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Novel Fluorescent Phospholipids for Assays of Lipid Mixing between Membranes†

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Received October 28, 1986; Revised Manuscript Received January 29, 1987

ABSTRACT: A series of fluorescent phospholipids has been synthesized, by a general and versatile procedure, with various fluorescent groups attached to the methyl-terminal half of one acyl chain in an otherwise normal phospholipid structure. Phospholipids labeled with (dialkylamino)coumarin moieties, and to a slightly lesser extent those labeled with a bimane group, exhibit a strong and stable blue fluorescence in phospholipid dispersions that is relatively insensitive to the physical state of the lipid phase. The fluorescence of these labeled phospholipids is efficiently quenched by resonance energy transfer to lipids labeled with a [[(dimethylamino)phenyl]azo]phenyl or a methyl(nitrobenzoxadiazolyl)amino group when these acceptors are incorporated into the same bilayer as the donor species. Acyl chain labeled phospholipid probes, both of whose chains are at least sixteen carbons in length, exchange extremely slowly between lipid vesicles (<1% exchange/h). These properties allow various donor–acceptor combinations of probes to be employed in sensitive and reliable assays of lipid mixing accompanying membrane fusion. We demonstrate that, in two particularly demanding applications (assays of the calcium-mediated coalescence of phosphatidylserine vesicles and of the proton-triggered coalescence of phosphatidylethanolamine vesicles), some combinations of acyl chain labeled probes offer substantial advantages over the commonly used *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine/*N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine pair to monitor accurately the progress of lipid mixing between vesicles.

The fusion of a variety of artificial and biological membranes has become an object of intensive study in recent years. Research in this area has been advanced considerably by the development of sensitive fluorometric assays that can monitor either intermixing of the aqueous compartments bounded by two initially distinct membranes or the intermixing of lipids that occurs when two membranes coalesce [for a review, see Düzgünes and Bentz (1986)].

In principle, assays of the mixing of fluorescent lipid probes between membranes can provide a convenient and sensitive method to monitor interactions between two membranes during the process of fusion. For some systems, this approach is more practical than measurements of aqueous contents mixing for monitoring certain aspects of membrane fusion, as lipid-mixing assays are not complicated by the effects of membrane leakiness, and they do not require prior manipulation of the internal aqueous compartments bounded by both membranes (Bental et al., 1984; Morris & Bradley, 1984; Harmsen et al., 1985). Moreover, assays of lipid mixing can be profitably employed to provide information complementary to that provided by contents-mixing assays in the study of systems for which both types of assays are feasible (Ellens et al., 1985; Düzgünes et al., 1985; Leventis et al., 1986).

While a number of assays have been devised to monitor membrane coalescence through lipid mixing, often with considerable success (Gibson & Loew, 1979a; Vanderwerf & Ullman, 1980; Owen, 1980; Struck et al., 1981; Uster & Deamer, 1981; Morgan et al., 1983; Gad & Eytan, 1983; Hoekstra et al., 1985; Pryor et al., 1985; Parente & Lentz,

† This work was supported by grants from the Medical Research Council of Canada (MA-7776) and les Fonds pour la Formation de Chercheurs et l'Aide á la Recherche du Quêbec (86-EQ-2954) to J.R.S. and from the Natural Science and Engineering Research Council of Canada to M.Z.
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0006-2960/87/0426-4279$01.50/0

1986; Hong & Vacquier, 1986), such assays have also frequently been reported to suffer from significant limitations or potential artifacts arising from specific undesirable properties of the probe(s) employed. A reasonable set of criteria for an optimal probe to be employed in such assays would be the following: (1) The probe closely resembles natural membrane lipids in its structure and physical properties, including its miscibility with other lipids and its rate of spontaneous exchange between membranes. (2) The probe gives rise to a signal that can be monitored simply and continuously and with high sensitivity, and this signal can be modulated by lipid mixing between membranes but is not directly affected by changes in the physical properties of an isolated membrane or by the agent(s) used to trigger fusion. (3) The probe can be prepared economically and reasonably simply and is sufficiently stable to light, oxygen, and chemical decomposition that it can conveniently be handled in a variety of conventional biochemical and cell-culture techniques.

To date, no probe or set of probes has been reported that clearly meets all of the above criteria. In this study, we have prepared a series of novel acyl chain labeled phospholipids, which can be used as probes to monitor fusion-induced lipid mixing between membranes through measurements of fluorescence energy transfer. We demonstrate that several combinations of these probes are highly suitable for use in simple, reliable, and versatile assays of lipid mixing during membrane coalescence.

Materials and Methods

Preparation of Methylamino and N-t-Boc-methylamino Fatty Acids. The syntheses of 12-(methylamino)stearic acid and its *N-t*-Boc[1] derivative are representative and are described below.

Methyl 12-hydroxystearate (Sigma) (586 mg, 2 mmol) was converted to the methanesulfonate derivative (Crossland & Servis, 1970) and reacted with 6 mL each of methanol and 40% aqueous methylamine for 24 h at 60 °C in a sealed tube. Ethanol (200 mL) was added and evaporated off in vacuo, and the residue was dissolved in 6 mL of ethanol plus 6 mL of 2.5 M aqueous KOH. The solution was heated to 75 °C for 58 h and then acidified by bubbling in CO_2 gas. After repeated addition and evaporation of ethanol to remove water, the residue was triturated twice with 1:1 methanol/$CHCl_3$ (20 mL) and filtered. The combined filtrates were dried down in vacuo. The crude methylamino acid thus recovered was either converted to the *N-t*-Boc derivative by treatment with di-*tert*-butyl dicarbonate (Morodor et al., 1976) or treated with methanolic HCl to give the methyl ester. Either compound (but not the insoluble methylamino acid itself) could readily be purified by chromatography on a column of Bio-Sil A packed in $CHCl_3$, eluting with a 0–5% gradient of methanol in $CHCl_3$. Both compounds were obtained in final yields of 40–65%. The methyl ester species could be converted quantitatively to 12-(methylamino)stearic acid by saponification with 1 M methanolic KOH (50 °C, 1 h), followed by acidification with CO_2 gas and removal of precipitated inorganic solids after addition of an equal volume of $CHCl_3$.

The procedures just described were also applied to generate 16-(methylamino)palmitic acid and its *N-t*-Boc derivative, starting from methyl 16-hydroxypalmitate, and 11-(methylamino)undecanoic acid and its *N-t*-Boc derivative, starting from 11-bromoundecanoic acid. In the latter case, the bromo acid was reacted directly with methanol and 40% aqueous methylamine, and the subsequent alkaline digestion step could be omitted. 16-Aminopalmitic acid was prepared in the same manner as 16-(methylamino)palmitic acid, by using 30% aqueous ammonia in place of 40% aqueous methylamine to react with the methanesulfonate derivative of methyl 16-hydroxypalmitate.

Preparation of Mercapto Fatty Acids. 11-Mercaptoundecanoic acid was prepared from 11-bromoundecanoic acid by the procedure of Pan and Fletcher (1968) and purified on a column of Bio-Sil A packed in hexane, eluting with a 0–10% gradient of diethyl ether in hexane. 12-Mercaptostearic acid was prepared from the methanesulfonate derivative of methyl 12-hydroxystearate (prepared as above) by reaction with a 50% molar excess of thiourea in ethanol at 70 °C for 18 h, followed by treatment with 4 volumes of 2 N aqueous NaOH for 4 h at 20 °C. After acidification of the aqueous phase, the crude sulfhydryl fatty acid was extracted into hexane and purified as for 11-mercaptoundecanoic acid. Both mercapto fatty acids could be obtained in 70–90% final yield on the basis of the starting bromo or hydroxy fatty acid.

[(Mercaptomethyl)thio]alkanoic acids were prepared from the above species by treatment with methyl methanethiosulfonate in dry $CHCl_3$ containing 1% pyridine (Ganong & Bell, 1984). After treatment with *N*-ethylmaleimide in methanol for 1 h at room temperature to derivatize remaining traces of unprotected mercapto acid, the protected fatty acid was purified on a column of Bio-Sil A, eluting with a 0–10% gradient of ethyl ether in hexane.

Preparation of Precursor Phosphatidylcholines. *N-t*-Boc- or *S*-(mercaptomethyl)-protected fatty acids, NBD-methylamino fatty acids, 16-[(trinitrophenyl)amino]palmitic acid, or bimanylthio fatty acids were converted to symmetric acyl anhydrides and coupled to 1-palmitoyl-*lyso*-phosphatidylcholine according to the procedure of Mason et al. (1981). The products were purified either by chromatography on silicic acid columns, eluting with a 30–60% gradient of methanol in chloroform, or by preparative thin-layer chromatography on silica gel G.

1-Palmitoyl-2-[(*t*-Boc-methylamino)acyl]-PC's were deprotected with HCl in dry $CHCl_3$ at 0 °C (Silvius et al., 1986), and 1-palmitoyl-2-[(mercaptomethyl)thio]acyl-PC's were

[1] Abbreviations: *t*-Boc, *tert*-butoxycarbonyl; CA, *N*-[2-[7-(dimethylamino)coumarin-4-yl]acetyl]methylamino; CPS, [[*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]methyl]thio; CPT, *N'*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]-*N*-methylthioureidyl; DAB, 4-[[4-(dimethylamino)phenyl]azo]phenyl; DABITC, 4-[[4-(dimethylamino)phenyl]azo]phenyl isothiocyanate; DABS, [[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino; DABSyl chloride, 4-[[4-(dimethylamino)phenyl]azo]benzenesulfonyl chloride; DABT, *N'*-[4-[[4-(dimethylamino)phenyl]azo]phenyl]-*N*-methylthioureidyl; DEPA, 1,2-dielaidoyl-*sn*-glycerol 3-phosphate; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DPH-PC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid trisodium salt; LUV, large unilamellar vesicles; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBDMA, methyl(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino; C_{12}-NBD-PC, 1-palmitoyl-2-[12-[methyl(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PA, 1,2-diacyl-*sn*-glycerol 3-phosphate; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; (11-X)-11-PC, 1-palmitoyl-2-(11-X-undecanoyl)-*sn*-glycero-3-phosphocholine (substituent groups X are given in Figure 1); (16-X)-16-PC, 1-palmitoyl-2-(16-X-hexadecanoyl)-*sn*-glycero-3-phosphocholine; (12-X)-18-PC, 1-palmitoyl-2-(12-X-octadecanoyl)-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, bovine brain phosphatidylserine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; DTT, dithiothreitol; TLC, thin-layer chromatography.

deprotected by treatment with a thirtyfold molar excess of dithiothreitol in 2:1:0.5 methanol/chloroform/100 mM aqueous EDTA, pH 7.4, for 12 h at 20 °C. The deprotected PC's with a 2-[(methylamino)acyl]chain were used directly for synthesis of fluorescent probes, while the deprotected PC's with a 2-(mercaptoacyl) chain were first separated from residual DTT by chromatography on silicic acid.

Labeling of Probe Precursors. All reactions of methylamino or mercapto fatty acids and their PC derivatives with fluorescent or chromophoric reagents were carried out at 25 °C under nitrogen and with exclusion of light. 1-Palmitoyl-2-[(methylamino)acyl]-PC's were labeled with 4-[[4-(dimethylamino)phenyl]azo]benzenesulfonyl chloride (DABSyl chloride), 4-[[4-(dimethylamino)phenyl]azo]phenyl isothiocyanate (DABITC), 7-(diethylamino)-3-(4-isothiocyanatophenyl)-4-methylcoumarin, or succinimidyl 7-(dimethylamino)coumarin-4-acetate by overnight reaction of a 10–20 mM solution of the PC, dissolved in dry chloroform containing 1% triethylamine, with 1.2–1.5 molar equiv of the fluorescent or chromophoric reagent. 1-Palmitoyl-2-(mercaptoacyl)-PC's were labeled with 7-(diethylamino)-3-[4-[(iodoacetyl)amino]phenyl]-4-methylcoumarin under similar conditions. The PC products were purified by preparative TLC in 65:30:4 (v/v/v) $CHCl_3$/methanol/H_2O. In this solvent system, it was possible to resolve any remaining unreacted [(methylamino)acyl]- or (mercaptoacyl)-PC from the labeled products.

Bimanylthio fatty acids were synthesized by reacting 1 equiv of monobromobimane for 4 h at 25 °C with 1.25 equiv of mercapto fatty acid in 1:1 methanol/water containing 2 equiv of $NaHCO_3$. NBD-labeled methylamino fatty acids were prepared similarly, except that a 50% molar excess of NBD-chloride was used. The NBD- and bimane-labeled fatty acids were recovered from the reaction mixtures by extraction into 2:1 hexane/ethyl ether after acidification to pH ~3 and then purified by preparative TLC on silica gel G, with 95:5 $CHCl_3$/methanol as the developing solvent. 16-[(Trinitrophenyl)amino]palmitic acid was synthesized from 16-aminopalmitic acid by the procedure of Goldberg et al. (1978).

Methods. (*A*) *Vesicle Preparations.* Vesicles for use in assays of fusion, or to obtain control measurements for such assays, were prepared by the reverse-phase evaporation procedure and filtered through 0.1-μm Nucleopore filters (Wilschut et al., 1980), or by bath sonicating dried lipid samples in buffer for 2 min, as indicated in the text. Liposomes for measurements of probe fluorescence spectra, energy transfer, and environmental effects on probe fluorescence were prepared by vortexing thoroughly dried lipid mixtures in buffer above the transition temperature and then bath sonicating under N_2 for 5 min. Vesicles for use in measurements of probe exchange rates were prepared similarly, but the lipids were initially vortexed in distilled water and bath sonicated for 2 min (to facilitate dispersal as unilamellar vesicles), then mixed with an equal volume of a twofold-concentrated buffer solution, and sonicated for a further 3 min to equilibrate the compositions of the aqueous phases inside and outside the vesicles.

For preparation of vesicles from PC, PA/PC mixtures, or PS, the buffer used was 150 mM NaCl, 5 mM histidine, 5 mM Tes, and 0.1 mM EDTA, pH 7.4. For preparation of vesicles from PE, 20 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5, was used as the buffer, and 1 molar equiv of glycine (adjusted to pH 9.5) was added per mole of PE to the medium used to disperse the lipids initially.

(*B*) *Fluorescence Measurements.* All fluorescence experiments were carried out on a Perkin-Elmer LS-5 spectrofluorometer, whose excitation source is a pulsed Xenon flash

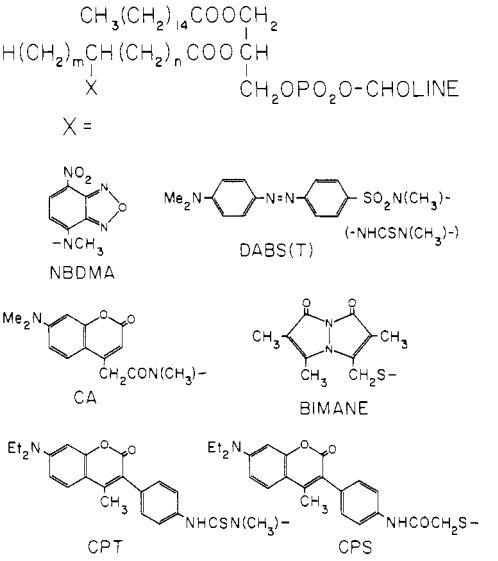

FIGURE 1: Structures of fluorescent phosphatidylcholines prepared in this study. For probes designated in the text as (11-X)-11-PC's, $m = 0$ and $n = 9$; for (16-X)-16-PC's, $m = 0$ and $n = 14$; and for (12-X)-18-PC's, $m = 6$ and $n = 10$.

lamp with an output intensity of 8.3 W. Fluorescence spectra were collected by using excitation and emission slit widths of 3 and 5 nm, respectively. For all fluorescence measurements, control samples were prepared without fluorescent probes to correct for possible contributions of light scattering to the observed fluorescence signal. For measurements of energy transfer and self-quenching, probes were incorporated at various concentrations into POPC vesicles, and the fluorescence of the donor species was measured before and after solubilization of the vesicles in 1% Triton X-100. The direct effect of Triton on the fluorescence of the donor species was determined by using vesicles labeled with the donor under conditions where fluorescence quenching was negligible.

Results and Discussion

Probe Fluorescence Characteristics. In Figure 1 are shown the structures of the chromo- and fluorophoric groups that were incorporated into the novel phospholipid probe molecules generated in this study. These residues are attached to three types of acyl chains in 1-palmitoyl-2-acyl-phosphatidylcholines: to the 11-position of a 2-undecanoyl chain [giving probes abbreviated as (11-X)-11-PC's], to the 16-position of a 2-palmitoyl chain [abbreviated as (16-X)-16-PC's], or to the 12-position of a 2-stearoyl chain [abbreviated as (12-X)-18-PC's]. To synthesize these probes, the appropriate mercapto- or methylamino-substituted fatty acids are first prepared from commercially available bromo- or hydroxy-substituted fatty acids. Most of the PC probes are then synthesized by acylating 1-palmitoyl-*lyso*-phosphatidylcholine with a protected (mercaptomethyl)thio fatty acid or *t*-Boc-methylamino fatty acid, which can be deprotected under mild conditions and then coupled to an appropriate fluorescent or chromophoric reagent. The bimane- and NBDMA-labeled PC's are synthesized by first preparing the fluorescent-labeled fatty acids, which are then used to acylate 1-palmitoyl-*lyso*-phosphatidylcholine.

The bimane-, NBD-, and coumarin-labeled phosphatidylcholines described above were incorporated at low concentrations (≤1 mol %) into liquid-crystalline POPC vesicles at 25 °C, and their fluorescence spectra were recorded. The excitation and emission spectra for most of these compounds showed single rather broad maxima (not shown), the peak positions of which are summarized in Table I. Also given in

Table I: Excitation and Emission Maxima Measured for Fluorescent Phospholipids in POPC Bilayers[a]

fluorescent lipid	λ_{ex} (nm)	λ_{em} (nm)
NBD-PE	470[b]	537
Rho-PE	525	597
C_{12}-NBD-PC	472[b]	544
(11-NBDMA)-11-PC	480[c]	530
(16-NBDMA)-16-PC	477[c]	533
(12-NBDMA)-18-PC	481[c]	538
(12-CA)-18-PC	390	472
(12-CPT)-18-PC	396	477
(12-CPS)-18-PC	390	473
(16-CPT)-16-PC	392	477
(11-bimanyl)-11-PC	392	468
(12-bimanyl)-18-PC	394	471
(12-DABS)-18-PC	447[d]	
(12-DABT)-18-PC	433[d]	
DPH-PC	355	445

[a] Bath-sonicated POPC vesicles containing 0.5 mol % fluorescent probe were prepared and their fluorescence spectra measured, as described under Materials and Methods. [b] A smaller excitation peak is seen at ~335 nm, with a minimum in the excitation spectrum at ~370 nm, as reported previously by Monti et al. (1978). [c] A smaller excitation peak is seen at ~350 nm in the excitation spectrum, with a minimum at 380–390 nm. [d] Measured for the absorption spectrum; these species are nonfluorescent.

Table II: Effects of Environmental Perturbations on Fluorescence Intensities for Various Labeled Phosphatidylcholines

	% change in fluorescence intensity in		
species	Triton X-100 vs. POPC[a]	DEPA/PC ($+Ca^{2+}$) vs. DEPA/PC ($-Ca^{2+}$)[b]	DOPE (pH 4.5) vs. DOPE (pH 9.5)[c]
NBD-PE	−44.3	+53.5	+49.0
C_{12}-NBD-PC	−46.3	−9.2	+40.3
(11-NBDMA)-11-PC	−21.2	+2.5	+46.1
(16-NBDMA)-16-PC	+1.2	+13.6	+134.3
(12-NBDMA)-18-PC	+2.1	+9.0	+52.9
(12-CA)-18-PC	−4.4	+2.6	+7.5
(12-CPT)-18-PC	+4.1	−0.6	−1.0
(12-CPS)-18-PC	−1.8	0.0	+1.8
(16-CPT)-16-PC	+23.0	−3.5	−1.0
(11-bimanyl)-11-PC	−34.3	+3.9	+9.1
(12-bimanyl)-18-PC	−34.0	+2.7	+10.3

[a] Bath-sonicated POPC vesicles (30 μM) labeled with 0.5 mol % probe were treated with Triton X-100 (final detergent concentration 1%) at 25 °C. [b] DEPA/DEPC vesicles (30 μM lipid, comprising 84.5 mol % PA and 15 mol % PC plus 0.5 mol % probe) were treated with 10 mM $CaCl_2$ (final concentration) at 42 °C. [c] Bath-sonicated DOPE vesicles (30 μM) labeled with 0.5 mol % probe were prepared at pH 9.5, and the pH was abruptly reduced to 4.5 at 37 °C.

Table I are the positions of the maxima of absorbance spectra recorded for dispersions of 1 mol % of the nonfluorescent species (12-DABS)-18-PC and (12-DABT)-18-PC in POPC vesicles at 25 °C. The emission spectra for the bimane- and coumarin-labeled PC's show strong overlap with the absorption spectra for the NBD-, DABS-, and DABT-labeled species. The fluorescence of the coumarin-labeled species was roughly five- to sevenfold brighter than that of NBD-PE at comparable instrumental sensitivity settings, while the bimane- and NBDMA-labeled species gave fluorescence comparable to and slightly less than that of NBD-PE, respectively.

In many potential applications of these fluorescent lipids, it can be important that the intensity of probe fluorescence remains stable even when the physical properties of the membrane environment change markedly. Therefore, we examined the stability of the fluorescence intensity for various probes during three treatments in which the physical properties of the probe environment were abruptly and substantially altered. First, probes initially incorporated at ≤1 mol % into POPC vesicles at 25 °C were solubilized into Triton X-100 micelles by addition of a roughly 800-fold excess of the detergent. Second, probes were incorporated into sonicated DOPE vesicles at pH 9.5, and the pH was abruptly lowered to 4.5 at 37 °C to initiate the aggregation of the vesicles and the formation of nonlamellar arrangements of the lipids (Ellens et al., 1986). Finally, probes were incorporated into vesicles of 85:15 dielaidoyl-PA/dielaidoyl-PC, which are liquid crystalline at 42 °C in the absence of divalent cations, and 10 mM $CaCl_2$ was rapidly added to convert the vesicles to the gel state (Graham et al., 1985). These perturbations of probe-containing vesicles induced relatively rapid changes in the measured intensities of probe fluorescence, which typically restabilized within a few minutes. In Table II we summarize the magnitudes of the fluorescence changes (as percentages of the initial fluorescence intensity) that are observed when vesicles containing various fluorescent PC's are subjected to the three types of perturbations described above. It can be seen that the fluorescence intensities measured for NBDMA- and bimane-labeled PC's can vary markedly in response to certain perturbations of the probe environment. By contrast, the coumarin-labeled PC's prepared in this study generally showed very good stability of their fluorescence intensity in response to all of the environmental perturbations employed in our experiments. (12-CPS)-18-PC and (12-CPT)-18-PC proved to be particularly outstanding in this regard (Table II).

The fluorescent phospholipid DPH-PC has recently been introduced as a useful probe of the dynamics and the phase behavior of membrane lipids (Morgan et al., 1983; Parente & Lentz, 1985, 1986). Since DPH-PC is highly fluorescent and closely resembles natural phospholipids in its structure, we examined the fluorescence properties of this species in the lipid systems discussed above. The intensity of fluorescence of DPH-PC in lipid bilayers proved to be quite stable in response to various perturbations of the lipid environment, and the fluorescence could be efficiently quenched by energy transfer to NBDMA-, DABS-, and DABT-labeled PC's (data not shown). However, lipid dispersions labeled with DPH-PC invariably showed a steady decline in fluorescence intensity with time, which could be slowed but not eliminated by reducing the excitation slit width and which appears to reflect a gradual photobleaching of the probe, as other workers have described previously for DPH itself (Shinitzky & Barenholz, 1974; Duportail & Weinreb, 1983). DPH-PC therefore does not appear to be suitable for lipid-mixing assays that monitor the intensity of probe fluorescence during membrane fusion.

Energy Transfer between Probes. As we have noted above, the emission spectra observed for coumarin- and bimane-labeled probes in lipid bilayers strongly overlap the excitation spectra for NBD-, DABS-, and DABT-labeled probes in the same environments. As a result, labeled lipids of these last three types can serve as efficient energy-transfer acceptors with coumarin- or bimane-labeled lipids. An illustration of this property is shown in Figure 2, where we have plotted the efficiency of energy transfer (measured as the extent of quenching of the donor fluorescence) when (12-bimanyl)-18-PC is incorporated into POPC vesicles together with various molar percentages of (12-NBDMA)-18-PC. The fluorescence of the bimane-labeled PC is quenched half-maximally when the NBDMA-labeled PC comprises 0.47 mol % of the total phospholipid. The results of a number of similar experiments are summarized in Table III, where we list the bilayer concentrations of various acceptors that are required to quench half-maximally the fluorescence of different bimane- and coumarin-labeled PC's in POPC vesicles. For comparison,

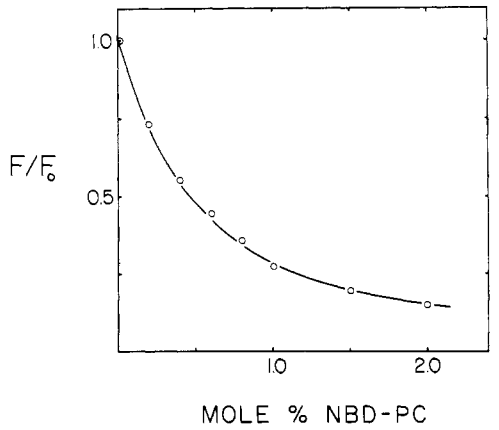

FIGURE 2: Quenching of the fluorescence of (12-bimanyl)-18-PC, incorporated at 1 mol % into bath-sonicated POPC vesicles, by varying molar percentages of (12-NBDMA)-18-PC incorporated in the same vesicles. Fluorescence was measured before and after the addition of 1% Triton X-100 to the vesicles to relieve quenching due to energy transfer; the fractional quenching of fluorescence $[=1 - (F/F_0]$ was determined from these measurements with a correction for the direct effect of Triton X-100 on the fluorescence of (12-bimanyl)-18-PC.

Table III: Bilayer Concentrations of Energy-Transfer Acceptor PC Species Giving Half-Maximal Quenching of Donor Fluorescence in POPC Vesicles[a]

donor	acceptor	mol % acceptor giving half-maximal quenching of donor fluorescence
(12-CA)-18-PC	(12-DABS)-18-PC	0.39
(12-CA)-18-PC	(12-DABT)-18-PC	0.38
(12-CA)-18-PC	(12-NBDMA)-18-PC	0.40
(12-CPT)-18-PC	(12-DABS)-18-PC	0.38
(12-CPT)-18-PC	(12-DABT)-18-PC	0.38
(12-CPT)-18-PC	(12-NBDMA)-18-PC	0.47
(12-CPS)-18-PC	(12-DABS)-18-PC	0.34
(12-CPS)-18-PC	(12-NBDMA)-18-PC	0.31
(12-bimanyl)-18-PC	(12-DABS)-18-PC	0.45
(12-bimanyl)-18-PC	(12-NBDMA)-18-PC	0.47
NBD-PE	Rho-PE	0.33
(11-NBDMA)-11-PC	(self)	5.3
(12-NBDMA)-18-PC	(self)	4.6

[a] Bath-sonicated POPC vesicles were labeled with 0.5 mol % donor and varying levels of acceptor probes, and quenching was determined at 25 °C as a function of the acceptor concentration as for Figure 3B.

results obtained with the NBD-PE/Rho-PE donor–acceptor pair are also shown. In all of these experiments, the efficiency of energy transfer depended only on the concentration of the acceptor species and was independent of the concentration of the donor species at least up to a level of 2 mol % donor, the highest level routinely tested.

Experimental results such as those presented in Figure 2 can be compared to results from theoretical simulations of energy transfer in a two-dimensional array of randomly distributed donors and acceptors [see, for example, Figure 1 of Wolber and Hudson (1979)] to estimate an apparent Förster energy-transfer length R_0 for each donor–acceptor pair listed in Table III. These estimated values for R_0 lie in the range 50–60 Å for all of the pairs of lipid probes presented in this table. These estimates must be regarded as only approximate ones, however, as the analysis of the data ignores the possibility of energy transfer between lipid probes in opposite leaflets of the bilayer, which is clearly possible for chain-labeled probes when the energy-transfer length begins to approach the full thickness of the bilayer. Nonetheless, it is clear that several pairs of acyl chain labeled phospholipids prepared in this study, like the previously characterized NBD-PE/Rho-PE energy-transfer pair, can exhibit highly efficient energy transfer even at very modest concentrations of the acceptor species in the bilayer.

NBD-labeled phospholipids have previously been shown to exhibit significant self-quenching of fluorescence when incorporated into lipid bilayers at levels above a few mole percent (Nichols & Pagano, 1981). The NBDMA-labeled phospholipids generated in this study also exhibit self-quenching of fluorescence, which can be quite significant at probe concentrations greater than 1–2 mol % in the bilayer (Table III).

Exchange of Probes between Bilayers. In order to use fluorescent lipid analogues as probes to assay lipid mixing accompanying membrane coalescence, it is essential that the rate of spontaneous exchange of the probes between separate lipid bilayers be very slow. We accordingly measured the rates of transfer of several fluorescent PC probes between sonicated unilamellar vesicles composed of POPC and DOPS (80:20 mol/mol) at 25 °C, using various procedures to monitor the exchange of different types of probes. First, NBD-labeled PC's were incorporated into POPC/DOPS vesicles at a high bilayer concentration (20 mol %), at which the probe fluorescence is largely self-quenched, and we monitored the rate of fluorescence increase due to probe exchange when these vesicles were incubated with a large excess (10–40-fold) of unlabeled vesicles (Nichols & Pagano, 1981). Using this method, we measured exchange rates of 5.5%/min and 4.5%/min for (11-NBDMA)-11-PC and C_{12}-NBD-PC, respectively. Variation of the concentration of acceptor vesicles over a fivefold range had no effect on the measured exchange rates. Essentially no intervesicle transfer of (16-NBDMA)-16-PC or (12-NBDMA)-18-PC could be detected in similar experiments, which allowed us to estimate a rate of 1.0%/h as an upper limit to the rate of spontaneous exchange of these probes between vesicles.

In a related series of exchange measurements, donor vesicles containing 1 mol % (11-bimanyl)-11-PC or (11-CPT)-11-PC plus 2 mol % (12-DABS)-18-PC, which acts as a nonexchangeable fluorescence quencher (see below), were incubated with a large excess of unlabeled vesicles. The rate of probe exchange was then estimated from the observed rate of dequenching of the probe fluorescence, an approach similar to that described previously by Nichols and Pagano (1982). The rates of exchange measured by this method for (11-bimanyl)-11-PC and (11-CPT)-11-PC were 13.6%/min and 0.11%/min, respectively. It is apparent that the nature of the fluorescent group, as well as the length of the acyl chain to which it is attached, can strongly influence the rate of spontaneous exchange of these PC probes between distinct lipid bilayers.

The experiments just described are very suitable to detect probe exchange between bilayers at rates as low as ~0.1%/min. To measure slower rates of probe exchange, we incubated donor vesicles, containing coumarin- or bimane-labeled PC's, with acceptor vesicles containing NBD-, DABS-, or DABT-labeled PC's, for periods up to several hours, before measuring the extent of fluorescence change brought about through probe transfer. In principle, fluorescence quenching developed during these coincubations of donor and acceptor vesicles could be attributable to exchange of either or both of the labeled PC's present in the mixture. In general, however, the rates of exchange of labeled (16-X)-16- and (12-X)-18-PC's between lipid vesicles were found to be too slow to produce accurately quantifiable fluorescence changes even after 4 h of incubation of the vesicles under our experimental conditions. From our results, we calculate that all of the (12-X)-18-PC's listed in

Table I, as well as (16-NBDMA)-16-PC, exchange between lipid bilayers at rates less than 1.0%/h, a rate too slow to influence results obtained in most studies of membrane fusion.

Applications to Lipid-Mixing Assays. The most widely used fluorescence assays of lipid mixing between interacting membranes employ a "lipid-mixing/dequenching" design, in which membranes colabeled with a fluorescent donor lipid and an appropriate energy-transfer acceptor are allowed to interact with a second, unlabeled membrane population (Düzgünes & Bentz, 1986). As the colabeled membranes mix lipids with unlabeled membranes, the lipid probes become diluted, leading to a reduction in the efficiency of energy transfer which is usually monitored as an enhancement of the fluorescence of the donor species [Vanderwerf & Ullman, 1980; Struck et al., 1981; Uster & Deamer, 1981; Morgan et al., 1983; for a related procedure measuring fluorescence lifetimes, see Parente and Lentz (1986)]. When the ratio of unlabeled to labeled membrane vesicles is high, lipid-mixing/dequenching assays can reliably monitor lipid mixing between membranes without interference from artifacts resulting from simple membrane aggregation. To employ these assays reliably to monitor lipid mixing, it is very important that the fluorescence signal measured can be accurately calibrated as a function of the extent of lipid mixing in a given system. In the light of these considerations, we examined the usefulness of different combinations of phospholipid probes to monitor the divalent cation triggered interactions of unilamellar vesicles formed from PS (Wilschut et al., 1980, 1981, 1985; Struck et al., 1981; Hoekstra, 1982a; Rosenberg et al., 1983; Parente & Lentz, 1986) and the proton-triggered coalescence of sonicated vesicles prepared at high pH from POPE [for previous studies with related PE systems, see Pryor et al. (1985) and Ellens et al. (1986)].

Our first experiments examined the effects of calcium addition on the fluorescence of (12-CPS)-18-PC and NBD-PE incorporated into PS LUV. While calcium at 2.5 mM has only a small effect on the fluorescence of either probe in PS vesicles, addition of 5 mM calcium to vesicles labeled with NBD-PE produces first a sizable increase (~30%) and then a slow decline of fluorescence intensity (Figure 3A). By contrast, the fluorescence of vesicles labeled with (12-CPS)-18-PC shows only a slow decrease (~0.8%/min), due to precipitation of very large vesicle aggregates, when 5 mM calcium is added.

When PS vesicles are labeled with (12-CPS)-18-PC plus (12-DABS)-18-PC, or with NBD-PE plus Rho-PE, the effects of calcium addition on the vesicle fluorescence are rather different from those observed when only the donor species is present (Figure 3B). Addition of 5 mM calcium to vesicles labeled with NBD-PE plus 0.4 mol % Rho-PE causes a rapid rise and then a gradual decrease in fluorescence (~2.5%/min). PS vesicles labeled with (12-CPS)-18-PC plus 0.4 mol % (12-DABS)-18-PC show a gradual decline in fluorescence intensity, to 39% of the initial value after 5 min in the presence of 5 mM calcium. In both cases, the rate of the slow decrease in fluorescence was directly proportional to the vesicle concentration (data not shown), implying that vesicle–vesicle interactions are important in the mechanism of this process.

Other workers have previously suggested that fluorescent-labeled phosphatidylcholines incorporated in PS vesicles undergo lateral redistribution, leading to an enhancement of probe–probe interactions, in the presence of millimolar levels of calcium (Hoekstra, 1982b; Parente & Lentz, 1986). Such effects, if present, could obviously account for at least part of the decrease in fluorescence observed when PS vesicles labeled with (12-CPS)-18-PC and (12-DABS)-18-PC are

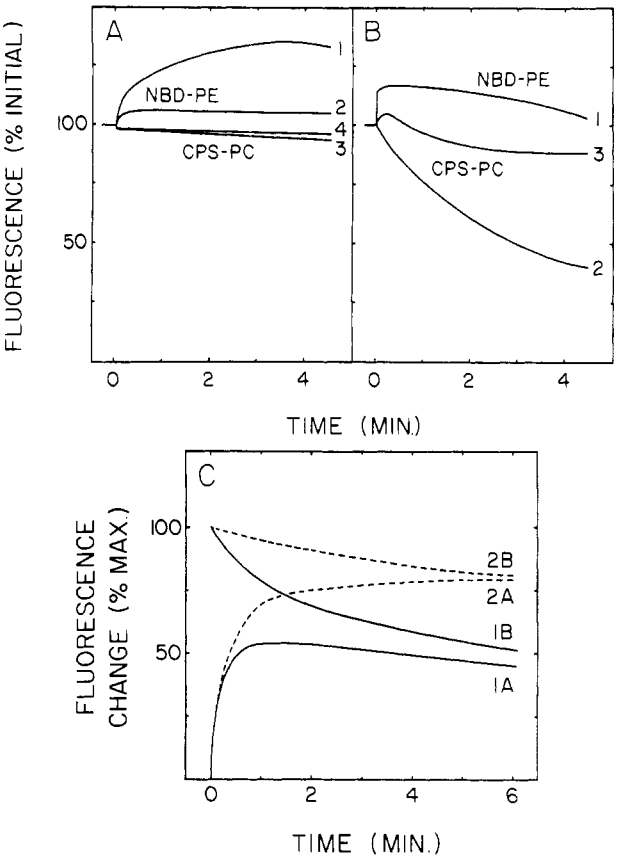

FIGURE 3: (A) Time courses of fluorescence measured when bath-sonicated PS vesicles labeled with 0.5 mol % NBD-PE were exposed to 5 mM calcium (curve 1) or 2.5 mM calcium (curve 2) or when similar vesicles labeled with 0.5 mol % (12-CPS)-18-PC were treated with 5 mM calcium (curve 3) or 2.5 mM calcium (curve 4). (B) Time courses of fluorescence observed upon addition of 5 mM calcium to bath-sonicated PS vesicles colabeled with 0.4 mol % each of NBD-PE and Rho-PE (curve 1), (12-CPS)-18-PC and (12-DABS)-18-PC (curve 2), or (12-CPS)-18PC and (12-DABS)-18-PA (curve 3). (C) Solid traces: Time courses of fluorescence observed when 5 mM calcium was added to a 7:1 mixture of unlabeled PS vesicles and PS vesicles labeled with 0.5 mol % (12-CPS)-18-PC plus 1 mol % (12-DABS)-18-PC (curve 1A) or to PS vesicles labeled with 0.0625 mol % (12-CPS)-18-PC and 0.125 mol % (12-DABS)-18-PC (curve 1B). Dashed traces: Time courses of fluorescence recorded when 5 mM calcium was added to a 7:1 mixture of unlabeled PS vesicles and PS vesicles labeled with 0.5 mol % (12-CPS)-18-PC plus 1 mol % (12-DABS)-18-PA (curve 2A) or to PS vesicles labeled with 0.0625 mol % (12-CPS)-18-PC plus 0.125 mol % (12-DABS)-18-PA (curve 2B). For all runs, the lipid concentration was 40 μM, and the temperature was 25 °C. The traces shown here and in Figures 4 and 5 are redrawn from experimental traces whose noise level was <1% of the signal in all cases.

exposed to 5 mM calcium. Accordingly, we examined the effects of calcium on the fluorescence of PS vesicles labeled with (12-CPS)-18-PC and (12-DABS)-18-PA, or with (12-CPS)-18-PA and (12-DABS)-18-PA (0.4 mol % each). As shown in Figure 3B, calcium causes only a modest quenching of fluorescence (~12% after 10 min), which is largely attributable to vesicle precipitation, for PS vesicles labeled with (12-CPS)-18-PC plus (12-DABS)-18-PA. Vesicles labeled with (12-CPS)-18-PA plus (12-DABS)-18-PA showed a fluorescence decrease of ~30% after 10 min in the presence of calcium (not shown). The effect of calcium on the fluorescence of PS vesicles labeled with (12-CPS)-18-PA alone was very similar to that shown in Figure 3A for vesicles labeled with (12-CPS)-18-PC alone. Taken all together, these results indicate that the effects of calcium on the fluorescence of PS vesicles containing CPS- and DABS-labeled phospholipids are

highly dependent on the nature of the probe head groups, although this is not true for vesicles labeled with the donor species alone.

In Figure 3C are shown time courses of fluorescence observed when bath-sonicated PS vesicles, labeled with 0.5 mol % (12-CPS)-18-PC plus 1 mol % of either (12-DABS)-18-PC or (12-DABS)-18-PA, were incubated with a sevenfold excess of unlabeled vesicles in the presence of calcium (lower curves). As controls for these experiments, "premixed" vesicles labeled with (12-CPS)-18-PC plus 0.125 mol % of either acceptor probe were treated with calcium at the same total lipid concentration (40 μM), giving the upper curves shown in Figure 3C. During the initial fast phase of lipid mixing, the fluorescence signals corresponding to complete mixing of lipids (upper curves) decay only very slightly from their initial value. We can thus estimate the initial rate of lipid mixing with good accuracy directly from the solid traces shown in Figure 3C. From these data, we calculate that the initial rate of lipid mixing between bath-sonicated PS vesicles at 40 μM lipid and 5 mM $CaCl_2$ is 5.5% of maximum/s (using data obtained with vesicles containing CPS- and DABS-labeled PC's) or 6.2% of maximum/s (using data obtained with vesicles containing CPS-labeled PC and DABS-labeled PA).[2] In comparable experiments using reverse-phase evaporation vesicles of PS that were filtered through a 0.1-μm Nucleopore filter, we estimated initial lipid-mixing rates of 3.3% of maximum/s or 3.0% of maximum/s, respectively, using the same two combinations of lipid probes.[2] Other workers have previously reported similar rates of lipid mixing between PS vesicles using comparable experimental conditions but different lipid probes (Wilschut et al., 1985; Parente & Lentz, 1986).

The time courses of fluorescence shown in Figure 3C indicate that it can become important at long times, when calcium-induced lipid mixing between PS vesicles is well advanced, to correct the fluorescence signal for the effects of vesicle precipitation and possible lateral redistribution of lipid probes in order to calculate accurately the extent of lipid mixing. As the results shown in this figure suggest, the necessary corrections could in principle be calculated from time courses of fluorescence obtained by using samples of premixed vesicles, whose fluorescence represents the signal corresponding to 100% lipid mixing. An alternative procedure would be to read the fluorescence of the samples after resequestering calcium with an excess of EDTA to reverse the effects noted above. The latter approach, while simpler, assumes that the abrupt sequestration of calcium with EDTA does not itself promote further mixing of lipids within the system. Using either of these procedures, we found in a series of experiments like those shown in Figure 3C that, at long times ($\sim$10 min), the fluorescence values attained by mixtures of labeled and unlabeled PS vesicles in the presence of calcium closely paralleled the values obtained for the corresponding premixed samples (results not shown). This was found to be true both

[2] Proper calibration of lipid-mixing assays requires a consideration of the fluorescence changes expected through the various stages of vesicle coalescence ("rounds of fusion") under the specific experimental conditions employed [see Wilschut et al. (1985) and Düzgünes and Bentz (1986)]. To facilitate direct comparison of our results with the primary data presented by other workers, we have not corrected our estimates of lipid-mixing rates to account for this fact but have calculated the rates simply as a percentage of the maximum expected fluorescence change per second. In our experiments from which the quoted rates were determined, a single round of vesicle fusion would produce a dequenching of fluorescence equal to 40–45% of that expected upon complete mixing of lipids. In the other studies cited, a similar fraction of the total possible fluorescence change is expected in the first round of fusion.

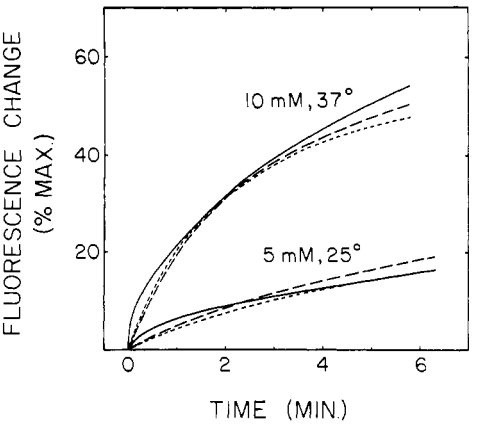

FIGURE 4: Time courses of fluorescence recorded when PS LUV, labeled with different donor and acceptor phospholipid probes, were treated with magnesium in the presence of a sevenfold excess of unlabeled PS LUV (total lipid concentration = 40 μM). The labeled vesicles contained 0.5 mol % each of (—) NBD-PE and Rho-PE, (---) (12-CPS)-18-PC and (12-DABS)-18-PA, or (– –) (12-CPS)-18-PC and (12-DABS)-18-PC. For the upper three traces, the temperature was 37 °C, and 10 mM magnesium was added at time zero. The lower three traces were recorded at 25 °C and 5 mM magnesium.

for vesicles labeled with (12-CPS)-18-PC plus varying amounts of (12-DABS)-18-PC and for vesicles labeled with (12-CPS)-18-PC plus varying amounts of (12-DABS)-18-PA, using various ratios of labeled to unlabeled vesicles (1:1 to 1:15). These results indicate that, at long times as well as in the initial stages of vesicle coalescence, (12-CPS)-18-PC can be used together with (12-DABS)-18-PA or (12-DABS)-18-PC to examine the calcium-promoted mixing of lipids between PS vesicles, so long as the fluorescence signal is analyzed with reference to the behavior of an appropriate premixed control sample.

When PS LUV were treated with 5 or 10 mM magnesium at 25 or 37 °C, we observed a measurable rate of lipid mixing, which was detected by using various combinations of fluorescent probes (Figure 4). This finding was somewhat surprising, as previous studies have reported that magnesium produces negligible rates of lipid mixing between PS LUV at 25 °C (Rosenberg et al., 1983; Wilschut et al., 1985). After considerable effort, we were unable to find any evidence that our result is attributable to the presence of any lipid or metal ion contaminants in our experimental system. It is possible that the PS preparations used here (obtained from Supelco) may differ somewhat in their properties from those used in previous studies (obtained from Avanti Polar Lipids). Of greatest interest here is the fact that the rate of the magnesium-induced probe mixing that we observe between PS vesicles is independent of the vesicle concentration in the range examined (10–50 μM PS), implying that this process is not aggregation-limited. Under these conditions, we can compare the rates at which different lipid probes redistribute between already aggregated lipid vesicles. As shown in Figure 4, the rate of magnesium-induced redistribution of lipids between PS LUV is quite similar as monitored by either the NBD-PE/Rho-PE, the (12-CPS)-18-PC/(12-DABS)-18-PC, or the (12-CPS)-18-PC/(12-DABS)-18-PA donor–acceptor pairs. It thus appears that all of the probes are transferred between vesicles by a "bulk" mechanism that is insensitive to the details of the probe structure and physical properties. There is no indication that any of these probes can exchange between aggregated vesicles by some special mechanism that does not function with comparable efficiency for the other lipid species in the same membrane. It will be of interest to make similar comparisons of the transfer rates of various lipid probes in

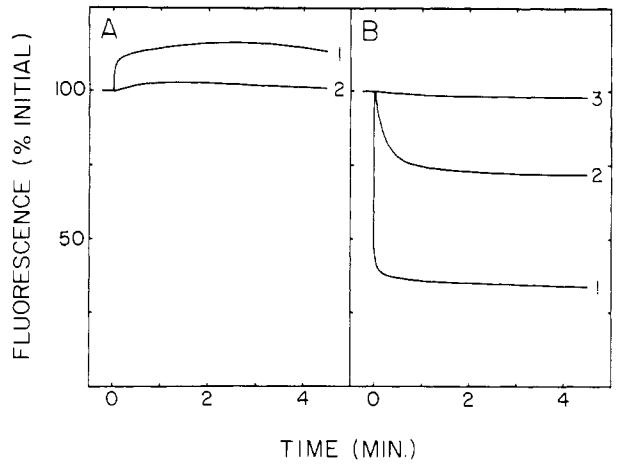

FIGURE 5: (A) Time courses of fluorescence recorded when POPE vesicles, labeled with 0.4 mol % NBD-PE (curve 1) or 0.5 mol % (12-CPS)-18-PC (curve 2) and bath-sonicated at pH 9.5, were mixed with acetate buffer to reduce the pH to 4.5. (B) Time courses of fluorescence recorded when similar POPE vesicles, labeled with 0.4 mol % each of NBD-PE and Rho-PE (curve 1), 0.5 mol % each of (12-CPS)-18-PC and (12-DABS)-18-PC (curve 2), or 0.5 mol % (12-CPS)-18-PC and 1.0 mol % 1-palmitoyl-2-[16-[(trinitrophenyl)amino]palmitoyl]-PC (curve 3), were subjected to a reduction of the pH from 9.5 to 4.5. The bilayer concentrations of the acceptor species in (B) were adjusted to give comparable quenching of the fluorescence of the donor species in all three samples at pH 9.5 (~60% for curves 1 and 2, ~50% for curve 3). All runs were carried out at 37 °C and with a lipid concentration of 30 μM.

other systems where lipid mixing between membranes is not aggregation-limited.

In a second series of experiments, we examined the suitability of (12-CPS)-18-PC, together with various energy-transfer acceptors, and the NBD-PE/Rho-PE energy-transfer pair to monitor the proton-induced mixing of lipids between vesicles that are prepared from POPE at pH 9.5. Bath-sonicated POPE vesicles, dispersed in 20 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5, were induced to aggregate and to coalesce by injecting a concentrated acetate buffer to a final pH of 4.5. When vesicles labeled only with NBD-PE or with (12-CPS)-18-PC were abruptly exposed to pH 4.5, the measured fluorescence increased by 17% or 4%, respectively (Figure 5A). When POPE vesicles were colabeled with 0.4 mol % each of NBD-PE and Rho-PE, reduction of the pH from 9.5 to 4.5 caused a very rapid reduction in the fluorescence by 55%, as shown in Figure 5B. The rate and amplitude of this fluorescence decrease was independent of the vesicle concentration (not shown). Vesicles colabeled with (12-CPS)-18-PC and (12-DABS)-18-PC (0.5 mol % each) showed a reduction in fluorescence by 25% when the pH was lowered to 4.5 (Figure 5B, curve 2). The rate of the proton-induced fluorescence decrease in this case was directly proportional to the lipid concentration (results not shown), indicating that vesicle interactions are important in the mechanism of this phenomenon.

Results quantitatively comparable to those shown in Figure 5B for POPE vesicles labeled with (12-CPS)-18-PC and (12-DABS)-18-PC were also obtained in similar experiments using vesicles labeled with (12-CPS)-18-PC plus either (12-DABT)-18-PC, (12-NBDMA)-18-PC, or (12-DABS)-18-PE (not shown). The proton-induced decrease in fluorescence of POPE vesicle labeled with (12-CPS)-18-PC plus an acceptor phospholipid thus appears to reflect some aspect of the behavior of the vesicles themselves, not simply an idiosyncracy in the behavior of one particular donor–acceptor probe combination. The simplest explanation for this behavior is that the massive aggregation and collapse of PE vesicles at acidic pH (Pryor et al., 1985; Ellens et al., 1986) allows significant energy transfer to develop between probes in distinct but apposed surfaces (Gibson & Loew, 1979b; Wolber & Hudson, 1979), since the equilibrium separation of neutral PE bilayers is very small (Lis et al., 1982; McIntosh & Simon, 1986). In this case it should be possible to reduce the extent of energy transfer between aggregated vesicles by using a donor–acceptor probe combination with a shorter effective energy-transfer length (Wolber & Hudson, 1979). Consistent with this suggestion, we found that POPE vesicles labeled with (12-CPS)-18-PC plus 1-palmitoyl-2-[16-[(trinitrophenyl)-amino]palmitoyl]-PC, a somewhat weaker energy-transfer acceptor than the DAB- and NBDMA-labeled species discussed above, showed almost no fluorescence decrease upon aggregation at pH 4.5 (Figure 5B, curve 3).

The results described above suggest that (12-CPS)-18-PC and (12-DABS)-18-PC can be used to monitor the proton-induced coalescence of POPE vesicles in a straightforward manner, ideally in a lipid-mixing/dequenching assay using a high ratio of unlabeled to labeled vesicles to minimize the possible development of energy transfer between probes in apposed bilayers. This conclusion was confirmed in a series of experiments (not shown) in which POPE vesicles labeled with 0.4 or 1.0 mol % each of (12-CPS)-18-PC and (12-DABS)-18-PC were induced to fuse at pH 6.5 with different amounts of unlabeled vesicles, calibrating the fluorescence signal corresponding to 100% lipid mixing by using premixed control samples as described above for the PS (calcium) system. Using a 9:1 ratio of unlabeled vesicles to vesicles labeled with 0.4 mol % each of (12-CPS)-18-PC and (12-DABS)-18-PC, we estimate that over the entire time course of proton-induced lipid mixing between POPE vesicles, the fluorescence signal will need to be corrected by an amount equivalent to no more than 10% of the maximum fluorescence change in order to determine accurately the extent of lipid mixing at any time. In principle, almost no correction of the fluorescence signal would be needed to monitor lipid mixing in an assay using POPE vesicles labeled with (12-CPS)-18-PC plus 1-palmitoyl-2-[16-[(trinitrophenyl)amino]palmitoyl]-PC, although a higher level of the acceptor probe (1–2 mol %) would be required in the labeled vesicle population in this case.

CONCLUSIONS

The primary goal of this study has been to develop and to characterize phospholipid analogues that offer fluorescence characteristics highly favorable for assays of lipid mixing between membranes, as well as a relative simplicity and versatility of preparation and a close resemblance to natural phospholipids in certain key properties, such as rates of spontaneous exchange between membranes. Of all the fluorescent lipid probes examined in this study, those that best fulfill our initial criteria appear to be the CPS- and CPT-labeled phospholipids with two long (C_{16} or C_{18}) acyl chains. The intense blue fluorescence, low environmental sensitivity, and good photostability of these species make them highly suitable as probes for lipid-mixing assays (in combination with energy-transfer acceptors such as DAB-labeled lipids), as well as for fluorescence microscopy (J. Bentz, personal communication). The CPS-labeled phospholipids are particularly attractive from a synthetic point of view, as a suitably protected mercapto fatty acid can be incorporated into any desired phospholipid structure, then deprotected under very mild conditions, and specifically labeled with the fluorescent moiety at the final stage of the preparation. The bimane-labeled PC's offer many of the same advantages as the coumarin-labeled

PC's and possess a smaller fluorescent group, although the bimane-labeled species exhibit a less intense fluorescence and a slightly greater environmental sensitivity.

The labeled phospholipids described in this study, and a wide variety of analogous species that can readily be prepared with different head groups, acyl chains, and sites of attachment of the fluorescent groups, offer a useful and versatile tool for reliable and sensitive assays of lipid mixing between membranes. Further experiments are currently in progress to explore these and other applications of these novel lipid probes.

Registry No. C_{12}-NBD-PC, 108535-66-6; (11-NBDMA)-11-PC, 108535-67-7; (16-NBDMA)-16-PC, 108535-68-8; (12-NBDMA)-18-PC, 108535-69-9; (12-CA)-18-PC, 108535-70-2; (12-CPT)-18-PC, 108560-82-3; (12-CPS)-18-PC, 108535-71-3; (16-CPT)-16-PC, 108535-72-4; (11-bimanyl)-11-PC, 108535-73-5; (12-bimanyl)-18-PC, 108535-74-6; (12-DABS)-18-PC, 108535-75-7; (12-DABT)-18-PC, 108535-76-8; DPH-PC, 98014-38-1; POPC, 26853-31-6; DEPA, 98574-23-3; DOPE, 4004-05-1; 12-(methylamino)stearic acid, 107743-38-4; *N*-Boc-12-(methylamino)stearic acid, 108535-77-9; methyl 12-hydroxystearate, 141-23-1; 11-mercaptoundecanoic acid, 71310-21-9; 11-bromoundecanoic acid, 2834-05-1; 12-mercaptostearic acid, 108535-78-0; methyl 12-hydroxystearate (methanesulfonate derivative), 108535-79-1.

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