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Communication

Sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate modulate phosphatidylserine homeostasis in glioma C6 cells★✪

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The effect of sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate on L-[U-^{14}C]serine incorporation into phosphatidylserine and phosphatidylserine-derived phosphatidylethanolamine was investigated in intact glioma C6 cells. Sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate are potent signalling molecules which, due to their physicochemical features, may function as amphiphilic compounds. It has been found that sphingosine and sphingosylphosphorylcholine (amphiphilic cations) significantly increase [^{14}C]phosphatidylserine synthesis and decrease the amount of ^{14}C-labeled phosphatidylethanolamine. Sphingosine 1-phosphate (an amphiphilic anion) was without effect on phosphatidylserine synthesis but, similarly as sphingosine and sphingosylphosphorylcholine, reduced the conversion of phosphatidylserine to phosphatidylethanolamine. These results strongly suggest that sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate can modulate cellular phospholipid homeostasis by stimulation of phosphatidylserine synthesis and an interference with phosphatidylserine decarboxylase.

Sphingolipids are natural and important membrane constituents, particularly concentrated in brain and nerve tissues. Sphingolipids breakdown products, such as sphingosine,

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Abbreviation: TLC, thin-layer chromatography.

sphingosylphosphorylcholine and sphingosine 1-phosphate have been identified as second messengers acting on the regulation of cell growth, differentiation and programmed cell death [1-3]. Recent observations have shown that sphingosine 1-phosphate and sphingosylphosphorylcholine may also act as first messengers, binding to specific plasma membrane receptors [4-6]. Thus, sphingolipids have to be regarded as intracellular and extracellular messengers.

Sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate are composed of a sphingoid backbone (which predominantly is sphingosine), consisting 2-amino-1,3-diol hydrophilic head domain and a long (15 carbon atom) domain of hydrophobic alkyl chain. Sphingosylphosphorylcholine, a lysosphingolipid, and sphingosine 1-phosphate contain highly polar head structures which are esterified at the 1-hydroxy position by phosphocholine (sphingosylphosphorylcholine), or by a phosphate group (sphingosine 1-phosphate) [3]. Hydrophilic heads of sphingosine and sphingosylphosphorylcholine can be positively charged in their aminogroups at physiological pH, and therefore, together with two distinct hydrophobic and hydrophilic groups, may be categorized as endogenous amphiphilic cations [7]. On the contrary, sphingosine 1-phosphate can be regarded as bearing a net negative charge.

We have recently reported 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 the microsomal fraction from glioma C6 cells and rat liver [8]. We have also shown that sphingosine stimulates phosphatidylserine synthesis in intact glioma cells [8]. It was therefore of interest to check what effect could be observed for sphingosylphosphorylcholine and sphingosine 1-phosphate in glioma C6 cells.

In animal cells, phosphatidylserine is solely synthesized by the base exchange reaction in which serine is directly exchanged for the amino alcohol moiety of preexisting phospholipids, particularly phosphatidylethanolamine [9]. This reaction occurs mainly in the endoplasmic reticulum, whereas conversion of phosphatidylserine into phosphatidylethanolamine, *via* the decarboxylation pathway, occurs in mitochondria [10].

The aim of the present study was to examine whether sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate could affect phosphatidylserine synthesis and its further metabolic conversion to phosphatidylethanolamine in intact glioma C6 cells.

MATERIALS AND METHODS

Materials. Glioma C6 cells were from the American Type Culture Collection (U.S.A.). Minimum Essential Medium, calf serum, trypsin solution and phosphate-buffered saline were from Gibco BRL (U.K.). Penicillin and streptomycin were from Polfa (Tarchomin, Poland). Bovine serum albumin, D-(+)-erythro-*trans*-sphingosine and sphingosylphosphorylcholine were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Sphingosine 1-phosphate was from Calbiochem (La Jolla, U.S.A.). L-[U-^{14}C]Serine was purchased from Amersham (U.K.). Silica 60 plates for thin-layer chromatography (TLC) were from Merck (Darmstad, Germany). All other chemicals used were of analytical grade.

Cell culture. Glioma C6 cells (passages 40-60) were used. The cells were cultured in Minimum Essential Medium supplemented with 10% (v/v) calf serum and antibiotics (culture medium) under a humidified atmosphere of 5% CO_2 at 37°C, as described previously [11]. The cells were passed when confluent using trypsin (0.25%), and the medium was changed twice a week. The cells had reached confluence at the time of experiment.

Assay for the serine base-exchange activity. Confluent cultures of cells (3×10^6 cells per dish, 60 mm diameter) were incu-

bated in 2 ml of the culture medium with L-[U-^{14}C]serine (158 mCi/mmol; 0.1 μCi/ml) in the presence or absence of sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate. Sphingosine and sphingosylphosphorylcholine were added as a complex with equimolar concentration of bovine serum albumin to minimize the cytotoxicity [12]. Stock solution of sphingosine 1-phosphate was made in methanol. Immediately before use the solution was dried and sphingosine 1-phosphate was dissolved in phosphate buffered saline and added to dishes. All incubations were carried out at 37°C for 30 min. After the incubation, the medium was removed and the cells were washed, scrapped off and collected as described [11]. Lipids were extracted using methanol/chloroform (2:1, v/v) according to Bligh & Dyer [13] and phospholipids were analyzed by two dimensional TLC as described [11]. Phospholipid spots were visualized with iodine vapor and ninhydrin and scrapped off for radioactivity counting.

Assay of cell viability. Cell viability was assayed by Trypan Blue exclusion determined microscopically after incubation of the cells without (control) or with sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate. In each case, Trypan Blue exclusion showed about 97% viability.

Data presentation. The results are mean values ±S.E.M. of at least triplicate determinations. Statistical significance of differences were estimated by Student's *t*-test.

RESULTS

Figure 1 shows the action of amphiphilic cations sphingosine and sphingosylphosphorylcholine on the incorporation of radioactive serine into phosphatidylserine in glioma C6 cells. As it is shown, both amphiphilic cations increased significantly phosphatidylserine synthesis at a concentration-dependent manner. However, sphingosine, at low, 15 μM concentration was practically without effect and at 25 μM concentration increased [^{14}C]serine incorporation only negligibly (Fig. 1A). Twofold or even threefold increase occurred only at 100 μM or 150 μM sphingosine added to the assay medium (Fig. 1A). On the contrary, sphingosylphosphorylcholine even at 15 μM

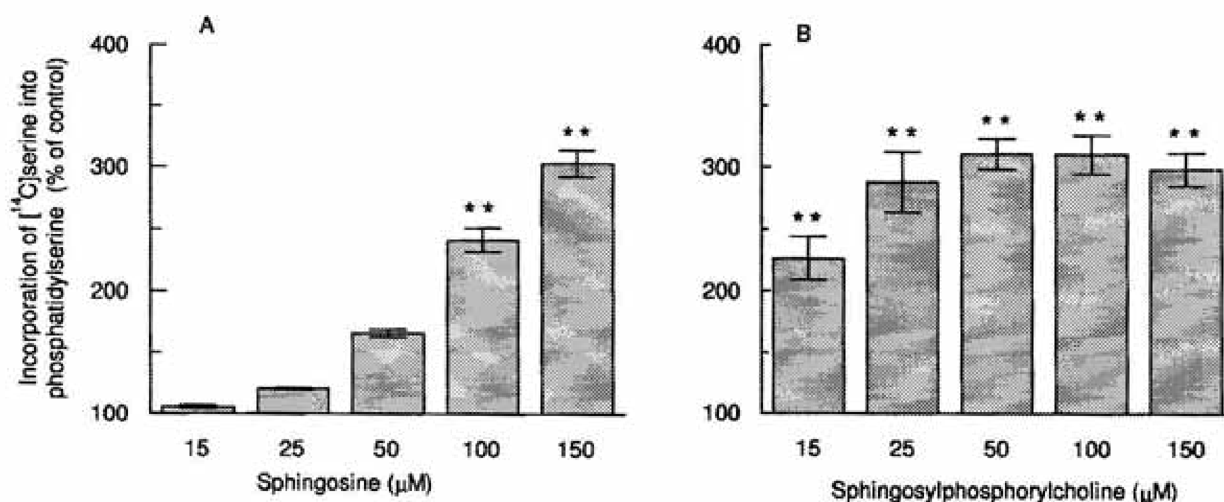

Figure 1. Effect of various concentrations of sphingosine (A) and sphingosylphosphorylcholine (B) on [^{14}C]serine incorporation into phosphatidylserine in glioma C6 cells.

The cells were incubated under conditions described in Materials and Methods. Results are expressed as percentage of the incorporation in untreated (control) cells which amounted to 1500 ± 200 d.p.m./dish. All values are means ±S.E.M. of three experiments done in triplicate. Asterisks indicate statistical significance with respect to the control (**$P < 0.01$, Student's *t*-test).

concentration increased phosphatidylserine synthesis more than twofold and such high response was maintained at all concentrations examined (Fig. 1B). Still, it seemed that the difference in the profile of this increase for both compounds was quantitative rather than qualitative; to obtain the same effect much more sphingosine than sphingosylphosphorylcholine was needed. The increase in phosphatidylserine synthesis in the presence of sphingosine and sphingosylphosphorylcholine was observed in the absence of change in the total uptake of [^{14}C]serine by the cells (not shown).

Figure 2A shows a lack of the effect of the amphiphilic anion, sphingosine 1-phosphate, on [^{14}C]serine incorporation into phosphatidylserine. Sphingosine 1-phosphate, at all concentrations used (1, 10 and 100 μM), was without effect. Small differences with respect to control (untreated cells) were statistically insignificant (Fig. 2A). On the other hand, addition of sphingosine 1-phosphate to the assay medium strongly decreased the formation of phosphatidylethanolamine (Fig. 2B). A comparison between control and sphingosine 1-phosphate treated cells indicated a significant decrease in labeling of phosphatidylethanolamine at 100 μM sphingosine 1-phosphate ($P < 0.05$) (Fig. 2B).

The reduced conversion of phosphatidylserine into phosphatidylethanolamine was also observed in cells treated with sphingosine or sphingosylphosphorylcholine. The percentage of phosphatidylserine metabolized into phosphatidylethanolamine was similarly decreased in cells treated with 100 μM concentration of either compound (Table 1). It is worth noting that, in the experimental protocol used, phosphatidylserine may be decarboxylated to phosphatidylethanolamine and the latter can be methylated to phosphatidylcholine. However, during 30 min incubation of the cells with [^{14}C]serine, labeling in phosphatidylcholine was not detectable. Thus, the percentage of radioactivity found in labeled phosphatidylethanolamine represents the activity of phosphatidylserine decarboxylase. Table 1 shows that all amphiphilic compounds tested inhibit this metabolic pathway.

DISCUSSION

Studies on microsomal membranes from rat brain [14], rat liver [8], human platelets [15],

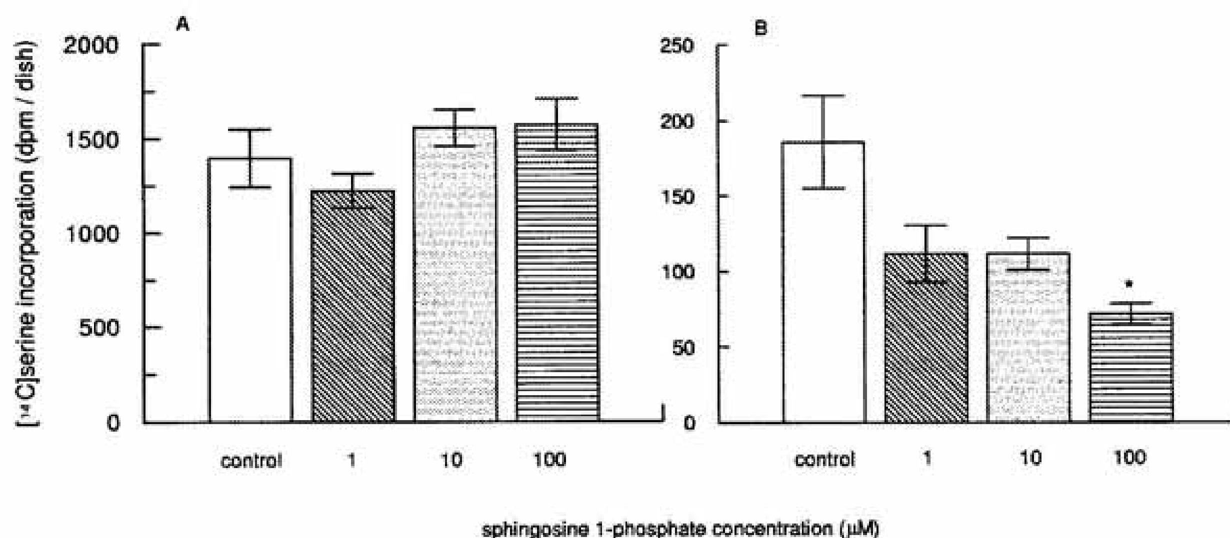

Figure 2. Effect of various concentration of sphingosine 1-phosphate on phosphatidylserine (A) and phosphatidylethanolamine (B) formation in intact glioma C6 cells.

The cells were incubated with [^{14}C]serine and with or without of sphingosine 1-phosphate as described in Materials and Methods. All values are means ±S.E.M. of three experiments done in triplicate. Asterisks indicate statistical significance with respect to the control group (*$P < 0.05$, Student's *t*-test).

and glioma C6 [8] have demonstrated that a number of drugs belonging to amphiphilic cations have a stimulatory, and anions an inhibitory, effect on phosphatidylserine synthesis. The regulation of serine base exchange activity by amphiphilic cations has been explained by Kanfer & McCartney [14] as a result of rearrangement of membrane phospholipids, serving as substrates for the serine base exchange enzyme. In addition, we have suggested [8] that amphiphilic cations interact electrostatically with such phospholipids, thereby reducing the negative charge on their phosphate groups and facilitating the enzyme action. Amphiphilic anions would interfere with such interaction [8].

Nevertheless, one can assume that amphiphilic compounds added exogenously to intact cells could affect phosphatidylserine synthesis in a quite different manner than that found in the microsomal fraction. Although it has been demonstrated that cationic amphiphilic drugs (and among them sphingosine) stimulate the incorporation of labeled serine into phosphatidylserine in various cell lines [8, 16-19], such phenomenon may not occur for compounds being quickly metabolized in the cell. A representative example is sphingosine 1-phosphate which, added exogenously to cells, may be rapidly dephosphorylated to sphingosine by phosphatidate phosphohydrolase [20].

Brindley & Waggoner [20] have recently described that this multifunctional phosphohydrolase, identified in the plasma membrane, can specifically dephosphorylate phosphatidate, lysophosphatidate, ceramide 1-phosphate and sphingosine 1-phosphate. The active site of the enzyme was found in the outer leaflet of the plasma membrane. Thus, sphingosine 1-phosphate present in blood and acting as the first messenger [4-6], or added externally to cultured cells, may be rapidly converted into sphingosine which is characterized by other signalling and physicochemical properties [20]. Moreover, sphingosine, the product of this reaction, inhibits phosphatidate phosphohydrolase and can be phosphorylated by sphingosine kinase to form sphingosine 1-phosphate [1, 20]. Thus, phosphatidate phosphohydrolase and sphingosine kinase could regulate the balance of sphingosine and sphingosine 1-phosphate in the cell.

Table 1. Effect of various concentrations of sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate on phosphatidylethanolamine formation

Treatment	μM	[^{14}C]Phosphatidylethanolamine formed (% of total incorporation)
Control cells, no addition		10 ± 1
+ sphingosine	25	7 ± 1
	50	5 ± 0.5
	100	3 ± 0.4
+ sphingosylphosphorylcholine	15	5 ± 0.5
	25	4 ± 0.4
	50	4 ± 0.5
	100	4 ± 0.5
+ sphingosine 1-phosphate	1	8 ± 1
	10	6 ± 0.5
	100	4 ± 0.5

The cells were incubated for 30 min with [^{14}C]serine as described in Materials and Methods in the absence or presence of various concentrations of sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate. After incubation phospholipids were extracted and separated on TLC. The sum of radioactivity found in phosphatidylserine and phosphatidylethanolamine was taken as 100%. The data are means ±S.E.M. for five (sphingosine) and three (sphingosylphosphorylcholine and sphingosine 1-phosphate) individual experiments done in triplicate.

The results of the present study are compatible with the above data. Sphingosylphosphorylcholine, even at low concentration, is able to increase a phosphatidylserine synthesis, whereas sphingosine needs a much higher concentration to exert a similar effect (Fig. 1). We suggest that this result and the lack of the response of the cells to sphingosine 1-phosphate (Fig. 2A) may be explained in the light of the conversion of sphingosine to sphingosine 1-phosphate and sphingosine 1-phosphate to sphingosine and the balance between substrates and products of both reactions, regulated by the activity of enzymes and the amount of substrates added to the cells.

The present study also shows that all amphiphilic compounds strongly diminish the conversion of phosphatidylserine to phosphatidylethanolamine (Table 1). A similar effect of amphiphilic cationic drugs has been reported in neuronal cell line, LA-N-2 [18]. Amphiphilic compounds alter properties of the membrane, affecting fluidity, order and charge [21]. Most probably, such changes make difficult the physical contact between the endoplasmic reticulum and mitochondria [22] and prevent phosphatidylserine decarboxylation.

Thus, it seems that bioactive endogenous amphiphilic compounds such as sphingosine, sphingosylphosphorylcholine and sphingosine 1-phosphate may not only activate or inhibit several enzymes [23] but, due to their physicochemical features, may subtly modify lipid metabolism including that of phosphatidylserine. Since phosphatidylserine is involved in protein kinase C activation [24], stimulation of its synthesis may play a modulatory role in cell signalling and hence in cell function.

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