Dual Inhibitory Effect of Gangliosides on Phospholipase C-Promoted Fusion of Lipidic Vesicles†

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ABSTRACT: The effect of a variety of gangliosides has been tested on the phospholipase C-induced fusion of large unilamellar vesicles. Bilayer composition was phosphatidylcholine:phosphatidylethanolamine: cholesterol (2:1:1 mole ratio) plus the appropriate amounts of glycosphingolipids. Enzyme phosphohydrolase activity, vesicle aggregation, mixing of bilayer lipids and mixing of liposomal aqueous contents were separately assayed. Small amounts (<1 mol %) of gangliosides in the lipid bilayer produce a significant inhibition of the above processes. The inhibitory effect of gangliosides increases with the size of the oligosaccharide chain in the polar head group. Inhibition depends in a nonlinear manner on the ganglioside proportion, and is complete at ∼5 mol %. Inhibition is not due to ganglioside-dependent changes in vesicle curvature or size. Ganglioside inhibition of vesicle fusion is due to two different effects: inhibition of phospholipase C activity and stabilization of the lipid lamellar phase. Enzyme inhibition leads to a parallel decrease of vesicle aggregation and lipid mixing rates. Mixing of aqueous contents, though, is depressed beyond the enzyme inhibition levels. This is explained in terms of the fusion pore requiring a local destabilization of the lipid bilayer, the lamellar structure being stabilized by gangliosides. ^{31}P-NMR and DSC experiments confirm the inhibitory effect of gangliosides in various lamellar-to-nonlamellar transitions.

Cell membrane fusion is an important physiological and pathophysiological process, in whose understanding at the molecular level considerable progress has been made in recent years. In particular, there is growing experimental support for the hypothesis that, underlying the multiform processes of cell and model membrane fusion, a single molecular mechanism involving certain structural changes in the membrane lipids is actually operating [for reviews see Monck and Fernandez (1994), Chernomordik and Zimmerberg (1995), and Chernomordik *et al*. (1995a)]. Those changes in lipid architecture are usually described in terms of the so-called "stalk hypothesis", where the stalk is a transient, highly-bent lipid intermediate (Chernomordik *et al*., 1987; Siegel, 1993). The newer experimental results (Stegmann, 1993; Siegel *et al.*, 1994) are not in agreement with the previously proposed existence of inverted micellar intermediates. Former work from this laboratory has dealt with the fusion of large unilamellar liposomes by the catalytic action of phospholipase C (Nieva *et al*., 1989, 1993, 1995; Burger *et al*., 1991). This model is unique among membrane fusion systems in that fusion is induced by a catalytic agent and thus it is susceptible of fine modulation. In particular, it is self-regulated by diacylglycerol, a product of the enzyme reaction and the putative fusogenic agent in the system (Nieva *et al*., 1993). Phospholipase C-induced liposome fusion is otherwise very sensitive to bilayer lipid composition (Nieva *et al*., 1989, 1995) and it is inhibited by lysophosphatidylcholine (Nieva *et al*., 1993), in common with other, different fusion systems (Chernomordik *et al*. 1993, 1995b; Yeagle *et al*., 1994).

Glycosphingolipids contain a sphingosine base in which the amino group forms an amide linkage with a fatty acyl residue to form ceramide. To the hydroxyl group in carbon 1′ of the ceramide moiety a number of carbohydrate residues, joined by glycosidic linkages, are linked [see Maggio (1994) for a review]. Gangliosides constitute an important group of glycosphingolipids, containing hexose(s), hexosamine(s), and sialic acid(s) in their sugar moieties. Glycosphingolipids are relevant to our studies of phospholipase C-promoted vesicle fusion in two ways, namely, that they may modulate phospholipase C activity and that they may modify the self-assembly and topology of phospholipid aggregates. Inhibition of phospholipase C by gangliosides, and stimulation by sulfatides, has been described in monolayer studies (Bianco *et al*., 1990; Perillo *et al*., 1994a) and also, more recently, in micellar and vesicular systems (Maggio *et al*., 1994; Daniele *et al*., 1996). Gangliosides are also known to have significant effects on the membrane curvature (Maggio *et al*., 1988; Thomas & Poznansky, 1989) and to stabilize the lamellar phase under circumstances favouring otherwise the lamellar−hexagonal (H_{II})[1] transition (Perillo *et al*., 1994b). In fact, there are several instances in which glycosphingolipids cause inhibition of model fusion processes (Hoekstra & Düzgünes, 1986; Melikyan *et al*., 1990).

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonate, Ch, cholesterol; DG, diacylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylenebis(pyridinium bromide); G, ganglioside; H_{II}, inverted hexagonal phase; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; R18, octadecylrhodamine B.

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Table 1: Structure and Nomenclature of Glycosphingolipids Pertinent to This Study

abbreviation	class	polar headgroup[a]	
	cerebroside	gal	(0)
	sulphatide	gal-SO_3^-	(-1)
GM3	monosialoganglioside	glu-gal(sia)	(-1)
Gg4Cer	tetraglycosylceramide	glu-gal-NAc-gal	(0)
GM1	monosialoganglioside	glu-gal-NAc-gal(sia)	(-1)
GD1a	disialoganglioside	glu-gal(sia)-NAc-gal(sia)	(-2)
GT1b	trisialoganglioside	glu-gal(sia,sia)-NAc-gal(sia)	(-3)

[a] Abbreviations: gal, galactose; glu, glucose; sia, sialic acid; NAc, *N*-acetylglucosamine. (In parentheses, net electric charge at neutral pH).

In view of the above results and hypotheses, we have undertaken a detailed study of the effects of a variety of glycosphingolipids (Table 1) on the enzyme activity of phospholipase C on large unilamellar vesicles basically consisting of PC:PE:Ch (2:1:1 mole ratio), i.e., the system in which optimum fusion activities were found (Nieva *et al*., 1989). The effects of adding various amounts and species of glycosphingolipids to that mixture have also been separately detected on the vesicle aggregation, lipid mixing and vesicle contents mixing processes elicited by the phospholipase C activity. As a result, we demonstrate that even small proportions of gangliosides (<1 mol %) significantly decrease the rate and extent of liposome fusion through the combined effect of enzyme inhibition and stabilization of the lipid lamellar phase. Some preliminary results have been previously published in a review on phospholipase C-induced liposomal fusion (Goñi *et al*., 1994).

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Lipid Products (South Nutfield, England); DOPC and DOPE were from Avanti Polar Lipids (Birmingham, AL); cholesterol (Ch) was from Sigma (St. Louis, MO). Octadecylrhodamine B (R18), 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), and *p*-xylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR).

Glycosphingolipids were purified from bovine brain as described previously (Fidelio *et al*., 1991). Briefly, crude gangliosides from beef brain gray matter were obtained from the upper phase after Folch's partitioning. This crude fraction was treated with 0.1 M NaOH in methanol, dialyzed against bidistilled water and freeze-dried. The resulting purified total ganglioside (Gtot) preparation contained 21% GM1, 42% GD1a, 18% GD1b, and 19% GT1b. Individual gangliosides were isolated by chromatography on Iatrobeads and Sephadex LH-20 columns (Fidelio *et al*., 1991, "Method D"). Gangliosides were at least 98% pure according to thin-layer chromatographic analysis (resorcinol-hydrochloride stain). Single batches of each glycolipid, or glycolipid mixture, were used throughout this work.

Vesicle Preparation and Vesicle Size Measurements. Large unilamellar vesicles (LUV) prepared by the extrusion method of Mayer *et al*. (1986) were used as the phospholipase C substrate. The lipid composition of these liposomes was PC/PE/Ch (2:1:1 mole ratio). When required, the appropriate amounts of glycosphingolipids were added. These amounts are indicated as *additional* mole percentages; thus a liposome composition containing 1% ganglioside is PC:PE:Ch:ganglioside (50:25:25:1 mol %). The aqueous lipid suspensions were extruded through Nuclepore filters, 0.1-μm pore diameter, unless otherwise stated. Average vesicle diameters were measured by quasielastic light scattering using a Malvern Zeta-Sizer instrument. Vesicles with diameters ranging between ca. 80 and 130 nm were obtained, according to the experimental conditions, as described under Results.

Enzyme Assays. Phospholipase C activity was assayed by determining phosphorus contents in the aqueous phase of an extraction mixture (chloroform:methanol, 2:1) after addition of aliquots from the reaction mixture at different times. Phosphorus was assayed according to Bartlett (1959). Protein concentration was assayed after Lowry *et al*. (1951).

Aggregation and Fusion Assays. Liposome aggregation and fusion was induced in suspensions 0.3 mM in lipid; the process was started by adding 1.6 units of enzyme/mL, corresponding approximately to the optimum ratio of 10 enzyme molecules/vesicle (Nieva *et al*., 1989). Vesicle aggregation was estimated by the increase in scattered light in an LS-50 Perkin Elmer spectrofluorimeter, by fixing the excitation and emission wavelengths at 520 nm.

Lipid mixing was measured by dilution in the bilayer of the self-quenching probe R18, as described by Hoekstra *et al*. (1984). The 0% fluorescence level (or 0% mixing) was determined from a 1:4 mixture of 8 mol % R18 containing liposomes and R18-free liposomes. The fluorescence of the same amount of liposomes with the diluted probe uniformly distributed, i.e., 1.6 mol % R18-containing liposomes, was taken as the 100% fluorescence level, or 100% lipid mixing. The use of fluorescent phospholipid derivatives of the kind rhodamine-PE for lipid mixing studies was precluded because those phospholipid probes were also good substrates for phospholipase C.

Mixing of aqueous vesicle contents, as well as vesicle leakage, was estimated using the ANTS/DPX fluorescent probe system described by Ellens *et al*. (1985). Three liposome preparations were used, loaded with (a) 50 mM ANTS, 90 mM NaCl, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0; (b) 180 mM DPX, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0; or (c) 25 mM ANTS, 90 mM DPX, 45 mM NaCl, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.0. Nonencapsulated material was removed from the vesicles using a Sephadex G-75 column, with 10 mM HEPES, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.0, as the elution buffer. This buffer was also used in all the fusion and enzyme assays. The osmolalities of all solutions were measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany) and adjusted to 0.4 osm/kg by adding NaCl.

Fluorescence scales were calibrated for fusion and release assays as described previously (Nieva *et al*., 1989). Briefly, the 100% fluorescence level (or 0% fusion) was set by using a 1:1 mixture of ANTS and DPX liposomes. The fluorescence level corresponding to 100% mixing of contents was determined from 0.3 mM liposomes containing coencapsulated ANTS and DPX; the value so obtained corresponds to either 100% fusion of 0% leakage. The 100% fluorescence level for leakage was obtained by detergent lysis of the liposomes containing both ANTS and DPX. Corrections for differences in the amount of entrapped solutes in the various vesicle preparations were routinely carried out after measuring the ratio of ANTS fluorescence before and after the addition of excess detergent (5 mM Triton X-100). The fluorescence change of a preparation containing 0.15 mM ANTS liposomes plus 0.15 mM "empty" liposomes (i.e., buffer-loaded) was routinely subtracted from the ANTS/DPX fluorescence signal in order to account for scattering and other possible artifacts. Since the aggregates, under our measuring conditions, may involve a large number of vesicles, fusion rates and maximal fusion values were directly estimated from the degree of ANTS quenching at the required time point. No further corrections were made over those values. The lag times were calculated on the time course curves as the time for the maximum slope line to intersect with the 0% effect level base line. Assays were performed in thermostated cuvettes with constant stirring, in an LS-50 Perkin Elmer spectrofluorimeter. Excitation light was adjusted to 355 nm, and emission light was adjusted to 520 nm. An interference filter (450 nm) was used to avoid scattered excitation light.

Differential Scanning Calorimetry. For high-sensitivity differential scanning calorimetry the lipid dispersions (2−8 mg/mL) in 10 mM TES, 10 mM $MgCl_2$, 100 mM NaCl, 5 mM EDTA, pH 7.4 buffer, were kept at 0 °C for 2 days before scanning with a Microcal MC2D calorimeter at a rate of 0.5 °C/min, as described by Maggio *et al*. (1985). Similar results were found at a scan rate of 1 °C/min.

^{31}P-NMR Spectroscopy. ^{31}P-NMR spectra were recorded in a KM360 Varian spectrometer, operating at 300 MHz for protons. Spectral parameters were as follows: 45° pulses (10 μs); pulse interval, 3 s; sweep width, 16 kHz; full proton decoupling. 1000 FID were routinely accumulated for each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature and contained ~0.2 M lipid in 5 mm tubes.

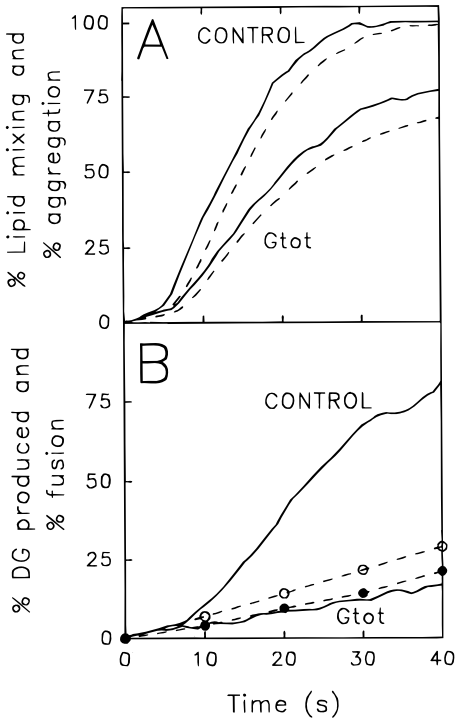

FIGURE 1: Ganglioside inhibition of phospholipase C-promoted vesicle fusion. Control: large unilamellar vesicles consisting of PC:PE:Ch (2:1:1 mole ratio). Gtot: plus 0.1 mol % total gangliosides. A. Continuous lines, vesicle aggregation; broken lines, lipid mixing. B. Continuous lines, mixing of vesicle aqueous contents (fusion); (○) % diacylglycerol production, control; (●) % diacylglycerol production in the presence of 0.1 mol % total gangliosides.

RESULTS

Phospholipase C, when acting on vesicles made of PC:PE:Ch (2:1:1), yields diacylglycerols (DG) which, in turn, lead to vesicle aggregation, intervesicle lipid mixing and mixing of vesicle aqueous contents. These observations are taken together as indicative of vesicle−vesicle fusion (Nieva *et al*., 1989). When the lipid membranes are "doped" with small amounts of glycosphingolipids, both the enzyme activity and the changes in vesicle architecture are clearly inhibited. Figure 1 shows the effect of adding 0.1 mol % of total gangliosides (Gtot) to the original lipid mixture: phosphohydrolase activity, vesicle aggregation, and lipid and aqueous contents mixing are all noticeably depressed, particularly the latter parameter, as will be discussed below.

It is known that incorporation of gangliosides to lipid mixtures leads to increased radii of curvature in the corresponding vesicles, i.e., to smaller liposomes (Maggio *et al*., 1988). In fact, in our system, the presence of glycosphingolipids does lead to vesicles of slightly smaller diameters (Table 2). In turn, the initial diameter may greatly influence the outcome of the experiment (Alonso *et al*., 1981), so that glycosphingolipids might be thought to act through changes in the initial vesicle size. However, the extrusion method of Mayer *et al*. (1986) allows a rather fine tuning of vesicle size, irrespective of composition, by using different filter pore sizes. Thus two vesicle preparations were made, one containing the standard lipid composition, of average diameter 128.9 nm, and another containing in addition 1 mol % total gangliosides, of average diameter 131.7 nm. In this case the presence of glycosphingolipid decreased the ag-

Table 2: Inhibitory Effect of Glycolipids on the Rates of Phospholipase C Phosphohydrolase Activity, Vesicle Aggregation, and Vesicle Fusion (Mixing of Aqueous Contents)[a]

lipid composition	mol % glycosphingolipid	average diameter (nm)	hydrolysis rate	aggregation rate	fusion rate
control	0	128.7 ± 1.3	100	100	100
+GM3	0.25	119.5 ± 1.5	84.7	90.1	26.8
+GM3	1	115.3 ± 2.5	21.3	24.9	3.2
+Gg4Cer	0.25	118.7 ± 2.0	56.1	67.2	18
+GM1	0.25	120.1 ± 6.5	50.0	55.1	10.5
+GM1	1	112.1 ± 1.7	14.3	15.5	1.8
+Gtot	0.1	122.1 ± 1.5	77	75.2	14.5
+Gtot	0.25	110.3 ± 1.8	16.9	24.4	3.1
+Gtot	1	103.6 ± 2.8	6.1	8.3	0.3
+GT1b	0.25	107.2 ± 0.7	10.4	12.8	1.2
+GT1b	1	101.7 ± 2.1	3.6	5.5	0.1

[a] Percent values relative to a glycolipid-free liposome composition, PC:PE:CHOL (2:1:1). Data taken from experiments as shown in Figure 3.

gregation rate to 6.0% of the control, thus showing that inhibition by glycolipid is not due to changes in vesicle diameter. Moreover, it was found that decreasing vesicle size, at constant lipid composition, led to an increased aggregation rate, e.g., vesicles of diameter 84.7 ± 6.1 nm aggregated at rates ~5-fold higher than vesicles 128.9 ± 1.2 nm in diameter, thus actual inhibition would be in any case larger than measured in our system. The initial size argument could also be applied to the experiments in which inhibition of contents mixing is observed, since smaller vesicles would mean smaller volumes and smaller amounts of fluorescent probes, thus smaller fluorescence changes and apparently inhibited fusion. However, it can be readily calculated that, in the extreme case of ganglioside concentrations leading to a 20% reduction in diameter, the reduction in spherical volume would be of ~49%, while much larger inhibitions of the extent of aqueous contents mixing (by >95%) are observed (data not shown).

The inhibitory phenomenon is dependent on the amount and nature of the glycolipid. Figure 2A shows the inhibition of phospholipase C-induced increase in light scattering (vesicle aggregation) in the presence of 1 mol % of various pure glycosphingolipids and of a glycolipid mixture. When these results are analyzed in the light of the glycolipid structural data (Table 1), it is clear that the inhibitory potency of glycosphingolipids is mainly related to the number of the sugar units in their polar headgroup. In addition to the glycolipids mentioned in Figure 2A, cerebrosides (e.g., galactosylceramide) and sulfatides (e.g., sulfogalactosylceramide) were tested in a similar way, but concentrations of these reagents of up to 5 mol % in the bilayer did not have any inhibitory effect (actually, 5% sulfatide produced some enhanced aggregation). These results strengthen and extend previous conclusions, using vesicles with different lipid composition (Maggio *et al*., 1994), that the inhibition of various stages of membrane bilayer interactions by gangliosides depends on a relatively large (Hoekstra & Düzgünes, 1986; Maggio & Yu, 1992) and hydrated (Montich *et al*., 1985; 1988; Mueller & Blume, 1993; Bagatolli *et al*., 1995) oligosaccharide head group in the glycolipid.

As mentioned above, gangliosides produce already a detectable inhibition when mixed at 1/1000 mole ratio with the other lipids. Figure 2B shows a nonlinear dose-dependent inhibitory effect of the aggregation rate, with 100% inhibition observed at ~5 mol %. The proportions producing 50% inhibition for each glycolipid or mixture are as follows: GM3, 0.67%; Gg4Cer, 0.57%; GM1, 0.51%; Gtot, 0.37%, and GT1b, 0.32%.

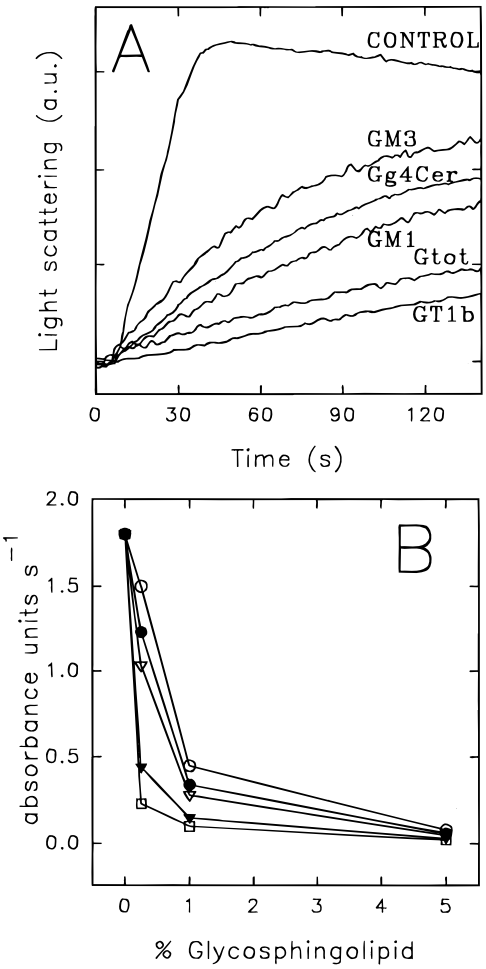

FIGURE 2: Role of the sugar moiety in glycosphingolipid inhibition of phospholipase C-induced vesicle aggregation. A. Time course of vesicle aggregation in the absence (control) or in the presence of 1 mol % of various glycosphingolipids in the lipid bilayer. See Table 1 for lipid structures. B. Rate of vesicle aggregation as a function of glycolipid concentration in the lipid bilayers; (○) GM3, (●) Gg4Cer, (▽) GM1, (▼) Gtot, (□) GT1b.

As noted in Figure 1, the inhibitory effect of gangliosides on vesicle fusion is particularly remarkable when the mixing of aqueous contents is considered, i.e., inhibition of contents mixing is much more pronounced than inhibition of vesicle aggregation, or lipid mixing, or enzyme activity. This is exemplified in more detail in Figure 3. Some degree of parallelism appears to exist between inhibition of phosphohydrolase enzyme activity (Figure 3A) and inhibition of

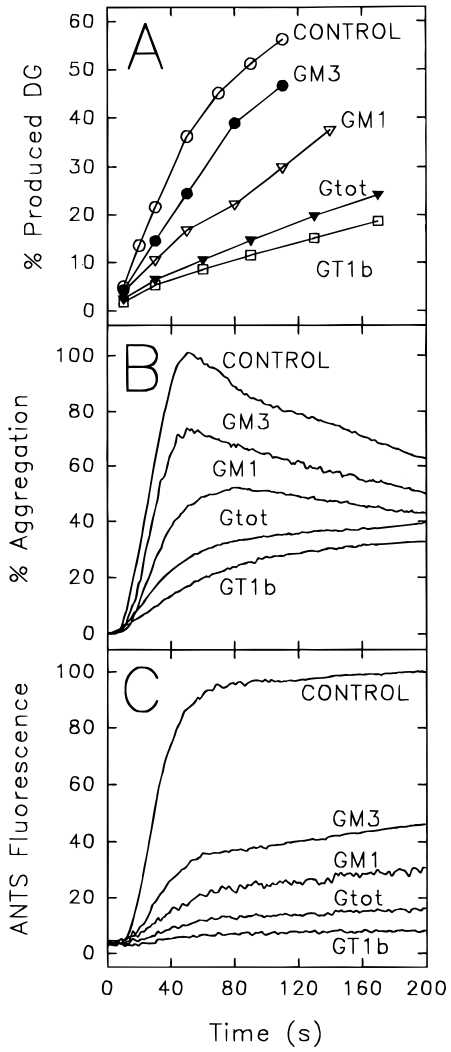

FIGURE 3: Correlation between (A) inhibition of phosphohydrolase activity, (B) inhibition of vesicle aggregation, and (C) inhibition of mixing of liposomal aqueous contents. See Table 1 for lipid structures

vesicle aggregation[2] (Figure 3B). However, the extent and rate of mixing of aqueous contents (Figure 3C) are inhibited beyond parallel. The same effect is shown, for a variety of experimental conditions, in Table 2. In all cases, the presence of inhibitor leads to fusion (contents mixing) rates dramatically lower than the corresponding hydrolysis or aggregation rates.

In summary the above data show that gangliosides exert a twofold inhibitory effect on phospholipase C-promoted liposome fusion: an inhibition of enzyme activity that leads apparently to a similar decrease in vesicle aggregation and lipid mixing and a further inhibitory process that prevents vesicles in close contact from mixing their aqueous contents. As detailed in the Discussion, ganglioside inhibition of phospholipase C has been described earlier in monolayers (Bianco *et al*., 1990; Perillo *et al*., 1994b) and may be explained by the regulation of the glycosphingolipids at different steps of the interfacial catalysis (Bianco *et al*., 1990; Maggio *et al*., 1994; Perillo *et al*., 1994b). With respect to the specific inhibition of contents mixing, and considering the proposed involvement of isotropic lipid structures in the vesicle−vesicle contact patches during fusion (Nieva *et al*., 1995), it has recently been shown that gangliosides at very low proportions modulate in a dual manner the formation of hexagonal II phases induced either by temperature or by compositional variation, depending on their relative mole fraction in the lipid phase; at proportions above 0.5 mol % ganglioside GD1a and 2 mol % GM1 they stabilize the lamellar phase (Maggio, 1994; Perillo *et al*., 1994a), thus inhibiting the formation of water-permeable isotropic phases (i.e., inhibiting contents mixing by a mechanism that is independent of but superimposed with enzyme inhibition).

This hypothesis was tested for the case of phospholipase C-induced fusion by examining the effect of glycosphingolipids on the thermotropic lamellar-to-nonlamellar transitions in an aqueous lipid dispersion containing PC:PE:Ch:DG (47:23:25:5). Although the parent mixture PC:PE:Ch (2:1:1) is known to be lamellar between 20 and 80 °C, the one containing 5% DG displays a lamellar to isotropic transition centered at 52.5 °C (Nieva *et al*., 1995). As shown in Figure 4, when total gangliosides are added up to 1 mol % to the mixture containing 5% DG, the transition is shifted towards higher temperatures by ∼10 °C. Thus, similar to their effect in different lipid mixtures containing PE or DG (Maggio, 1994; Perillo *et al*., 1994a), defined proportions of gangliosides can also stabilize the lipid lamellar phase of the mixture employed in the present studies, making more difficult the formation of nonlamellar and, particularly, of isotropic phases. The effect is observed for a variety of pure and mixed glycosphingolipids (Table 3) and the temperature shift is seen to be dose-dependent, as was the case with fusion inhibition.

As a further test of the lamellar phase-stabilizing properties of glycolipids, using a different system and different techniques, the lamellar-to-hexagonal phase transition of a DOPE:DOPC (5:1) mixture was studied. This is known to occur as a narrow transition centered at ∼32 °C and is shown in Figure 5A as detected by differential scanning calorimetry. DOPC alone does not reveal any thermotropic transition in the temperature range under study. The lamellar-to-hexagonal transition is also detected by a maximum of phospholipase C activity (Figure 5C), while again DOPC fails to show any temperature effect. The effect of ganglioside GD1a on this transition is shown in panels B and D of Figure 5. At 1 mol % GD1a (DOPE:DOPC:GD1a 5:1:0.06) the thermotropic transition becomes wider and is shifted to ∼38 °C, while at 3 mol % GD1a the transition is no longer detected. Enzyme activity follows exactly the same pattern. Thus the stabilizing effect of the lamellar phase, concomitant to the inhibition of phosphohydrolase activity by ganglioside, is further confirmed by two other techniques with different lipid systems.

DISCUSSION

The main observation in this paper is that gangliosides inhibit phospholipase C-promoted liposomal fusion both by directly inhibiting the enzyme and by stabilizing the lipid bilayer. In turn, these findings may be relevant to some of the current ideas on the mechanism of membrane fusion.

Gangliosides as Phospholipase Inhibitors. Bianco *et al*. (1989) showed that several glycosphingolipids, which are

[2] Time-dependent curves of light scattering sometimes show a maximum value and a decrease afterward. This should not be interpreted as a stop and/or reversal of the aggregation process but rather as an artefact produced when the aggregate size becomes large with respect to the wavelength of the incident light and conditions for Rayleigh scattering do no longer prevail (Viguera *et al*., 1995).

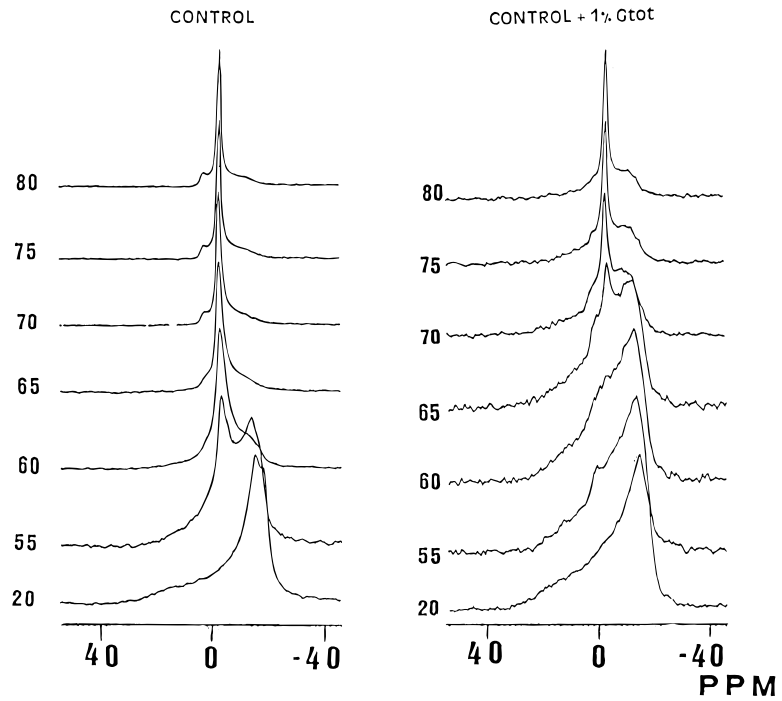

FIGURE 4: ^{31}P-NMR spectra of phospholipid:cholesterol aqueous dispersions, recorded at different temperatures. Control, PC:PE:Ch:DG (47:23:25:5 mole ratio). Spectral shifts in ppm relative to ortophosphoric acid. Lipid concentration ~0.2 M. Temperatures (in °C) are given by each curve.

Table 3: Lamellar-to-Isotropic Midpoint Transition Temperatures of Ganglioside-Containing Lipid Mixtures, as Derived from ^{31}P-NMR Spectra

lipid composition	T_m (°C)
0% gangliosides (control)[a]	52.5
0.5% total gangliosides	55
1% total gangliosides	60
3% total gangliosides	65
1% monosialogangliosides	57.5
1% disialogangliosides	60
1% trisialogangliosides	65
3% monosialogangliosides	62.5
3% disialogangliosides	65

[a] Control: PC/PE/CHOL/DG (47:23:25:5).

not substrates for phospholipases, can modulate the activity of phospholipase A_2 without acting directly on the active centre or the interfacial recognition region of the enzyme. In particular, gangliosides exhibited markedly inhibitory effects. These observations were later extended to the phospholipase C activity from *Clostridium perfringens* (Bianco *et al*., 1990). All of the above experiments were carried out with the lipids in monolayer form. The glycosphingolipid effects were observed at high proportions, i.e., >10% of the total lipid. More recently, Maggio *et al*. (1994), Perillo *et al*. (1994b), and Daniele *et al*. (1996) reported ganglioside inhibition respectively of phospholipase A_2 and of phospholipase C (from *B. cereus*) activities when the substrate was in the form of small unilamellar vesicles. In the latter studies, gangliosides were generally used at 10% concentration with respect to total lipids. In all cases, the inhibitory effect of gangliosides increases with the complexity of the polar head group (Bianco *et al*., 1989, 1990; Maggio *et al*., 1994). For phospholipase A_2, the glycosphingolipid effect has been correlated with the type of electrostatic field configuration across the interface brought about by the ganglioside polar headgroup dipoles (Perillo *et al*., 1994a). In general, glycosphingolipids appear to be acting at the level of the lipid−water interface, by modifying the catalytic activity of the adsorbed enzyme and/or by altering the availability of substrates in an appropriate conformation (Daniele *et al*., 1996). The results in the present paper (Figures 1 and 3) show that, when the substrate is present in the form of LUV, the inhibitory potency of gangliosides is much higher, since they inhibit all enzyme activity at or below 5%, i.e., at mole fractions that still allow reasonable activities in monolayers and small unilamellar vesicles. Otherwise, our observations on the effect of glycosphingolipids on phospholipase C activity are in complete agreement with the previous studies.

Gangliosides and the Stability of Lamellar Phases. The packing properties of lipids in bilayers and non-bilayer structures have been rationalized, among others, by Israelachvili *et al*. (1980) in terms of molecular shapes and by Helfrich (1973) according to the curvatures of the various aggregates. Gangliosides would be lipids of conical shapes in the former nomenclature and lipids of positive curvature in the latter. This refers to a propensity of gangliosides to form micelles, which they do indeed exhibit, either as pure or in combination with "cylindrical" (i.e., bilayer-forming) phospholipids, such as phosphatidylcholine (Maggio *et al*., 1988; Corti *et al*., 1988). The conical shape of complex gangliosides, with a large cross-sectional area in relation to the length of the hydrocarbon portion, opposes the curvature distortion ("negative curvature") required for the formation of hexagonal II or certain cubic phases (Seddon, 1990; Mariani *et al*., 1988). Along these lines, Perillo *et al*. (1994b) have shown that gangliosides inhibit the lamellar-to-H_{II} hexagonal phase transition in various lipid mixtures. The above results (Figures 4 and 5, Table 3) show the lamellar stabilizing properties of gangliosides in a lamellar-to-H_{II} hexagonal transition (Figure 5) and also in a lamellar-to-isotropic [presumably cubic; see Nieva *et al*. (1995)]

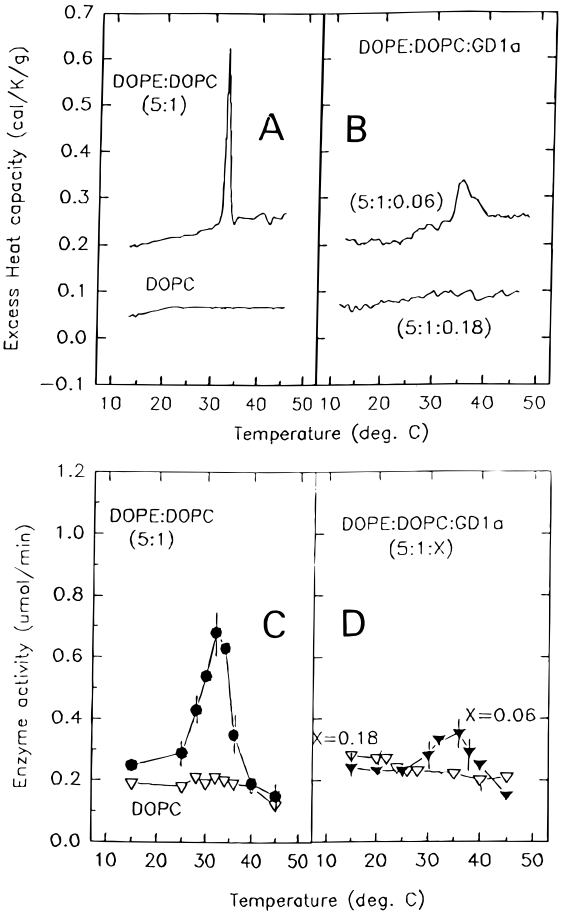

FIGURE 5: Effect of ganglioside GD1a on the lamellar-to-H_{II} phase transition of DOPE:DOPC (5:1 mole ratio). A. Differential scanning calorimetry thermograms of DOPE:DOPC (5:1) and of pure DOPC. B. Thermograms of DOPE:DOPC:GD1a mixtures containing, respectively, 1% and 3% ganglioside. C. Phospholipase C activity; lipid preparations as in A. D. Phospholipase C activity; lipid preparations as in B.

transition (Figure 4 and Table 3) in accordance with the results and hypotheses just mentioned.

It should be emphasized that the effect of gangliosides is exerted synergistically but independently on the topological level of the bilayer recombination and on phospholipase activity since either process can be regulated by gangliosides in the absence of the other (Maggio & Yu, 1992; Bianco *et al*., 1990, 1991; Maggio *et al*., 1994; Perillo *et al*., 1994a,b).

Gangliosides in Model and Cell Membrane Fusion. It is now generally accepted that the membrane fusion process can be divided into several stages (Bentz *et al*., 1988), namely, membrane apposition (or vesicle aggregation), mixing of lipids in the outer bilayers ["hemifusion", Chernomordik *et al*. (1995b), or "close apposition", Viguera *et al*. (1993)], and formation of the fusion intermediate (stalk, pore, etc.), leading to full lipid mixing and mixing of aqueous contents from both compartments. Our results with the *B. cereus* enzyme reproduce qualitatively earlier results on the marked inhibition by gangliosides of bilayer merging (hemifusion) and fusion induced by myelin basic protein or melittin and calcium in bilayer vesicles (Hoekstra & Düzgünes, 1996; Maggio & Yu, 1989, 1992). Myelin basic protein is one of the first membrane-associated proteins described to induce cell membrane fusion (Monferrán *et al*., 1979). It is interesting that both of these proteins can induce hexagonal II phases (Smith & Cornell, 1985; Batemburg *et al*., 1988), whose formation is modulated in a composition-dependent manner by the proportion of gangliosides in the lipid bilayer (Maggio, 1994; Perillo *et al*., 1994). The data in Figures 1 and 3, and in Table 2, show that the ganglioside-dependent inhibition of phospholipase C leads to a parallel inhibition of vesicle aggregation and lipid mixing and to a much stronger inhibition of contents mixing. This has at least two important consequences. One is that diacylglycerol formation, i.e., the result of enzyme activity, is directly linked to vesicle aggregation. This was suggested by us previously (Nieva *et al*., 1993), and the present parallelism between phosphohydrolase activity and liposome aggregation rates strongly confirms that hypothesis. The second important point is that the inhibitory effect of gangliosides at the level of lipid topology appears to affect content mixing but not lipid mixing in the vesicles. This helps to establish hemifusion and pore formation as two entities that can be separately studied and shows that it is only at the latter stage when a propensity toward a negative curvature is required from the lipids. In fact, in terms of the stalk hypothesis (Chernomordik *et al*., 1987; Siegel, 1993), a strongly-bent structural intermediate should be formed involving the two outer monolayers of the apposed vesicles. Maggio *et al*. (1988b) and Thomas and Poznansky (1989) showed that in SUV or in vesicles the size of our LUV gangliosides exhibited some preference for the outer monolayer, this fact also contributing to an enhanced resistance to negative bending. Theoretical work by Siegel (1993) showed that factors stabilizing the spontaneous curvature of the membrane should inhibit fusion pore formation to a larger extent than lipid mixing. This is also in agreement with the results presented here.

Also noteworthy is the observation that studies on lipid phase transitions, as shown in Figures 4 and 5, can serve as a guide to predict the behavior of a given lipid composition in a fusion event (e.g., Figures 1 and 3). This extends our previous observations with phospholipid:cholesterol:diacylglycerol systems (Nieva *et al*., 1995) and reinforces as well the idea of a fusion "structural intermediate" (Nieva *et al*., 1993) that may well correspond to the previously mentioned stalk.

The inescapable question on the biological relevance of the above observations cannot receive a fully satisfactory answer as yet. However, phospholipase C (and Ca^{2+} in millimolar concentrations) has been found to be involved in a physiological fusion process, namely, sperm exocytosis, and is probably important in other exocytotic processes (Roldán & Harrison, 1989; Spungin *et al*., 1995). Moreover, the growing consensus on a single topological mechanism underlying the various fusion processes (see introduction) increases the validity and relevance of studies involving model systems. In this context, the variety, ubiquity, and varied physiological roles of glycosphingolipids (Maggio, 1994) make them excellent candidates for regulating membrane lipid topology.

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