Influence of Glycolipid Oligosaccharide and Long-Chain Base Composition on the Thermotropic Properties of Dipalmitoylphosphatidylcholine Large Unilamellar Vesicles Containing Gangliosides†

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ABSTRACT: The thermotropic behavior of dipalmitoylphosphatidylcholine large unilamellar vesicles containing gangliosides has been studied by high-sensitivity heating and cooling differential scanning calorimetry. These studies have been directed to identify and evaluate the influence of both the ganglioside lipidic portion and oligosaccharide moiety on the physical properties of phospholipid bilayers containing gangliosides. The influence of the ganglioside lipidic portion has been evaluated by studying the behavior of vesicles containing different GD1a molecular species carrying homogeneous lipid moieties (C20 or C18 sphingosine or sphinganine and stearic acid). The influence of the ganglioside saccharide portion was evaluated by investigating the thermotropic behavior of vesicles containing different gangliosides (GM1, Fuc-GM1, GD1a, GT1b) carrying the same homogeneous long-chain base moiety (C20 sphingosine and stearic acid). These studies, in conjunction with previous studies using homogeneous lipidic portion ganglioside GM1 and phosphatidylcholines of various chain lengths [Masserini, M., & Freire, E. (1986) *Biochemistry 25*, 1043–1049], indicate that, for a given oligosaccharide composition, gangliosides exhibit lateral phase separation in an extent dependent upon the length and unsaturation difference between the ganglioside long-chain base and phosphatidylcholine acyl chains. For a given ganglioside lipidic composition the extent of phase separation is dependent upon the number of sugar units present in the glycolipid. The addition of Ca^{2+} induces or enhances phase separation in a manner dependent on the long-chain base and oligosaccharide composition. Cooling differential scanning calorimetry experiments showed that the ganglioside property to form aggregates within the membrane is independent of the initial physical state of the bilayer. The analysis of the lipidic and saccharidic composition dependence of the thermotropic behavior suggests that geometrical and chemical factors contribute to determine the aggregative properties of gangliosides.

Gangliosides, sialic acid containing glycosphingolipids, occur in the plasma membrane of vertebrate cells and especially in brain (Leeden, 1978; Tettamanti et al., 1980) as different species, a number of which have been isolated and structurally characterized [for a review see Wiegandt (1985)]. The biological functions of gangliosides such as the recognition of external ligands and the biotransduction of membrane-mediated information (Brady & Fishman, 1978; Ando, 1983) are generally viewed in function of the oligosaccharide portion of these molecules, although the importance of the lipidic portion in determining the physicochemical and biochemical properties of these molecules has already been recognized (Kannagi et al., 1982; Yohe et al., 1976).

In a previous paper (Masserini & Freire, 1986) the ganglioside lipidic portion was shown to affect the thermotropic properties of phospholipid vesicles containing gangliosides, particularly the lateral phase separation of the glycosphingolipid within the membrane. To continue the investigation aiming at elucidating the role of the ganglioside ceramide portion in membrane organization and function, we have studied the thermotropic behavior of fused unilamellar vesicles composed of dipalmitoylphosphatidylcholine containing highly purified ganglioside species, homogeneous both in the ceramide and oligosaccharide moieties. These studies have been performed by using heating and cooling high-sensitivity differential scanning calorimetry (DSC) to evaluate the influence of the gel and fluid phases of the membrane on ganglioside phase separation. The membrane perturbation induced by these glycosphingolipids has been evaluated as a function of the oligosaccharide portion for a given ceramide composition and as a function of the ceramide portion for a given oligosaccharide composition. For these experiments the gangliosides molecules were incorporated into the outer layer of phospholipid vesicles (Felgner et al., 1980), mimicking the asymmetric distribution found in the plasma membrane (Wiegandt, 1982).

Materials and Methods

Commercial chemicals were of analytical grade. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Biochemicals (Birmingham, AL) and used without further purification provided that it gave a single spot when assayed by thin-layer chromatography (TLC) [solvent system, chloroform/methanol/water 60:35:4 (v/v/v); 2-h run at 20 °C; spots revealed with iodine].

Gangliosides GM1, GD1a, and GT1b were extracted and purified from bovine brain according to the method of Tettamanti et al. (1973). Fucose-GM1 (Fuc-GM1) was extracted and purified from pig brain as described by Sonnino et al. (1978). Identification, structural analysis, and purity were assayed as described by Sonnino et al. (1978). During the purification step by silica gel column chromatography only

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Table I: Lipid Composition of the Gangliosides Used in the Present Investigation

	long-chain base (%)				fatty acids (%)			
ganglioside	C18:1	C18:0	C20:1	C20:0	16:0	18:0	20:0	others, C18:1
GD1a	>99				3.2	91	2.5	3.3
C18:0 GD1a		>99			2.8	94	2.1	1.1
C20:1 GD1a			>99		1.3	95.6	1.9	1.2
C20:0 GD1a				>99	1.2	92	3	3.8
C20:1 GM1			>99		1.8	93	3.1	2.1
C20:1 Fuc-GM1		>99			1.6	94	3	1.4
C20:1 GT1b			>99		0.5	91.1	4.2	4.2

the fractions corresponding to the central portion of the eluted peaks were further processed. These fractions contained the highest percentage of stearic acid. The final purity of GM1, Fuc-GM1, GD1a, and GT1b preparations, not yet homogeneous in the lipidic composition, was over 99% referred to the oligosaccharide moieties. Further separation of GM1, Fuc-GM1, GD1a, and GT1b gangliosides into molecular species with homogeneous long-chain base composition was attained by reverse-phase HPLC as described by Gazzotti et al. (1984) with the modifications introduced by Sonnino et al. (1986). The eluted fractions were dried, dissolved in water, dialyzed, and centrifuged at 40000 rpm for 30 min. The supernatants were lyophilized and further purified by precipitation with cold acetone as described by Ghidoni et al. (1980).

By use of the procedure described, gangliosides species carrying long-chain bases with 20 or 18 carbon atoms in the unsaturated form (C20:1 GM1; C20:1 Fuc-GM1; C20:1 GD1a; C20:1 GT1b; C18:1 GM1; C18:1 GD1a) were prepared. Preparation of saturated species (C20:0 GD1a; C18:0 GD1a) was attained by hydrogenation of the corresponding unsaturated species, following the procedure described by Sonnino et al. (1984). The final purity of gangliosides was better than 99% regarding both the oligosaccharide portion and long-chain base composition. The fatty acid composition was over 91% stearic acid. Table I summarizes the lipidic composition of the purified molecular species carrying the C20 and C18 sphingosine and sphinganine used in the present investigation.

Vesicle Preparation. Fused unilamellar vesicles (FUV) were prepared as described by Schullery et al. (1980). DPPC, dried and lyophilized from a chloroform/methanol solution, was suspended and vortexed in 50 mM KCl (containing 0.02% NaN_3) at a final concentration of 50 mg/mL. The suspension sonicated for 1 h in a bath sonicator (G112SPIG, Laboratory Supplies, Hicksville, NY) at 50 °C (above the lipid phase transition) was centrifuged at 15000*g* for 60 min. Upon incubation at low temperature (4 °C) for 3 weeks, a homogeneous population of large unilamellar vesicles (LUVs) of about 900-Å diameter is produced (Wong et al., 1982). Size and homogeneity of the vesicle preparation were checked by negative staining electron microscopy and differential scanning calorimetry.

Incorporation of gangliosides into the lipid bilayer was achieved by addition of micellar dispersions of the single ganglioside species (from concentrate aqueous solution of 10–20 mg/mL) to dilute aliquots of the vesicle preparations (5 μmol/mL) followed by overnight incubation at 50 °C. By this procedure a stable population of fused unilamellar vesicles containing ganglioside molecules asymmetrically located only into the outer layer of the vesicles is obtained (Felgner et al., 1981; Masserini & Freire, 1986). By use of this method, it is possible to completely transfer the ganglioside molecules from the micelles to the vesicles provided that the ganglioside mole percent in the mixture does not exceed 12% of the total lipid (Felgner et al., 1981). For these studies ganglioside mole percents of up to 10% of the total lipid were used.

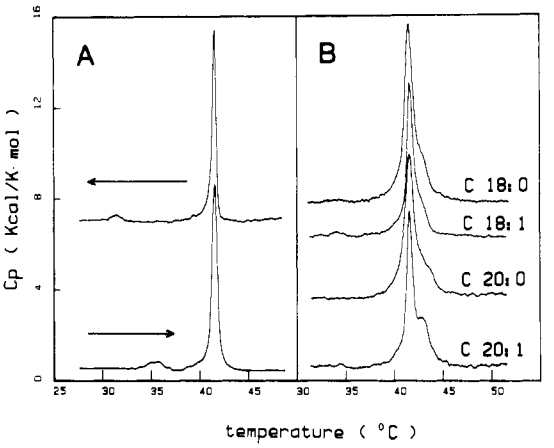

FIGURE 1: (A) Heat capacity function vs temperature for DPPC large unilamellar vesicles. Arrow = direction of the scan. (B) Heat capacity function vs temperature for DPPC large unilamellar vesicles containing 10% (molar) C18:0 GD1a, C18:1 GD1a, C20:0 GD1a, or C20:1 GD1a ganglioside (heating scans). For better detail, the curves in panel B have been enlarged by a factor of 2. In this and all figures the curves have been shifted in the *y* axis for presentation purposes.

Differential Scanning Calorimetry. Calorimetric experiments were performed with a Microcal MC2D differential scanning calorimeter interfaced to a microcomputer with a Data Translation DT-2801 card for data acquisition and instrument control. The instrument has been modified for cooling operation as described before (Myers et al., 1987).

For these studies, the concentration of the lipid was 1–2 mM. The calorimetric scans were performed at a scanning rate of 20 °C/h. When required, Ca^{2+} was added to the vesicle preparations from a concentrated $CaCl_2$ stock solution directly into the calorimeter cell. The addition of Ca^{2+} was always done at a temperature above the phospholipid gel–liquid crystalline phase transition and the calorimeter allowed to cool very slowly to permit a reequilibration of the cation across the membranes. In all cases the pH of the samples was between 6.4 and 6.7, well above the p*K* of the ganglioside sialic acid (2.5). All the calorimetric data are repeated scans of the sample to assure equilibration and reproducibility.

Other Techniques. Phospholipid concentration was assayed from phosphate analysis by a modification of Bartlett's procedure as described by Marinetti (1962). Ganglioside-bound sialic acid was determined according to the method of Svennerholm (1964).

Results

To evaluate the influence of the ganglioside–ceramide portion on the thermotropic properties of ganglioside-containing DPPC vesicles, four GD1a ganglioside species, carrying long-chain base moieties with 20 or 18 carbon atoms in the saturated or unsaturated form (C18:1 GD1a, C18:0 GD1a, C20:1 GD1a, C20:0 GD1a) and stearic acid as the main fatty

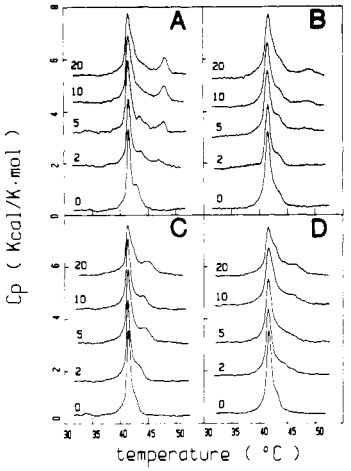

FIGURE 2: Heat capacity function vs temperature for DPPC large unilamellar vesicles containing 10% (molar) GD1a molecular species in the presence of increasing Ca^{2+} concentrations (heating scans). (A) C20:1; (B) C20:0; (C) C18:1; (D) C18:0. The numbers next to the calorimetric traces represent the millimolar Ca^{2+} concentration.

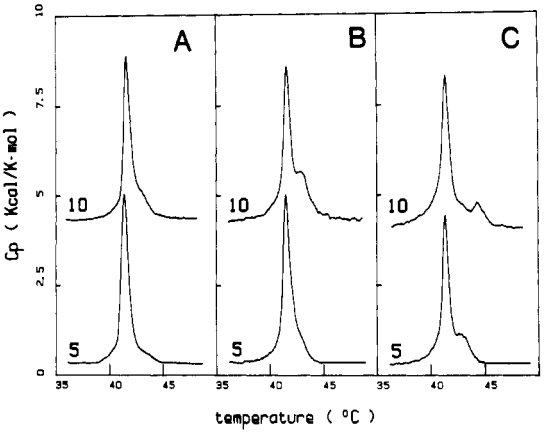

FIGURE 3: Heat capacity function vs temperature for DPPC large unilamellar vesicles containing 5 or 10% (molar) C20:1 GM1 (A), C20:1 GD1a (B), or C20:1 GT1b (C) in the absence of Ca^{2+} (heating scans).

acid (see Table I for compositional analysis), were asymmetrically inserted into DPPC FUVs and studied by high-sensitivity heating and cooling differential scanning calorimetry.

The heating and cooling calorimetric scans of DPPC fused unilamellar vesicles are shown in Figure 1A. As shown in the figure, the peaks corresponding to the main gel–liquid crystalline transition are independent of the scanning direction and characterized by an enthalpy change of 8.4 kcal/mol, a T_m of 41.5 °C, and a half-height width of 0.5 °C. The pretransition, however, is centered at 35 °C in the heating scan and at 31 °C in the cooling scan, as a consequence of the slow kinetics of this transition (Lentz et al., 1978).

DPPC Vesicles Containing Different Ganglioside GD1a Species. As shown in Figure 1B, the scan of DPPC vesicles containing 10 mol % of C20:1 GD1a shows the presence of a small overlapped peak, besides the main phospholipid transition, centered at a slightly higher temperature (42.8 °C). This minor peak decreases in amplitude with both the chain length and degree of unsaturation of the ganglioside long-chain base. So, for C18:0 GD1a it appears only as a slight shoulder toward the high-temperature end of the transition. In all cases the temperature of the main transition and the total enthalpy change remained constant and equal to those of pure dipalmitoylphosphatidylcholine within the experimental error. These data and the data below suggest that in DPPC–GD1a vesicles the ganglioside unsaturated species (C20:1 GD1a and C18:1 GD1a) possess a higher tendency to segregate within the DPPC lipid bilayer. The saturated species (C20:0 GD1a and C18:0 GD1a) appear to be more uniformly dispersed within the bilayer judging from the shape of the calorimetric melting profiles.

The effects of the addition of $CaCl_2$ at increasing concentrations on the thermotropic properties of DPPC vesicles containing 10 mol % of different GD1a gangliosides species are shown in Figure 2. Upon addition of Ca^{2+}, highly remarkable effects are observed in the membrane system. As a general trend, the main transition peak gradually broadens and a new peak appears at higher temperatures. In the case of C20:1 GD1a species (Figure 2A), the small high-temperature peak, centered at 42.8 °C in the absence of Ca^{2+}, is shifted to a slightly higher temperature (43.5 °C) while its height decreases until it is undetectable at Ca^{2+} concentrations higher than 5 mM. At the same time a new peak, detectable at Ca^{2+} concentrations as low as 2 mM, appears at a higher temperature (47 °C). The area under this new peak, and its position, increase upon increasing Ca^{2+} concentration at the expenses of the main transition peak. The total enthalpy of the process remains constant within the experimental error. At excess Ca^{2+} concentration (20 mM) the new high-temperature peak is centered at 47.2 °C and its area represents 25% of the total.

In the case of the C18:1 GD1a species (Figure 2C) the shoulder at the high-temperature end of the main transition peak becomes a distinct peak, and its position and area increase as the Ca^{2+} concentration is increased. In the presence of 20 mM Ca^{2+}, the high-temperature peak is centered at 44.5 °C and its area represents 20% of the total.

The behavior of the saturated C20:0 GD1a species (Figure 2B) follows a similar qualitative trend even though not as pronounced as in the case of the unsaturated species. A new, and distinct, high-temperature peak is detectable only at Ca^{2+} concentrations equal to or higher than 5 mM. At excess Ca^{2+} (20 mM) concentration, the new peak is centered at 48 °C and its area represents 15% of the total. In the case of the C18:0 GD1a species (Figure 2D) the main transition peak becomes gradually broader upon the addition of $CaCl_2$ and, at cation concentrations higher than 10 mM, a second peak broad and overlapped, centered at 46 °C, becomes evident. The area of this peak increases with Ca^{2+} concentration at the expense of the main transition peak.

In addition to the above results, it should be noted that the area of the phase-separated peaks increased upon increasing the molar fraction of GD1a ganglioside in the vesicles, at a given Ca^{2+} concentration (see also Figures 3 and 4 and discussion below). These data imply that for a given ganglioside species and constant Ca^{2+} concentration the chemical composition of the ceramide moiety plays an important role in determining the thermotropic properties of the membrane. We have previously (Masserini & Freire, 1986) reported a similar trend for ganglioside GM1, using only unsaturated species (C18:1 GM1 and C20:1 GM1).

Effect of Oligosaccharide Moiety. To ascertain the influence of the oligosaccharide moiety on the thermotropic properties of ganglioside-containing DPPC vesicles, the thermotropic behavior of vesicles containing one of the highly purified ganglioside species C20:1 GT1b, C20:1 GD1a, and C20:1 GM1 was compared. These gangliosides differ from each other in the number of sialic acid residues present on the oligosaccharide chain, while the composition of their lipidic portion is almost identical (see Table I). Therefore, differences

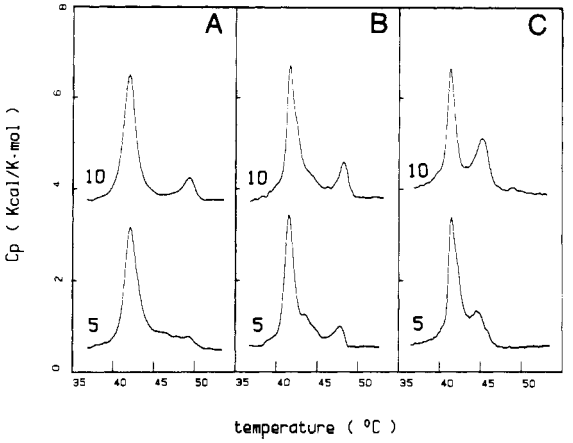

FIGURE 4: Heat capacity function vs temperature for DPPC large unilamellar vesicles containing 5 or 10% (molar) C20:1 GM1 (A), C20:1 GD1a (B), or C20:1 GT1b (C) in the presence of excess Ca^{2+} concentration (heating scans).

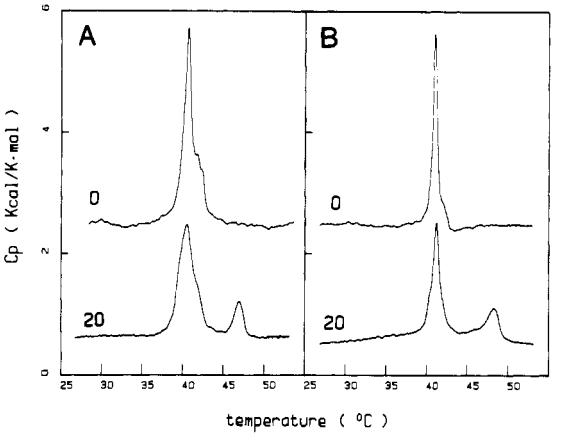

FIGURE 5: Heat capacity function vs temperature for DPPC large unilamellar vesicles containing 9% (molar) C20:1 GD1a (A) or C20:1 GM1 (B) in the absence or in the presence of excess (20 mM) Ca^{2+} concentration (cooling scans).

in their thermotropic behavior can be ascribed to the different oligosaccharide moieties.

Figure 3 shows the calorimetric scans of DPPC vesicles containing 10% or 5% (molar) of these gangliosides. In the presence of the monosialoganglioside C20:1 GM1 (Figure 3A) only a small skewness toward the high-temperature end of the DPPC main transition is present, while in the presence of the disialo (C20:1 GD1a) ganglioside species a partially overlapped but recognizable peak at 42.8 °C is present. In the presence of the trisialo C20:1 GT1b species the phase-separated peak is well-defined and centered at a higher temperature (44.2 °C).

The enthalpy change is virtually identical (8.3 kcal/mol) in all cases to that of pure DPPC. The extent of phase separation follows the order GT1b > GD1a > GM1. In addition, the data also indicate a direct relationship between the magnitude of phase separation and the ganglioside molar content. These data strongly suggest a direct relationship between the extent of ganglioside phase separation and the number of ganglioside sugar units in DPPC–ganglioside asymmetric vesicles. A similar trend was previously noted by the authors (Bertoli et al., 1981; Myers et al., 1984) using native gangliosides carrying a mixture of the naturally occurring ceramide moieties.

The addition of $CaCl_2$ to the above membrane systems produced dramatic changes in the heat capacity function. As shown in Figure 4C, for the case of 10% (molar) C20:1 GT1b-DPPC vesicles, the small high-temperature peak became broader and its T_m and area increased in the presence of 20 mM Ca^{2+}. The effect of Ca^{2+} addition could be detected at concentrations as low as 2 mM. The calorimetric profiles for the membrane systems containing 5 or 10% (molar) of C20:1 GM1 or C20:1 GD1a at excess Ca^{2+} concentration (20 mM) are reported in Figure 4A,B.

In the presence of 20 mM Ca^{2+}, the phase-separated peak in the case of the trisialoganglioside GT1b is centered at 45.3 °C (area = 30% of the total) and at 47.2 °C for GD1a ganglioside (area = 25% of the total). In the case of GM1 ganglioside, the new high-temperature peak is centered at 49 °C and its area accounted for 20% of the total, in agreement with previous data (Masserini & Freire, 1986). The total enthalpy of the transition remains the same for all samples, either in the absence or in the presence of Ca^{2+}. It is noted that the addition of Ca^{2+} caused a slight increase of the phospholipid main transition from 41.5 to 41.8 °C in the systems containing GD1a or GM1 but not in those containing GT1b ganglioside.

Remarkably, the higher the tendency of the system toward phase separation in the absence of Ca^{2+} (GT1b > GD1a > GM1) for a given ganglioside lipidic composition, the higher is the amount of phospholipid molecules that phase separate at a given Ca^{2+} concentration. Also, in the presence of Ca^{2+}, the transition temperature of the phase-separated domains is inversely related to the number of sugar units in the ganglioside molecule. To check whether this dependence is due to the different number of sialic acid residues in the ganglioside molecule, the thermotropic behavior of vesicles containing 10% of C20:1 Fuc-GM1 was investigated. Fuc-GM1 carries the same number of sugar units as GD1a ganglioside, but only one sialic acid residue. C20:1 Fuc-GM1/DPPC vesicles showed a thermotropic behavior identical with that of C20:1 GD1a/DPPC vesicles; i.e., in addition to the main phospholipid gel–liquid transition peak, the calorimetric scans are characterized by the presence of a small phase-separated peak centered at 42.5 °C in the absence of calcium ions and by the presence of a small peak centered at 48 °C at excess (20 mM) Ca^{2+} concentration (data not shown).

Cooling Scans. Figure 5 shows calorimetric cooling scans for vesicle samples containing 9% (molar) C20:1 GD1a (panel A) and 9 mol % C20:1 GM1 (panel B) in the absence and in the presence of 20 mM Ca^{2+}. A comparison of the cooling scans with the corresponding heating scans indicates that these curves are identical within the experimental error, independent of the direction of temperature scanning, with regard to the area, curve shape, and temperature of the corresponding peaks. These data suggest that, under these experimental conditions, the calorimetric scans represent an equilibrium situation being unaffected by any slow relaxation process reflecting different degrees of species separation in the gel or liquid-crystalline state.

Discussion

The calorimetric data presented in this paper indicate that the degree of phase separation existing in ganglioside/phosphatidylcholine is a complex process that depends on several factors: (a) the nature of the oligosaccharide head group; (b) the difference between the lipidic portion of the ganglioside and the host lipid bilayer, with regard to both chain length and unsaturation; and (c) the existence of external physicochemical factors like divalent cations (Ca^{2+}) that trigger or enhance phase separation processes. Together these results indicate that gangliosides, as many other membrane components, constitute a rich family of molecules whose properties

are determined both by their oligosaccharide head groups and by their lipidic portion.

The calorimetric data obtained by using different molecular species of GD1a ganglioside incorporated into DPPC FUVs indicate that the degree of phase separation is highly dependent on the chemical composition of the hydrophobic portion of the ganglioside. The higher the difference in length or degree of unsaturation of the ganglioside ceramide from the phospholipid fatty acyl matrix (16:0 for DPPC), the higher is their tendency to phase separate. Since in all cases the appearance of the phase-separated peaks is made at the expense of the main transition peak, it is possible to conclude that the phase-separated region represents mixed phosphatidylcholine–ganglioside domains rather than pure glycolipid clusters. These results, together with those previously obtained with GM1 ganglioside (Masserini & Freire, 1986) and confirmed in the present investigation, support and strengthen the idea that the main driving force for ganglioside lateral phase separation in ganglioside/phospholipid bilayers is the difference in the chemical composition of the lipidic portion of the glycolipid and the phospholipid bilayer matrix. In agreement with this view are also the data obtained with GD1a ganglioside saturated species (C18:0 and C20:0 GD1a). In fact, the cooperative unit associated with the main phospholipid transition is lower in the vesicular preparation containing these species, in agreement with their more ideal miscibility with the phospholipid, compared to that of the unsaturated species.

The presence of Ca^{2+} ions enhances the ganglioside tendency to phase separate in all the experimental systems utilized. Even though the molecular origin of this effect is not clear, it has been previously reported that Ca^{2+} rigidifies the phospholipid bilayer matrix (Borle & Seelig, 1985; Akutsu & Seelig, 1981). This effect could lead to a "passive" exclusion of the ganglioside from the more rigid, more ordered phospholipid matrix as these molecules are not well structurally matched with the lipid bilayer. A passive exclusion of this type, rather than active clustering, appears to be the driving force for ganglioside phase separation since, for a given oligosaccharide moiety, the exclusion is proportional to the dissimilarity between the lipidic portion of the ganglioside and the bilayer matrix. This idea is consistent with previous studies using a single ganglioside and phosphatidylcholines of various chain lengths (Masserini & Freire, 1986) and with ganglioside–Ca^{2+} binding experiments reporting that gangliosides do not have a particularly high affinity toward Ca^{2+} (McDaniel & McLaughlin, 1985). As mentioned before, the phase-separated domains appear to be mixed ganglioside–phospholipid domains. Judging from the areas under the phase-separated peaks, these domains range in composition from 3:1 to 1:1 lipid–ganglioside at excess Ca^{2+} concentrations.

Previous literature data have not been sufficient to understand the role of the oligosaccharide portion in determining the thermotropic behavior of the ganglioside–phospholipid vesicles, the reason being that native gangliosides, commonly used in these investigations, are a mixture of different molecular species with prevalence of the C20:1 and C18:1 long-chain base moieties. Given the different tendency of the various ganglioside molecular species to phase separate, only a comparison between the properties of vesicles containing gangliosides with different oligosaccharide portion but identical lipid composition can provide information to answer this question. The data obtained in this paper show that, for a given lipidic composition, gangliosides have a tendency to phase separate within the bilayer in a fashion proportional to the number of sugar units on the molecule. This phenomenon can be monitored by the appearance of the phase-separated calorimetric peaks besides the main phospholipid gel–liquid crystalline transition peak and seems to be independent of the number of sialic acid residues as suggested by the comparative studies of vesicles containing GD1a or Fuc-GM1 ganglioside. The number of sugar units of the ganglioside influences at least two features of the thermotropic behavior of the membrane systems investigated: first, there is a direct correlation between the number of sugar units (GT1b > GD1a > GM1) and the temperature at which the phase-separated peaks are centered in the absence of Ca^{2+}, signaling the presence of more ordered, higher melting microdomains; second, there is also a correlation between the number of sugar units in the ganglioside molecule and the number of phospholipid molecules that phase separate at any given ganglioside concentration. These effects could be attributed to the presence of specific interactions between the ganglioside oligosaccharide and the phosphorylcholine headgroups or to differences in the oligosaccharide headgroup volume which increases proportionally to the number of saccharide residues/molecule (Maggio et al., 1978). The larger volume of the ganglioside headgroup may allow a higher number of phosphatidylcholine molecules to assemble with the ganglioside, giving rise to larger phase-separated peaks in the calorimetric scans.

For a given ganglioside/phospholipid ratio and long-chain base composition, the temperature location of the phase-separated peaks induced by calcium ions decreases upon increasing the number of sialic acid residues (GM1 > GD1a > GT1b). Within this context, the gangliosides with bulkier headgroup volume (GT1b) are presumably prevented from undergoing a massive phenomenon of passive exclusion due to sterical constraints. The phase-separated domains associated with this ganglioside are relatively richer in phosphatidylcholine than those associated with gangliosides having smaller headgroup (GM1). This accounts for the larger calorimetric area under the phase-separated peaks of the gangliosides having bulkier headgroups.

Registry No. DPPC, 2644-64-6; GD1a, 12707-58-3; GM1, 37758-47-7; Fuc-GM1, 82785-21-5; GT1b, 59247-13-1; Ca, 7440-70-2.

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Pyridoxal Phosphate as a Probe of the Cytoplasmic Domains of Transmembrane Proteins: Application to the Nicotinic Acetylcholine Receptor†

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ABSTRACT: A novel procedure has been developed to specifically label the cytoplasmic domains of transmembrane proteins with the aldehyde pyridoxal 5-phosphate (PLP). *Torpedo californica* acetylcholine receptor (AcChR) vesicles were loaded with [^{3}H]pyridoxine 5-phosphate ([^{3}H]PNP) and pyridoxine-5-phosphate oxidase, followed by intravesicular enzymatic oxidation of [^{3}H]PNP at 37 °C in the presence of externally added cytochrome *c* as a scavenger of possible leaking PLP product. The resulting Schiff's bases between PLP and AcChR amino groups were reduced with $NaCNBH_3$, and the pyridoxylated proteins were analyzed by fluorography. The four receptor subunits were labeled whether the reaction was carried out on the internal surface or separately designed to mark the external one. On the other hand, the relative pyridoxylation of the subunits differed in both cases, reflecting differences in accessible lysyl residues in each side of the membrane. Proteinase K treatment of labeled AcChR vesicles generated a peptide of 13 kDa that could be detected with anti-PLP antibodies only when the pyridoxylation was carried out on the internal surface of the vesicles. Even though there are no large differences in the total lysine content among the subunits and there are two copies of the α-subunit, internal surface labeling by PLP was greatest for the highest molecular weight (δ) subunit, reinforcing the concept that the four receptor subunits are transmembranous and may protrude into the cytoplasmic face in a fashion [Strader, C. D., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807-5811] that is proportional to their subunit molecular weight. Yet, the labeling data do not fit well to any of the models proposed for AcChR subunit folding. The method described can be used for selective labeling of the cytoplasmic domains of transmembrane proteins in sealed membrane vesicles.

Knowledge of the topology of the proteins associated with biological membranes is of basic importance to the understanding of structure–function relationships of membrane proteins. Several procedures for the identification and localization of surfaces of membrane proteins have been developed. Those methods include a number of membrane-impermeable agents, both anionic and cationic, that label proteins on the external surface of membrane vesicles (Rifkin et al., 1972; Steck & Dawson, 1974; Cabantchick & Rothstein, 1974; Thompson et al., 1987; Kyte et al., 1987; Dwyer, 1988). These probes have been designed to react with either nucleophilic or electrophilic groups of proteins on the membrane surface (Jackson, 1975; Berg, 1969). However, ligands that are inaccessible from the outside cannot be directly identified in the intact cell by such impermeant probes. Pyridoxal 5-phosphate (PLP)[1] is one of these probes used to label the outer surfaces

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