Ricinus communis Agglutinin-Mediated Agglutination and Fusion of Glycolipid-Containing Phospholipid Vesicles: Effect of Carbohydrate Head Group Size, Calcium Ions, and Spermine†

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ABSTRACT: The glycolipids galactosylcerebroside (GalCer), lactosylceramide (LacCer), and trihexosylceramide (Gb_3) were inserted into phospholipid vesicles, consisting of phosphatidylethanolamine and phosphatidic acid. The extent to which their carbohydrate head groups protruded beyond the vesicle surface and their interference with membrane approach were examined by determining vesicle susceptibility toward type I *Ricinus communis* agglutinin (RCA_1) induced agglutination and Ca^{2+}- and spermine-induced aggregation and fusion either in the presence or in the absence of the lectin. The initial agglutination rates increased in the order GalCer $\ll$ LacCer $<$ Gb_3, while a reversed order was obtained for Ca^{2+}- and spermine-induced aggregation and fusion, indicating an enhanced steric interference on close approach of bilayers with increasing head group size. The lectin-mediated agglutination rates for LacCer- and Gb_3-containing vesicles increased by an order of magnitude when Ca^{2+} was also included in the medium, at a concentration that did not induce aggregation per se. Charge neutralization could not account for this observation as the polyvalent cation spermine did not display this synergistic effect with RCA_1. Addition of Ca^{2+} to preagglutinated vesicles substantially reduced the threshold cation concentration for fusion (micromolar vs. millimolar). Quantitatively, this concentration decreased with decreasing carbohydrate head group size, indicating that the head group protrusion determined the interbilayer distance within the vesicle aggregate. The distinct behavior of Ca^{2+} vs. spermine on RCA_1-induced agglutination on the one hand and fusion on the other indicated that Ca^{2+} regulates the steric orientation of the carbohydrate head group, which appears to be related to its ability to dehydrate the bilayer. As a result, lectin agglutinability becomes enhanced while fusion will be interrupted as the interbilayer distance increases, the threshold head group size being three carbohydrate residues (Gb_3). Finally, GalCer-containing vesicles were not agglutinated by RCA_1 at ambient temperature, irrespective of the presence of Ca^{2+}. Above 25 °C, RCA_1 facilitated Ca^{2+}-induced fusion of the vesicles, which was abolished by the haptenic sugar lactose. Since Gb_3- and LacCer-containing vesicles displayed a similar behavior, a temperature-induced alteration in the supporting lipid matrix is suggested, which apparently affects lectin/glycolipid interaction.

The presence of glycoconjugates, covalently linked to either lipids or proteins, at the membrane surfaces of cells and subcellular organelles is considered to be important in a variety of membrane-mediated phenomena. At the level of the plasma membrane, their role as recognition sites for hormones (Shechter et al., 1979), toxins (Kelly et al., 1979), and viruses (White et al., 1983) as well as their involvement in cellular adhesion (Düzgüneş, 1975; Rauvala, 1983) has been well established. The dynamic properties of glycolipids and/or glycoproteins as mobile membrane receptors can be conveniently studied by incorporating these complex macromolecules into relatively simple artificial membranes and investigating their interaction with lectins [for a recent review, see Grant & Peters (1984)]. Multidentate molecules such as lectins (Goldstein & Hayes, 1978; Barondes, 1981; Olden et al., 1982) can display a potential for receptor cross-linking when the artificial bilayers (lipid vesicles) contain the appropriate surface carbohydrate, and as a result, vesicle agglutination will be induced. Despite the presence of the suitable carbohydrate receptor, other factors may, in addition, determine the ability of a lectin to interact with its receptor. These factors include receptor density (Surolia et al., 1975; Curatolo et al., 1978; Goodwin et al., 1982), the physical properties of the supporting lipid matrix surrounding the receptor (Hampton et al., 1980; Sundler, 1984; Utsumi et al., 1984), and the extent to which the carbohydrate head group protrudes from the bilayer surface (Slama & Rando, 1980; Sundler, 1984).

Specific recognition, in conjunction with the establishment of close approach of adjacent bilayers, has also made lectin–glycolipid/glycoprotein interaction studies an interesting area of research in relation to membrane fusion events, particularly in light of the presence of lectins and lectin-like molecules both at the cell surface and in the intracellular environment (Barondes, 1981; Olden et al., 1982).

Recently, we have shown that lectins can modulate Ca^{2+}-induced fusion of glycolipid-containing phospholipid vesicles (Düzgüneş et al., 1984; Hoekstra et al., 1985a). We demonstrated that the major neutral glycolipid of the erythrocyte membrane globoside Gb_4 and the disialoganglioside

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GD_{1a} inhibit membrane fusion of vesicles containing these glycolipids while the addition of lectins specific for the glycolipids greatly enhanced the rate of fusion. In addition, these studies revealed that the carbohydrate head group per se may sterically interfere with the process of close approach of adjacent bilayers, a necessary though not sufficient condition for fusion to occur. Furthermore, during fusion, a Ca^{2+}-induced reorientation of the glycolipid head group may take place that regulates the interbilayer distance and thereby membrane fusion (Hoekstra et al., 1985a). These results have prompted us to continue our investigations on the steric interference of glycolipid carbohydrate head groups on intermembrane contact, using lipid vesicles consisting of phosphatidylethanolamine (PE),[1] phosphatidic acid (PA), and one of the following glycolipids: galactosylcerebroside ("GalCer vesicles"), lactosylceramide ("LacCer vesicles"), or trihexosylceramide ("Gb_3 vesicles"). We examined their ability to agglutinate in the presence of the divalent lectin of *Ricinus communis* agglutinin (RCA_1) and to aggregate and fuse upon addition of Ca^{2+} and the polyamine spermine (Schuber et al., 1983), both in the presence and in the absence of RCA_1.

Experimental Procedures

Materials. *Ricinus communis* agglutinin I (RCA_1, M_r 120 000) and spermine (hydrochloride) were obtained from Sigma (St. Louis, MO). Egg phosphatidylethanolamine (PE) and phosphatidic acid (PA), derived from egg phosphatidylcholine, were purchased from Avanti Polar Lipids (Birmingham, AL). Galactosylcerebroside [GalCer, Gal(β1→1)Cer] and trihexosylceramide [Gb_3, Gal(α1→4)Gal(β1→4)Glc(β1→1)Cer] were from Supelco Inc. The major fatty acids in GalCer and Gb_3 are $C_{24:0}$, $C_{22:0}$, and $C_{24:1}$, comprising 60–75% of the total fraction (manufacturer's source). *N*-Lignoceroyldihydrosphingosyllactosylceramide [LacCer, Gal(β1→4)Glc(β1→1)Cer] was a product from Miles-Yeda Ltd., Rehovot, Israel. The lipids were routinely checked for purity by thin-layer chromatography. $TbCl_3 \cdot 6H_2O$ was obtained from Alfa (Danvers, MA); dipicolinic acid (DPA, sodium salt) and nitrilotriacetic acid (NTA) were from Sigma.

Vesicle Preparation. Large unilamellar glycolipid-containing PE/PA vesicles with an average diameter of 0.1 μm, were prepared by reverse-phase evaporation and subsequent extrusion through Unipore polycarbonate filters (Bio-Rad) as previously described (Düzgüneş et al., 1983; Hoekstra et al., 1985a) The molar ratio of the lipids comprising the vesicle bilayer was 3.5:1:0.45 (PE, PA, and glycolipid, respectively). The aqueous contents, encapsulated in the vesicles, were (i) 2.5 mM $TbCl_3$/50 mM NTA, (ii) 50 mM DPA/20 mM NaCl, and (iii) 1.25 mM $TbCl_3$/25 mM NTA/25 mM DPA/10 mM NaCl. The media were buffered with 5 mM TES, pH 7.4. Nonencapsulated material was removed by gel filtration on Sephadex G-75 (elution buffer 100 mM NaCl/5 mM TES, pH 7.4, containing 1 mM EDTA).

Vesicle Agglutination and Aggregation. Agglutination of the vesicles induced by RCA_1 or cation-induced aggregation was followed continuously by measuring the increase in the absorbance of the vesicle suspension at 450 nm in a Beckman Model 34 spectrophotometer. The initial rates were calculated from the tangents drawn to the curves at time zero.

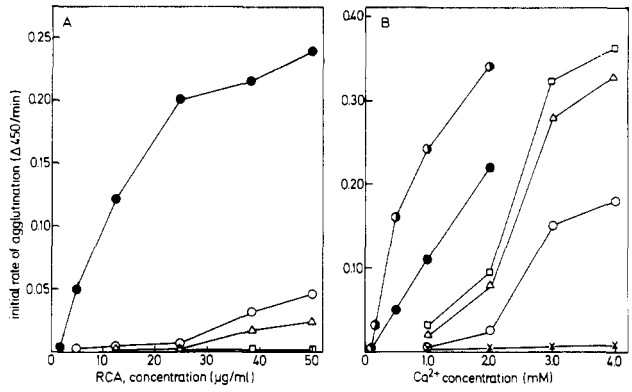

FIGURE 1: Initial rates of vesicle aggregation, induced by RCA_1, Ca^{2+}, and RCA_1/Ca^{2+}. (A) PE/PA vesicles, containing galactosylcerebroside [GalCer (□)], lactosylceramide [LacCer (△)], or trihexosylceramide [Gb_3 (○)] were suspended in 100 mM NaCl/0.1 mM EDTA/5 mM TES, pH 7.4, at a final lipid concentration of 50 μM. The incubation temperature was 23 °C. Various amounts of RCA_1 were injected into the medium and the turbidity change was monitored at 450 nm. The initial rates of agglutination were determined as described under Experimental Procedures and plotted as a function of the (final) RCA_1 concentration. The effect of 1 mM Ca^{2+} on RCA_1-induced agglutination of Gb_3-containing vesicles (●) was determined by injecting Ca^{2+} into the medium 2 min after addition of RCA_1 (the rate thus obtained was corrected for RCA_1-induced agglutination in the absence of Ca^{2+}) or 2 min prior to the addition of RCA_1. Under both conditions the calculated rates were essentially identical. (B) Vesicle aggregation was induced by injecting Ca^{2+} to the desired final concentration into the glycolipid vesicle containing medium [(□) GalCer; (△) LacCer; (○) Gb_3]. Experimental conditions were as in (A). For comparison, the initial rates of Ca^{2+}-induced aggregation of globoside Gb_4 containing vesicles (×) were also included (see text). Alternatively, Gb_3-containing vesicles were incubated with 12.5 (●) or 50 μg/mL RCA_1 (◑); after 2 min, various Ca^{2+} concentrations were injected, and the initial aggregation rates thus obtained were plotted as a function of the Ca^{2+} concentration.

Fusion Measurements. Tb- and DPA-containing vesicles were mixed at a ratio of 1:1 in 100 mM NaCl/5 mM TES/0.1 mM EDTA, pH 7.4. The final lipid concentration was 50 μM. The incubation temperature was 23 °C, unless indicated otherwise. The fluorescent $Tb(DPA)_3^{3-}$ complex, formed upon fusion (Wilschut et al., 1980), was monitored continuously (λ_{ex} = 276 nm, λ_{em} = 545 nm), on an SLM-4000 fluorometer. The cuvette holder was equipped with a magnetic stirring device. A cutoff filter (>530 nm) was placed between the sample and the emission monochromator. Calibration of fluorescence was done by lysing 25 μM Tb-containing vesicles (after elimination of the external EDTA by gel filtration of a portion of the vesicles with 100 mM NaCl/5 mM TES as the elution buffer) with 0.5% sodium cholate in the presence of excess (25 μM) DPA. The fluorescence intensity thus obtained was set at 100%, and the rate of fusion was calculated as described above for agglutination and expressed as the percentage of the total $Tb(DPA)_3^{3-}$ fluorescence obtained per minute (%/min).

Membrane permeability changes caused by lectin/glycolipid interaction or resulting from bilayer merging were determined by monitoring in parallel experiments the release and subsequent fluorescence quenching of the entrapped $Tb(DPA)_3^{3-}$ complex, as described elsewhere (Bentz et al., 1983; Hoekstra et al., 1985a,b).

Results

Effect of Carbohydrate Head Group Exposure on RCA_1 and Ca^{2+}-Induced Aggregation of Glycolipid-Containing Vesicles. The carbohydrate moieties of galactosylcerebroside (GalCer), lactosylceramide (LacCer), and trihexosylceramide (Gb_3) protruded to different extents from the PE/PA bilayer,

[1] Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; GalCer, galactosylcerebroside [Gal(β1→1)Cer]; LacCer, lactosylceramide [Gal(β1→4)Glc(β1→1)Cer]; Gb_3, trihexosylceramide [Gal(α1→4)Gal(β1→4)Glc(β1→1)Cer]; DPA, dipicolinic acid; RCA_1, *Ricinus communis* agglutinin, type I; NTA, nitrilotriacetic acid; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

as revealed by lectin- and Ca^{2+}-induced vesicle aggregation (Figure 1). As shown in Figure 1A (open symbols), the lectin *Ricinus communis* agglutinin (RCA_1), which specifically interacts with D-galactosyl moieties (Nicolson et al., 1974), agglutinated both Gb_3- and LacCer-containing PE/PA bilayers in a concentration-dependent manner, the trihexosylceramide vesicles being most susceptible as indicated by the higher rate of agglutination. By contrast, addition of increasing amounts (up to 150 μg/mL) of RCA_1 to GalCer-containing PE/PA vesicles did not result in agglutination, suggesting that the monogalactosyl residue did not sufficiently extend beyond the phospholipid bilayer/water interface. In principle, the inaccessibility of GalCer toward an interaction with RCA_1 under these conditions, can be explained by various reasons. Apart from steric hindrance, it seems possible that the properties of the surrounding lipid matrix, such as the presence of negative charges provided by PA, may interfere with RCA_1–GalCer interaction. Furthermore, it has been reported that a single galactose residue as in the cerebroside constitutes a relatively poor hapten for RCA_1 (Kabat, 1978). However, as will be shown below, under appropriate experimental conditions RCA_1 does display binding affinity for GalCer in PE/PA bilayers, ruling out the latter possibility. It was of interest, therefore, to examine next the ability of Ca^{2+} to aggregate the vesicles and the effect of the divalent cation on RCA_1-induced agglutination. As demonstrated in Figure 1B (open symbols), additions of Ca^{2+} caused aggregation of the vesicles, the rate being dependent on the glycolipid structure incorporated in the bilayer. Compared to RCA_1-induced agglutination, the order of aggregation susceptibility was reversed, i.e., the larger the glycolipid carbohydrate head group, the lower the rate of Ca^{2+}-induced aggregation. For comparison, previous data (Hoekstra et al., 1985a) on the Ca^{2+}-induced aggregation of PE/PA vesicles, containing globoside Gb_4 (a glycolipid containing four carbohydrate moieties: GalNAc-Gal-Gal-Glc-ceramide), were included in Figure 1B (×). It thus becomes possible to define a "threshold" carbohydrate head group size beyond which the ability of the vesicles to come into sufficiently close proximity for Ca^{2+}-induced aggregation is abolished. This threshold carbohydrate head group size appears to be three carbohydrate residues (Gb_3) under our experimental conditions.

In spite of the presence of a rather bulky carbohydrate group on the vesicle bilayer, as is the case for Gb_3 vesicles, both the initial rates (Figure 1B) and the extents (cf. Figure 10A) of RCA_1-induced agglutination were substantially lower than those measured for Ca^{2+}-induced aggregation (Figure 1, panel A vs. panel B). However, the efficiency of agglutination became markedly enhanced when Ca^{2+} was also included in the incubation medium besides the lectin, as shown in Figure 1B (filled symbols). As a function of the RCA_1 concentration, the presence of 1 mM Ca^{2+} (which induces negligible aggregation by itself; cf. Figure 1B) increased the rate of aggregation of Gb_3 vesicles by a factor of 5 at 50 μg/mL RCA_1 up to ~40-fold at 12.5 μg/mL [Figure 1A, (●) vs. (○)]. Thus, as a result of the combined action of RCA_1 and Ca^{2+}, vesicle aggregation became apparent under conditions where separate addition barely revealed a change in turbidity (1 mM Ca^{2+} or 12.5 μg/mL RCA_1, respectively). The stimulating effect of Ca^{2+} on vesicle aggregation was already detectable at a Ca^{2+} concentration as low as 0.15 mM Ca^{2+} at an RCA_1 concentration of 50 μg/mL [Figure 1B, (◑)]. This observation implies that the actual threshold Ca^{2+} concentration is in the order of 50 μM, taking into account the presence of 0.1 mM EDTA in the medium (see below). The enhancement in the rate of vesicle aggregation, as induced by the combined action of RCA_1 and Ca^{2+}, was independent of the order of lectin and cation addition; i.e., virtually identical rates were seen when Ca^{2+} was added prior to RCA_1. Hence, these results indicated that Ca^{2+} modified the bilayer properties such that the ability of RCA_1 to interact with the glycolipid receptor became enhanced. Experimental support for this suggestion was obtained when the effect of inhibitors on RCA_1/Ca^{2+}-induced aggregation was examined.

When EDTA (2-fold molar excess) was injected into the medium, 2 min after the addition of Ca^{2+} to Gb_3 vesicles, the chelator immediately arrested the aggregation process and caused a concomitant drop of the turbidity (~20–60%, depending on the final Ca^{2+} concentration), indicating a partial dissociation of the aggregated vesicle complex. Alternatively, when vesicle agglutination was mediated by RCA_1 (in the absence of Ca^{2+}), addition of the haptenic sugar lactose (10 mM) resulted in a complete dissociation of the agglutinated vesicle complex. However, when vesicle aggregation was induced by the combined action of RCA_1 and Ca^{2+}, addition of EDTA resulted only in an immediate inhibition of vesicle aggregation at a low RCA_1 concentration (12.5 μg/mL) while at higher RCA_1 concentrations (≥20 μg/mL) aggregation proceeded with a rate as seen in the presence of lectin alone; i.e., under these conditions EDTA did *not* induce dissociation of the vesicle complex. Finally, when lactose was added instead of EDTA, a partial disruption of the aggregates was observed between 40 and 60% when aggregation had been induced by 1.0 and 0.5 mM Ca^{2+}, respectively, and 50 μg/mL RCA_1. These results indicated that the vesicle aggregates formed by the combined action of RCA_1 and Ca^{2+} were held together by the lectin, whose activity was apparently facilitated by the cation while the only partial dissociation of the vesicle complex by lactose could be indicative for the occurrence of vesicle fusion (see below).

The combined effect of RCA_1 and Ca^{2+} on LacCer vesicles was very similar to that observed for Gb_3 vesicles (not shown), although the absolute rates were lower. The differences in rate were proportionally comparable to the differences seen between these vesicles for RCA_1-induced agglutination in the absence of Ca^{2+} (Figure 1A). As far as GalCer vesicles are concerned, also the combined action of RCA_1 and Ca^{2+} was ineffective in inducing aggregation of these vesicles. Hence, it appears that the inaccessibility of RCA_1 to the galactosyl residue of the galactosylcerebroside, when incorporated into PE/PA bilayers, is apparently not determined by charge interference but rather by poor exposure of the monosaccharide beyond the vesicle surface. This notion does not necessarily imply, however, that RCA_1/receptor interaction is not at all affected by charge: if the receptor does not protrude beyond the vesicle surface, charge neutralization will not likely alter the exposure of the receptor. Therefore, two possibilities have to be taken into account to explain the observed enhancement of RCA_1 agglutination activity in the presence of Ca^{2+}, as observed for LacCer- and Gb_3-containing vesicles: (i) RCA_1/receptor interaction *is* affected by charge, and as a result of (partial) charge neutralization by Ca^{2+}, the accessibility of the lectin toward its receptor will become enhanced; (ii) as a result of the presence of Ca^{2+}, the properties of the bilayer become modified such that the spatial orientation of the carbohydrate head group changes, which could result in an increased distance of the terminal galactosyl residue relative to the bilayer/water interface, thus leading to a diminished steric interference of the bilayer per se on receptor/lectin interaction. Relevant insight into these various possibilities was obtained

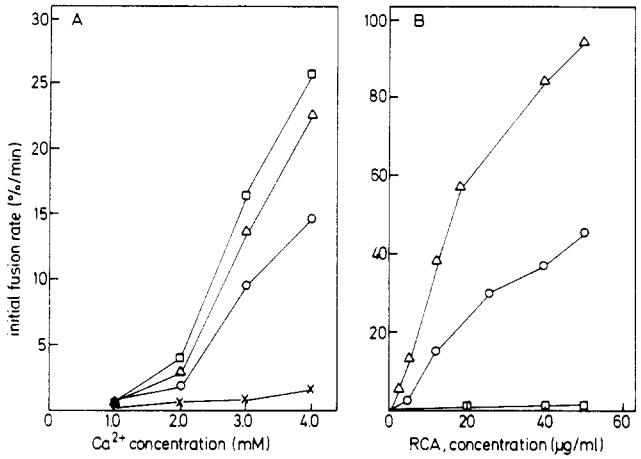

FIGURE 2: Initial rates of Ca^{2+}-induced vesicle fusion in the absence (A) and presence (B) of RCA_1. (A) Various Ca^{2+} concentrations were injected into a medium containing an equimolar mixture of Tb- and DPA-containing vesicles, composed of PE/PA and one of the following glycolipids: GalCer (□); LacCer (△); Gb_3 (○); globoside Gb_4 (×; data from Hoekstra et al., 1985a). The incubation conditions were as described in the legend of Figure 1. The formation of the fluorescent Tb/DPA complex was monitored continuously, and the initial fusion rates were determined. (B) Tb- and DPA-containing glycolipid vesicles were incubated with various concentrations of RCA_1. After 2 min, 1 mM Ca^{2+} was added to the medium. The initial fusion rates were plotted as a function of the RCA_1 concentration. The vesicle composition was as described above: GalCer (□); LacCer (△); Gb_3 (○).

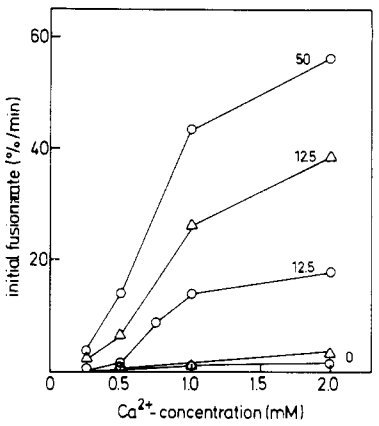

FIGURE 3: Effect of glycolipid composition and lectin concentration on Ca^{2+}-induced fusion. PE/PA vesicles, containing Gb_3 (○) or LacCer (△), were preincubated with RCA_1 (0, 12.5, and 50 μg/mL, as indicated). After 2 min, various Ca^{2+} concentrations were injected into the medium to induce fusion. The initial fusion rates were plotted as a function of the Ca^{2+} concentration. The incubation temperature was 23 °C.

by experiments on the fusogenic properties of the glycolipid-containing vesicles.

Effect of Carbohydrate Head Group Protrusion on Membrane Fusion. Besides its physiological significance, investigating the fusogenic properties of glycolipid-containing vesicles may also reveal relevant information on the dynamic behavior of the carbohydrate head group structure (Hoekstra et al., 1985a). Within an aggregated state induced either by Ca^{2+} or by RCA_1, the actual distance between apposed bilayers, which needs to be overcome before membrane merging occurs, can still be considerable when taking into account the molecular sizes of the carbohydrate units and the lectin (Sundler & Wijkander, 1983).

As shown in Figure 2A, the initial rates of Ca^{2+}-induced fusion followed a similar pattern as that observed for Ca^{2+}-induced aggregation, and the rates decreased with increasing head group size. Furthermore, fusion was virtually abolished when the head group consisted of more than three carbohydrate units. At 1 mM Ca^{2+}, negligible fusion was observed. However, prior addition of RCA_1 had a pronounced effect on the fusion rate of both Gb_3 and LacCer vesicles, upon subsequent addition of Ca^{2+}, in a manner very similar to that seen for vesicle aggregation (Figure 2B). The enhancement in the initial fusion rate was only seen when Ca^{2+} was added *after* addition of the lectin, in contrast to the observation that vesicle aggregation was stimulated by RCA_1 and Ca^{2+} independent of the order of addition. Furthermore, a comparison between the kinetics of Ca^{2+}-induced aggregation and Ca^{2+}-induced fusion (Figure 1B vs. Figure 2A) suggests that fusion represents the rate-limiting step in the overall process, since at higher Ca^{2+} concentrations the rate of aggregation tends to saturate while the rate of fusion still increases linearly. It is also interesting to note that the combined action of RCA_1/Ca^{2+} displays a higher fusion rate for LacCer vesicles than for vesicles containing the trihexosylceramide, contrary to the order of effectiveness during agglutination. Hence, these results indicate that Gb_3 interferes with the process of close approach of membranes, as required for membrane fusion, and tends to keep the bilayers separated within the aggregated state. Indeed, from a comparison of the kinetics of membrane fusion of both vesicle types, LacCer-vesicles fuse rapidly and extensively, showing a fusogenic behavior typical for pure PE/PA vesicles [not shown; cf. Schuber et al. (1983)], while RCA_1/Ca^{2+}-induced fusion of Gb_3 vesicles abruptly levels off (cf. Figure 9). Finally, as anticipated, no combined effect of RCA_1/Ca^{2+} was seen on the initial fusion rate of GalCer vesicles (Figure 2B), which is in accordance with a similar lack of effect seen when the agglutination experiments (Figure 1A) were performed.

In addition to an increase in the initial fusion rate, prior addition of RCA_1 also caused a considerable reduction in the Ca^{2+} threshold concentration, i.e., the minimal concentration required to induce fusion of LacCer and Gb_3 vesicles. In the absence of RCA_1, this threshold concentration was centered around 1 mM Ca^{2+}, whereas in the presence of the lectin significant fusion was already detectable at ~0.15 mM Ca^{2+} (Figure 3), i.e., at an effective Ca^{2+} concentration of ~50 μM, since the medium contained 0.1 mM EDTA, necessary for preventing Tb/DPA complex formation in the external medium (Wilschut et al., 1980; Hoekstra, 1982c). As shown in Figure 3, the threshold cation concentration was dependent on the RCA_1 concentration and decreased with increasing lectin concentration, ranging from ca. 1 to 0.2 mM Ca^{2+} (as determined by extrapolation) for Gb_3 vesicles in the presence of 0–50 μg/mL RCA_1. The size-dependent interference of the glycolipid carbohydrate head group structure with close, i.e., fusion-susceptible, apposition of membranes as noted above was also reflected by differences in the Ca^{2+} threshold concentrations. In spite of a higher extent of agglutination, the threshold Ca^{2+} concentration for fusion of Gb_3 vesicles in the presence of 12.5 μg/mL RCA_1 was ~0.45 mM (Figure 3), while under comparable conditions, a 4-fold lower cation concentration sufficed to initiate the fusion of LacCer vesicles.

Effect of Temperature on Ca^{2+}- and Ca^{2+}/RCA_1-Induced Fusion of Glycolipid-Containing Vesicles. The initial fusion rates of LacCer- and Gb_3-containing PE/PA vesicles, induced by Ca^{2+}, either in the presence or in the absence of RCA_1, increased as a function of temperature. The data, shown in Figure 4, were plotted as the ratio of the initial fusion rate obtained in the presence of RCA_1 over that obtained in its absence (at a fixed Ca^{2+} concentration) and normalized to the same ratio obtained at 37 °C. It is apparent that the effect

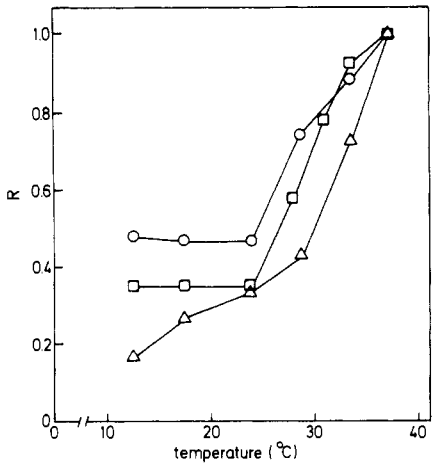

FIGURE 4: Ca^{2+}-induced fusion of glycolipid-containing vesicles as a function of temperature, effect of RCA_1. Equimolar amounts of Tb- and DPA-containing glycolipid vesicles [PE/PA and GalCer (□), LacCer (△), or Gb_3 (○), respectively] were mixed in 100 mM NaCl/0.1 mM EDTA/5 mM TES, pH 7.4, at various temperatures. A total of 12.5 (LacCer and Gb_3 vesicles) or 100 μg/ml RCA_1 (GalCer vesicles) was subsequently injected, followed after 2 min by 2 mM Ca^{2+} (final concentration). Alternatively, fusion was induced in the absence of RCA_1 by injecting 2 mM Ca^{2+} in the medium. The initial fusion rates, in the presence (f_i^+) and absence (f_i^-) of RCA_1, were determined at the temperatures indicated, and the ratio $(f_i^+/f_i^-)_T$ normalized to the ratio at 37 °C, $(f_i^+/f_i^-)_{37°C}$, was plotted (R) as a function of temperature: $R = (f_i^+/f_i^-)_T/(f_i^+/f_i^-)_{37°C}$.

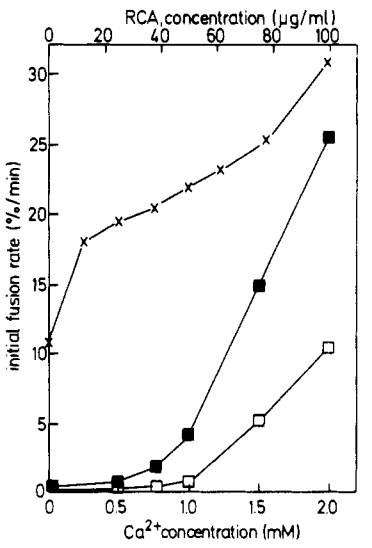

FIGURE 5: Effect of RCA_1 on Ca^{2+}-induced fusion of galactosylcerebroside-containing PE/PA vesicles. Two populations of PE/PA/GalCer vesicles, containing Tb and DPA, respectively, were incubated at 37 °C in the presence of various concentrations RCA_1 (upper scale). After 2 min, 2 mM Ca^{2+} was added to induce fusion. The initial fusion rates were determined and plotted as a function of the RCA_1 concentration (×). In addition, fusion was induced by various Ca^{2+} concentrations (lower scale), either in the absence (□) or presence (■) of RCA_1 (75 μg/mL). In the latter case, the vesicles were preincubated with the lectin for 2 min, prior to the addition of Ca^{2+}.

of temperature on RCA_1/Ca^{2+}-induced fusion was much more pronounced for the fusion properties of LacCer vesicles than for vesicles containing the trihexosylceramide. Over the temperature range studied, the initial fusion rate of the former was stimulated 5-fold, while that of the latter increased by a factor of about 2. The distinct fusogenic behavior of LacCer and Gb_3 vesicles as a function of temperature cannot be explained by a temperature-dependent modification of the lectin per se, as it should have affected fusion of both vesicle types in a proportional manner. Rather, the results suggest that the apparent exposure of the lactosyl moiety improved substantially with increasing temperature, allowing lectin–receptor interaction to occur with higher efficiency. Enhanced exposure may have been accomplished by a temperature-induced alteration in the orientation of the lactosyl moiety, relative to the plane of the bilayer, and/or may have resulted indirectly from an effect of temperature on the physical properties of the supporting lipid matrix. Particularly, the latter suggestion seems attractive as both curves (Figure 4, (○ and △)] display a break around 25–30 °C. This remarkable temperature-dependent increase of RCA_1/Ca^{2+}-induced fusion of LacCer vesicles, relative to that in the absence of RCA_1, prompted us to investigate whether GalCer vesicles might display similar temperature-dependent properties.

Temperature-Dependent Exposure of Cerebroside Galactosyl Residues. At 37 °C, the initial rates of Ca^{2+}-induced fusion of GalCer-containing PE/PA vesicles increased with increasing RCA_1 concentration, indicating that the lectin did affect interbilayer contact between GalCer vesicles at an elevated temperature (Figure 5). Furthermore, the RCA_1-dependent increase in the initial fusion rate was completely abolished when the haptenic sugar lactose was included in the incubation mixture prior to the lectin, implying that the enhancement was mediated by the specific interaction between RCA_1 and the galactose receptor. The threshold temperature at which the stimulating effect of RCA_1 on Ca^{2+}-induced fusion of GalCer vesicles became apparent was centered around 26 °C (Figures 6 and 4). Similar to observations for

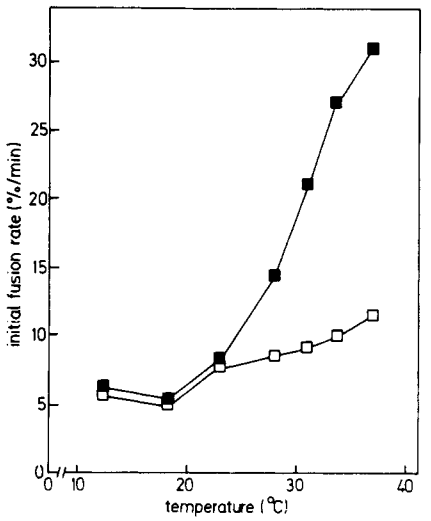

FIGURE 6: Temperature dependence of Ca^{2+}-induced fusion of galactosylcerebroside-containing PE/PA vesicles, effect of RCA_1. Equimolar amounts of Tb- and DPA-containing vesicles (50 μM lipid) were suspended in the fusion medium and equilibrated at the indicated temperatures. Fusion was induced by adding Ca^{2+} (2 mM) to the vesicle suspension as such (□) or to a suspension that had been pretreated with RCA_1 (100 μg/mL) for 2 min (■).

LacCer- and Gb_3-containing PE/PA vesicles at ambient temperature, the threshold Ca^{2+} concentration for fusion of GalCer vesicles was reduced under conditions where RCA_1 became effective (Figure 5). Thus, in the presence of 75 μg/mL RCA_1 a Ca^{2+} threshold concentration of ~0.2 mM was obtained, while in the absence of the lectin fusion required an initial total Ca^{2+} concentration of ~0.8 mM.

Effect of Spermine. Polyamines, in particular spermine, readily aggregate and fuse PE/PA vesicles (Schuber et al., 1983). Since it has been proposed that polyamines are involved in regulating physiologically significant membrane fusion phenomena, it was of interest to examine the effect of spermine on the glycolipid-containing vesicles and to establish whether

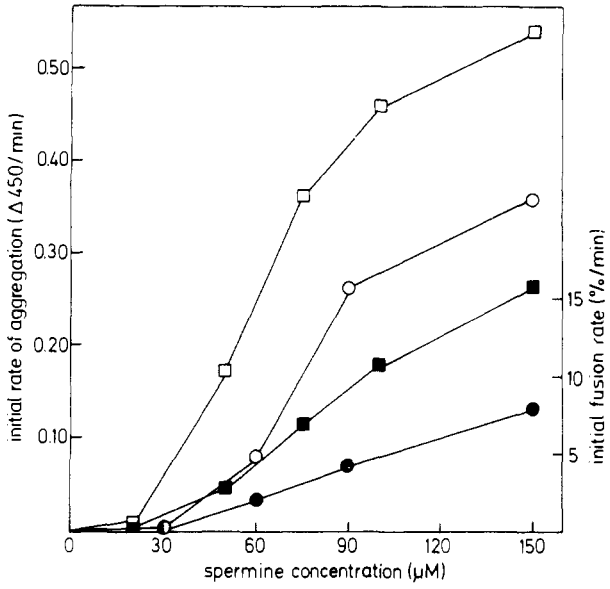

FIGURE 7: Spermine-induced aggregation and fusion of glycolipid-containing vesicles. Aggregation (open symbols) and fusion (filled symbols), induced by various concentrations of spermine, was monitored as described in the legends of Figures 1 and 2. The incubation temperature was 23 °C. (□) GalCer vesicles; (○) Gb_3 vesicles.

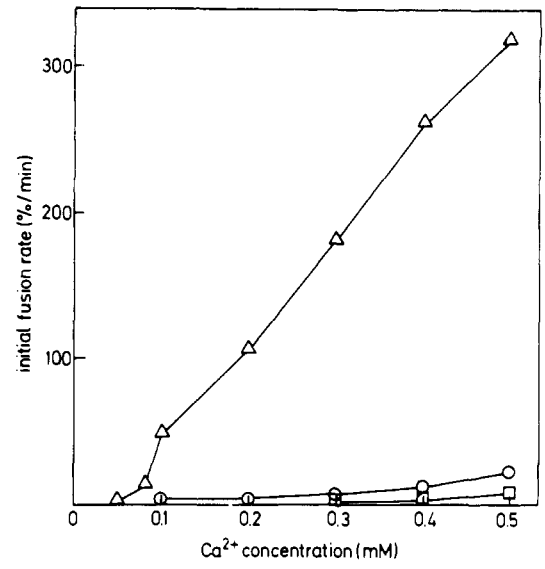

FIGURE 8: Effect of RCA_I/spermine on Ca^{2+}-induced fusion of glycolipid vesicles. Vesicles were incubated at 23 °C with 12.5 μg of RCA_I (2 min) followed by spermine (30 μM). At 3 min after addition of spermine, various Ca^{2+} concentrations were injected into the medium, and fusion was monitored. The PE/PA vesicles contained LacCer (△), Gb_3 (○), or GalCer (□).

a polycation like spermine could affect the dynamic behavior of a glycolipid in fusogenic processes, similarly as observed for Ca^{2+}. As shown in Figure 7, the presence of carbohydrate protrusions on the vesicle surface also affected spermine-induced aggregation of the glycolipid-containing vesicles, and as observed for Ca^{2+}, the efficiency decreased with increasing size of the carbohydrate moiety. Spermine became already effective at concentrations in the micromolar range (vs. millimolar for Ca^{2+}; cf. Figure 1B). On the other hand, the relative ability of both cations to aggregate GalCer vs. Gb_3 vesicles appeared to be very similar, as revealed by plotting the ratio of the initial aggregation rates of GalCer vesicles/Gb_3 vesicles as a function of the Ca^{2+} and spermine concentration (not shown). Furthermore, spermine-induced aggregation was accompanied by fusion (Figure 7, filled symbols), but in this case the rates were lower than those observed for Ca^{2+}. Interestingly, in contrast to Ca^{2+}, spermine at a near threshold concentration (30 μM) only marginally affected RCA_I-induced agglutination (not shown). Thus, whereas a threshold concentration of 1 mM Ca^{2+} increased the rate of RCA_I-induced agglutination by 5–40-fold (Figure 1A), an increase of only 10–30% in the initial rate of RCA_I-induced agglutination was seen when similar experiments were performed in the presence of spermine. These results indicate that the consequences of binding of the divalent cation vs. that of the polyvalent polyamine differ with respect to the subsequent possibility of lectin–receptor interaction. The small increase in the agglutination rate, resulting from the combined action of spermine and RCA_I was, however, exclusively due to an enhanced lectin–receptor interaction as addition of the haptenic sugar lactose completely dissociated the vesicle aggregates. Also, at higher spermine concentrations, the combined action of RCA_I and spermine did not appreciably stimulate the initial rate of vesicle aggregation relative to the rate seen in the absence of the lectin.

A rather intriguing observation was made, which depended strongly on the glycolipid structure inserted in the PE/PA bilayer, when the various glycolipid-containing vesicles were incubated with threshold concentrations of RCA_I (12.5 μg/mL) and spermine (30 μM), followed by Ca^{2+}. As noted

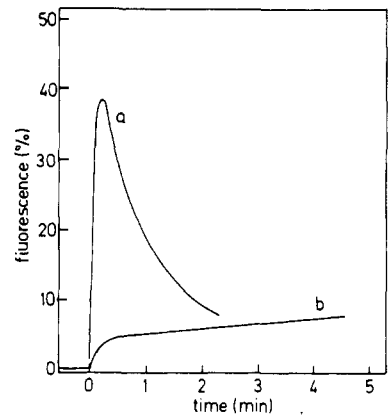

FIGURE 9: Kinetics of Ca^{2+}-induced fusion of glycolipid vesicles, pretreated with RCA_I and spermine. PE/PA vesicles, containing LacCer (curve a) or Gb_3 (curve b) were preincubated with RCA_I (12.5 μg/mL, 2 min) and spermine (30 μM, 3 min), respectively. Fusion was induced by injecting 0.5 mM Ca^{2+} into the medium at $t = 0$, and the fluorescence development was monitored as a function of time. The incubation temperature was 23 °C.

before, significant aggregation was not observed in the presence of these concentrations of RCA_I and spermine and neither was fusion (not shown). However, when LacCer vesicles were used, subsequent addition of only trace amounts of Ca^{2+} sufficed to induce fusion, which proceeded with a very high initial fusion rate when the Ca^{2+} concentration was further increased (Figures 8 and 9). Relative to the rates obtained in the absence of spermine, i.e., in the presence of RCA_I and Ca^{2+} only (cf. Figure 3), the divalent cation on a molar basis proved to be 20–40 times more effective (when corrected for the presence of 0.1 mM EDTA). For example, in the presence of 12.5 μg/mL RCA_I, LacCer vesicles fused with an initial rate of ~40%/min upon addition of 2 mM Ca^{2+} (see Figure 3) or 1.9 mM Ca^{2+} when corrected for the presence of EDTA. Approximately 0.15 mM Ca^{2+} (50 μM, after correction) sufficed to induce a similar rate when the medium contained in addition 30 μM spermine. The stimulating effect of RCA_I/spermine on Ca^{2+}-induced fusion was independent of the order of addition of the lectin and polyamine but required

Ca^{2+} to be added *after* addition of spermine and RCA_1. The fusion process was instantaneously inhibited when relative to the Ca^{2+} concentration a 2-fold molar excess of EDTA was injected into the medium.

For Gb_3 vesicles, the combined action of RCA_1/spermine on Ca^{2+}-induced fusion appeared to be less outspoken (Figures 8 and 9). Although fusion became apparent at very low Ca^{2+} concentrations (~0.25 mM, or 0.15 mM after correction for the presence of EDTA), the enhancement in the initial fusion rates, relative to the rates observed in the absence of spermine (cf. Figure 3), was relatively marginal (1.5–2.0-fold) when compared to the enhancement observed for LacCer vesicles. Finally, consistent with the observations that RCA_1 does not agglutinate GalCer vesicles at 23 °C, while spermine does not induce significant vesicle aggregation at 30 μM, Ca^{2+}-induced fusion of these vesicles was barely detectable under comparable experimental conditions as described for LacCer and Gb_3 vesicles (Figure 8).

Effect of Aggregation and Fusion on Membrane Stability. Except for differences in the initial fusion rates, the overall Ca^{2+}-induced fusion kinetics of the glycolipid vesicles, as reflected by Tb/DPA fluorescence development (Figure 9), were very similar when monitored in the presence of RCA_1 (as noted above) or RCA_1/spermine. After an initial rapid phase, the fluorescence leveled off during Ca^{2+}-induced fusion of Gb_3 vesicles (curve b) in the presence of RCA_1/spermine. The transient nature of the fluorescence development observed during fusion of LacCer vesicles (curve a) indicated that these vesicles rapidly lost their contents during fusion. The fluorescence decrease thus corresponds with a destabilization of the bilayer, as a result of which EDTA, present in the external medium, rapidly dissociates the fluorescent Tb/DPA complex either in the external medium or, after its permeation across the perturbed vesicle bilayer, within the vesicle interior. The leakage event was monitored in parallel experiments, using PE/PA/glycolipid vesicles, containing the (preformed) fluorescent Tb/DPA complex. Under identical experimental conditions as those in Figure 9, the rapid and extensive leakage of the LacCer vesicles was confirmed, showing that within 3 min after addition of Ca^{2+} essentially all fluorescence had disappeared (not shown). The lower level of fluorescence observed during fusion of Gb_3 vesicles was apparently due to their limited fusion capacity, since no release of vesicle contents could be detected under these conditions, as revealed by the leakage assay. Neither did membrane destabilization become apparent during RCA_1-induced agglutination of Gb_3- (Figure 10B, curve, c) or LacCer-containing PE/PA vesicles (not shown). In Figure 10, the time courses of aggregation, fusion, and leakage are shown as obtained at the highest concentrations of RCA_1 (50 μg/mL), Ca^{2+} (4 mM), and spermine (150 μM) used in this study to induce agglutination or fusion of Gb_3 vesicles. Qualitatively, similar data were obtained for LacCer vesicles (not shown). In contrast to RCA_1 and spermine, Ca^{2+} induced substantial leakage, as reflected by the fusion curve per se (Figure 10A, curve b) and the leakage curve (Figure 10B, curve b). The kinetics of Ca^{2+}-induced leakage displayed a typical biphasic behavior. The initial rate of leakage appeared to be fairly modest, but after 2–3 min a rapid and substantial increase in the rate of leakage was observed. Quantitatively, the rate of leakage was dependent on the Ca^{2+} concentration and the kinetics were very similar when the vesicles had been agglutinated by RCA_1 prior to the addition of Ca^{2+}. The leakage rates during RCA_1/Ca^{2+}-induced fusion of Gb_3 vesicles are shown in Figure 11. When these results are compared to those obtained for fusion (Figure 3),

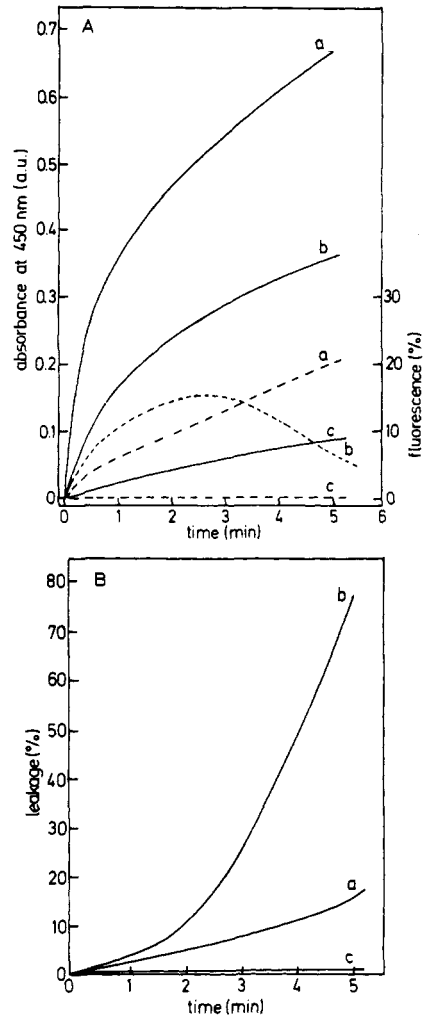

FIGURE 10: Kinetics of vesicle aggregation, fusion, and leakage of vesicle contents induced by spermine, Ca^{2+}, and RCA_1. Gb_3-containing vesicles were suspended in the incubation medium. Aggregation (solid curves, A), fusion (dashed curves, A), and leakage of vesicle contents (B) were induced by adding 150 μM spermine (a) 4 mM Ca^{2+} (b), or 50 μg/mL RCA_1 (c). Leakage of vesicle contents was determined by monitoring the release of the preformed fluorescent Tb/DPA complex, encapsulated in the aqueous interior of the vesicles. Release of contents is revealed as fluorescence quenching as a result of complex dissociation by EDTA and, if present, Ca^{2+} in the external medium, as described under Experimental Procedures.

it is then interesting to note that in the presence of 1 mM Ca^{2+} essentially no bilayer destabilization was observed despite the occurrence of fusion under these conditions. These results, showing that fusion of Gb_3-containing PE/PA vesicles proceeds essentially as a nonleaky process at low Ca^{2+} concentrations, are consistent with the results presented in Figure 9, demonstrating that nonleaky fusion occurred in the presence of RCA_1 and spermine at a Ca^{2+} concentration of 0.5 mM.

Discussion

RCA_1- and Cation-Induced Aggregation of Glycolipid-Containing Bilayers. As demonstrated in this study, the lectin/receptor interaction is facilitated when the distance of the carbohydrate-receptor moiety relative to the plane of the bilayer increases while this interaction is further influenced by the properties of the bilayer per se. The extent to which the various glycolipids protruded beyond the PE/PA bilayer surface was also revealed by the diminished tendency of the vesicles to aggregate upon addition of Ca^{2+} and spermine, when the carbohydrate head group size increased. As a result of charge neutralization, trihexosylceramide-containing vesicles

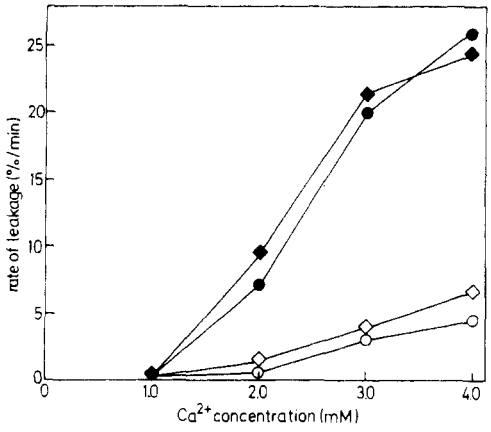

FIGURE 11: Rates of Ca^{2+}-induced leakage of Gb_3-containing vesicles, in the presence and absence of RCA_1. PE/PA/Gb_3 vesicles, containing the fluorescent Tb/DPA complex, were incubated at 23 °C in the presence (◇, ◆) or absence (ηop, ●) of RCA_1 (12.5 μg/mL). After 2 min, various Ca^{2+} concentrations were injected into the medium, and leakage was monitored as a function of time (cf. Figure 10B). The initial rates of leakage were calculated (open symbols) from the tangents drawn to the curves at $t = 0$, while the maximal rates of leakage (filled symbols) were calculated from the tangents drawn to the steepest part of the curves (b in Figure 10B). The rates were plotted as a function of the Ca^{2+} concentration.

still aggregated, whereas almost complete inhibition was seen when the bilayer contained the globoside Gb_4, which contains four carbohydrate residues (Hoekstra et al., 1985a). Thus, with respect to the ability of the glycolipid vesicles to aggregate, the trisaccharide head group of Gb_3 can be defined as the threshold carbohydrate head group size. Although RCA_1 was able to interact with the terminal galactosyl residue of lactosylceramide, the presence of the dissacharide on the vesicle surface did not substantially interfere with Ca^{2+}-induced aggregation, as the rates were very similar to those obtained for Ca^{2+}-induced aggregation of galactosylcerebroside-containing vesicles, the monosaccharide not being recognized by RCA at ambient temperature. This would indicate that the lactosyl head group of lactosylceramide must be near the vicinity of the phospholipid head group region.

RCA_1 and Ca^{2+} showed a synergistic effect on the agglutinability of Gb_3- and LacCer-containing PE/PA bilayers, as subthreshold concentrations of the cation and the lectin, when given together, yielded substantial agglutination rates. At ambient temperature, such an effect was not observed for GalCer vesicles. Interestingly, this synergism was not observed when Ca^{2+} was replaced by the polyvalent cation spermine, which implies that charge neutralization of PA cannot account for the enhanced lectin agglutinability. It can also be ruled out that Ca^{2+} modified the lectin per se; conceivably, a preincubation of RCA_1 and Ca^{2+} would then have resulted in Ca^{2+}-induced protein aggregation, which would have led to a diminished receptor/lectin interaction, instead of the observed enhancement in lectin reactivity. In addition, it has been reported that RCA_1 does not require divalent cations for binding of carbohydrates (Goldstein & Hayes, 1978). It is concluded, therefore, that stimulation of RCA_1-induced agglutination in the presence of Ca^{2+}, but *not* spermine, was accomplished by a secondary effect, involving a Ca^{2+}-specific modification of the supporting lipid matrix. Furthermore, it seems reasonable to assume that the subtle (local) change in the lipid matrix will be sensed most effectively by those receptors that are closest to the phospholipid head group region. Indeed, whereas Ca^{2+} concentrations in excess of 1 mM were required to facilitate soybean agglutinin-induced agglutination of globoside Gb_4-containing PE/PA bilayers (Hoekstra et al., 1985a), cation concentrations of 0.15–0.5 mM Ca^{2+} sufficed to yield substantial agglutination of LacCer- and Gb_3-containing bilayers. These observations would provide further support for an effect of Ca^{2+} on the lipid bilayer rather than on lectin/receptor interaction per se. Others (Rendi et al., 1979; Hampton et al., 1980; Sundler, 1982) have also reported that lectin-induced agglutination of negatively charged bilayers can be stimulated upon addition of Ca^{2+}, and the effect has been explained in terms of reduced electrostatic repulsion between glycolipid-containing bilayers and alterations in membrane hydration. Previously, when investigating lectin-induced agglutination of globoside-containing vesicles, we suggested (Hoekstra et al., 1985a) that the effect induced by Ca^{2+} can be explained as a cation-induced modulation of the steric orientation of the carbohydrate head group. The fusion experiments, in particular, corroborate and extend this suggestion.

Ca^{2+}-Induced Steric Modulation of Carbohydrate Head Groups: Effect on Membrane Fusion. The steric interference of glycolipid head groups with membrane approach was indicated by the apparent differences between the initial rates of agglutination vs. fusion. In spite of higher agglutination rates, the initial fusion rates of Gb_3 vesicles were substantially lower than the rates observed for LacCer vesicles. In fact, the overall kinetics of Ca^{2+}-induced fusion of LacCer and Gb_3 vesicles, in the presence of either RCA_1 or RCA_1/spermine, differed remarkably. LacCer vesicles displayed a fusogenic behavior that closely resembles that of pure PE/PA vesicles; i.e., they undergo multiple fusion events and eventually loose their aqueous contents, as revealed by the transient nature of the fluorescence development. In contrast, fusion of Gb_3 vesicles halts rather abruptly after an initial increase, as previously observed for globoside Gb_4-containing vesicles (Hoekstra et al., 1985a). This observation could not be attributed to leakage of vesicle contents but rather to a steric interference of the carbohydrate head group structure with the process of close approach of bilayers. Initially, membrane contact between Gb_3 vesicles is sufficiently close to allow rapid fusion. However, membrane fusion can only be sustained when the interbilayer distance remains sufficiently close to be effective for subthreshold Ca^{2+} concentrations. Apparently, as Ca^{2+} binding proceeds the interbilayer distance between Gb_3 vesicles subsequently increases, abolishing the process of membrane fusion. It should be noted that the aggregated vesicles do not dissociate but increase their mutual distance within the aggregated vesicle complex. As the interbilayer distance increases, the high-affinity binding sites for Ca^{2+} (Ekerdt & Papahadjopoulos, 1982) would be expected to submerge, and concomitantly, membrane fusion at (sub)-threshold Ca^{2+} concentrations will cease. It appears that lectin-induced agglutination allows the appearance of intermembrane structures that present a high affinity for Ca^{2+} and which, therefore, decrease the threshold cation concentration. In a quantitative sense, the decrease in threshold Ca^{2+} concentration necessary to induce fusion should depend, a priori, on the interbilayer distance established by lectin-induced agglutination, and hence on the extent to which the glycolipid carbohydrate head group protrudes beyond the bilayer surface. In fact, we have observed this dependency, as the threshold Ca^{2+} concentrations required to induce fusion after lectin-induced agglutination decreased in the order globoside Gb_4 > Gb_3 > LacCer (Figures 3 and 8; Hoekstra et al., 1985a).

Interestingly, subthreshold concentrations of spermine did not facilitate RCA_1-induced agglutination, nor was spermine-induced fusion facilitated by RCA_1, indicating that its

mode of interaction with the bilayer differs from that of Ca^{2+}. These differences between Ca^{2+} and spermine were also reflected by their distinctly different effect on membrane stability, as Ca^{2+} induced extensive leakage of vesicle contents, in contrast to spermine (Figure 10B). Taken together, these results can be explained in terms of the different capacity of spermine and Ca^{2+} to (locally) dehydrate (apposed) bilayers, a necessary condition for triggering membrane fusion (Wilschut et al., 1980; Hoekstra, 1982a; Wilschut & Hoekstra, 1984; Düzgüneş, 1985). This different dehydrating capacity can be correlated to the enhanced lectin-induced vesicle agglutination in the presence of Ca^{2+}, as opposed to spermine. A picture thus emerges that involves a (partial) dehydration of the bilayer upon binding of Ca^{2+} to PA, which in turn induces a reorientation of the strongly hydrophilic carbohydrate head group by forcing it to tilt toward a more aqueous environment, i.e., by increasing the distance of the terminal sugar residue relative to the bilayer/water interface. As a result, fusion will be interrupted when the glycolipid contains at least three carbohydrate residues, i.e., Gb_3 and globoside Gb_4. Because of its relatively short carbohydrate chain length (Grant & Peters, 1984), the lactosyl head group will, even in its extended form, still provide a close intermembrane contact and fusion will be sustained. The same mechanism also explains the enhanced lectin agglutinability.

Effect of Temperature on RCA_1–Glycolipid Interaction. At temperatures above 25 °C, an RCA_1-mediated enhancement of Ca^{2+}-induced fusion became apparent also for GalCer vesicles. The direct involvement of an RCA_1/galactosyl moiety interaction was revealed by abolishment of the lectin-mediated effect by the haptenic sugar lactose. This result suggests that the galactosyl moiety of the cerebroside was insufficiently exposed at room temperature, while with increasing temperature a modification was induced, involving either the glycolipid structure per se or a temperature-induced alteration in the lipid matrix, thus affecting GalCer/RCA_1 interaction in an indirect manner. Since the phase transition of the glycolipid is centered around 60 °C (Maggio et al., 1981), the latter possibility seems more likely, particularly in light of the observation that a similar temperature-dependent effect was seen on both LacCer and Gb_3 vesicles (Figure 4). Up to 25 °C, the fusion rates for GalCer vesicles, either in the presence or in the absence of RCA_1, were essentially the same (Figure 6), while these rates increased proportionally for Gb_3 vesicles. However, in the case of LacCer vesicles, the initial fusion rate in the presence of RCA_1 increased faster than in its absence, indicating an enhanced glycolipid/receptor interaction with increasing temperature. Differences in lectin/receptor affinity as a result of temperature-dependent modifications of the lectin can be excluded since such an effect should have been also reflected by an enhanced interaction with Gb_3 vesicles. Rather, it would appear that the relative enhancement of LacCer vesicle fusion in the presence of RCA_1 can be attributed to physical alterations in the vesicle bilayer. In line with the above notion on Ca^{2+}-facilitated agglutination, such alterations will be most sensitive to the carbohydrate head group (disaccharide vs. trisaccharide), which is relatively close to the vicinity of the phospholipid head group region. Presumably, above 25 °C, these bilayer modifications become even more pronounced as the initial fusion rates in the presence of RCA_1 relative to the rates in the absence of the lectin became also enhanced for Gb_3 and GalCer vesicles. This temperature-dependent effect may be attributed to the transformation of egg PE from a lamellar to a hexagonal phase, which takes place at a temperature around 28 °C (Cullis & De Kruijff, 1979). As a result, lipid phase separation (Papahadjopoulos, 1978; Hoekstra, 1982b) may subsequently increase the local density of glycolipid receptors in the lateral plane of the bilayer, which in turn will increase lectin agglutinability (Surolia et al., 1975; Curatolo et al., 1978; Goodwin et al., 1982; Grant & Peters, 1984).

Conclusions

The present results, in conjunction with those previously reported on globoside Gb_4 containing vesicles (Hoekstra et al., 1985a), indicate that close approach of adjacent bilayers can be regulated by the size of the glycolipid carbohydrate head group. The effective protrusion can be further regulated by Ca^{2+}, when the glycolipids are inserted into negatively charged bilayers. This effect is indirectly accomplished by Ca^{2+} binding to the supporting matrix, which causes the head group to adopt a more perpendicular orientation relative to the lateral plane of the bilayer; the results indicate this to be related to the ability of Ca^{2+} to dehydrate the bilayer. As a consequence, the distance between the terminal sugar receptor and the plane of the bilayer will increase, while within the agglutinated vesicle complex the interbilayer distance increases, which causes the arrest of membrane fusion. The latter phenomenon becomes apparent for glycolipids containing at least three carbohydrate residues such as trihexosylceramide (this study) and globoside Gb_4 (Hoekstra et al., 1985a).

Micromolar amounts of Ca^{2+} sufficed to induce fusion after preagglutination of the vesicles with the lectin, or when in addition, a subthreshold (30 μM) concentration of spermine was used. In the latter case, i.e., under conditions that may bear physiological relevance, very high fusion rates were observed. Spermine has been suggested to function as an intracellular modulator of membrane fusion occurring during exocytotic events or membrane biosynthesis (Schuber et al., 1983). The routing of such and other intracellular vesicles to their specific sites of action would presumably require a specific recognition system that could involve an interaction between carbohydrate structures and specific membrane localized lectins. That certain glycolipids show a particular tendency to regulate intermembrane distances and thus membrane fusion is rather intriguing and merits further investigation.

Acknowledgment

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Registry No. Ca, 7440-70-2; GalCer, 85305-88-0; Gb_3, 71965-57-6; LacCer, 4682-48-8; spermine, 71-44-3.

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X-ray Diffraction Evidence for Fully Interdigitated Bilayers of 1-Stearoyllysophosphatidylcholine†

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ABSTRACT: X-ray diffraction experiments have been performed on 1-stearoyllysophosphatidylcholine or C(18):C(0)PC as a function of hydration at temperatures below the order/disorder transition (T_m = 26.2 °C). At these temperatures, hydrated C(18):C(0)PC forms lamellae. The bilayer thickness, as determined by the saturation hydration method and electron-density profile, is 35–36 Å, and the average area per C(18):C(0)PC molecule at the lipid/water interface is 45.5 Å^2. The packing geometry of C(18):C(0)PC in the lamella is proposed to adopt a fully interdigitated model in which the long C(18) acyl chain extends across the entire hydrocarbon width of the bilayer. Thus far, three different types of interdigitated bilayers are known for phosphatidylcholines. These various types of chain interdigitation are discussed in terms of the chain length difference between the *sn*-1 and *sn*-2 acyl chains.

It is generally recognized that in biological membranes most phospholipids self-assemble into two-dimensional arrays of the noninterdigitated lamellar type; that is, each phospholipid molecule is oriented with its polar head group toward the membrane surface and its chain methyl groups toward the geometric center of the hydrophobic core in the membrane interior. Recently, several spectroscopic studies have been directed to the investigation of aqueous dispersions of mixed-chain phospholipids including sphingomyelin, whose two hydrocarbons chains are inequivalent in terms of carbon number (Huang et al., 1984; Hui et al., 1984; Levin et al., 1985; McIntosh et al., 1984). Results of these studies indicate that in the gel state the packing mode of pure mixed-chain phospholipids in the two-dimensional array of lamellae is that of a highly ordered interdigitated bilayer, which is a marked deviation from the generally recognized noninterdigitated bilayer form of symmetric phospholipids. It is now established that at temperatures below the main order/disorder phase transition, mixed-chain phospholipid molecules can be accommodated in at least three different types of interdigitated packing modes, depending on the chain-length difference

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