Articles

Role of the Polar Head Group Stereoconfiguration in the Cation-Induced Aggregation of Dimyristoylphosphatidylglycerol Vesicles†

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ABSTRACT: Cation-induced aggregation of small unilamellar vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol (1′-DMPG), the corresponding 3′ stereoisomer (3′-DMPG), and their 1:1 mixture was studied as a function of the concentration of different mono- and divalent cations. The order of efficiency, $Na^+ > Li^+ > K^+ > Cs^+$, of the monovalent cations to induce the aggregation of DMPG vesicles is the same for both stereoisomers and their mixture. However, significant differences in the Na^+-induced aggregation of 1′-DMPG and 3′-DMPG were evident. The threshold concentration of aggregation by Na^+ was 0.35 M for 3′-DMPG, 0.55 M for 1′-DMPG, and 0.50 M for the mixed liposomes. Such difference in the aggregation of DMPG stereoisomers was not observed for the other mono- and divalent cations. The higher affinity of 3′-DMPG for Na^+ is suggested to be due to a slightly different favored conformation of the head group glycerol moiety. Aggregation of the stereoisomers by 1 M NaCl was identical, indicating that the differences in the affinity of 1′-DMPG and 3′-DMPG for sodium can be overcome by very high ionic strength. Inclusion of 20 mol % cholesterol in vesicles enhanced the aggregation of 1′-DMPG and decreased the aggregation of 3′-DMPG by Na^+ and thus abolished the difference between the two stereoisomers.

Cation-induced aggregation of acidic phospholipid vesicles has recently gained increasing interest. This is due to observations that in addition to divalent cations (Lansman et al., 1975; Ohki et al., 1982) aggregation can be induced also by high concentrations of monovalent cations (Ohki et al., 1982, 1984; Yoshimura et al., 1985; Day et al., 1980). The aggregation induced by monovalent cations is fully reversible and depends on temperature (Day et al., 1980), phospholipid composition (Yoshimura et al., 1985), and on the monovalent cation species (Ohki et al., 1982, 1984).

Studies on the mechanisms of aggregation of phospholipid vesicles are relevant in providing understanding of various membrane processes such as fusion, intermembrane adhesion, and communication between cells. The exact mechanism of vesicle aggregation is not clear. It is found to consist of two processes: the forward process of aggregation and the backward deaggregation (Day et al., 1980). It is not known whether the mere screening of the polar head group negative charges is adequate to induce aggregation or if specific adsorption of the cations to phospholipid membrane is needed. Changes in the degree of hydration may also be involved.

Phospholipids are chiral molecules having at least one asymmetric carbon atom. Until recently very little attention has been given to the effect of phospholipid stereoconfiguration on the properties of membranes. The thermodynamic behavior of racemic phosphatidylethanolamine has been found to be complex (Tenchov et al., 1984). The absence of subtransition (Boyanov et al., 1983) and a shift of pretransition to lower temperatures (Eklund et al., 1984) have been reported for DL-phosphatidylcholines. In addition to the central *sn*-2 carbon of the glycerol backbone, phosphatidylglycerol has also LD configuration in the polar head group glycerol moiety. Thus four different stereoconfigurations are possible. Of these the only naturally occurring enantiomer is the *sn*-3-phosphatidyl-*sn*-1′-glycerol. Most studies on synthetic phosphatidylglycerols have employed lipid mixtures that are racemic with respect to the polar head group glycerol moiety.

Our preliminary space-filling model-building studies indicated such structural differences in the polar head group structure of 3′-DMPG[1] and 1′-DMPG, which may result in differing affinities for Na^+. Vesicle aggregation was used to compare the binding of cations to small unilamellar liposomes of 3′-DMPG and 1′-DMPG.

Materials and Methods

Ammonium salts of synthetic 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol and the corresponding 3′ stereoisomer were purchased from KSV Chemicals (Helsinki, Finland). No impurities were detected in these lipids on thin-layer chromatography on silica gel (Merck, Darmstadt, West Germany) developed with chloroform/methanol/water/ammonia (65:20:2:2). Lipid concentrations were determined by phosphorus analysis (Chen et al., 1956). All chemicals used were of reagent grade, and the water used in experiments was freshly deionized in a Milli-RO/Milli-Q (Millipore) water-filtering system.

Stock solutions of the lipids were made in chloroform. The lipids were pipetted in this solvent, which was thereafter removed under a stream of nitrogen, and the samples were kept

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[1] Abbreviations: 1′-DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-1′-glycerol; 3′-DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidyl-*sn*-3′-glycerol; *sn*, stereochemical notation; DMPC, dimyristoylphosphatidylcholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine.

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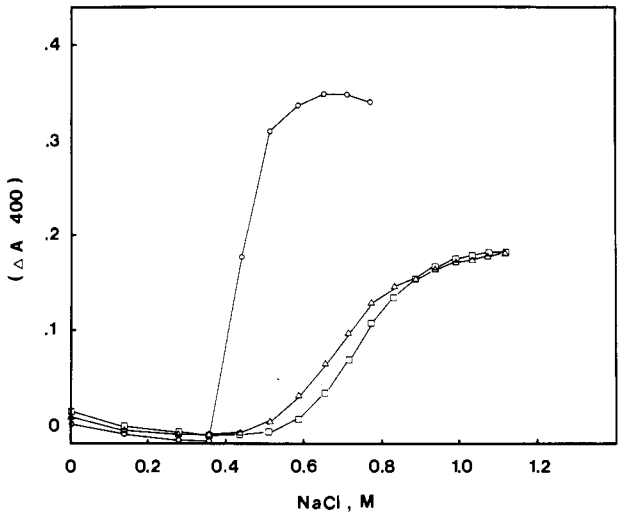

FIGURE 1: Turbidity change of phospholipid vesicles at 400 nm (ΔA_{400nm}) as a function of NaCl concentration. Each data point represents the average over five separate experiments: (○) 3′-DMPG; (□) 1′-DMPG; (△) 1:1 mixture.

under reduced pressure overnight. Phospholipids were suspended in 20 mM Tris-HCl, pH, 7.4, and 0.1 mM EDTA buffer to yield a final lipid concentration of 1 mg mL^{-1}. In studies on divalent cation induced aggregation EDTA was omitted. The hydrated lipid was sonicated for 1 h with a Bransonic 221 bath-type sonicator under nitrogen at 30 °C. To remove multilamellar vesicles, the preparation was centrifuged for 60 min at 35000*g*, and the supernatant was used in aggregation experiments within 2 days of preparation. Final lipid concentration was adjusted to 0.1 mg mL^{-1}.

Turbidity changes were measured with a Shimadzu Graphicord spectrophotometer with a thermostated cuvette compartment. The threshold concentrations for aggregation (Ohki et al., 1982) were measured by adding small aliquots (35 μL) of concentrated salt (3 M for monovalent cations and 50 mM for divalent cations) solutions. After each addition, the samples were mixed, and the absorbance at 400 nm was measured 3 min after the addition of the salt. Unless otherwise indicated, turbidity measurements were carried out at 10 °C.

RESULTS

The turbidity changes of vesicles consisting of either 1′-DMPG, 3′-DMPG, or their 1:1 mixture as a function of increasing NaCl concentration are shown in Figure 1. The threshold concentration of aggregation taken as the intercept of the slope of ΔA_{400nm} vs cation is 350 mM for 3′-DMPG, 550 mM for 1′-DMPG, and 500 mM for the racemic lipid. The slope of aggregation is considerably steeper and the change in absorbance more pronounced for the 3′-DMPG vesicles than that for 1′-DMPG or the mixed liposomes.

Aggregation of 3′-DMPG vesicles by Li^+, K^+, and Cs^+ (added as their Cl salts) was also studied (Figure 2a). Their efficiency in aggregating DMPG vesicles is significantly less than that of Na^+. No significant differences were observed between 3′-DMPG, 1′-DMPG, and their 1:1 mixture (data not shown). The overall order of efficiency, $Na^+ > Li^+ > K^+ > Cs^+$, of the monovalent cations to induce aggregation of DMPG vesicles is the same for both enantiomers and for their mixture.

The aggregation of the 3′-DMPG vesicles as a function of the concentration of different divalent cations is shown in Figure 2b. The aggregation threshold concentrations for Ca^{2+}, Mn^{2+}, and Mg^{2+} are 3.0, 3.5, and 7.0 mM, respectively. Compared to monovalent cations, the differences of about 2

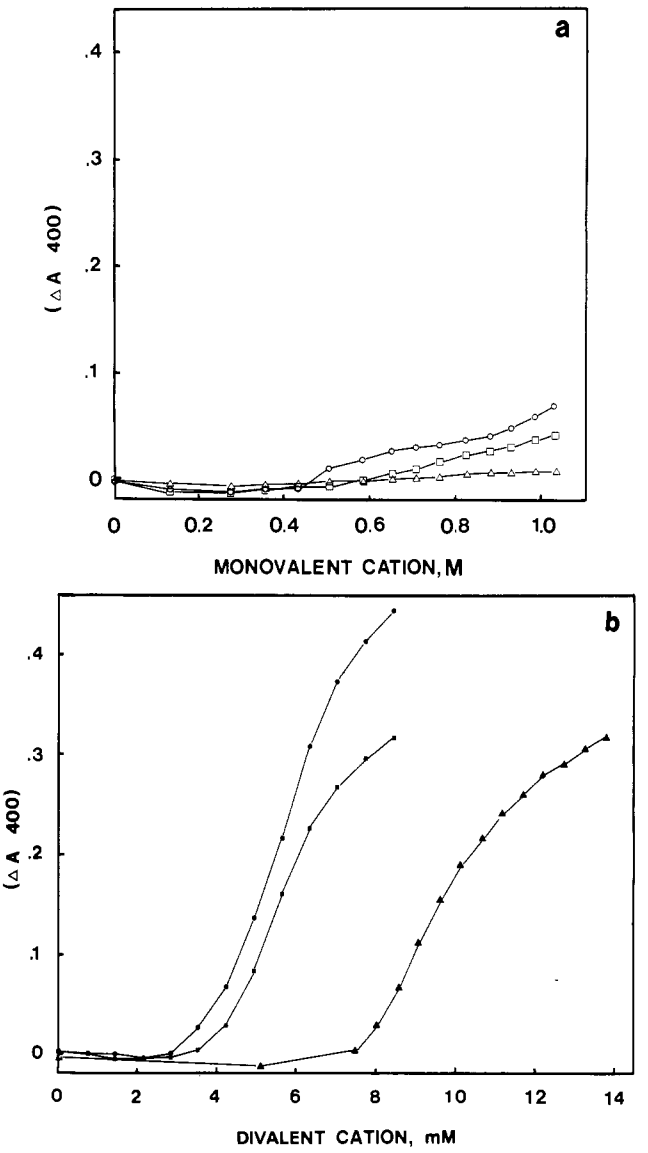

FIGURE 2: (a) Aggregation of 3′-DMPG vesicles by LiCl (○), KCl (□), and CsCl (△). The aggregation of 1′-DMPG and the mixed vesicles is nearly identical and therefore is not shown. (b) Change in turbidity (ΔA_{400nm}) of 3′-DMPG vesicles as as function of $CaCl_2$ (●), $MnCl_2$ (■), and $MgCl_2$ (▲) concentration. Temperature is 10 °C; 20 mM Tris-HCl, pH 7.4.

orders of magnitude may suggest that specific binding of the divalent cations to the phosphate might also be involved, resulting in the formation of poorly water soluble complexes. The slopes of ΔA_{400nm} vs ion and the threshold values of aggregation of the different enantiomeric forms of DMPG are very similar for all the divalent cations studied, although the change in turbidity of mixed vesicles aggregated by Mg^+ is somewhat less than that for the pure enantiomers (data not shown). The order of efficiency of the divalent cations to induce aggregation of DMPG vesicles is $Ca^{2+} > Mn^{2+} > Mg^{2+}$.

The kinetics of aggregation of DMPG vesicles at 10 °C by 1.0 M NaCl was then studied (Figure 3). At this salt concentration the rates of aggregation of the different enantiomers and their 1:1 mixture are identical indicating that the differences in the affinity of 1′-DMPG and 3′-DMPG for sodium ion can be overcome by high salt concentration.

The sodium-induced aggregation of DMPG vesicles was also studied at 30 °C, i.e., above the phase transition temperature of DMPG (Figure 4a). At this temperature no increase in turbidity was observed up to 1 M NaCl during a 2-h incu-

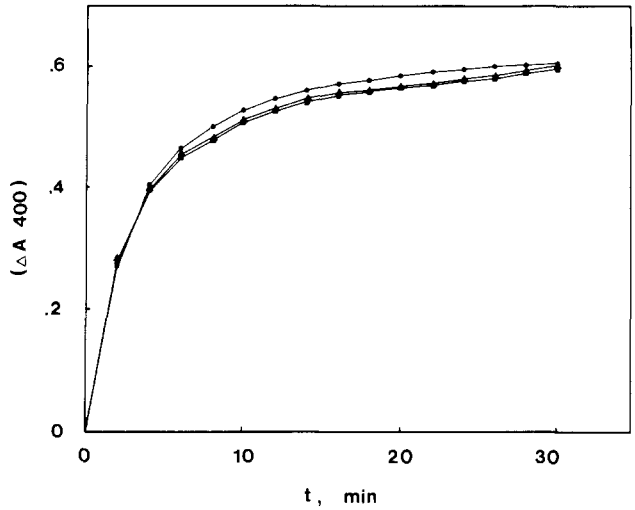

FIGURE 3: Time course for the aggregation of 3′-DMPG (●), 1′-DMPG (■), and the mixed (▲) vesicles by 1 M NaCl at 10 °C.

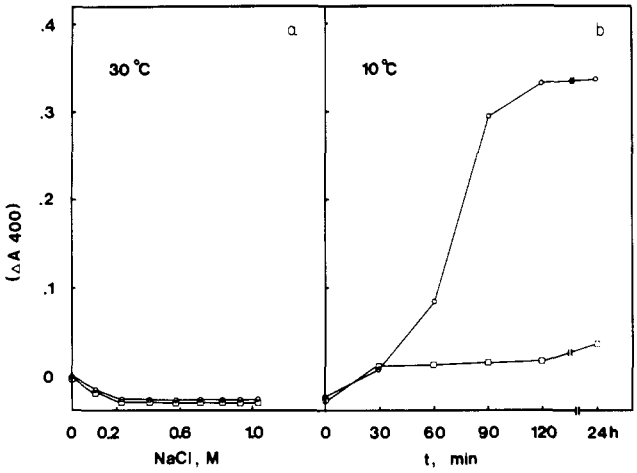

FIGURE 4: Aggregation of 1′-DMPG (□) and 3′-DMPG (○) vesicles as a function of NaCl concentration at 30 °C (a). In panel b the temperature was lowered to 10 °C, whereafter turbidity was followed as a function of time.

bation. When the temperature was then reduced to 10 °C (Figure 4b), a gradual increase in the turbidity of 3′-DMPG was evident and after 2 h the turbidity was at the same level as that seen in the control experiment performed at 10 °C. In contrast, turbidity of 1′-DMPG vesicles increased slowly, requiring several days. After incubation for 2 h at 10 °C, only a slight increase in A_{400nm} was observed (Figure 4).

The reversibility of Na^+-induced aggregation was studied as a function of temperature. Vesicles were aggregated by 0.6 M NaCl at 10 °C, whereafter the temperature was raised to 30 °C. No decrease in turbidity of 1′- or 3′-DMPG vesicles was observed during a 2-h incubation (data not shown), indicating that the aggregates are very stable.

Finally, the effect of cholesterol on the aggregation of DMPG vesicles by Na^+ was studied. Inclusion of 20 mol % cholesterol into the DMPG vesicles enhanced the aggregation of 1′-DMPG vesicles and decreased the aggregation of 3′-DMPG vesicles, thus abolishing the difference between the stereoisomers (Figure 5). In the presence of 50 mol % cholesterol optically clear preparations could not be obtained even after extensive sonication. This is most probably due to a tendency for larger liposomes to form in the presence of high cholesterol content (Johnson et al., 1973; Gent & Prestegard, 1974). Therefore, results allowing unambiguous comparison could not be obtained.

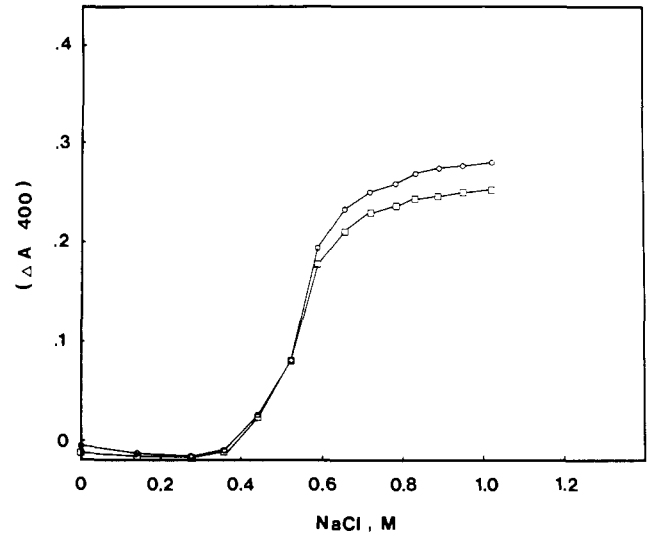

FIGURE 5: NaCl-induced turbidity increase for 1′-DMPG (□) and 3′-DMPG (○) vesicles containing 20 mol % cholesterol.

DISCUSSION

The objective of the present study was to investigate the significance of the PG polar head group glycerol stereoconfiguration in ion binding and in vesicle aggregation.

The efficiency of monovalent cations to induce aggregation of DMPG vesicles at 10 °C was $Na^+ > Li^+ > K^+ > Cs^+$. This pattern was the same for both *sn*-1′ and *sn*-3′ stereoisomers and is identical with that reported for the aggregation of small vesicles of PS and PA (Ohki et al., 1982, 1984). It is possible that this pattern depends more on the properties of the specific monovalent cations, such as dehydration energy and effective charge, than on the properties of the different acidic phospholipid species. It is perhaps also worth noting that there appears to be an analogy between vesicle aggregation and the flocculation of hydrophobic colloids.

In an earlier study no major structural differences in the NMR spectra of 1′ and 3′ stereoisomers of DPPG were found (Wohlgemuth et al., 1980). As illustrated in Figure 1, however, significant differences were evident in the aggregation of 3′-DMPG and 1′-DMPG-vesicles by Na^+. Likewise, our differential scanning calorimetry studies also indicate that the thermotropic phase behavior of the *sn*-1′- and *sn*-3′-DMPG enantiomers differs only if the samples have been incubated in the presence of NaCl for at least 1 h at a temperature below the pretransition temperature of DMPG.[2]

Figure 6a illustrates the CPK models of tentative conformations for the polar head groups of 1′-DMPG and 3′-DMPG. As the NMR spectrum of PG closely resembles that of PC and PE (Wohlgemuth et al., 1980), we based the molecular conformations shown on the available X-ray data on DMPC (Pearson & Pascher, 1979). In the tentative conformations shown it is assumed that similarly to PC (Pearson & Pascher, 1979) the *sn*-3 carbon of the glycerol backbone and the first carbon of the polar head group are at the opposite sides of the phosphate group. It follows that the two polar oxygens of the 3′-DMPG head group glycerol become vicinal to the negative charge of the phosphate oxygens, thus forming a polar negatively charged site with proper dimensions to specifically accommodate Na^+. 1′-DMPG can adopt a similar conformation as a mirror image, but if the *sn*-3 carbon and the first carbon of the polar head group glycerol are at the opposite sides of the phosphate group, as with DMPC, the favored conformation

[2] I. S. Salonen, K. K. Eklund, J. A. Virtanen, and P. K. J. Kinnunen, unpublished data.

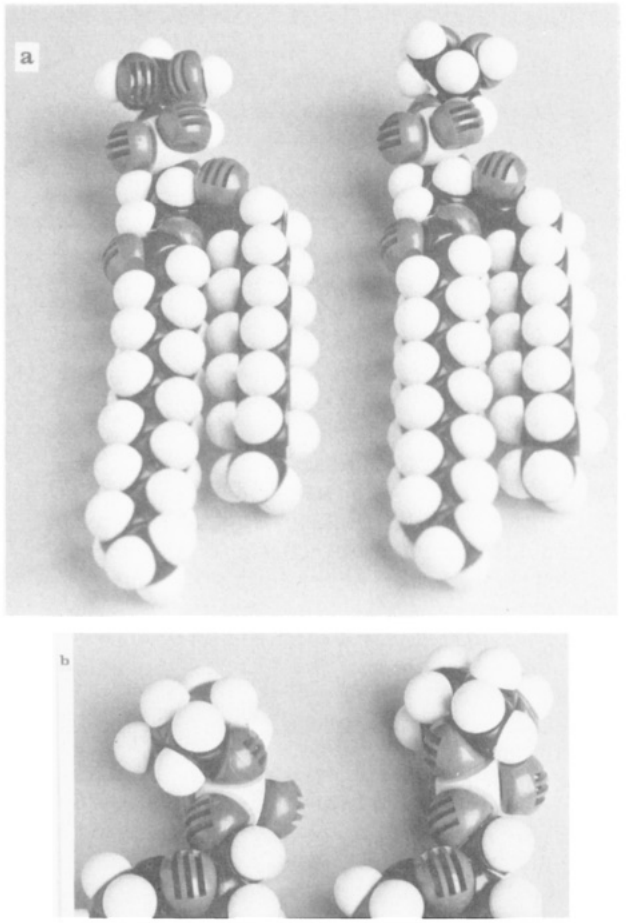

FIGURE 6: (a) Tentative conformations of the polar head groups of 3′-DMPG (left) and 1′-DMPG (right) illustrated with CPK space-filling models. The head groups of 3′-DMPG and 1′-DMPG are viewed from the side opposite that in (b). 1′-DMPG in (b) is in a conformation similar to that of 3′-DMPG (a mirror image), and in this conformation the *sn*-3 carbon and the first carbon of the head group glycerol moiety of the 3′-DMPG (left) are at the opposite sides and those of the 1′-DMPG (right) are at the same side of the phosphate group. See Discussion for details.

of 1′-DMPG would be closer to that shown in Figure 6a. The positions of the *sn*-3 carbon and the first carbon of the head group glycerol moiety are further illustrated in Figure 6b.[3]

When Na^+ was added at 30 °C instead of 10 °C, no increase in turbidity of vesicles of either stereoisomer was observed (Figure 4a). This is consistent with the work of Day et al. (1980), who showed that the rate of aggregation of small PS vesicles decreases as temperature is increased. They suggested it to be due to an increased rate of deaggregation at elevated temperatures. However, when DMPG vesicles are first aggregated at 10 °C and temperature is thereafter increased to 30 °C, only a slight decrease in turbidity of 3′-DMPG and 1′-DMPG vesicles is observed (data not shown). This indicates that once formed the salt-induced aggregates of DMPG are rather stable. This is supported by our preliminary results from experiments employing vesicles containing pyrene-labeled lipids,[4] which seem to indicate that, not unlike PS vesicles (Day et al., 1980), significant fusion (Chong & Colbow, 1976; Papahadjopoulos et al., 1974) of the DMPG vesicles at 10 °C is not induced by Na^+.

The opposite effects of cholesterol on the Na^+-induced aggregation of 1′- and 3′-DMPG liposomes cannot be adequately explained at present, and more systematic studies are needed. However, changes in the degree of hydration in the presence of cholesterol could be involved (Chauhan et al., 1986).

The origin of chirality of biomolecules, the unique property of nature, is still unknown (MacDermott, 1986). In the case of PG, however, a clear difference is evident in the interactions of 3′-DMPG and 1′-DMPG with Na^+. It is possible that this difference has proved important also in the course of evolution and has led to the selection of 1′-PG by nature. It is also of interest that both 1′-PG and 3′-PG head group configurations are present in cardiolipin. The possible role of the 3′ stereoconfiguration for the function of this mitochondrial lipid warrants further studies.

Finally, the marked difference in the Na^+ binding properties of 1′- and 3′-DMPG suggests that the feasibility of use of *rac*-phosphatidylglycerol in liposome formulations for therapeutics should probably be reconsidered.

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We thank Drs. T. Thuren and I. Salonen for several stimulating discussions and E. Saarikalle for technical assistance.

Registry No. 1′-DMPG, 57618-28-7; 3′-DMPG, 110797-69-8; DMPG, 61361-72-6; Na, 7440-23-5; Li, 7439-93-2; K, 7440-09-7; Cs, 7440-46-2; Ca, 7440-70-2; Mn, 7439-96-5; Mg, 7439-95-4; cholesterol, 57-88-5.

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[3] After this paper was communicated, an X-ray diffraction study on crystals of racemic DMPG appeared (Pascher et al., 1987). The results presented in this work do indicate that the conformations of the 3′-DMPG and 1′-DMPG head groups differ and are in general agreement with those proposed by us for liposomal DMPG.

[4] K. K. Eklund, J. A. Virtanen, and P. K. J. Kinnunen, unpublished results.