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Effects of calcium-induced aggregation on the physical stability of liposomes containing plant glycolipids

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Abstract

Membranes containing either negatively charged lipids or glycolipids can be aggregated by millimolar concentrations of Ca^{2+}. In the case of membranes made from the negatively charged phospholipid phosphatidylserine, aggregation leads to vesicle fusion and leakage. However, some glycolipid-containing biological membranes such as plant chloroplast thylakoid membranes naturally occur in an aggregated state. In the present contribution, the effect of Ca^{2+}-induced aggregation on membrane stability during freezing and in highly concentrated salt solutions ($NaCl \pm CaCl_2$) has been determined in membranes containing different fractions of uncharged galactolipids, or a negatively charged sulfoglucolipid, or the negatively charged phospholipid phosphatidylglycerol (PG), in membranes made from the uncharged phospholipid phosphatidylcholine (PC). In the case of the glycolipids, aggregation did not lead to fusion or leakage even under stress conditions, while it did lead to fusion and leakage in PG-containing liposomes. Liposomes made from a mixture of glycolipids and PG that approximates the lipid composition of thylakoids were very unstable, both during freezing and at high solute concentrations and leakage and fusion were increased in the presence of Ca^{2+}. Collectively, the data indicate that the effects of Ca^{2+}-induced aggregation of liposomes on membrane stability depend critically on the type of lipid involved in aggregation. While liposomes aggregated through glycolipids are highly stable, those aggregated through negatively charged lipids are severely destabilized.

Keywords: Ca^{2+}-effect; Freezing; Galactolipid; Liposome; Non-bilayer lipid; Osmotic stress

1. Introduction

While all biological membranes serve as boundaries between cellular compartments, or between different cells, some membranes occur naturally in a highly aggregated state, such as grana stacks of plant chloroplast thylakoid membranes or the myelin sheath around nerve cells. Both types of membranes contain a relatively high proportion of glycolipids and it has been shown that interactions between these glycolipids in opposing monolayers of adjacent membranes play an important role in stabilizing this aggregated physiological state.

From model membrane studies, it appears that in myelin, aggregation may at least in part be mediated by Ca^{2+}-induced interactions between galactosylceramide or glucosylceramide, and cerebroside sulfate [1–4]. For thylakoids, similar model membrane studies implicated digalactosyldiacylglycerol (DGDG) in such interactions [5,6], as well as the light-harvesting protein complex of Photosystem II and monogalactosyldiacylglycerol (MGDG) (see Ref. [7] for a review). Whether the third thylakoid glycolipid, sulfoquinovosyldiacylglycerol (SQDG) is also involved in Ca^{2+}-induced aggregation, has not been reported. Native thylakoid membranes are composed of approximately 50% proteins and 50% lipids by weight. Approximately 50% of the polar membrane lipids are made up of the non-bilayer lipid MGDG, 25% of the bilayer lipid DGDG, and the remaining 25% are comprised of the negatively charged bilayer lipids SQDG and phosphatidylglycerol (PG) [8]. The physiological role of the plant galactolipids, as revealed by the study of knock-out mutants in the biosynthetic pathways of both MGDG and DGDG, has recently been reviewed [9].

Aggregation by Ca^{2+} has also been extensively studied in membranes containing negatively charged lipids. In liposomes made from phosphatidylserine (PS), addition of Ca^{2+} leads to aggregation, followed by vesicle fusion and

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leakage of aqueous content [10]. In contrast, aggregation of liposomes made from DGDG by Ca^{2+} is completely reversible by addition of EDTA [5] and the same is true for the aggregation of liposomes containing galactosylceramide and cerebroside sulfate [4]. From these data, it can be concluded that aggregation of membranes through interactions between glycolipids is not accompanied by membrane fusion events. However, the question remains, how aggregation influences the physical stability of such membranes, as they might become more prone to fusion and leakage under conditions of dehydration. Freezing and drought are two stresses that are known to damage the thylakoid membrane system in plant leaves (see Ref. [11] for a review). Such stresses lead to the leakage of soluble proteins from the thylakoid lumen both in vitro [12,13] and in vivo [14]. It was therefore investigated, which thylakoid lipids in addition to DGDG could contribute to Ca^{2+}-induced aggregation and how this influences the stability of liposomes during freezing or during osmotic stress in the presence of high concentrations of NaCl.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, AL), egg phosphatidylglycerol (EPG) from Sigma. *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), *N*-(lissamine Rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rh-PE) and carboxyfluorescein (CF) were obtained from Molecular Probes (Leiden, The Netherlands). CF was purified according to Ref. [15]. The chloroplast glycolipids SQDG, MGDG and DGDG were purchased from Lipid Products (Redhill, Surrey, UK).

2.2. Preparation of liposomes

Liposomes were composed of EPC and different fractions of other lipids as indicated in the figures. Lipids were mixed in chloroform, dried under a stream of N_2 and stored under vacuum overnight to remove traces of solvent. Mixtures of different lipids were made by weight and are expressed as % (w/w). Liposomes for leakage studies were made as previously described [16]. Briefly, 5 mg of lipid were hydrated in 250 μl of 100 mM CF, 10 mM TES and 0.1 mM EDTA (pH 7.4) and extruded using a Liposofast hand-held extruder (Ref. [17]; Avestin, Ottawa, Canada) with 100 nm pore filters. To remove external CF, the liposomes were passed over a Sephadex G-25 column (NAP-5, Pharmacia) in 10 mM TES, 0.1 mM EDTA and 50 mM NaCl (TEN buffer, pH 7.4). Liposomes for fusion assays were made with the same lipid compositions as for leakage, with the addition of 1 mol% each of the fluorescent probe pair NBD-PE and Rh-PE.

2.3. Freezing of liposomes

Equal volumes of liposomes (10 mg lipid/ml) and concentrated $CaCl_2$ solutions in TEN were combined (40 μl/sample) to reach the final $CaCl_2$ concentrations indicated in the figures. Samples were frozen rapidly in an ethylene glycol bath pre-cooled to −20 °C. After 3 h of incubation, samples were warmed quickly to room temperature in a water bath. Controls were incubated on ice for 3 h.

2.4. Leakage and fusion measurements

CF fluorescence is self-quenching when the dye is trapped inside the liposomes at high concentrations and fluorescence is increased when the dye is released into the medium. Leakage was determined as described previously [16] by measuring fluorescence at room temperature with a Kontron SFM 25 fluorometer (Bio-Tek Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 460 and 550 nm, respectively.

Liposome fusion after freezing and thawing was determined using fluorescence resonance energy transfer [18] as described in detail previously [16]. Briefly, two liposome samples were prepared: one sample was labeled with both NBD-PE and Rh-PE, while the other sample was unlabeled. The two samples were combined after extrusion in a 1:9, (labeled/unlabeled) ratio, resulting in a final lipid concentration of 10 mg/ml. The liposomes were mixed with $CaCl_2$ solutions in the same manner as for the leakage experiments. Fusion was measured by fluorescence resonance energy transfer [18] with a Kontron SFM 25 fluorometer at excitation and emission wavelengths of 450 and 530 nm, respectively.

Leakage and fusion values reported in the figures always represent means ± S.D. from three parallel samples. Where no error bars are visible, they are smaller than the symbols.

2.5. Liposome aggregation

The aggregation of liposomes of different lipid composition was investigated by measuring changes in light scattering after addition of $CaCl_2$. Extruded vesicles were suspended in TEN buffer at a concentration of 100 μg/ml in a fluorimeter cuvette. The solution was constantly stirred with a magnetic stir bar in the cuvette. Changes in light scattering were observed 30 s after addition of small aliquots of a 1 M $CaCl_2$ solution in a fluorimeter at a 90° angle with both excitation and emission set to 600 nm. Fluorescence values were stable 30 s after the addition of each aliquot.

3. Results and discussion

As described in the Introduction, vesicles made from different glycolipids or from negatively charged lipids can

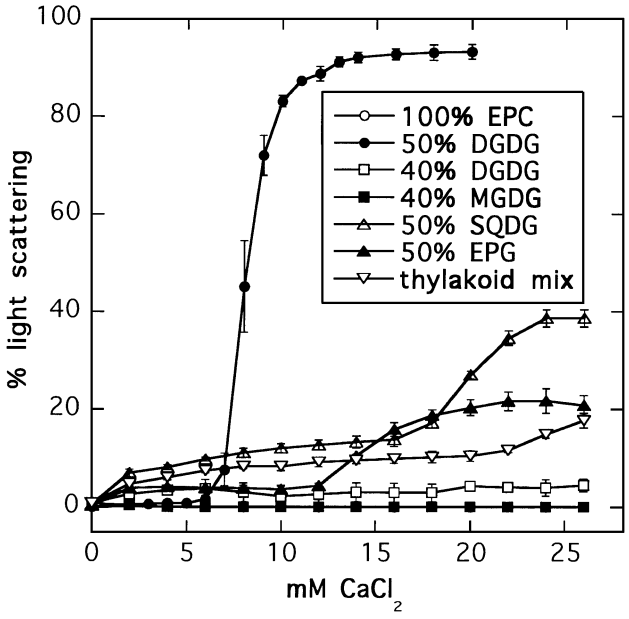

Fig. 1. Aggregation of liposomes measured as light scattering at 600 nm in a fluorimeter. The liposomes were composed of EPC and the indicated weight fractions of the other lipids. The liposomes denoted "thylakoid mix" contained 40% MGDG, 30% DGDG, 15% SQDG, and 15% EPG. Small volumes of a concentrated $CaCl_2$ solution were added successively to obtain the indicated concentrations. The data represent the means ± S.D. from three titrations each.

be aggregated by Ca^{2+}. In the case of the chloroplast glycolipids, only DGDG has been investigated and only with vesicles made exclusively from this lipid. Physiologically, this is of doubtful relevance, since thylakoid membranes contain a mixture of lipids, of which DGDG is only a minor part (20–30 mol%, [8]). We have therefore investigated the aggregation behavior of liposomes containing a smaller fraction of the different lipids present in thylakoid membranes and of a mixture of theses lipids that resembles the lipid composition of the native biological membrane. The effects of different concentrations of the single lipids on aggregation and stability were investigated with membranes composed of these lipids and EPC. As in a previous study [16], EPC was used, because it carries no net charge and the behavior of such mixtures during stress treatments is well characterized [19].

Fig. 1 shows that liposomes composed of 50% DGDG and 50% EPC were aggregated by $CaCl_2$ at concentrations above approximately 6 mM. This was critically dependent on the presence of DGDG, as membranes composed exclusively of EPC were not aggregated by $CaCl_2$ at concentrations up to 26 mM. The same was true for membranes containing 40% DGDG, indicating that 50% DGDG is the threshold concentration for the aggregation of liposomes by $CaCl_2$. Similarly, there was no aggregation of liposomes containing 40% MGDG. Whether higher concentrations of MGDG would lead to aggregation could not be determined directly, as a higher fraction of MGDG in the membranes would lead to the formation of non-bilayer structures and therefore preclude a clear interpretation of the results. However, since the thylakoid lipid mix, which contains 70% galactolipids (40% MGDG and 30% DGDG) showed only weak aggregation, we conclude that MGDG did not contribute significantly to liposome aggregation in the presence of $CaCl_2$. In an earlier investigation, it was shown that liposomes made from a total polar lipid extract from thylakoids showed strong aggregation under similar conditions [20]. In this case, however, experiments were performed with small sonicated vesicles, which showed extensive fusion under these conditions. Increased fusion in small unilamellar vesicles compared to large unilamellar vesicles has also been reported for other membrane systems [21]. In our case, no significant increase in fusion was observed under non-stressed conditions in the presence of $CaCl_2$ (see Fig. 4).

The negatively charged lipids SQDG and EPG at concentrations of 50% in EPC membranes only led to a small degree of aggregation. It should be noted, however, that aggregation was stronger in the presence of SQDG than in the presence of EPG. This may indicate that the glucose moiety also contributed to the aggregation effects. It should be mentioned at this point that aggregation is not only dependent on lipid composition and Ca^{2+} concentration, but also on lipid concentration. Light scattering measurements have to be performed at relatively low lipid concentrations (0.1 mg/ml in Fig. 1). At much higher lipid concentrations (50 mg/ml), all lipid combinations except

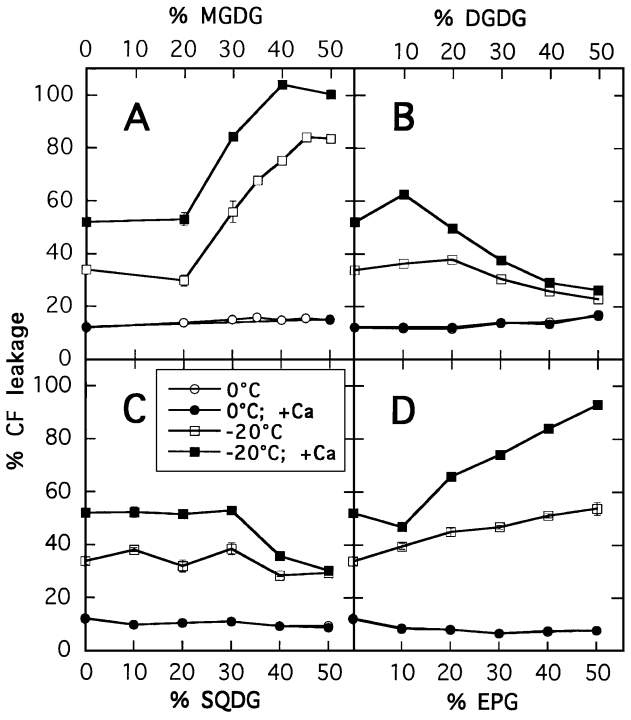

Fig. 2. CF leakage from liposomes made from EPC and different fractions of the indicated lipids. Samples were either frozen at −20 °C or stored unfrozen at 0 °C for 3 h. Samples indicated by +Ca contained 5 mM $CaCl_2$ in addition to the TEN buffer.

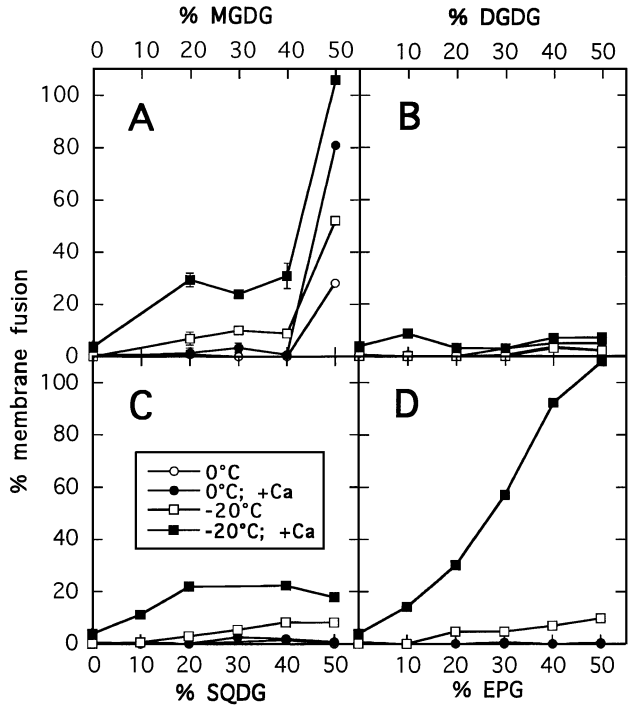

Fig. 3. Stability of liposomes of different lipid compositions in the absence or presence of 5 mM $CaCl_2$, measured as membrane fusion. See legend to Fig. 2 for further details.

100% EPC and 40% MGDG/60% EPC led to visible aggregation in the presence of 25 mM $CaCl_2$. While 40% DGDG/60% EPC still showed aggregation at this higher lipid concentration, samples containing 30% or 20% DGDG did not. Those liposomes that showed aggregation at the higher lipid concentration are expected to also aggregate during freezing (see below), when water is removed as ice and solutes and membranes are concentrated in the remaining unfrozen volume.

Ca^{2+} interacts with PG through electrostatic binding to the PO_2^- groups of the phosphodiester moiety, leading to dehydration of the phosphate group and a change in the overall conformation of the headgroup [22,23], similar to what had been found earlier for complexes between PS and Ca^{2+} [24]. Glycolipids such as DGDG are uncharged, so that electrostatic interactions with Ca^{2+} can be excluded. It has been shown that Ca^{2+} leads to a marked dehydration of the interfacial region of membranes containing DGDG [5,25] or galactosylceramide and cerebroside sulfate [1]. This dehydration reduces the repulsive hydration forces that otherwise prevent the close approach of bilayers [26] and decreases the interfacial polarity [25]. This allows the sugars in the lipid headgroups of opposing bilayers to interact [1,27], presumably through hydrogen bonding and hydrophobic interactions [5,28].

A question that has until now not been addressed in the literature is, whether the Ca^{2+}-induced aggregation of glycolipid-containing liposomes leads to a destabilization of the membranes, either directly or during stress. Fig. 2 shows that in the absence of a stress treatment (0 °C) none of the lipids led to increased leakage in the presence of 5 mM $CaCl_2$. Freezing, however, led to much more complex responses (Fig. 2; − 20 °C). Leakage was slightly increased in the presence of $CaCl_2$ in pure EPC vesicles. Increasing amounts of MGDG (Fig. 2A) or EPG (Fig. 2D) destabilized the membranes during freezing. This effect was strongly magnified by Ca^{2+} in the case of EPG, while the difference between samples in the absence or presence of $CaCl_2$ remained constant at all investigated MGDG concentrations. Quite strikingly, leakage during freezing was reduced when liposomes containing increasing amounts of DGDG (Fig. 2B) or SQDG (Fig. 2C) were frozen in the presence of $CaCl_2$.

The primary effect of vesicle aggregation is that membranes of different vesicles come into close contact. It has been shown in liposomes containing the negatively charged lipids PS [10] or cardiolipin [29], that aggregation can lead to vesicle fusion which may be accompanied by leakage. Both processes, however, can also occur independently of each other [30]. We therefore investigated the lipid-dependent effect of $CaCl_2$ on vesicle membrane fusion (Fig. 3). The most striking result of this investigation was that DGDG, which had the strongest effect on aggregation, did not lead to an increase in fusion, either at 0 or − 20 °C (Fig. 3B). In the case of MGDG (Fig. 3A), fusion remained low, both in the absence or presence of $CaCl_2$, up to 40% MGDG. The

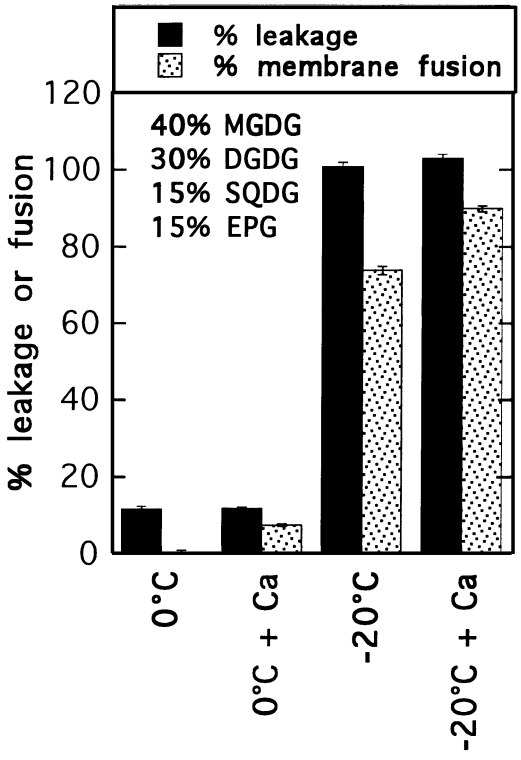

Fig. 4. Effect of $CaCl_2$ (5 mM) on CF leakage and membrane fusion in membranes containing the indicated mixture of lipids, which approximates the lipid composition of plant thylakoid membranes.

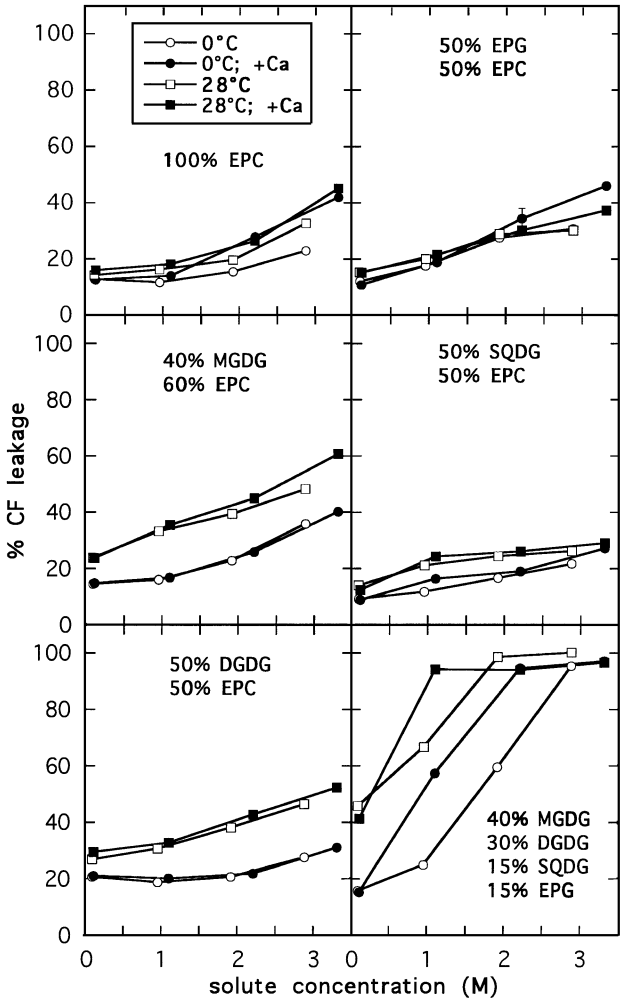

Fig. 5. Effects of high solute concentrations on the stability of liposomes containing the indicated lipids. Samples contained either only increasing concentrations of NaCl (50 mM to 1.5 M) or (+Ca) NaCl at these concentrations and in addition $CaCl_2$ (50 mM NaCl/5 mM $CaCl_2$ to 1.5 M NaCl/150 mM $CaCl_2$). The total solute concentration assuming complete dissociation of the salts is indicated. After samples were incubated for 3 h either at 0 or 28 °C, CF leakage was determined.

inclusion of 50% MGDG led to increased fusion, which was dependent on the presence of $CaCl_2$ and on freezing. The data confirm earlier observations that freezing of liposomes containing small amounts of MGDG leads to leakage without corresponding fusion [16,31]. This is unexpected for a non-bilayer lipid such as MGDG, as it has been shown that fusion proceeds through non-bilayer intermediates in the membranes [32] and that non-bilayer lipids increase the propensity of membranes to undergo fusion [33,34]. The inclusion of the non-bilayer lipid PE in liposomes, for instance, increases both leakage and fusion during freezing [16]. It has been shown for membranes containing lactosyl ceramide, that the sugar headgroups of opposing bilayers interdigitate during aggregation [35]. A similar phenomenon may also occur in the case of the two chloroplast glycolipids, although there is no experimental evidence for this yet. This could lead to complexes that are stable enough to prevent membrane fusion even under severe stress conditions. In contrast, the negatively charged lipid EPG induced massive membrane fusion in the presence of $CaCl_2$ during freezing (Fig. 3D).

A mixture of lipids that resembles the composition of thylakoid membranes is very unstable during freezing (Fig. 4), already in the absence of Ca^{2+}. Therefore, no strong effects of Ca^{2+} could be observed. However, without freezing, these liposomes were quite stable, even in the presence of 5 mM $CaCl_2$, which led to a limited degree of aggregation (Fig. 1). The instability during freezing of liposomes made from similar lipid mixtures has been reported previously [16].

Several physical changes take place during freezing that could potentially lead to the destabilization of membranes. In addition to lowering the temperature, ice crystallization occurs, which could have an impact on the physical behavior of the membranes either directly [36], or indirectly through the increased solute concentration and closer prox-

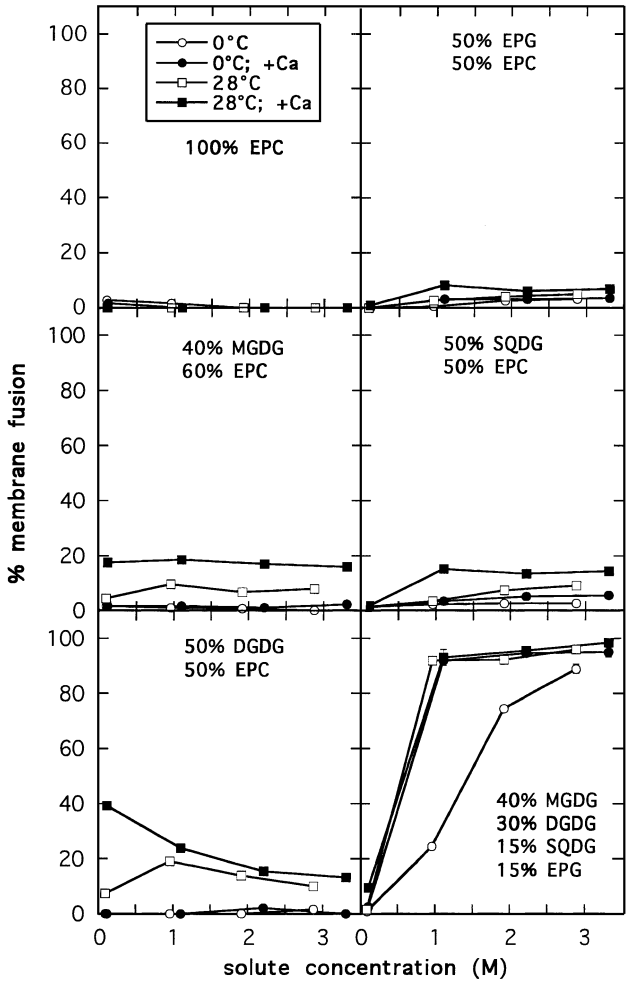

Fig. 6. Effects of high solute concentrations on membrane fusion in liposomes of the indicated compositions. See legend to Fig. 5 for experimental details.

imity of membranes to each other. To elucidate the role of osmotic stress in the observed effects of freezing (Figs. 2–4), we incubated liposomes of different lipid compositions in solutions containing up to 1.5 M NaCl. To mimic the effect of freeze-induced solute concentration in the presence of $CaCl_2$, we also used solutions that contained NaCl and $CaCl_2$ at the same molar ratio used in the freezing experiments (10:1). In order to make the results of the two solute systems directly comparable, leakage (Fig. 5) and fusion (Fig. 6) data were plotted as a function of the total solute concentration, assuming complete dissociation of the salts (i.e., 10 mM NaCl = 20 mM solutes; 10 mM $CaCl_2$ = 30 mM solutes). Temperature effects during osmotic stress were evaluated by incubating the samples at two different temperatures, 0 and 28 °C. Higher temperatures increase the propensity of lipids to undergo a transition to a non-bilayer phase [37]. As membrane fusion is believed to proceed through non-bilayer intermediates [33,34], we would expect higher temperatures to facilitate fusion under osmotic stress conditions.

Fig. 5 shows that increased solute concentrations resulted in increased leakage for all lipid compositions, both in the absence and presence of Ca^{2+}. In most cases, leakage was higher at the higher temperature. The most pronounced effects of temperature were seen for liposomes containing galactolipids or the thylakoid lipid mixture. As during freezing this mixture was the most unstable of all investigated lipid compositions and the only one where the presence of Ca^{2+} had a clearly destabilizing effect.

The same was true when membrane fusion was measured under these conditions (Fig. 6). Strong fusion was only observed with liposomes containing the thylakoid lipid mixture, while all other liposomes showed no significant increase in fusion with increased solute concentration, temperature, or addition of $CaCl_2$. Increased fusion has also been reported for other complex lipid mixtures in the presence of osmotic stress induced by high concentrations of PEG [38]. The present experiments indicate that, except in the case of the thylakoid lipid mixture, even in the presence of $CaCl_2$, osmotic stresses are not the predominant cause of freezing-induced destabilization. Most likely, ice crystallization leads to additional stresses, either directly, or indirectly through the removal of liquid water and the resultant dramatic increase in membrane concentration in the residual unfrozen volume.

In conclusion, it has been shown that membrane aggregation has opposing effects on membrane stability, depending on whether Ca^{2+}-induced aggregation occurs via negatively charged lipids or glycolipids. Liposomes containing glycolipids are as stable in the aggregated state as in the non-aggregated state, while aggregation clearly destabilizes liposomes containing negatively charged lipids. Complex mixtures that approach the composition of a biological membrane are more unstable than would be predicted from their single components. In biological membranes, the protein complement can also play a role in membrane stability. It has, for instance, been shown recently that the chlorophyll *a*/*b* light-harvesting protein of Photosystem II from pea thylakoids stabilized the non-bilayer forming lipid MGDG in the lamellar phase in model membrane experiments [39].

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