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Calcium-Induced Lipid Phase Separations and Interactions of Phosphatidylcholine/Anionic Phospholipid Vesicles. Fluorescence Studies Using Carbazole-Labeled and Brominated Phospholipids†

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ABSTRACT: A novel method that uses a carbazole-labeled fluorescent phosphatidylcholine, which partitions preferentially into liquid-crystalline lipid domains, to monitor the kinetics and the extents of thermotropic and ionotropic lateral phase separations in vesicles combining brominated and nonbrominated phosphatidylcholines (PCs), phosphatidic acids (PAs), and phosphatidylserines (PSs) is described. The calcium-induced segregation of several nonbrominated PA species in liquid-crystalline brominated PC bilayers behaves as a well-defined lateral phase separation; the residual solubility of the PA component in the PC-rich phase in the presence of calcium can vary severalfold depending on the PA acyl chain composition. PC/PS mixtures show a pronounced tendency to form metastable solutions in the presence of calcium, particularly when they contain less than equimolar proportions of PS. This metastability is not readily relaxed by repeated freeze–thawing of vesicles in the presence of calcium, by avidin-mediated contacts between PC/PS vesicles containing biotinylated lipids, or by calcium-induced lateral segregation of PA in the same vesicles. Different PS species exhibit different apparent residual solubilities in liquid-crystalline PC bilayers, ranging from <10 mol % for dimyristoyl-PS to ca. 45 mol % for dioleoyl-PS, after prolonged incubations of PC/PS multilamellar vesicles with excess calcium. Results are presented, obtained by using the above lipid-segregation assay and parallel assays of intervesicle lipid mixing, that raise questions concerning the relevance of the equilibrium behavior of calcium-treated PS/PC mixtures to the relatively rapid interactions (fusion and lipid mixing) of PC/PS vesicles that follow initial exposure to calcium.

Since most biological membranes contain a wide variety of different lipid species, an understanding of the interactions and the lateral distributions of different lipids in multicomponent bilayer membranes is important to understand many aspects of membrane behavior. Several physical techniques, including calorimetry, nuclear magnetic resonance, electron spin resonance, vibrational spectroscopy, X-ray diffraction, and

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freeze-fracture electron microscopy, have been applied extensively to characterize the interactions of different lipid species in relatively simple systems containing a small number of lipid components [for reviews, see Lee (1977), Boggs (1980), Ohnishi and Tokutomi (1980), McElhaney (1982), Jain (1983), Casal and Mantsch (1984), Cullis et al. (1985), and Thompson and Tillack (1985)].

Fluorescence spectroscopy offers in principle an attractive alternative method to characterize the interactions of different lipid species in multicomponent bilayer membranes. Fluorescence-based experiments offer high sensitivity and selectivity, rapid temporal resolution, and considerable versatility in the choice of probes and samples employed. Moreover, measurements of the efficiencies of distance-dependent interactions between different lipid probes (e.g., energy transfer or static quenching) can provide useful information concerning the interactions and the lateral distributions of different lipid species in a given lipid mixture. Successful application of this approach requires careful choices of lipid probes, however, to ensure that the probe molecules reflect faithfully the behavior of the unlabeled species whose properties are of primary interest. Several recent studies have described the use of fluorescent lipid probes to monitor the lateral distributions of different lipid species in multicomponent lipid bilayer systems (Somerharju et al., 1985; Graham et al., 1985; Jones & Lentz, 1986; Parente & Lentz, 1986; Leventis et al., 1986; Haverstick & Glaser, 1987, 1988, 1989) and in natural membranes (de Bony et al., 1989).

The present study describes a novel fluorescence-based method, employing measurements of the contact-dependent interactions between carbazole-labeled fluorescent lipids (Lackowicz & Hogen, 1980) and brominated lipid quenchers (East & Lee, 1982; McIntosh & Holloway, 1987), to study the mutal lateral distributions of different lipid species in multicomponent lipid bilayers. The method offers a very favorable combination of sensitivity, versatility, and experimental convenience for the study of lipid lateral distributions and phase separations in systems containing an arbitrary number of components. In this study, this approach has been applied to characterize the calcium-induced lateral segregation of anionic lipids in a variety of phosphatidylcholine/phosphatidic acid (PC/PA) and phosphatidylcholine/phosphatidylserine (PC/PS) mixtures and to investigate the relationship between lipid lateral segregation and the calcium-mediated interactions of PC/PS vesicles.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-, dioleoyl-, and dielaidoylphosphatidylcholines and egg yolk phosphatidycholine, as well as lysopalmitoylphosphatidylcholine, were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). 11,12-Dibromooctadecanoic acids were prepared from *cis*- and *trans*-vaccenic acid by treatment with a slight excess of bromine in dry CCl_4 at 0 °C in the dark. After a 15-min reaction, excess bromine was destroyed with aqueous potassium metabisulfite, and the organic phase was washed twice with 1% aqueous NaCl and then dried (Na_2SO_4) and concentrated under N_2. 1-Palmitoyl-2-(11,12-dibromooctadecanoyl)phosphatidylcholines and 1-palmitoyl-2-(11′-carbazolylundecanoyl)phosphatidylcholine were prepared by acylation of 1-palmitoyllysophosphatidylcholine with the appropriate fatty acid anhydrides, using methods described previously (Mason et al., 1981).

Phosphatidylcholines were prepared from the corresponding phosphatidic acids by phospholipase D digestion as described previously (Graham et al., 1985). Phosphatidylserines were prepared from the pyridinium salts of the corresponding phosphatidic acids as described elsewhere (Silvius & Gagné, 1984a). All anionic phospholipids were finally twice precipitated from chloroform/methanol with barium acetate (Comfurius & Zwaal, 1977), and the barium was subsequently removed by successive Folch washes (Folch et al., 1957) using 100 mM EDTA,[1] 100 mM Na_2SO_4, and 100 mM EDTA as the aqueous components.

Methods

Vesicle Preparations. Lipid samples were dried from chloroform solution, first under nitrogen and then for at least 2 h under high vacuum. The dried lipid mixtures were then dispersed in 150 mM KCl, 5 mM Tes, and 0.2 mM EDTA, pH 7.4, above the transition temperature of the higher melting component by one of the following procedures: (1) vortexing by hand for 1 min; (2) bath sonication for 2–5 min under nitrogen to produce a consistently low turbidity in all samples; or (3) bath sonication followed by eight cycles of freezing in a dry ice/ethanol bath and thawing at room temperature. Trapped-volume measurements, using carboxyfluorescein as a marker of the internal aqueous space (Wilschut et al., 1980), indicated that bath-sonicated vesicles of various compositions exhibited a roughly 4-fold increase in trapped volume per unit of lipid after freeze–thawing. Extended sample incubations were carried out in polypropylene tubes to minimize sample losses due to adhesion to the walls of the incubation vessel. Vesicles containing ≥85 mol % PS or PA aggregated massively on prolonged exposure to calcium and could not be readily redispersed sufficiently well to permit accurate fluorescence readings after incubation in glass vessels.

Fluorescence Measurements. The fluorescence of lipid dispersions was measured in a Perkin-Elmer LS-5 spectrofluorometer, using excitation and emission wavelengths of 293 and 358 rm, respectively, to monitor carbazole-PC fluorescence and of 390 and 468 rm, respectively, to monitor (12-CPS)-18-PC fluorescence. Except where otherwise indicated, fluorescence measurements were carried out at 37 °C using continuously stirred lipid samples dispersed in 150 mM KCl, 5 mM Tes, and 0.2 mM EDTA, pH 7.4, at a lipid concentration of 20 μM. To standardize the amount of fluorescent probe in each sample, the fluorescence was also measured for a duplicate aliquot of each sample that was dispersed into methanol.

Lipid mixing between vesicles was assayed by a resonance energy transfer based assay as described previously (Silvius et al., 1987). For these assays "labeled" vesicles (2 μM lipid), containing 1 mol % (12-CPS)-18-PC and 0.35 mol % (12-

[1] Abbreviations: Bio-PE, *N*-[(6-biotinyl)amino]caproylphosphatidylethanolamine; carbazole-PC, 1-palmitoyl-2-(11′-carbazolylundecanoyl)-*sn*-glycero-3-phosphocholine; cBr_2-PC (-PS/-PA) or tBr_2-PC (-PS/-PA), 1-palmitoyl-2-(11,12-dibromooctadecanoyl)-*sn*-glycero-3-phosphocholine (-3-phosphoserine/-3-phosphatidic acid) whose dibromooctadecanoyl moieties are derived by bromine addition to 11-*cis*- or 11-*trans*-octadecenoic acid, respectively; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; (12-CPS)-18-PC, 1-palmitoyl-2-[12-[[*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]-methyl]thiostearoyl]phosphatidylcholine; (12-DABS)-18-PC, 1-palmitoyl-2-[12-[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylaminostearoyl]phosphatidylcholine; DE, 1,2-dielaidoyl; DM, 1,2-dimyristoyl; DO, 1,2-dioleoyl; DSPC, 1,2-distearoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid trisodium salt; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PO, 1-palmitoyl-2-oleoyl; PS, phosphatidylserine; NBD, *N*-(7-nitrobenz-oxadiazol-4-yl); Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt.

Table I: Maximum Emission Wavelengths Measured for Carbazole-PC in Different Organic Solvents and Egg Phosphatidylcholine Vesicles

solvent	dielectric constant	max emission wavelengths[b] (nm)
hexane + 3% ether[a]	1.96	357.6, 374.5
cyclohexane	2.02	356.9, 373.8
diethyl ether	4.34	356.8, 373.7
1-butanol	17.1	355.6, 372.8
methanol	32.6	355.1, 372.8
phosphatidylcholine vesicles[c]		357.2, 374.4

[a] Carbazole-PC was first dissolved in a small volume of dry, alcohol-free diethyl ether, which was then diluted 33-fold with hexane. [b] Sample spectra were recorded at 25 °C by using an excitation wavelength of 293 nm and excitation and emission slits set at 10 nm and 5 nm, respectively. [c] Very similar emission wavelengths were measured for carbazole-PC in 70:30 POPS/POPC or 50:50 POPA/POPC vesicles in the presence or absence of 10 mM calcium.

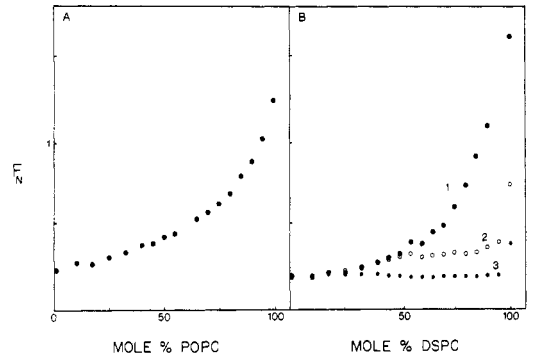

FIGURE 1: Variation of normalized carbazole-PC fluorescence with mole fraction of cBr_2-PC in multilamellar vesicles combining POPC and cBr_2-PC (panel A) or distearoyl PC and cBr_2-PC (panel B). Curves 1–3 in panel B represent data obtained at 55, 45, and 20 °C, respectively. The normalized intensity of carbazole-PC fluorescence is expressed as the ratio of the intensity of carbazole-PC fluorescence recorded for a given sample in buffer divided by the fluorescence intensity measured for an equal aliquot of the same sample dissolved in methanol.

DABS)-18-PC, were incubated with a 9-fold excess of "unlabeled" vesicles, containing 1.5 mol % carbazole-PC, and the fluorescence of (12-CPS)-18-PC was monitored. For parallel measurements of lipid lateral redistributions under the same conditions, the latter vesicles alone were used at a concentration of 20 μM, and the fluorescence of carbazole-PC was monitored.

Results

Fluorescence Properties of Carbazole-PC. In Table I are listed the wavelengths of maximum fluorescence emission measured for carbazole-PC in several organic solvents and in egg PC vesicles. The emission wavelengths for this probe in the phospholipid vesicles are intermediate between those measured in hexane and in cyclohexane and are significantly longer than those measured in more polar solvents, consistent with a localization of the carbazole moiety in the hydrocarbon interior of the bilayer.

As is illustrated in Figure 1, the fluorescence of carbazole-PC is efficiently quenched by 1-palmitoyl-2-(11,12-dibromooctadecanoyl) phospholipids such as cBr_2-PC in bilayer lipid vesicles. As shown in Figure 1A, the fluorescence measured for carbazole-PC in cBr_2-PC/POPC vesicles at 20 °C increases smoothly with increasing mole fractions of the nonbrominated species. A similar result is observed for carbazole-PC in cBr_2-PC/DSPC vesicles at 55 °C, as shown in Figure 1B (curve 1). Markedly different results are observed,

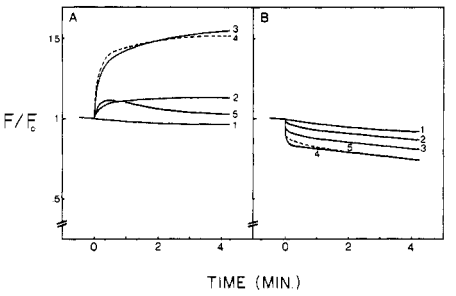

FIGURE 2: Time courses of fluorescence recorded when sonicated/freeze–thawed PA/PC vesicles, labeled with carbazole-PC (1.5 mol %), were exposed to calcium (10 mM) at 37 °C. (Panel A) cBr_2-PA/POPC vesicles; (panel B) POPA/cBr_2-PC vesicles. In each panel, curves 1–5 were recorded for vesicles containing 0, 25, 50, 75, and 90 mol % PA, respectively. Calcium was added at time zero with rapid stirring to vesicle samples containing 20 μM lipid in 150 mM KCl, 10 mM Tes, and 0.1 mM EDTA, pH 7.4.

however, when the fluorescence of carbazole-PC is measured in the latter vesicles at lower temperatures (Figure 1B, curves 2 and 3). Under these conditions, the fluorescence of carbazole-PC no longer increases smoothly with increasing DSPC content but instead levels off above a critical DSPC mole fraction, which is ca. 50 mol % at 45 °C and ca. 20 mol % at 20 °C. This behavior is readily understandable (see Appendix) if we assume that the carbazole probe partitions only very weakly into DSPC-enriched gel-phase domains that coexist with a cBr_2-PC-enriched liquid-crystalline phase at lower temperatures. From the data shown in Figure 1B, we estimate that such segregated gel-state domains appear when the bilayers contain more than ca. 20 mol % DSPC in cBr_2-PC at 20 °C and more than ca. 50 mol % DSPC in cBr_2-PC at 45 °C.

Calcium-Induced Phase Separations in PA/PC Vesicles. In Figure 2 are shown time courses of fluorescence observed when sonicated/freeze–thawed vesicles, labeled with carbazole-PC and prepared from various mixtures of either POPC and cBr_2-PA (panel A) or cBr_2-PC and POPA (panel B), are exposed to 10 mM calcium. In both cases, the addition of calcium leads to rapid and substantial changes in fluorescence, which differ in direction for the two types of vesicles (enhancement when the brominated component is PA, diminution when it is PC) and which vary in magnitude with the vesicles' PA content. The addition of equal concentrations of calcium to carbazole-PC-labeled vesicles containing equimolar amounts of POPC and POPA produced only a very slow and modest decrease in fluorescence, probably as a consequence of gradual vesicle precipitation (not shown). These results, and further findings presented below, suggest that carbazole-PC faithfully reports a rapid lateral redistribution of brominated and nonbrominated lipids in PA/PC vesicles upon addition of calcium.

The effects of several experimental factors on the calcium-induced fluorescence changes reported by carbazole-PC in cBr_2-PA/POPC and in POPA/cBr_2-PC vesicles were examined, with results that can be summarized as follows. Sonicated/freeze–thawed vesicles gave relatively rapid fluorescence changes upon calcium addition, which were comparable in their final amplitudes to the considerably slower fluorescence changes observed with vortexed vesicles of the same composition were used. Maximal amplitudes of fluorescence change were observed for these vesicles at calcium concentrations of 5–10 mM and at pH values of 6.5 or higher. Finally, the fluorescence changes induced by calcium in cBr_2-PA/POPC vesicles were not detectably accelerated by addition of a calcium ionophore, bromo-A23187 (20 nM), with or without the protonophore CCCP (30 nM) (result not shown). Higher

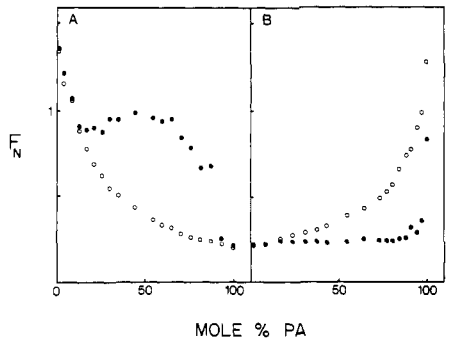

FIGURE 3: Normalized fluorescence intensities measured when sonicated/freeze–thawed vesicles, labeled with 1.5 mol % carbazole-PC and composed of mixtures of cBr_2-PA in POPC (panel A) or POPA in cBr_2-PC (panel B), were incubated for 48 h in the presence (solid circles) or absence (open circles) of 10 mM calcium. Details of sample incubation and data collection were as given under Materials and Methods.

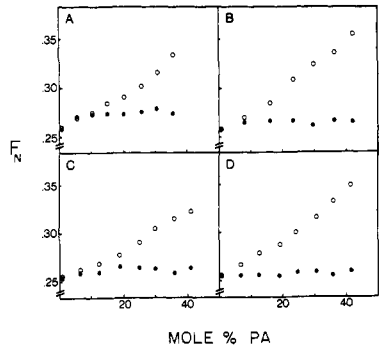

FIGURE 4: Normalized fluorescence intensities measured when sonicated/freeze–thawed vesicles, labeled with 1.5 mol % carbazole-PC and combining cBr_2-PC with the indicated molar percentages of DOPA (panel A), POPA (panel B), PA derived from egg yolk PC (panel C), or DEPA (panel D), were incubated for 48 h in the presence (solid circles) or absence (open circles) of 10 mM calcium. Details of sample incubation and data collection are given under Materials and Methods.

levels of bromo-A23187 were not employed, as they caused significant calcium-dependent quenching of carbazole-PC fluorescence.

In further experiments, the fluorescence of carbazole-PC was recorded in sonicated/freeze–thawed vesicles containing varying proportions of cBr_2-PA in POPC, or of POPA in cBr_2-PC, after prolonged incubations in the presence or absence of calcium (10 mM). The results of these measurements are plotted in Figure 3 as a function of the mole fraction of the PA component (panel A, cBr_2-PA in POPC; panel B, POPA in cBr_2-PC). In both cases, the normalized fluorescence measured in the absence of calcium increases continuously with increasing mole fraction of the nonbrominated lipid species. By contrast, the corresponding fluorescence curves measured in the presence of calcium show extended regions that are nearly horizontal, beginning at ca. 11 mol % cBr_2-PA in POPC and <10 mol % POPA in cBr_2-PC. This behavior is precisely that expected if calcium induces lateral segregation of a PA-rich phase in vesicles containing mole fractions of PA higher than these values, assuming that the carbazole-PC probe partitions strongly into the PC-rich phase (see Appendix). It is noteworthy that vesicles containing mole fractions of PA that exceed only slightly these apparent "solubility thresholds" nonetheless show rapid, readily observable fluorescence changes upon addition of calcium (see, e.g., Figure 2). There is thus little indication that these PA species form metastable solutions in liquid-crystalline PC bilayers in the presence of calcium.

To assess to what extent the acyl chain composition of PA affects the proclivity of this lipid to segregate from PC in the presence of calcium, we examined the effect of calcium on the fluorescence of carbazole-PC in vesicles combining cBr_2-PC with increasing molar percentages of different nonbrominated PA species. As shown in Figure 4, calcium-induced lateral segregation of the PA component, assessed by the appearance of a near-horizontal region in the plot of normalized carbazole-PC fluorescence vs PA content, is observed for vesicles containing more than roughly 10 mol % DOPA, 7 mol % egg PA, 5 mol % POPA, or 1–2 mol % DEPA. In control experiments, calcium induced no significant fluorescence changes in similar vesicles in which the brominated PC component was replaced by POPC (not shown).

Calcium-Induced Lipid Lateral Segregation in PC/PS Vesicles. (a) Rapid Effects of Calcium. In Figure 5A are shown time courses of fluorescence observed when carbazole-PC-labeled (sonicated/freeze–thawed) vesicles containing cBr_2-PS and POPC in varying proportions were exposed to

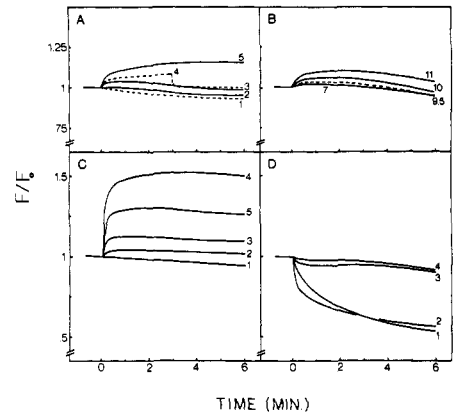

FIGURE 5: Time courses of fluorescence recorded after addition of calcium to PC/PS vesicles labeled with 1.5 mol % carbazole-PC. (Panel A) Bath-sonicated vesicles, containing cBr_2-PS and POPC in varying proportions (curves 1–5: 0, 60, 70, 80, and 90 mol % PS, respectively), were exposed to 10 mM calcium at time zero. Curve 4 (dashed) indicates the effects of addition of excess citrate (20 mM) 3 min after the addition of calcium to vesicles containing 80 mol % cBr_2-PS. (Panel B) Sonicated/freeze–thawed vesicles composed of cBr_2-PS, POPC, and carbazole-PC in a 75:25:1.5 molar ratio were exposed to calcium (10 mM) at time zero in buffers of the indicated pH. Solutions of pH 8.5 or lower were buffered with 10 mM Tes, while solutions of pH 9–11 were buffered with glycine. (Panel C) Bath-sonicated vesicles, containing 0, 10, 25, 60, or 90 mol % cBr_2-PS (curves 1–5, respectively), were incubated in 150 mM KCl, 10 mM Tes, 2.5 mM K_2HPO_4, and 0.1 mM EDTA, pH 7.4, before calcium was added to 4 mM at time zero. The addition of calcium under these conditions produced only a small increase in sample turbidity; very large turbidity increases were observed when higher concentrations of phosphate (5 mM) or calcium (10 mM) were employed. (Panel D) Bath-sonicated vesicles, combining cBr_2-PC with 75 mol % DEPS (curve 1), DMPS (curve 2), brain PS (curve 3), or DOPS (curve 4), were exposed to 10 mM calcium at time zero. Results essentially identical with those shown for DOPS-containing vesicles were also observed for similar vesicles containing POPS (not shown).

10 mM calcium. The addition of calcium induces a modest amount of rapid fluorescence dequenching for vesicles containing more than ca. 50 mol % cBr_2-PS, while very little calcium-induced dequenching is observed for vesicles with lower PS contents. Essentially complete reversal of the calcium-induced changes in carbazole-PC fluorescence was observed when a 2-fold excess of the weak calcium chelator citrate (Durham, 1983) was added to vesicles containing <80 mol % PS within several minutes after calcium addition (Figure 5A, curve 4). However, citrate could not completely reverse the fluorescence changes observed in PS/PC vesicles at longer times (several hours) after calcium addition, although

the stronger calcium chelator EDTA could do so (not shown).

Several approaches were explored in an effort to enhance the rapid fluorescence changes induced by calcium in vesicles combining cBr_2-PS, POPC, and carbazole-PC in 75:25:1.5 or 60:40:1.5 molar proportions. Bath-sonicated vesicles of these compositions gave slightly greater extents of rapid calcium-induced fluorescence dequenching than did sonicated/freeze–thawed vesicles, while vesicles dispersed by gentle vortexing showed little rapid dequenching of fluorescence under the same conditions. These calcium-induced fluorescence changes were not enhanced by drastically reducing the ionic or the osmotic strengths of the intra- or extravesicular media or by adding bromo-A23187 (20 nM) to the vesicles, with or without 30 nM CCCP. The inclusion of cholesterol in the vesicles, in an equimolar proportion with the POPC component, also had no observable effect on the calcium-induced lateral segregation of cBr_2-PS (not shown).

Two modifications of the standard aqueous medium used in the above experiments (150 mM KCl, 5 mM Tes, 0.1 mM EDTA, pH 7.4) were found to increase significantly the degree of fluorescence dequenching observed on addition of calcium to cBr_2-PS/POPC/carbazole-PC vesicles. First, increasing the pH above 9.5 modestly increased the extent of calcium-induced fluorescence dequenching in these vesicles, as illustrated in Figure 5B. Second, and more dramatically, the presence of millimolar concentrations of phosphate in the medium substantially increased the extent of fluorescence dequenching observed when the vesicles were exposed to calcium (Figure 5C). These effects of phosphate were not observed with cBr_2-PS/POPC vesicles without carbazole-PC or with POPC/POPS vesicles labeled with carbazole-PC. In other experiments (not shown), low millimolar concentrations of phosphate were also found to potentiate substantially the calcium-induced mixing of lipids between vesicles containing 10–50 mol % PS in PC, which showed little or no lipid mixing in the presence of calcium alone. These effects of phosphate probably reflect the nucleation of calcium phosphate precipitation on the vesicle surfaces, as the effects increase dramatically, at a fixed (millimolar) concentration of calcium, when the phosphate concentration rises above a threshold value in the low millimolar range.

The results presented in Figure 5D illustrate the effects of calcium addition on the fluorescence of carbazole-PC in vesicles combining cBr_2-PC with various species of PS in a 25:75 molar ratio. While very little rapid fluorescence quenching is observed upon addition of calcium to vesicles containing cis-unsaturated PS species (POPS, brain PS, or DOPS), significant quenching is observed for vesicles containing saturated or trans-unsaturated PS species (DMPS or DEPS). Phosphate (2.5 mM, combined with 4 mM calcium) significantly enhanced the rapid fluorescence changes induced by addition of calcium to cBr_2-PC/DOPS and cBr_2-PC/POPS vesicles, although this effect was less dramatic than in the case of POPC/cBr_2-PS vesicles (not shown).

(*b*) *Effects of Prolonged Calcium Exposure.* In further experiments, carbazole-PC fluorescence was used to monitor the lateral redistributions of the lipid species in several types of PC/PS mixtures after prolonged incubations (48–120 h) in the presence of calcium. The data shown in Figure 6A–E were obtained by using multilamellar vortexed vesicles, which in some cases showed more extensive lateral segregation of PS under these conditions than did unilamellar vesicles of the same compositions. The results shown in Figure 6A for cBr_2-PC/DEPS vesicles are representative of those obtained for the other systems examined and will be discussed in detail.

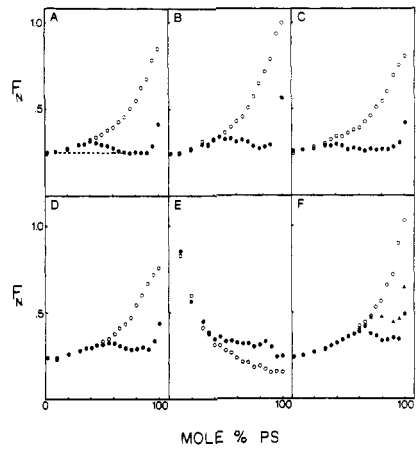

FIGURE 6: Normalized fluorescence intensities measured when multilamellar PC/PS vesicles, labeled with 1.5 mol % carbazole-PC, were incubated for 48 h in the presence (solid circles) or absence (open circles) of 10 mM calcium. The following PC/PS mixtures, containing the indicated molar percentages of the PS component, were examined: (panel A) cBr_2-PC/DEPS; (panel B) cBr_2-PC/POPS; (panel C) cBr_2-PC/DMPS; (panel D) cBr_2-PC/bovine brain PS; (panel E) POPC/tBr_2-PS; (panel F) cBr_2-PC/DOPS. Solid triangles in panel F represent data obtained by using bath-sonicated rather than vortexed lipid dispersions. Further details of sample incubation and data collection are given under Materials and Methods.

In the absence of calcium, cBr_2-PC and DEPS appear completely miscible, giving a smooth variation of the carbazole-PC fluorescence intensity, without discernible breaks or plateaus, as the mole fraction of the brominated lipid component is varied (Figure 6A, open circles). Even in the presence of calcium (10 mM), lateral segregation of the PS component cannot be observed for vesicles containing less than ca. 45 mol % DEPS (solid circles). However, as the vesicle PS content rises above this value, the normalized carbazole-PC fluorescence measured in the presence of calcium does not simply become constant, as would be expected for a well-defined lateral phase separation (see e.g., Figure 2B), but instead decreases to reach a constant minimum value between 70 and 90 mol % DEPS before rising again as the DEPS content approaches 100 mol %. A plausible explanation for this behavior, which is consistent with previous findings for other PC/PS systems (Florine & Feigenson, 1987; Feigenson, 1989; Coorssen & Rand, 1989) is that the "true" solubility of DEPS in liquid-crystalline bilayers of cBr_2-PC in the presence of calcium is significantly less than 45 mol % but that cBr_2-PC and DEPS can form metastable solutions in vesicles with DEPS contents lower than this value.

In the light of the above results, a reasonable maximum estimate for the true equilibrium solubility of DEPS in cBr_2-PC bilayers in the presence of calcium can be obtained as illustrated in Figure 6A: a horizontal line is extrapolated leftward from the minimum in the high-PS region of the fluorescence curve to intersect the left-hand rising phase of the curve at ca. 10 mol % DEPS. Applying this type of analysis to the data presented in Figure 6, we can extract the following maximum estimates for the equilibrium solubilities of the PS component in various PC/PS mixtures in the presence of calcium: ca. 45 mol % DOPS, 30 mol % brain PS, 20 mol % POPS, 10 mol % DEPS, and <10 mol % DMPS in cBr_2-PC and ca. 40 mol % tBr_2-PS and 65 mol % cBr_2-PS (not shown) in POPC. Significantly, neither initial freeze–thawing (up to six cycles at −10 or −70 °C) in calcium-containing buffer nor the addition of bromo-A23187 (20 nM) was able to induce any indication of lipid lateral segregation in multilamellar vesicles containing <40 mol % DOPS in cBr_2-PC or cBr_2-PS in POPC

after incubation for 48–120 h in the presence of 10 mM calcium at 37 °C.

As noted above, the extent of lipid redistribution in PC/PS vesicles was in some cases significantly different when unilamellar (sonicated or sonicated/freeze–thawed) vs multilamellar (gently vortexed) vesicles were incubated in the presence of calcium. This point is illustrated by the results shown in Figure 6F, which were obtained with vortexed (solid triangles) and bath-sonicated (solid circles) DOPS/cBr_2-PC vesicles. The other PC/PS mixtures examined in this study gave less marked differences in the behavior of sonicated vs vortexed dispersions when incubated with calcium under the same conditions.

Calcium-Induced Phase Separations and Intervesicle Interactions. The results presented above suggest that prolonged interbilayer apposition (as would occur in multilamellar dispersions), as opposed to simple transient collisions between lipid vesicles, may be important to initiate lateral segregation of PS in the presence of calcium. In agreement with this conclusion, rapid calcium-induced lateral redistribution of cBr_2-PS and tBr_2-PS was observed in vesicles containing as little as 10 mol % of these species in POPE or 30 mol % of these species in *N*-methyl-POPE (not shown). Both of these neutral lipid species support much stronger interbilayer interactions (vesicle aggregation and membrane coalescence) than does POPC (Uster & Deamer, 1981; Düzgünes et al., 1981; Silvius & Gagné, 1984a,b; Gagné et al., 1985; Ellens et al., 1986a,b).

To address more directly the question of whether lateral segregation of PS in PC/PS bilayers can be promoted by factors that promote interbilayer associations, two approaches were explored. First, vesicles were prepared by combining POPC and cBr_2-PS with POPA, a lipid that promotes calcium-mediated interaction between vesicles when present even in relatively low molar proportions (Leventis et al., 1986). Second, vesicles combining various PC/PS mixtures with small amounts of biotinylated PE (Bio-PE) were preaggregated with avidin before exposure to calcium. In both cases, intervesicle lipid mixing and lateral redistribution of the lipid species were monitored in parallel samples, as described under Materials and Methods, to characterize the interactions of these vesicles upon the addition of calcium.

As shown in Figure 7A (curve 1), bath-sonicated vesicles containing cBr_2-PS and POPC in equimolar proportions show a limited amount of lipid mixing on a time scale of minutes when exposed to 10 mM calcium. In a parallel experiment, monitoring of carbazole-PC fluorescence shows evidence for only slight lateral redistribution of the lipid species in these vesicles on this time scale (Figure 7C, curve 1). When the above experiment is repeated with vesicles containing equimolar cBr_2-PS and POPC plus 20 mol % POPA, the rate and extent of calcium-induced lipid mixing between the vesicles are markedly increased (Figure 7A, curve 2), but the calcium-induced change in carbazole-PC fluorescence (Figure 7C, curve 2) is no more extensive. Several related experiments led to the same general conclusion: in the presence of calcium, vesicles combining up to 20 mol % POPA with a given mixture of POPC and cBr_2-PS showed better lipid mixing, but no more extensive lateral redistribution of the brominated PS component, than did PA-free vesicles containing POPC and cBr_2-PS in the same relative proportions. Related experiments (not shown) likewise revealed no marked synergy in the calcium-induced lateral segregation of cBr_2-PS and of cBr_2-PA from POPC in vesicles combining these three lipid species.

Another series of experiments examined how avidin-mediated preaggregation affected the processes of calcium-promoted lipid mixing and lipid lateral redistribution for PC/PS vesicles containing a small proportion of biotinylated PE (Bio-PE). In parts B and D of Figure 7 are shown representative results obtained with vesicles containing 40:60 mixtures of DOPS/cBr_2-PC, DMPS/cBr_2-PC, or cBr_2-PS/POPC together with 2 mol % Bio-PE. In all three cases, the addition of 10 mM calcium leads to a slow and limited mixing of lipids between the vesicles (Figure 7B), which is accompanied by negligible lateral redistribution of the lipid components (Figure 7D). If the same vesicles are first incubated with avidin, at a concentration sufficient to produce a severalfold increase in turbidity, neither the time course of lipid mixing nor the lateral redistribution of lipids upon subsequent addition of calcium is significantly altered. Similar vesicles containing lower molar proportions of PS (≤35 mol %) showed negligible extents of calcium-induced lipid mixing and lipid lateral redistribution in the presence or absence of avidin.

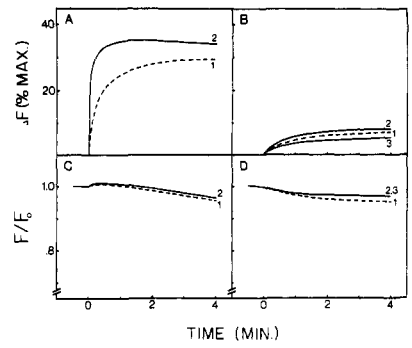

FIGURE 7: (Panels A and C) Time courses of lipid mixing (panel A) and lipid lateral redistribution, as monitored by carbazole-PC fluorescence (panel C) when sonicated vesicles containing either 50:50 cBr_2-PS/POPC (curves 1) or 40:40:20 cBr_2-PS/POPC/POPA (curves 2) were exposed to calcium (10 mM) at time zero. (Panels B and D) Time courses of lipid mixing (panel B) and lipid lateral redistribution, as monitored by carbazole-PC fluorescence (panel D) when sonicated PS/PC vesicles containing 2.5 mol % Bio-PE were exposed to calcium (10 mM) at time zero with (solid curves) or without (dashed curves) prior incubation with avidin. Vesicles (1 mM lipid) were preincubated for 30 min at 25 °C with or without avidin (1 nmol/10 nmol of Bio-PE) and then diluted to 20 μM lipid and warmed to 37 °C for fluorescence measurements. (Curves 1) Vesicles combining DMPS with cBr_2-PC in a 40:60 molar ratio, preincubated without avidin; (curves 2) vesicles combining DMPS with cBr_2-PC in a 40:60 molar ratio, preincubated with avidin; (curves 3) vesicles combining cBr_2-PS with DOPC in a 40:60 molar ratio, preincubated with avidin. Experimental traces for vesicles combining DOPS with cBr_2-PC in a 40:60 molar ratio were essentially indistinguishable from those shown for DMPS/cBr_2-PC vesicles (curves 1 and 2) in panels B and D. Other experimental details are given under Materials and Methods.

Discussion

The potential to use fluorescence measurements to monitor the lateral distributions of different lipid species in multicomponent lipid bilayers is an attractive one, and several types of lipid probes, including diphenylhexatrienyl-, pyrenyl-, anthracenyl-, and NBD-labeled phospholipids (Somerharju et al., 1985; Graham et al., 1985; Jones & Lentz, 1986; Parente & Lentz, 1986; Leventis, et al., 1986; Haverstick & Glaser, 1987, 1988, 1989; de Bony et al., 1989) have in fact been used in the past to monitor lipid lateral distributions in multicomponent lipid and lipid/protein bilayers. The present combination of a carbazole-labeled fluorescent phospholipid probe and a brominated quencher species offers some particular advantages for the study of lipid lateral distributions in multicomponent lipid bilayer membranes. First, both the fluorescent reporter group and the corresponding quencher moiety are relatively nonpolar entities that are expected to associate reasonably well with the hydrocarbon interior of a

lipid bilayer, in agreement with the results presented in this and previous papers (Lackowicz & Hogen, 1980; McIntosh & Holloway, 1987). Second, since the quenching of carbazole–phospholipid fluorescence by brominated phospholipids is a contact-dependent phenomenon, measurements of the efficiency of quenching of carbazole–phospholipid fluorescence by brominated phospholipids may allow detection of even small-scale inhomogeneities in lipid mixing (e.g., "clustering" of particular species), as well as outright lateral segregation of well-defined phases, in bilayers combining brominated and nonbrominated phospholipids. Finally, the brominated quencher species employed in this study are simple to prepare in large quantities and are very similar to natural phospholipids in many important physical properties. It should in fact be possible, for critical applications, to construct brominated and fluorescent-labeled phospholipid probes that resemble natural phospholipids even more closely in their physical properties than do the species employed here [see, e.g., Lala et al. (1988)].

Calcium-induced phase separations have been demonstrated in different binary mixtures of PA and PC by using a variety of techniques, including electron spin resonance (Ito & Ohnishi, 1974; Galla & Sackmann, 1975), calorimetry (Jacobson & Papahadjopoulos, 1975; van Dijck et al., 1978; Graham et al., 1985; Smaal et al., 1987), fluorescence measurements (Graham et al., 1985; Haverstick & Glaser, 1987, 1988; Leventis et al., 1986), freeze-fracture electron microscopy (Hartmann et al., 1977), and vibrational spectroscopy (Kouaouci et al., 1985). The present study demonstrates that the isothermal lateral segregation of PA induced by calcium in liquid-crystalline PA/PC vesicles is rapid and behaves as a well-defined equilibrium phase separation for a variety of PA/PC mixtures. While all of the PA species examined exhibit a relatively low solubility in PC bilayers in the presence of calcium, this solubility limit varies significantly with the acyl chain composition of the PA, ranging from <2 mol % for DEPA to ca. 10 mol % for DOPA. The limit of solubility of PA in PC bilayers in the presence of calcium has been investigated in only a few cases, notably for a spin-labeled PA, which appears to be soluble to the extent of slightly less than 10 mol % in egg PC bilayers in the presence of calcium (Ito & Ohnishi, 1974; Feigenson, 1983).

Previous studies of calcium-induced lipid phase separations in PC/PS systems have produced a less consistent picture than have similar studies of PC/PA mixtures. Early spin-label studies (Ohnishi & Ito, 1974; Tokutomi et al., 1981; Ohki et al., 1981), and the more recent calcium-binding studies of Feigenson (1989), have suggested that calcium induces lateral phase separations in Millipore filter supported multibilayers containing as little as 15 mol % brain PS in egg PC, or 5 mol % DOPS in DOPC, at physiological temperatures. By contrast, lateral segregation of PS has generally been observed in multilamellar PC/PS vesicles only when the vesicles contain at least 30–35 mol % PS (van Dijck et al., 1978; Hui et al., 1983; Silvius & Gagné, 1984b; Florine & Feigenson, 1987). Studies to date using unilamellar vesicles have provided divergent results. Coorssen and Rand (1989) have reported that unilamellar PC/PS vesicles containing 20–70 mol % DOPS in DOPC show calcium-induced phase segregation by X-ray diffraction only when "stressed" (e.g., by mild osmotic dehydration), while Hoekstra (1982) concluded from fluorescence measurements that sonicated vesicles containing >20 mol % bovine brain PS in DOPC exhibit calcium-induced lateral segregation of lipids at equilibrium. The present results suggest that lateral redistribution of PS can occur when unilamellar as well as multilamellar PC/PS vesicles are incubated with calcium even in the absence of external stresses but that vesicles with intermediate PS contents may readily form long-lived metastable solutions in the presence of even high levels of calcium.

The calcium-dependent lateral segregation of PS appears to be strongly restricted, in two different senses, in vesicles containing less than ca. 50 mol % PS in PC. First, the calcium-induced lateral segregation of PS appears to be kinetically restricted in vesicles containing <50 mol % of various PS species in PC, even though some of these samples (e.g., DMPS/cBr_2-PC vesicles) show extensive lipid lateral redistribution after prolonged incubations in the presence of calcium. Avidin/biotinyl-PE-mediated preaggregation of such vesicles failed to enhance either rapid intervesicle lipid mixing or rapid lateral segregation of lipids upon subsequent addition of calcium. The addition of moderate amounts of PA to unilamellar vesicles containing 10–50 mol % PS in PC considerably enhanced calcium-induced mixing of lipids between the vesicles but nonetheless also failed to promote a rapid calcium-induced lateral segregation of the PS component. Therefore, neither the promotion of interbilayer apposition per se nor the formation of laterally segregated domains of another acidic phospholipid (PA) appears to be able to facilitate the ready segregation of the PS component in such mixtures. The observation that phosphate can act in synergy with calcium to promote rapid lateral segregation of PS in bilayers containing as little as 10 mol % PS is intriguing and agrees with the previous suggestions of Fraley et al. (1980). Further study will be required, however, to determine whether phosphate produces this effect in a purely catalytic manner (e.g., by promoting apposition and/or collapse of vesicles on the surfaces of calcium phosphate particles) or by comprising an integral element of the laterally segregated PS-rich domains that form under these conditions.

The calcium-induced segregation of PS/PC vesicles is also restricted in a second sense, namely, that the lateral segregation of unsaturated PS species in particular from PC appears to be far from absolute, even in samples that contain high mole fractions of PS and that are incubated for prolonged periods (days) in the presence of calcium. The apparent "residual solubilities" of different PS species in cBr_2-PC bilayers in the presence of calcium vary with the structure of the PS component, in the order DMPS < DEPS < POPS < brain PS < DOPS. This result is not surprising, as PS species with different acyl chains have been shown to differ significantly in several other aspects of their interactions with divalent cations, including calcium-binding affinity (Feigenson, 1986), the mean molecular area of the PS/calcium complex (Mattai et al., 1989), and the ability of magnesium to elicit acyl chain crystallization (Casal et al., 1989). Further work will be required to define how strongly other factors, such as the calcium concentration, the nature of the PC component, and the experimental temperature, affect the apparent residual solubility of PS in liquid-crystalline PC bilayers.

The quantitative conclusions presented above must be tempered by the consideration that the apparent solubilities of PS in PC bilayers in the presence of calcium have been reported to vary depending on the morphology as well as the composition of the PC/PS dispersion studied. Certain PS species have been reported to phase-separate more extensively from PC, in the presence of calcium, in Millipore-supported PC/PS multibilayers than in lipid vesicles of like composition (Ito & Ohnishi, 1974; Florine & Feigenson, 1987; Feigenson, 1989). Feigenson (1989) has suggested that this difference reflects

primarily a better equilibration of calcium between lamellae in the supported-multibilayer system than in preparations of lipid vesicles. If this is the case, the present estimates of the residual solubilities of different PS species in calcium-treated PC/PS vesicles must be considered to be only maximum or apparent values. If so, it is nonetheless noteworthy that a variety of treatments and incubation conditions fail to produce calcium-induced segregation of PS in PS/PC vesicles (even those with high PS contents) that is as extensive as that reported previously for supported-multibilayer systems. It is also possible, however, that supported PC/PS multibilayers and PC/PS vesicles exhibit genuinely different equilibrium properties in the presence of calcium, reflecting distinctive features of the supported-multibilayer system (e.g., the large number of closely and uniformly apposed lamellae or the presence of the cellulose filter substrate). We are exploring experimental approaches to address this issue directly.

Considerable interest has been directed to the possible role(s) of lipid lateral segregation in the divalent cation mediated interactions of membranes that combine anionic lipids with "fusion-refractory" species such as choline phospholipids (Hoekstra, 1982; Silvius & Gagné, 1984b; Düzgünes et al., 1984; Leventis et al., 1986; Feigenson, 1989). Our present results are consistent with the idea that *limited*, and possibly reversible, rapid lateral redistributions of anionic lipids accompany the calcium-mediated interactions (lipid mixing and fusion) of PC/PS as well as PC/PA vesicles. However, some observations suggest that the rapid lateral redistributions of lipids observed in PC/PS vesicles on addition of calcium may not reflect a smooth and direct evolution of the system toward the equilibrium state that is observed after prolonged incubations (days). First, the rapid (~minutes) and the long-term (~days) fluorescence changes reported by carbazole-PC in PC/PS vesicles upon addition of calcium differ markedly in their susceptibility to reversal by the weak chelator citrate. This is true even for vesicles containing relatively high mole fractions of PS in PC (e.g., 80 mol % PS), for which the process of vesicle coalescence is well advanced after even a few minutes' exposure to calcium. These results suggest that the initial binding of calcium between PS/PC bilayers may be relatively weak, in contrast to the much stronger binding observed between apposed bilayers at equilibrium in multibilayer systems (Feigenson, 1986, 1989), yet can nonetheless mediate efficient coalescence of the vesicle membranes. Second, vesicles combining cBr_2-PC with a fixed, near-equimolar proportion (40 or 50 mol %) of different PS species (e.g., DOPS vs DMPS) show very similar time courses of calcium-induced lipid mixing (and of lipid lateral redistribution), even when the different PS species vary markedly in their propensities to segregate from cBr_2-PC after prolonged incubations with calcium. These results raise some question, as Hoekstra (1982) has also queried previously, whether the behavior of PS/PC mixtures after long incubations in the presence of calcium is directly relevant to the comparatively rapid interactions of PS/PC vesicles (fusion and lipid mixing) that follow calcium addition.

While the experiments described here have utilized the combination of carbazole-labeled and brominated phospholipid probes mainly to study lipid lateral phase separations, similar approaches can be used to examine how homogeneously various lipid species intermix in lipid bilayers that comprise a single phase. One potential approach to this latter question is to compare the efficiencies with which a given brominated lipid quenches the fluorescence of different carbazole-labeled phospholipids in a single-phase lipid mixture. We are currently exploring this approach for various lipid mixtures of biological interest.

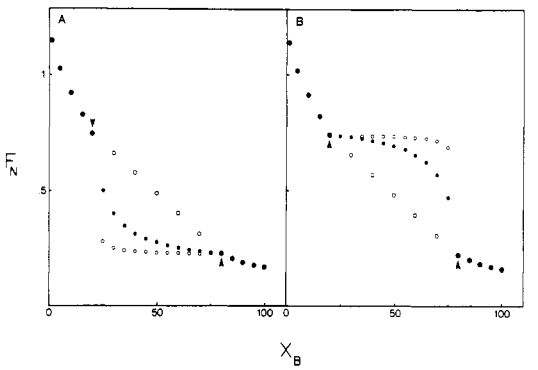

FIGURE 8: Predicted variation of carbazole-PC fluorescence intensity with bilayer composition in binary mixtures of a nonbrominated species A and a brominated species B, in the hypothetical case where such mixtures exhibit phase separation when the mole fraction of B (X_B) lies between 0.2 and 0.8 (arrowheads). (Panel A) Carbazole-PC partitions in favor of the B-rich phase II, with partition coefficient K (defined as in the Appendix) equal to 1.0 (upper open circles), 10 (solid circles), and 100 (lower open circles). (Panel B) Carbazole-PC partitions in favor of the A-rich phase I, with partition coefficient K (defined as in the Appendix) equal to 1.0 (lower open circles), 0.1 (solid circles), and 0.01 (upper open circles). It is assumed that the molar proportion of carbazole-PC is small enough not to affect the phase behavior of the A/B mixtures.

Appendix. Predicted Variation of Carbazole-PC Fluorescence with Composition in Phase-Separated Binary Mixtures of Brominated and Nonbrominated Lipids

For a carbazole-labeled phospholipid in a homogeneous mixture of brominated and nonbrominated lipids, we assume that the normalized fluorescence intensity measured for the probe will vary smoothly with the mole fraction of the brominated component (e.g., as in Figure 1A). Of interest here is how the probe fluorescence intensity will vary with composition when the probe is incorporated into a binary mixture of brominated and nonbrominated lipids that forms two well-defined phases, designated I and II, when the mole fraction of the brominated component (B) varies from a minimum value X_B^I to a maximum value X_B^{II}. We define the following additional quantities: F_N represents the normalized fluorescence intensity measured for the probe in a sample containing an arbitrary mole fraction X_B^0 of the brominated species. F_N^I and F_N^{II} represent the value of F_N measured at $X_B = X_B^I$ and at $X_B = X_B^{II}$, respectively. K is the partition coefficient describing the distribution of the probe between the two coexisting phases:

K = mole fraction of probe in phase II/mole fraction of probe in phase I

Application of the lever principle and the above definitions leads, by a straightforward derivation, to the relationship

$$F_N = \frac{F_N^I(X_B^{II} - X_B^0) + KF_N^{II}(X_B^0 - X_B^I)]}{[(X_B^{II} - X_B^0) + K(X_B^0 - X_B^I)]}$$

which can be rearranged to the alternative form

$$F_N = \frac{[X_B^0(KF_N^{II} - F_N^I) + (F_N^I X_B^{II} - KF_N^{II} X_B^I)]}{[X_B^0(K - 1) + (X_B^{II} - KX_B^I)]}$$

from which the hyperbolic dependence of the fluorescence intensity on X_B^0 in the region of phase separation can be clearly seen.

In Figure 8 are shown representative curves illustrating the variation of carbazole-PC fluorescence intensity with composition for a hypothetical binary mixture of brominated and nonbrominated phospholipids, with the region of phase separation extending from $X_B^I = 0.2$ to $X_B^{II} = 0.8$. It can be seen that as the value of the partition coefficient deviates more markedly from unity, the fluorescence curves more dramatically reflect the phase separation by exhibiting a broad plateau region and a narrow region in which the fluorescence varies sharply with composition.

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