Biochimica et Biophysica Acta, 1112 (1992) 105–114

BBAMEM 75821

Modulation by glycosphingolipids of membrane–membrane interactions induced by myelin basic protein and melittin

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(Received 29 April 1992)
(Revised manuscript received 3 September 1992)

Key words: Ganglioside; Sulfatide; Glycosphingolipid; Membrane fusion; Myelin basic protein; Melittin

The effect of glycosphingolipids (GSLs) with oligosaccharide chains of different length and charge on membrane–membrane interactions induced by myelin basic protein (MBP) or melittin (Mel) was comparatively investigated with small unilamellar vesicles. MBP induces a fast vesicle aggregation and close membrane apposition. Merging of lipid bilayers and vesicle fusion induced by MBP are slower and less extensive processes compared to membrane apposition. The changes of membrane permeability concomitant to these phenomena are small. The Trp region of MBP remains in a rather polar environment when interacting with vesicles; its accessibility to NO_3^- or acrylamide quenching depends on the type of GSLs in the membrane. The Trp region of Mel is inserted more deeply into the lipid bilayer and its accessibility to the aqueous quenchers is less dependent on variations of the oligosaccharide chain of the GSLs. Mel induces a faster and more extensive membrane apposition and bilayer merging than does MBP. Extensive vesicle disruption occurs in the presence of Mel. Negatively charged GSLs facilitate membrane proximity and vesicle aggregation but an increase of the oligosaccharide chain length of either neutral or acidic GSLs decreases the interaction among vesicles that are induced by either protein. This effect is independent of the different mode of insertion of MBP and Mel into the membrane. Our results suggest that the modulation by the oligosaccharide chain on the protein-induced interactions between bilayers containing GSLs is probably exerted beyond the level of local molecular interactions between the basic proteins and the lipids.

Introduction

In the differentiation, development and degeneration of the nervous system the composition in glycosphingolipids (GSLs) of neural membranes undergoes dramatic qualitative and quantitative changes [1–4]. The variability of the composition and expression of GSLs is important for the recognition, adhesiveness and communication among cell membranes [5,6]. It has been suggested that gangliosides and membrane-associated proteins can mediate surface interactions [4,7]. However, the influence of the oligosaccharide chain of GSLs on membrane–membrane interactions induced by proteins has not been systematically studied. In this work, we investigate comparatively how variations of the oligosaccharide chain length and charge affects membrane interactions induced by two basic proteins, myelin basic protein (MBP) and Melittin (Mel), as representative models for proteins that are either partially embedded (MBP) or that insert deeply into the lipid bilayer (Mel).

These proteins were chosen because, different to others, a reasonable amount of information exists on their effects on the organization and stability of biomembranes. MBP is the main encephalitogenic protein of the central nervous system [8,9]. It has been used as a model of extrinsic membrane protein and for its role in myelin stability during physiological and pathological

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Abbreviations: GSLs, glycosphingolipids; Cer, ceramide (*N*-acylsphingoid); GalCer, Galβ1–1Cer; LacCer, Galβ1–4Glcβ1–1Cer; Gg_4Cer (asialo-G_{M1}), Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1Cer; NeuAc, *N*-acetylneuraminate; Sulf, Gal(3-sulfate)β1–1Cer; G_{M1}, Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1Cer; G_{D1a}, NeuAcα2–3Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1Cer; PC, egg phosphatidylcholine; PA, egg phosphatidic acid; MBP, myelin basic protein; Mel, melittin; RET, resonance energy transfer; Trp, tryptophan residue; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, rhodamine phosphatidylethanolamine; NTA, nitrilotriacetic acid; DPA, dipicolinic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CF, carboxyfluorescein.

conditions [10–12]. The small amphipathic peptide Mel, the main constituent of bee venom [13], has been considerably studied as a model of amphipathic peptides that affect the structural integrity of membranes [13–17]. MBP and Mel induce membrane aggregation, permeability changes, vesicle fusion, and affect the formation of hexagonal II phases [16–24]. Both are basic proteins that interact well with either neutral or zwitterionic lipids but the effects are more marked with negatively charged phospholipids and GSLs [16,17,25–29]. Native MBP induces chicken erythrocyte fusion [30] and this effect is abolished by acetylation or succinylation of its basic amino-acid residues [31]. Succinylated Mel can induce vesicle fusion in a pH-dependent manner [32]. The interactions of MBP and Mel with GSLs depend on the type of oligosaccharide chains in the lipid polar headgroup [25–27,33]. We previously found that Sulf and GalCer, two GSLs with short polar headgroups that are enriched in myelin, can modulate the rate and extent of membrane interactions induced by MBP [34]. The results of the present work indicate that the length and charge of the oligosaccharide chain of GSLs are major factors that modulate the interactions among membrane surfaces induced by MBP and Mel.

Materials and Methods

Reagents. Egg PC, PA, NBD-PE and Rh-PE were from Avanti Polar Lipids (Whithling, AL). They were over 99% pure as checked by HPTLC. GSLs were prepared as described elsewhere [35]. Gangliosides were submitted to the additional purification steps necessary for monolayer work [36]. Porcine MBP was from Ely Lilly, and it was further purified as previously described [30]. Mel was from Sigma (St. Louis, MO, USA), and further purification was according to Mollay et al. [37] as described previously [25]. CF was from Eastman Kodak (Rochester, NY). NTA, Tes, DPA, and Sephadex G-75/120 were from Sigma (St. Louis, MO). $TbCl_3 \cdot 6H_2O$ was from Alfa (Danvers, MA). EDTA disodium salt, and other chemicals were of analytical reagent grade, solvents were freshly redistilled before use and water was bidistilled in an all-glass apparatus.

Vesicles. Small unilamellar vesicles were prepared by evaporating under N_2 lipid solutions of egg PC with GSLs in the desired proportions in chloroform/methanol (2:1, v/v). The dry lipid was lyophilized for at least 4 h. The lipid was suspended in 100 mM NaCl-5 mM Tes buffer (pH 7.4), vortexed and subsequently sonicated under N_2 for 1 h in a cup-horn sonicator above the transition temperature previously determined for each lipid system [38]. The suspension was centrifuged to remove undispersed lipid [34]. Negative staining (1% uranyl acetate) of the preparations revealed small unilamellar vesicles of 20–50 nm diameter. Vesicles were stored above their transition temperature and used within two days. For fluorescence RET assays the vesicles contained 0.6 mol% (probe dilution assay) or 2 mol% (probe mixing assay) of NBD-PE and Rh-PE in the same or in separate vesicles, respectively [34,39]. Mixing of aqueous compartments or leakage was determined using different populations of vesicles entrapping one of the following solutions: (a) 15 mM $TbCl_3$; (b) 50 mM DPA and 20 mM NaCl; (c) 1.25 mM $TbCl_3$, 25 mM NTA, 25 mM DPA and 10 mM NaCl; (d) 50 mM CF. All solutions were buffered in 5 mM Tes at pH 7.4. The buffers for measuring the fluorescence and for the elution of the vesicles from the column contained EDTA [40]. Unencapsulated material was eliminated by gel filtration through a column of Sephadex G-75. Between 95–98% of PC and GSLs was recovered in the solvent front for all vesicle preparations.

Measurements. Different conclusions may be reached depending on the particular fluorescent assay employed to evaluate intermembrane interactions [40]. Due to this, we employed light scattering, absorbance changes and six different fluorescence assays to monitor lipid bilayer merging, vesicle aggregation, fusion, permeability changes, and vesicle–protein interactions; several of these assays were performed simultaneously with the same vesicle preparation [34,40,41]. Fluorescence measurements were made continuously as a function of time with an SLM-4800C fluorometer at 22 ± 1°C under continuous stirring. The final lipid concentration was 50 μM. MBP or Mel were added from a concentrated solution (10 mg/ml). Calibration of fluorescence, excitation and emission wavelengths and filters employed for the different assays was done as described recently [34]. The tables and figures show mean values of duplicate or triplicate experiments. For all the assays the S.E. of these values was within a maximum of $\pm 6\%$. The λ_{max} were reproducible to ± 1.5 nm and the K_{SV} values to ± 1 M^{-1}.

Tb, DPA and CF entrapment was between 2.3–3.8 nmol/μmol lipid, 18.7–25.3 nmol/μmol lipid, and 16.2–23.7 nmol/μmol lipid, respectively, for the different preparations. Spontaneous leakage of about 4% per day for Tb, 12% per day for DPA and 3% per day for CF was observed. The different preparations were used only during the period in which no significant differences were observed in the initial light scattering or absorbance at 450 nm. In the cases where the absorbance at 450 nm increased above 0.1 after adding the proteins, the fluorescence intensities were corrected for inner filter effects [42]. No changes of RET were found when the vesicles were mixed in the absence of proteins. Also, the proteins did not modify the fluorescence of vesicles containing only one lipid probe incorporated in the membrane or the entrapped

water-soluble probes. Changes of the intrinsic fluorescence of MBP and Mel upon interacting with the lipid vesicles was measured by recording the emission spectra of their single Trp residue with $\lambda_{exc} = 280$ nm [31].

Protein binding. The binding of MBP to the different lipid vesicles was evaluated by incubating 50 nmol of lipid, in a total volume of 0.5 ml of NaCl-Tes buffer containing 1 mM EDTA, with different aliquots of a 0.1 mM solution of MBP for 30 min at 22°C under continuous stirring. The preparation was subsequently centrifuged at 200000 × *g* for 1 h. The original supernatant was separated, the pellet was washed with a same volume of buffer and spun down as before, the second supernatant was added to the first. The lipids and MBP were determined in both the combined supernatants and the washed pellet. PC and GSLs were determined colorimetrically or by HPTLC as previously described [35,36,42]. MBP was determined fluorometrically with *o*-phthalaldehyde [44]. The recovery of MBP and of the different lipids was between 95 and 99%. More than 95% of all the lipids was always recovered in the pellet. With Mel, the centrifugation method only gave reliable results at relatively large lipid/protein molar ratios above 200. As this ratio approached about 50 to 100, lipid was increasingly found in the supernatant. This is in agreement with results from others [16,18] and is due to the formation of mixed micelles or discoidal complexes with lipids induced by Mel [45,46]. Thus, with Mel we used the shift of λ_{max} of its Trp residue and the relative fluorescence intensity enhancement upon binding as a measure of its interaction with lipids [17].

Results

Effect of myelin basic protein

Vesicles of PC or PC-GalCer bound 1 molecule of MBP per 80–100 molecules of total lipid. The MBP/lipid molar ratio that saturated MBP binding to lipid vesicles containing 20 mol% GSLs increased to about 1:60 for PC-LacCer, 1:45 for PC-Gg4Cer, 1:28 for PC-Sulf, 1:22 for PC-G_{M1}, and 1:17 for PC-G_{D1a}. These values are in good agreement with the few studies that have reported binding of MBP to vesicles containing some of these lipids [10]. These results indicate that the presence of more complex or negatively charged GSLs generally facilitate the interaction with and incorporation of MBP into the lipid interface. Similar conclusions were previously reached from studies of MBP-GSLs interactions done with lipid monolayers [25,26] and by calorimetry [47].

A rapid increase of light scattering occurs after adding MBP (Table IA). This is followed by a slower increase of absorbance at 450 nm (Fig. 1a, Table I).

TABLE I

A. Initial rate of vesicle interactions induced by MBP

Lipid	Aggr. (OD_{450}/min)	Probe mix ($\%F_{max}$/min)	Probe dilut ($\%F_{max}$/min)	Fusion ($\%F_{max}$/min)	Leak TbDPA ($\%F_{max}$/min)	Leak CF ($\%F_{max}$/min)
PC	3.00	300	49	38	12	7
PC/GalCer	2.20	89	28	18	5	3
PC/LacCer	0.70	68	16	6	3	2
PC/Gg4Cer	0.21	56	6	1	2	2
PC/Sulf	6.20	520	30	13	3	1
PC/G_{M1}	3.60	370	21	6	10	4
PC/G_{D1a}	3.00	270	15	2	14	7

B. Extent of effects induced by MBP after 3 min

Lipid	Aggr. (OD 450 nm)	Probe mix ($\%F_{max}$)	Probe dilut ($\%F_{max}$)	Fusion ($\%F_{max}$)	Leak [a] TbDPA ($\%F_{max}$)	Leak [a] CF ($\%F_{max}$)	λ_{max} (nm)	K_{SV}^{nit} (M^{-1})	K_{SV}^{acr} (M^{-1})
PC	0.81	33	14	10	31	10	346	11	4
PC/Galcer	0.56	29	8	6	13	9	347	10	-
PC/LacCer	0.43	15	5	2	6	10	348	10	-
PC/Gg4Cer	0.18	9	3	1	4	8	347	12	5
PC/Sulf	1.20	88	10	7	10	5	344	15	6
PC/G_{M1}	0.80	67	8	4	33	2	345	17	7
PC/G_{D1a}	0.64	53	7	2	36	2	346	19	7

MBP was 5.4 μM and lipid was 50 μM (GSLs are 20 mol% of total phospholipid). λ_{max} for Trp fluorescence corresponds to the maximal blue shift. This occurs at lipid/MBP ratios of between 100 and 40 for systems with GalCer, LacCer and Gg4Cer and between 28 and 17 for those with Sulf and gangliosides. λ_{max} of Trp for MBP in buffer alone is 348 nm. The Stern-Volmer constant for Trp fluorescence quenching of MBP (no lipids) by NO_3^- is $K_{SV}^{nit} = 32.3\ M^{-1}$ and by acrylamide is $K_{rSV}^{ac} = 10.1\ M^{-1}$. Values for% F_{max} were rounded to nearest integer. [a] The leakage values are those taken after 10 min.

Negative charges on the GSLs facilitate an initial rapid aggregation induced by MBP. On the other hand, an increase of the oligosaccharide chain length induces a decrease of both the rate and extent of aggregation induced by MBP (Tables IA and IB). The presence of negative charges at the surface is not the only factor modulating the membrane interactions. In vesicles containing PA, in addition to the neutral GSLs (not shown), the protein-induced vesicle aggregation is moderately higher than in the vesicles of PC with the corresponding GSLs and without PA but the interfering effect of the oligosaccharide chain was very similar.

The initial rates of RET among probes located in different vesicle populations (probe mixing assay, indicative of both membrane proximity and bilayer merging) are very rapid (Fig. 1b and Table IA) and immediately follow the changes of light scattering and absorbance. For the probe dilution assay (indicative of bilayer merging only) both the rate and extent of RET are smaller than in the probe mixing assay (Fig. 1b). Negative charges provided by the presence of either PA or GSLs facilitate the rate and extent of membrane approximation induced by MBP but have little effect on bilayer merging. An increase of the length of the oligosaccharide chain in either neutral or acidic GSLs interferes with both processes (Tables IA and IB). Bilayer merging (Fig. 1b) occurs immediately prior to, and to a greater extent than whole vesicle fusion (Fig. 2). The latter is retarded and diminished in extent when the membrane contains GSLs with longer oligosaccharide chains (Tables IA and IB).

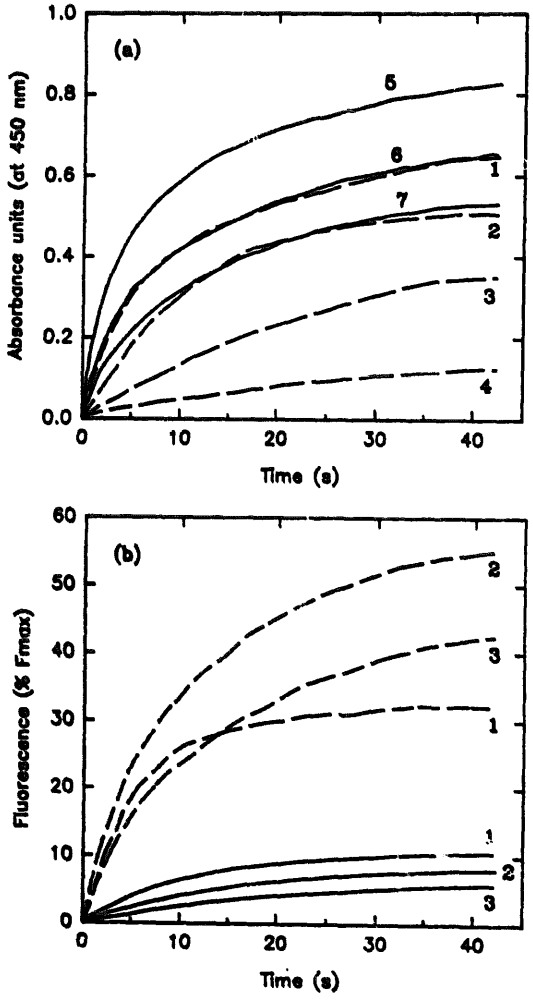

Fig. 1. Absorbance changes and fluorescence RET induced by MBP. (a) Kinetics of the absorbance changes at 450 nm of vesicles of: PC (1), PC/GalCer (2), PC/LacCer (3), PC/Gg_4Cer (4), PC/Sulf (5), PC/G_{M1} (6), and PC/G_{D1a} (7). (b) The percentage of fluorescence change (see text) in the probe mixing (dashed lines) or probe dilution assay (full lines) is shown for vesicles of: PC (1), PC/G_{M1} (2), and PC/G_{D1a} (3). MBP was added at time zero at a final concentration of 5.4 μM. The mole fraction of GSLs is 0.2 and the total lipid concentration is 50 μM.

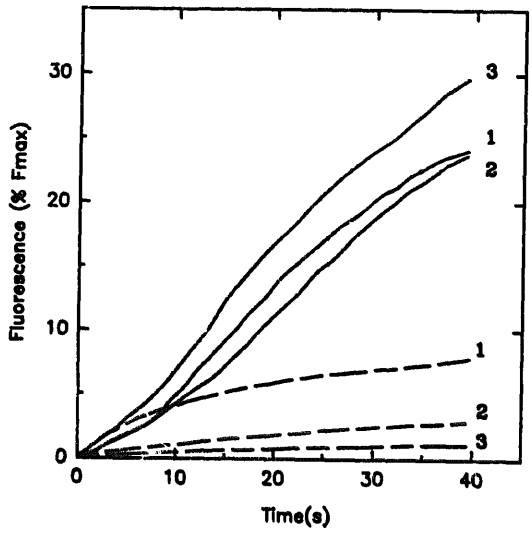

Fig. 2. Fusion and permeability changes induced by MBP. The percentage of fluorescence change (see text) due to the formation of the Tb-DPA complex by intermixing (fusion) of the vesicle aqueous compartments (dashed lines) or the leakage of the preformed and entrapped Tb-DPA complex (full lines) are shown as a function of time for vesicles of: PC (1), PC/G_{M1} (2), and PC/G_{D1a} (3). MBP was added at time zero at a final concentration of 5.4 μM. The mole fraction of GSLs is 0.2 and the total lipid concentration is 50 μM.

The leakage of aqueous markers (Fig. 2) is the slowest process. This indicates that the vesicle permeability changes little during the membrane interactions induced by MBP. The presence of neutral GSLs results in even less leakage; for vesicles containing negatively charged GSLs both the rate and extent of leakage increase when the oligosaccharide chain is increased or contains sialosyl groups (Fig. 2, Tables IA and IB).

The above changes depend on the MBP concentration and on the proportion of GSLs. At about 3 μM MBP the different effects are near the maximum. Figs. 3 and 4 illustrate this for the probe mixing and fusion assays. A plateau level is reached when the mole fraction of GSLs is between 0.1 and 0.2. The magnitude of the effects is very sensitive to small variations of the proportions of GSLs below these values (Figs. 3b

and 4b) but this can be offset by variations in the concentration of MBP with respect to the lipids (Figs. 3a and 4a).

The Trp fluorescence of MBP shows only a small blue shift and a very slight increase of the quantum yield upon its association to the lipid vesicles (Table IB). This is in agreement with previous findings [38,40]. The decrease of intrinsic fluorescence of the protein in buffer (no vesicles) induced by the collisional aqueous quenchers NO_3^- or acrylamide, occurs with a Stern-Volmer quenching constant of 32.3 M^{-1} for NO_3^- and 10.2 M^{-1} for acrylamide (Table IB). The K_{SV} values are diminished when the protein interacts with vesicles. This indicates a reduced accessibility of the quenchers to the Trp region of MBP. When the protein is associated tp GSLs-containing vesicles. The K_{SV} constants become larger for vesicles containing Sulf and, even more, for vesicles containing gangliosides. Compared to vesicles with neutral GSLs, this indicates that the Trp of MBP becomes more accessible to the aqueous quenchers in the membranes with acidic GSLs (Table IB).

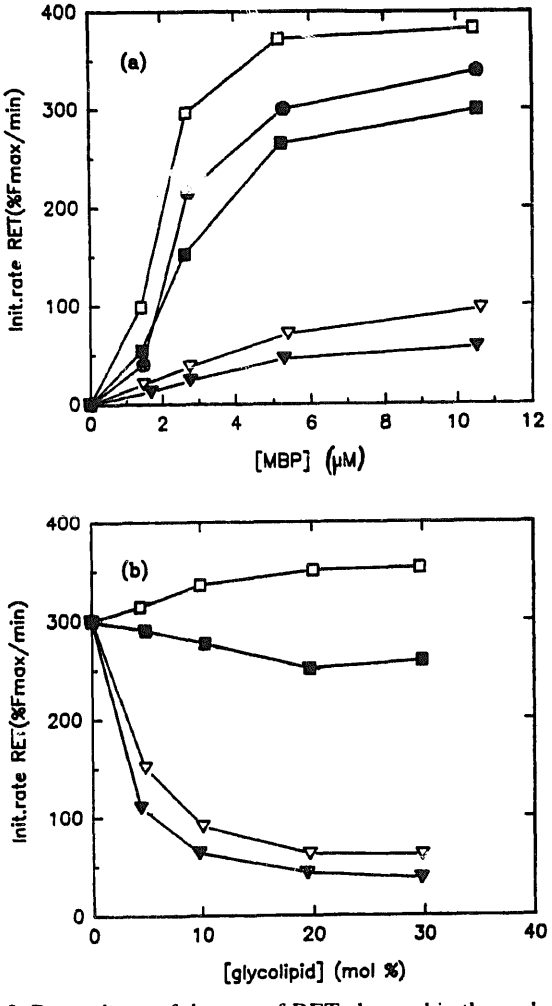

Fig. 3. Dependence of the rate of RET observed in the probe mixing assay with the concentration of MBP (a) and the proportion of GSLs (b). The initial rate of RET is shown for vesicles of PC (●); PC/LacCer (▽); PC/Gg_4Cer (▼); PC/C_{M1} (□), and PC/G_{D1a} (■).

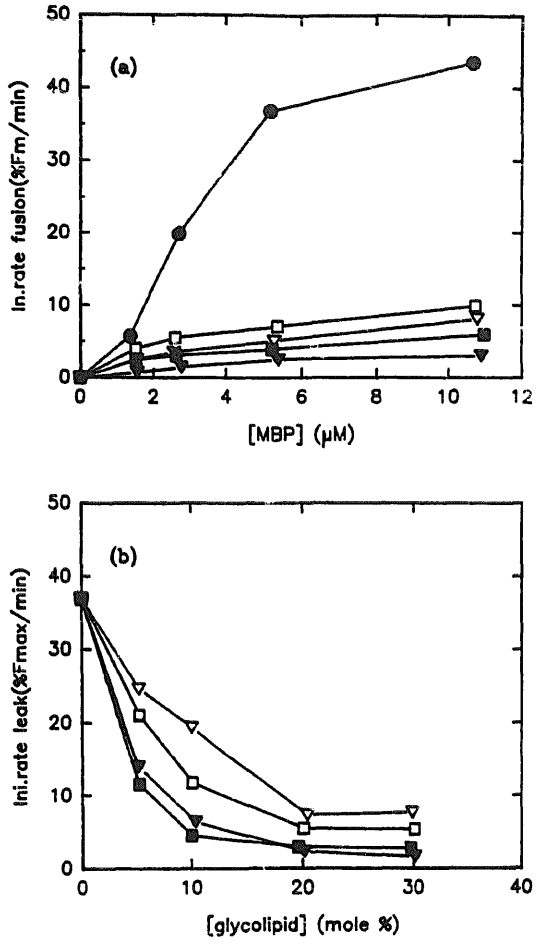

Fig. 4. Dependence of the rate of fusion with the concentration of MBP (a) and the proportion of GSLs (b). The initial rate of fusion is shown for vesicles of PC (●), PC/LacCer (▽), PC/Gg_4Cer (▼), PC/G_{M1} (□), and PC/G_{D1a} (■).

Effect of melittin

The binding of Mel to the vesicles was practically independent on the oligosaccharide chain length of the GSLs but was sensitive to the presence of negative charges. The protein/lipid molar ratio at which the Mel binding saturates for vesicles of PC only or for those containing 20 mol% of neutral GSLs was between 1:40 and 1:50. The amount of protein bound increased to between 1:18 and 1:22 for PC vesicles containing 20 mol% of Sulf or gangliosides. This is also in agreement with previous studies [25,26].

The turbidity changes induced by Mel are much less and more similar among vesicles containing different GSLs than those found for MBP (Fig. 5a, Tables IIA and IIB). Compared to MBP, the initial rates of RET for either the probe mixing or the probe dilution assays and the extent of probe dilution induced by Mel are markedly increased (Fig. 5b). On the other hand, the extent of probe mixing induced by Mel is similar to

that caused by MBP in vesicles containing neutral GSLs but is about half of that induced by MBP in vesicles containing Sulf or gangliosides (compare Figs. 1 and 5 and Tables IB and IIB).

The initial rate and extent of leakage (Fig. 6) induced by Mel are considerably greater than for MBP. The fusion assay exhibits a fast maximum and then a decrease, in correspondence to the changes in permeability (Fig. 6), both the initial rate and the extent of fusion are higher than those caused by MBP. However, in any case and similar to MBP, the increase of the oligosaccharide chain length of GSLs interferes with the interactions between membranes (Tables IIA and IIB).

The quenching of the Trp fluorescence of Mel in buffer alone by NO_3^- (K_{SV} 54.1 M^{-1}) or acrylamide (K_{SV} 14.2 M^{-1}) is higher than for MBP. This indicates that, in the absence of lipids, the Trp region is more accessible in Mel than in MBP to the aqueous quenchers. The quenching is markedly decreased in the presence of vesicles but different GSLs cause little differences in the accessibility of the aqueous quenchers to the Trp region of Mel. The blue shift of the single Trp of Mel and the increase of quantum yield are considerable compared to the changes observed for MBP (Tables IB and IIB) but the shift of λ_{max} in the presence of vesicles is similar regardless of the lipid composition (Table IIB).

Discussion

Previous work showed that MBP can induce aggregation and fusion of PC vesicles [19,20]. The interaction of MBP with phospholipid liposomes leads to vesicle dimers that subsequently coalesce into more compact structures [48] and, with myelin lipids MBP induces the formation of multilayered structures [11,24,49].

An initial aggregation of the vesicles is the fastest kinetic event induced by MBP. The high rate and extent of RET in the probe mixing assay (probes located initially in different membranes) shows that this occurs with close proximity of the lipid bilayers. According to the Forster equation [42], the membrane surfaces should be at no more than about 5 nm in order to have efficient energy transfer of the magnitude observed. The slow initial rate and small extent of interactions found with the probe dilution assay indicates that most of the RET induced by MBP is due to bilayer proximity and not to bilayer merging. The even slower rate and extent of whole vesicle-vesicle fusion induced by MBP indicates that only a small part of the

TABLE II

A. Initial rate of vesicle interactions induced by Mel

Lipid	Aggr. (OD_{450}/min)	Probe mix ($\%F_{max}$/min)	Probe dilut ($\%F_{max}$/min)	Fusion ($\%F_{max}$/min)	Leak TbDPA ($\%F_{max}$/min)	Leak CF ($\%F_{max}$/min)
PC	0.22	850	910	310	60	57
PC/GalCer	0.23	410	625	218	75	72
PC/LacCer	0.22	397	510	256	38	35
PC/Gg4Cer	0.16	291	295	225	33	31
PC/Sulf	0.28	880	900	293	45	42
PC/G_{M1}	0.19	343	460	300	150	135
PC/G_{D1a}	0.19	310	330	268	180	167

B. Extent of effects induced by Mel after 3 min

Lipid	Aggr. (OD 450 nm)	Probe mix ($\%F_{max}$)	Probe dilut ($\%F_{max}$)	Fusion ($\%F_{max}$)	Leak TbDPA ($\%F_{max}$)	Leak CF ($\%F_{max}$)	λ_{max} (nm)	K_{SV}^{mit} (M^{-1})	K_{SV}^{act} (M^{-1})
PC	0.06 (0.18)	29	51	38 (58)	67	43	335	11	4
PC/GalCer	0.05 (0.17)	34	46	34 (52)	62	59	335	10	-
PC/LacCer	0.04 (0.15)	26	35	20 (41)	49	29	336	10	-
PC/Gg4Cer	0.04 (0.12)	19	23	16 (29)	38	30	336	8	5
PC/Sulf	0.05 (0.12)	33	50	36 (56)	62	52	336	10	6
PC/G_{M1}	0.03 (0.10)	28	40	30 (36)	65	50	338	8	4
PC/G_{D1a}	0.03 (0.08)	20	30	22 (36)	48	35	338	8	5

Mel was 5.3 μM and lipid was 50 μM (GSLs are 20 mol% of total phospholipid). Values in brackets correspond to the peak values that are obtained between 15–25 s after adding the protein. The λ_{max} for Trp fluorescence corresponds to the maximal blue shift. This occurs at at a lipid/Mel ratios between 40 and 50 for vesicles of PC or with neutral GSLs and between 18 and 22 for acidic GSLs. λ_{max} of Trp for Mel in buffer without vesicles is 348 nm. The Stern-Volmer constant Trp fluorescence quenching in buffer alone by NO_3^- is $K_{SV}^{mit} = 54.1\ M^{-1}$ and by acrylamide is $K_{SV}^{act} = 14.2\ M^{-1}$. Values for $\%F_{max}$ were rounded to nearest integer.

vesicles that undergo bilayer merging proceed to complete fusion. Most of the vesicles probably remain in close apposition, some with fused bilayers, but with separate aqueous vesicular compartments. The initial rate of permeability change is small. Comparison of its time-course and extent with that of vesicle fusion suggests that MBP induces a preferential increase of permeability in other vesicles than those actually involved in fusion. The larger rate of leakage in the Tb-DPA assay compared to that of CF may be due to the entry of EDTA into the vesicle. This suggests a certain degree of selective permeability changes but without marked disruption of the vesicle integrity.

The Trp region of MBP appears to reside in a rather polar environment even after interacting with the lipid vesicles but its accessibility to the aqueous quenchers is impaired. The increased shielding can not

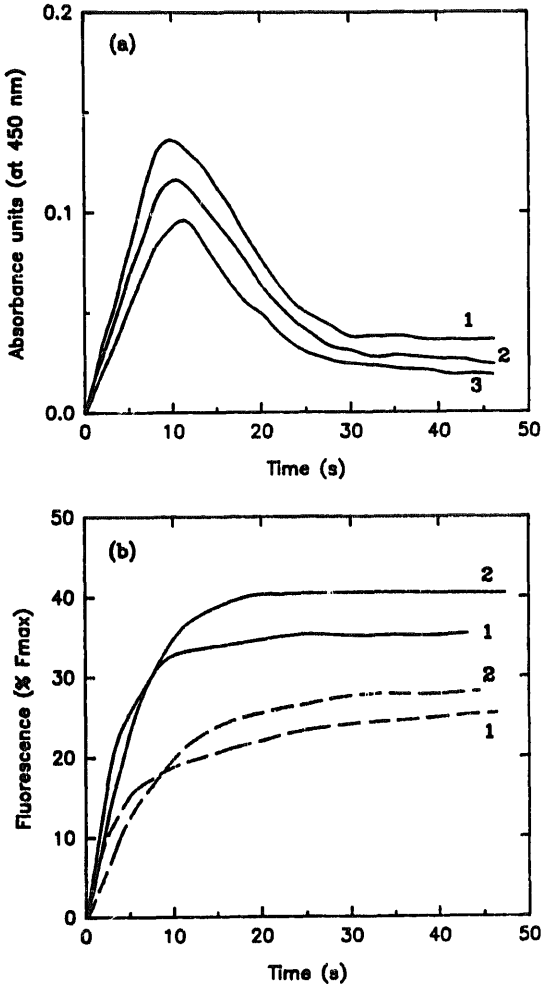

Fig. 5. Absorbance changes and fluorescence RET induced by Mel. (a) The absorbance at 450 nm is shown for vesicles of PC/LacCer (1), PC/Sulf (2), and PC/G_{M1} (3). (b) The kinetics of the percentage of RET in the probe mixing (dashed lines) or probe dilution (full lines) assays (see text) are shown for vesicles of PC/LacCer (1), and PC/G_{M1} (2). Mel was added at time zero at a final concentration of 5.3 μM. The mole fraction of GSLs is 0.2 and the total lipid concentration is 50 μM.

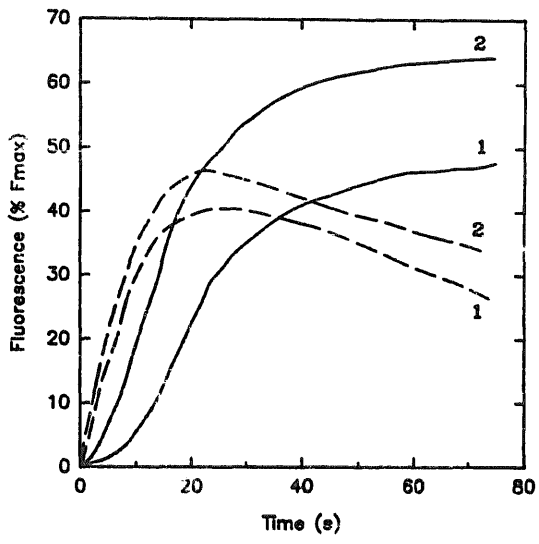

Fig. 6. Fusion and permeability changes induced by Mel. The percentage of fluorescence change due to the formation of the Tb-DPA complex after vesicle fusion (dashed lines) or the leakage of the preformed and entrapped Tb-DPA complex (full lines) (see text) are shown for vesicles of PC/LacCer (1), and PC/G_{M1} (2). Mel was added at time zero at a final concentration of 5.3 μM. The mole fraction of GSLs is 0.2 and the total lipid concentration is 50 μM.

derive from simple variations of the surface electrostatics caused by the lipids because similar relative results are found for NO_3^- and acrylamide. However, the latter is less discriminatory, probably because of some partitioning into the membrane phase [50]. The presence of asialo-G_{M1}, with four neutral carbohydrates in the oligosaccharide chain, increases only slightly the accessibility of the Trp region of MBP to the quenchers, compared to when the protein is bound to vesicles of PC alone. On the other hand, even though the K_{SV} values remain low compared to that of the protein in absence of vesicles, the presence Sulf and gangliosides significantly increase the Trp accessibility to the quenchers. MBP probably resides mostly at the surface of lipid vesicles, with some hydrophobic regions penetrating the bilayer [25,26,51,52], where it induces changes of the lipid molecular packing [26], the phase state [24,47] and the interfacial micropolarity [53,54]. It was previously shown in lipid monolayers that, depending on the lateral surface pressure, MBP can be squeezed out of the hydrocarbon chain portion of the interface while remaining associated to the lipid polar headgroup region [26]. Studies by high sensitivity differential scanning calorimetry were consistent with a partial embedding of MBP into the lipid phase of vesicles formed by PC and the negatively charged GSLs [47]. Together with these previous findings, our results suggest that in membranes containing these GSLs the Trp region of MBP is located in an environment of reduced polarity compared to the bulk aqueous phase and becomes more accessible to the aqueous quenchers

in proportion to the length of the oligosaccharide chain of the acidic GSLs.

It is clear that an increase of the oligosaccharide chain length of GSLs interferes with close intermembrane interactions. The longer the chain the smaller the membrane interactions induced by MBP. All Negatively charged GSLs facilitate vesicle aggregation and membrane apposition. On the other hand, all the GSLs interfere with bilayer merging and complete vesicle fusion. These effects are not due to a reduced binding of MBP to the vesicle. To the contrary, the presence of GSLs with negatively charged or longer oligosaccharide chains lead to a greater amount of MBP bound to the vesicle surface.

The rate and extent of turbidity changes induced by Mel are much less than those caused by MBP. On the other hand, the initial rate of membrane apposition and bilayer merging occur faster than with MBP. In principle, it would seem logical that the rate of bilayer apposition should always precede and its extent exceed bilayer merging. However, with Mel both the rate and extent of RET measured by the probe mixing assay (indicative of membrane proximity and/or bilayer merging) are *less* than those detected by the probe dilution assay (indicative of bilayer merging only). These two assays depend critically on the relative rates at which the vesicles interact among themselves in relation to the velocity of their disruption, reorganization or intermediate stabilization. Mel induces a fast vesicle disruption, with rapid recombination of lipids and formation of non-vesicular structures [45,46,55,56], before slower processes such as substantial bilayer apposition, merging or vesicle fusion can take place. Thus, a large change of RET is measured by the probe dilution assay because both lipid probes originally in the *same* membrane are rapidly dispersed (probably into smaller micellar structures as shown by the light scattering changes) and recombined with unlabelled lipids derived from similarly disrupted vesicles not containing the probe when the two populations are mixed. A rapid and extensive decrease of RET (also measured by a probe dilution assay) induced by Mel was reported previously for unilamellar vesicles of cardiolipin [17]. The smaller changes in the rate and extent of RET measured by the probe mixing assay can also be explained by Mel inducing a very rapid reorganization of each *separate* population of vesicles (NBD-PE or Rh-PE type) before detectable (i.e., stable in the time of the experiment) intermembrane contacts occur.

The turbidity changes are consistent with these effects. The variation of absorbance caused by Mel is smaller and kinetically different than for MBP. It shows an initial increase followed by a rapid decrease to very low values. This, and the high rate and extent of leakage, are due to a considerable proportion of vesicles being rapidly disrupted. On the other hand, a certain fraction of the vesicle population reaches a transient stabilization (cf. Ref. 57) through complete membrane fusion as shown by the fast intermixing of the vesicle aqueous compartments. This exhibits a maximum at about 15–25 s after adding the protein and, subsequently, the vesicle content is slowly lost. About 40–60% of the rapidly fused vesicles become unstable.

It was previously reported that the oligosaccharide chain of GSLs reduces the rate of interaction and fusion between bilayer vesicles induced by lectins and Ca^{2+} [58,59]. Similar to MBP, an increase of the length of the oligosaccharide chain of GSLs causes a reduction of the intermembrane interactions induced by Mel. However, Mel penetrates more deeply into the lipid interface [17,25] than MBP. This is also shown by the larger shift of the wavelength maximum, by the increase of relative fluorescence of Trp, and by the lower quenching by NO_3^- or acrylamide. The length of the oligosaccharide chain of GSLs has little effect on the penetration of Mel into the lipid bilayer. Similar to MBP, the effects of Mel are not due to a reduced binding of the protein to the vesicles containing the more complex GSLs since more protein is bound to the latter interfaces.

The more general conclusion of our results is that negative charges in the GSLs facilitates and stabilizes membrane proximity induced by the basic proteins while, at the same time, an increased length of the oligosaccharide chain interferes with close membrane–membrane interactions. The possibilities for the apposition and recombination of lipid bilayers is determined by a complex balance of forces. Among others, the surface free energy, membrane curvature, electrostatic, van der Waals and hydration forces are of paramount importance [11,60,61]. Studies done with monolayers [33,60], bilayers [62], X-ray diffraction [63] and two-dimensional NMR [64] have consistently pointed out that the oligosaccharide chain of GSLs is approximately perpendicular to the surface. This notwithstanding the oscillations of a certain amplitude that continuously occurs due to the molecular kinetic energy and fluctuations of the lateral surface pressure [33]. At the local level, the conformation of the oligosaccharide chain is constrained by specific glycosidic linkages that determine the relative position of the carbohydrate residues with respect to the membrane interface [65]. However, even for GSLs with marked distortions in the oligosaccharide chain conformation [66], the measured intermolecular spacings are inconsistent with the carbohydrate residues lying flat on the membrane surface either for simgle GSLs or when these are mixed with other lipids and proteins [66].

The modulation of membrane interactions by GSLs is not markedly dependent on the mode of insertion of the protein into the membrane and is inversely correlated with the amount of protein bound. This suggests

that the regulation of protein-mediated membrane contacts by GSLs probably occurs at a distance from the interface where it is controlled by the length and protrusion of the polar headgroup. In GalCer or LacCer, the polar headgroup length is similar to that of PC [33,60] while in the more complex GSLs the polar headgroup protrudes by at least 2 nm into the aqueous medium [37,60]. In all cases, the GSLs are highly hydrated [63,68] and the size of their hydration shell is highly sensitive to the long range order of the bulk phase aqueous environment [69]. On these bases, the oligosaccharide chain appears to act as a very sensitive sensor of membrane proximity or 'spacer' on the basis of its length and hydration. On the other hand, the specific type of carbohydrate residues (presence of negative charges, residue and glycosidic linkage configuration) influences the amount of protein bound and the local lipid–protein interactions [26,68,69]. The simultaneous thermodynamic and geometric optimization (cf. Ref. 67) of these factors results in two membrane surfaces facing each other being kept in proximity but at a certain distance (probably at the energy minimum where the attractive and repulsive forces of different origin balance each other [33,61,67,70]). The results of fluorescence RET suggest that this should be in the range of probably not less than 5 nm and no more than 10 nm. This is right in the range of distances spanned by two approximately straight oligosaccharide chains having 2–5 carbohydrate units plus their immediately associated hydration shell [67]. At the same time, the oligosaccharide chains impede extensive membrane recombination.

Acknowledgments

This work was supported in part by a National Multiple Sclerosis Society Grant RG-2170-A-2 (B.M.) and NIH Grant NS-23102 (R.K.Y.).

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