LATERAL DIFFUSION OF GANGLIOSIDE G_{M1} IN PHOSPHOLIPID BILAYER MEMBRANES

B. GOINS,* M. MASSERINI,*‡ B. G. BARISAS,§ AND E. FREIRE*

University of Tennessee, Department of Biochemistry, Knoxville, Tennessee 37996; ‡University of Milan, Department of Biochemistry, Milan, Italy; and §Colorado State University, Department of Chemistry, Fort Collins, Colorado 80523

ABSTRACT The lateral diffusion coefficient of ganglioside G_{M1} incorporated into preformed dimyristoylphosphatidylcholine (DMPC) vesicles has been investigated under a variety of conditions using the technique of fluorescence photobleaching recovery. For these studies the fluorescent probe 5-(((2-Carbohydrazino)methyl)thio)acetyl) amino eosin was covalently attached to the periodate-oxidized sialic acid residue of ganglioside G_{M1}. This labeled ganglioside exhibited a behavior similar to that of the intact ganglioside, and was able to bind cholera toxin. The lateral diffusion coefficient of the ganglioside was dependent upon the gel-liquid crystalline transition of DMPC. Above T_m the lateral diffusion coefficient of the ganglioside was 4.7×10^{-9} cm^2 s^{-1} (with >80% fluorescence recovery). This diffusion coefficient is significantly slower than the one previously observed for phospholipids in DMPC bilayers. The addition of increasing amounts of ganglioside, up to a maximum of 10 mol %, did not have a significant effect on the lateral diffusion coefficient or in the percent recovery. At 30°C, the lateral mobility of ganglioside G_{M1} was not affected by the presence of 5 mM Ca^{2+}, suggesting that, at least above T_m, Ca^{2+} does not induce a major perturbation in the lateral organization of the ganglioside molecules. The addition of stoichiometric amounts of cholera toxin to samples containing either 1 or 10 mol % ganglioside G_{M1} produced only a small decrease in the measured diffusion coefficient. The fluorescence recovery after photobleaching experiments were complemented with excimer formation experiments using pyrene-phosphatidylcholine. Above the transition temperature the presence of 10 mol % ganglioside G_{M1} induced a large decrease in the rate of excimer formation. These results also indicated that the addition of ganglioside G_{M1} to phospholipid bilayer vesicles induces a significant restriction in the lateral mobility parameters of the lipid bilayer and that the presence of Ca^{2+} does not have a further effect in the mobility of the probe molecules.

INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids that are associated exclusively with the outer leaflet of the plasma membrane and synaptic membrane (1–3). Because their oligosaccharide portion is exposed on the cell surface, this class of glycolipids is thought to be important in cell growth regulation and cellular interactions (4). Gangliosides have been implicated as cellular recognition sites (5) for viruses, protein hormones, and bacterial toxins, of which cholera toxin is the best documented example (6). Studies with cholera toxin-ganglioside G_{M1} have shown that internalization of the toxin molecule is by receptor-mediated endocytosis and that lateral movement of the complex is an intermediate step (7). Reports of lateral redistribution induced by cholera toxin in lymphocytes by both indirect immunofluorescence with labeled toxin antibody (8) and by incorporation of exogenous fluorescently labeled gangliosides have been previously reported in the literature (9–11). A major cellular interaction with which gangliosides have been linked is that of synaptic transmission (12). The divalent cation Ca^{2+} is thought to have a part in this function by complexing with the negatively charged head group of the ganglioside, thereby destabilizing the synaptic membrane to release its transmitters (13). The association between sialic acid and Ca^{2+} has been well documented (14, 15). Previous calorimetric studies have revealed that millimolar amounts of Ca^{2+} can induce solid phase separation in dipalmitoylphosphatidylcholine (DPPC) fused unilamellar vesicles containing ganglioside G_{M1} (16) or ganglioside Gt_{1b} (17).

In this paper we have studied the role that the head group region of ganglioside G_{M1} plays in its lateral diffusion within the bilayer. Using fluorescence photobleaching recovery we have quantitatively determined the lateral mobility of fluorescently labeled ganglioside G_{M1} incorporated into preformed dimyristoylphosphatidylcholine (DMPC) vesicles. This method of incorporation allows for an assymmetrical distribution of the gangliosides, which mimics that distribution found in the plasma membrane (18). The fluorescence photobleaching recovery experiments have been complemented by excimer formation experiments using pyrene-phosphatidylcholine (pyrene-Pc) as a probe to measure molecular collisions within the lipid bilayer matrix.

MATERIALS AND METHODS

Materials

Ganglioside G_{M1} prepared by sialidase treatment of the crude ganglioside fraction from beef brain was a kind gift of Dr. T. E. Thompson (University of Virginia). The fatty acid composition of this ganglioside G_{M1} is primarily C 18:0 (~80%), the rest being primarily a mixture of C 20:0, C 22:0, and C 24:0 fatty acid chains. The aldehyde reactive probe 5(((2-Carbohydrazino)methyl)thio)acetyl) amino eosin (CMTAA-eosin) was a generous gift from Richard Haugland (Molecular Probes, Junction City, OR). Cholera toxin was purchased from Sigma Chemical Co. (St. Louis, MO). The protein was supplied as a lyophilized powder and was reconstituted with distilled water at a concentration of 0.5 mg/ml in 50 mM Tris-HCl, 0.2 M NaCl, 3 mM NaN_3, 1 mM Na_2EDTA, pH 7.5. DMPC and DPPC were obtained from Avanti Biochemicals (Birmingham, AL). 1-Palmitoyl-2-pyrene decanoyl PC was obtained from KSV Chemicals (Helsinki, Finland). The purity of all lipids was checked by thin layer chromatography (TLC).

Ganglioside Labeling with CMTAA-Eosin

The method of Cherry et al. (19) was used to label the sialic acid moiety of ganglioside G_{M1}. The ganglioside was first dissolved in 2 ml PBS buffer, pH 7.4. An additional 2 ml PBS buffer containing 2 mM sodium periodate was added to the ganglioside solution to give a final concentration of 1 mg/ml. After a 30-min incubation on ice, the periodate was removed by an overnight dialysis using Spectrapor dialysis tubing (MW cutoff 1,500) against PBS buffer pH 7.4 at 4°C. The following day a 1.5 M excess of CMTAA-eosin was added and the solution incubated for 2 h on ice in the dark. To remove the excess label, the conjugated ganglioside was dialyzed against PBS buffer at 4°C in the dark for 2 d with a change of buffer. Following dialysis, the sample was passed through a G-25 column using a rapid filtration-centrifugation procedure (20). To check for the presence of the labeled ganglioside a TLC plate (21) was run. An aliquot of the sample was N_2 dried and redissolved in chloroform/methanol (1:1) and spotted on an E. Merck silica gel 60 plate (Alltech Associates, Deerfield, IL). The plate was developed in chloroform/methanol/0.02% $CaCl_2$ (55:45:10). After air drying, the pink bands which migrated with standard G_{M1} were detected by either spraying the plate with primulin or resorcinol (21). To separate the labeled ganglioside from any additional unconjugated probe preparative TLC plates were run under the same conditions described above. The labeled gangliosides were eluted from the silica gel by adding excess chloroform/methanol/distilled H_2O (5:5:1), vortexed vigorously, and allowed to sit for at least 30 min (21). The solution was centrifuged 10 min at 3,000 rpm and the labeled ganglioside containing supernatant was removed. The solvent was evaporated under N_2. To remove any excess silica gel, 0.5 ml of chloroform/methanol (1:1) was added to the dried sample and recentrifuged. The solvent was again removed and the labeled ganglioside redissolved in PBS buffer and stored at 4°C. The concentration of label could be measured by using the extinction coefficient of eosin at 540 nm of 83 $mM^{-1}cm^{-1}$ (22). Ganglioside concentration was checked using a total sialic acid assay (23).

Vesicle Preparation

Large bilayer vesicles (>2 μm in diameter) were prepared following a method similar to that described by Fahey and Webb (24). 0.4 ml DMPC solution consisting of a concentration of 5 mg/ml in chloroform/methanol (2:1) was pipetted slowly on to the bottom of a 150-ml beaker. The solvent was allowed to dry under a stream of N_2. After complete evaporation 5 ml of double distilled H_2O was pipetted slowly onto the dried lipid film at a temperature above the phase transition temperature. After several hours the vesicles were collected. Large DPPC unilamellar vesicles of 900-Å diameter used in the pyrene experiments were prepared by fusing small sonicated vesicles at 4°C as described elsewhere (16, 17). Total phospholipid phosphorous was assayed using a modified Bartlett procedure (25).

Fluorescence Photobleaching Recovery

The instrumentation and data analysis used in these experiments have been described in more detail elsewhere (26, 27, 28). 514-nm excitation was provided by a Coherent Radiation CR-4 argon ion laser. A universal microscope with vertical fluorescence illuminator and a 40× dry objective was used (Carl Zeiss Inc., Thornwood, NY). Fluorescence was measured by a photomultiplier tube (model C31034A; RCA, Lancaster, PA) coupled to a photon counter. The experiments in this paper were done using bleach times of 5 ms with a $1/e^2$ spot radius of 0.56 μm. Raw fluorescence recovery data were processed on line by a Nova 3/12 computer to yield the diffusion coefficient and fraction of mobile fluorescent molecules. Temperature was maintained by using a thermoelectrically controlled thermal stage. All the values presented in this paper are the mean of at least 12 measurements.

Excimer Formation Experiments

Excimer formation experiments were performed by measuring the fluorescence emission spectrum of pyrene-PC incorporated into vesicles containing the desired amount of ganglioside G_{M1}. All fluorescence measurements were performed in a spectrofluorometer (model LS-5; Perkin-Elmer Corp., Norwalk, CT) equipped with a red-sensitive photomultiplier and a thermostated cuvette holder connected to a Precision RDL 20 bath circulator. The temperature of the samples was monitored during the experiments with a Keithley digital thermometer. In these experiments the samples were excited at 320 nm, and the emissions of monomer and dimer were taken at their maxima at 395 and 480 nm, respectively.

RESULTS

Ganglioside Binding to Cholera Toxin

The chemical structure of the fluorescent labeled ganglioside G_{M1} (CMTAA-Eosin-G_{M1}) is shown in Fig. 1. To check the integrity and biological activity of the labeled ganglioside molecules, their ability to bind cholera toxin was examined. It has previously been reported (16, 29) that the binding of cholera toxin to its receptor, ganglioside G_{M1}, is accompanied by a blue shift in the tryptophan emission spectrum of the toxin. The tryptophan emission spectrum (excitation wavelength = 280 nm) was measured in the absence and in the presence of stoichiometric amounts of CMTAA-eosin ganglioside G_{M1}. As shown in Fig. 2, the emission maximum shifted from 347 to 341 nm after addition of the labeled ganglioside. This fluorescence

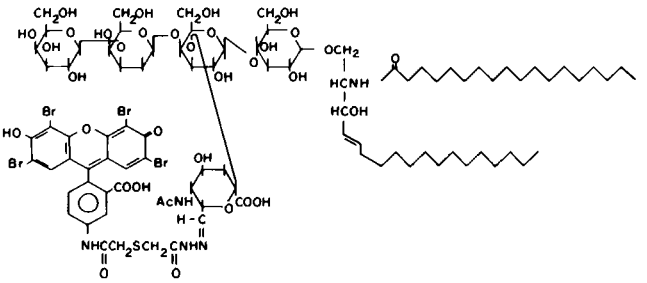

FIGURE 1 Structure of 5 (((2-Carbohydrazino)methyl)thio)acetyl) amino eosin (CMTAA-eosin) modified ganglioside G_{M1}.

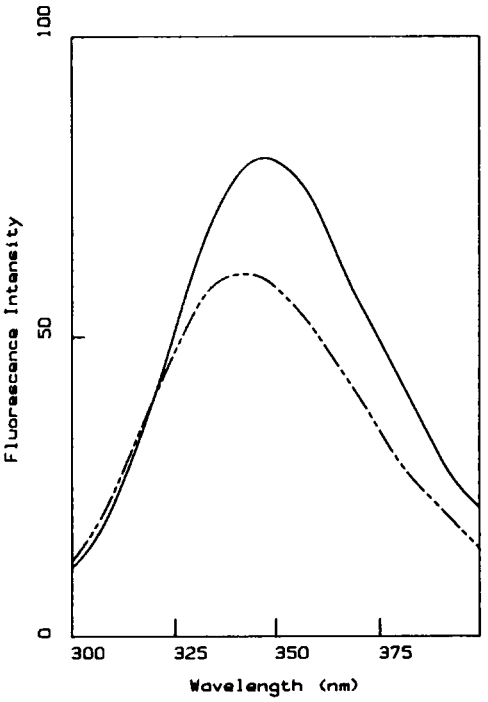

FIGURE 2 Tryptophan emission spectrum of cholera toxin in the absence (solid line) or presence of stoichiometric amounts of CMTAA-eosin labeled ganglioside G_{M1} (dashed line).

change is similar to the one obtained with the native ganglioside and indicates that the tryptophan residues in the protein have become exposed to a more hydrophobic environment. It should be noted that neither the addition of phospholipid vesicles without ganglioside nor the addition of other gangliosides induced any changes in the fluorescence spectrum of the toxin molecule.

Ganglioside Lateral Diffusion as a Function of Temperature and Concentration

Before the photobleaching experiments, the phospholipid vesicles were observed by both phase contrast and epifluorescence microscopy to confirm that the labeled ganglioside had associated with the vesicles. The fact that the labeled ganglioside is quite mobile in the bilayer as judged by diffusion coefficients and recovery values provides further evidence that the ganglioside did incorporate into the bilayer and had not just adhered to the vesicle surface. These observations agree with previous reports in which gangliosides have been shown to spontaneously incorporate within bilayers of model membrane systems (9) and cells (10–11).

The lateral diffusion of ganglioside G_{M1} incorporated into DMPC vesicles was measured as a function of temperature and mole fraction of ganglioside. The results are shown in Fig. 3. At all three ganglioside concentrations studied (1, 5, and 10 mol %), the diffusion was dependent upon the gel-liquid crystalline state of the bilayer. At 25°C,

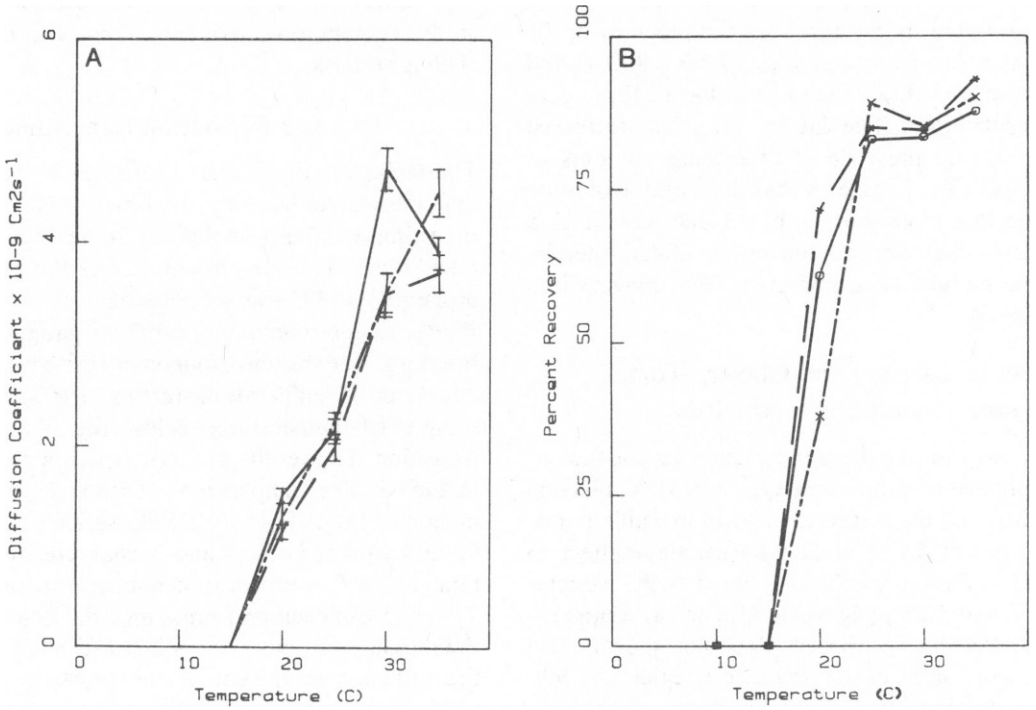

FIGURE 3 *Panel A:* Lateral diffusion coefficient versus temperature for CMTAA-eosin labeled ganglioside G_{M1} incorporated into DMPC bilayers at either 1% (—), 5% (--) or 10% (---) total mole fraction of G_{M1}. In each case, the concentration of labeled ganglioside G_{M1} was constant. *Panel B* depicts the temperature dependence of the fluorescence percent recovery for DMPC bilayers containing CMTAA-eosin ganglioside G_{M1} at either 1% (O), 5% (*), or 10% (×) total G_{M1}.

slightly above the phase transition temperature for DMPC (T_m = 24.5°C) but still within the transition region, the diffusion coefficients were ~2.0 × 10^{-9} cm^2s^{-1} for all ganglioside-containing samples. This value is significantly slower than the coefficient ($D = 1.1 \times 10^{-8} cm^2s^{-1}$) found for the fluorescent lipid analogue diI in DMPC vesicles without ganglioside. This latter value is similar to the one previously obtained by Fahey and Webb (24) under equivalent conditions. The addition of ganglioside also resulted in a reduced lateral diffusion coefficient (2.7 × 10^{-9} cm^2 s^{-1}) of diI at this temperature. Previously, we have found, using high sensitivity differential scanning calorimetry, that in the presence of ganglioside G_{M1} the heat capacity peak of the phospholipid becomes broader and skewed towards the high temperature end of the transition, and that this effect is probably due to a higher melting temperature of the ganglioside containing regions of the membrane (16). These results are consistent with the present observations and further suggest that ganglioside molecules only become fully mobile after the bulk of the phospholipid has melted. A twofold increase ($D = 4.7 \times 10^{-9} cm^2s^{-1}$) in the GM_1 diffusion coefficient occurred when the DMPC vesicles containing ganglioside were incubated at 35°C, which is above the phase transition temperature. This value is still slower than the one obtained for pure DMPC bilayers at this temperature ($D = 1.6 \times 10^{-8} cm^2s^{-1}$) using the fluorescent lipid analogue diI. This restricted movement on the part of the ganglioside in the fluid state of the bilayer is not due to the amount of total ganglioside present in the bilayer. This is further substantiated by the fact that the recovery in the fluorescence after bleaching (see Fig. 3 *B*) is >80% at and above the phase transition temperature for all three ganglioside concentrations. The lateral diffusion decreased only slightly by the presence of increasing amounts of unlabeled ganglioside. It appears that the addition of more ganglioside up to a mole fraction of 0.1 does not cause a measurable crowding or immobilization effect, thereby preventing the mobile-labeled fraction from moving into the bleached area.

Effect of Calcium and Cholera Toxin on Ganglioside Lateral Mobility

The effect of calcium on the temperature dependence of the lateral diffusion of ganglioside G_{M1} in DMPC bilayers was also studied and the results are shown in Table I. The addition of up to 5 mM Ca^{2+} did not have a major effect on the measured diffusion coefficients for DMPC vesicles containing 1% and 10% ganglioside G_{M1} for all temperatures studied. Percent recoveries (data not shown) also remained the same even in the presence of calcium. Only the samples containing 1 mol % ganglioside showed a slight but statistically significant decrease in the lateral diffusion coefficient when Ca^{2+} was added above the phase transition temperature. Under all the remaining experimental conditions Ca^{2+} did not have a significant effect on the ganglioside mobility.

The substitution of the unlabeled ganglioside fraction for gangliosides G_{d1a} ($D = 3.44 \times 10^{-9} cm^2s^{-1}$) and G_{t1b} ($D = 3.83 \times 10^{-9} cm^2s^{-1}$) at a 10 mol % concentration gave similar results. In the presence of Ca^{2+} the diffusion coefficients for G_{d1a} and G_{t1b} were 4.0 × $10^{-9} cm^2s^{-1}$ and 3.33 × $10^{-9} cm^2s^{-1}$ at 30°C, respectively. These gangliosides differ only in their number of sialic acid residues. It has been suggested that Ca^{2+} ions might have a crossbridging effect on gangliosides and that this effect may restrict the lateral movement of the ganglioside molecules (30). From the photobleaching results it can be seen that the lateral mobility of gangliosides G_{m1}, G_{d1a}, and G_{t1b} is somewhat insensitive to the presence of Ca^{2+} ions.

The results obtained after the addition of stoichiometric amounts of cholera toxin to samples containing 1% or 10% ganglioside G_{M1} are shown in Fig. 4. In both cases the presence of the toxin caused a decrease in the measured lateral diffusion coefficient. However, this decrease in the lateral diffusion coefficient was not very large and was not accompanied by a decrease in the percent recovery. Previously, we have also observed a small decrease in the rate of pyrene excimer formation after the addition of cholera toxin to phospholipid vesicles containing ganglioside G_{M1} (16). Examination of the lipid vesicles under the fluorescence microscope did not reveal any evidence of patching or clustering after the addition of cholera toxin. These observations are in agreement with the results of Thompson et al. (37) using freeze-etch electron microscopy to study the organization of ganglioside G_{M1} in phosphatidylcholine vesicles.

Excimer Formation Experiments

The technique of excimer formation by pyrene phospholipid derivatives has been used by several authors (31, 32) to estimate lateral mobilities in artificial and natural membranes. For our present studies the excimer forming probe pyrene-PC was incorporated into large unilamellar DPPC vesicles containing different ganglioside G_{M1} mole fractions. The excimer/monomer ratios were measured as a function of temperature starting at 60°C and cooling the sample to temperatures below the phospholipid phase transition. The results of these experiments are illustrated in Fig. 5. The temperature dependence of the excimer/monomer ratios for pure DPPC vesicles is similar to that found by other authors and is characterized by an inflection at 41.5°C, the transition temperature of DPPC. Above T_m the rate of excimer formation is diffusion controlled and the measured excimer/monomer ratios are proportional to the diffusion coefficient of the probe in the membrane (32). Above the phase transition temperature the addition of 10 mol % ganglioside G_{M1} causes a large decrease in the excimer/monomer ratio, consistent with a large restriction in the lateral mobility of the probe. At 55°C the excimer/

TABLE I
EFFECT OF CA^{2+} ON THE LATERAL DIFFUSION COEFFICIENT OF GANGLIOSIDE G_{M1} IN GEL OR FLUID DMPC BILAYERS

Temperature (°C)	1% G_{M1}*		10% G_{M1}	
	Control	Ca^{2+}‡	Control	Ca^{2+}
20	1.51 ± 0.074	1.81 ± 0.211	1.29 ± 0.068	2.18 ± 0.309
25	2.15 ± 0.122	2.36 ± 0.126	2.23 ± 0.106	2.46 ± 0.130
30	4.72 ± 0.206	3.36 ± 0.306	3.51 ± 0.190	3.56 ± 0.245

*Percent ganglioside depicts moles of ganglioside to total moles of lipid.
‡5 mM Ca^{2+} was used throughout the experiment.
All values are $\times 10^{-9}$ cm^2 s^{-1} and are represented as mean values ± standard errors.

monomer ratio is 0.46 for pure DPPC vesicles and decreases to 0.33 after incorporation of 10 mol % ganglioside. The addition of Ca^{2+} did not produce any significant difference in the excimer/monomer ratios above the phase transition temperature as indicated in Fig. 5 *B*. These results are in agreement with the fluorescence recovery after photobleaching experiments in which no change in the measured diffusion coefficients were observed after the addition of Ca^{2+}.

DISCUSSION

Gangliosides have been implicated as cell surface membrane recognition sites for viruses, polypeptide hormones, and bacterial toxins (5). In particular, ganglioside G_{M1} has been identified as the receptor for cholera toxin (6). The cholera toxin molecule is a multisubunit protein composed (33, 34) of five binding (B) subunits (M = 11,000 per subunit) and an A subunit. This A subunit is composed of an active hydrophobic portion termed A_1 (M = 21,000) linked via a disulfide bond to a smaller subunit, A_2, (M = 5,000), which is responsible for holding the B and A_1 subunits together. The binding subunits are able to bind ganglioside G_{M1} in a stoichiometric fashion and the intact toxin binds a maximum of five ganglioside molecules (35). The association of cholera toxin to intact cells such as lymphocytes has been shown to induce a massive redistribution and capping of the gangliosides molecules on the cell surface (8, 9, 11). The molecular mechanism of this process still remains unclear. The results presented in this communication were designed to elucidate the dynamic parameters of ganglioside diffusion in well defined single component lipid bilayers, and to test whether or not the association of cholera toxin or the presence of Ca^{2+} has a significant effect on the distribution and mobility of gangliosides in a cell-free system.

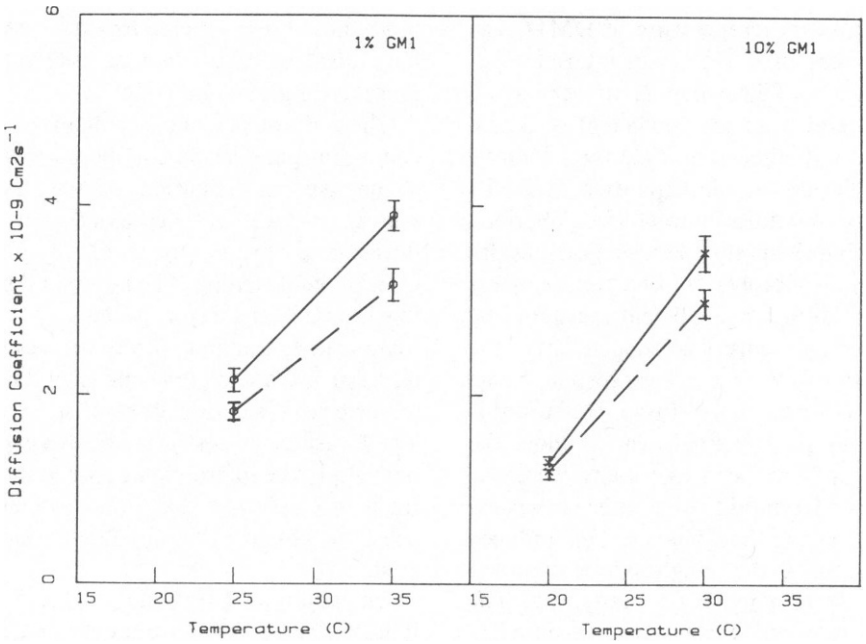

FIGURE 4 Lateral diffusion coefficient vs. temperature for DMPC bilayers containing a total of either 1% G_{M1} (O) or 10% G_{M1} (×) in the presence (– – –) or absence (——) of stoichiometric amounts of cholera toxin. In both cases a constant amount of CMTAA-eosin labeled ganglioside G_{M1} was added.

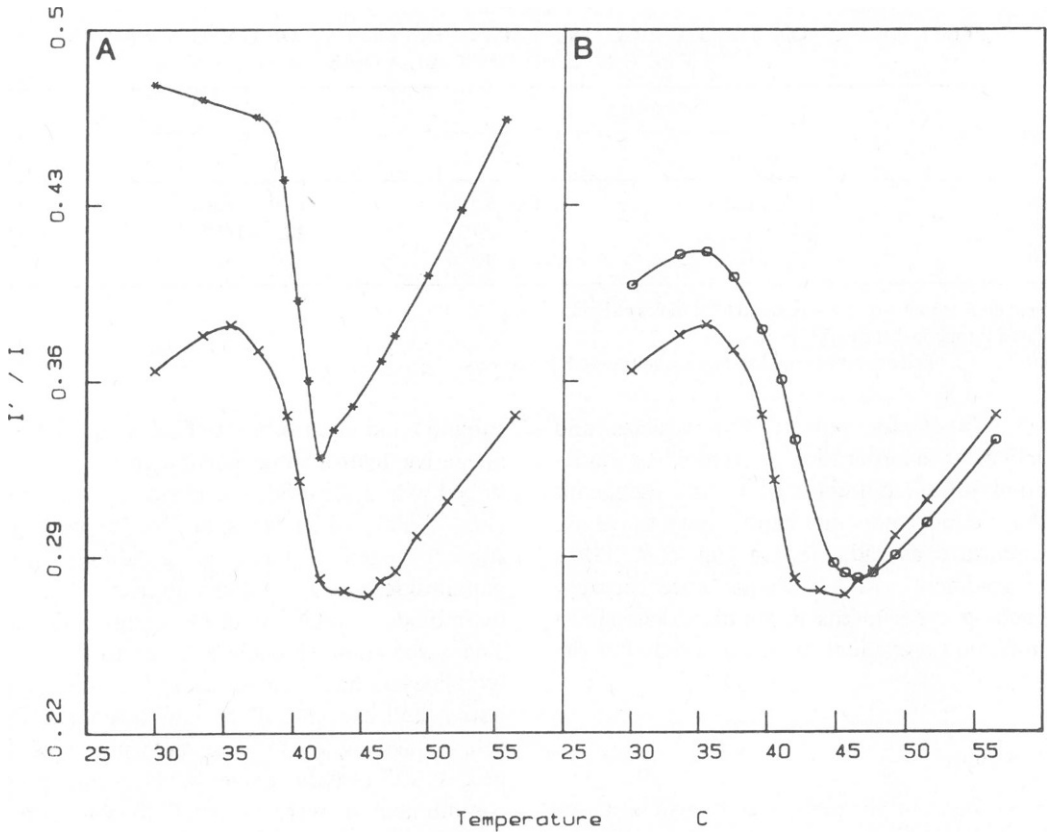

FIGURE 5 *Panel A:* Excimer/monomer ratios vs. temperature of pyrene-PC incorporated into large unilamellar DPPC vesicles in the absence (*) or presence (×) of 10 mol % ganglioside G_{M1}. The lipid concentration was 3 μM and the amount of pyrene-PC was 3 mol % of the total lipid. *Panel B:* The temperature dependence on the excimer/monomer ratios of pyrene-PC incorporated into large unilamellar DPPC vesicles containing 10% G_{M1} in the absence (×) or presence (O) of 20 mM Ca^{2+}. Experimental conditions were the same as described in *panel A*.

Above the phase transition temperature of DMPC the lateral diffusion coefficient of CMTAA-ganglioside G_{M1} was ~4.7 × 10^{-9} cm^2s^{-1}. This value is in very close agreement with the lateral diffusion coefficient of 5.5 × 10^{-9} cm^2 s^{-1} obtained by Reidler et al. (36) for a fluorescein-labeled lyso ganglioside G_{M1} incorporated into 3T3 cells and also with the lateral diffusion coefficient obtained by Spiegel et al. (11) using rhodamine-labeled gangliosides incorporated into human fibroblasts. Our experiments indicate that the lateral diffusion coefficient measured by fluorescence photobleaching recovery is independent of the ganglioside mole fraction over the concentration range studied (1–10 mol %), however this diffusion coefficient is about half of that of pure phospholipid vesicles under the same conditions. Excimer formation experiments were also consistent with a reduced lateral diffusion after incorporation of ganglioside G_{M1} in the lipid bilayer. This reduced lateral diffusion coefficient of the ganglioside is expected on the basis of the bulkier ganglioside headgroup. The oligosaccharide headgroup of ganglioside G_{M1} has a molecular weight of 1,016 compared to the molecular weight of 153 for phosphatidylcholine. The additional hydrodynamic drag will slow down the lateral diffusion of the ganglioside molecules. Previous electron spin resonance studies (30) have also indicated an increased bilayer rigidity in the presence of glycosphingolipids.

The addition of cholera toxin in the phospholipid vesicles containing ganglioside G_{M1} did not produce any evidence of a massive redistribution of the fluorescence with the bilayer surface and decreased only slightly the lateral diffusion coefficient measured by fluorescence recovery after photobleaching. The lack of a distinguishable clustering in artificial bilayer systems after addition of cholera toxin is in agreement with recent results using freeze etch electron microscopy (37) and should be contrasted with the massive redistribution observed in natural cell membranes. These results suggest that the driving force for capping is not simply the interaction between the ganglioside and the toxin molecule but that other elements present in intact cells, yet absent in phospholipid vesicles, must be considered.

The addition of Ca^{2+} did not cause a significant change in lateral diffusion above the phase transition temperature as indicated by the fluorescence photobleaching recovery experiments as well as the excimer formation experiments. These observations are in agreement with recent electro-

phoretic mobility and binding experiments by McDaniel and McLaughlin (38). These authors concluded that the intrinsic association constant of calcium with G_{M1} is quite low, $<100\ M^{-1}$. The absence of a strong direct Ca^{2+}-ganglioside interaction accounts for the absence of a Ca^{2+}-induced ganglioside clustering in the fluid phase of the bilayer. Recent calorimetric experiments (Masserini and Freire, in preparation) using homogeneous chain length gangliosides indicate that the Ca^{2+} induced solid phase immiscibility observed in calorimetric scans of ganglioside-phosphatidylcholine mixtures (16, 17) is ganglioside chain length dependent and is probably triggered by a tightening of the phosphatidylcholine bilayer matrix followed by ganglioside passive exclusion from the phosphatidylcholine rich phase, rather than by active crossbridging of ganglioside molecules by Ca^{2+}.

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