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Fusion and Phase Separation Monitored by Lifetime Changes of a Fluorescent Phospholipid Probe†

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ABSTRACT: The sensitivity of the fluorescence lifetime of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) to its local concentration in lipid bilayers was used to monitor both lipid mixing and phase separation occurring during membrane vesicle fusion. Vesicles containing 2 mol % DPHpPC were mixed with a 10-fold excess of vesicles devoid of probe. Upon addition of a fusogen, mixing of bilayer lipids associated with fusion was followed as an increase in the fluorescence lifetime of DPHpPC. Ca^{2+}-induced fusion of phosphatidylserine vesicles served to test the method and was shown to have an exponential half-time of 7 s. Phase separation (between the phosphatidylserine head groups of bulk lipid and the phosphatidylcholine head groups of the probe) was monitored by DPHpPC under the same conditions used to follow lipid mixing due to fusion. Phase separation was not significant until 10 min after Ca^{2+} addition and was completely reversible by disodium ethylenediaminetetraacetate addition. Vesicle aggregation induced by Ca^{2+} addition to mixed phosphatidylserine/phosphatidylcholine vesicles did not alter the DPHpPC lifetime, indicating that close association of vesicles did not promote intervesicular exchange of the probe. In addition, we have investigated the effects of Ca^{2+} on the fluorescence properties of this probe and of the head-group-labeled fluorescent probes *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine and *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine, which are used in the fluorescence energy transfer assay of Struck et al. [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099]. Ca^{2+} was shown to quench the fluorescence intensity of the head-group-labeled probes, while the hydrophobic environment surrounding the fluorescent moiety of DPHpPC appeared to protect it from the direct influence of such water-soluble fusogens. Overall, monitoring of DPHpPC fluorescence lifetime offered some significant advantages over current methods for detecting phospholipid reorganizations accompanying membrane fusion.

An understanding of the membrane fusion process is essential to the development of a molecular appreciation of a wide range of cellular phenomena, including exocytosis and endocytosis. The molecular events of fusion are difficult to examine in complex natural membranes; therefore, well-defined synthetic and purified native lipid vesicles are the preferred system for studying bilayer changes associated with fusion.

Two classes of fluorescent assays have been used to study vesicle fusion. One detects the mixing of internally trapped vesicle contents, and the other detects the mixing of lipid bilayer components between two vesicle populations. Both types of assays are essential for unequivocal monitoring of membrane fusion, since lipid mixing could result from phospholipid exchange and contents mixing could be confused with vesicle leakage. Several assays fall into the first of these two categories (Vanderwerf & Ullman, 1980; Ellens et al., 1985; Kendall & MacDonald, 1982; Wilschut et al., 1980). The popular lipid mixing assay of Struck et al. (1981) makes use of changes in intensity resulting from fluorescence energy transfer between *N*-(4-nitro-2,1,3-benzoxadiazolyl) (NBD) and *N*-(lissamine Rhodamine B sulfonyl) (Rh) attached to the head-group region of phosphatidylethanolamine (PE)[1] molecules. Both NBD-PE and Rh-DOPE are placed in the same vesicle population at concentrations which allow energy transfer from NBD to Rhodamine. Fusion of these vesicles

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; $DC_{15}PC$, 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; NBD-PE, *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine; Rh-DOPE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; REV, reverse-phase evaporation vesicle(s); LMV, large, multilamellar vesicle(s); Na_2EDTA, disodium ethylenediaminetetraacetate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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with non-probe-containing vesicles results in dilution of the probes and an increase in NBD fluorescence accompanied by a decrease in Rhodamine fluorescence. However, both the NBD and Rhodamine moieties are shown here to be quenched by fusogenic agents added to the aqueous medium surrounding the vesicles under study. This quenching seriously complicates quantitative interpretation of lipid mixing data.

We report an alternative approach for monitoring the mixing of lipid during membrane fusion. In addition, the same method can report changes in the local concentration of a particular phospholipid species and, therefore, is capable of detecting local domains or redistributions of lipids that may be crucial to the fusion process. The proposed assay is based on the observation that the fluorescence lifetime of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) is sensitive to its concentration in the bilayer. The use of an intensive as opposed to an extensive property of the probe (fluorescence lifetime vs. fluorescence intensity) facilitates comparison of results obtained under different conditions. These results can be quantitatively interpreted in terms of the molecular events involved in the fusion process. While our new assay does not allow for continuous monitoring of membrane changes, the ease of interpretation of lifetime data and the inaccessibility of the fluorescent moiety of DPHpPC to the outside medium make it an attractive alternative to conventional lipid mixing assays.

Materials and Methods

Materials

Bovine brain phosphatidylserine, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC), 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine ($DC_{15}PC$), and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) as well as the fluorescent probes *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine (NBD-PE), *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-DOPE), and 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)-phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). These compounds were found to be greater than 98% pure by thin-layer chromatography (Analtech GHL plates; 0.01 M dipotassium oxalate). TLC plates were developed in 65:25:4 $CHCl_3/CH_3OH/H_2O$ (v/v) and stained with iodine. Fluorescent compounds were also viewed under near-UV or visible light before iodine staining. Stock solutions of DPPC in $CHCl_3$ were filtered over Norit-A neutral activated charcoal to remove trace fluorescent contaminants.

Ultrapure KCl was obtained from Heico, Inc. (Delaware Water Gap, PA; lot 2179). *N*-[Tris(hydroxymethyl)-methyl]-2-aminomethanesulfonic acid (TES) was purchased from Research Organics Inc. (Cleveland, OH; lot 1344). Other materials were of the highest quality available. Buffer solutions were filtered twice through 0.22-μm GS filters (Millipore Corp., Bedford, MA) prior to use.

Methods

Vesicle Preparation. In general, two vesicle samples were used for each experiment: one containing pure lipid or lipid mixtures and the other containing lipid and probe molecules at the specified molar ratio. Vesicles devoid of probe were nonfluorescent and are referred to as "unlabeled" vesicles. Preparations made with the probe DPHpPC were kept in amberized glassware (Reliance Glass Works, Inc., Bensenville, IL) due to the light sensitivity of this compound (Parente & Lentz, 1985). Reverse-phase evaporation vesicles (REV) were prepared in 50 mM KCl, 10 mM TES, and 0.1 mM EDTA, pH 7, as described by Szoka et al. (1980) and filtered through 0.4- and 0.2-μm filters (Nuclepore Corp., Pleasanton, CA). Isopropyl ether for REV preparation was distilled over $LiAlH_4$ and passed over an alumina column before use to remove impurities (Parente & Lentz, 1984). Large, multilamellar vesicles (LMV) were prepared in 50 mM KCl by gentle agitation of a hydrated lipid sample as described previously (Lentz et al., 1976).

Fluorescence. An SLM 4800 spectrofluorometer (Urbana, IL) equipped with a modified, three-position, multitemperature cuvette holder was used for all fluorescence measurements (Barrow & Lentz, 1985). DPHpPC-, NBD-PE-, and Rh-DOPE-containing samples were excited by using the 366-, 435-, and 547-nm mercury lines, respectively, of a 200-W mercury–xenon light source (Canrad-Hanovia, Newark, NJ).

For lifetime measurements, emission was monitored through a high-pass KV-450 filter (50% transmittance at 450 nm; Schott Optical Glass, Duryea, PA). Values of phase angle shifts and modulation ratios were recorded (Barrow & Lentz, 1983), and the derived lifetimes were calculated by the method of Spencer and Weber (1969) using a modulation frequency of 30 MHz and an isochronal reference standard (Barrow & Lentz, 1983) of DPH in heptane (τ = 6.78 ns; 2×10^{-7} M). The reference standard was maintained at 23 °C while the temperature in the other two positions of the cuvette holder was variable. Corrections were routinely made for background intensities of nonfluorescent vesicle samples when they were greater than 0.5% of the intensity of the fluorescent samples (Barrow & Lentz, 1985). Lifetimes of samples with an apparent absorbance greater than 0.2 OD unit were corrected for instrumental anomalies reflective of high sample turbidity (Barrow & Lentz, 1985). This correction was necessary in most of the fusion studies described, since sample turbidity changed over the time course of the experiment. To make these corrections, vesicle samples containing DPHpPC were adjusted by adding varying amounts of glycogen to give a known turbidity. A standard curve of lifetime vs. turbidity (measured at 366 nm on a Hitachi Model 100-20 spectrophotometer) was constructed with these samples. For *our* phase fluorometer, this correction amounts to −3.75 ns/ODU, which can be significant for a sample with apparent absorbance of 0.3 or 0.4 ODU. To correct an observed lifetime value, sample turbidity was measured, and the change in lifetime at this optical density was read from the standard curve. This difference was then subtracted from the observed lifetime to give the corrected value.

Results

Concentration Dependence of DPHpPC Lifetime. The spectroscopic and physical properties of DPHpPC in lipid bilayers were described previously (Parente & Lentz, 1985). This phospholipid analogue differs from a normal dipalmitoylphosphatidylcholine molecule at the 2-position of the glycerol carbon backbone where (carboxyethyl)diphenylhexatriene replaces the saturated 16-carbon fatty acid. The fluorescence lifetime of DPHpPC was measured in DPPC LMV (0.15 mM) containing various lipid to DPHpPC ratios in order to quantitatively examine the effect of membrane probe concentration on its fluorescence lifetime. In Figure 1, we show the DPHpPC lifetime derived from phase shift measurements vs. lipid:probe ratio in DPPC vesicles above their phase transition temperature (48 °C). Only the phase-derived lifetime measured at 30 MHz is shown, although similarly shaped curves were obtained for both phase and modulation lifetimes recorded at 6-, 18-, and 30-MHz modulation fre-

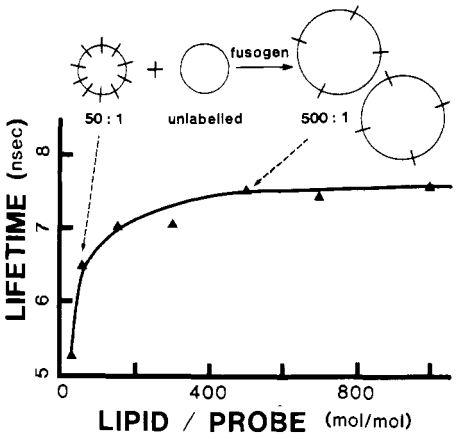

FIGURE 1: Dependence of the DPHpPC fluorescence lifetime on the lipid to probe ratio in DPPC LMV. The lipid concentration was 0.15 mM, and the vesicles contained varying amounts of DPHpPC. Phase and modulation lifetime measurements were recorded at 6-, 18-, and 30-MHz modulation frequencies for all samples. For simplicity, only data from the phase-derived lifetime at 30 MHz are plotted. Above this plot is a drawing illustrating how lifetime changes at varying lipid to probe ratios can be used as an assay of intervesicular lipid mixing during fusion. Arrows point to the expected lifetime values of vesicles at the beginning and end of the fusion process.

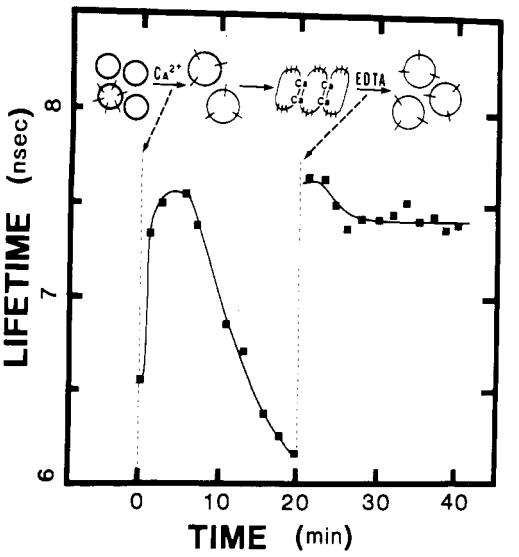

FIGURE 2: DPHpPC lifetime vs. time after Ca^{2+} addition to induce membrane fusion of bovine PS REV. Vesicles containing 2 mol % DPHpPC (0.01 mM total lipid) were mixed with a 10-fold excess of nonfluorescent vesicles (0.1 mM). Dashed lines indicate times of Ca^{2+} and Na_2EDTA addition, respectively. The accompanying diagram summarizes our interpretation of the Ca^{2+}-induced fusion process in bovine PS REV. The decline in lifetime from 10 to 20 min is attributed to a phase separation of PS by Ca^{2+} which is reversible by Na_2EDTA addition.

quencies (data not shown). The absolute values of these measurements differed by ±0.5 ns at the different frequencies. These differences are small since the fluorescence decay of DPHpPC is nearly single exponential at all temperatures (Parente & Lentz, 1985). A sharp decline in the fluorescence lifetime was observed at lipid to probe ratios less than 200:1, as shown in Figure 1. Above 500 lipids per probe, the lifetime remained constant at different lipid to probe ratios. Varying the lipid species (i.e., bovine PS or mixtures of bovine PS/POPC and dioleoyl-PG/POPC) resulted in an identical concentration dependence of the probe lifetime. The value of the probe lifetime was temperature dependent (Parente & Lentz, 1985), although the shape of the DPHpPC concentration curve was not (data not shown). A similar pattern of lifetime behavior has been observed for the parent fluorophore diphenylhexatriene (Barrow & Lentz, 1985).

Principle of Lipid Mixing Assay. The reduction of DPHpPC lifetime described above has been exploited to develop an assay for the mixing of bilayer components during fusion. The design of this assay is illustrated by the diagram in the upper portion of Figure 1. One vesicle population containing 2 mol % probe (fluorescence lifetime roughly 6.5 ns) is combined with a 10-fold excess of a second, nonfluorescent vesicle population, as indicated by the left-hand arrow of Figure 1. Bilayer mixing induced by the addition of Ca^{2+}, a known fusogenic agent, is expected to result in a reduction of the probe concentration in the newly formed bilayer and, thus, an increase in lifetime to roughly 7.5 ns (right-hand arrow of Figure 1). Errors in our lifetime measurements were typically in the range of 0.05–0.1 ns, which means that this technique was sensitive to small changes in the probe environment. Each reported lifetime value was obtained by averaging 10 individual measurements made in the course of 1–2 min. The fluorescence lifetime is an intensive quantity and is not dependent on the concentration of vesicles present in any given experiment. This greatly facilitated comparison of data from different experiments and allowed us to distinguish between fusion reactions involving the mixing of bilayer components from several vesicles and those involving only two vesicles.

Aggregation and Probe Transfer. To establish the viability of this assay, aggregation and probe transfer must be shown not to interfere with detection of fusion and phase separation. The fusion of bovine PS-containing vesicles has been reported in previous studies of internal contents and lipid mixing (Wilschut et al., 1980; Struck et al., 1981). Conditions for reversible Mg^{2+}- or Ca^{2+}-induced aggregation of either pure PS or mixed PS/PC vesicles have also been detailed (Wilschut et al., 1981; Fraley et al., 1980). To differentiate aggregation from fusion, Fraley et al. (1980) have used the Tb^{3+}/dipicolinic acid contents mixing assay to show that the contents of two populations of 50:50 bovine PS/egg PC REV did not mix in the presence of Ca^{2+} even though vesicle aggregation did occur. We have extended these results to a 50:50 bovine PS/POPC REV system and have used these vesicles to test our lipid mixing assay for interference due to probe transfer between aggregated vesicles. In the presence of 5–10 mM Ca^{2+}, no change in the lifetime of DPHpPC was observed when vesicles containing 2 mol % probe were mixed with a large excess of unlabeled vesicles, even though sample turbidity increased substantially under these conditions. This result indicates that vesicle aggregation did not result in a fluorescence lifetime shift and that probe exchange did not occur between vesicles which came into close contact but did not fuse.

Fusion and Phase Separation. For fusion studies, bovine PS REV containing 2 mol % DPHpPC (0.01 mM total lipid) were mixed at room temperature with bovine PS REV (0.1 mM) devoid of probe. Mixing of the unlabeled and fluorescently labeled vesicles did not alter the initial low fluorescence lifetime value. Ca^{2+} was added to a final concentration of 6.5 mM at time zero (Figure 2). Within 5 min, the lifetime of DPHpPC increased to a value observed for vesicles containing greater than 500 lipid molecules per probe molecule (7.5 ns). This increase indicates a dilution of probe molecules and the occurrence of lipid mixing associated with fusion. An unexpected decline in lifetime was observed after 10 min (Figure 2). According to our standard curve of lifetime vs. lipid:probe ratio (Figure 1), this lower value implied an increased local concentration of DPHpPC molecules within some portion of the bilayer. The observed lifetime decline is consistent with

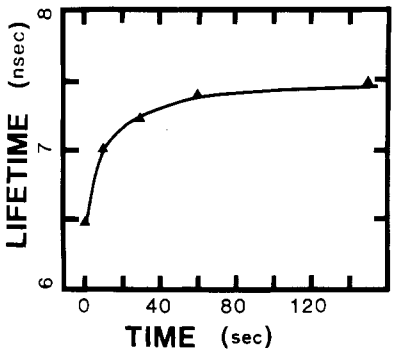

FIGURE 3: Time course of the early events of Ca^{2+}-induced PS fusion monitored by DPHpPC lifetime changes. Vesicle populations were mixed as described in Figure 2, and 6.5 mM Ca^{2+} was added at time zero to initiate fusion. Each time point represents the lifetime at the time of Na_2EDTA addition (11 mM) to halt the fusion process.

the previously reported phase separation induced by Ca^{2+} sequestering of phosphatidylserine molecules (Papahadjopoulos, 1978).

Addition of Na_2EDTA (11 mM) after 20 min to chelate Ca^{2+} increased the DPHpPC lifetime to that expected for complete mixing of the lipids in the labeled and unlabeled vesicle populations, as observed at the 5-min time point (see Figure 2). This final lifetime value remained unchanged over the next 20 min. The events presumed to occur in conjunction with the observed lifetime changes are diagrammed in the upper portion of Figure 2, with arrows to indicate the addition of Ca^{2+} and Na_2EDTA.

As mentioned earlier, the individual time points for our bilayer mixing assay represent an average of 10 lifetime measurements made during a time span of 1–2 min. For this reason, the rapid initial rate of lipid mixing during vesicle fusion had to be studied by stopping the Ca^{2+}-induced reaction (final Ca^{2+} concentration 6.5 mM) with the addition of Na_2EDTA (11 mM) at various time points before lifetime measurements were recorded. No change in lifetime was evident when measured for up to 30 min after the fusion reaction was haltered. From the resulting time course of lipid mixing shown in Figure 3, the half-time of the fusion process was determined to be 7 s.

The reversible reduction in the DPHpPC lifetime 10 min after Ca^{2+} addition, noted above, suggested a phase separation induced by Ca^{2+} sequestering of PS molecules. We were able to test directly for phase separation (independent of fusion) by slightly altering our assay design. As indicated in Figure 4, Ca^{2+} was added at time zero (6.5 mM final Ca^{2+} concentration) to a suspension of bovine PS REV containing 0.2 mol % probe (500:1 lipid to probe ratio). The DPHpPC lifetime was initially at its maximum value (according to Figure 1) and remained relatively constant until about 10 min (see Figure 4). At this point, the lifetime declined, indicating an increase in the local probe concentration (refer to Figure 1). Na_2EDTA was added (final concentration 11 mM) 20 min after Ca^{2+} addition. This immediately reversed the change associated with the presence of Ca^{2+} and returned the lifetime reported by DPHpPC back to its initial value for vesicles containing a 500:1 lipid to probe ratio (7.5 ns). A model of the events presumed to occur is depicted in Figure 4, with arrows to indicate the addition of Ca^{2+} and Na_2EDTA.

In Figures 2 and 4, the lifetime rose above the maximum value obtained from the standard curve of Figure 1 (approximately 7.5 ns) at the 8- and 20-min time points. These increases occurred at points where the turbidity of the vesicle solutions, measured in an identical but parallel experiment, was in rapid flux. As a result, the turbidity at the time of the

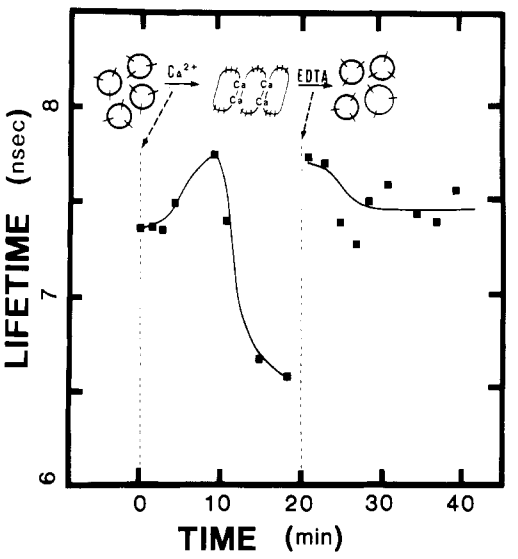

FIGURE 4: Change in DPHpPC lifetime with time to monitor Ca^{2+}-induced phase separation. Bovine PS REV (0.1 mM) containing 0.2 mol % DPHpPC were treated with Ca^{2+} (6.5 mM) at time zero (dashed line). Na_2EDTA (11 mM) was added at 20 min (dashed line) to disaggregate vesicles. The diagram represents our interpretation of the data in terms of Ca^{2+}-induced phase separation.

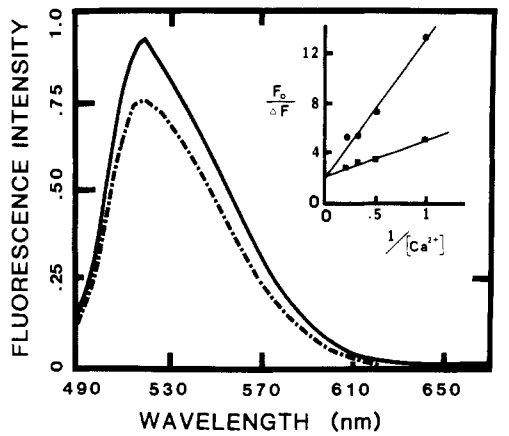

FIGURE 5: Effect of Ca^{2+} addition on the fluorescence intensity of $DC_{15}PC$ REV containing 5 mol % NBD-PE. The solid line represents the emission spectrum recorded in the absence of Ca^{2+}. The dashed line is the spectrum resulting from vesicles incubated with 5 mM Ca^{2+} for 10 min. Excitation wavelength = 435 nm. Spectra were recorded at room temperature from 490 to 700 nm. The inset shows the dependence of Rh-DOPE (squares) and NBD-PE (circles) relative fluorescence intensity on the concentration of added Ca^{2+} plotted according to a modified Stern–Volmer equation (Lehrer, 1971). F_0 is the fluorescence intensity in the absence of Ca^{2+}, and ΔF is the difference between F_0 and the fluorescence observed in the presence of indicated Ca^{2+} concentrations.

lifetime measurement may have been overestimated, and the required turbidity corrections (see Methods) may have overcompensated for the apparent decrease in lifetime resulting from sample turbidity. Such errors would be expected to be greatest at times of rapid changes in sample turbidity.

Interaction of Probes with Ca^{2+}. To determine if Ca^{2+} alters the measured fluorescence properties of probes used in lipid mixing assays, we chose to look at NBD-PE and Rh-DOPE as well as the DPHpPC probe used here. NBD-PE and Rh-DOPE are used in the popular fluorescence energy transfer assay of Struck et al. (1981). Ca^{2+} quenched the fluorescence intensity of the head-group-labeled probes incorporated in both negatively charged and neutral lipid vesicles. Figure 5 shows the uncorrected fluorescence intensity spectrum of $DC_{15}PD$ REV containing 5 mol % NBD-PE recorded in the absence

of Ca^{2+} (solid line). The dashed line in Figure 5 is the spectrum obtained 10 min after Ca^{2+} was added to a final concentration of 5 mM. Additional spectra were also recorded 10 min after Ca^{2+} was added to identical vesicle samples or to vesicles containing 5 mol % Rh-DOPE at several different final concentrations. The inset to Figure 5 summarizes the results of these sequential quenching experiments in terms of modified Stern–Volmer plots (Lehrer, 1971). The y intercept of these plots, $1/f_a$, is equal to the inverse of the fraction of probe molecules accessible to the quencher, and the slope equals $1/f_aK$, where K is the Stern–Volmer quench constant of the accessible fraction. For the large, unilamellar vesicles used, $f_a = 0.5$, which means that probe molecules are equally distributed in both bilayer leaflets. The quench constant obtained for NBD-PE was 1.8×10^{-4} M^{-1}, while that for Rh-DOPE was 7.1×10^{-4} M^{-1}. At this point, we have insufficient data to distinguish between a static or collisional quenching mechanism.

Similar results were obtained in vesicles composed of negatively charged phospholipids. When 2 mol % NBD-PE or Rh-DOPE was present separately in bovine PS REV, a 50% or 82% drop in fluorescence intensity was observed, respectively, 10 min after addition of 5 mM Ca^{2+}. With either lipid species, addition of Na_2EDTA (10 mM final concentration) resulted in a 80–95% return of the initial fluorescence intensity.

Hoekstra (1982a) has reported that Ca^{2+} quenching of the NBD-PE probe is due to Ca^{2+}-induced phase separation in vesicles rich in bovine PS. Our results demonstrate that this is not the only explanation of fluorescence quenching, since Ca^{2+} added to either NBD-PE- or Rh-DOPE-containing phosphatidylcholine vesicles resulted in substantial fluorescence quenching (Figure 5 inset). In pure phosphatidylcholine vesicles, Ca^{2+} should not induce vesicle fusion or phase separation. Therefore, quenching must be due to direct interactions of Ca^{2+} with the fluorescent moiety of the probe. This Ca^{2+}-induced quenching can cause difficulties in making quantitative interpretations of lipid mixing data with the NBD-PE and Rh-DOPE probes. Morris and Bradley (1984) have also reported interactions of these probes with Ca^{2+} when incorporated in chromaffin granules. Although they saw an enhancement, rather than a decrease, in fluorescence intensity when using the Rh-DOPE probe, this study further emphasizes the possibility of misinterpreting data obtained by using these probes unless the effects of agents added via the aqueous medium can be quantitated.

In related experiments designed to determine if Ca^{2+} interacts with DPHpPC to alter its fluorescence lifetime, up to 10 mM Ca^{2+} was added to DPPC REV containing 2 mol % probe. No change in lifetime was observed, indicating no Ca^{2+}–phospholipid interaction capable of perturbing the fluorescent properties of the fluorophore buried in the hydrophobic region of the bilayer. This is not surprising, since the binding constant of phosphatidylcholine vesicles for Ca^{2+} has been reported to be 13.8 M^{-1} (Altenbach & Seelig, 1984).

Discussion

Advantages of the DPHpPC Lipid Mixing Assay. We have developed an assay which utilizes the concentration-dependent fluorescence lifetime of a relatively new phospholipid probe, DPHpPC, to detect lipid mixing and lipid segregation associated with vesicle–vesicle fusion and phospholipid phase separation, respectively. While we have taken advantage of its lifetime behavior, other features of DPHpPC were equally important in making it a useful probe of membrane fusion. The fluorescent moiety of DPHpPC, diphenylhexatriene, is located in the hydrophobic region of the bilayer where it is inaccessible to hydrophilic molecules in the aqueous medium. Its position relative to the bilayer plane is well-defined, since it is covalently attached to the glycerol backbone of a lipid molecule. DPHpPC is also highly fluorescent (with an extinction coefficient of 60 000 ODU M^{-1} cm^{-1} at 355 nm in $CHCl_3$; Parente & Lentz, 1985), making it easy to monitor in dilute membrane suspensions. Finally, Morgan et al. (1982) have shown that DPHpPC does not spontaneously transfer between vesicles, an important requirement if the probe is to be used to monitor fusion-induced lipid mixing.

The basis for this assay is the change in fluorescence lifetime observed when DPHpPC is located in different local environments. When starting with vesicles containing greater than 1 mol % probe, fusion with nonfluorescently labeled vesicles results in a decrease in the local concentration of probe molecules, as reflected by an increase in probe fluorescence lifetime. Utilizing this property of the lifetime, one can quantitate the extent of lipid mixing at early times in the fusion process (i.e., when only one or two rounds of fusion have occurred). This is accomplished by comparing the observed lifetime to the standard curve (Figure 1) to obtain the extent of probe dilution resulting from the fusion process. Because of the ambiguities associated with Ca^{2+} quenching and because the exact efficiency of energy transfer will be quite sensitive to the detailed design of an experiment, obtaining such a quantitative interpretation would be much more difficult, if not impossible, with the NBD-PE/Rh-DOPE assay (Struck et al., 1981; Hoekstra, 1982a,b).

We note also that our assay cannot rigorously be interpreted quantitatively when fusion occurs in an inhomogeneous fashion (e.g., when significantly less than one round of fusion occurs). This is because our calibration curve (Figure 1) was constructed by using a homogeneous population of vesicles containing uniformly distributed probe molecules. However, since the average lifetime observed from a mixture of two closely spaced lifetimes is roughly the intensity-weighted average of the two components (Barrow & Lentz, 1985), extension of a quantitative interpretation to a slighly inhomogeneous population should not be seriously in error.

Fusion and Phase Separation. In the fusion process induced by Ca^{2+}, one cannot easily distinguish between reversible vesicle aggregation and the irreversible events leading to fusion (Papahadjopoulos, 1978). In the specific case of PS and Ca^{2+}, a rigid anhydrous interbilayer complex forms which eventually leads to the development of large cochelated cylinders (Papahadjopoulos et al., 1975). Papahadjopoulos and co-workers [for a review, see Papahadjopoulos (1978)] have proposed that separation of the rigid Ca^{2+}–PS phase could destabilize the bilayer structure in a manner favoring the fusion process. Other lipid species present presumably should be excluded from this rigidly packed matrix. With our lipid mixing assay, one can separate the fusion process from other lipid structural changes (Figures 2 and 4). We can discern the formation of patches of phosphatidylcholine containing high concentrations of probe molecules by a dramatic drop in DPHpPC lifetime when Ca^{2+} sequesters PS molecules.

Others (Hoekstra, 1982b; Silvius & Gagne, 1984a,b; Bentz et al., 1985; Wilschut et al., 1985) have raised the question of whether this structural PS–Ca^{2+} complex initiates vesicle fusion in this system. Hoekstra (1982b) followed the kinetics of phase separation by monitoring NBD quenching when Ca^{2+} was added to bovine PS/NBD-PE small, unilamellar vesicles. Likewise, fusion kinetics were followed by looking at the decrease in NBD fluorescence when bovine PS/NBD-PE and bovine PS/Rh-DOPE vesicles were mixed in the presence of

Ca^{2+}. He determined the half-time of the fusion reaction to be 5 s, while that of phase separation was 1 min. The half-time that we have measured for fusion in PS REV (see Figure 3) was 7 s, in good agreement with Hoekstra's report. While we did not measure the half-time of the phase separation event, we did not see substantial indication of its occurrence until 10 min after Ca^{2+} addition. Our use of larger vesicles may be responsible for the apparently slower onset of phase separation. Despite ambiguities associated with Ca^{2+} quenching of NBD-PE and Rh-DOPE fluorescence (Figure 5), we agree wtih Hoekstra that substantial phase separation occurred only some time after fusion and, therefore, is not a prerequisite for fusion.

Only extensive phase separation resulting in patches of highly concentrated probe molecules (greater than 1 mol %) will be detected by our method or by the method of Hoekstra (1982a,b; greater than 5 mol %). The extensive phase separation detected by us and by Hoekstra (1982b) probably reflects the anhydrous, interbilayer complex described by Papahadjopoulos et al. (1975). It is not surprising that formation of this extensive and highly organized structure is a slow process relative to fusion. Local patch formation would be expected to be much faster (controlled by the rate of lipid lateral diffusion) and could still be an important component of the fusion process, despite the results reported here and by Hoekstra (1982b).

Photophysics of Concentration-Dependent Lifetime Decline. The exact nature of the decrease of DPHpPC lifetime at high concentrations is unknown, but several explanations may be considered. First, simple collisional quenching interactions between probe molecules could account for the decline. Second, the probe may alter bilayer structure when incorporated into vesicles at high concentrations, thereby changing the properties of its local environment. Third, several excited-state species could contribute to the observed fluorescence decay of DPHpPC. The distribution of these species could shift with probe concentration, changing the observed decay parameters. Finally, reduction in lifetime at high probe concentrations may be caused by energy transfer between excited- and ground-state probe molecules, yielding an altered excited state with a different extrinsic lifetime. Although the latter of these possibilities seems the least plausible, some evidence exists to argue against the former ones. First, the decrease in lifetime at greater than 1 mol % probe was not accompanied by an equivalent decline in fluorescence intensity (Parente & Lentz, 1985), which rules out a simple collisional quenching mechanism. Second, differential scanning calorimetry was used to examine the phase behavior of DPPC LMV containing 0, 0.25, and 2 mol % of DPHpPC (Parente & Lentz, 1985). Transition parameters (temperature and width at half-height) of samples containing probe differed only slightly from those observed with the sample containing no probe. This is consistent with the behavior of DPHpPC as a minor impurity in the bilayer and rules out the possibility of substantial disruption of bilayer structure by the probe. Finally, two-component heterogeneity analysis of phase and modulation data collected at 48 °C for vesicle samples containing a range of probe concentrations (0.14–2 mol %) yielded a constant fractional intensity of the low-lifetime component at all probe concentrations (R. A. Parente and B. R. Lentz, unpublished results). These observations leave homogeneous energy transfer as a possible mechanism to account for the fluorescence lifetime of DPHpPC, although more work on the photophysical properties of DPHpPC will be needed to establish this. In the absence of further photophysical studies, one can still make use of empirical changes in DPHpPC lifetime. By taking advantage of these empirical observations, we have demonstrated that changes in local lipid concentrations associated with membrane fusion or phase separation can be quantitated.

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