The Dissimilar Effect of Diacylglycerols on Ca^{2+}-Induced Phosphatidylserine Vesicle Fusion

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ABSTRACT We have studied the effect of physiological concentrations of different diacylglycerols on Ca^{2+}-induced fusion between phosphatidylserine vesicles. We monitored vesicle fusion as mixing of membrane lipids under conditions where the limiting factor was the aggregation and also in conditions where this aggregation was not the limiting factor. We found that diacylglycerols have a different modulating effect on the Ca^{2+}-induced fusion: i) depending on their interfacial conformation, so that 1,2-isomers of diacylglycerols containing unsaturated or short saturated acyl chains stimulated fusion and their 1,3-isomers did not, and ii) depending on their specific type of bilayer interior perturbation, so that diacylglycerols containing unsaturated or short chain saturated acyl chains stimulated fusion but those containing long-chain saturated acyl chains did not. These requirements resembled those required for the diacylglycerol activation of protein kinase C, suggesting that diacylglycerol acts in both the specific activation of this enzyme and the induction of membrane fusion through the same perturbation of lipid structure. We found that polylysine affected the stimulatory role of 1,2-dioleoylglycerol differently, depending on whether aggregation was the limiting factor of fusion. When we studied the effect of very low concentrations of diacylglycerols on the bulk structural properties of phosphatidylserine, we found that they neither significantly perturbed the thermotropic transitions of phosphatidylserine nor affected the interaction of Ca^{2+} with the phosphate group of phosphatidylserine. The underlying mechanism of fusion between phosphatidylserine vesicles is discussed.

INTRODUCTION

Diacylglycerols (DGs) produced in the receptor-mediated hydrolysis of inositol phospholipids play important roles in signal transduction of a variety of extracellular messengers taking place at the cell surface through activation of protein kinase C (PKC) (Nishizuka, 1984; Berridge, 1987). On the other hand, DGs are known to induce a variety of structural changes in membranes, including alteration of membrane curvature (Ohki et al., 1982), lateral phase separation (Ortiz et al., 1988), the spreading apart of the polar head groups of the phospholipids, the production of nonlamellar phases (Dawson et al., 1984; Das and Rand, 1986; Epand, 1985; Siegel et al., 1989a; De Boeck and Zidovetzki, 1989; López-García et al., 1994a), and the formation of ripple phases (López-García et al., 1994b). Prevalent models of PKC activation suggest that DG-induced membrane bilayer perturbations allow regulatory domains of PKC to insert into the membrane (Zidovetzki and Lester, 1992).

DGs have been found long ago to be able to promote the fusion of certain membranes (Ahkong et al., 1973), and recently much attention has been focused on the study of the effect of DGs on the process of membrane fusion. These studies include systems that form nonlamellar phases like that composed of phosphatidylethanolamine (Siegel et al., 1989b; Van Gorkom et al., 1992), and those that by themselves have no tendency to form nonlamellar structures like that containing phosphatidylserine (PS) (Gómez-Fernández et al., 1992; Ortiz et al., 1992; Walter et al., 1994). It is interesting that PS vesicles have been studied in the presence of DGs and Ca^{2+}, revealing important effects of the DGs on the lipid-water interface with and enhancement of the interaction between Ca^{2+} and PS (López-García et al., 1993, 1994a).

The study of the effect of DGs on fusion of PS-containing vesicles deserves particular attention. The addition to Ca^{2+} to unilamellar PS causes the vesicles to fuse (Papahadjopoulos et al., 1975); PS systems are probably the most widely studied model system for membrane fusion, and the study of the role that DGs may have on this process is of interest to get insight into the molecular mechanism of membrane fusion. In the present work, we have studied the effect of physiological concentrations of different DGs on Ca^{2+}-induced fusion (observed as mixing of vesicle lipid) between PS unilamellar vesicles, its interrelation with polylysine, and the possible effect os DGs on the structural properties of PS. We found that DGs have an important modulating effect on the Ca^{2+}-induced fusion. The effect of DGs on Ca^{2+}-induced PS fusion resembles that of DGs activation of PKC (Kishimoto et al., 1980) in the sense that 1,2-isomers of unsaturated or short saturated acyl chains stimulated fusion and

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Abbreviations used: DGs, diacylglycerols; 1,2- and 1–3-DC_8G, 1,2- and 1,3-dicapryloylglycerol; 1,2-$DC_{10}G$, 1,2-dicaprylglycerol; 1,2- and 1,3-DOG, 1,2- and 1,3-dioleoylglycerol; 1,2-DPG, 1,2-dipalmitoylglycerol; DSC, differential scanning calorimetry; FT-IR, Fourier-transformed infrared spectroscopy; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine; PKC, protein kinase C; PS, phosphatidylserine; T_m, midpoint temperature of the gel-to-liquid-crystaline phase transition.

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1,3-isomers, and 1,2-long saturated acyl chain showed no effect or even an inhibitory one on the process of the fusion of PS vesicles. In the study of the interaction between water-soluble proteins and membranes, polylysine has been fre-quently used as a model for basic extrinsic proteins (Hong et al., 1991). We have studied the possible interaction of polylysine and the stimulatory role of 1,2-dioleoylglycerol on Ca^{2+}-induced fusion of PS vesicles.

MATERIALS AND METHODS

Chemicals

Bovine brain phosphatidylserine (PS), 1,2- and 1,3-dioleoylglycerol (1,2- and 1,3-DOG), 1,2- and 1,3-dicapryloylglycerol (1,2- and 1,3-DC_8G), 1,2-dicaprylglycerol (1,2-$DC_{10}G$), 1,2-dipalmitoylglycerol (1,2-DPG), *N*-(7-nitro, 2, 1, 3-benzoxadiazol-4-yl) phosphatidylethanolamine (N-NBD-PE), and *N*-(lissamine rhodamine B-sulfonyl) phosphatidylethanolamine (N-Rh-PE) were obtained from Avanti Polar Lipids, (Birmingham, AL). Polylysine hydrobromide (average M_r 7100) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All other reagents were of the highest purity available. Twice-distilled and deonized water was used. The purity of 1,2-diacylglycerols with respect to acyl chain migration during storage was checked by thin-layer chromatography on silica gel plates using diethylether-petroleum ether-acetic acid (50:50:1, by volume), and all the samples used in this work showed a single spot.

Vesicle fusion

Vesicles for fusion experiments were prepared by combining chloroform solutions of PS, N-NBD-PE, and N-Rh-PE when necessary, and the appropriate amounts of DGs to give different final concentrations as indicated in each case. Organic solutions were dried under nitrogen stream and stored for 3 h under vacuum to remove the last traces of solvent. The dry lipid mixtures were dispersed in aqueous buffer (100 mM NaCl, 10 mM HEPES, 0.1 mM EDTA, pH 7.4). The resulting multilamellar vesicles (MLV) suspension was used to form large unilamellar vesicles (LUV) by extrusion through two stacked polycarbonate filters (Nuclepore, 0.1 μm pore size) using an Extruder (Lipex Biomembrane Inc, Vancouver, Canada) essentially as described by Hope et al. (1985). Lipid vesicle samples were analyzed for organic phosphorous according to the method of Bötcher (Bötcher et al., 1961). The size of the vesicles was estimated by negative staining electron microscopy. In agreement with Walter et al. (1994), it was found that the average diameter of the vesicles (0.1 μm) was not significantly affected by the incorporation of DGs at the concentration used in this work.

Vesicle aggregation was followed by changes in 90° light scattering at 400 nm. Vesicle fusion was monitored by the resonance energy transfer (RET) assay for lipid mixing, essentially as described previously by Struck et al. (1981) using two populations of PS vesicles, one containing 0.7 mol% of both N-NBD-PE and N-Rh-PE and the other devoid of fluorescent probes at a 1:1 ratio. The extent of lipid mixing was measured by following the emission of the fluorescence of N-NBD-PE (530 nm) upon excitation ar 450 nm. All measurements were carried out at 25°C in a BioLogic Modular Optical System (Grenoble, France) equipped with a built-in stirring device.

The overall fusion or lipid mixing process can be described (Nir, 1991; Walter and Siegel, 1993) by the forward and reverse aggregation rate constants (k_1 and k_{-1}) between two single unilamellar vesicles (V) to form a dimer aggregate (VV) that will fuse to form a fusion or lipid mixing product (F) with a fusion rate constant (k_f):

$$V + V \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} VV \xrightarrow{k_f} F$$

Both aggregation and lipid mixing rates contribute to the overall rate of reaction. The initial rate of aggregation is proportional to the square of the initial phospholipid vesicle concentration. The rate of lipid mixing is directly proportional to the concentration of dimer aggregates. At low initial phospholipid vesicle concentrations, the rate of aggregation is slow relative to lipid mixing, and the overall rate of fusion is controlled by the rate of aggregation. At high vesicle concentration, the rate of aggregation may be fast relative to lipid mixing so that the overall rate of fusion is controlled by the rate of fusion (k_f).

DSC and FT-IR

Lipid vesicles for DSC and FT-IR were prepared as follows. 5 μmol of PS and the appropriate amount of different DGs were used. In the case of samples studied in the absence of Ca^{2+}, MLV prepared as described above were used. For samples containing Ca^{2+}, LUV were prepared as described above and 10 mM $CaCl_2$ was added to the suspension. The suspension was centrifuged at 10,000 rpm in a bench microfuge, and the pellets were used for measurements.

For DSC measurements, pellets were collected and placed into small aluminum pans. Pans were sealed and scanned in a Perkin-Elmer DSC-4 calorimeter at a heating rate of 4°C/min.

For FT-IR measurements, pellets were suspended in a 40 μl of final buffer volume. Infrared spectra were obtained in a Philips PU 9800 Fourier transform infrared spectrometer equipped with a DTGS detector. Samples were examined at 25°C in a thermostated Specac 20710 cell equipped with CaF_2 windows and using 25 μm teflon spacers (all from Specac, Kent, U.K.). Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using a sample shuttle accessory to average background spectra over the same time period. Substraction from buffer was performed interactively using Spectra-Calc (Galactic Industries, Salem, MA)

RESULTS

The PS concentration dependence of fusion has been described previously (Wilschut et al., 1980; Walter and Siegel, 1993). It has been shown that at low PS concentrations the fusion rate was almost second order in PS (slope ~ 2) indicating that the rate of aggregation was rate-limiting and that at high concentration of PS the observed overall rate was nearly first order in PS concentration (slope ~ 1), indicating that these fusion rates are in the regime where the rate of lipid mixing, rather than aggregation, was rate-limiting. The aggregation and fusion rates are independently susceptible to exogenous factors such as temperature, so that the phospholipid vesicle concentration at which one or the other process is predominant will vary with the experimental conditions. We have performed the PS vesicle concentration dependence of lipid mixing to know the range of PS concentration, which in our experimental conditions determines a process of fusion where aggregation or fusion per se are the rate-limiting step. This is presented in Fig. 1, where the initial rates of lipid mixing (presented as percent of maximal fluorescence per min) were corrected for the actual lipid concentration, relative to 10^3 μM, and plotted in a double-logarithmic manner against the lipid concentration. The fitting of the rate of fusion × concentration to the PS concentration gave a slope of 1.9 for the region of low PS concentration and 0.9 for the region of high PS concentration (correlation coefficient 0.99). These values are in close agreement with those reported previously (Wilschut et al., 1980; Walter and Siegel,

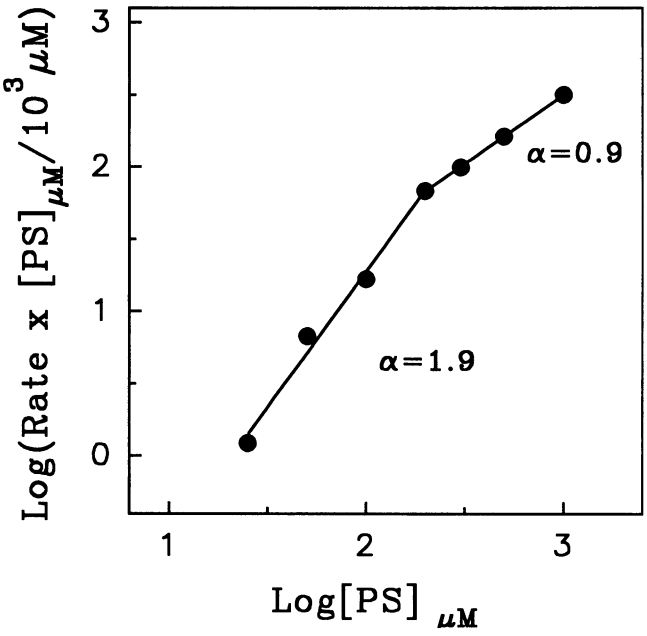

FIGURE 1 Dependence of the initial rate of lipid mixing for pure phosphatidylserine vesicles on vesicle concentration. Initial rates of lipid mixing (presented as percent of maximal fluorescence per min) were corrected for the actual lipid concentration, relative to 10^3 μM, and plotted in a double-logarithmic manner against the lipid concentration. The numbers under the curve indicate the slope of each portion. Calcium concentration was 5 mM. Points represent the average of three experiments (error bars were smaller than the size of symbols).

1993). In the following experiments, we have selected a PS concentration of 50 μM (which in our experimental conditions is clearly in the branch of the curve where the apparent rate constant is near 2) to study the PS fusion under conditions where aggregation is the limiting step, and selected a PS concentration of 300 μM (which is clearly in the branch of the curve where the apparent rate constant is near 1) to study PS fusion under conditions where lipid mixing per se is the rate-limiting step.

The effects of 1,2-DOG and 1,2-DPG on Ca^{2+}-induced PS vesicles lipid mixing are shown in Fig. 2 *A*. The presence of 2 mol% 1,2-DOG increased the rate of lipid mixing when compared with control PS vesicles, whereas the presence of 2 mol% 1,2-DPG decreased the rate of fusion. Fig. 2 *B* shows that the presence of 2 mol% either 1,2-DOG or 1,2-DPG did not significantly change the rate of Ca^{2+}-induced aggregation of PS vesicles, although differences in the final extent of aggregation were observed. The initial rate of Ca^{2+}-induced lipid mixing of PS vesicles containing 2 and 5 mol% 1,2-DOG or 1,2-DPG as a function of Ca^{2+} concentration is depicted in Fig. 3. The Ca^{2+} threshold concentration was not changed by the presence of DGs. 1,2-DOG clearly increased the rate of lipid mixing, and the increase of the amount of 1,2-DOG produced a further increase in lipid mixing at all Ca^{2+} concentrations. 1,2-DPG produced the opposite effect, which is clearer at a concentration of 5 mol%. Lower concentrations of DGs produced negligible effects when compared with the fusion of PS control vesicles containing no DGs.

The above experiments were carried out at a relatively diluted (50 μM) lipid concentration. Fig. 2 *B* showed that the presence of DGs did not affect the initial rate of vesicle aggregation, but differences in the final extent of aggregation were found; hence, it can be still argued that the aggregation step is limiting the overall process. After this observation, all of the following experiments were performed at 300 μM lipid concentration at which aggregation of vesicles is clearly not rate-limiting, so that the observed effects of DGs on membrane fusion will not be due to changes in aggregation rate.

We studied the effect of 1,2-DOG and 1,2-DPG to compare with the results shown above at diluted vesicle concentration, and extended the study to other DGs differing in chain length and position. The dependence of the rate of lipid mixing on the Ca^{2+} concentration for vesicles composed of PS and those containing different amounts of DGs is shown in Fig. 4. The effect of 1,2-DOG and 1,3-DOG on Ca^{2+}-induced lipid mixing is shown in Fig. 4 *A*. It is clearly seen that 1,2-DOG produced an enhancement of the rate of lipid mixing that is higher when the 1,2-DOG concentration is increased. The effect of 1,3-DOG was clearly different because no dramatic change in lipid mixing rate was observed. Qualitatively similar results are shown in Fig. 4 *B* for DC_8G,

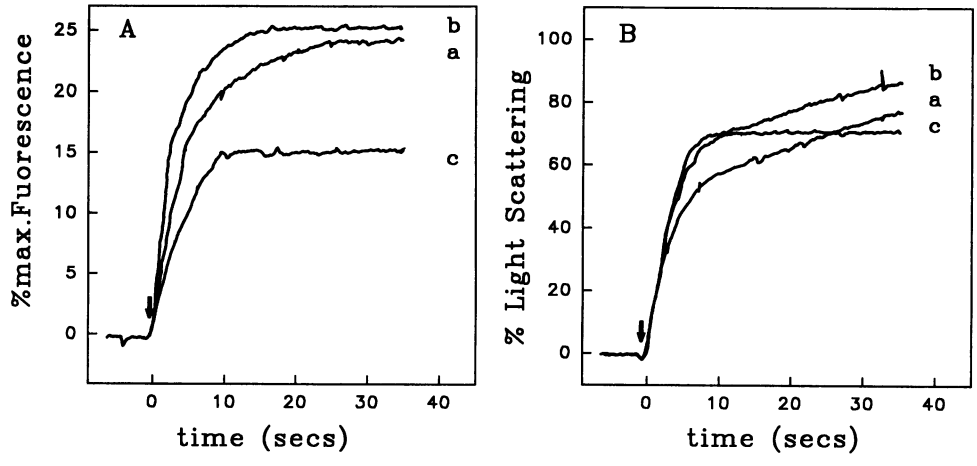

FIGURE 2 Time-dependent vesicle lipid mixing (*A*) and vesicle aggregation (*B*) for pure phosphatidylserine vesicles (a) and phosphatidylserine containing 2 mol% of 1,2-dioleoylglycerol (b) or 1,2-dipalmitoylglycerol (c). Total phospholipid concentration was 50 μM, and calcium was added at zero time at a final 10 mM concentration. 100% light scattering correspond to the maximum extent of aggregation obtained with pure phosphatidylserine vesicles at longer times.

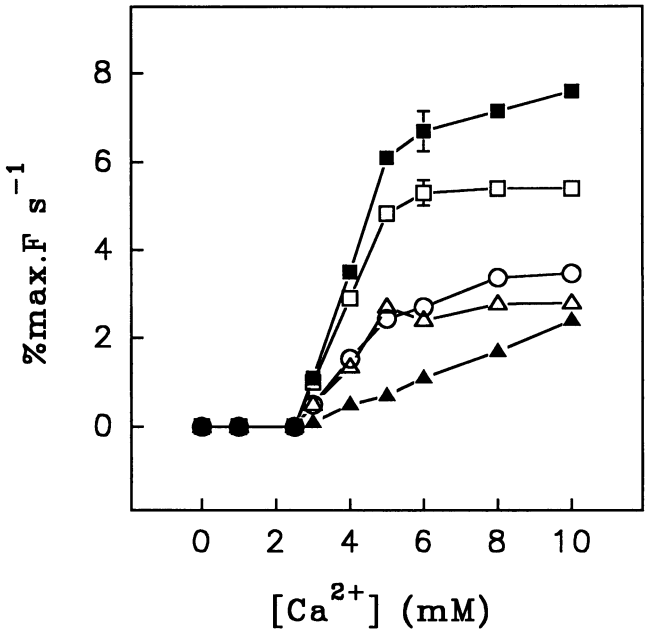

FIGURE 3 Dependence of the initial rate of vesicle lipid mixing on the calcium concentration for vesicles made of pure phosphatidylserine (○) and phosphatidylserine containing 2 mol% (□) and 5 mol% (■) 1,2-dioleoylglycerol or 2 mol% (△) and 5 mol% (▲) 1,2-dipalmitoylglycerol. Total phospholipid concentration was 50 μM. Points represent average of three experiments (error bars are shown when larger than the symbols).

because the 1,2-DC_8G isomer produced an increase in the lipid mixing rate whereas the 1,3-DC_8G produced even a slight decrease of the fusion rate. Fig. 4 *C* shows that although 1,2-$DC_{10}G$ had a stimulatory effect on fusion, its larger homolog 1,2-DPG produced an inhibition of fusion, thus confirming the results carried out at dilute vesicle concentration.

Fig. 5 *A* shows the effect of polylysine on the role of 1,2-DOG in Ca^{2+}-induced vesicle fusion at dilute vesicle concentration, i.e., where aggregation is the rate-limiting step. If we compare Fig. 5 *A* in the presence of polylysine with Fig. 3 in the absence of polycation, two effects of polylysine become clear (either in control PS systems or in those containing 1, 2-DOG). First, it decreased the Ca^{2+} threshold concentration for fusion from 2.5 mM in the absence of polycation to 0.1–0.2 mM in the presence of polylysine, and second, it increased the rate of fusion at all Ca^{2+} concentrations. However, Fig. 5 *A* clearly shows that in the presence of polylysine the stimulatory effect of 1,2-DOG is attenuated, because no significant difference was found between the samples containing 2 and 5 mol% 1,2-DOG, and there was no stimulatory effect of 1,2-DOG at high Ca^{2+} concentration. The effect of polylysine on the role of 1,2-DOG under conditions where aggregation is not the rate-limiting step of fusion, i.e., high vesicle concentration, is depicted in Fig. 5 *B*. Comparing Fig. 5 *B* with Fig. 4 *A* in the absence of polycation, it can be observed that polylysine did not significantly affect lipid mixing of vesicles composed only of PS, but it produced an inhibition of fusion of vesicles containing 1,2-DOG. When the effect of 1,2-DOG in the presence of polylysine is observed (Fig. 5 *B*), it is seen that 2 mol% 1,2-DOG produced a slight increase of the rate of fusion at all Ca^{2+} concentrations, but the presence of higher amounts of 1,2-DOG (5 mol%), although producing a moderate increase at low Ca^{2+} concentration, actually inhibited fusion at high Ca^{2+} concentration.

We next studied the effect of the different DGs on the structural properties of PS. Fig. 6 shows the thermograms for the gel-to-liquid-crystalline phase transition of PS and PS containing 5 mol% different DGs isomers, in the absence (Fig. 6 *A*) and in the presence (Fig. 6 *B*) of Ca^{2+}. Pure PS undergoes its gel-to-liquid-crystalline phase transition at 5°C, and the presence of either 5 mol% 1,2- and 1,3-DOG isomers produced a slight shift of the transition temperature, T_m, to 6.2°C. The effect of saturated DGs, like 1,2- and 1,3-DC_8G and 1,2-$DC_{10}G$ at the same concentration, is opposite because they produce a slight decrease of T_m to 4.2°C. The effect of 1,2-DPG is more pronounced than that of its shorter homologs because it shifted the main endotherm to 3.5°C and, in addition, a second peak is clearly observed at higher temperatures (24.2°C). The complex PS/Ca^{2+} shows a gel-to-liquid-crystalline phase transition with T_m at 115°C. The presence of the different DGs, at 5 mol%, did not seem to change significantly the phase transition temperature (subtle changes are difficult to observe because the endotherms are very wide) except for 1,2- and 1,3-DOG, which showed a shoulder located at the upper part of the thermogram.

To check whether the presence of DGs alters the interaction of Ca^{2+} with the phosphate group of PS, we carried out a control by using FT-IR. The asymmetric stretching band of the $(PO_2)^-$ group was monitored for vesicles containing pure PS and PS plus 5 mol% of one of the DGs (all of the DGs used in this work were tested in this way), in the presence and in the absence of Ca^{2+}. It was found (results not shown) that at the concentrations used here, none of the DGs were capable of producing an alteration of the dehydration of the $(PO_2)^-$ group of PS that could be detected through FT-IR. The dehydration of the $(PO_2)^-$ group would give rise to the disappearance of the band centered at 1222 cm^{-1} (hydrated) and to the appearance of a different band at 1238 cm^{-1} (Dluhy et al., 1983).

DISCUSSION

The present work was initiated to extend our previous studies on the role of DOG on Ca^{2+}-induced fusion of a system (PS/PC, 1:1) that was unable to undergo fusion in the absence of DGs, by studying a system composed solely by PS that can be triggered to fuse by Ca^{2+} in the absence of DGs. Similar to what was observed by us PS/PC (Ortiz et al., 1992), our results have shown that physiological concentrations of 1,2-DOG were able to stimulate Ca^{2+}-induced lipid mixing of PS vesicles. 1,2-DOG has been reported very recently to produce an increase of lipid mixing rate induced by divalent cation (Walter et al., 1994). We have carried out a comparative study of the effect of DGs differing in acyl chain length, saturation, and isomerization, and the results ob-

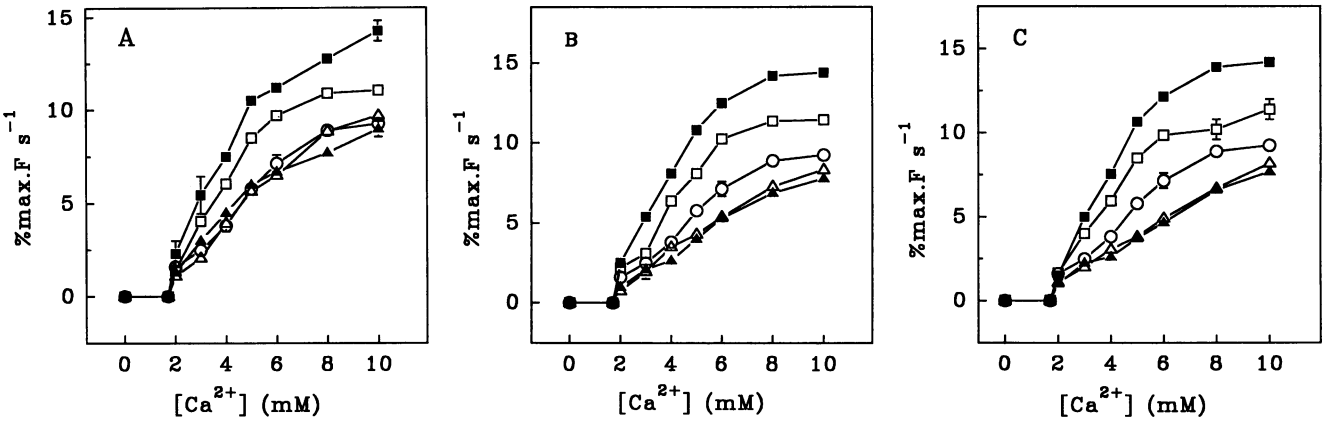

FIGURE 4 Dependence of the initial rate of lipid mixing on the calcium concentration for (*A*) vesicles made of pure phosphatidylserine (○) and phosphatidylserine containing 2 mol% (□) and 5 mol% (■) 1,2-dioleoylglycerol or 2 mol% (△) and 5 mol% (▲) 1,3-dioleoylglycerol; (*B*) vesicles made of pure phosphatidylserine (○) and phosphatidylserine containing 2 mol% (□) and 5 mol% (■) 1,2-dicapriloylglycerol or 2 mol% (△) and 5 mol% (▲) 1,3-dicapryloylglycerol; and (*C*) vesicles made of pure phosphatidylserine (○) and phosphatidylserine containing 2 mol% (□) and 5 mol% (■) 1,2-dicaprylglycerol or 2 mol% (△) and 5 mol% (▲) 1,2-dipalmitoylglycerol. Total phospholipid concentration was 300 μM. Points represent average of three experiments (error bars are shown when larger than the symbols).

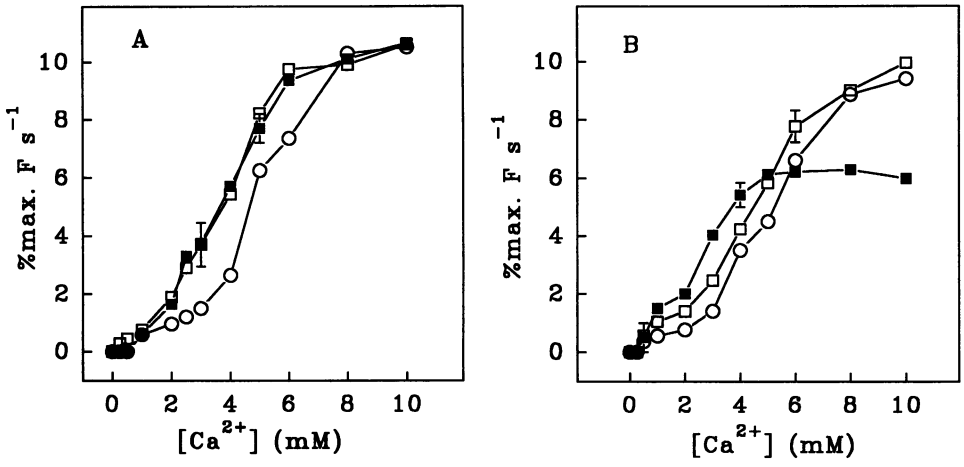

FIGURE 5 Dependence of the initial rate of lipid mixing on the calcium concentration for vesicles made of pure phosphatidylserine (○) and phosphatidylserine containing 2 mol% (□) and 5 mol% (■) 1,2-dioleoylglycerol in the presence of 0.25 μM polylysine. Total phospholipid concentration was 50 μM (*A*) or 300 μM (*B*). Points represent average of three experiments (error bars are shown when larger than the symbols).

tained, as discussed below, suggested that the effects of DGs are specific and depend on both their interfacial and hydrophobic (acyl chain) properties, i.e., that the effect of DGs on Ca^{2+}-induced fusion of PS vesicles might depend on the specific type of bilayer perturbation induced.

Although the mechanism of Ca^{2+}-induced fusion of PS vesicles is not fully elucidated, there is evidence suggesting that it occurs via the involvement of dehydration of the polar head group and generation of defects in molecular packing of the phospholipids at the region of interbilayer contact (Wilschut, 1991). Factors affecting stability of bilayer membranes would be important to modulate the process of fusion. 1,2-DOG is a molecule with a small polar head group that upon incorporation in PS membranes should be expected to decrease the density of polar head groups, and this could facilitate the fusion process by disruption of the bilayer interface. We have previously shown that DGs perturb the lipid/water interface of PS vesicles (López-García et al., 1993, 1994a) so that the dehydrating effect of Ca^{2+} on PS was enhanced by the presence of DGs. Furthermore, we have observed previously by using FT-IR that the presence of DGs significantly reduced the hydration of the carbonyl ester group of the phospholipid (López-García et al., 1994b). If these effects at the lipid-water interface were the only cause, because the interfacial structure of 1,2-DOG and 1,2-DPG are indistinguishable (Hamilton et al., 1991), both DGs should be expected to similarly activate membrane fusion. Far from that, we found that 1,2-DPG inhibited PS vesicle fusion, and this suggests that the events taking place in the bilayer palisade are highly relevant to the mechanism that modulates the fusion process. In fact, the hydrophobic interactions between the lipid side chains is a function of the length of the chains and also of the degree of chain unsaturation. The long fully saturated chains of 1,2-DPG will have great hydrophobic interactions and will make the bilayer interior more rigid, leading to stabilization of the phospholipid packing and, thus, inhibiting fusion. Unsaturated chains, containing *cis*-double bonds like those of 1,2-DOG, would have the opposite effect, because these unsaturated fatty acid chains do not pack as tightly as the saturated ones and, there-

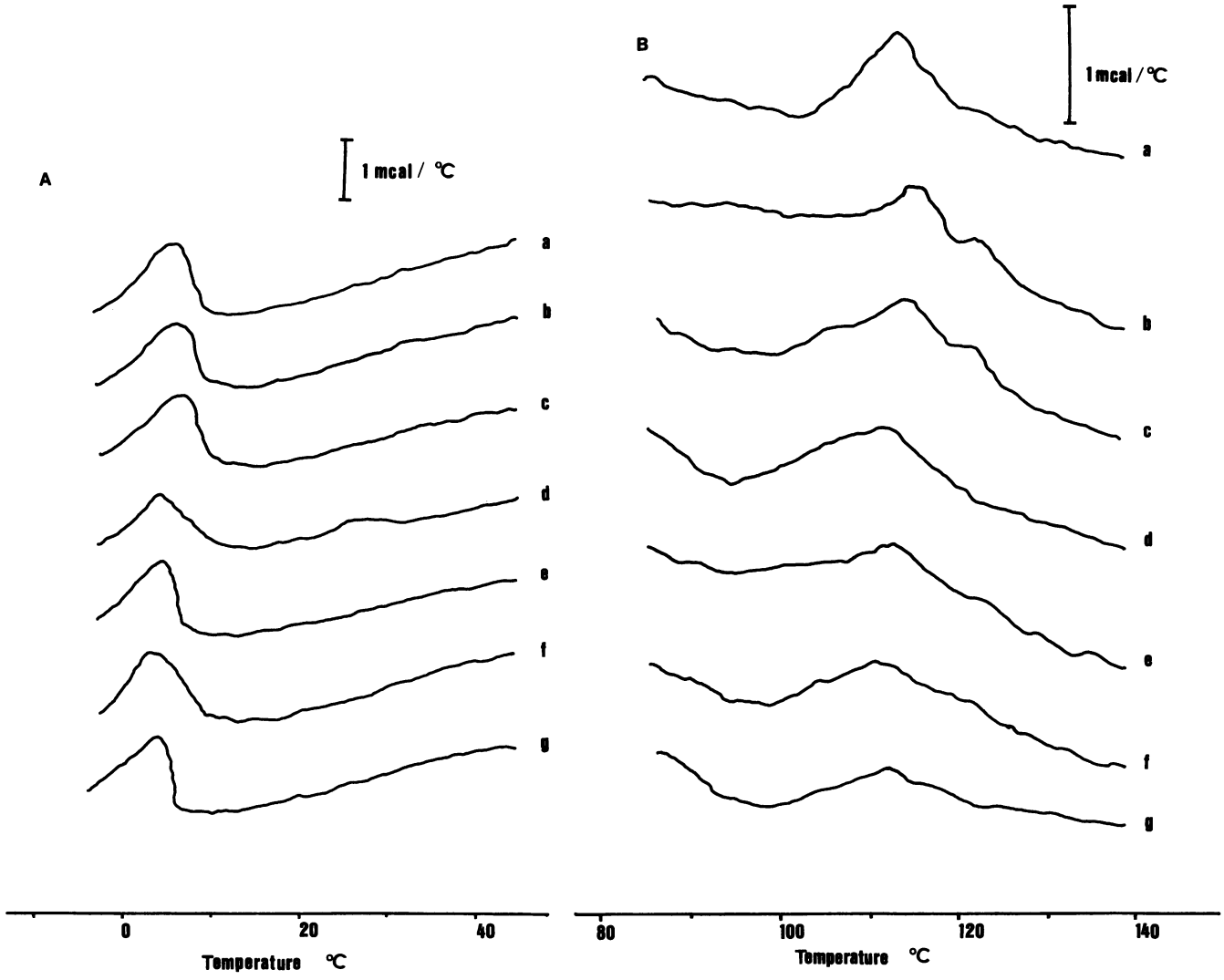

FIGURE 6 DSC thermograms for the gel to liquid-crystalline phase transition of pure phosphatidylserine vesicles (a) and phosphatidylserine vesicles containing 5 mol% 1,2-dioleoylglycerol (b), 1,3-dioleoylglycerol (c), 1,2-dicapryloylglycerol (d), 1,3-dicapryloylglycerol (e), 1,2-dicaprylglycerol (f) and 1,2-dipalmitoylglycerol (g) in the absence (*A*) and presence (*B*) of 10 mM calcium.

fore, the attractive forces between the lipid side-chains are decreased, thus facilitating the formation of defects in phospholipid packing and, hence, intermembrane contact and fusion. The modulatory effects of 1,2-DOG and 1,2-DPG do not significantly change the rate of vesicle aggregation under conditions where vesicle contact is the rate-limiting step of fusion, but they produced modulatory effects under conditions where aggregation was not rate-limiting, indicating that these effects are exerted at the level of the actual process of fusion.

To test the importance of the interfacial conformation of DGs on the process of fusion, we studied the effect of 1,3-DOG. The 1,3-isomer of DOG would perturb the PS acyl chains to the same extent that the 1,2-isomer, but we found that 1,3-DOG lacked the stimulatory effect on PS fusion (Fig. 4 *A*). It has been reported that the interfacial structure of 1,2- and 1,3-DOG are different (Hamilton et al., 1991). Both carbonyl groups of 1,2-DOG are H-bonded to water, but the secondary ester (*sn*-2) carbonyl is relatively more hydrated than the primary ester (*sn*-1) carbonyl, suggesting that its interfacial conformation resembles that found in the liquid-crystalline lamellar of certain phosphatidylcholines in which the glycerol backbone is perpendicular to the bilayer plane (Hamilton et al., 1991; Smith et al., 1992), and contrary to this, the glycerol backbone of 1,3-DOG lies normal to acyl chains with the carbonyls equivalently hydrated (Hamilton et al., 1991). Our results suggest that these differences at the lipid-water interface are crucial for modulating PS vesicle fusion.

To get insight into the importance of the acyl chain length on the modulatory effect of DGs on PS fusion, we studied the effect of two disaturated short acyl chain DGs, namely 1,2-DC_8G and 1,2-$DC_{10}G$. As shown in Fig. 4, *B* and *C*, both stimulated PS vesicle fusion. It was shown using the fluorescence probe diphenyl hexatriene that DGs with short chains may increase the order of the bilayer at temperatures

above the gel-to-liquid-crystalline phase transition (Ortiz et al., 1988). It was described by using NMR that short chain DGs like 1,2-DC_8G produced "transverse" perturbation of the bilayer (De Boeck and Zidovetzki, 1989; Goldberg et al., 1994) consisting of the DG-induced increase of the order parameters of the phospholipid side chains in the region adjacent to the glycerol backbone, with the concomitant decrease of the order in the lower region of the chains, close to the bilayer interior. This will increase structural fluctuations in the bilayer interior and, thus, it will stimulate fusion. Again this effect on the hydrophobic part of the phospholipid has to be complemented by an appropriate interfacial conformation, because 1,3-DC_8G (Fig. 4 *B*) lacked stimulatory effect and produced even an inhibitory effect on PS vesicle fusion; it should be remarked here that 1,3-DC_8G was substantially different from 1,2-DC_8G with respect to its effects on the order of the membrane (Ortiz et al., 1988).

The above observations suggest that an absolute requirement for DGs to stimulate PS vesicle fusion is a specific stereochemical configuration at the region of the glycerol backbone: only 1,2-*sn*-DG, but not 1,3-*sn*-DG, stimulates PS vesicle fusion. 1,2-*sn* configuration is necessary but not sufficient, and a specific bilayer perturbation by the DGs is also necessary like that produced by *cis*-double bond or short acyl chains. It is interesting to note that the above requirements for stimulating PS fusion are the same requirements that are necessary for PKC activation, because it has been shown that DGs with unsaturated chains activate PKC, although long chain-saturated do not (Kishimoto et al., 1980; Goldberg et al., 1994), and also that short chain-saturated DGs also activate the enzyme (Lapetina et al., 1985) and that 1,2-*sn*-DG, but not 1,3-*sn*-DG, activates PKC (Rando and Young, 1984; Nomura eta al., 1986). The requirements for stimulation of PS vesicle fusion can be just fortuitously similar to PKC activation, or it might be that the perturbation of the bilayer that led to promotion of fusion of PS vesicles is the same that facilitates PKC insertion into lipid membranes and activation. In this regard, it has been shown recently that the different bilayer perturbation exerted by DGs with various lengths and saturated acyl chains on PS/phosphatidylcholine systems correlates with their different effects on the activity of PKC (Goldberg et al., 1994).

We next studied the interaction of polylysine with the stimulatory role of 1,2-DOG on PS vesicle fusion. Polylysine has been used frequently as a typical model for basic extrinsic protein in liposome fusion (Gad et al., 1982; Uster and Deamer, 1985), producing an enhancement of the rate of vesicle fusion and a reduction of the Ca^{2+} threshold concentration. Polylysine, because of its positively charged character, can decrease the surface density and surface potential of negatively charged lipid bilayers (Hong et al., 1991). Polylysine-induced fusion of PS-containing vesicles is optimal when the number of sites for vesicle-peptide-vesicle interaction is maximized and when the net charge of the aggregated system is near zero (Walter et al., 1986). The stimulatory effect on the fusion process is thought to be exerted exclusively at the level of the initial aggregation of the vesicles (Gad et al., 1985) through charge neutralization due to the polycationic feature of polylysine. In accordance with this, we found that polylysine decreased the Ca^{2+} threshold and increased the rate of fusion of PS and PS containing 1,2-DOG when aggregation of vesicles was the rate-limiting step. However, when the actual fusion step was rate-limiting, we found that the presence of 1,2-DOG may even, under certain conditions, decrease the rate of fusion. Many authors have suggested that the binding of polylysine to acidic lipids also involves hydrophobic forces in addition to the straightforward electrostatic interaction and that the polypeptide penetrates into the bilayer (Hartmann and Galla, 1978; Carrier and Pêzolet, 1986) and perturbs the lipid packing (Hammes and Schullery, 1970). How this perturbation of the bilayer by polylysine counteracts the stimulatory effect of 1,2-DOG is not evident from our results; but clearly when aggregation is rate-limiting, the role of polylysine as counterion of PS, which will decrease the surface charge density and surface potential, overwhelms any other effect on the bilayer and enhances aggregation of vesicles, leading to an enhancement of fusion. That under certain conditions 1,2-DOG may change its stimulatory role by an inhibitory one has been described in phospholipase C-promoted vesicle fusion (Nieva et al., 1993) and PKC activation (Lapetina et al., 1985; Epand et al., 1992). Our results suggest that DGs modulate not only the interaction of PS with Ca^{2+} but also that of PS with a model fusion-promoting protein.

Quantitative measurements of DGs present in stimulated cells have shown that they may reach 1.45 mol% over total lipid (Preiss et al., 1986) or about 2 mol% (Takuwa et al., 1987). So the concentrations of DGs used in this work can be considered physiological and well within the range of DGs concentrations used in standard procedures for PKC activation assays that use 11.5 mol% with respect to total lipid (Ogita et al., 1991) and may reach values as high as 19 mol% (Wooten at el., 1987) or 25 mol% (Bolen and Sando, 1992). When we studied the effect of these low concentrations of DGs, such as 2–5 mol%, on the structural properties of PS, we found that they did not significantly affect the thermotropic transition of PS except for 1,2-DPG, which we found to give rise to a second peak at temperatures higher than that of liquid-crystalline phase transition of pure PS, suggesting the formation of gel-like complexes between 1,2-DPG and PS that laterally separates from the bulk lipid in the liquid-crystalline phase. These immiscibilities of 1,2-DPG in the liquid-crystalline phase do not provide interspacing for the bulk lipids and, together with the stabilization of the bilayer interior, result in a lack of promotion of fusion.

None of these DGs, at the concentrations used here, significantly affected the interaction of Ca^{2+} with the phosphate group of PS, as seen through FT-IR. From these observations, we suggest that DGs, at the low concentrations used in this study, do not grossly affect the structural organization of the bulk of PS. This does not exclude that some molecules of PS could be affected by the presence of DGs, but other physical techniques would be necessary to detect this. In any case, the stimulation of fusion could be induced simply by

the presence of some active points in the membrane, and DGs molecules should be expected to give place to these points.

There is an apparent controversy concerning the mechanism by which 1,2-DOG might stimulate PS vesicle fusion. We have previously suggested, on the basis of ^{31}P-NMR, FT-IR, and freeze fracture electron microscopy, that 1,2-DOG promoted fusion of PS/phosphatidylcholine (1:1) vesicles via nonlamellar intermediates (Ortiz et al., 1992). However, in the present work and in that of Walter et al. (1994) using pure PS vesicles, 1,2-DOG does not seem to act by stabilizing nonlamellar phases. It could be thought, as suggested by Walter et al. (1994), that in a PS/phosphatidylcholine/1,2-DOG/Ca^{2+} system lipids were not ideally mixed and that the effect of 1,2-DOG could have been similar to those described in the present work and in that previous paper (Walter et al., 1994), i.e., the PS component of the membrane. However, there are two pieces of evidence in favor of the participation of a nonlamellar intermediate in the 1,2-DOG promotion of fusion in PS/phosphatidylcholine system: (i) we have shown (Ortiz et al., 1992) that the presence of a high amount of 1,2-DOG promotes fusion but does not (as seen by FT-IR) induce the formation of a Ca^{2+}/PS complex, or at least not a complex with the same characteristics of that formed with pure PS (or as shown above for PS containing low amounts of 1, 2-DOG); (ii) it has been reported recently by Goldberg et al. (1994) that in a similar PS/phosphatidylcholine (1:4) system containing high amount of 1,2-DOG, Ca^{2+} induced the presence of a nonbilayer lipid phase observed by ^{2}H-NMR and corresponding to isotropic lipid as seen by ^{31}P-NMR, which is more evident when observing the PS component.

We believe that the bilayer lipid composition is a factor that may determine the apparent mechanism by which 1,2-DOG exerts its stimulatory effects on Ca^{2+}-induced membrane fusion. Pure PS vesicles containing physiological concentration of DGs, as studied in this work and in that of Walter et al. (1994), is a system very prone to undergo fusion induced by Ca^{2+}, so that subtle perturbations of the bilayer produced by DGs are able to modulate the rate of the process but are not able to alter drastically the structural organization of PS vesicles, at least as seen by physical techniques like those used here, which only detect changes affecting a significant proportion of the membrane lipids. Apart from that, the possibility remains that these low amounts of DGs alter the structure of PS *during* the interaction with Ca^{2+} and it is not observed at equilibrium. What seems clear is that in this system, fusion proceeds similarly to the case of pure PS vesicles. On the other hand, it is known that phosphatidylcholine antagonizes the tendency of PS to undergo fusion induced by Ca^{2+}. Hence, a PS containing a high proportion of phosphatidylcholine (Ortiz et al., 1992; Goldberg et al., 1994) is a system with no tendency to fuse by Ca^{2+}; thus, low amounts of 1,2-DOG are not able to induce fusion, and very high amounts of 1,2-DOG are necessary for inducing fusion, and in this case they seem to act through nonlamellar intermediates.

CONCLUSIONS

We have shown that different DGs can modulate differently the rate of Ca^{2+}-induced fusion of PS vesicles, depending on their interfacial conformation, so that 1,2-DG stimulates fusion but 1,3-DG does not stimulate or even inhibit fusion, and the specific type of bilayer interior perturbations such that unsaturated or short chain-saturated DGs stimulate fusion but long chain-saturated DGs do not. These requirements resemble those needed for PKC activation, supporting the early suggestion (Das and Rand, 1984) that the bilayer perturbation exerted by DGs can lead either to promote Ca^{2+}-induced fusion of PS or to PKC activation through the same underlying mechanism. In our system, the physiological amounts of DGs used do not alter significantly the structural organization of PS bilayers to an extent large enough to be appreciated through the physical techniques used. Probably the specific bilayer perturbation exerted by stimulatory DGs increases fluctuations of the lipid phase facilitating the formation of packing defects and results in an accelerated fusion, probably through a mechanism similar to that present in pure PS vesicles. Polylysine is able to affect the role of 1,2-DOG such that increasing the 1,2-DOG concentration can lead, under certain conditions, to an inhibition of Ca^{2+}-induced fusion.

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