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Studies on glucosylceramidase binding to phosphatidylserine liposomes: the role of bilayer curvature

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The influence of phosphatidylserine (PS) liposome size on their capacity to activate and bind purified glucosylceramidase was investigated. Gel filtration and flotation experiments showed that large unilamellar vesicles (LUV) of either pure PS or PS in admixture with phosphatidylcholine (PC) are unable to tightly bind purified glucosylceramidase, and thus, to fully stimulate its activity. By contrast, small unilamellar vesicles (SUV) of PS adsorb glucosylceramidase on their surface, the smaller vesicles within the SUV preparation being the most effective. Reconstitution of glucosylceramidase can either be favoured or inhibited by factors affecting the bilayer curvature of PS liposomes. An increase of PS vesicle size induced by a fusogenic agent such as poly(ethylene glycol) (PEG), decreased enzyme binding and activity. On the contrary, the reduction of PS LUV size by sonication increased their stimulating ability. Enzyme association with PS SUV is reversible. In fact, glucosylceramidase bound to PS SUV was released from the lipid surface when the SUV were transformed into larger vesicles by PEG; dissociation from the vesicles resulted in a dramatic decrease of enzyme activity. Although PS LUV are unable to reconstitute glucosylceramidase, their association with oleic acid (OA) promotes the interaction with glucosylceramidase. This phenomenon is best explained in terms of OA-induced surface defects of PS LUV, with consequent exposure of the more hydrophobic part of the membrane and hence the improved binding of hydrophobic region/s of glucosylceramidase. Our data indicate that the physical organization of the PS-containing liposomes is of critical importance for glucosylceramidase reconstitution. The observation that physical changes of the lipid surface can markedly affect the enzyme activity offers a new approach to the study of glucosylceramidase regulation.

Introduction

Glucosylceramidase (EC 3.2.1.45) is the enzyme that hydrolyzes glucosylceramide to ceramide and glucose. The defective activity of this enzyme is the cause of Gaucher's disease, an autosomal recessive disorder highly heterogeneous in its clinical manifestations [1–3]. Glucosylceramidase is tightly bound to the lysosomal membrane [4]. One of the main characteristics of the purified enzyme is its requirement of acidic phospholipids such as phosphatidylserine (PS) for the reconstitution of its activity [5–7]. The effect of PS can be enhanced by an endogenous activator protein, called Sap-2 [8,9].

It has been known for quite some time that residual glucosylceramidase in most of the non-neurologic cases of Gaucher's disease is markedly stimulated by PS, whereas residual enzyme in neurologic cases is relatively unresponsive to this acidic phospholipid [6,7,10]. Recently, several mutations in the gene for glucosylceramidase which give rise to deficient enzyme activity have been described in Gaucher patients [11–13]. One of the most frequently observed single point mutations causes a substitution of serine for asparagine at amino-acid position 370. This mutation is usually associated with a mild phenotype without neurologic symptoms [14]. The 370 mutant protein has a greatly enhanced sensitivity to PS. Cells carrying the 370 codon mutation can be stimulated to near normal activity in vitro by the combination of PS and SAP-2 [15].

Considering the important role of acidic phospholipids as modulators of enzyme activity, the interaction

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Abbreviations: MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; OA, oleic acid; TC, sodium taurocholate; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-L-α-phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-L-α-phosphatidylethanolamine; PEG, poly(ethylene glycol).

between PS and glucosylceramidase from controls and Gaucher's patients has been extensively studied [16–18]. Despite this relatively large research effort, some structural aspects of the enzyme stimulation mechanism by acidic phospholipids remain unclear. In previous reports from this laboratory direct evidence was obtained that the reconstitution of purified glucosylceramidase occurs through the spontaneous incorporation of glucosylceramidase into preformed bilayers containing an acidic phospholipid [19,20]; upon association, glucosylceramidase becomes active on both glucosylceramide, the natural lipid substrate and 4-methylumbelliferyl-β-D-glucopyranoside (MUGlc), an artificial water-soluble substrate. Enzyme association depends on several factors, the most important being the percentage of acidic phospholipids present on the lipid surface [19,20].

In a continuous effort to discover the conditions influencing the binding of glucosylceramidase to acidic phospholipid-containing liposomes, we investigated whether the physical characteristics of phospholipid vesicles used for studying the activation of purified glucosylceramidase might play a role in this phenomenon. Several reports have shown that the properties of unilamellar vesicles can markedly vary with the bilayer curvature [21]. Here we present a study on the effects of variation of PS liposome size on their capacity to stimulate and bind glucosylceramidase.

Materials and Methods

Materials. Oleic acid (OA), sodium taurocholate (TC) (synthetic, > 98% pure), poly(ethylene glycol) (PEG) average molecular weight 8000 and 3-*sn*-phosphatidylcholine (PC) from egg yolk were from Sigma (St. Louis, MO, USA). PS from bovine spinal cord and Triton X-100 were from Calbiochem (San Diego, CA, USA). MUGlc was obtained from Koch-Light (Colnbrook, UK). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-L-α-phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-L-α-phosphatidylethanolamine (*N*-Rh-PE) were from Avanti Polar-Lipids (Alabaster, AL, USA). L-α-Dipalmitoyl[2-palmitoyl-9,10^{3}H(N)]-PC (50 Ci/mmol), (1-^{14}C)OA (55 mCi/mmol) and [*methoxy*-^{3}H]inulin were from DuPont de Nemours (Germany), NEN Research Products. 1,2-Dioleoyl-3-*sn*-phosphatidyl-L-[3-^{14}C]serine (54 mCi/mmol) was from Amersham International (UK). The radiopurity of labelled compounds, determined by thin-layer chromatography followed by counting of radioactivity, exceeded 98%. The other chemicals were of the purest available grade.

Enzyme preparation. Glucosylceramidase was purified from human placenta following the procedure described by Furbish et al. [22] with a few modifications [23].

Glucosylceramidase assay. Glucosylceramidase was assayed either in the presence or in the absence of detergents. In the former case, the assay mixture contained, in a final volume of 0.2 ml: 0.1/0.2 M citrate/phosphate buffer (pH 5.6), 2.5 mM MUGlc, 0.1% (v/v) Triton X-100, 0.25% (w/v) TC and glucosylceramidase. In the latter case, the assay mixture contained, in a final volume of 0.2 ml: 10 mM acetate buffer (pH 5.8), 150 mM NaCl, 1 mM dithioerythritol and 1mM EDTA (buffer A), 2.5 mM MUGlc, different amounts of lipid vesicles as specified in the experiments and glucosylceramidase. To preserve the shape of the vesicles, the incubation medium in the second assay (buffer A) had a composition similar to the buffer of the vesicle preparation (buffer B, see below) except for the pH value. The mixtures were incubated for 30 min at 37°C. The extent of reaction was estimated fluorometrically as previously described [20]. All assays were carried out in duplicate and agreed within 5%.

1 U of glucosylceramidase is defined as the amount of enzyme which hydrolyzes 1 nmol of MUGlc/h in the presence of detergents.

Vesicle preparation. Small unilamellar vesicles (SUV) were prepared by dispersing the dried lipids (4 mg/ml) supplemented with radiolabelled PS as tracer in 2 mM L-histidine and 2 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes) adjusted to a final pH of 7.4, 150 mM NaCl, 1 mM dithioerythritol and 1 mM EDTA (buffer B). The lipid suspension was submitted to sonication under nitrogen in a Branson B 15 Sonifier (3 min with a Cup Horn at a power setting of 100 W, followed by 6 min with a Microtip at a power setting of 30 W). The sonication temperature was about 30°C. The preparation was centrifuged at 110 000 × *g* for 30 min and the supernatant used for the experiments. More than 95% of the radioactivity present in the dried lipid was found in the final preparation.

Large unilamellar vesicles (LUV) were prepared by filter exclusion [24,25] using a high pressure extrusion apparatus (Lipex Biomembranes, Vancouver, Canada), equipped with a water-jacketed 'thermobarrel' attached to a circulating water bath. The dry lipids supplemented with radiolabelled PS or PC as tracer were dispersed in buffer B by vortex mixing at a concentration of 4 mg/ml. The resulting suspension was subjected to five cycles of freezing in liquid nitrogen followed by thawing in warm water. The lipid suspension was then extruded at 40°C through two stacked 0.1-μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA, USA), under a nitrogen pressure of up to 200 psi. The extrusion procedure was repeated 10 times.

The oleic acid (OA) dispersion was prepared by suspending in buffer B the dried compound (2 mg/ml) supplemented with radiolabelled OA as tracer and

sonicating under nitrogen with a Microtip (power setting 30 W) until a clear suspension was obtained.

Determination of LUV internal volume. To determine the internal volume, PS LUV without radiolabelled phospholipid were prepared as indicated above in the presence of a trace amount of [*methoxy*-^{3}H]inulin (1 μCi/ml). Non-encapsulated inulin was removed from the liposomes by gel chromatography on a Bio-Gel A-15m column (10 × 370 mm). The internal volume of the liposomes, expressed as μl/μmol of phospholipid, was assessed by phosphate determination according to Bartlett [26] and radioactivity counting.

Vesicle fusion assay. Vesicle fusion induced by PEG was measured by the resonance energy transfer assay described by Struck et al. [27] using a Perkin-Elmer MPF 44 spectrophotofluorometer, equipped with constant temperature cell holder and stirrer. A mixture of PS SUV, containing both *N*-NBD-PE and *N*-Rh-PE at 1 mol% each, and PS SUV without the fluorescence probes at a molar ratio of 1:4 was added with PEG. After 30 min incubation at 37°C, the mixture was diluted 10-fold with buffer A and the fusion determined by monitoring the NBD fluorescence increase compared to a sample treated in the same way but in the absence of PEG. Samples were excited at 475 nm and monitored at 530 nm. The 100% fusion level was set with liposomes containing 0.2% each of *N*-NBD-PE and *N*-Rh-PE at the same total lipid concentration as in the fusion experiment.

Chromatography on Bio-Gel A-15m and Bio-Gel A-50m columns. To fractionate PS SUV or PS LUV, the vesicles supplemented with labelled PS as tracer were loaded on a Bio-Gel A-15m column (10 × 370 mm; Bio-Rad, Richmond, CA, USA) preequilibrated and eluted with buffer A.

To analyze the physical interaction of glucosylceramidase with lipid vesicles, the samples were incubated for 10 min at 37°C and then loaded on shorter Bio-Gel A-15m or Bio-Gel A-50m columns (10 × 200 mm). After elution with 17.5 ml of buffer A, 2% (w/v) TC was added to the eluent. A new column was used for each experiment. Fractions were tested for enzyme activity in the presence of detergents (see glucosylceramidase assay) immediately after elution. To examine the combined effect of OA and PS LUV on glucosylceramidase, the two lipid preparations were premixed and incubated for 10 min at 37°C.

The flow rate was 0.3 ml/min for all columns. Fractions of 0.5 ml were collected. Chromatographic procedures were conducted at room temperature. The lipid distribution was determined by measuring the fraction radioactivity. When two labelled lipids were present, double isotope counting conditions were adopted.

Ultracentrifugation analysis of the physical interaction between vesicles and glucosylceramidase. Aliquots of vesicles and glucosylceramidase were diluted to 0.25 ml with buffer A adjusted to pH 5.4. To examine the combined effect of OA and PS LUV, the two lipid preparations were premixed as for the gel permeation experiments (see above). After 10 min incubation at 37°C, the enzyme-vesicle mixtures were added with equal volumes of 30% (w/v) Dextran T40 (Pharmacia LKB Biotechnology, Uppsala, Sweden) and placed into an Ultra-clear™ centrifuge tube (13 × 51 mm) over a cushion of 1 ml of 30% Dextran. 2.3 ml of 10% Dextran were carefully layered over, followed by 1 ml of buffer A adjusted to pH 5.4. All Dextran solutions were made up in this buffer. Centrifugation was performed in a Beckman SW 55 Ti rotor for 1 h at 300 000 × *g* at 25°C. Fractions of 0.2 ml were pumped out from the bottom of the centrifuge tubes. To determine the enzyme content of the fractions, aliquots were added to the assay mixture containing detergents (see glucosylceramidase assay). The presence of vesicles was determined by radioactivity counting.

Reversibility of glucosylceramidase binding to PS liposomes. Glucosylceramidase (2800 U) was mixed with PS SUV (320 μg) supplemented with labelled PS and incubated at 37°C for 10 min in 200 μl of buffer A. Enzyme activity was determined by diluting 4 μl to 200 μl with buffer A containing 2.5 mM MUGlc. An aliquot of the sample was analyzed on a Bio-Gel A-15m column (10 × 200 mm; see above) to determine the percent of enzyme bound to the PS SUV. The glucosylceramidase-PS SUV mixture was added to two volumes of buffer A containing 45% (w/v) PEG and incubated at 37°C for 30 min. Enzyme activity was determined once again by diluting 12 μl to 200 μl with buffer A containing 2.5 mM MUGlc. To determine the enzyme bound to the PS vesicles after treatment with PEG, the sample was diluted with 6 volumes of buffer A and the fused liposomes were pelletted by centrifugation at 190 000 × *g* for 30 min. The pellet was resuspended in the original volume of buffer A. The amount of glucosylceramidase and vesicles in the supernatant and pellet was assessed by measuring the enzyme activity in the presence of detergents (see glucosylceramidase assay) and by radioactivity counting.

Results

Effect of PS liposome size on glucosylceramidase stimulation

Sonicated PS dispersions, the phospholipid preparations most commonly used to stimulate purified glucosylceramidase [5–7], consist of small unilamellar vesicles (SUV) slightly heterogeneous in size [28]. The liposome size distribution of sonicated PS was checked on a Bio-Gel A-15m column and the fractionated liposomes were assayed for their ability to activate glucosylceramidase. Fig. 1A shows that the liposomes had

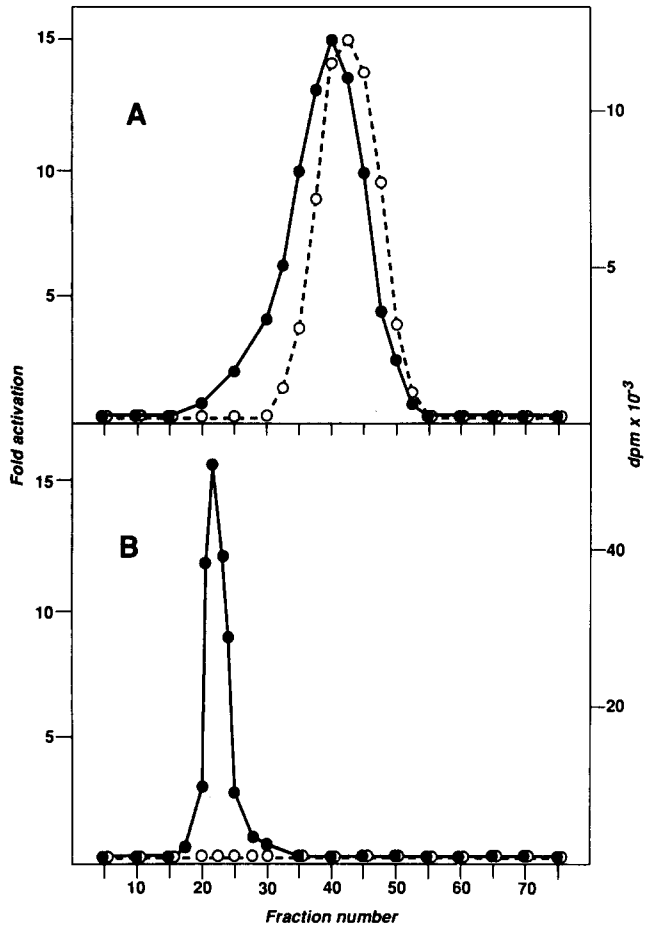

Fig. 1. Stimulation of glucosylceramidase activity by PS SUV and LUV fractionated on a Bio-gel A-15m column. 2 mg of either PS SUV (A) or LUV (B), supplemented with labelled PS as tracer, were loaded on a Bio-gel A-15m column (10×370 mm) and eluted with buffer A. An aliquot (1 μl) of the fractions was diluted to 200 μl with buffer A containing 2.5 mM MUGlc and 20 U of purified glucosylceramidase. The enzyme activity was measured without further additions. The fold-activation (○) refers to the ability of PS to stimulate the enzymatic reaction above the reaction with no PS. Vesicle distribution (●) was determined by measuring the fraction radioactivity.

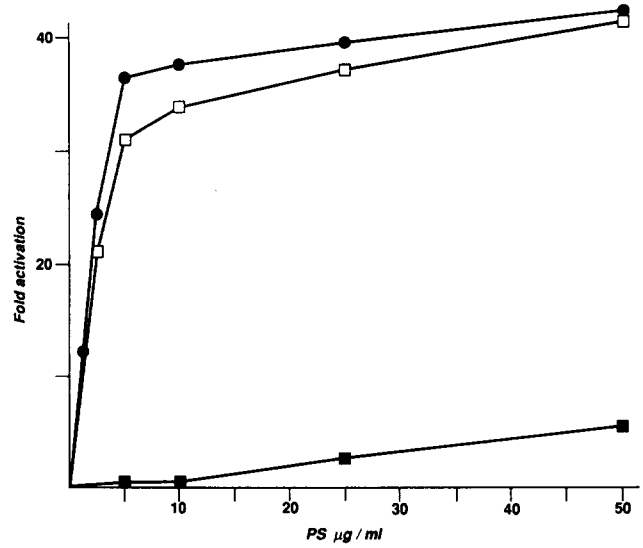

Fig. 2. Comparison of the activating capacity of PS SUV and LUV. Different amounts of PS SUV (●) and LUV (■) were added to 200 μl of buffer A containing 2.5 mM MUGlc and 20 U of purified glucosylceramidase. Enzyme activity was measured without further additions. PS LUV were tested also after 6 min sonication (□). The fold-activation refers to the ability of PS to stimulate the enzymatic reaction above the reaction with no PS.

different stimulating capacities, the activation of glucosylceramidase being higher with the fractions in the trailing edge of the peak. This result suggests that the larger PS liposomes of the SUV preparation are less effective than the smaller ones. To confirm this observation, the stimulation exerted by a homogeneous preparation of large unilamellar vesicles (LUV) was tested. PS LUV were generated by repetitive extrusion of multilamellar vesicles through polycarbonate filters with a pore size of 100 nm [24,25]. Their internal volume was 1.6 μl/μmol of phospholipid, a value in good agreement with that reported for these type of vesicles [24,25]. LUV, analyzed by gel permeation on Bio-Gel A-15m, eluted in a narrow peak close to the void volume and were found nearly devoid of stimulating capacity on the enzyme (Fig. 1B). To verify that this result was due to vesicle size and not to vesicle concentration – the latter decreases when the vesicle mean size increases – we determined the dependence of glucosylceramidase activation on the amount of PS in the assay (Fig. 2). Even at high concentrations, PS LUV were unable to induce activation comparable to that produced by SUV. A marked reduction of PS LUV size, obtained by extensive sonication and checked by chromatography on Bio-Gel A-15m (data not shown), caused a dramatic activation increase (Fig. 2). The opposite effect, namely a decreased activation by passing from the very effective PS SUV to larger PS vesicles, was obtained by incubating PS SUV with high concentrations of PEG (20–30%; Table I), a compound that causes extensive fusion of phospholipid SUV [29]. PS SUV fusion was ascertained by monitoring the increase of fluorescence produced by reduction in the efficiency of resonance energy transfer between *N*-NBD-PE and *N*-Rh-PE incorporated into PS SUV [27]. Table I shows that 30% PEG caused an almost complete fusion of the liposomes. Incubation of PS SUV with 10% PEG neither induced fusion nor decreased their activating capacity. As control, glucosylceramidase was incubated 30 min in 30% PEG. The enzyme's activity, measured after dilution in the assay mixture, was not affected by this treatment.

PS, sonicated in admixture with a non activating phospholipid such as PC [20], maintains its stimulating power provided the molar PS fraction is higher than 60% (Fig. 3). Below this percentage, the activation markedly decreases and glucosylceramidase can not be

TABLE I

Effect of PEG-induced fusion on the ability of PS vesicles to activate glucosylceramidase

A mixture (0.65 mM) of PS SUV containing both *N*-NBD-PE and *N*-Rh-PE at 1 mol% each, and PS SUV without the fluorescence probes at a molar ratio of 1:4 was incubated for 30 min at 37°C in buffer A, either in the presence or in the absence of different PEG concentrations. An aliquot (10 μl) of the mixture was then diluted to 200 μl with buffer A containing 2.5 mM MUGlc and 20 U of purified glucosylceramidase. Enzyme activity was measured without further additions. PEG-induced fusion of PS SUV was assessed as reported in Materials and Methods.

PEG addition % (w/v)	Fusion (%)	Enzyme activity (nmol/h)
0%	0	20
10%	2	25
20%	85	13
30%	98	2

effectively stimulated by the mixed liposomes even at high PS concentrations in the assay.

The activation exerted by PS/PC liposomes depended again on the mode of preparation of the vesicles; at a 25 μg PS/ml concentration sonicated PS/PC SUV (8:2) stimulated the enzyme 40-fold (Fig. 3), while the corresponding LUV prepared by extrusion gave a 5-fold activation.

Effect of PS liposome size on glucosylceramidase binding

We have previously shown that adsorption of the enzyme on acidic phospholipid-containing vesicles is a

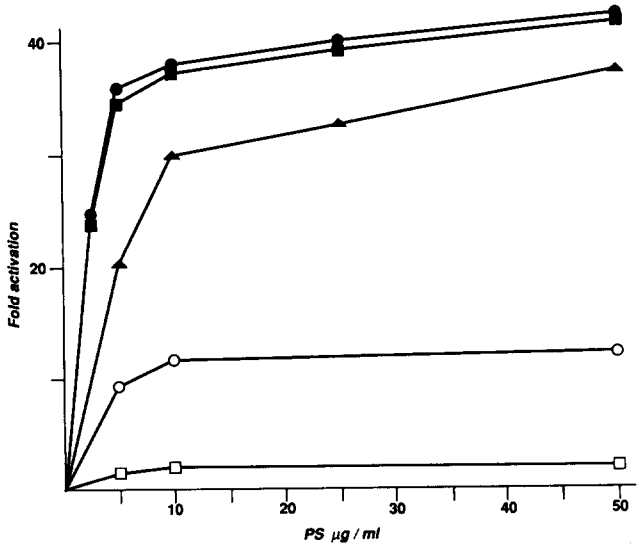

Fig. 3. Comparison of the activating capacity of PS/PC SUV containing different molar PS fractions. Different amounts of PS/PC SUV were added to 200 μl buffer A containing 2.5 mM MUGlc and 20 U purified glucosylceramidase. Enzyme activity was measured without further additions. The percentage of PS in the liposomes was either 20% (□), 40% (○), 60% (▲), 80% (■), or 100% (●). The fold-activation refers to the ability of PS/PC SUV to stimulate the enzymatic reaction above the reaction with no PS/PC SUV.

prerequisite for the reconstitution of purified glucosylceramidase [19,20]. Most likely, the poor activation obtained with PS LUV is the consequence of their inability to bind the enzyme. To determine the amount of glucosylceramidase associated with the liposomes, we have taken advantage of the anomalous behaviour of glucosylceramidase in gel permeation chromatography. In fact, it was observed that glucosylceramidase is retained by several gel-permeation matrices, the Bio-gel matrix included, and that this interaction is competed by the association of the enzyme with appropriate liposomes [20,33]. Fig. 4A shows that the enzyme incubated with PS SUV coelutes with the liposomes, while it is retained by a column of Bio-Gel A-50m when incubated with PS LUV (Fig. 4B). Noteworthy, the enzyme is preferentially bound to the smallest PS SUV, namely the most efficient as activators (see Fig. 1A). Thus, we conclude that no strong interactions occur between glucosylceramidase and large PS vesicles.

To investigate the reversibility of enzyme binding to the PS SUV surface, glucosylceramidase was first bound to PS SUV. Subsequently, the complex was incubated with 30% PEG, a treatment that induces liposome fusion (see Table I). The formation of larger vesicles resulted in the loss of enzyme activation. After centrifugation of the PS-enzyme mixture treated with PEG, 96% of the fused liposomes pelletted, while 90% of the enzyme remained in the supernatant. This result indicates the release of glucosylceramidase from the lipid surface. The released enzyme could be reactivated either by adding PS SUV or detergents.

Effect of OA on glucosylceramidase binding to PS LUV

The poor interaction of glucosylceramidase with PS LUV indicates that the presence of PS is not sufficient to promote glucosylceramidase reconstitution. We have previously shown that fatty acids are able to synergistically enhance the stimulatory power of acidic phospholipids [19,20]. In an effort to find a condition that could promote the binding of glucosylceramidase also to large liposomes, an OA dispersion was added to the PS LUV. The association of the fatty acid with PS LUV was indicated by the co-elution of the two compounds from a Bio-Gel A-50m column (Fig. 4C). Glucosylceramidase incubated with premixed PS LUV and OA also co-eluted with the lipids, a result indicating a tight association of the enzyme with the vesicle surface (Fig. 4C). In gel-permeation chromatography both OA and glucosylceramidase are retained by the Bio-Gel matrix. Therefore, it was impossible to establish whether OA alone could bind the enzyme. Flotation provided an alternative procedure suitable to examine the interactions between OA, PS LUV and glucosylceramidase. Experiments were performed by layering enzyme-vesicles mixtures near the bottom of a discontinuous

Dextran gradient. After incubation with premixed PS LUV and OA, the enzyme floated with the lipids (Fig. 5A). In the absence of OA, the enzyme remained near the starting position, while PS LUV moved towards the top of the gradient (Fig. 5B). The floating OA dispersion was also unable to bring glucosylceramidase to the upper part of the gradient (Fig. 5C). These results show the poor affinity of glucosylceramidase not only

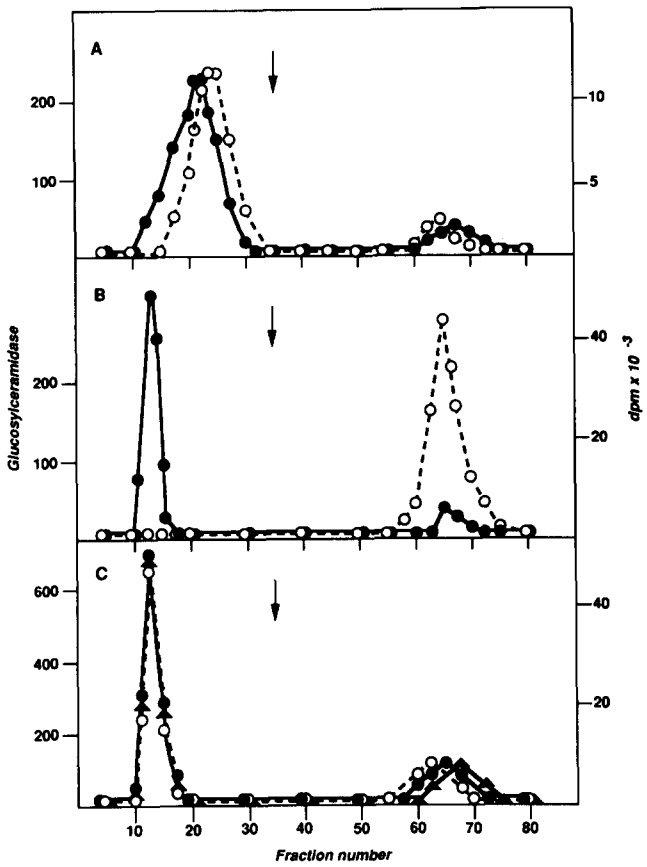

Fig. 4. Physical interaction of glucosylceramidase with PS SUV and LUV analyzed by chromatography on Bio-Gel columns. Purified glucosylceramidase (1000–1500 U) was incubated with PS SUV (200 μg) or with PS LUV (400 μg) or with a mixture of PS LUV (400 μg) and OA (100 μg) (see Materials and Methods). The first sample (A) was prepared in buffer A and loaded on a Bio-Gel A-15m column, the second (B) and third (C) samples were prepared in buffer A adjusted to pH 5.4 and loaded on Bio-Gel A-50m columns. The elution was performed as reported in Materials and Methods. The arrows indicate when TC was added to the elution buffer. PS vesicles were supplemented with trace amounts of labelled [^{14}C]PS (A and B) or labelled [^{3}H]PC (C) and their distribution (●) was determined by measuring either the carbonium (A and B) or the tritium radioactivity (C). The OA dispersion was supplemented with [^{14}C]OA and the fatty-acid distribution (▲) was determined by measuring the carbonium radioactivity. An aliquot of the fractions was also tested for enzyme activity (○) in the presence of detergents (see Materials and Methods). When the PS LUV-OA mixture was chromatographed in the absence of the enzyme, the elution of the two lipids was identical to that shown in (C). When the PS LUV-glucosylceramidase mixture was analyzed on a Bio-Gel A-15m instead of a Bio-Gel A-50m column, the vesicles and enzyme distribution was identical to that shown in (B).

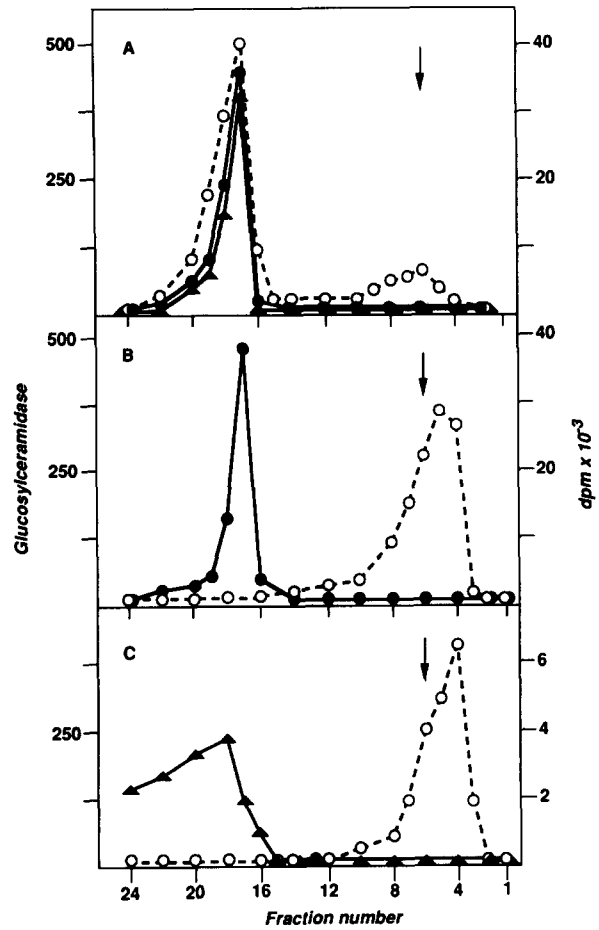

Fig. 5. Physical interaction of glucosylceramidase with PS LUV and OA analyzed by ultracentrifugation. Purified glucosylceramidase (1000–1500 U) was incubated with a mixture of PS LUV (400 μg) and OA (100 μg) (A) or only with PS LUV (400 μg) (B) or OA (100 μg) (C). The ultracentrifugation was performed as described in Materials and Methods. The arrows indicate where the samples were originally layered. The glucosylceramidase (○), PS (●) and OA (▲) distributions were determined as in Fig. 4. When glucosylceramidase was centrifuged in the absence of vesicles, the enzyme distribution was identical to that shown in (B). When the PS LUV-OA mixture was centrifuged in the absence of the enzyme, the distribution of the two lipids was identical to that shown in (A).

for PS LUV but also for the OA dispersion. The enzyme associates with the vesicles only after mixing of the two lipid preparations. The OA-concentration-dependence of glucosylceramidase binding to PS LUV shows that a tight association was obtained when the PS or PS/PC LUV were mixed with an equimolar amount of OA (Table II).

As expected, the association of glucosylceramidase with the PS LUV-OA complex was accompanied by activation of the enzyme (Table II); in the presence of OA, both PS or PS/PC LUV could stimulate glucosylceramidase to the same extent than PS SUV with only a slight difference in the pH optimum (pH 5.8 for PS SUV and pH 5.4 for the PS LUV-OA complex). When LUV were prepared from an equimolar mixture of PS

TABLE II

Effect of OA on the glucosylceramidase reconstitution by PS-containing LUV

Glucosylceramidase (5000 U/ml) was incubated at 37°C for 10 min with either PS LUV (1.25 mM) or PS/PC(6:4) LUV (2.1 mM total phospholipid) in buffer A adjusted to pH 5.4. PS-containing LUV were preincubated at 37°C for 10 min in the absence or in the presence of the indicated amounts of OA before glucosylceramidase addition. An aliquot of the enzyme-vesicle mixtures (10 μl) was diluted to 200 μl with buffer A (pH 5.4), containing 2.5 mM MUGlc, and the enzyme activity was measured without further additions. The percent of enzyme bound to the liposomes was determined by analyzing an aliquot of the samples on columns of Bio-Gel A-50m (see Fig. 4B and C).

Additions	Enzyme activity (nmol/h)	Percent bound enzyme
PS LUV	6	0
+ OA (0.62 mM)	14	20
+ OA (1.25 mM)	50	90
PS/PC LUV	5	0
+ OA (0.89 mM)	10	5
+ OA (2.4 mM)	54	95

and OA, their activating capacity was the same than that observed when OA was added exogenously to PS LUV. The OA dispersion itself did not exert a significant stimulation of the enzyme activity.

Discussion

In previous papers we demonstrated that purified glucosylceramidase reconstitutes its activity by binding to anionic phospholipid-containing vesicles of appropriate composition [19,20]. The results in this paper show for the first time that not only the composition but also the physical characteristics of the vesicles are crucial parameters in glucosylceramidase association and activation. We have found that, in contrast with PS SUV that adsorb glucosylceramidase at their surface, LUV of pure PS or PS in admixture with PC are unable to tightly bind the enzyme and to fully stimulate its activity. Treatments that modify the bilayer curvature, such as sonication of LUV or PEG-induced fusion of SUV, likewise affect enzyme binding and activity. Since the stimulation of the enzyme by PS/PC liposomes depends on the PS molar fraction, glucosylceramidase activity can be modulated by varying either the PS percentage or the size of PS-containing liposomes. Interestingly, an efficient activation by PS/PC SUV is achieved with a much higher PS percentage (60–80%) than that of PA or PS (20–40%) previously found necessary for activation in cholesterol-containing vesicles [20,30]. This difference might be related to a cholesterol-induced phase separation, a phenomenon observed in mixed phospholipid-cholesterol vesicles [31,32]; the formation of PA or PS-rich areas on the membrane most probably allow enzyme reconstitution at a lower PA or PS molar fraction.

The explanation of the different ability of PS SUV and LUV to reconstitute glucosylceramidase most likely resides in the packing difference between the highly curved outer surface of SUV and the relatively flat outer surface of LUV. The packing constraint of SUV translates into a partial exposure of the hydrophobic membrane interior, probably facilitating the interaction of the vesicles with hydrophobic regions of glucosylceramidase. Not only hydrophobic but also electrostatic interactions likely contribute to the association of glucosylceramidase with SUV, as indicated by the drastically different findings on glucosylceramidase interaction with negatively-charged phospholipids in contrast with neutral phospholipids [19,20].

We have found that the association between enzyme and PS liposomes is reversible. When PS SUV are transformed into larger structures by a fusogenic agent such as PEG, glucosylceramidase desorbs from their surface, loosing its ability to act on the substrate. In other words, a change of the bilayer organization can dramatically reduce glucosylceramidase activity. Several compounds, which inhibit the enzyme activity in the presence of PS or detergents, were employed to examine the properties of the glucosylceramidase active site [34,35]. Enzyme inhibition by a fusogen such as PEG – that does not influence the Michaelis parameters K_m and k_{cat} but rather affects the amount of glucosylceramidase present on the lipid surface by inducing a structural change of the PS liposomes – has never been envisaged before.

The finding that glucosylceramidase binds to PS SUV but not LUV may help to better understand the basic requirements of glucosylceramidase reconstitution. On the other hand, the properties of LUV more closely approach those of natural membranes, which usually do not exhibit the degree of curvature typical of SUV. Glucosylceramidase can also bind to PS-containing LUV but only after their association with an OA dispersion. How the presence of an unsaturated fatty acid can promote the glucosylceramidase binding to the PS LUV surface is not yet clear. In our experiments, OA interacts with preformed liposomes, most likely altering the phospholipid packing characteristics of the liposomal membrane. The formation of organizational defects in the bilayer surface following the association of long-chain fatty acids to phospholipid vesicles is well-documented [36–38]. Defect sites of the PS LUV bilayer induced by a perturbing agent such as OA could accomodate a hydrophobic part of the glucosylceramidase molecule. This interpretation is consistent with the fact that OA is not essential for the binding of the enzyme to PS SUV where some degree

of irregular packing already exists due to the high curvature of these bilayers.

Not only lipids but also proteins can play a role in the binding of glucosylceramidase to lipid surfaces. Actually, an endogenous protein(s), identified by us in placenta [23], reactivates the purified enzyme by promoting the binding of glucosylceramidase to glucosylceramide-containing vesicles [39].

From our past [19,20,23,39] and present data it appears that enzyme activation is highly complex, depending on the presence of lipids and proteins capable of promoting the glucosylceramidase association with vesicles. In all the cases we examined, the glucosylceramidase activation was correlated to the promotion of enzyme binding to an appropriate vesicle surface. The present results indicate that enzyme association occurs when the lipid surface containing acidic phospholipids also possesses packing irregularities. The observation that enzyme binding to PS-containing vesicles depends on the physical characteristics of the bilayer offers a new dimension in the regulation of glucosylceramidase activity.

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