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Thermodynamic and phase characterizations of phosphatidylethanolamine and ganglioside G_{D1a} mixtures

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By employing diphenylhexatriene steady-state fluorescence anisotropy, pyrenedecanoic acid excimer formation, and high sensitivity scanning calorimetry we have demonstrated that the liposomes containing phosphatidylethanolamine (PE) and various mole fractions of ganglioside G_{D1a} had a gel-liquid crystalline phase transition between 15 and 25 °C. Calorimetric measurements indicated that these phase transitions were broad and centered between 17 and 21 °C. The enthalpy change of the transition was linearly dependent on the ganglioside concentration up to 10.0 mol% and plateaued between 11.4–16.2 mol%. The high enthalpy change (37 kcal/mol of G_{D1a} added into the PE bilayer) indicates the existence of PE-G_{D1a} complex structure in the liposomal membrane. It is proposed that semi-fluid domains containing six PE and one ganglioside molecule are present in the PE-G_{D1a} membranes at temperatures above gel-liquid crystalline phase transition. The Sendai virus induced leakage of PE-G_{D1a} liposomes has been investigated by using an entrapped, self-quenching fluorescent dye, calcein. The leakage rate was dependent on the mole fraction of ganglioside G_{D1a} and was maximal at 6.3 mol%. Arrhenius plots of the leakage rates showed breaks in the 20–25 °C temperature range, which correspond to the gel-liquid crystalline phase transition of the target liposomes. These data suggest that the rate of Sendai virus-induced leakage can be regulated via fluidity modulation by changing the PE to G_{D1a} ratio at constant temperatures.

Introduction

Gangliosides belong to a class of lipids with many important functions in cellular recognition and other physiological processes [1]. They mediate the binding and internalization of hormones, interferons, lectins, toxins and viruses into cells. Since there are indications that gangliosides can reduce the fluidity of biological membranes [2], they may also be involved in the regulation of the activities of membrane-bound enzymes, such as adenylate cyclase, phosphodiesterase, and acetylcholinesterase [1,3,4]. Although the interactions between gangliosides and phosphatidylcholines have been demonstrated by using various physical means [5–7], the interactions between gangliosides and other membrane lipids have not yet been rigorously studied [8]. Phosphatidylethanolamine (PE) has potential roles in the dynamic feature of the biological membranes, such as membrane fusion, exocytosis, transbilayer transport and inter-

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Abbreviations: PE, phosphatidylethanolamine; TPCK, L-1-(tosylamido)-2-phenylethylchloromethyl ketone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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membrane communication [9]. Interestingly, both PE and gangliosides coexist in cellular membranes and are especially enriched in membranes which have frequent fusion activities, such as synaptosomal membranes and chromaffin granules [10]. The involvement of gangliosides in the regulation of fusion activities via membrane fluidity modulation remains undetermined.

In the present study we have employed three techniques with different sensitivities, i.e., diphenylhexatriene steady-state fluorescence anisotropy, pyrenedecanoic acid excimer formation, and high sensitivity scanning calorimetry to study the physical state of PE-ganglioside G_{D1a} membranes. A model of PE-G_{D1a} packing in the membranes has been proposed. We have also investigated the effects of varying membrane fluidity (by changing the temperature and/or PE/G_{D1a} ratio) on Sendai virus-induced leakage of PE-G_{D1a} liposomes. The latter is thought to be one of the concomitant processes of Sendai virus-liposome fusion [11]. These studies serve as a starting point for a better understanding of a model as a target membrane for Sendai virus action.

Materials and methods

Materials

PE which was transesterified from egg phosphatidylcholine was purchased from Avanti (Birmingham, AL). TPCK-trypsin was purchased from Sigma. Ganglioside G_{D1a} was purchased from Supelco. The purity of G_{D1a} was confirmed with TLC using chloroform/methanol/2.5 N ammonium hydroxide (60 : 40 : 9, v/v) as a developing solvent system [12,13]. Hexadecyl[^{3}H]cholestanyl ether was synthesized and purified as described [14,15]. Diphenylhexathene was purchased from Eastman. Pyrenedecanoic acid was purchased from Molecular Probes.

Methods

Liposome preparations. Liposomes were prepared by sonication followed by freeze and thaw according to Tsao and Huang [11] with some modifications. 20 mM of sonicated lipid mixture including PE, G_{D1a} and a tracer amount of lipid marker hexadecyl[^{3}H]cholestanyl ether was mixed with same volume of 140 mM calcein in Hepes-buffered saline (for leakage experiments only) or with same volume of Hepes-buffered saline (for all other experiments). Nine cycles of freeze and thaw of the liposomes allowed calcein to be entrapped into the aqueous compartment of the liposomes. The liposome suspensions were chromatographed on a Bio-Gel A-0.5 m column (1 × 12 cm) to separate liposomes from untrapped calcein.

Liposome characterizations. For electron microscopic studies, the liposomes were negatively stained with potassium phosphotungstate, pH 7.5, for 3 min according to Tsao and Huang [16]. The trap volume of liposomes was calculated from the number of calcein molecules entrapped in the liposomes and the concentration of calcein in the liposomes. The concentration of calcein entrapped in the aqueous compartment of the liposomes was calculated from the percent fluorescence quenching of calcein by using a concentration-quenching standard curve of this self-quenching dye. Lipid mass was calculated from the radioactivity of hexadecyl[^{3}H]cholestanyl ether, which labeled liposomal lipids.

Virus preparations and hemagglutination, hemolysis assays. The preparation of intact and trypsin-treated Sendai virus and assays for their protein integrity (SDS-polyacrylamide gel electrophores), functions (hemagglutination, hemolysis) were done according to Tsao and Huang [11].

Liposome leakage assay. Liposome leakage assay has been described previously [11]. 5 μl of Sendai virus (8 mg viral protein/ml) preincubated to the desired temperature was added to 2 ml of reaction mixture (1.8 μg/ml of lipid and 750 μM of EDTA in Hepes-buffered saline) at the same temperature to initiate the reaction. To monitor the calcein leakage, a Perkin-Elmer LS-5 spectrofluorometer was used. Excitation was at 490 nm, while emission was measured at 520 nm. At the end of the experiment concentrated deoxycholate solution was added to a final concentration of 0.06% to completely release calcein. The leakage rate was obtained from the slope of the plots, ln $(1 - F(t))$ vs. t., where

$$F(t) = \frac{I(t) - I_o}{I_f - I_o}$$

and I_o = initial fluorescence, I_f = total fluo-

rescence measured in the presence of deoxycholate, and $I(t)$ = fluorescence at time t.

High sensitivity differential scanning calorimetry. Calorimetric measurements were performed using a Microcal MC1 differential calorimeter equipped with two separate Keithley amplifiers connected to the heat capacity and temperature output, respectively. The calorimeter data were automatically collected and processed by an interfaced IBM PC microcomputer and digitized by a Data Translation DT-805 A/D converter. The measurements of PE-G_{D1a} vesicles were performed at a scanning rate of 15 Cdeg/h at a concentration of 10 mM lipid/ml with total sample volume of 0.7 ml.

Steady-state fluorescence spectroscopy. Steady-state fluorescence anisotropy measurements were performed using a Perkin-Elmer LS-5 spectrofluorometer equipped with a red sensitive photomultiplier tube, a polarization accessory and a thermostated cuvette holder connected to a Neslab RTE-8 bath circulator. Diphenylhexathene was dissolved in acetonitrile and added to the vesicle suspensions at a final ratio of 1 probe molecule/1000 lipid molecules. Lipid concentration was 0.3 mM. Probe incorporation was allowed to continue for 5 h at 35 °C prior to fluorescence measurements. Fluorescence was measured by excitation of diphenylhexatriene at 360 nm, while emission was monitored at 430 nm. Anisotropy $r(t)$ was calculated according to [17]:

$$r(t) = \frac{I_{\parallel} - I \times G}{I_{\parallel} - 2I_{\perp} \times G}$$

where G is the correction factor obtained using horizontally polarized light ($G = I_{\parallel}/I_{\perp}$).

Excimer formation. Excimer formation studies were performed with a Perkin-Elmer LS-5 spectrofluorometer. Pyrenedecanoic acid was dissolved in acetonitrile and added to the vesicle suspensions at a final ratio of 1 probe molecule/20 lipid molecules. Lipid concentration was 15 μM. Probe incorporation was allowed to continue for 2 h at 35 °C prior to fluorescence measurements. Fluorescence was measured by excitation of pyrenedecanoic acid at 320 nm, while emission for monomers and excimers were monitored at 395 nm and 480 nm, respectively. Excimer to monomer ratio (I'/I) was obtained from the fluorescence peak height ratio at 480 nm to 395 nm, according to Galla and Hartmann [18].

Results

Characterization of PE-G_{D1a} liposomes

As revealed with negative stain electron microscopy (photograph not shown), both PE and PE-G_{D1a} (6.3% G_{D1a}) liposomes were oligo- or multilamellar liposomes of 500–3000 nm in diameter. The trap volume was found to be 0.2 and 0.3 l/mol for PE and PE-G_{D1a} liposomes, respectively, as calculated from the calcein-trapping efficiency of these liposomes. The calcein entrapped inside PE and PE-G_{D1a} liposomes showed a 57% and 55% self-quenching, respectively, which corresponds to approximately 70 mM of calcein inside the aqueous compartment of the liposomes. Percent fluorescence quenching did not change for several days when stored at 4 °C indicating that calcein was stably entrapped in the liposomes.

Steady state fluorescence anisotropy

The membrane fluidity of PE-G_{D1a} liposomes was investigated by diphenylhexatriene steady-state fluorescence anisotropy as a function of temperature and G_{D1a} mole fraction. Fluorescence anisotropy, which has an inverted relationship with membrane fluidity, is sensitive to the structural order of lipids, i.e., the degree to which the probe rotations are restricted by the molecular packing of membrane lipids [19–20]. All the Arrhenius plots, ln (anisotropy) vs. 1/temperature (Fig. 1), showed broken straight lines consistent with the existence of a phase transition between 20 and 25 °C for each PE-G_{D1a} liposome tested. The activation energies were between −7.0 and −8.8 kcal/mol at the temperatures below the transition and between −5.3 and −6.1 kcal/mol at the temperatures above the transition. The anisotropy increased as temperature decreased or as the G_{D1a} mole fraction increased, except for the 3.2% G_{D1a} sample that had an anisotropy comparable to or slightly higher than the 6.3% G_{D1a} sample depending on the temperature. As part of the experiments to study the functional role of PE-G_{D1a} membrane (see below) we also investigated the membrane fluidity of Sendai virus, trypsin-treated

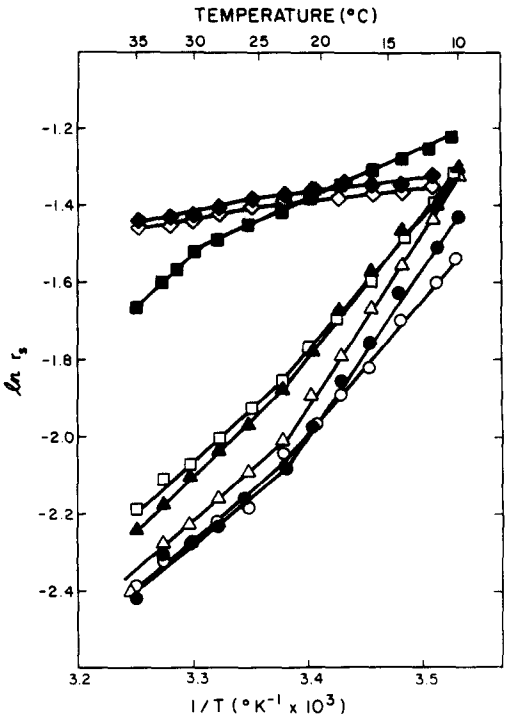

Fig. 1. Temperature dependence of fluorescence anisotropy of diphenylhexatriene in liposomal membranes containing 3.2% (○), 6.3% (●), 11.4% (△), 16.2% (▲), and 20.5% (□) of G_{D1a} as well as in G_{D1a} micelles (■), Sendai viral membranes (◆) and trypsin-treated Sendai viral membranes (◇). Transitions occurred between 20 and 25 °C for liposomal membranes alone.

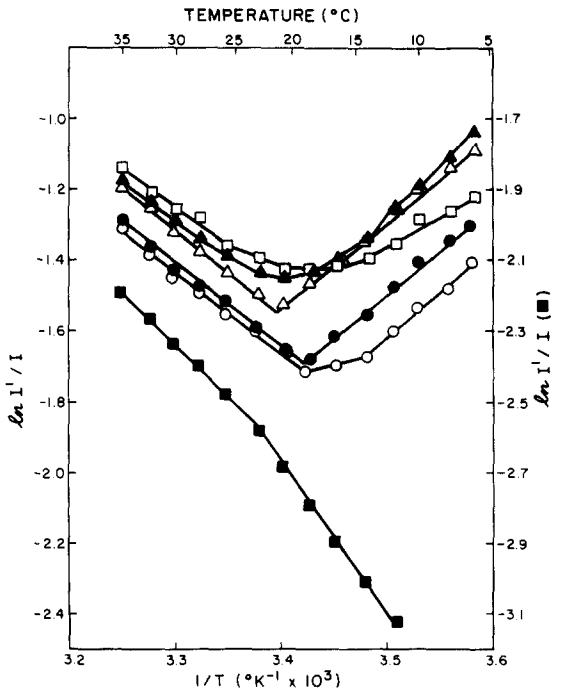

Fig. 2. Temperature dependence of pyrenedecanoic acid excimer formations in liposomal membrane containing different mol% of G_{D1a} as well as in G_{D1a} micelles. Symbols are the same as in the legend of Fig. 1. Most of the transitions occurred between 15 and 21 °C for liposome samples.

Sendai virus and G_{D1a} micelles. The latter had much higher anisotropy values than PE-G_{D1a} liposomes and had no phase transition between 20 and 25 °C. The data indicate that PE-G_{D1a} membrane may have a gel-liquid crystalline phase transition between 20 and 25 °C.

Excimer formation

The physical state of the PE-G_{D1a} liposomes were further explored with pyrenedecanoic acid excimer formation. Excimer formation depends upon the lateral motion and collision of monomer probes to form excimer (excited dimer) under proper light excitation. It is therefore sensitive to the local concentration and the fluidity in the vicinity of the probe [21]. A high excimer-to-monomer ratio indicates either a more fluid membrane or the formation of pyrenedecanoic acid-enriched domains, which are squeezed out of crystalline phase of lipid matrix into the remaining fluid part of the membrane [18]. Arrhenius plots, ln (excimer to monomer ratio) vs. 1/temperature of each PE-G_{D1a} samples are shown in Fig. 2. From 30 to 35 °C all liposomes with 3.2, 6.3, and 11.4% G_{D1a} still kept the same activation energies until an abrupt phase transition occurred at 19, 19, and 21 °C, respectively, while liposomes with 16.2 and 20.5% G_{D1a} had a gradual change in activation energies with a range of transition temperature between 5 and 35 °C. Below the midpoint of the transitions all the liposomes had an increased excimer to monomer ratio and from 7.5 to 14 °C they had activation energies between 4.9 and 5.6 kcal/mol, except for 3.4 kcal/mol for 20.5% G_{D1a} liposomes. All of the samples showed a temperature dependence which was typical for a gel-liquid crystalline phase transition. However, samples with different G_{D1a} mole fraction had different values of excimer formation even above transition temperature. Sendai virus and G_{D1a} micelles did not

show phase transition between 10 and 25 °C. They also had very low excimer-to-monomer ratio in all temperatures tested, especially for G_{D1a} micelles.

High sensitivity scanning calorimetry

The thermodynamic properties of the PE-G_{D1a} liposomes were investigated by high sensitivity differential scanning calorimetry. Calorimetry provides a direct estimate of the overall enthalpy change, which is sensitive to the close van der Waal's contact of aliphatic side chains in a gel-liquid crystalline phase transition [22]. All the heat capacity vs. temperature plots with different G_{D1a} mole fractions had broad phase transitions peaked between 17.3 and 21.0 °C depending on the G_{D1a} mole fraction (Fig. 3). The phase transitions share the same temperature range, while the peak temperature shifted up with the G_{D1a} mole fraction until 16.2%. The enthalpy change associated with the phase transition initially increased linearly with G_{D1a} mole fraction and leveled off between 10 and 16% G_{D1a} and dropped drastically at higher mole fractions (Fig. 4). The initial slope of the enthalpy dependence on the ganglioside mole fraction was 37 kcal/mol. This number should be proportional to the number of PE molecules which undergo a gel-liquid crystalline phase transition between 15 and 25 °c under the influence of a single ganglioside molecule.

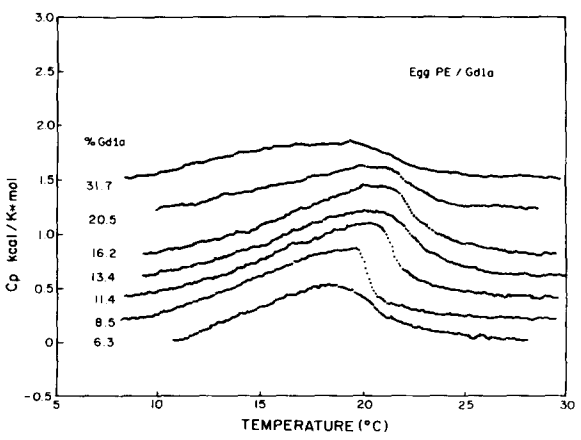

Fig. 3. Temperature dependence of heat capacity of the gel-liquid crystalline phase transitions for liposomes containing different mol% of G_{D1a}.

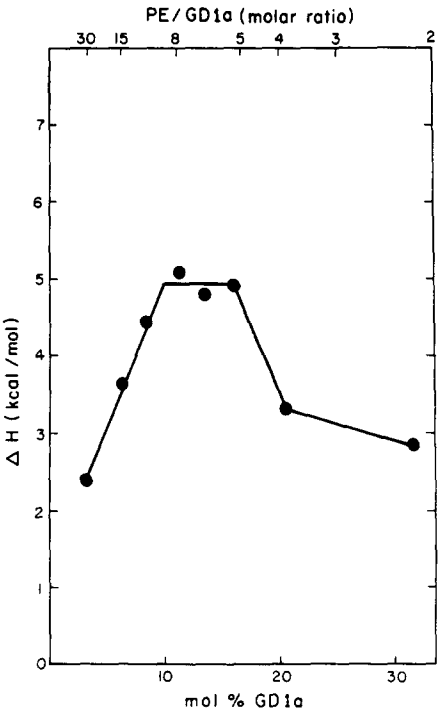

Fig. 4. G_{D1a} concentration dependence of heat capacity change of the gel-liquid crystalline phase transitions for liposomes containing different mol% of G_{D1a}.

Temperature dependence of the leakage

The fluorescence intensity increase as a result of Sendai virus-induced calcein leakage from liposomes was monitored as a function of time. The leakage was found to follow first-order kinetics by fitting the data into linear ln $(1 - F(t))$ vs. t plots (data not shown). The temperature dependence of the first-order leakage rate is shown in the Arrhenius plots in Fig. 5. The leakage rate of all the samples tested showed a discontinuity in the temperature dependence between 20 and 25 °C (although it is not as obvious as others for the 20.5% G_{D1a} sample). The activation energies, obtained from the slopes of the Arrhenius plots, were between 32 and 37 kcal/mol (except for 47.8 kcal/mol for the 6.3% G_{D1a} sample) at temperatures below the transition and between 15 and 21 kcal/mol (except 34.5 kcal/mol for the 20.5% G_{D1a} sample) at temperature above the transition. The leakage rate was not only dependent on temperature but also on the mole fraction of G_{D1a}. It decreased as the G_{D1a} mole fraction increased.

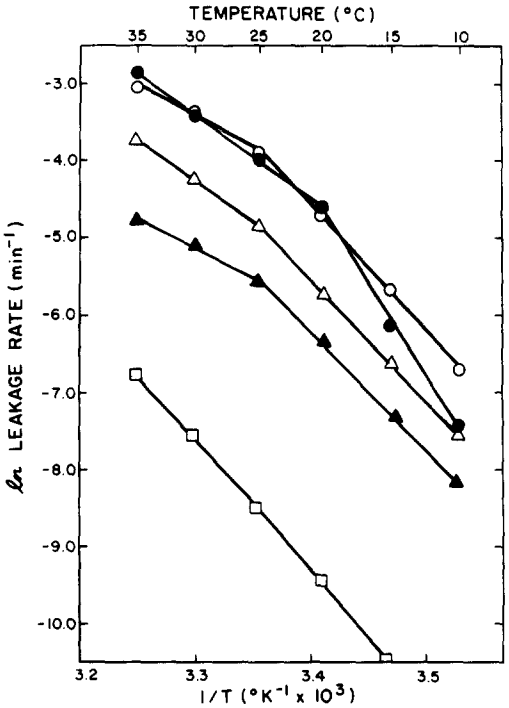

Fig. 5. Temperature dependence of calcein leakage from liposomes containing different mol% of G_{D1a}. Symbols are the same as in the legend of Fig. 1. The activation energies were different at higher and lower temperatures. Transitions occurred between 20 and 25 °C.

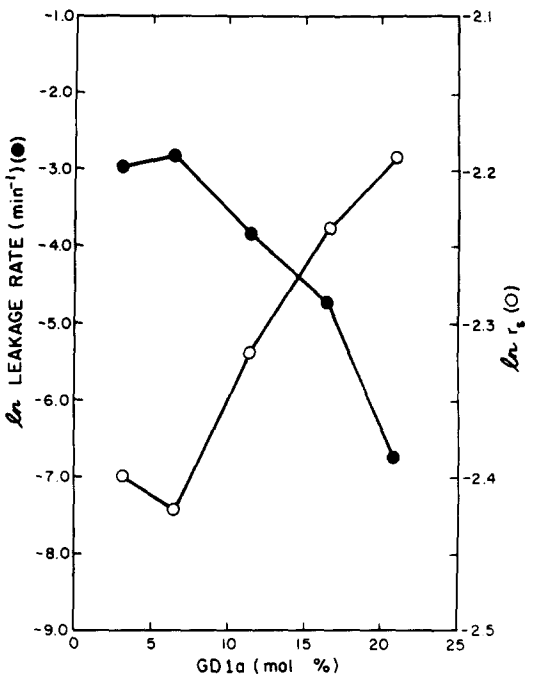

Fig. 6. Comparisons of the G_{D1a} concentration dependence of the calcein leakage (●) and fluorescence anisotropy (○) of liposomal membranes at 35 °c. The calcein leakage was inversely proportional to the fluorescence anisotropy. The standard deviations of the measurement did not exceed 2% and had an average of 1.2%.

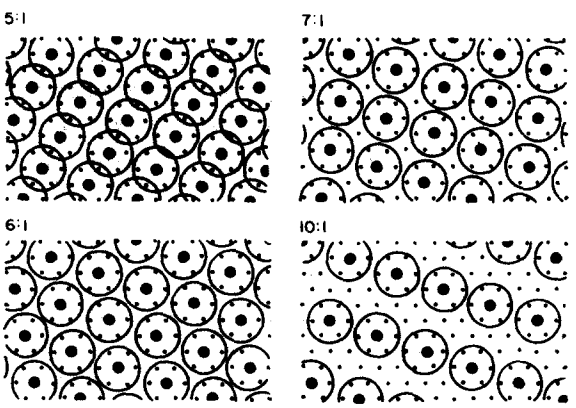

Fig. 7. A proposed model for the lipid packing of the liposomes containing different PE-to-G_{D1a} ratios. The PE-G_{D1a} ratio is shown at the upper left corner of each panel. Each G_{D1a} molecule (●) is surrounded by six boundary PE molecules (•) to form a semi-fluid 'complex' (defined by a large circle). The areas outside the large circle are fluid PE domains.

This is particularly apparent for G_{D1a} mole fractions between 6.3 to 20.5%. Fig. 6, in which the leakage rates and the anisotropy of the liposomes at 35 °C are plotted against the G_{D1a} mole fraction, shows the opposite relationship between the anisotropy and the leakage rate. The leakage rate was also F-protein dependent, since trypsin-treated Sendai virus, from which part of F protein was cleaved [11], did not induce leakage of any liposomes at any temperature tested (data not shown).

Discussion

Gangliosides can not form bilayer by themselves and only form micelles at concentrations above critical micellar concentration [23,24]. However, we found that ganglioside G_{D1a} could be incorporated into PE bilayers to form liposomes,

which have an average diameter ranging from 500 to 3000 nm as revealed with negative stain electron microscopy. These liposomes were very stable in that they could retain 70 mM calcein in a hyposmotic condition (592 mosmol/l in the aqueous interior vs. 242 mosmol/l in the external medium) for several days. There is little doubt that under the experimental conditions used, PE-G_{D1a} membranes were in the lamellar phase which served as an excellent permeability barrier for hydrophilic molecules and ions [9]. This notion is further supported by the fact that in our experimental temperature range PE-G_{D1a} membranes had a gel-liquid crystalline phase transition which only exists in the lamellar phase of a given membrane [25,26].

The observed phase transition of PE-G_{D1a} liposomes was a transition from a gel to a liquid-crystalline state of the lipid mixtures. Diphenylhexatriene fluorescence anisotropy studies showed that there was a noticeable change in membrane fluidity at the transition (Fig. 1). Pyrenedecanoic acid excimer formation studies (Fig. 2) showed a temperature dependence which is typical for a gel-liquid crystalline phase transition observed in other well-established lipid systems [18]. The large values in the enthalpy change during the transition (5 kcal/mol for 10–16% G_{D1a} samples), revealed by the high-sensitivity scanning calorimetry (Fig. 4), further ruled out the possibility of having a bilayer-to-hexagonal phase transition or phase separation [27–29]. The small discrepancy (within 4°C) in phase transition temperature measured with different techniques is likely due to the fact that these techniques are sensitive to different parameters of the phase transition, i.e., structural order, fluidity, or close van der Waal's contact of membrane lipids.

The physical state and the structure of PE-G_{D1a} membranes are further described by the following observations. Fluorescence anisotropy data showed that the membrane fluidity above the gel-liquid crystalline phase transition was G_{D1a}-concentration dependent (Fig. 1). Pyrenedecanoic acid excimer formation studies (Fig. 2) indicated that the G_{D1a}-dependent changes above the transition were due to the formation of semi-fluid domains in the membranes. The pyrenedecanoic acid probes could be expelled from the semi-fluid domains and moved into the remaining fluid part of the membrane, resulting in an increase in the excimer formation [18]. These semi-fluid domains probably resulted from the complex formation among PE and G_{D1a} molecules because the transition enthalpy change (resulted largely from the close van der Waal's contact) and the peak transition temperature (resulted largely from head group interactions) of PE-G_{D1a} thermotropic phase transition (Fig. 3) were also ganglioside-concentration dependent [10,22]. It is interesting to note that although the enthalpy change during the phase transition depends on the G_{D1a} mole fraction initially, it reaches a maximum at a PE/G_{D1a} ratio of 8:6 (11–14% G_{D1a}) and drops abruptly when the ratio is below 6 (Fig. 4). It indicates that the basic unit ('complex') which can undergo a gel-liquid crystalline phase transition in the range of 15–25°C is likely to be composed of PE and G_{D1a} with a ratio of 6. The existence of PE-G_{D1a} 'complexes' is further supported by the fact that 1 mol of G_{D1a} could add 37 kcal/mol of enthalpy change under the gel-liquid crystalline phase transition (Fig. 4). The enthalpy change, 37 kcal/mol, is too large to be provided by any single lipid, which is no greater than 15 kcal/mol [10,27,28], and should be attributed to the 'complexes' formed between PE and G_{D1a}. All of these observations are consistent with the following model for PE-G_{D1a} membrane packing (Fig. 7): (a) 'complexes' that contain one G_{D1a} molecule and six surrounding 'boundary PE' with a structure analogous to the association between membrane proteins and their boundary-lipid counterparts are located randomly in the 'free PE' matrix of the bilayer membranes; (b) the boundary PE is in a semi-fluid state at the temperature above the transition, whereas free PE stays in fluid state; (c) in the PE-G_{D1a} membranes, primarily the boundary PE or 6:1 'complexes' and not free PE or G_{D1a} alone, undergo a gel-liquid crystalline phase transition between 15 and 25°C. Our data are consistent with the model. For fluorescence anisotropy and excimer formation studies (Figs. 1 and 2) this model predicts a phase transition between 15 and 25°C for all liposome compositions, and a higher anisotropy and excimer formation at higher G_{D1a} mole fractions even at temperatures above the phase transition. It has to be noted that

the ganglioside effects are apparent even at low G_{D1a} concentrations, since one G_{D1a} molecule can 'rigidify' six PE molecules according to our model. For differential scanning calorimetry studies (Fig. 4) this model predicts an initial linear increase in the enthalpy change of phase transition with increased G_{D1a} content of the membrane due to the increasing amount of the 6:1 'complex' formation. The enthalpy change reaches a maximum at higher G_{D1a} content (10–16%) due to the interactions between 'complexes' as a result of close contact among 'complexes'. At even higher G_{D1a} content (PE/G_{D1a} ratio equals 5 or smaller) the enthalpy change decreases due to the overlapping boundary PE among the neighboring 'complexes'. This model also predicts a maximal boundary circumference between domains of PE-G_{D1a} complex and free PE at about 10% G_{D1a} (Fig. 7). The boundary between different phases is though to be one of the major causes of membrane fusion [30,31]. It may be the structural basis of the observation that maximal fusion activity between Sendai virus and these membranes occurs at 11.4% G_{D1a} [16].

The nature of the interactions within a PE-G_{D1a} complex remains unclear; however, several possibilities are conceivable. According to the 'Shape Concept' [32], inverted cone-shaped G_{D1a} may be spatially complementary to adjacent cone-shaped PE molecules to form the complex. The complexes are likely to endow PE-G_{D1a} membranes with physical properties which are completely different from the PE membranes. Intermolecular hydrogen bonding, which is known to increase the gel-liquid crystalline phase transition temperature for many lipids [10], may exist among the phosphate and amine groups of PE, and the hydroxyl and amide groups of G_{D1a}. It should be noted that the proposed PE-G_{D1a} complex is a weak complex which may undergo dynamic dissociation and reassociation. These complexes are likely to exist in biological membranes, particularly in those membranes enriched with both PE and gangliosides such as the synaptosomal membrane and chromatin granules [10]. Thus, the regulation of the relative amounts of PE and ganglioside in these membranes may be crucial to certain membrane activities, e.g., membrane fusion. To illustrate such a possibility, we chose to study the interactions between Sendai virus and the PE-G_{D1a} liposomes as model target membranes. Virus-induced leakage of the target membrane is thought to be a direct result of virus-target fusion [11]. Sendai virus-induced leakage of liposomes containing PE and G_{D1a} showed an abrupt change in the leakage between 15 and 25 °C which closely corresponded to the phase transition temperatures of the PE-G_{D1a} membranes (Fig. 5). The fact that only target membranes, but not viral membrane, have a gel-liquid crystalline phase transition in the same temperature range (Figs. 1–3) indicates that the target membrane fluidity plays a determining role in Sendai virus-induced leakage process. Interestingly, the leakage rate at 35 °C can be regulated in the range of four order of magnitude via the fluidity modulation by changing PE-to-G_{D1a} ratio (Fig. 6). These data strongly suggest the possibility of regulating biological membrane activities at physiological temperatures by regulating the ratio of PE to G_{D1a} in the membranes enriched with these lipid species [33]. Since the size of PE-G_{D1a} complex is small, this kind of fluidity modulation could be highly localized and yield fine-tuned activity regulation. Furthermore, our results also indicate that a minimum model system for the viral target membrane can be constructed with the combination of a receptor (G_{D1a}) and a matrix lipid (PE). With this simple model system, it is now possible to study the detailed molecular events of virus-membrane fusion. One such study on the kinetic analysis has already been demonstrated [16].

Acknowledgements

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