying of the endoplasmic reticulum calcium stores, either through agonist-receptor interactions, or artificially, by the action of calcium ionophors or thapsigargin, results in a decreased activity of the serine base exchange enzyme system [10,12,20-22]. However, sphingosine, proposed as a modulator of Ca^{2+} signals in cell mediating Ca^{2+} release from intracellular stores [8], increased PS synthesis. This discrepancy can be explained by our recent observation [23] that in glioma C6 cells treated with 100 μM sphingosine, Ca^{2+} was not completely released from the intracellular stores and some amounts of these ions were trapped within the endoplasmic reticulum. In contrast, thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{2+}-pumping ATPase [24], completely depleted Ca^{2+} stores in glioma C6 cells, that cannot be refilled again [23,25]. The present study shows that the depletion of the endoplasmic reticulum stores by thapsigargin suppressed the stimulatory effect of sphingosine on PS synthesis. These data demonstrate that the influence of sphingosine on PS synthesis is secondary towards the action of the base exchange enzyme that requires Ca^{2+} for its activity.

TABLE 2

Effect of Sphingosine, Sphingosylphosphorylcholine and Cholesterol 3-Sulfate on Serine Incorporation into Rat Liver Microsomal Phospholipids

Additions	L-[U-^{14}C]serine incorporation	
	pmol/mg protein/min	%
None	119 ± 15	100
+ sphingosine		
30 μM	165 ± 6**	139
50 μM	249 ± 28***	209
100 μM	175 ± 22**	147
+ sphingosylphosphorylcholine		
50 μM	189 ± 14***	159
100 μM	207 ± 12***	174
150 μM	172 ± 6**	145
+ cholesterol 3-sulfate		
50 μM	62 ± 6***	52
100 μM	29 ± 2**	24

Note. Rat liver microsomes were incubated for 20 min with [^{14}C]-serine as described in Materials and Methods. The data are means ± SEM of triplicate determination from four experiments. Statistical significance was assessed by Student's *t*-test (**$p < 0{,}001$; ***$p <$ 0,001).

The physicochemical features of sphingosine, as well as commercial amphiphilic drugs, allow them to intercalate into phospholipid bilayers of artificial and biological membranes and to alter their properties affecting fluidity, order and charge. The regulation of the serine base exchange activity by amphiphilic cations and anions has been explained by Kanfer and McCartney [9] as a result of change of the order of the endoplasmic reticulum membrane. The authors have found that both stimulators and inhibitors have no effect on the K_m value towards serine but only on the V_{max} value of the reaction and proposed that amphiphilic cations enhance the interaction of the membrane-bound cosubstrate (mainly PE) with the membrane bound enzyme, while the amphiphilic anions prevent this interaction. According to this hypothesis, this interaction occurs by exposing phospholipid headgroups into a more hydrophilic environment, accessible to the catalytic site of the enzyme.

Amphiphilic cations can shift the surface charge of artificial and natural membranes to less negative values [26]. It has been known that CADs possess special properties to associate with negatively charged polar groups of phospholipids and are able to prevent the binding to the membrane of other positive molecules. It has been speculated that CADs might interact physically with membrane phospholipids by electrostatic binding with their head groups [27,28]. In 1995, Lopez-Garcia et al. [29] have presented evidence that sphingosine and stearylamine anchor to artificial membranes containing dipalmitoylphosphatidylserine and interact electrostatistically with the phospholipids. The authors documented that not only the apparent dissociation constant of the carboxyl group, but also the percentage of dehydrated phosphate groups of phospholipids, was significantly lower.

Thus, we suggest that sphingosine and other amphiphilic cations not only alter the orientation of phospholipid cosubstrate headgroups in the membrane exposing them into more hydrophilic environment, as proposed by Kanfer and McCartney [9], but also interact electrostatically with such exposed headgroups thereby reducing the negative charge on their phosphate groups. One may assume that through this interaction a charge redistribution on the phosphate group could be achieved and that the enzyme might more easily interchange a serine molecule with the base moiety of the cosubstrate phospholipids. Amphiphilic anions would interfere with such interaction. This hypothesis is in good agreement with our observation that introduction of phosphate groups into membrane proteins caused by PKC activation, making the membrane more negative, decreased PS synthesis in glioma C6 cells. This effect was also observed under conditions of PKC down-regulation. Sphingosine, making the membrane more positive, completely reversed these effects. Phosphorylation of membrane proteins by protein kinases was reported to alter surface potential and enzyme activities by changing the surface charge density [30].

Moreover, PS synthase I and II, two different enzymes catalyzing the serine base exchange in Chinese hamster ovary cells [31,32], encoded by pssA and pssB genes, are composed of amino acid sequences that possess C- and N-terminal fragments with high number of positively charged amino acids. The amino acid sequences of the pssB gene product, and the pssA gene product, PS synthase I, show that both of them have several potential membrane-spanning domains, and their N- and C-terminals are predicted to be situated on the cytosolic side of the endoplasmic reticulum membrane, respectively. pssA protein has a unique double lysine motif at its C-terminus, whereas pssB, at its N-terminus, has a double arginine motif [32]. Both of these motifs are known as the specific endoplasmic reticulum targeting signals [33,34]. Therefore, we propose that these positively charged amino acid sequences might play an important role in both the *in vivo* and *in vitro* mechanisms of the serine base exchange reaction, interacting directly with the cosubstrate phospholipid headgroups of the membrane and causing a charge redistribution on their phosphate groups. This change would facilitate the enzymatic reaction leading to the exchange of serine with the base moiety of phospholipids. Ca^{2+} might act allosterically through binding to the enzyme on the luminal side, but influencing the active site localized on the cytosolic side of the membrane.

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