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β-Galactosidase-induced destabilization of liposome composed of phosphatidylethanolamine and ganglioside G_{M1}

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A novel type of liposome bilayer destabilization catalyzed by the enzyme, β-galactosidase, is described. Unsaturated phosphatidylethanolamine (PE), an H_{II}-phase-forming lipid, does not form stable liposomes at physiological temperature and pH. However, stable unilamellar liposomes can be prepared by mixing PE with a minimum of 5 mol% ganglioside G_{M1}, a micellar-phase-forming lipid. Treatment of these G_{M1}/PE liposomes with β-galactosidase induces a rapid leakage (3–6 min) of the entrapped fluorescent dye, calcein. The studies indicate that liposome destabilization is the result of catalytic degradation of G_{M1}, rather than a stoichiometric binding of G_{M1} by β-galactosidase. Kinetic data indicate that the destabilization takes place via liposome collision. This simple, rapid method of liposome destabilization by β-galactosidase will be useful in designing a liposome-based signal amplification mechanism for assays involving enzymes.

Introduction

It has been well established that unsaturated phosphatidylethanolamine (PE) such as DOPE does not form stable bilayer by itself at physiological temperature and pH [1]. According to the 'shape-concept' of Cullis and De Kruijff [1], elaborated by Israelachvili et al. [2] and reviewed by Gruner et al. [3], the shape of the lipid molecule determines the equilibrium configuration of lipid aggregation (bilayer or non-bilayer structure). Furthermore, the cone-shaped molecules such as unsaturated phosphatidylethanolamine can be complemented with the inverted cone-shaped molecules such as micellar-phase-forming lipids to form stable bilayer structures [1,4]. This was demonstrated by the observations that the bilayer phase of unsaturated PE can be stabilized by a number of amphiphiles including gangliosides [5,6], haptenated lipid [7], fatty acids [8], fatty acyl amino acids [9], cholesteryl hemisuccinate [10], and other protonatable double-chain amphiphiles [11]. Amphipathic proteins such as glycophorin [12,13] and acylated antibody [14] can also be used as a stabilizer [15].

Recently, a novel type of liposome called target-sensitive immunoliposome has been designed based on the principle of shape complementarity [7,15]. The liposomes are mainly composed of PE but are stabilized with acylated antibody [15] or amphipathic antigens [7,13]. Lysis of the liposome occurs when the stabilizer of the liposome makes a specific immune complex with a multivalent target membrane. Uses of the target-sensitive immunoliposome for in vitro diagnosis

Abbreviations: PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; TPE, transphosphatidylated PE prepared by transphosphatidylation of egg PC; DOC, deoxycholate.

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[16,17] and drug delivery [18] have been demonstrated. Furthermore, it has been shown that the proteolytic cleavage of the stabilizer causes the destabilization of the otherwise stable target-sensitive immunoliposomes [13,15], indicating a special requirement for the structure of the stabilizer molecule.

We have previously characterized the thermodynamic and phase properties of PE and ganglioside G_{D1a} mixtures [6]. A composition model has been proposed for the interactions between PE and G_{D1a}. In the present study, we have compared the stabilization effects of two more gangliosides; i.e., G_{M1} and G_{M2}. Furthermore, enzymatic conversion of G_{M1} of the G_{M1}/PE liposomes to G_{M2} by incubation with the enzyme, β-galactosidase, has led to a rapid change of the liposome stability. These observations have shed some light into the structural requirement of an amphipathic stabilizer molecule for PE liposomes.

Materials and Methods

Transphosphatidylated PE (TPE), DOPE and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). Gangliosides, G_{M1} and G_{M2}, were from Supelco (Houston, TX) or Calbiochem (San Diego, CA). The purity of the gangliosides was confirmed with TLC using chloroform/methanol/2.5 M ammonium hydroxide (60 : 40 : 9) as a developing solvent [19,20]. Calcein and *Escherichia coli* β-galactosidase were from Sigma Chemicals (St. Louis, MO). All other reagents were of analytical grade.

Liposome preparation

Liposomes were prepared by sonication or by a fast extrusion method [21]. In routine experiments, 3 μmol of DOPE/TPE (2 : 1 molar ratio) or DOPC with 5 mol% G_{M1}, were mixed with a trace amount of [^{3}H]cholestanyl ether (final spec. act. $1 \cdot 10^{12}$ cpm/mol lipid) and dried under a stream of N_2 and vacuum desiccated for no less than 1 h. The lipid mixture was then hydrated with phosphate-buffered saline (300 μl) containing 50 mM calcein and 0.02% azide (pH 8.0). The mixture was allowed to stand at room temperature for 2–3 h with occasional mixing. The lipid mixture was then vortexed and allowed to stand at room temperature for an additional hour. Then, the suspension was extruded quickly and forcefully 20 times through a 28 gauge needle attached to a 0.5 ml B-D insulin syringe. Two additional cycles of extrusion were performed with a 4–12 h resting interval [21]. The non-entrapped calcein was removed by chromatography on a Bio-Gel A-0.5 m column. The fractions were measured for ^{3}H cpm and calcein fluorescence in the presence or absence of 0.15% DOC. The peak fractions were stored at 4 °C at a lipid concentration of 500 μM.

Ninety-degree light scattering of liposomes

To test for the liposome formation, sonicated liposomes were used. Routinely, 1 μmol of dried lipids containing [^{3}H]cholestanyl ether (final spec. act. $1 \cdot 10^{12}$ cpm/mol of lipid) and G_{M1} were hydrated with 100 μl of phosphate-buffered saline (pH 8.0) containing 0.02% sodium azide and sonicated for two 10 min cycles with an intervening rest period of 3 h at room temperature. The resulting liposome suspensions were diluted 100-fold in phosphate-buffered saline. Light scattering at 90 ° was measured in a Perkin-Elmer LS5 spectrofluorometer with excitation and emission at 660 nm with a slitwidth of 3 nm.

Fluorescence quenching measurements of liposomes

The integrity of the liposomes was tested by measuring the calcein fluorescence of the liposomes in the absence or presence of 0.15% DOC. Sonicated liposomes prepared as described above, were used, except that the hydration mixture contained 50 mM calcein. Free calcein was removed by gel filtration using Bio-Gel A-0.5 m column chromatography. The fluorescence was measured with a Perkin Elmer LS5 spectrofluorometer with $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm. The total liposome concentration used for the fluorescence measurements was 1 μM in phosphate-buffered saline containing 1 mM EGTA. Percent quenching was calculated from the formula:

$$\% \text{ quenching} = (1 - F_0/F_t) \times 100$$

where F_0 and F_t are the fluorescence of the liposome samples before (F_0) and after (F_t) addition of 0.15% DOC.

Electron microscopy

Liposomes (0.4 μmol/ml) containing 5 mol% G_{M1} were negatively stained with 0.5% uranyl acetate and viewed in a Hitachi 600 electron microscope operating at 75 kV. The size of the liposomes were determined using photographically enlarged micrographs.

β-Galactosidase-induced release of calcein from liposomes

Liposomes (200 μM) containing entrapped calcein were incubated at 37°C with β-galactosidase (1–5 mg/ml). At various time intervals, 1 μM aliquot of liposomes was diluted into 2 ml phosphate-buffered saline (pH 8.0), containing 1 mM EGTA and 500 mM lactose (to stop the enzymatic reaction of β-galactosidase) for the fluorescence measurement. The percentage of calcein released was calculated using the formula:

$$\% \text{ release} = \frac{(F - F_0)}{(F_t - F_0)} \times 100$$

where F_0 and F are the calcein fluorescence before and after the addition of β-galactosidase, respectively, and F_t is the total fluorescence after the addition of DOC.

Inhibition experiments were carried out as described above, except that the liposomes were preincubated with 500 mM lactose for 5 min before the addition of β-galactosidase. All measurements were done in triplicate and the variations were within 6% of the mean.

Results

Strategy of the approach

The equilibrium phase of the unsaturated PE at physiological conditions is the hexagonal phase (H_{II}). However, the propensity of forming the H_{II} phase differs greatly among the different species of the PE, depending on the fatty acid composition. For example, the L_α to H_{II} phase transition temperature (T_H) is 12°C for DOPE, 32–45°C for egg PE, and 63°C for TPE [22]. Thus, under the conditions used for the present study, i.e., phosphate-buffered saline and room temperature, one would expect that the bilayer liposomes could be prepared with TPE alone, but not with DOPE alone. This is in fact the case (data not shown). Furthermore, if a micelle-forming lipid such as G_{M1} is to stabilize the bilayer phase of the unsaturated PE, one would expect that it is more difficult to do so for DOPE alone than for a mixture of DOPE and TPE. Thus, our strategy is to first find out an appropriate mixing ratio of DOPE/TPE, such that the mixture by itself is at the verge of being capable of forming stable liposomes. Such a mixture would present an excellent chance to be stabilized for liposome formation by the addition of G_{M1}. Furthermore, we have also used a minimal amount for G_{M1} for stabilization, such that the enzymatic degradation of G_{M1} by β-galactosidase would lead to a sensitive destabilization of the liposomes.

Stabilization of DOPE / TPE bilayer by G_{M1}

Formation of intact liposomes was monitored by light scattering and fluorescence quenching of calcein entrapped in liposomes. Initially, different lipid compositions were tested. Of the three ratios tested, DOPE/TPE (1 : 1 molar ratio) formed stable liposomes without a stabilizer. However, 2 : 1 and 3 : 1 ratios (DOPE/TPE) resulted in leaky liposomes, hence the 2 : 1 (molar ratio) DOPE/TPE lipid composition was selected for further studies. To determine the minimal concentration of G_{M1} that could stabilize the otherwise unstable DOPE/TPE bilayer, various amounts of G_{M1} were mixed with the PE mixture and liposomes were prepared by sonication. Low turbidity, as measured by 90° light scattering, of the resulting liposome suspension was taken as an indication of the formation of stable, small liposomes. At molar fractions of G_{M1} above 5%, stable liposomes could be formed (Fig. 1A). At molar fractions of G_{M1} between 1–4%, the liposome suspension was very turbid, hence resulting in a higher light scattering. On the other hand, below 1 mol% of G_{M1}, the light scattering was again low due to the formation of large aggregates (H_{II} phase) of the lipids. Pure DOPE or DOPE/TPE (2 : 1) formed large aggregates, in contrast to DOPC which formed stable liposome regardless of the addition of G_{M1} (Fig. 1A). Fig. 1B shows that the trapping efficiency of the liposomes increased with increasing molar fraction of G_{M1}. DOPE/TPE liposomes containing 5 mol% G_{M1} were stable for calcein

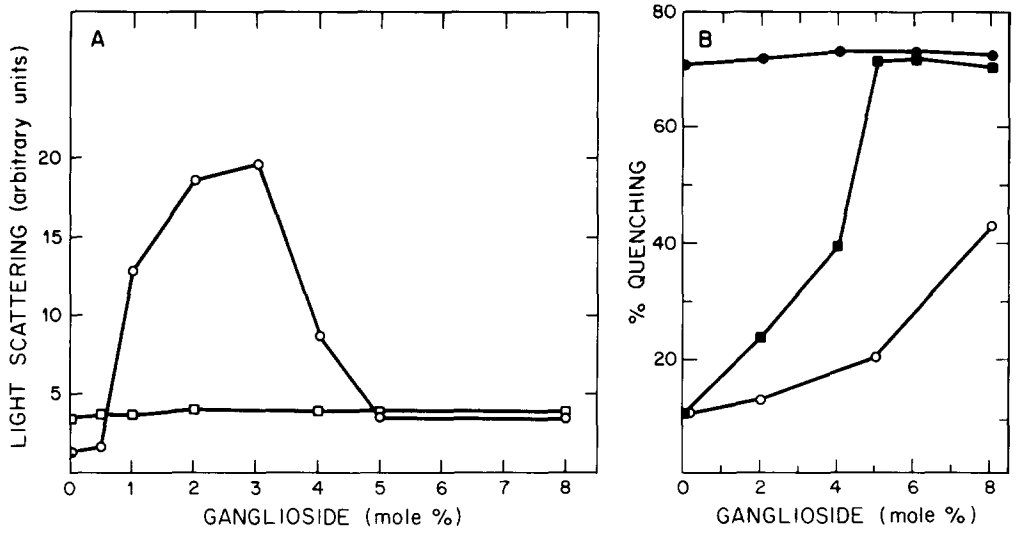

Fig. 1. (A) Stabilization of DOPE/TPE liposomes with ganglioside G_{M1}. 90° light scattering of the sonicated liposomes containing various mole% of G_{M1} were measured for DOPE/TPE (2:1, molar ratio) (○) or DOPC (□) lipids. (B) Fluorescence quenching of DOPC (●) or DOPE/TPE (2:1, molar ratio) (■, ○), sonicated liposomes with different mol% of ganglioside G_{M1} (■) or G_{M2} (○).

entrapment with about 72% fluorescence quenching, which compared very well with fluorescence quenching of the DOPC liposomes containing 0–8 mol% G_{M1}. We concluded that a minimum of 5 mol% G_{M1} was required for the formation of stable, intact DOPE/TPE (2 : 1) liposomes (hereafter called 'G_{M1}/PE liposomes'). Extruded liposomes also required a minimum of 5 mol% G_{M1} for stable calcein entrapment. This composition was used in all subsequent experiments. The stabilization effect of G_{M2}, degalactosylated product of G_{M1}, was also studied by fluorescence quenching (Fig. 1B). It was clear that G_{M2} was not as good a stabilizer as G_{M1}. Nevertheless, G_{M2} had some partial stabilization activity.

Size of liposomes

As revealed by negative-stain electron microscopy, the G_{M1}/PE extruded liposomes were mainly unilamellar with an average diameter of 1500 ± 400 Å ($n = 150$) (Fig. 2).

Liposome destabilization induced by β-galactosidase

Calcein was encapsulated in the liposomes at a self quenching concentration (50 mM). Liposome destabilization was measured by the fluorescence enhancement of calcein as the dye was released

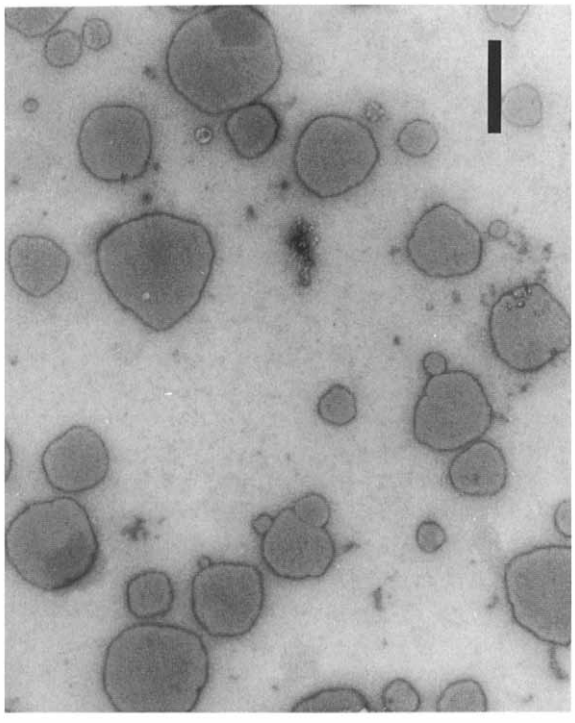

Fig. 2. Electron micrograph of G_{M1}/PE liposomes. 0.4 μmol/ml of liposome suspension was negatively stained with 0.5% uranyl acetate and viewed under a Hitachi 600 electron microscope. The bar represents 2000 Å.

from liposomes [7]. We tested the ability of β-galactosidase to destabilize the G_{M1}/PE liposomes. When the reaction was carried out at room temperature, the $t_{1/2}$ for the dye release was about 120–140 min (data not shown). However, when the reaction was carried out at 37°C, the $t_{1/2}$ of the reaction was decreased to less than 10 min, depending on the β-galactosidase concentration. The dye-release kinetics were distinctively biphasic, with a well-defined lag period preceding the fast phase (Fig. 3). The lag period could be shortened by increasing the enzyme concentration. However, even at a saturation concentration (5 mg/ml) of β-galactosidase there was still an initial lag period of approx. 2 min. This is probably because the cleavage of substantial fraction of G_{M1} residues is necessary to cause the destabilization of the liposome. G_{M1}/PC liposomes were very stable to β-galactosidase digestion, as there was no significant dye release from these liposomes even at the highest β-galactosidase concentration tested. The G_{M1}/PE liposomes with no β-galactosidase treatment were stable at least up to 1 h at 37°C. However, the β-galactosidase

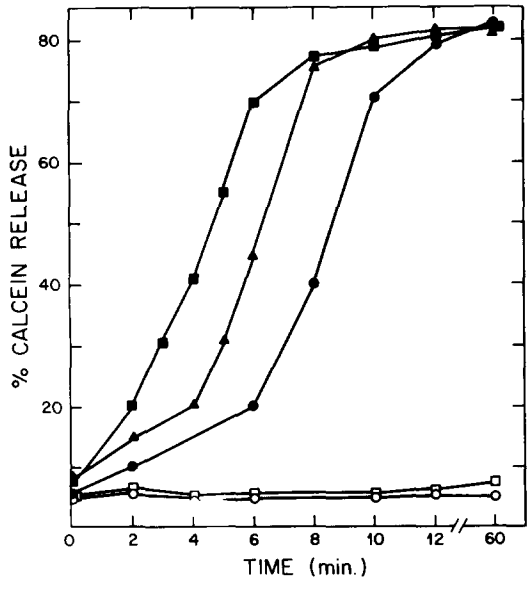

Fig. 3. β-Galactosidase-induced lysis of G_{M1}/PE liposomes. G_{M1}/PE (■, ▲, ●, □) or G_{M1}/PC (○) liposomes containing 50 mM entrapped calcein were incubated at 37°C for various times in the presence of 0 (□), 1 (●), 2 (▲, ○) or 5 (■), mg/ml β-galactosidase, and the percentage of calcein release was measured.

treatment of the G_{M1}/PE liposomes maximally released about 80–85%, never 100% of the encapsulated dye. This is consistent with the observation that the degalactosylated G_{M1}, i.e., G_{M2}, was able to partially stabilize the bilayer phase of PE as shown in Fig. 1B. To confirm the fact that the destabilization of the G_{M1}/PE liposome was due to the conversion of G_{M1} to G_{M2} by β-galactosidase, the liposomal lipids were extracted after treatment with β-galactosidase for different lengths of time, separated on TLC (solvent chloroform/methanol/2.5 M ammonium hydroxide, 50 : 40 : 10) and the spots were visualized by iodine vapor. Although the detection method was not sensitive enough to detect very low amounts of gangliosides, the first appearance of G_{M2} was detected after 4 min. The amount of G_{M2} increased with longer incubation time and at the time of maximal dye release, the amount of G_{M2} present was roughly equal to that of G_{M1}. The rate of G_{M1} to G_{M2} conversion was estimated to be 0.8 μmol/mg β-galactosidase per min. These data indicate that the enzymatic conversion of G_{M1} to G_{M2} by β-galactosidase caused the otherwise stable G_{M1}/PE liposome to release the entrapped dye marker.

Stoichiometric vs. catalytic enzyme reaction

To further rule out the possibility that the enzyme action may be due to a stoichiometric binding to liposomes rather than a catalytic reaction, the following experiment was performed. First, the calcein-entrapped G_{M1}/PE liposomes in excess (200 μM) were incubated with a sub-saturation concentration of β-galactosidase (0.8 mg/ml) for 30 min, at 37°C. After the maximal release of the dye marker (80% of total release), a second aliquot of liposomes (200 μM) was added and the dye release was monitored with time. Although the initial lag period was 8 min compared to a 6 min lag previously, as shown in Fig. 4, the second batch of liposomes also maximally released the entrapped calcein (up to 80% of total) with time. Addition of the third aliquot of liposomes (200 μM) showed a similar result. If the three aliquots of liposome (600 μM) were added together with the same amount of β-galactosidase (0.8 mg/ml), it showed a maximum dye release with a longer lag period of about 15 min.

If the liposome destabilization is due to a stoichiometric binding of β-galactosidase to liposomes, upon addition of the second aliquot of excess liposomes, one would expect to observe less than the maximal level of dye release at the steady state. Similarly, addition of a third batch of excess liposomes would have resulted in even lower level of release of the dye marker in the liposomes. However, the data presented in Fig. 4 clearly argue against a stoichiometric mechanism, as the addition of each aliquot of excess liposomes still resulted in maximal release of dye.

Cleavage of a certain fraction of G_{M1} residues of the G_{M1}/PE liposome may be necessary to cause the destabilization of the liposome resulting in the release of the entrapped dye marker. In the previous experiment, the liposomes were in great excess, and therefore the increased lag period may be due to the fact that the amount of enzyme available per liposome was low, hence taking a longer time to cleave a sufficient amount of G_{M1} resulting in the dye release. Keeping in mind that the rate of the catalytic degradation of G_{M1} is slow (0.8 μmol/mg enzyme per min) compared to that of lactose (40 μmol/mg enzyme per min) or *o*-nitrophenyl-β-D-galactoside (240 μmol/mg enzyme per min) as the substrate, indicating that the liposomal G_{M1} is a poor substrate of β-galactosidase. Also shown in Fig. 4 is that the β-galactosidase-induced liposome destabilization could be inhibited by the addition of excess lactose, probably due to a competitive inhibition of the enzyme activity.

Liposome destabilization as a function of liposome concentration

It has been suggested earlier that contact between the individual PE immunoliposomes is required for the destabilization of the PE bilayers [10,23]. Furthermore, the destabilization of glyco-

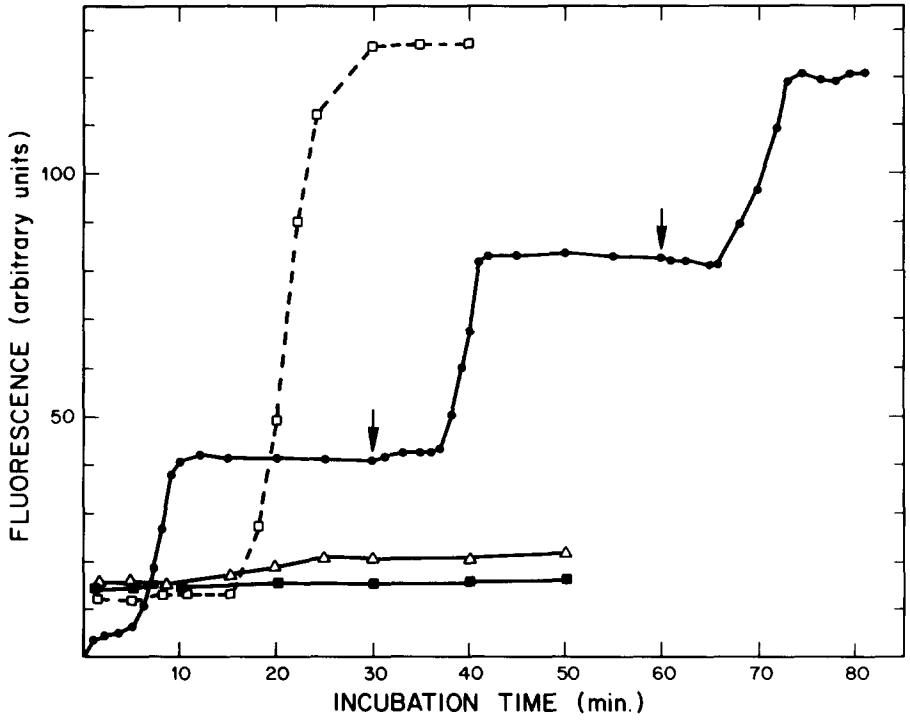

Fig. 4. β-Galactosidase-induced release of calcein from G_{M1}/PE liposomes in the presence of excess liposomes or lactose. Liposomes (200 μM (●) or 600 μM (□, ■)) were incubated in the presence (●, □) or absence (■) of 0.8 mg/ml β-galactosidase and the dye leakage was measured as a function of time. The arrows (↓) indicate the addition of excess liposomes (200 μM). A second set of experiments were carried out where the G_{M1}/PE liposomes (600 μM) were preincubated with lactose (△) and the fluorescence enhancement was measured in the presence of β-galactosidase (0.8 mg/ml).

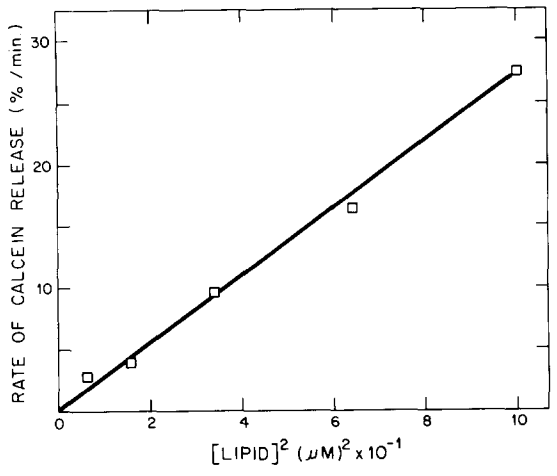

Fig. 5. Calcein release as a function of lipid concentration. Various concentrations of liposomes were incubated with β-galactosidase (1 mg/ml) and the maximal rate of fluorescence increase was measured after the lag period.

phorin-DOPE liposomes induced by trypsin digestion is a second-order reaction requiring collision of the liposomes [13]. These observations prompted us to investigate the possibility that collision of G_{M1}/PE liposomes is required for the bilayer destabilization. The maximal rate of calcein release was measured after the initial lag period at various liposome concentrations. As shown in Fig. 5, the rate of dye release increased linearly with the square of the liposome concentration indicating a second-order reaction requiring collision between the liposomes.

Discussion

Our data clearly show that G_{M1} is a better PE bilayer stabilizer than G_{M2} (Fig. 1B). Enzymatic conversion of G_{M1} to G_{M2} by incubation of the G_{M1}/PE liposomes with β-galactosidase leads to a rapid destabilization of the liposomes (Figs. 3 and 4). G_{M1} differs from G_{M2} by one galactose residue at the terminal position. Thus, one hydrophilic, neutral saccharide residue makes a significant difference in terms of the stabilization effect of ganglioside. Saccharides are excellent H-bonding molecules. The presence of saccharide residues at the bilayer surface would increase the interfacial hydration level by H-bonding with H_2O. Interfacial hydration is thought to be one of the principal parameters which determines whether phospholipids aggregate to a bilayer or a non-bilayer structure [24,26,27]. PE has a much less interfacial hydration than PC and is thus much more in favor of the H_{II} phase than PC [3,25,28]. The presence of G_{M1} brings more H_2O molecules to the interfacial region and stabilizes the bilayer phase. Removal of the galactose residue would reduce the interfacial hydration and destabilize the bilayer phase.

Although the bilayer stability is thermodynamically controlled by the interfacial hydration, the bilayer destabilization process is also kinetically controlled. It is well-established that the destabilization of PE liposomes requires collision among the liposomes. This is because the destabilization process involves the formation of the 'intermembranous intermediate' (IMI) structure [23] or the 'isotropic phase' [29]. Data in Fig. 5 indicate that the destabilization of the G_{M1}/PE liposomes induced by β-galactosidase also requires collision of the liposomes. Thus, removal of galactose residue from G_{M1} has to be followed by liposome collision in order to bring about the destabilization. Degalactosylated liposomes would readily convert into the IMI or isotropic phase upon collision due to a relatively weak repulsive hydration force [26,27] as compared to the original liposomes. Note that the rate analyzed in Fig. 5 is the maximal rate after the initial lag period. This is the time when a substantial amount of G_{M1} has already been converted to G_{M2}. The destabilization process at this period of time is collision controlled. During the lag period, destabilization is likely to be limited by the slow rate of G_{M1} conversion to G_{M2}.

The observation that β-galactosidase induces a relatively rapid liposome lysis has some biotechnological implications. Liposome lysis in response to the immune complex formation, whether mediated by the complement or not, is the basis of the homogeneous immunoliposome assays [30]. Enzymes are often entrapped in the liposomes such that a colorigenic reaction can be catalyzed after liposome lysis. β-Galactosidase has previously been used for this purpose [31,32]. Due to the fact that a large number of enzyme molecules can be released from a given liposome, liposome-based

assays often provide high sensitivity. The sensitivity of the assay could be further enhanced with a second liposome which is destabilized by β-galactosidase released from the first liposome. Reporter molecules, another enzyme or a fluorescent dye such as calcein, are entrapped in the second liposome. Such a cascade reaction for the enhanced assay sensitivity has recently been demonstrated by us using the CEDIA digoxin assay as an example [33]. Active β-galactosidase activity is reconstituted in proportion to the amount of digoxin in the test sample. We have prepared G_{M1}/PE liposomes encapsulating glucose-6-phosphate dehydrogenase as a reporter enzyme. The overall sensitivity of detection was increased by 40-fold. It is likely that the signals of other assays using β-galactosidase as the final readout step could also be enhanced with the G_{M1}/PE liposomes described in this paper.

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