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Cholesterol Modulation of Lipid Intermixing in Phospholipid and Glycosphingolipid Mixtures. Evaluation Using Fluorescent Lipid Probes and Brominated Lipid Quenchers†

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ABSTRACT: Carbazole- and indole-labeled phospholipids have been used to monitor the homo- or heterogeneity of lipid mixing in several types of lipid bilayers combining a brominated and a nonbrominated lipid with varying amounts of cholesterol. Experimental quenching curves (relating the normalized probe fluorescence intensity to the mole fraction of brominated lipid) show a characteristic smooth, monophasic form for homogeneous liquid–crystalline lipid mixtures. However, for mixtures exhibiting lipid lateral segregation, such curves show marked perturbations in form over the region of composition where segregation occurs. Using this approach, it is found that high mole fractions of cholesterol (40–50 mol %) promote the formation of apparently homogeneous solutions in mixtures of disaturated and monounsaturated phosphatidylcholines (PCs) that exhibit extensive thermotropic phase separations in the absence of sterol. At only slightly lower levels of cholesterol, however, these systems exhibit inhomogeneous lipid mixing over a wide range of relative proportions of the two PC components. Mixtures of cerebroside and monounsaturated PCs, even at high bilayer cholesterol contents, exhibit significant inhomogeneity in lipid mixing over a wide range of cerebroside/PC ratios. Phase-separating PC/PC and PC/cerebroside mixtures can readily form long-lived metastable solutions when the level of the higher-melting component in the liquid–crystalline phase exceeds its equilibrium solubility by as much as 20–30 mol %; this tendency is significantly increased by cholesterol. Cholesterol shows no significant ability to enhance lipid intermixing in a third type of phase-separating lipid system, combining a monounsaturated PC with a monounsaturated phosphatidic acid–calcium complex. Experiments using cleavable phospholipid conjugates, linking a fluorescent lipid to a brominated lipid, suggest that each fluorescent molecule probes a local lipid domain comprising $\lesssim$40–50 nearby acyl chains.

The lateral organization of different lipid species in complex lipid mixtures, including those found in biological membranes, remains an important issue in membrane research. While various thermodynamic and spectroscopic methods have been used to characterize the organization of a number of binary lipid mixtures [for reviews, see Lee (1977), Silvius (1982), Thompson and Tillack (1985), Cullis et al. (1985), Knoll et al. (1991), and Caffrey et al. (1991)], such methods are often much more complicated to apply to ternary or higher-order lipid mixtures. This problem can be still more acute for systems containing cholesterol [reviewed in Yeagle (1985), Presti (1985), Vist and Davis (1990), and Finean (1990)], which reduces the cooperativity of lamellar phase transitions and may potentially reduce the sizes of individual domains in phase-separated lipid mixtures.

In spite of the limitations just noted, the properties of ternary and higher-order systems containing cholesterol are of considerable interest to model and to characterize better the

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complex lipid mixtures found in biological membranes. Several ternary mixtures of phospholipids and cholesterol have been studied using calorimetry and freeze-fracture electron microscopy (de Kruijff et al., 1974; van Dijck et al., 1976; Demel et al., 1977; van Dijck, 1979; Calhoun & Shipley, 1979; Finegold & Singer, 1991), NMR (Cullis & de Kruijff, 1978; Cullis et al., 1978; Cullis & Hope, 1980; Tilcock et al., 1982, 1984, 1988; Bally et al., 1983), and ESR (Shin & Freed, 1989; Shin et al., 1990), chiefly to examine possible selectivity in the interactions of cholesterol with phospholipids of different structures. X-ray diffraction and calorimetry have been applied to examine the phase behavior of phosphatidylcholine (PC)[1]/cerebroside/cholesterol mixtures at high sterol/PC ratios (Ruocco & Shipley, 1984; Johnston & Chapman, 1988), providing evidence that at sufficiently high bilayer concentrations at least some cerebroside species can segregate laterally from choline phospholipids even in the presence of cholesterol. For the most part, the methods employed in the above studies have been particularly well-suited to detect the segregation of extensive, well-ordered domains in lipid bilayers.

Measurements of the quenching of lipid (or protein) probe fluorescence by brominated or spin-labeled lipid molecules provide a promising alternative to the above approaches to examine the lateral distributions of different molecular species in multicomponent bilayers (London & Feigenson, 1978, 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986; Simmonds et al., 1982, 1984; Florine & Feigenson, 1987; Silvius, 1990; Yeager & Feigenson, 1990; Bolen & Holloway, 1990). Quenching-based methods monitor intermolecular interactions over relatively short scales of time and distance, potentially allowing the detection of subtle inhomogeneities in lipid lateral distributions as well as large-scale phase separations. In this study, measurements of the quenching of indolyl- and carbazolyl-labeled lipid probes have been used to examine the effects of cholesterol on lipid intermixing in three types of brominated/nonbrominated lipid mixtures (PC/PC, PC/cerebroside, and PC/[phosphatidic acid–calcium]) that extensively phase-separate in the absence of sterol. It is found that cholesterol is a relatively inefficient promoter of stable lipid mixing in these systems at low-to-moderate bilayer concentrations. At high concentrations, cholesterol produces stable, microscopically homogeneous mixing of the different lipid species only in PC/PC mixtures, possibly reflecting the fact that only for these systems, among those studied here, does the sterol interact strongly with each of the individual lipid components in isolation under the same conditions.

Materials and Methods

Materials

16-(Indol-1-yl)palmitic acid/16-hydroxypalmitic acid was converted to 16-(methylsulfonyl)palmitic acid as described previously (Kimura & Regen, 1983) and then to 16-iodopalmitic acid by refluxing for 6 h with a 1 M solution of NaI in dry acetone (10 mL/g of fatty acid), following which the fatty acid was recovered by partitioning between 1% aqueous NaCl and hexane and evaporating the latter phase. The crude iodo fatty acid was coupled to indole by a modification of the method of Guida and Mathre (1980) as described previously for the synthesis of 8-(indol-1-yl)octanoic acid (Shin et al., 1991) but with extension of the reaction time to 120 h.

16-Bromopalmitic acid was synthesized by the method of Kimura and Regen (1983), and 11,12-dibromooctadecanoic acid was prepared by bromination of *trans*-vaccenic acid in dry CCl_4 in the dark at −10 °C. Fluorescent-labeled or brominated phosphatidylcholines (PCs) were prepared by acylating the appropriate 1-acyl-lysophosphatidylcholine with the anhydride of the labeled fatty acid, using 4-pyrrolidinopyridine as the catalyst, as described previously (Mason et al., 1981). Phosphatidic acids (PA) and phosphatidylethanolamines (PE) were prepared from the corresponding PC species by cabbage phospholipase D-mediated hydrolysis and transphosphatidylation, respectively (Comfurius & Zwaal, 1977; Graham et al., 1985; Leventis et al., 1991). Bovine brain sulfatides and cerebrosides were isolated as described previously (Radin, 1976; Momoi et al., 1976); hydroxy and nonhydroxy fatty acyl cerebrosides were separated by flash chromatography on silica gel 60 (Still et al., 1978), eluting first with 90/10 (v/v) then 88/12 (v/v) methanol in chloroform. Galactosylpsychosine was prepared from cerebroside by refluxing for 4 days with 1 M KOH in 9/1 methanol/water; the desired product was purified from the partially hydrolyzed preparation by chromatography on Bio-Sil A, using a 10–20% gradient of chloroform in methanol. (16-Bromopalmitoyl)-galactocerebroside was synthesized by condensing galactosylpsychosine with the *N*-hydroxysuccinimide ester of 16-bromopalmitic acid (Lapidot et al., 1967) for 60 h at 37 °C in 90/10/1 chloroform/methanol/triethylamine.

Disulfide-linked conjugates of brominated and fluorescent-labeled phospholipids were prepared as follows. Fluorescent PEs were first reacted overnight at 25 °C with a 3-fold molar excess of SPDP in dry chloroform containing 1% triethylamine. The resulting (pyridyldithiopropionyl)-substituted PEs were purified by thin-layer chromatography on silica gel 60, developing with 80/20 chloroform/methanol. Brominated phosphatidylcholines were converted to phosphatidyl-2-mercaptoethanols by stirring for 5 min at 30 °C in a 1/1 (v/v) emulsion of diethyl ether (distilled from P_2O_5) and an aqueous solution of 5% 2-mercaptoethanol, 100 mM sodium acetate, 5 mM $CaCl_2$, pH 6.8, in the presence of phospholipase D from *Streptomyces* sp. (Imamura & Horiuti, 1979) at 15 units/μmol of lipid. The products were partitioned between chloroform and 1/1 (v/v) methanol/100 mM aqueous EDTA, and the material recovered by concentration of the chloroform phase was purified by thin-layer chromatography on silica gel 60, developing with 80/20 chloroform/methanol. Both of the above reactions proceeded in nearly quantitative yield. To form the disulfide-linked conjugates, fluorescent (pyridyldithiopropionyl)-substituted PEs (1 equiv) were incubated with freshly prepared brominated phosphatidylmercaptoethanols

[1] Abbreviations: (16/11-carbazole)-PC, 1-palmitoyl-2-(11-(carbazol-9-yl)undecanoyl)-*sn*-glycero-3-phosphocholine; 16Br-cerebroside, *N*-(16-bromopalmitoyl)galactocerebroside; di16BrPC, 1,2-di(16-bromopalmitoyl)-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid (trisodium salt); HFA- or NFA-cerebroside, *N*-(2-hydroxyacyl)- or *N*-(nonhydroxyacyl)galactocerebroside fractions isolated from bovine brain; (16/8-indolyl)-PC, 1-palmitoyl-2-(8-indol-1-yl)octanoyl)-*sn*-glycero-3-phosphocholine; (16/12-indolyl)-PC, 1-palmitoyl-2-(12-(indol-1-yl)dodecanoyl)-*sn*-glycero-3-phosphocholine; (16/16-indolyl)-PC, 1-palmitoyl-2-(16-(indol-1-yl)palmitoyl)-*sn*-glycero-3-phosphocholine; ($18c^9$/16-indolyl)-PC,1-oleoyl-2-(16-(indol-1-yl)palmitoyl)-*sn*-glycero-3-phosphocholine; mBrPA, 1-oleoyl-2-(16-bromopalmitoyl)-*sn*-glycerol 3-phosphate; mBrPC, 1-oleoyl-2-(16-bromopalmitoyl)-*sn*-glycero-3-phosphocholine; PA, 1,2-diacyl-*sn*-glycerol 3-phosphate; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; tBr_2PC, 1-palmitoyl-2-(11,12-dibromooctadecanoyl)-*sn*-glycero-3-phosphocholine, prepared using the product of bromine addition to *trans*-11-octadecenoic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, sodium salt; $x_{NS}(A)$, mole fraction of lipid species *A* relative to the total *nonsterol* lipid in a given mixture.

(0.8 equiv) overnight in dry chloroform under nitrogen. The resulting conjugates were purified by thin-layer chromatography on silica gel 60 in 80/20/1 chloroform/methanol/water (R_f = 0.5 vs ca. 0.65 for the precursor species).

Cholesterol was obtained from Nu-Chek Prep (Elysian, MN), and 11-carbazolylundecanoic acid was from Molecular Probes (Eugene, OR). Lysophosphatidylcholines, distearoyl-PC, and 1-palmitoyl-2-oleoyl-PC were obtained from Avanti Polar Lipids (Alabaster, AL). Phospholipase D from *Streptomyces* sp. was the generous gift of Dr. S. Imamura (Toyo Jozo Trading Co., Tokyo, Japan). All common chemicals were of reagent grade or better; solvents were redistilled before use.

Methods

Lipid mixtures for analysis (normally 50–100 nmol of total lipid/sample, labeled with 0.5 mol % of a carbazolyl- or 1–1.5 mol % of an indolyl-labeled PC) were dried down under nitrogen from chloroform or 8/2 chloroform/methanol and then incubated for at least 6 h under high vacuum to remove residual solvent. PC and PC/cerebroside mixtures were redispersed by hand agitation in buffer (150 mM NaCl, 5 mM Tes, 0.1 mM EDTA, pH 7.4) above the phase transition temperature of the higher-melting component and then allowed to cool at ca. 0.5 °C/min from 50 °C (for cerebroside- or DSPC-containing mixtures) or 37 °C (for other lipid mixtures) to a final temperature of 20 °C except where otherwise indicated. PC/PA and PC/PG samples were initially redispersed at 25 °C by hand agitation in 150 mM KCl, 5 mM Tes, 0.1 mM EDTA, and then bath-sonicated to near-clarity and finally freeze-thawed three times. Samples treated with calcium were first dispersed as above and then mixed with $CaCl_2$ to a final calcium concentration of 10 mM. All samples were finally incubated, for 18 h at 20 °C where not indicated otherwise, before the fluorescence of replicate aliquots was read (at excitation/emission wavelengths of 281 nm/321 nm for indolyl- and 293/258 nm for carbazolyl-labeled samples) dispensed into buffer or methanol. The latter values allowed the fluorescence measured for each sample in buffer to be corrected for possible variations in the amount of probe present. Lipid samples for calorimetry were prepared as above but at a lipid concentration of 3–8 mM. Samples were analyzed on a Microcal MC-2 differential scanning calorimeter at a scan rate of 24 °C/h. Lipid concentrations were determined as described previously (Lowry & Tinsley, 1974), extending the sample digestion time to 5 h, or by drying to constant weight in vacuo for nonphospholipid samples.

Disulfide-linked conjugates, incorporated at 0.25 mol % (indolyl-labeled) or 0.125 mol % (carbazolyl-labeled) into 80/20 POPC/POPG vesicles, were reduced by treatment with dithiothreitol (50 mM) in buffer for 48 h at room temperature under nitrogen and in the dark, conditions established as sufficient to release completely the intramolecular fluorescence quenching in such conjugates.

RESULTS

In Figure 1 are shown the structures of the fluorescent lipid probes and brominated lipid quenchers used in this study. As shown previously (Silvius, 1990), the fluorescence of (16/11-carbazole)-PC in lipid bilayers is efficiently quenched by dibrominated phospholipids such as 1-palmitoyl-2-(11,12-dibromooctadecanoyl)-PC (tBr_2PC). Representative quenching profiles, illustrating the variation of probe fluorescence with the mole fraction of brominated phospholipid in liquid–crystalline POPC/tBr_2PC bilayers, are shown in Figure 2A,B. As shown in Figure 2A, very similar quenching profiles are observed for this system at 20 °C and at 37 °C, with only a slight enhancement in quenching efficiency at the higher temperature. Incorporation of cholesterol at a level of 45 mol % (Figure 2B) or 33 mol % (not shown) modestly increases both the intensity of probe fluorescence in POPC bilayers and the efficiency of fluorescence quenching by the brominated phospholipid. Similar effects of cholesterol are observed for the quenching of (18c^9/16-indolyl)-PC fluorescence by 1-oleoyl-2-(16-bromopalmitoyl)-PC (mBrPC) in POPC bilayers (Figure 2C). These relatively small effects of cholesterol probably represent a complex summation of distinct effects of the sterol on various aspects of probe fluorescence (spontaneous nonradiative deexcitation, quenching mediated by oxygen and by brominated lipids, etc.) through both modulation of lipid acyl chain packing and dynamics (Yeagle, 1985; Presti, 1985) and lateral dilution of the lipid acyl chains.

In all of the systems examined above, the quenching profiles are approximately hyperbolic in form, so that the reciprocal of the normalized fluorescence, $(1/F_N)$, varies nearly linearly with the mole fraction of brominated lipid, $x_{NS}(Br)$[2] (Figure 2C, inset). While neither the experimental quenching profiles

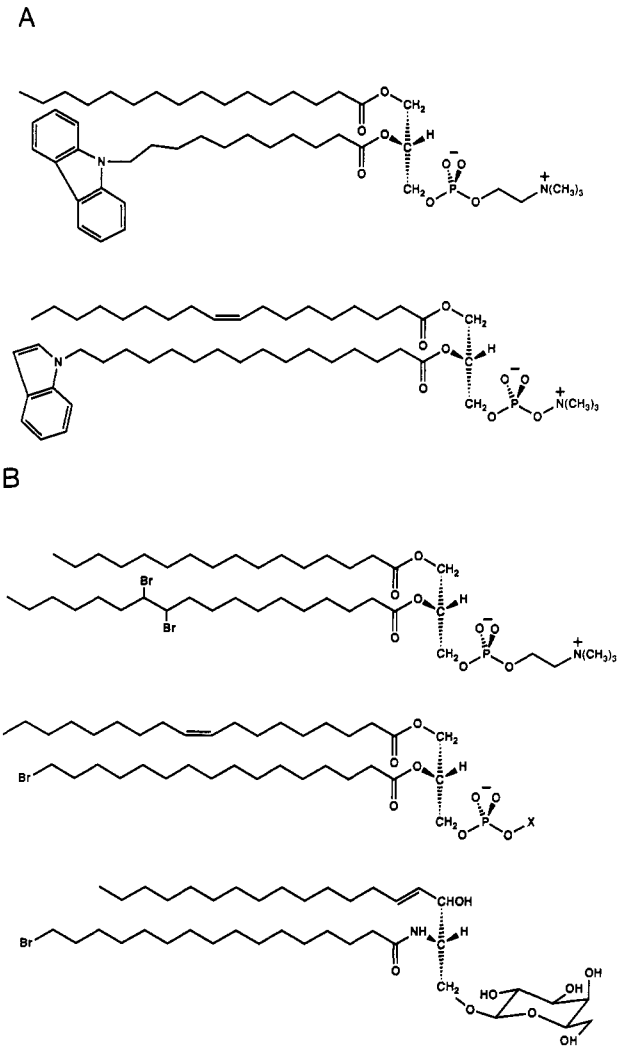

FIGURE 1: (A) Structure of the fluorescent lipid probes used in this study: upper, (16/11-carbazole)-PC; lower, (18c^9/16-indolyl)-PC. (B) Structures of the brominated lipid quenchers used in this study: upper, tBr_2PC; middle, mBrPC (X = choline) or mBrPA (X = H); bottom, 16Br-cerebroside.

[2] In this paper, the abbreviation x_{NS} is used to denote the mole fraction of a phospho- or sphingolipid relative to the total *nonsterol* lipid in a given mixture.

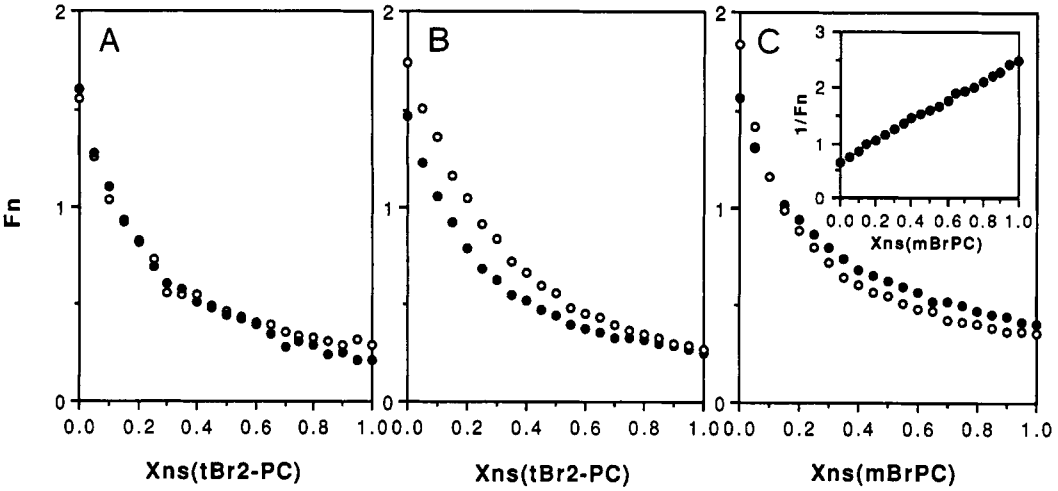

FIGURE 2: (A) Quenching curves measured at 20 °C (○) or 37 °C (●) for (16/11-carbazole)-PC in bilayers containing the indicated mole fraction of tBr_2PC in POPC. (B) Quenching curves measured at 37 °C for (16/11-carbazole)-PC in bilayers containing the indicated proportions of tBr_2PC in POPC and either 0 mol % cholesterol (●) or 45 mol % cholesterol (○). In this and all following figures, the proportion of one nonsterol component (here, of tBr_2PC) is specified as its mole fraction within the total *nonsterol* fraction (x_{NS}). (C) Quenching curves measured at 37 °C for ($18c^9$/16-indolyl)-PC in bilayers containing the indicated proportions of mBrPC in POPC and either 0 mol % cholesterol (●) or 45 mol % cholesterol (○). Inset: reciprocal transformation of quenching curve for cholesterol-free samples.

nor those predicted from theory (Yeager & Feigenson, 1990) are perfectly hyperbolic in form, both are reasonably well-approximated by this functional form. In practice, the homogeneity of lipid intermixing in a given mixture is best assessed by comparing its experimental quenching curve to that obtained for a related but homogeneous system. However, for systems where appropriate reference curves are difficult to obtain, inhomogeneous lipid mixing can also be detected as a strong deviation of the experimental quenching curve from a hyperbola of the form ($F_N = F_0/[1 + ((x_{NS}[Br])(F_0 - F_{Br})/F_{Br})]$), were F_0 and F_{Br} are the values of F_N measured for $x_{NS}(Br) = 0$ and 1.0, respectively.

Estimation of the Sampling Domain Size for Fluorescence Quenching. To interpret fully the results of fluorescence-quenching measurements like those above, it is useful to estimate the size of the sampling domain surrounding an individual fluorescent probe molecule, i.e., the total number of acyl chains around the probe which, if brominated, could contribute significantly to the overall probability of fluorescence quenching during a single excitation/deexcitation event. This question was addressed using disulfide-linked conjugates with the structures shown in Figure 3. When one of these conjugates is highly diluted in a nonbrominated lipid matrix, the probe fluorescence will be quenched by a single proximal brominated chain (that of the same conjugate molecule), and this quenching can be released by DTT cleavage of the conjugate. As detailed in the appendix, this effect can be used to estimate the efficiency with which a single brominated acyl chain quenches a nearby fluorescent acyl chain. Knowing this quantity and the extent of fluorescence quenching for the same probe in a bilayer composed entirely of brominated lipids, we can estimate the size of the sampling domain as defined above. For quenching of a 16-indolylpalmitoyl chain by lipids with 16-bromopalmitoyl chains, the number of acyl chains sampled by each probe is estimated to be 41 ± 7 (mean ± SEM; $N = 3$), while for quenching of a 11-carbazolylundecanoyl chain by lipids with 11,12-dibromooctadecanoyl chains, the corresponding estimate is 51 ± 3 chains (see appendix).

The estimates just presented are derived using two important assumptions. The first is that the sampling domain can reasonably be approximated as a discrete number of acyl chains around each probe molecule. This assumption can be relaxed using a more diffuse picture of the sampling domain, in which

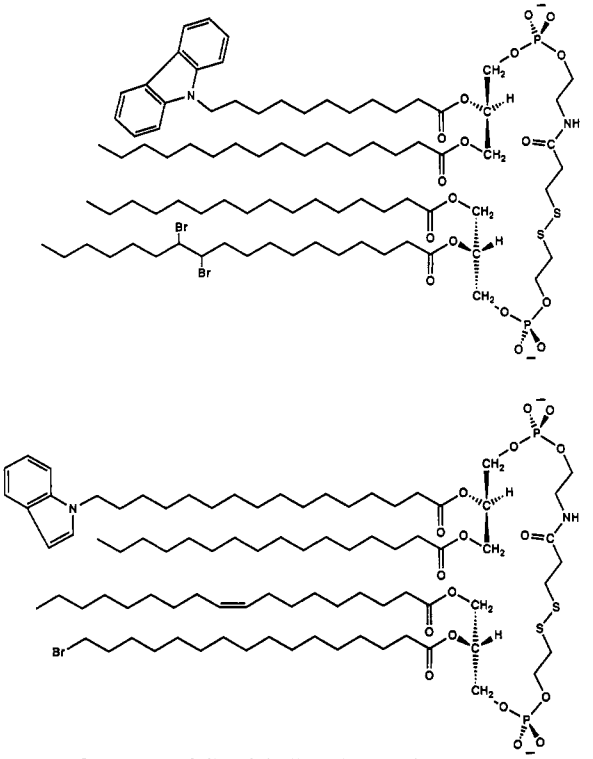

FIGURE 3: Structure of disulfide-linked probe/quencher conjugates used to estimate efficiencies of probe fluorescence quenching by a single proximal brominated acyl chain. Analogous conjugates were also prepared in which the brominated diacylglycero- moiety shown was replaced by a 1-palmitoyl-2-oleoylglycero- moiety.

the entire sampling domain is somewhat larger than the above estimates suggest but a subset of chains nearest the probe (*fewer* in number than the above estimates) contributes most of the observed quenching. The second key assumption used above is that the ability of a single brominated acyl chain to quench the fluorescence of a proximal fluorescent group, estimated using a fluorescent/brominated lipid conjugate as just described, is reasonably representative of the value of this parameter averaged over all positions in the sampling domain. This assumption may not be valid if the structure of the fluorescent/brominated lipid conjugate hinders close approach of brominated and probe-labeled acyl chains or if it allows the

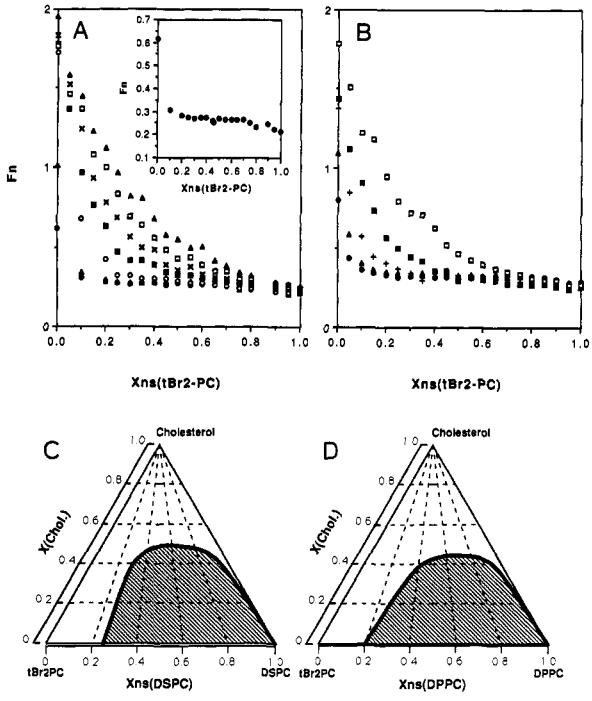

FIGURE 4: (A) Quenching curves measured at 37 °C for (16/11-carbazole)-PC in DSPC/tBr_2PC bilayers containing (●) 0 mol %, (△) 10 mol %, (○) 30 mol %, (■) 35 mol %, (×) 40 mol %, (□) 45 mol %, or (▲) 50 mol % cholesterol. Inset: Replot of quenching curve for cholesterol-free samples. (B) Quenching curves measured at 20 °C for (16/11-carbazole)-PC in DPPC/tBr_2PC bilayers containing (●) 0 mol %, (△) 10 mol %, (+) 20 mol %, (■) 35 mol %, or (□) 45 mol % cholesterol. Samples were prepared as described in Materials and Methods. (C) Schematic phase diagram for the DSPC/tBr_2PC/cholesterol system at 37 °C, indicating the region of phase separation (shaded) into DSPC-rich and tBr_2PC-rich domains observed by fluorescence quenching. Omitted from this phase diagram are other regions of phase separation where the segregated phases differ mainly in cholesterol content [e.g., crystalline cholesterol/cholesterol-saturated bilayer at mole fractions of cholesterol > 0.5, and gel/intermediate bilayer phases (Vist & Davis, 1990) at low mole fractions of tBr_2PC]; these features of the phase diagram do not directly impinge on the phase boundary indicated. (D) Schematic phase diagram for the DPPC/tBr_2PC/cholesterol system at 20 °C, indicating the region of phase separation into DPPC-rich and tBr_2PC-rich domains observed by fluorescence quenching. Other details are as described for panel C.

two acyl chains to attain separations much larger than the radius of the sampling domain. In such cases, the above estimates nonetheless provide an upper bound for the size of the sampling domain.

Phase Separation in PC/Cholesterol Bilayers. The quenching profiles shown in Figure 2 were obtained for phospholipid probes in purely lipid–crystalline bilayers, where brominated and nonbrominated lipids should be fairly homogeneously intermixed. As shown previously (Silvius, 1990), markedly different quenching profiles are expected for bilayers where the lateral distribution of brominated and nonbrominated phospholipids is inhomogeneous, as in the phase-separated DSPC/tBr_2PC system whose behavior is illustrated in Figure 4A,C. Within the phase-separation region the carbazolyl-labeled PC partitions strongly in favor of liquid–crystalline (tBr_2PC-rich) domains, and the quenching curve in this region deviates markedly (bowing out in the direction of greater quenching) from that expected for a homogeneous system [see Silvius (1990)]. By this criterion, at 37 °C and in the absence of cholesterol (Figure 4A, inset) the region of phase separation in DSPC/tBr_2PC mixtures appears to extend from x_{NS}(DSPC) = ca. 0.25 to 1.00. In agreement with this conclusion, scanning-calorimetric analysis (not shown) indicated that at 37 °C this system exhibits phase separation at DSPC contents x_{NS}(DSPC) ≥ 0.25–0.27. Fluorometric and calorimetric estimates of the "saturating" content of DSPC in liquid–crystalline tBr_2PC domains at 25 °C (x_{NS}[DSPC] = ca. 10 mol % and 8–9 mol %, respectively) were also in good agreement. The calorimetric behavior of the DSPC/tBr_2PC mixtures was very similar to that of DSPC/POPC mixtures with the same DSPC content.

As the proportion of cholesterol in DSPC/tBr_2PC bilayers increases, the quenching curves measured at 37 °C gradually shift toward that expected for homogeneous intermixing of the brominated and nonbrominated lipid components (compare Figure 4A with, e.g., Figure 2A) but attain the latter form only at a cholesterol level of 45–50 mol %. At moderate levels of cholesterol (e.g., 30–35 mol %), inhomogeneous lipid mixing is still observed over a wide range of relative proportions of DSPC and tBr_2PC. Interestingly, cholesterol does not appear to promote significant incorporation of tBr_2PC into the DSPC-rich gel phase until essentially complete miscibility of the components is attained. This conclusion is inferred from the fact that none of the quenching curves in Figure 4A shows a clear downward break or slope change indicative of the gel/(gel + liquid–crystalline) phase boundary, suggesting that this boundary lies at very low mole fractions of tBr_2PC even in mixtures with relatively high sterol contents. These conclusions about the behavior of the DSPC/tBr_2PPC/cholesterol system are summarized in the partial phase diagram shown in Figure 4C. The behavior of the ternary system tBr_2PC/DPPC/cholesterol at 20 °C (Figures 4B,D) or 25 °C (not shown) is qualitatively very similar to that of the tBr_2PC/DSPC/cholesterol system described above, with 40–45% cholesterol required to produce homogeneous intermixing of the components.

The quenching curves obtained for the above PC mixtures provide a particularly sensitive reflection of the lipid lateral distribution for samples enriched in the high-melting component. A complementary view of a thermotropic (PC/PC) phase separation is provided by quenching curves for ($18c^9$/16-indolyl)-PC in POPC/di16BrPC bilayers, where the brominated PC species is the higher-melting component. In this system, where the liquid–crystalline domains favored by the fluorescent probe are enriched in the nonbrominated component, phase separation is manifested as a bowing out of the quenching curve in the direction of decreased quenching in the phase-separation region.

At 20 °C and in the absence of cholesterol, phase separation is observed in POPC/di16BrPC mixtures at equilibrium when x_{NS}(di16BrPC) exceeds roughly 0.05 (Figure 5A inset, filled circles). This conclusion agrees well with that obtained from parallel calorimetric experiments (not shown), which showed phase separation at 20 °C when x_{NS}(di16BrPC) ≥ 0.04–0.05. Interestingly, however, samples containing substantially higher proportions of di16BrPC (up to roughly x_{NS}[di16BrPC] = 0.4) form metastable solutions, which require at least several hours to relax to equilibrium, when cooled to 20 °C after initial dispersal in the liquid–crystalline state (Figure 5A inset, open circles). This metastability is relieved by briefly cooling the samples to 0 °C or 10 °C before incubating at 20 °C (Figure 5A inset, filled circles). Similar, but still more persistent, metastability is observed in POPC/di16BrPC mixtures containing cholesterol (Figure 5A). Such metastability, which again is most pronounced for samples containing slightly supersaturating levels of di16BrPC, was substantially eliminated only when the samples were incubated for 24 h at 4 °C and then for 96 h at 20 °C.

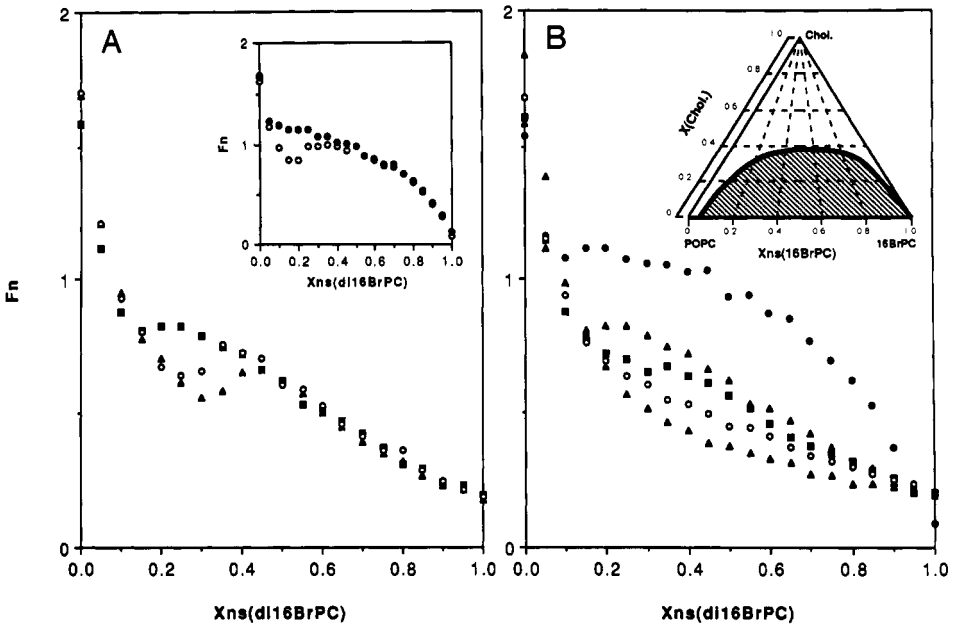

FIGURE 5: (A) Quenching curves measured at 20 °C for (18c^9/16-indolyl)-PC in POPC/di16BrPC bilayers containing 20 mol % cholesterol. (▲) Samples were incubated for 18 h at 20 °C after dispersal at 60 °C; (○) samples were incubated for 90 h at 20 °C after dispersal at 60 °C; (■) samples were incubated for 24 h at 4 °C and then for 90 h at 20 °C after initial dispersal at 60 °C. Inset: Quenching curve measured at 20 °C for (18c^9/16-indolyl)-PC in bilayers containing the indicated proportions of di16BrPC in POPC. (○) Samples were dispersed at 60 °C and then slowly cooled to 20 °C and incubated for 18 h at the latter temperature; (●) after dispersal at 60 °C, samples were slowly cooled to 0 °C, held at this temperature for 15 min, and then incubated at 20 °C for 18 h. (B) Quenching curves measured at 20 °C for (18c^9/16-indolyl)-PC in POPC/di16BrPC bilayers containing (●) 0 mol %, (△) 20 mol %, (■) 25 mol %, (○) 30 mol %, or (▲) 40 mol % cholesterol after incubation for 24 h at 4 °C and then for 90 h at 20 °C after initial dispersal at 60 °C. Inset: Schematic phase diagram for the POPC/di16BrPC/cholesterol system at 20 °C, indicating the region of phase separation into POPC- and di16BrPC-rich domains observed by fluorescence quenching. Other details are as described for Figure 4C.

Using samples incubated by the last protocol just noted (Figure 5B), it can be seen that cholesterol at levels up to 30 mol % modestly increases the limiting solubility of di16BrPC in homogeneous liquid–crystalline POPC bilayers (from x_{NS}[di16BrPC] = 0.05 at 0 mol % cholesterol to 0.15–0.20 at 30 mol % cholesterol), although the opposite boundary of the phase-separation region remains near x_{NS}(di16BrPC) = 1.00 (Figure 5C). Cholesterol also decreases markedly the apparent heterogeneity of lipid mixing within the phase-separation region, as the quenching curves in Figure 5B suggest. In principle, the latter effect may reflect the influence of multiple factors. The most important of these is almost certainly a convergence of the phospholipid compositions of the coexisting phases at higher cholesterol contents. However, a strong cholesterol-induced reduction in the average size of the segregated lipid domains or (less likely) in the preferential partitioning of the unsaturated PC probe into liquid-crystalline domains may also influence the shapes of the quenching curves in the phase-separation region, which therefore must be interpreted with some care.

PC/Brain Glycosphingolipid Mixtures. The myelin membrane contains substantial proportions of *N*-alkanoyl- and *N*-(2-hydroxyalkanoyl)galactocerebrosides (NFA- and HFA-cerebroside, respectively) and their 3′-sulfated (sulfatide) derivatives. Previous studies have found that both HFA- and NFA-cerebrosides, which exhibit much higher phase transition temperatures than do typical membrane phospholipids, readily phase-separate from phosphatidylcholines at physiological temperatures but can show complex metastable mixing with PC in the presence of high levels of cholesterol (Ruocco et al., 1983; Ruocco & Shipley, 1984; Maggio et al., 1985; Curatolo, 1986a,b; Johnston & Chapman, 1988; Bunow & Levin, 1988). Fluorescence-quenching measurements were applied to characterize in more detail the dependence of these phenomena on the bilayer cholesterol and cerebroside contents and on the

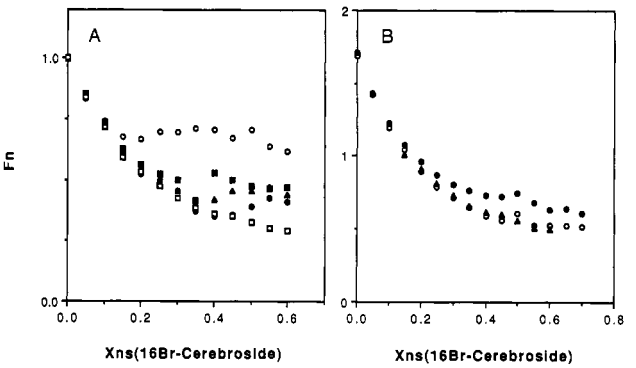

FIGURE 6: Quenching curves measured at 37 °C for (18c^9/16-indolyl)-PC in mixtures of POPC and 16Br-cerebroside plus cholesterol. (A) Samples containing the indicated proportions of 16Br-cerebroside and (○) 0 mol %, (■) 25 mol %, (△) 30 mol %, (●) 35 mol %, or (□) 45 mol % cholesterol were dispersed at 85 °C and then preincubated for 90 h at 20 °C. To facilitate comparison, all quenching curves have been scaled to originate at F_N = 1.00 for x_{NS}(16Br-cerebroside) = 0. (B) Samples containing the indicated proportions of 16Br-cerebroside and 45 mol % cholesterol were dispersed at 85 °C and then preincubated (▲) for 18 h at 20 °C, (○) for 90 h at 20 °C, or (●) for 24 h at 4 °C and then for 90 h at 20 °C.

composition of the cerebroside(/sulfatide) fraction.

In Figure 6 are shown fluorescence quenching curves measured at 37 °C for (18c^9/16-indolyl)-PC in bilayers composed of POPC and *N*-(16-bromopalmitoyl)galactocerebroside (16Br-cerebroside) plus varying amounts of cholesterol. In the absence of cholesterol, phase separation is observed at x_{NS}(16Br-cerebroside) ≥ 0.13–0.15 in samples preincubated for 18 or 90 h at 20 °C, although there is some tendency for metastable mixing at just-supersaturating levels of cerebroside (Figure 6A). In samples preincubated for only 30 min at 20 °C, phase separation was detected only for samples containing >30 mol % cerebroside (not shown). The estimated equilib-

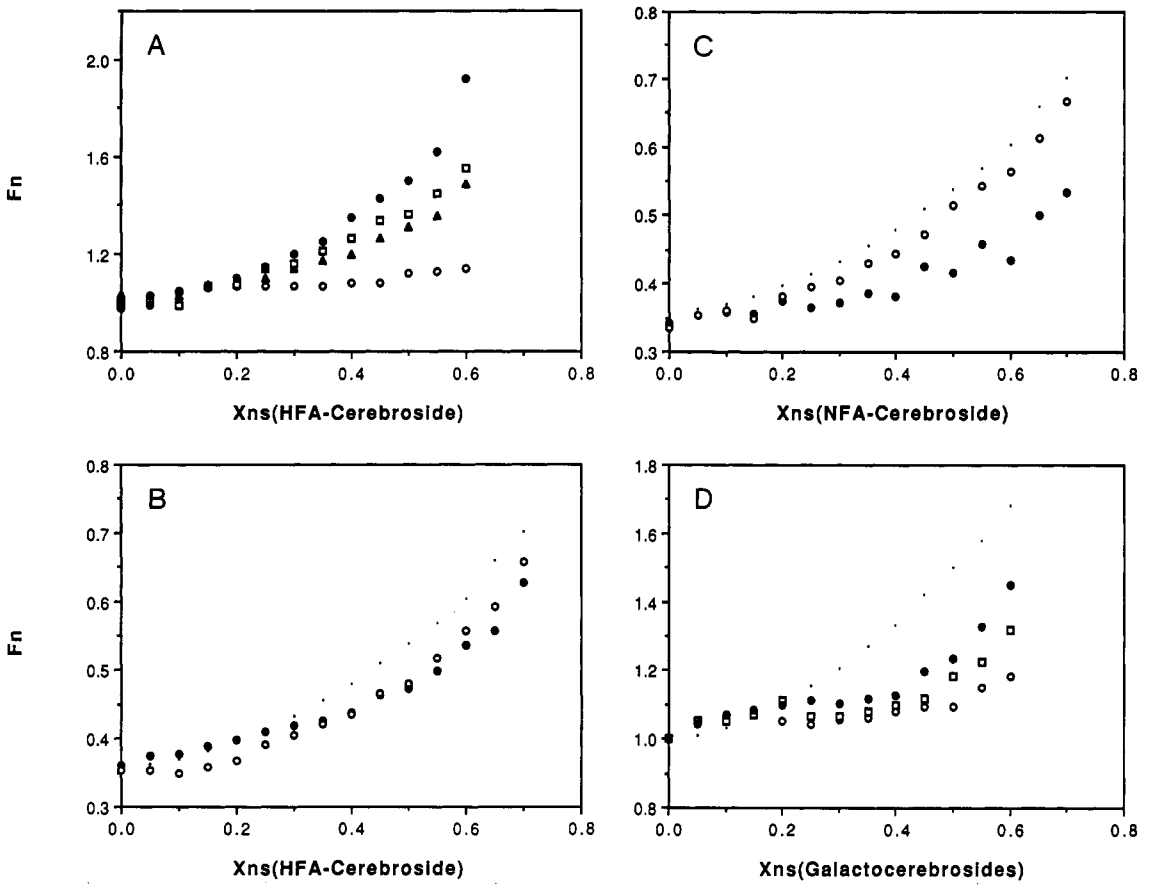

FIGURE 7: Quenching curves measured at 37 °C for (18c^9/16-indolyl)-PC in mixtures combining mBrPC with bovine brain galactosphingolipids and cholesterol. (A) mBrPC/HFA-cerebroside mixtures containing (○) 0 mol %, (▲) 25 mol %, (□) 35 mol %, and (●) 45 mol % cholesterol were incubated for 18 h at 20 °C after initial dispersal at 85 °C. To facilitate comparison, quenching curves have been scaled to converge to 1.00 for x_{NS}(cerebroside) = 0. Very similar quenching curves were obtained for comparable mixtures containing NFA- in place of HFA-cerebroside. (B) mBrPC/HFA-cerebroside mixtures containing 45 mol % cholesterol were dispersed at 85 °C and then incubated for (○) 90 h at 20 °C or (●) 24 h at 4 °C followed by 90 h at 20 °C. (·) The quenching curve is shown for (18c^9/16-indolyl)-PC in homogeneous mBrPC/POPC mixtures containing 45 mol % cholesterol and the same proportions of mBrPC as the cerebroside samples shown. (C) mBrPC/NFA-cerebroside mixtures containing 45 mol % cholesterol were dispersed at 85 °C and then incubated for (○) 90 h at 20 °C or (●) 24 h at 4 °C followed by 90 h at 20 °C. The quenching curve for similar samples incubated for 18 h at 20 °C (not shown) was essentially identical to that for samples incubated for 90 h at 20 °C. (·) The quenching curve is shown for comparable (homogeneous) POPC/mBrPC mixtures (see legend to panel B). (D) Mixtures combining mBrPC with a 3/2/1 (molar proportions) mixture of HFA-cerebroside, NFA-cerebroside, and bovine brain sulfatide and containing (○) 0 mol %, (□) 35 mol %, and (●) 45 mol % cholesterol were incubated for 18 h at 20 °C after initial dispersal at 85 °C; similar samples incubated for 24 h at 4 °C and then for 90 h at 20 °C gave essentially identical quenching curves (not shown). (·) The quenching curve is shown for comparable homogeneous POPC/mBrPC mixtures (see legend to panel B). To facilitate comparison, quenching curves have been scaled to converge to 1.00 at x_{NS}(cerebroside) = 0.

rium solubility of 16Br-cerebroside in POPC at 37 °C is similar to that reported for other galactocerebrosides in liquid–crystalline PC bilayers at similar temperatures (Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986a,b). Unlike cholesterol-free samples, samples containing 25–45 mol % cholesterol show more extensive lipid segregation when preincubated for 90 h at 20 °C (Figure 6A) than when preincubated for only 18 h at this temperature. The quenching curves for cholesterol-containing samples were further altered when the 90-h incubation at 20 °C was preceded by a 24-h incubation at 4 °C (Figure 6B). When preincubated under the latter conditions, samples containing even 45 mol % cholesterol show truly homogeneous mixing of the lipid components only up to x_{NS}(cerebroside) = 0.15, a level only marginally exceeding the solubility of the cerebroside in POPC in the absence of cholesterol.

In Figure 7 are shown a series of quenching curves measured for (18c^9/16-indolyl)-PC in bilayers combining mBrPC with bovine brain HFA-cerebroside, NFA-cerebroside, or a mixture of HFA-cerebroside, NFA-cerebroside, and sulfatide in the molar proportions found in bovine brain myelin (3/2/1). For all of these systems, in the absence of cholesterol the limit of solubility of the galactosphingolipids in mBrPC lies between 10 and 15 mol % (Figure 7A,D). Increasing proportions of cholesterol increase the apparent miscibility of mBrPC with NFA- or HFA-cerebroside in samples equilibrated for 18 h at 20 °C after initial dispersal at 85 °C (Figure 7A). At 45 mol % cholesterol, samples combining mBrPC with either NFA- or HFA-cerebroside appear virtually homogeneous at all compositions examined when preincubated exclusively at 20 °C (Figures 7B,C). However, when the 20 °C preincubation step is preceded by a 24-h incubation at 4 °C, significant lipid lateral segregation is observed, even at 45 mol % cholesterol, in samples containing NFA-cerebroside at mole fractions x_{NS}(cerebroside) ≥ 0.15. The same is true, to a more modest extent, for comparable samples containing HFA-cerebrosides under the same conditions.

Interestingly, samples combining POPC with a physiological mixture of bovine brain cerebrosides and sulfatides exhibit extensive lipid segregation, apparently to equilibrium, after only 18 h of incubation at 20 °C, even at 45 mol % cholesterol (Figure 7D). In a separate experiment (not shown), physiological proportions of sulfatide were also found to enhance markedly the kinetics of segregation of a 3/2 mixture of HFA-

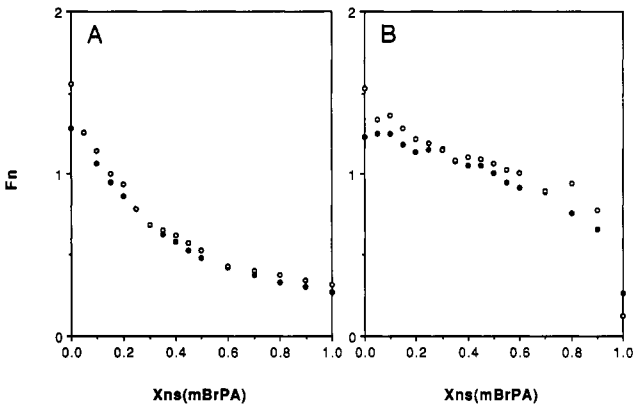

FIGURE 8: Quenching curves measured at 37 °C for (16/12-indolyl)-PC in POPC/mBrPA mixtures containing (●) 0 mol % or (○) 45 mol % cholesterol in (A) the absence or (B) the presence of 10 mM Ca^{2+}. Samples were first bath-sonicated to near-clarity in 150 mM KCl, 10 mM Tes, 0.1 mM EDTA, pH 7.4, then three times freeze-thawed, and finally incubated for 18 h at 20 °C with or without the addition of calcium to 10 mM.

and NFA-cerebroside from mBrPC in the absence of cholesterol. These results suggest, rather surprisingly, that the heterogeneous mixture of galactosphingolipids found in myelin may form segregated domains in PC(/cholesterol) bilayers more readily (at least in a kinetic sense) than do individual purified cerebroside fractions. We are currently investigating the basis for this phenomenon in more detail.

PC/Phosphatidic Acid Mixtures. A final series of experiments examined the effects of cholesterol on calcium-induced phase separation in bilayers of POPC and mBrPA labeled with (16/12-indolyl)-PC. In the absence of calcium and at 37 °C these species mix homogeneously in the presence or absence of cholesterol (Figure 8A). However, as illustrated in Figure 8B, addition of 10 mM calcium induces a strong phase separation, as observed previously in other PC/PA systems (Ito & Ohnishi, 1974; Galla & Sackmann, 1975; van Dijck et al., 1978; Graham et al., 1985; Silvius, 1990). Interestingly, this lateral segregation is neither abolished nor even markedly attenuated in bilayers containing as much as 45 mol % cholesterol (Figure 8B).

In preliminary experiments with the PC/PA(Ca^{2+}) system discussed above, (not shown) it was found that the quenching curves obtained for (16/12-indolyl)-PC or (16/8-indolyl)-PC showed a stronger flattening in the region of phase separation than did ($18c^9$/16-indolyl)-PC or (16/16-indolyl)-PC. These differences are unlikely to reflect differences in probe partitioning between phases in this system, as ($18c^9$/16-indolyl)-PC and (16/16-indolyl)-PC give essentially identical quenching curves, but are exactly those expected if a significant degree of transbilayer quenching occurs for the 16-indolylpalmitoyl-labeled phospholipid probes but is diminished or absent for the 12-indolyldodecanoyl- and 8-indolyloctanoyl-labeled species. Comparable differences in the quenching curves for 12- vs 16-indolyl-labeled lipid probes were not observed for the other phase-separated systems examined in this study.

Discussion

A previous study (Silvius, 1990) has demonstrated the utility of carbazolyl-labeled phospholipids to monitor lipid lateral segregation in binary mixtures of dibrominated and nonbrominated phospholipids. The present results demonstrate the feasibility of extending this approach to more complicated lipid mixtures, including in principle mixtures approaching the complexity of the lipid component of biological membranes. The introduction of indolyl-labeled phospholipids allows the technique also to employ monobrominated lipid quenchers, which can be made nearly isosteric with unlabeled lipid species. Both types of probe moieties are expected to be highly hydrophobic, and results obtained using the spin-label quenching parallax method of Chattophadhyay and London (1987) in fact indicate that they are both localized well within the bilayer interior (F. S. Abrams and E. London, manuscript in preparation). Recent studies of the intrabilayer disposition of bromo substituents on phospholipid acyl chains (McIntosh & Holloway, 1987; Wiener & White, 1991) indicate that these moieties also localize well within the membrane interior, at roughly the same depth as the corresponding methylene segments of an unsubstituted acyl chain.

The interpretation of the present quenching results raises three important questions concerning the nature of the quenching process. The first concerns the size of the sampling domain, i.e., of the domain around a lipid probe group that can potentially be sampled by the probe during the lifetime of the excited state. This quantity is estimated as ca. 40–50 acyl chains for the two types of probe/quencher combinations employed here, although as already noted, it is possible that these estimates actually represent upper bounds. Yeager and Feigenson (1990) have estimated a smaller sampling number of 12–18 chains in an analysis of the quenching of bilayer-associated tryptophan octyl ester by brominated phospholipids. Further study will be required to determine whether probe/quencher pairs such as those examined here, in which both fluorescent and bromo groups are attached to acyl chains buried in the bilayer interior, truly exhibit larger sampling domains in liquid–crystalline bilayers than do similar pairs in which at least one of the probe or quencher groups is confined to the bilayer/aqueous interface (Jacobs & White, 1989). Nonetheless, it seems clear that the probe/quencher combinations examined here can detect inhomogeneities in the lateral distributions of lipid species over a relatively fine scale of distances (of the order of a few molecular diameters).

A second question related to the above concerns the importance of lipid lateral diffusion in the fluorescence-quenching phenomena examined here. At first glance, the relatively large number of acyl chains estimated to be sampled by each probe molecule might suggest an important role for lateral diffusion in the quenching process. However, as Yeager and Feigenson (1990) have pointed out, phospholipid lateral diffusion is expected to be negligible during the lifetime of the excited state for fluorescent probes such as those examined here (Lackowicz & Hogen, 1980). The small effects of temperature on the quenching curves observed for these probes in liquid–crystalline lipid mixtures (Figure 2) also suggest that quenching in these systems is not primarily dependent on lipid lateral diffusion, which shows a considerably stronger temperature dependence (Wu et al., 1977). It is nonetheless clear that quenching in the systems examined here must involve some component of molecular motions, for otherwise the sampling domains for a probe molecule should comprise no more than 6–8 acyl chains, i.e., the immediate nearest neighbors of each probe group. It seems most plausible that these motions comprise mainly lateral excursions of acyl chains (wobble) and/or rotational movements of lipid molecules, rather than significant lateral diffusion of lipid molecules, over the lifetime of the excited state.

A final question regarding the mechanism of fluorescence quenching in the systems examined here is the extent to which probe and quencher moieties interact between the two halves of the membrane bilayer. Some level of transbilayer quenching

seems likely when 16-indolylpalmitoyl-labeled probes are used together with 16-bromopalmitoyl-labeled quenchers. Such transbilayer quenching, when present, will not mask the appearance of phase boundaries in a quenching curve but may modestly alter the curve shape within the phase-separation region *if* segregation of lipid domains occurs independently in the two halves of the bilayer. It was thus interesting to observe that PC/PA(Ca^{2+}) mixtures show evidence for such transbilayer-quenching effects, as judged from comparison of the quenching curves obtained for indolyl-labeled probes of different chain lengths, while phase-separated PC/PC and PC/cerebroside mixtures do not. We are currently investigating whether such differences can be correlated with differences in the nature of the segregated lipid domains (e.g., correlated or uncorrelated between the two bilayer halves) in the different systems examined.

A noteworthy feature of some of the phase-separating lipid mixtures examined here is a strong tendency to form long-lived metastable solutions upon cooling from the liquid–crystalline state, even when the bilayer content of the high-melting species exceeds its equilibrium solubility by as much as 40 mol %. The occurrence of such metastability in PC/PC(/cholesterol) and PC/cerebroside(/cholesterol) mixtures can be correlated with a second observation, namely that fluorescence-quenching measurements provide no evidence for preferential interactions of particular lipid species (e.g., clustering) in these systems outside the region of full-scale phase separation. For example, quenching curves obtained for liquid–crystalline POPC/16Br-cerebroside/($18c^9$/16-indolyl)-PC mixtures at subsaturating cerebroside levels are essentially superimposable on those obtained under the same conditions for similar mixtures labeled with (16-indolylpalmitoyl)*cerebroside* (not shown) and for POPC/mBrPC/($18c^9$/16-indolyl)-PC mixtures with equal brominated lipid contents. The inability to detect any preferential interactions of particular lipid species in these liquid–crystalline bilayers, even very near the phase-separation boundary, suggests that such preferential self-association is characteristic only of the solid state and may in fact require very long times to nucleate even in liquid–crystalline bilayers containing (moderately) supersaturating levels of the segregating species. We are currently using fluorescence-quenching measurements to examine the distribution of lipids in the liquid–crystalline phase for other systems whose phase diagrams suggest markedly nonideal lipid mixing.

The three general types of phase-separating systems examined here (PC/PC, PC/cerebroside, and PC/PA(Ca^{2+})) differ considerably with regard to the effects of cholesterol on the miscibility of the nonsterol lipid components. In all three systems, cholesterol at levels up to at least 30–35 mol % fails to promote completely homogeneous intermixing of lipids, in agreement with the conclusions of previous calorimetric and X-ray diffraction studies of related PC/PC and PC/cerebroside mixtures (de Kruijff et al., 1973, 1974; Ruocco & Shipley, 1984; Johnston & Chapman, 1988; Finegold & Singer, 1991). In PC/PC mixtures, however, cholesterol at these levels is found to modestly increase the solubility of the higher-melting PC in the liquid-crystalline phase. Higher cholesterol levels (40–50 mol %) promote the formation of microscopically homogeneous solutions of the disaturated and monounsaturated PCs examined here. This conclusion extends previous calorimetric findings that similar phospholipid mixtures exhibit no cooperative thermotropic transitions at high cholesterol levels (de Kruijff et al., 1973, 1974; Finegold & Singer, 1991). It seems clear that in the systems studied here the PC components become completely miscible at high cholesterol contents rather than forming segregated cholesterol-rich phases with distinct phospholipid compositions, an alternative possibility that would also be consistent with the calorimetric findings just noted. The present data do not rule out the formal possibility that the cholesterol-rich PC/PC mixtures studied form segregated phases with distinct sterol contents but very similar phospholipid compositions.

In contrast to its effects on phase-separating PC/PC mixtures, cholesterol appears to alter only slightly the equilibrium solubility of cerebrosides in homogeneous liquid–crystalline PC bilayers, although as noted above the sterol strongly enhances the tendency of such mixtures to form metastable solutions. The present results indicate that galactosphingolipids in the proportions found in the myelin membrane (ca. 20 mol %) could undergo lateral segregation from PC at physiological temperatures even in the presence of high levels of cholesterol, regardless of whether we assume that the galactosphingolipid fraction is exofacially or symmetrically distributed in the myelin membrane. However, to extend this conclusion to the myelin membrane, we must assess both the effects of the less-abundant myelin lipids on PC/cerebroside miscibility and, more importantly, the question of whether cerebroside can undergo lateral segregation even if it is asymmetrically distributed across the membrane bilayer.

Cholesterol shows even less ability to attenuate the tendency toward lateral segregation of lipids in PC/PA(Ca^{2+}) mixtures, a result consistent with previous observations that ≤33 mol % cholesterol cannot abolish calcium-induced lateral phase separations in PC/phosphatidylserine bilayers (Tilcock et al., 1984; Silvius, 1990). The strikingly poor ability of cholesterol to promote homogeneous incorporation of cerebrosides or PA(Ca^{2+}) into liquid–crystalline POPC bilayers may be related in part to the low solubility of the sterol in the stable solid phases of cerebrosides and of PA(Ca^{2+}), which obviously limits the ability of the sterol to destabilize these solid phases (Johnston & Chapman, 1988; Ruocco & Shipley, 1984; Bach & Wachtel, 1989). In mixtures of saturated and unsaturated PCs, cholesterol may exert a dual effect to promote lipid intermixing, altering the properties of both the solid phase preferred by the higher-melting component and the liquid–crystalline phase preferred by the lower-melting component (Yeagle, 1985; Presti, 1985; Ipsen et al., 1987; Vist & Davis, 1990; Finean, 1990). In PC/cerebroside or PC/(PA–Ca^{2+}) mixtures, by contrast, the influence of cholesterol will be confined primarily to the latter effect, which alone is clearly insufficient to promote efficient lipid mixing at temperatures where the higher-melting component in isolation prefers the crystalline state.

Appendix

Our starting point is the basic equation for fluorescence quenching:

$$F/F_0 = (k_f + k_n)/(k_f + k_n + k_{Br}) \quad (1)$$

where k_f, k_n, and k_{Br} represent respectively the rate constants for fluorescence emission, for all nonradiative decay processes unrelated to quenching by brominated residues, and for quenching by brominated acyl chains. For an individual fluorophore molecule, k_{Br} represents a sum of contributions from all brominated acyl chains lying sufficiently close to the fluorophore to exert a significant quenching effect. As noted previously [Yeager & Feigenson, 1990 (to which the reader is refered for a more complete treatment of the problem)], prediction and interpretation of k_{Br} can be simplified using the approximation that all brominated acyl chains lying within a sampling domain of M acyl chains around the fluorophore

have a comparable probability to quench the probe fluorescence, while for all more distal brominated chains this probability is zero.

In certain special cases, the effective quenching constant k_{Br} (determined by the microscopic distribution of brominated and nonbrominated acyl chains around a fluorophore molecule) may be assumed to be essentially the same for all fluorophores in the system. One such case arises when each fluorophore is accompanied by a single nearby brominated chain, as when one of the disulfide-linked conjugates shown in Figure 3 is highly diluted in a nonbrominated lipid matrix. If we designate as $\bar{k}_{Br}$ the contribution to the quenching rate constant made by a single brominated chain within the sampling domain, we expect that for a brominated conjugate before and after reductive cleavage with DTT

$$[F[+\mathrm{DTT}]/(F[-\mathrm{DTT}])]_{\mathrm{Br\ conj}} = (k_f + k_n + \bar{k}_{Br})/(k_{f'} + k_{n'}) \quad (2)$$

where unprimed and primed subscripts refer to the disulfide-linked and the reduced forms of the conjugates, respectively. Analogously, for the corresponding nonbrominated conjugate

$$[F[+\mathrm{DTT}]/(F[-\mathrm{DTT}])]_{\mathrm{non\text{-}Br\ conj}} = (k_f + k_n)/(k_{f'} + k_{n'}) \quad (3)$$

The difference of the left-hand terms given in eqs 2 and 3 is then simply equal to $(\bar{k}_{Br}/[k_{f'} + k_{n'}])$.

The overall quenching of probe fluorescence is also fairly simple to evaluate in a second special case, namely when the probe is dispersed in a matrix consisting entirely of brominated lipids. In this case, for the reduced form of one of the above conjugates

$$F_0/F = (k_{f'} + k_{n'} + M\bar{k}_{Br})/(k_{f'} + k_{n'}) = 1 + (M\bar{k}_{Br}/[k_{f'} + k_{n'}]) \quad (4)$$

where F_0 represents the normalized fluorescence measured for the same probe in a similar but nonbrominated lipid matrix. If the probe is dispersed in a matrix consisting entirely of lipids with one brominated and one nonbrominated acyl chain, it is correct to good approximation to replace M in the above expression by $(M/2)$ so long as M is not very small. By combining eqs 2, 3, and 4 and rearranging, we then obtain for one of the above conjugates

$$([F(\text{nonbrominated matrix})/F(\text{brominated matrix})] - 1)/([F[+\mathrm{DTT}]/(F[-\mathrm{DTT}])]_{\mathrm{Br\ conj}} - [F[+\mathrm{DTT}]/(F[-\mathrm{DTT}])]_{\mathrm{non\text{-}Br\ conj}}) = \alpha M \quad (5)$$

where $\alpha = {}^1/_2$ if the brominated "host" lipid used carries one brominated and one nonbrominated acyl chain and $\alpha = 1$ if this lipid carries instead two brominated acyl chains.

Applying the above calculations to data obtained for the indolyl-labeled conjugate shown in Figure 3 and its nonbrominated analogue, the numerator term in eq 5 was found to be 2.328 ± 0.534 using mBrPC as the brominated host lipid, and the denominator term was estimated as 0.1127 ± 0.0185, yielding an estimate of $M = 41 \pm 7$ acyl chains (mean ± SEM, the latter estimated by standard propagation-of-error methods; $N = 3$). For the carbazolyl-labeled probe shown in Figure 3 and its nonbrominated analogue, the numerator term was 5.258 ± 0.259 using tBr_2PC as the brominated host species and the denominator was 0.2070 ± 0.0151, giving an estimated value of $M = 51 \pm 3$ acyl chains (mean ± SEM; $N = 3$).

As already noted, the model used above to define and to estimate the parameter M is clearly an oversimplified picture of the detailed nature of the sampling domain. However, the above estimates of M provide an equally useful picture of the dimensions of the sampling domain if we can assume simply that the terms $\bar{k}_{Br}$ in eq 2 and $(M\bar{k}_{Br})$ in eq 4 represent *mean* values averaged in a similar manner over all sites sampled by the fluorophore. The interpretation of the estimated parameter M is discussed further in the text.

Registry No. (16/11-Carbazole)-PC, 122412-48-0; (18-C^9/16-indolyl)-PC, 139408-27-8; tBr_2PC, 96110-16-6; mBrPC, 139408-28-9; mBrPA, 139408-29-0; 16Br-cerebroside, 139408-30-3; DSPC, 816-94-4; DPPC, 63-89-8; POPC, 26853-31-6; di16BrPC, 139408-31-4; (16/11-carbazole)-PC disulfide linked to tBr_2PC, 139408-32-5; (16/12-indolyl)-PC disulfide linked to mBrPC, 139408-33-6; cholesterol, 57-88-5.

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