Diacylglycerol and Hexadecane Increase Divalent Cation-Induced Lipid Mixing Rates Between Phosphatidylserine Large Unilamellar Vesicles

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ABSTRACT Bovine brain phosphatidylserine (BBPS) vesicles were prepared with traces of dioleoylglycerol (18:1,18:1 DAG) or hexadecane (HD) to determine the influence of changes in headgroup or acyl chain packing on divalent cation-induced lipid mixing rates. A stopped-flow apparatus was used to combine vesicles with 3 mM Ca^{2+} or Ba^{2+}. Aggregation was monitored by light scattering and lipid mixing by lipid probe dilution. Neither 3–6 mol % 18:1,18:1 DAG nor up to 10 mol % HD significantly altered the BBPS chain melting temperature, vesicle diameter, or vesicle aggregation rates. Lipid mixing rates doubled by adding either 3 mol % 18:1,18:1 DAG or 6 mol % HD to BBPS with no change in the Ca^{2+} concentration threshold. The Arrhenius slopes of the lipid mixing rates for control, 3 mol % 18:1,18:1 DAG, and 6 mol % HD vesicles were identical. ^{2}H-nuclear magnetic resonance spectra of perdeuterated dipalmitoylglycerol and HD in BBPS in the absence and presence of Ca^{2+} and Ba^{2+} showed that the solutes occupied different time-averaged positions in the bilayer under each condition. These data suggest that: 1) the enhanced lipid mixing rate is related to the volume of the added alkyl chains; 2) 18:1,18:1 DAG and HD may alter the activation entropy or the attempt frequency at one or more steps in the lipid mixing process; 3) 18:1,18:1 DAG and HD are likely to act at a different spatial or temporal point than the divalent cation; and 4) it is unlikely that the effect of these solutes on lipid mixing is due to their equilibrium time-averaged positions in the bilayer. Others have shown that apolar lipids accelerate fusion in nonbilayer phase-forming systems, but BBPS does not form these phases under these conditions. Therefore, we propose that the effect of very small amounts of apolar substances may be very general, e.g., stabilizing the hydrophobic interstices associated with a variety of proposed intermediate structures.

INTRODUCTION

Although fusion between biological membranes is a ubiquitous and essential process, very little is understood about the molecular rearrangements of the membrane components that are involved. Studies with intact cells or reconstituted cell systems are particularly suited to describing the biochemical requirements for targeting and fusion. However, these preparations are not suitable, due to their complexity, for defining the parameters controlling the membrane lipid rearrangements required for fusion. Proposed pathways for lipid rearrangements during fusion include structures that function as intermediates in transitions between bilayer and inverted phases (Siegel, 1986) or L_β and L_α phases (Papahadjopoulos, 1978) or "stalk" structures that form under a wide variety of circumstances (Markin et al., 1984; Chernomordik et al., 1985). More recently, the data from studies describing the mechanism of action of virus fusion proteins and initial fusion pore behavior have been used to suggest new models for the lipid rearrangements (Bentz et al., 1990; Zimmerberg et al., 1991; Guy et al., 1992; Siegel, 1993a).

The physical chemical principles governing the rearrangement of lipids during biological fusion events are likely to be the same as those operating in simpler systems. Several factors have been proposed to be important in governing fusion in pure lipid systems. Changes in the short-range forces between apposed interfaces (McIntosh et al., 1989; Rand and Parsegian, 1989) determine the frequency and extent to which interfaces can become apposed closely enough for fusion to occur. The hydrophobicity of the lipid/water interface (Ohki and Arnold, 1990) and the ability of the interfaces to adopt small radii of curvature (Siegel, 1986; Markin et al., 1984; Gruner, 1985; Siegel, 1986, 1993b) may affect the ability of the apposed interfaces to rearrange into fusion sites. It has also been suggested that the free energy needed to stabilize the hydrophobic voids that form between leaflets of the bilayer when intermembranous structures form is another barrier to fusion (Siegel, 1993a).

Recently, much fusion research has explored lipid systems that form inverted hexagonal (H_{II}) or inverted cubic phases and how fusion rates can be facilitated by the addition of diacylglycerol (DAG) or alkane to these systems (e.g., Siegel et al., 1989b). However, membrane fusion occurs between biological membranes that are far from the H_{II} phase transition. Will the addition of DAG or alkane to lipids with little or no tendency to form nonbilayer phases alter the probability that these membranes will fuse? To answer this question, we chose a system that has no tendency to form nonbilayer phases and yet is susceptible to membrane fusion in the absence of proteins.

Phosphatidylserine (PS) vesicles can be induced to aggregate and fuse by the addition of millimolar levels of divalent cation (Ba^{2+} or Ca^{2+}) (e.g., Wilschut et al., 1980;

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Bentz et al., 1983; Walter and Siegel, 1993). Divalent cation binding to PS induces formation of the L_β phase but not H_{II} phase at neutral pH in well-hydrated systems. Bovine brain PS (BBPS) will form H_{II} phases only at low water activity (<65% water by weight) when 20 mol % or more DAG is added (Das and Rand, 1986) or in hydrated dioleyl-PS at very low pH (de Kroon et al., 1990). However, PS with 50 mol % DAG in excess water at neutral pH remains lamellar even in the presence of calcium (Das and Rand, 1986).

In the present work we have studied the effect of very small amounts of two types of hydrophobic substances, dioleoylglycerol (18:1,18:1 DAG) and hexadecane (HD), on divalent cation-induced aggregation and lipid mixing rates between PS vesicles. Neither additive significantly altered vesicle size, aggregation rates, or the main phase transition (T_m). ^{2}H-nuclear magnetic resonance (NMR) studies of perdeuterated analogues of these solutes indicated that they adopt different time-averaged orientations within PS bilayers both in the presence and absence of divalent cations. Yet, at the same concentration of alkyl chains, 18:1,18:1 DAG and HD increase the lipid mixing rate to the same extent, at least at the tested concentrations. We infer that either the time-averaged distributions of these two solutes are not representative of their true role(s) in facilitating lipid mixing and they act in similar ways or that the HD and 18:1,18:1 DAG act in different ways, but the quantitative effects are fortuitously similar under the conditions examined. These studies have been presented in preliminary form (Walter et al., 1992, 1993a).

MATERIALS AND METHODS

Chemicals and buffers

All phospholipids, 18:1,18:1 DAG, 16:0,16:0 DAG, and d_{62}-16:0,16:0 DAG were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), stored in chloroform under nitrogen at −80°C, and used without further purification. HD was from Aldrich (Milwaukee, WI) and d_{34}-HD was purchased from MSD Isotopes (St. Louis, MO). Deuterium-depleted water came from MSD Isotopes or Cambridge Isotopes (Woburn, MA). The salts, sodium chloride, calcium chloride, and barium chloride were supplied by Fisher. *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and ethylenediamine tetraacetic acid (EDTA) were from Sigma Chemical Co. (St. Louis, MO). The buffer was 100 mM NaCl, 10 mM HEPES, 0.1 mM EDTA, and 0.02% sodium azide titrated to pH 7.2 with NaOH.

Differential scanning calorimetry

Lipid phase transition temperatures were determined by differential scanning calorimetry (DSC) using a model MC2 calorimeter (Microcal Corp., Amherst, MA). Samples of PS with and without HD were prepared from stock solutions in chloroform. Chloroform was removed by rotary evaporation and subsequent application of vacuum for 1 h. The PS was hydrated at 12.5 mg PS/ml in the same buffer described above but without sodium azide. After incubation on ice for 1 h, the suspension was subjected to five freeze-thaw cycles (dry ice/30°C water bath), and then introduced into the calorimeter. For DSC measurements in the presence of divalent cations, the samples were processed in one of two ways. In both cases the PS was suspended in divalent cation-free buffer and was freeze-thawed three times. In the first method, an appropriate volume of divalent cation stock solution ($BaCl_2$ or $CaCl_2$) was added to both saturate the PS with divalent cation and provide an excess divalent cation concentration in the buffer of 3.0 mM. The samples were freeze-thawed three more times and introduced into the calorimeter. In the second method, the lipid suspension and divalent cation solution, at room temperature, were mixed in the DSC cell itself by syringe action and then scanned with no further treatment. The two different methods were used to check for divalent cation-induced phase separation of HD or DAG: if such separation occurred, there was a chance that the DAG or HD would have been lost from the sample using the first technique. With the second method, any apolar lipid separation from the bilayer structure would occur within the DSC cell and the transition of the neat HD or diglyceride would be observable in the thermogram. Both sample preparation methods yielded the same temperature dependencies.

^{2}H-NMR

BBPS samples (300 mg) were prepared with 3 mol % d_{62}-16:0,16:0 DAG or 6 mol % d_{34}-HD by combination in pentane. The pentane was removed by rotary evaporation and subsequent exposure to vacuum for several hours. The sample was hydrated in the NaCl buffer under argon for several hours or overnight at 4°C and then subjected to five freeze-thaw cycles. The pH was tested to ensure that it remained at 7.2 after the second and fifth freeze-thaw and adjusted if necessary. For samples with calcium or barium, the protocol was adjusted to ensure that these divalent cations were equilibrated with the BBPS. After the lipid was thoroughly hydrated, an appropriate amount of calcium was added from a stock solution prepared in deuterium-depleted water (100 mM $CaCl_2$, 100 mM NaCl, 10 mM HEPES, 0.1 mM EDTA, pH 7.2). The amount of calcium was determined to be enough to titrate the PS molecules and leave 3 mM calcium free in the buffer. After three freeze-thaw cycles in this calcium buffer, the sample was centrifuged, the buffer removed, and the lipid was resuspended in a second buffer containing 3 mM $CaCl_2$. All samples were held on wet ice under argon and examined within no more than 3 days of preparation. A similar procedure was used for the barium-containing samples.

NMR spectra were obtained with a JEOL FX270 Fourier-transform spectrometer in 10-mm tubes at 41.36 MHz using a solid echo with a 10-μs pulse width. Up to 400,000 transients were collected at 10 s^{-1}. A spectral width of 50 KHz was used and 2000 data points obtained. The data were processed with 50 Hz of line broadening, with the exception that for isotropic line shapes, 0 Hz of line broadening were used.

Vesicle preparation

Vesicles were prepared as described previously (Walter and Siegel, 1993) by sequential freeze-thaw and extrusion through 0.1-μm nuclepore filters (Nuclepore Corporation, Pleasanton, CA) using a Lipex Extruder (Lipex Biomembranes, Inc., Vancouver, BC, Canada). Lipid concentration was determined from hydrolyzable phosphate (Ames and Dubin, 1960). Vesicle size distribution was determined by elution over a TSK-G6000 gel filtration column (Walter et al., 1993b). The vesicles were stored at 5°C under a nitrogen atmosphere.

To prepare vesicles with 18:1,18:1 DAG or HD, stock solutions of these were prepared in chloroform, and the required amount was added to the lipid solutions before chloroform evaporation to ensure thorough mixing. Only insignificant amounts of 18:1,18:1 DAG or HD were lost to the aqueous phase. Using the HD partition coefficient between HD and water as an estimate for the membrane/water partition coefficient in vesicles containing 5 mol % HD, at a total PS dilution in the aqueous buffer to 50 μM, the estimated fraction of the total added HD that would be lost to the aqueous phase would be about $2.6 \cdot 10^{-7}$. With the use of [^{14}C]HD, we demonstrated that there were no detectable losses of HD from the vesicles to the aqueous phase in the presence and absence of divalent cations after several washes (data not shown).

Each control and experimental condition required three separate preparations of vesicles, i.e., unlabeled PS, PS with 0.7 mol % each of the energy transfer probes *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rho-PE), and PS with 0.35 mol % of each of these probes. The unlabeled and the 0.7 mol % NBD-PE/Rho-PE vesicles were combined

in a 1:1 lipid ratio to permit the measurement of lipid mixing. Under these conditions the fluorescence of NBD-PE, the energy donor, increases as the labeled lipids disperse among the unlabeled lipid. The 0.35 mol % NBD-PE/Rho-PE vesicles served as the reference for the fluorescence of a dimeric fusion product as well as a monitor for any contributions of scattered excitation light or precipitation-induced changes in the fluorescence signals. No scattering or precipitation was observed in the fluorescence signal over the time regime of interest. The probes did not alter the PS T_m or the size and size distribution of the resulting vesicles (Walter and Siegel, 1993).

Aggregation and lipid mixing rate determinations

Divalent cation induced aggregation and lipid mixing were monitored by light scattering increases and the increase in specific NBD-PE fluorescence as probes dilute subsequent to lipid mixing, respectively. The vesicles and divalent cation were combined by a 1:1 volume dilution with a stopped-flow mixing apparatus equipped with a fluorescence optical cell (SLM-Aminco) inserted in a fluorescence spectrometer (DMX-1000, SLM Instruments, Urbana, IL). The mixing time was 5–10 ms. Scattered light intensity was monitored at an angle of 90° to the excitation beam at 470 nm. NBD-PE fluorescence was measured at an excitation wavelength of 470 nm and emission wavelength of 535 nm with a 515 long-pass filter in the emission optical path to minimize stray light and scatter contributions to the signal. Three records were averaged for each scatter determination and at least 10 fluorescence records were averaged for each lipid mixing and maximum fluorescence sample.

Lipid vesicle aggregation must occur before lipid mixing or fusion between the membranes. The overall process can be described by the aggregation and disaggregation rate constants (k_1 and k_{-1}) between two vesicles (V) to form a dimeric aggregate (VV) that will fuse to form a new single vesicle (F) with a fusion rate constant (k_f):

$$V + V \underset{(k_{-1})}{\overset{(k_1)}{\leftrightarrow}} VV \xrightarrow{(k_f)} F \qquad (1)$$

The two rates may be separated by using conditions where one or the other dominates the overall rate, e.g., at low lipid concentration, aggregation is likely to be the slower step, whereas at high lipid concentration, the fusion step may be limiting. Since aggregation rates are relatively insensitive to temperature and lipid mixing rates increase steeply with temperature in this system (Walter and Siegel, 1993), we verified that the apparent initial lipid mixing rates were not aggregation-rate-limited over the temperature range tested. All fluorescence intensity (lipid-mixing curves) were compared to their respective scatter intensity curves, and each group of experiments included a series of lipid concentrations to assure that the lipid-mixing rate was the primary contribution to the overall observed rate.

Lipid mixing is one concomitant of fusion, but not all lipid mixing events are necessarily due to fusion. However, the lipid mixing process we observe is a least closely related to membrane fusion, if not the same process. It is observed under the same conditions as membrane fusion detected by contents mixing assays (e.g., Bentz et al., 1985). Lipid mixing did not occur under conditions not previously associated with contents mixing, e.g., when the vesicles were aggregated with Mg^{2+} (Bentz et al., 1983), when the lipids were below T_m (approximately 6°C), or in the absence of divalent cation, even after long times. The Ca^{+2} and Ba^{2+} concentration dependence for lipid mixing was similar to that observed for contents mixing (for review see Nir, 1991). Moreover, lipid mixing began in the absence of a discernible delay as expected if the process occurred by fusion rather than by exchange during vesicle collapse and cochleate structure formation. Two contents mixing assays were attempted but not used. The ANTS/DPX assay (Ellens et al., 1985) was abandoned due to significant perturbation of vesicle size and lipid mixing rates caused by DPX (Walter and Siegel, 1993). Dipicolinic acid (DPA), the chelator used in the Tb/DPA assay (Wilschut et al., 1980) was not encapsulated properly when the vesicles were prepared by extrusion (data not shown).

The extent of mixing at time t is expressed as a percentage of the fluorescence intensity expected if all the vesicles interacted in pairs to form dimers. These rates were calculated from the slope of the initial part of the fluorescence intensity curves where the lipid mixing is primarily between dimers. Although vesicle interactions ultimately include trimers, tetramers, etc., their contribution at such early stages in the process will be relatively small. The percentage of dimer formation at time t was calculated using:

$$\%\ \text{dimer}(t) = \frac{F_{1:1}(t) - F_0}{0.5 \times (F_{max}(t) - F_0} \times 100 \qquad (2)$$

where $F_{1:1}(t)$ and $F_{max}(t)$ are the matched fluorescence traces of the combined labeled and unlabeled vesicles (1:1 ratio) and the 0.35% labeled vesicles, respectively. The initial fluorescence intensity (F_0) was calculated from the first 3 points ($<0.01*t_{1/2}$) of the $F_{1:1}$ sample and the factor 0.5 included to correct for the probability that an encounter would occur between a labeled and an unlabeled vesicle. Values equivalent to less than 30% dimer formation were used to calculate the initial slopes.

RESULTS

18:1,18:1 DAG and HD did not affect the size of PS vesicles

Vesicles prepared with 18:1,18:1 DAG or HD were identical in size and size distribution when compared with control vesicles (Fig. 1). Thus, any effects of these additives cannot be explained by changes in vesicle diameter or in vesicle number. The mode diameter was 70 nm.

Effects of 18:1,18:1 DAG, 16:0,16:0 DAG and HD on the BBPS thermograms in the absence and presence of divalent cations

18:1,18:1 DAG at 3 mol % in BBPS had little or no effect on T_m (Fig. 2 *A*). The endotherm is slightly narrower in the

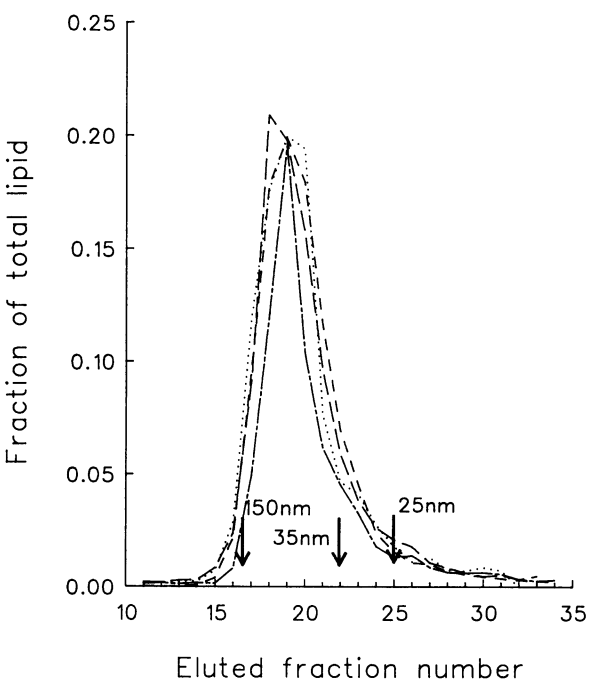

FIGURE 1 The size distribution of control vesicles (· · ·) compared with control vesicles containing the fluorescent probes NBD-PE and Rho-PE (- - - -), with vesicles containing 3 mol % 18:1, 18:1 DAG (– – –) and vesicles with 6 mol % HD (– - –). The vesicles were eluted at 0.5 ml/min over a TSK-G6000-PW gel filtration column that was calibrated using vesicles of known sizes.

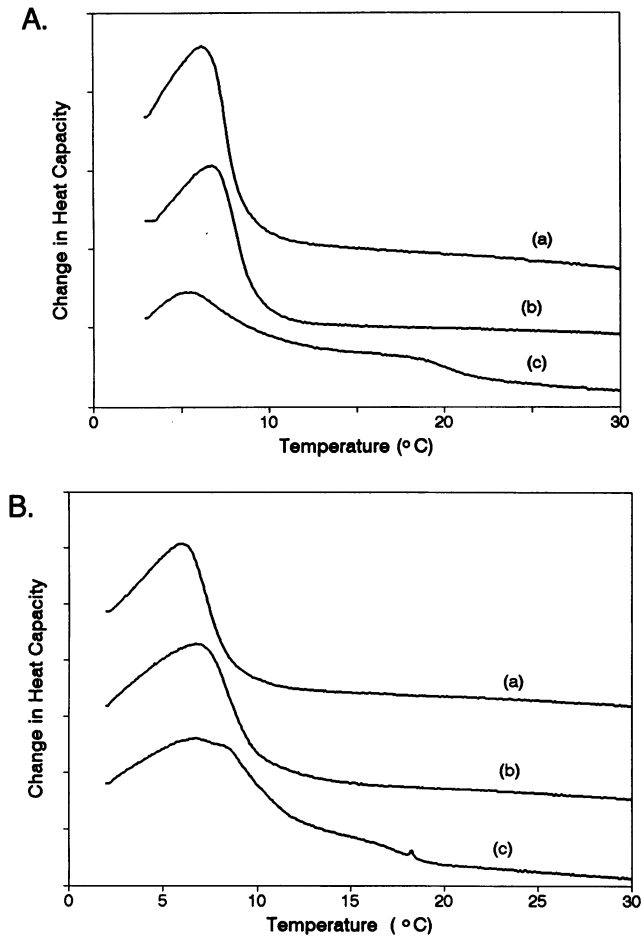

FIGURE 2 The effect of added DAG (*A*) and HD (*B*) on the phase transition temperature T_m of BBPS determined by DSC. (*A*) thermograms of PS (a), PS with 3 mol % 18:1,18:1 DAG (b), and PS with 3 mol % 16:0,16:0 DAG (c). (*B*) Thermograms are from control BBPS (a) BBPS with 5% HD (b), and BBPS with 10% HD (c). The thermograms have been offset for clarity.

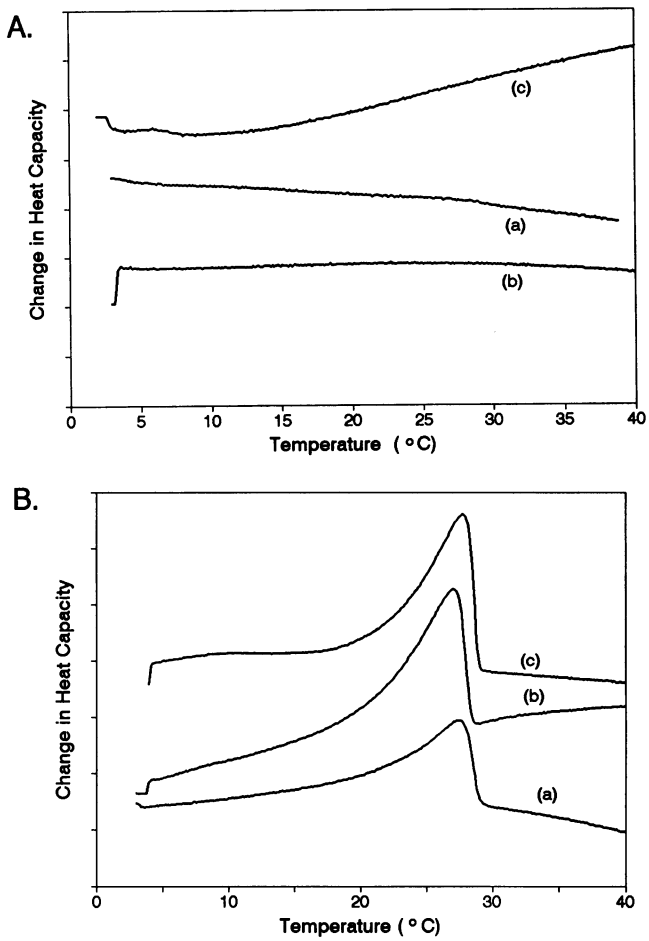

FIGURE 3 The effect of 18:1,18:1 DAG and HD on BBPS equilibrated with calcium (*A*) or barium (*B*) assessed by DSC. (*A*) Samples equilibrated with Ca^{2+} at a final unbound concentration of 3 mM or greater (see Materials and Methods) were compared using DSC. The samples are BBPS (a), BBPS with 3 mol % 18:1,18:1 DAG (b), and BBPS with 6 mol % HD (c). There are no obvious transitions up to 40°C, the temperature region explored in the lipid mixing experiments. (*B*) Thermograms from samples of BBPS equilibrated with 3 mM or more Ba^{2+}. Trace (a) is BBPS in the absence of added solute, trace (b) is BBPS with 3 mol % 18:1,18:1 DAG, and trace (c) is BBPS with 6 mol % HD.

presence of 3 mol % 18:1,18:1 DAG than that for pure BBPS. The thermogram of 3 mol % 16:0,16:0 DAG in BBPS showed that 16:0,16:0 DAG had very little effect on the T_m (decreases it by about 1°C), but there was also a second peak or shoulder at about 17°C (Fig. 2*A*). This latter does not arise from a region of pure 16:0,16:0 DAG (melting temperature 50°C; Small, 1986) but does indicate the presence of a 16:0,16:0 DAG-rich phase below 20°C. Addition of 5 mol % HD broadened the transition and slightly raised T_m (Fig. 2*B*). The effect was more pronounced at 10 mol %, where there was a small endothermic peak at about 18°C, indicating a small fraction of the HD had separated as neat HD (melting point 18.17°C; Small, 1986).

To determine whether either 18:1,18:1 DAG or HD altered the effect of divalent cations on PS, the T_m in the presence and absence of excess Ca^{2+} or Ba^{2+} was determined by DSC. No transition was observed for control or solute-containing BBPS equilibrated with excess calcium over the temperature range examined (4–80°C) (Fig. 3 *A*). The expected T_m for the BBPS/calcium system is about 120°C (Casal et al., 1987), so we may conclude from these data that the BBPS is in the gel state and that neither of these apolar solutes has been excluded into a neat phase.

In the presence of excess Ba^{2+} the endotherms with or without the additives were asymmetric (Fig. 3 *B*). However, neither HD (6 mol %) nor 18:1,18:1 DAG (3 mol %) altered the shape of the endotherm or the position of the peak. Nor did the addition of Ba^{2+} result in additional peaks, indicating that no regions of phase separated neat HD or 18:1,18:1 DAG were formed.

^{2}H-NMR spectra of d_{62}-16:0,16:0 DAG and d_{64}-HD in BBPS and BBPS-divalent cation complexes

The ^{2}H-NMR experiments were designed to determine the time-averaged orientation of 16:0,16:0 DAG (as an analog of 18:1, 18:1 DAG) and HD in BBPS and BBPS complexed

with Ca^{2+} or Ba^{2+}. Spectra were obtained above and below the T_m of the BBPS/Ba^{2+} complex. The ^{2}H-NMR powder patterns of chain perdeuterated d_{62}-16:0,16:0 DAG indicate that the acyl chains of this solute are in an ordered environment (Fig. 4 *A*). The widest quadrupole splitting decreases with increasing temperature from approximately 27.5 KHz at 20°C to 24 KHz at 40°C, reflecting the relative decrease in order of the acyl chain region. Spectra obtained at 20°C before and after data collection at 40°C were the same. This was true for all samples, indicating that they were at equilibrium.

When the BBPS is equilibrated with 3 mM Ca^{2+}, the powder pattern of the d_{62}-16:0,16:0 DAG was altered in two ways (Fig. 4 *B*). First, most of the resonance intensity was lost from the 50-KHz window, a limiting constraint of the instrument available for these measurements. This was indicative of a significant broadening of the powder pattern. The broadened powder pattern is likely due to 16:0,16:0 DAG in a rather immobile environment. This is to be expected since at these temperatures the BBPS/Ca^{2+} complex is about 80–100°C below its T_m. A small fraction of the d_{62}-16:0,16:0 DAG ^{2}H-NMR signal appears as a central isotropic resonance that is somewhat more intense at 40°C than at 16°C. It is unlikely that this represents pure phase-separated 16:0,16:0 DAG since 1) 16:0,16:0 DAG is solid at 40°C and would not yield an isotropic resonance; and 2) no such phase separation was detected between 4°C and 80°C by DSC. Alternatively, the isotropic signal may arise from a small fraction of the 16:0,16:0 DAG existing in disordered regions, such as small (even unimolecular) inclusions between the leaflets of the PS bilayers.

In the BBPS/Ba^{2+} samples with 3 mol % d_{62}-16:0,16:0 DAG there was no clear powder pattern either above or below T_m within the detection capability of the instrument used in these studies (data not shown). This observation is indicative of a broad powder pattern suggesting that the 16:0,16:0 DAG is in a very ordered environment. In contrast to BBPS/Ca^{2+}, no isotropic 16:0,16:0 DAG signal was evident at 15°C for the BBPS/Ba^{2+} sample, even though both samples were below their respective T_ms.

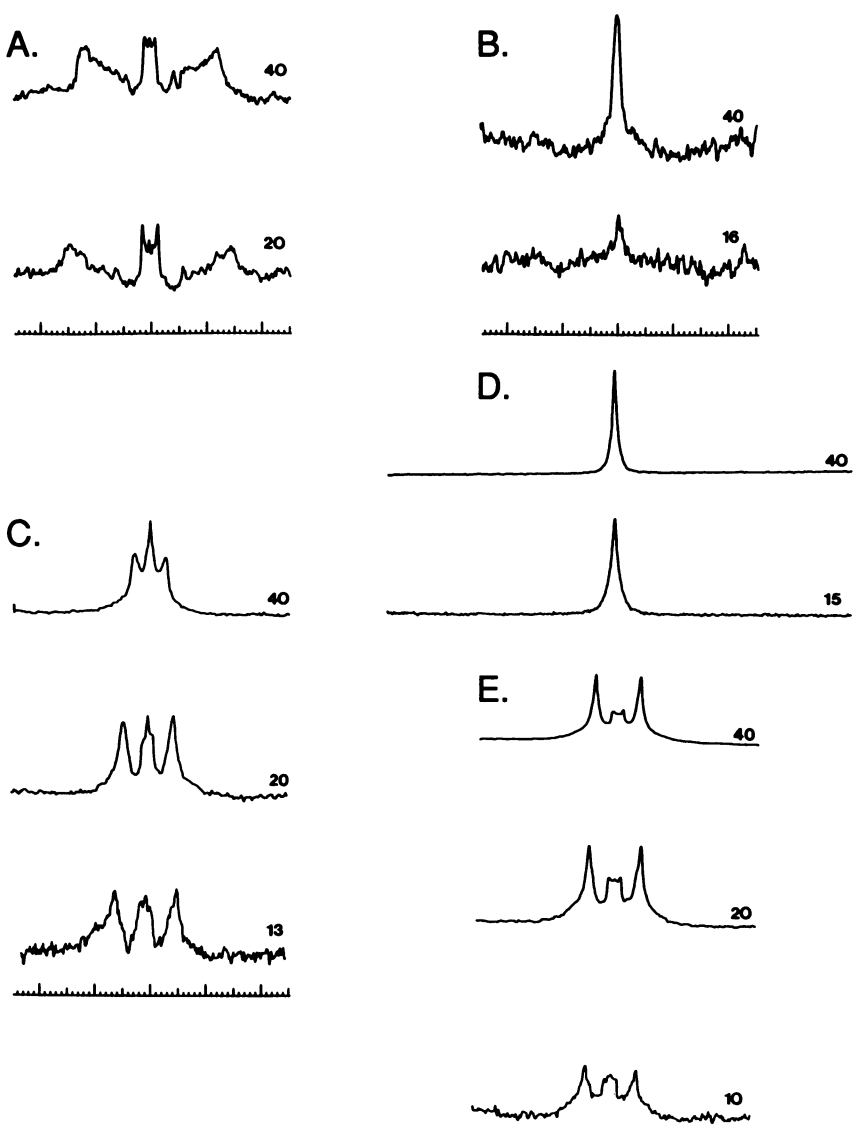

FIGURE 4 ^{2}H-NMR spectra of perdeuterated 16:0,16:0 DAG and perdeuterated HD in BBPS in the absence and presence of divalent cations. (*A*) 3 mol % d_{62}-16:0,16:0 DAG in BBPS. (*B*) 3 mol % d_{62}-16:0,16:0 DAG in BBPS equilibrated with 3 mM Ca^{2+}. (*C*) 6 mol % d_{34}-HD in BBPS. (*D*) 6 mol % d_{32}-HD in BBPS equilibrated with 3 mM Ca^{2+}. (*E*) 6 mol % d_{34}-HD in BBPS equilibrated with 3 mM Ba^{2+}.

The ^{2}H-NMR spectra for d_{34}-HD in BBPS with and without calcium or barium were also different. In the absence of divalent cations, the ^{2}H-NMR spectra for d_{34}-HD incorporated at 6 mol % in BBPS suggested that most of this alkane was in an ordered environment (Fig. 4 *C*). The largest quadrupole splittings were approximately 9.2 and 5 KHz at 20°C and 40°C, respectively, indicating a decrease in order and/or changes in average orientation (with respect to the director for axial rotation) with increasing temperature. A small portion of the HD gave rise to an isotropic resonance, indicating that it had access to more than one environment in the membrane. It is likely that the HD is distributed between two environments: one with the HD long axis primarily parallel to the acyl chains (more ordered) and one in the bilayer midplane represented by the isotropic resonance. The fraction of HD in the isotropic phase was greater at warmer temperatures (Fig. 4 *C*).

The ^{2}H-NMR spectra of d_{34}-HD at 6 mol % in BBPS in the presence of calcium consisted of a broad isotropic resonance (Fig. 4 *D*). The widths at half-height were 590 Hz and 880 Hz at 40°C and 15°C, respectively. Bulk HD has a width closer to 35 Hz at these temperatures (Siegel et al., 1989a) and was not expected to be present, since neat HD was not observed by DSC. Therefore, the HD was in a time-averaged environment that was ordered but significantly less ordered than its time-averaged environment in the absence of calcium. In a few examples, two peaks could be resolved in these spectra, suggesting the HD is equilibrated between two somewhat different relatively disordered environments. The environment may be similar to that in which a small fraction of the 16:0,16:0 DAG is located (Fig. 4 *B*), although the present data are insufficient to support this hypothesis.

The ^{2}H-NMR powder spectra for 6 mol % d_{34}-HD in BBPS equilibrated with 3 mM Ba^{2+} at 10°C, 20°C, and 40°C are shown in Fig. 4 *E*. The largest quadrupole splittings were, as expected, somewhat narrower at 40°C than at 20°C (6.9 and 9.4 KHz, respectively): these are similar to the widths observed in the absence of divalent cation (5 and 9.2 KHz). No major isotropic peak was apparent. The lack of an isotropic peak indicates that little or no HD was in a very disordered environment (as described for the calcium equilibrated system). The HD environment in Ba^{2+}/BBPS is more ordered than that in uncomplexed BBPS, even above T_m of the Ba^{2+}/BBPS system (24.6°C).

Calcium-induced aggregation and lipid mixing: 18:1,18:1 DAG and HD effects

The calcium-induced aggregation rates of PS vesicles were not altered by the presence of 18:1,18:1 DAG or HD at the levels tested (Fig. 5, *A* and *C*). Therefore, any differences in

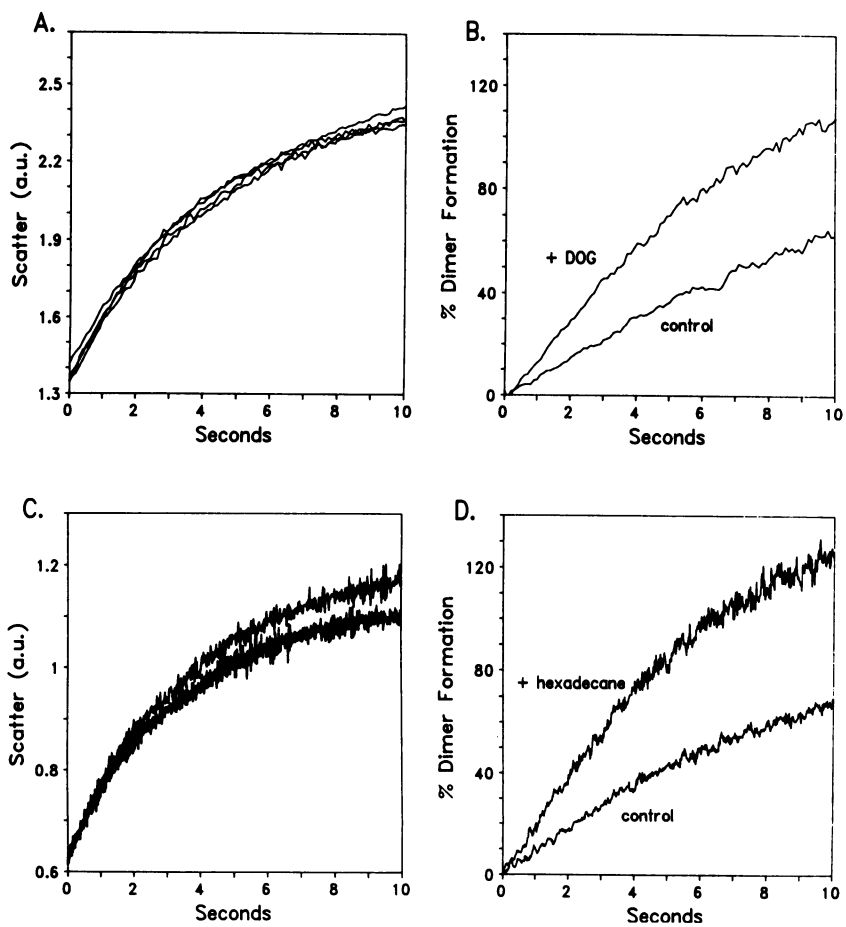

FIGURE 5 The effect of 18:1,18:1 DAG or HD on calcium-induced BBPS aggregation and lipid mixing rates. In these traces, BBPS lipid vesicles were combined at $t = 0$ with calcium by stopped-flow mixing to final concentrations of 100 μM PS and 3 mM Ca^{2+} at 25°C. The rise in scatter and percent dimer formation calculated from the change in energy transfer are plotted against time for control and +3 mol % 18:1,18:1 DAG vesicles in *A* and *B*. Similar experiments for PS vesicles and those containing 6 mol % HD are shown in *C* and *D*. The four curves in the scatter traces are the scatter intensity changes observed when the fluorescence-labeled and unlabeled vesicles are present in a 1:1 ratio for control and +18:1,18:1 DAG (or +HD) experiments, and the control and 18:1,18:1 DAG (or +HD) vesicles prepared with 0.35 mol % of both of the fluorescent probes. Each curve is not specifically labeled, since they are indistinguishable. Neither the NBD-PE and Rho-PE nor the 18:1,18:1 DAG or HD affected the rate of light scattering changes. Initial rates of lipid mixing were determined by fitting a line to data up to 30% of complete dimer formation.

the rates of lipid mixing will not be due to changes in aggregation rate.

The addition of 1.5 mol % and 3 mol % 18:1,18:1 DAG to PS did increase the rate of lipid mixing at all temperatures and lipid concentrations for which the lipid mixing step contributed significantly to the overall rate (Eq. 1). No differences were observed when the overall reaction was aggregation-rate-limited (e.g., 20 μM lipid, data not shown), supporting the conclusion made from the scatter curves in Fig. 5 *A* that these additives do not change the rate of aggregation. As shown in Fig. 5 *B*, lipid mixing rates for 3 mol % 18:1,18:1 DAG in PS vesicles were faster than those of the control vesicles. The average increases in rates calculated for a range of lipid concentrations (80–200 μM PS) and temperatures (15–40°C), were 1.3- and 1.9-fold that of control vesicles when 18:1,18:1 DAG was incorporated at 1.5 and 3.0 mol %, respectively (Table 1).

Vesicles with 6 mol % HD also demonstrated significantly greater rates of lipid mixing than control vesicles as indicated in the example (Fig. 5 *D*) with no alteration to aggregation rates (Fig. 5 *C*). Moreover, when the data were pooled across experiments that were primarily lipid mixing rate-limited, vesicles with 6 mol % HD showed an average increase in lipid mixing rates of 2.1 times the control values (Table 1).

18:1,18:1 DAG effects are independent of the divalent cation used

Previous experiments have indicated that the rate of lipid mixing is dependent on the type and concentration of divalent cation used to induce fusion among PS vesicles (e.g., Bentz et al., 1983; Nir, 1991, Walter and Siegel, 1993). Thus, to determine whether there was any interaction or synergism between the ion and 18:1,18:1 DAG effects, lipid mixing rates were determined as a function of the type of divalent cation for barium and calcium-induced interactions (Table 2). Regardless of the presence of 18:1,18:1 DAG, the lipid mixing rates with 3 mM Ca^{2+} were just over half of those induced by 3 mM Ba^{2+}. With both ions, the effect of 18:1,18:1 DAG was to double the lipid mixing rate. Neither 18:1,18:1 DAG nor HD altered the threshold concentrations of Ca^{2+} required for aggregation or lipid mixing (data not shown).

TABLE 1 Comparison of lipid mixing rates for control, 18:1,18:1 DAG, and HD PS vesicles + 3 mM calcium

Condition	Ratio of test to control*	Number of observations
1.5 mol% 18:1,18:1 DAG	1.3 (±0.2)	13
3.0 mol% 18:1,18:1 DAG	1.9 (±0.3)	15
6.0 mol% HD	2.1 (±0.2)	18

* Values represent the pooled averages for a range of lipid concentrations (80–200 μM PS) and temperatures (15–40°C). Conditions were selected so that the overall rate was dominated by lipid mixing rates and not by the rate of aggregation.

TABLE 2 Comparison of calcium and barium-induced lipid mixing rates for PS control and PS + 3 mol% 18:1,18:1 DAG LUVs at 20°C

Condition	Rate of dimer formation (% per s)		
	Control	+3 mol% DAG	+DAG/control
3 mM Ca^{2+}	3.9	7.3	1.9
3 mM Ba^{2+}	6.7	13.2	2.0
Ratio of Ca^{2+}/Ba^{2+}	0.58	0.55	—

Temperature dependence of lipid mixing for PS-18:1,18:1 DAG and PS-HD vesicles

Calcium-induced lipid mixing rates for PS vesicles increase rapidly with increasing temperature (Bentz et al., 1985; Walter and Siegel, 1993). The temperature dependencies of reaction rates can be used as an indicator of the nature of the rate-limiting process. To determine whether 18:1,18:1 DAG and HD were acting in a similar manner and if the magnitudes of the increases in lipid mixing rates were temperature-dependent, lipid mixing was measured as a function of temperature (10–50°C) for 80, 100, and 200 μM total PS and 3 mM Ca^{2+}. In every experiment and at every temperature tested, the rate of lipid mixing was increased by the addition of either 18:1,18:1 DAG or HD by a factor near 2 (Table 1). An example of one of these paired sets of experiments is shown in Fig. 6 for vesicles containing 18:1,18:1 DAG (3.0 mol %) or HD (6 mol %). Control, 18:1,18:1 DAG-, and HD-containing vesicle lipid mixing rates increased with temperature, yet the rate of the 3 mol % 18:1,18:1 DAG and 6 mol % HD vesicles were about twice that of the control vesicles over the entire temperature range. At the highest temperatures (50°C or more) the lipid mixing rates were partially aggregation-rate-limited. All data that were clearly aggregation-limited were eliminated from the calculations in Tables 1 and 3.

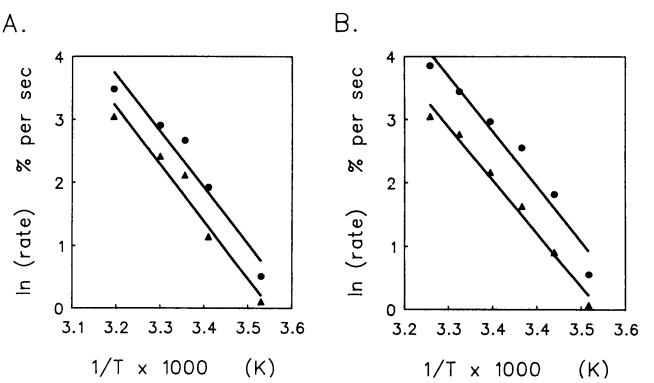

FIGURE 6 Arrhenius plots of the initial rate of lipid mixing for control, 18:1,18:1 DAG-containing, and HD-containing vesicles. (*A*) The natural logarithm of the rate of lipid mixing is plotted versus $1/T$ for control (trif) and 18:1,18:1 DAG-containing (circf) vesicles. (*B*) The natural logarithm of the rate of lipid mixing versus $1/T$ for control vesicles (trif) and vesicles containing 6 mol % HD in BBPS (circf). The solid lines represent the least squares fit to the data.

TABLE 3 Temperature dependence of lipid mixing rates for control, +18:1,18:1 DAG, and +HD BBPS vesicles

Conditions	Control	Activation energies (kJ/kmol) 1.5 mol% DAG	3 mol% DAG	6 mol% HD
200 μM BBPS	−90.6 (r = 0.995)	−96.4 (r = 0.995)		
100 μM BBPS	−87.3 (r = 0.986)		−90.6 (r = 0.986)	
200 μM BBPS	−86.5 (r = 0.999)		−84.0 (r = 0.988)	
200 μM BBPS	−98.9 (r = 0.997)			−98.9 (r = 0.997)
200 μM BBPS	−98.9 (r = 0.997)			−103 (r = 0.984)
100 μM BBPS	−94.8 (r = 0.997)			−98.9 (r = 0.995)
Average	−92.8 (n = 6)	−96.4 (n = 1)	−87.3 (n = 2)	−100 (n = 3)
SD	±5.5		±4.7	±2.4

Calcium was 3 mM.

The slope of these Arrhenius plots is related to the activation enthalpy of the reaction ($\Delta H^{\#}$) and the intercept is a function of the activation entropy ($\Delta S^{\#}$)and the attempt frequency. The lines represent the least squares fit to the data and appear to be parallel (Fig. 6). Parallel lines across a wide temperature range suggest that the additives effect primarily $\Delta S^{\#}$ or the attempt frequency. The values of the apparent $\Delta H^{\#}$ are tabulated in Table 3 and are not significantly different from each other regardless of the type of vesicle used in the experiment. The variation in slope across experiments was similar to or greater than the differences between the treatments: the paired data indicate that the difference with treatment is less than 2.6% of the control slope. Thus, although there may be solute-induced differences in the activation enthalpy that we could not detect, the present data is most consistent with the primary effect of the solutes being the on activation entropy or attempt frequency.

DISCUSSION

Low mole fractions of both 18:1,18:1 DAG and HD doubled the divalent cation-induced lipid mixing rate of PS large unilamellar vesicles (LUVs) with no apparent changes in lipid phase behavior or LUV aggregation rates. A surprising, and perhaps fortuitous, result is that the effect of these two very different additives was quantitatively the same for approximately the same volume of added alkyl chain. The temperature dependencies observed suggest that neither 18:1,18:1 DAG nor HD significantly altered the apparent overall activation enthalpy for lipid mixing, but that both seem to increase the apparent activation entropy or the attempt rate. The similar effects of these two apolar solutes suggest that their equilibrium dispositions in the bilayer in the absence or presence of divalent cations may not be directly relevant to their mode of action.

The effect of 18:1,18:1 DAG and HD on PS lipid mixing rates

Addition of either 3 mol % 18:1,18:1 DAG or 6 mol % HD results in a twofold increase in the divalent cation-induced lipid mixing rate of BBPS vesicles without altering aggregation rates (and, by inference, without altering the divalent cation binding constants; Bentz et al., 1985; Walter and Siegel, 1993). Neither solute has a major effect on the main phase transition temperature of BBPS either in the presence or absence of divalent cations, suggesting that the effect on lipid mixing is not mediated by some major structural modification of the BBPS bilayer. In contrast, in studies where 10–20 mol % DAG or DAG and phospholipase C are added to otherwise non-fusogenic lipids, these appear to work by inducing regions that can form isotropic phases (Ortiz et al., 1992) or via some complex involving Ca^{2+}, phospholipase C, DAG, and lipid (Nieva et al., 1993).

The effect of 18:1,18:1 DAG and HD was observed, regardless of whether the divalent cation used to induce lipid mixing was Ca^{2+} or Ba^{2+}, even though the equilibrium environments of HD and DAG are very different with these two ions. However, these apolar solutes seem to manifest their effect at a step that is common to both the calcium and barium lipid mixing pathways. The Ba^{2+}- and Ca^{2+}-induced mixing pathways differ in apparent activation enthalpies for the overall process (Walter and Siegel, 1993). Neither 18:1,18:1 DAG nor HD appear to have a major effect on the apparent activation enthalpy, suggesting that they are acting at a step different from the one(s) that distinguish the behavior of the two divalent cations.

The Arrhenius plots in Fig. 6 do not necessarily yield the true activation energies of the lipid mixing process, since the post-aggregation process very likely involves more than one step. Multistep and cooperative processes are not simply related to the overall activation energy (e.g., Bentz, 1992) so that under these conditions, an apparently linear Arrhenius-type temperature dependence could be fortuitous. However, subject to these caveats, we can make some qualitative inferences from these plots.

First, the temperature dependencies of the lipid mixing rate for 18:1,18:1 DAG-, HD-, and control vesicles are very similar over the entire temperature range. This implies that the overall mechanism of lipid mixing does not change fundamentally over the observed temperature range. Second, the major differences between the plots for control versus HD- or 18:1,18:1 DAG-containing vesicles are in the y intercepts. If the apparent rate constant has an Arrhenius temperature dependence, our results suggest that the activation entropy,

attempt frequency, or both are different in the presence versus the absence of HD and 18:1,18:1 DAG. The attempt frequency could reflect the rate of structural fluctuations within apposed bilayers and/or the rate of extremely close contact of the interfaces of aggregated vesicles. Changes in the activation entropy could reflect changes in the configurational freedom of the acyl chains in the intermediate(s), demixing effects, or other factors.

We think it is unlikely that 18:1,18:1 DAG or HD substantially changes the frequency of contacts between aggregated vesicles. Both solutes alter the PS inter-headgroup spacing, and hence the surface charge density and surface hydrophobicity of the LUV, which could affect the contact rate (Ohki and Arnold, 1990). However, the effect of 18:1,18:1 DAG on the surface potential is greater than the effect of HD (McIntosh et al., 1980; Pope et al., 1989; Cunningham et al., 1989), as expected, since the 18:1,18:1 DAG headgroup resides at the lipid-water interface even when equilibrated with Ca^{2+} (Hamilton et al., 1991), whereas HD resides primarily in the bilayer. These differences in solute distribution make it unlikely that the lipid mixing effects observed here are due to changes in the headgroup region.

From the arguments above, it seems more likely that these solutes affect the apparent activation entropy of lipid mixing. One hypothesis consistent with this is a role for HD and 18:1,18:1 DAG in stabilizing the transient hydrophobic voids within intermediates in the lipid mixing process. Such voids are thought to form between the monolayers as regions of the bilayers bend to form fusion intermediates (e.g., Siegel, 1986, 1993b). In the absence of apolar solutes, the acyl chains of surrounding phospholipids must adopt nearly all *trans* configurations of reduced entropy to fill these voids (e.g., Gruner, 1989; Sjoland et al., 1989). Both HD and 18:1,18:1 DAG, as additional hydrocarbons, could contribute to filling these voids, which would increase the entropy of the intermediate, increase the apparent activation entropy, and thereby increase the rate of lipid mixing.

Correlation between DAG and HD locations and their effect on lipid mixing rates

The ^{2}H-NMR results show that, at equilibrium, the time-averaged orientation/distribution in the bilayer differs for DAG and HD in BBPS/Ca^{2+} and BBPS/Ba^{2+} bilayers. Therefore the equilibrium positions in the bilayer of these solutes cannot explain the similarity of their effect on lipid mixing rates. One explanation is that the two solutes work in different ways to give what appears to be the same effect in this limited study. For example, the more surface active 18:1,18:1 DAG may facilitate bilayer bending by its effects on headgroup separation (Tate and Gruner, 1987; Siegel et al., 1989a). Alternatively, the effect of 18:1,18:1 DAG might be to change the membrane surface properties (Ohki and Arnold, 1990). In contrast, HD may stabilize the associated voids similar to what has been proposed for stabilization of the inverted H_{II} phase in phosphatidylethanolamines (PE) (Gruner, 1989; Sjoland et al., 1989).

A second explanation is that the time-averaged orientation/distribution of DAG and HD in the bilayer does not reflect their site of action during the lipid mixing process. Instead, it may be that the distribution of these molecules in the immediate vicinity of a fusion event, on the time scale of the event, is different from what is observed at equilibrium. Neither solute is rigidly held in position; e.g., both can rapidly exchange from one monolayer to the other (Hamilton et al., 1991; Pope and Dubro, 1986). In the present study, PS mixing occurs before the vesicles collapse and leak, which means that lipid mixing occurs when the divalent cation is asymmetrically distributed across the bilayer, making it likely that added solutes are not dispersed identically (or necessarily equally) in the two monolayers.

Differences in the HD environment in Ca^{2+} versus Ba^{2+}/BBPS

HD exists in very different time-averaged environments in Ca^{2+}/BBPS and in Ba^{2+}/BBPS (Fig. 4, *D* and *E*), which might be expected since the two divalent cation-lipid complexes are different at the relevant temperatures. Ca^{2+}/BBPS is far below its melting point (120°C; Casal et al., 1987) at the experimental temperatures, whereas Ba^{2+} is close to T_m as determined by DSC (about 25°C, Fig. 3 *B*). The structures of Ca^{2+}/BBPS and Ba^{2+} complexes are different: Ca^{2+} binding eliminates the chain tilt in PS bilayers, whereas Ba^{2+} does not (Hauser and Shipley, 1984). Similarly, Ca^{2+} appears to induce better lateral chain order than Ba^{2+} (at least in saturated-chain PS; Hauser and Shipley, 1984).

Models for divalent cation-induced PS lipid mixing

Although there are fairly explicit models for membrane fusion that involve intermediates in bilayer/nonbilayer transitions (Siegel, 1986, 1993b), these are relevant only for systems that are near the lamellar to cubic or inverted hexagonal phase transitions, such as PE or monomethyl-PE. Recent reports suggest that 20–30 mol % 18:1,18:1 DAG can induce non-lamellar phases in PC:PS (1:1) membranes and thereby induce divalent cation-dependent lipid mixing (Ortiz et al., 1992).[1] However, pure PS does not undergo lamellar to cubic or hexagonal phase transitions except at very extreme conditions of low water activity (Das and Rand, 1986) or very low pH (de Kroon et al., 1990). The addition of DAG and

[1] We note that the design of the study by Ortiz et al. (Ortiz et al., 1992) does not permit them to distinguish between many possible mechanisms by which the addition of these huge amounts of 18:1,18:1 DAG result in apparent fusion. First, the amounts of 18:1,18:1 DAG are too high to be ideally mixed in PC (Cunningham et al., 1989). Based on our data, the effect could have been similar to ours via the PS component of the membrane. The DAG could also have changed the surface hydrophobicity as proposed by Ohki and Arnold (1990) or it may have promoted intermediates associated with the non-lamellar phase which they observed in some fraction of their lipid at equilibrium.

calcium does not induce PS to form H_{II} structures (Das and Rand, 1986). We confirmed this for our system by x-ray analysis of Ca^{2+}-equilibrated BBPS/18:1,18:1 DAG: this system is lamellar throughout the temperature range of these experiments (data not shown). Therefore, HD and 18:1,18:1 DAG do not act by stabilizing exactly the same sort of intermediates that form near lamellar/inverted cubic or lamellar/inverted hexagonal phase transitions.

Discontinuities in the lipid packing between divalent cation-free and bound regions have been proposed as intermediates for PS vesicle fusion, although there is no definitive evidence supporting this hypothesis in pure PS systems (e.g., Bentz et al., 1985; Walter and Siegel, 1993). There is no evidence from the DSC experiments that 18:1,18:1 DAG and HD stabilize separations of solid/solid, solid/liquid, or liquid/liquid phases in PS bilayers at equilibrium. Effective dehydration of the apposed membrane interfaces has also been proposed as necessary and possibly sufficient for membrane fusion (Ohki and Arnold, 1990). Again, since the surface effects of HD and 18:1,18:1 DAG are different, it currently seems unlikely that these two solutes act by enhancing surface dehydration.

CONCLUSIONS

We have shown that the apolar solutes HD and 18:1,18:1 DAG substantially increase the rate of divalent cation-induced lipid mixing between BBPS vesicles. The effects of the two substances are the same for an equivalent added volume of each solute. Despite the different equilibrium dispositions of HD and 18:1,18:1 DAG within PS bilayers, and differences in the dispositions of each solute in the presence of Ca^{2+} compared with Ba^{2+}, both increased Ca^{2+}-induced lipid mixing rate by the same factor, and 18:1,18:1 DAG increased the rates similarly in the presence of Ca^{2+} and Ba^{2+}. 18:1,18:1 DAG and HD do not appear to alter the fundamental mechanism of lipid mixing, since the temperature dependencies of the Ca^{2+}-induced lipid mixing rates of control, 18:1,18:1 DAG-, and HD-containing LUV are very similar. The fact that 18:1,18:1 DAG increases the rate of Ba^{2+} and Ca^{2+}-induced lipid mixing by the same factor implies that it acts at a different spatial or temporal point than the divalent cations in the lipid mixing process. A role for HD and 18:1,18:1 DAG in filling voids within lipid mixing intermediates is consistent with our data, although exactly how this or other energetic gains are achieved at the molecular level is yet to be fully explained.

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