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Effects of cholesterol on the divalent cation-mediated interactions of vesicles containing amino and choline phospholipids

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We have used assays of lipid probe mixing, contents mixing and contents leakage to monitor the divalent cation-mediated interactions between lipid vesicles containing phosphatidylserine (PS) as a minority component together with mixtures of phosphatidylethanolamine (PE), phosphatidylcholine (PC) or sphingomyelin, and cholesterol in varying proportions. The initial rates of calcium- and magnesium-induced lipid probe quenching between vesicles, which reflect primarily the rates of vesicle aggregation, are strongly reduced as progressively higher proportions of PC or sphingomyelin are incorporated into PE/PS vesicles. The initial rates of divalent cation-induced contents mixing and contents leakage for PE/PS vesicles are also strongly reduced when choline phospholipids are incorporated into the vesicles in even low molar proportions. Sphingomyelin has a more potent inhibitory effect on these processes than does PC at an equal level in the vesicle membranes. The inclusion of cholesterol in these vesicles, at levels up to 1:2 moles sterol/mole phospholipid, has little effect on the rates of calcium- or magnesium-induced vesicle aggregation. However, cholesterol significantly enhances the initial rates of vesicle contents mixing and contents leakage in the presence of divalent cations when the vesicles contain choline as well as amino phospholipids. This effect is substantial only when the level of cholesterol exceeds the level of choline phospholipids in the vesicles. These results may have significance for the fusion of certain cellular membranes in mammalian cells, whose cytoplasmic faces have lipid compositions very similar to those of the vesicles examined in this study.

Introduction

A variety of studies in recent years have demonstrated that the efficiency of fusion between both artificial (lipid vesicle) and biological membranes can depend strongly on the membrane lipid composition [1–9]. Since animal cell membranes typically contain small amounts of anionic lipids together with larger amounts of neutral lipids, the effects of these latter species on membrane fusion are of considerable interest. Studies of the divalent cation-promoted fusion of vesicles combining anionic and neutral phospholipids have shown that phosphatidylcholine (PC) antagonizes apposition and fusion of bilayer lipid membranes, while phosphatidylethanolamine (PE) can support these processes [3,4,10,11].

Fewer studies have examined the effects of cholesterol on the interactions and fusion between phospholipid bilayer membranes. Uster and Deamer [3] have reported that replacement of a portion of the PC component in PC/phosphatidylserine (PS) vesicles by cholesterol en-

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hances the ability of the vesicles to undergo calcium-promoted lipid mixing. Braun et al. [12] have found that inclusion of cholesterol in PS vesicles slows the rate of vesicle aggregation but enhances the rate of calcium-induced fusion (contents mixing) between already aggregated vesicles. Other studies [13–15] have concluded that cholesterol may either inhibit the fusion of phospholipid vesicles or have no effect on fusion, depending on the phospholipid composition. In contrast, studies of the fusion of biological membranes have suggested that some fusion processes may be enhanced by, or may even require, the presence of cholesterol in the participating membranes [16–23].

The results described above suggest that the effects of cholesterol on membrane fusion may vary depending on the phospholipid compositions of the membranes involved. As noted above, animal cell membranes typically contain large amounts of neutral phospholipids, particularly PE, PC and sphingomyelin in varying proportions, together with smaller amounts of anionic lipids and varying amounts of cholesterol. In an effort to understand how cholesterol can affect the fusion competence of membranes with such phospholipid compositions, we have examined here the divalent cation-promoted interactions of vesicles that combine low mole fractions of PS (15 or 25 mol%) with varying proportions of PE, cholesterol and PC or sphingomyelin. Our results indicate that sphingomyelin is even more potent than PC in inhibiting the divalent cation-mediated interactions of PE/PS vesicles, but that cholesterol can significantly offset the inhibitory effects of choline phospholipids on vesicle association and destabilization, particularly when the molar ratio of cholesterol to choline phospholipids is greater than roughly 1 : 1.

Materials and Methods

Materials. Egg yolk phosphatidylcholine, bovine brain sphingomyelin and Triton X-100 were purchased from Sigma, and cholesterol was obtained from Nu-Chek Prep. (Elysian, MN). Ethanolamine hydrochloride (Gold Label) was obtained from Aldrich. 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes (Junction City, OR). Phospholipase D was extracted from Savoy cabbage by the procedure of Davidson and Long [24] as outlined previously [10]. Diethyl ether used for vesicle preparations was redistilled from P_2O_5, stabilized with 0.5% water and stored in the cold. All other organic solvents and inorganic chemicals used were of at least reagent grade.

Egg PE was prepared from egg PC by phospholipase D-mediated transphosphatidylation as described previously [25], with the modification that the reaction was carried out at pH 6.8. The crude PE was purified as described by Silvius and Gagné [10]. Dioleoylphosphatidylserine was prepared and purified as described previously [10]. The purity of the different lipids used was evaluated by TLC in chloroform/acetone/methanol/acetic acid/water (50 : 20 : 10 : 10 : 5, v/v) and $CHCl_3/CH_3OH/H_2O$/conc. NH_4OH (65 : 35 : 2.5 : 2.5, v/v). All lipid stocks were stored at −70 °C under nitrogen.

Methods. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation and pressure filtration through 0.1 μm pore-size Nucleopore membranes, as described by Wilschut et al. [27]. Lipid probe quenching between vesicles was measured by the procedure of Hoekstra [28], using vesicles labeled with 2 mol% of either NBD-PE or Rho-PE. Leakage of vesicle contents was measured by the procedure described by Ellens et al. [29], using vesicles containing 25 mM ANTS and 50 mM DPX. Mixing of vesicle contents was assayed by the procedure of Ellens et al. [30], using vesicles containing either 50 mM ANTS or 90 mM DPX, with a 1 : 4 ratio of ANTS- to DPX-containing vesicles. All solutions used to prepare vesicle samples were buffered with 5 mM histidine, 5 mM Tes (pH 7.4) and were adjusted with NaCl to an osmolarity equivalent to that of 150 mM NaCl, 5 mM histidine, 5 mM Tes, 1 mM EDTA (pH 7.4) (determined by direct osmometry). Except where otherwise indicated, all assays of vesicle fusion and leakage were carried out at 30 °C with vesicles suspended in this latter buffer at a lipid concentration of 30 μM. Phospholipid was assayed by the procedure of Lowry and Tinsley [26], but the digestion of the samples was prolonged to 4 h to ensure complete digestion.

Results

Fusion and destabilization of PS/PE/(PC or sphingomyelin) vesicles

The results of previous studies have demonstrated clearly that PE but not PC can support efficient divalent cation-mediated fusion of lipid vesicles that like natural membranes, contain anionic phospholipids as a minority component. In the present study, we examined systematically how changes in the balance of PE and PC (or sphingomyelin) influence the calcium- and magnesium-mediated destabilization and fusion of vesicles that combine these neutral phospholipids with low proportions (15 mol% or 25 mol%) of PS. Assays of vesicle contents mixing, lipid probe mixing and leakage were used to characterize the divalent cation-mediated interactions of these vesicles. The assay that we employed to monitor lipid probe mixing [28] can in principle be significantly affected by simple apposition of vesicle membranes as well as by true mixing of lipid components. While control experiments provided no evidence that vesicle aggregation per se was a major contributing factor for the fluorescence changes we observed in our experiments, we will refer to results obtained using this assay as 'lipid probe quenching' in the remainder of this paper. What is most important for our purposes is the fact that as discussed below, the rate of divalent cation-induced lipid probe quenching was found to be purely aggregation rate-limited for all types of vesicles examined here under standard conditions. Therefore, we regard the results obtained here using the lipid probe mixing assay as a measure of rates of vesicle aggregation, rather than as a measure of intrinsic rates of lipid mixing per se.

In Fig. 1A are shown time courses of fluorescence observed using the lipid probe quenching and leakage assays to monitor the effects of calcium (10 mM) on vesicles containing 25 mol% PS together with either 75 mol% PE ('PE/PS') or 60 mol% PE plus 15 mol% PC ('20% PC'). The results of parallel assays of contents mixing between vesicles of these compositions under similar conditions are shown in Fig. 4. It can be seen that the incorporation of even a modest molar percentage of PC (15 mol%) into PE/PS vesicles sharply reduces the rates of calcium-induced contents mixing and leakage, while the initial rate of lipid probe quenching is more modestly reduced. When these experiments are carried out using various calcium concentrations, it is apparent that the incorporation of PC into the PE/PS vesicles has two distinct effects on the calcium-mediated interactions of the vesicles, as the lipid probe

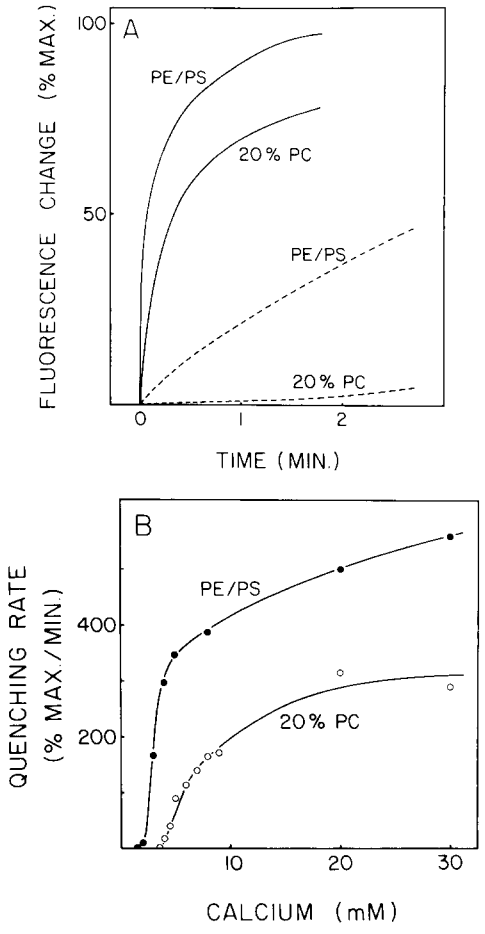

Fig. 1. (A) Time courses of lipid probe quenching (solid curves) and leakage of vesicle contents (dashed curves) when vesicles composed of either 25:75 PS/PE (PE/PS) or 25:60:15: PS/PE/PC (20% PC) are exposed to 10 mM calcium at time zero. Assays of lipid probe quenching and contents leakage were carried out as described in Materials and Methods. (B) Initial rates of lipid probe quenching, determined from time courses like those shown in (A), for 25:75 PS/PE (PE/PS) or 25:60:15 PS/PE/PC (20% PC) vesicles treated with varying concentrations of calcium. In this and all subsequent figures, the PC (or sphingomyelin) content of each vesicle preparation is expressed on all graphs as the molar percentage of PC in the neutral phospholipid fraction.

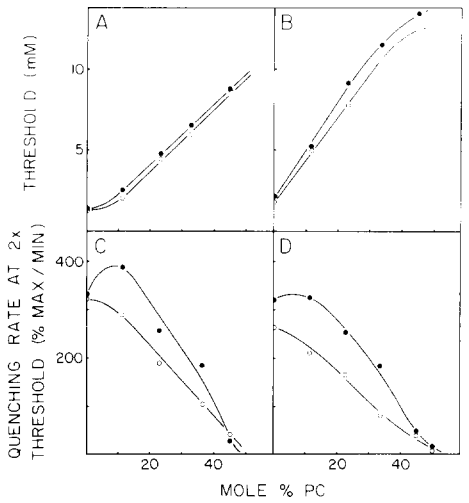

Fig. 2. (A, B). Threshold concentrations of (A) calcium and (B) magnesium required to induce lipid probe quenching between vesicles containing 15 mol% PS plus various mixtures of PE and PC with the indicated PC contents. (C, D). Efficiencies of lipid probe quenching induced by (C) calcium or (D) magnesium between PS/PE/PC vesicles of the indicated PC contents. In each panel, open and filled circles represent data points obtained for vesicles containing 0 or 33 mol% cholesterol, respectively. Initial rates of lipid probe quenching were determined as in Fig. 1.

quenching data shown in Fig. 1B illustrate. First, the presence of PC elevates the threshold concentration of calcium below which vesicle-vesicle interactions cannot be observed. Second, PC slows the rates of interactions between vesicles even at calcium concentrations well above the threshold. To quantitate this latter effect most economically in the discussion to follow, we will define a quantity termed the 'efficiency' of lipid probe quenching or leakage, which for a particular divalent cation and type of liposome represents the rate of lipid probe quenching or leakage measured at a divalent cation concentration equal to twice the threshold.

Lipid-mixing experiments of the type shown in Fig. 1 were carried out using vesicles composed of 15 mol% PS plus varying mole proportions of PE and PC, giving the results summarized in Fig. 2. As can be seen from Figs. 2A and 2B, the incorporation of increasing amounts of PC into PE/PS vesicles steadily increases the threshold calcium or magnesium concentrations required to initiate vesicle-vesicle contact and lipid probe quenching. At the same time, increasing levels of PC in the vesicles progressively diminish the efficiency of calcium- or magnesium-initiated lipid probe quenching to almost zero at an equimolar ratio of PC to PE in the bilayer (Figs. 2C and 2D). When these experiments were repeated using vesicles that contained cholesterol (1 : 2 mol/mol phospholipid) in combination with the above phospholipid mixtures, the effects of increasing PC content on divalent cation-mediated lipid probe quenching were similar (Fig. 2, filled circles). Cholesterol has almost no effect on the threshold divalent cation levels needed to initiate interactions between vesicles of a given phospholipid composition. However, the sterol modestly elevates the efficiency of lipid probe quenching, particularly for vesicles containing 10–30 mol% PC. Similar results were obtained using vesicles containing 25 mol% PS (not shown), although these vesicles generally gave somewhat faster rates of lipid probe quenching than did vesicles with the same PE/PC ratio that contained only 15 mol% PS.

In parallel with the lipid probe quenching experiments described above, we examined the rates of calcium- and magnesium-induced destabilization (measured as leakage of aqueous contents) for vesicles of the same lipid compositions. For every vesicle composition examined, the threshold concentrations of calcium and magnesium needed to elicit detectable leakage were very similar to the threshold concentrations required to induce lipid probe quenching. In Fig. 3 we summarize the efficiencies of contents leakage induced by calcium and magnesium from vesicles containing 25 mol% PS plus varying proportions of PC and PE. It can be seen that the presence of small amounts of PC in these vesicles sharply reduces the rate of contents leakage in the presence of either divalent cation. The presence of cholesterol (1 : 2 mol sterol/mol phospholipid) has little effect on the calcium-induced leakage of contents from PE/PS vesicles but strongly enhances the leakage from vesicles that also contain low mole fractions of PC (Fig. 3A). Cholesterol generally increases the rate of magnesium-induced contents leakage from PS/PE vesicles as well as from PS/PE/PC vesicles, but in this case also the magnitude of the enhancement is greater for vesicles containing 10 or 20 mol% PC than for vesicles containing only PS and PE (Fig. 3B).

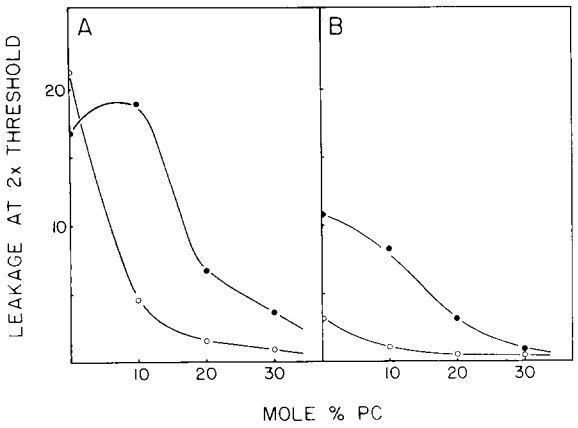

Fig. 3. Efficiencies of contents leakage induced by (A) calcium or (B) magnesium from vesicles containing 25 mol% PS plus various mixtures of PE and PC with the indicated PC contents (expressed as the molar percentage of PC in the neutral phospholipid fraction). In each panel, open and filled circles represent data points obtained for vesicles containing 0 or 33 mol% cholesterol, respectively. Leakage of vesicle contents was assayed as described in Materials and Methods.

The results shown in Figs. 1 to 3 demonstrate that the rate of lipid probe quenching between PS/PE/PC (/cholesterol) vesicles is considerably faster in the presence of divalent cations than is the leakage of vesicle contents. Moreover, the time courses of lipid probe quenching and contents leakage are significantly different in their overall shape, as well as in their initial rates, particularly for vesicles containing PC (Fig. 1A). These observations suggest that the destabilization of lipid vesicles under these conditions is a slower process than is the initial vesicle–vesicle interaction that leads to lipid probe quenching. In agreement with this suggestion, we found that the initial rate of lipid probe quenching between vesicles of various compositions was in all cases proportional to the second power of the lipid concentration (data not shown), suggesting that this process is aggregation-limited [31]. By contrast, the dependence of the leakage rate on the lipid concentration was more nearly first-order, particularly for vesicles containing PC, indicating that this process is rate-limited principally by processes occurring subsequent to vesicle aggregation.

As sphingomyelin represents a second major choline phospholipid in the plasma membranes of eukaryotic cells, we also examined how this lipid affects the divalent cation-mediated interactions between lipid vesicles containing PS and PE with or without cholesterol. Lipid probe quenching and leakage experiments were carried out using vesicles containing 25 mol% PS plus variable proportions of PE and sphingomyelin, giving the results summarized in Table I. Sphingomyelin, when incorporated into PS/PE vesicles at levels of 7.5–15 mol%, elevates the threshold concentrations of divalent cations required to initiate lipid probe quenching and leakage somewhat more strongly

TABLE I

EFFECT OF SPHINGOMYELIN ON DIVALENT CATION-INDUCED LIPID PROBE QUENCHING AND CONTENTS LEAKAGE OF PE/PS VESICLES

Initial rates of lipid probe quenching and leakage of contents from vesicles of the indicated compositions were assayed as described in Materials and Methods, using a phospholipid concentration of 30 μM. In the designations of vesicle lipid composition, Sph = sphingomyelin and (+Chol.) indicates that the vesicles contained cholesterol at a 1:2 molar ratio of sterol to phospholipid.

Lipid composition	Lipid probe quenching				Contents leakage			
	Threshold (mM)		Efficiency (%max/min)		Threshold (mM)		Efficiency (%max/min)	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
25:75 PS/PE	1.8	2.8	259	238	1.9	2.5	21.3	3.2
25:75 PS/PE (+Chol.)	1.6	2.8	206	359	1.9	2.8	16.6	10.8
25:67.5:7.5 PS/PE/Sph	4.0	5.0	59	48	3.5	4.2	3.4	0.66
25:67.5:7.5 PS/PE/Sph (+Chol.)	4.0	5.0	250	260	3.7	5.0	19.2	4.26
25:60:15 PS/PE/Sph	5.5	6.5	11.8	11.9	4.0	6.0	0.3	0.025
25:60:15 PS/PE/Sph (+Chol.)	5.2	6.1	100	40	4.0	6.0	1.2	0.60

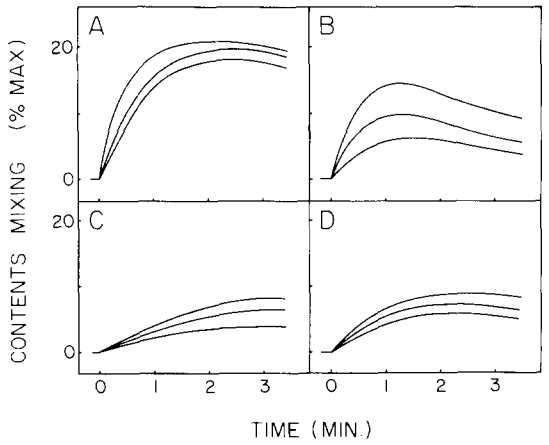

Fig. 4. Time courses of calcium-induced contents mixing between vesicles of the following lipid compositions: (A) 25:75 PS/PE, (B) 25:75 PS/PE plus cholesterol (1 mol/2 mol phospholipid), (C) 25:60:15 PS/PE/PC, or (D) 25:60:15 PS/PE/PC plus cholesterol (1 mol/2 mol cholesterol). Contents mixing between vesicles was assayed as described in Materials and Methods. In each panel, the lower, middle and upper time courses shown were recorded using 10, 20 and 30 mM calcium, respectively.

than does the equivalent amount of PC (compare Table I to Fig. 2). More strikingly, the efficiencies of divalent cation-induced lipid probe quenching and leakage for these vesicles are very dramatically reduced when even low mole fractions of sphingomyelin are incorporated. These inhibitory effects of sphingomyelin can be offset substantially by the incorporation of cholesterol (1:2 mol/mol phospholipid), particularly when the vesicles contain only 7.5 mol% sphingomyelin, in which case cholesterol restores the efficiencies of lipid mixing and leakage to levels near those observed for PE/PS vesicles.

Contents mixing experiments were also carried out with vesicles of the same lipid compositions as those examined in the lipid probe quenching and leakage experiments described above. Fluorescence time courses representing calcium-induced contents mixing between vesicles of four different lipid compositions are shown in Fig. 4, and results from a variety of experiments of this type are summarized in Table II. As for the lipid mixing and leakage assays, the rate of contents mixing increases as the level of calcium is increased above a threshold concentration that is very similar to that measured from the other two assays. The incorporation of even small amounts of PC into the PE/PS bilayers sharply decreases the rate of contents mixing at any given calcium concentration above threshold. The incorporation of cholesterol into PE/PS vesicles, in a 2:1 ratio of phospholipid to sterol, decreases slightly the rate of divalent cation-induced contents mixing. However, when PC is also present in the vesicles, the

TABLE II

INITIAL RATES OF DIVALENT CATION-MEDIATED CONTENTS MIXING BETWEEN VESICLES CONTAINING AMINO AND CHOLINE PHOSPHOLIPIDS

Contents mixing between lipid vesicles of the indicated compositions was assayed as described in Materials and Methods at a phospholipid concentration of 30 μM and the divalent cation concentrations shown. Vesicles whose listed compositions include the designation (+Chol.) contained cholesterol in a 1:2 molar ratio of sterol to phospholipid. Sph, sphingomyelin.

Vesicle lipid composition	Initial rate of contents mixing (% maximum/min) at			
	10 mM Ca^{2+}	30 mM Ca^{2+}	10 mM Mg^{2+}	30 mM Mg^{2+}
25:75 PS/PE	17.3	25.7	10.0	7.0
25:75 PS/PE (+Chol.)	7.3	19.6	7.3	4.5
25:60:15 PS/PE/PC	2.1	5.5	0.8	1.5
25:60:15 PS/PE/PC (+Chol.)	3.9	7.0	0.6	1.6
25:52.5:22.5 PS/PE/PC	0.6	2.4	0.1	0.3
25:52.5:22.5 PS/PE/PC (+Chol.)	1.7	5.1	0.1	0.8
25:67.5:7.5 PS/PE/Sph	2.6	5.4	0.6	1.7
25:67.5:7.5 PS/PE/Sph (+Chol.)	2.7	7.5	1.5	3.0

rate of contents mixing is increased by the presence of cholesterol. The actual enhancement of contents mixing by the presence of cholesterol in these latter vesicles may be considerably greater than the traces shown in Fig. 4D would suggest, since cholesterol also substantially increases the rate of contents leakage from 25 : 60 : 15 PS/PE/PC vesicles (see Fig. 3). However, we made no attempt to correct for this effect (see, for example, Ref. 31), as it is not clear in the present case whether contents leakage occurs only after contents mixing between two vesicles, in parallel with (but independently of) contents mixing, or both. In these different cases, the corrections necessary to account for the effects of leakage on measured contents mixing rates are quite different.

A pattern of results qualitatively similar to that shown in Fig. 4 was also observed when vesicles of the types examined in this figure were treated with magnesium instead of calcium (see Table II). However, in this case the absolute rates of contents mixing were somewhat slower than those observed at the same concentrations of calcium. Sphingomyelin strongly suppressed the divalent cation-promoted mixing of contents when incorporated at 15 mol% into PE/PS vesicles (not shown). Vesicles containing only 7.5 mol% sphingomyelin showed initial rates of contents mixing that were very similar to the rates observed for vesicles containing twice this amount of PC (see Table II). As for the PC-containing vesicles, the incorporation of cholesterol into the sphingomyelin-containing vesicles significantly increased the rate of divalent cation-induced contents mixing (Table II). The effects of choline phospholipids and cholesterol on divalent cation-induced contents mixing between PE/PS vesicles thus generally parallel the effects of these compounds on leakage of vesicle contents, as described above.

The results presented above indicate that cholesterol, at a level of 1 : 2 mol sterol/mol phospholipid, can enhance strongly the rates of divalent cation-induced contents mixing and leakage for vesicles containing PE and PS plus 7.5 mol% or 15 mol% of choline phospholipids. To examine in more detail the relationship between the cholesterol level and the rate of calcium-induced destabilization for such vesicles, we measured the rates of calcium-promoted leakage of contents from vesicles combining various amounts of cholesterol with each of the following lipid mixtures: 25 : 67.5 : 7.5 PS/PE/PC, 25 : 60 : 15 PS/PE/PC or 25 : 67.5 : 7.5 PS/PE/sphingomyelin. In Fig. 5 we show the rates of contents leakage, measured at twice the threshold concentration of Ca^{2+} needed to trigger vesicle aggregation, for vesicles containing these three phospholipid mixtures plus varying mole fractions of cholesterol. As the amount of cholesterol incorporated into the vesicles increases, the rate of vesicle contents leakage remains roughly constant until the ratio of cholesterol to choline phospholipids reaches a value near unity. When higher amounts of cholesterol are incorporated into the bilayers, the rate of contents leakage increases rapidly. The threshold concentrations of calcium needed to elicit vesicle leakage showed little change as the proportion of cholesterol in the vesicles was varied.

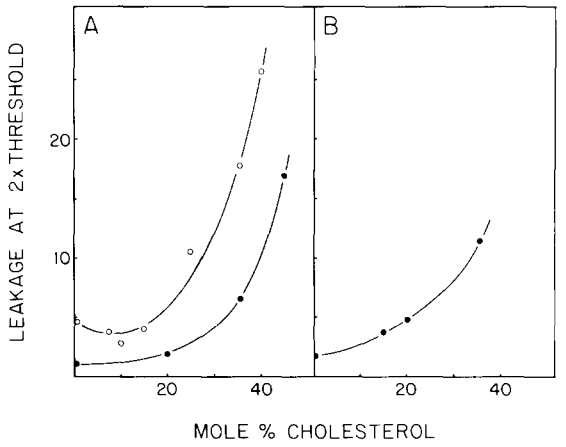

Fig. 5. (A) Efficiencies of leakage of contents induced by calcium from vesicles composed of either 25 : 67.5 : 7.5 PS/PE/PC (open circles) or 25 : 60 : 15 PS/PE/PC (filled circles) plus the indicated amounts of cholesterol, expressed as a molar percentage of the total vesicle lipid. (B) Efficiencies of calcium-induced contents leakage from vesicles composed of 25 : 67.5 : 7.5 PS/PE/sphingomyelin plus the indicated molar percentages of cholesterol. Initial rates of calcium-induced leakage of vesicle contents were determined as for Fig. 3.

Discussion

In this paper, we have examined various aspects of the divalent cation-mediated interactions be-

tween liposomes that contain physiologically low proportions of an anionic lipid, PS, together with mixtures of the major neutral polar lipids found in mammalian cell membranes. Previous studies of the properties of lipid vesicles of this type [3,4,10] have shown that highly fusion-competent vesicles can be prepared using PE in combination with as little as 15–25 mol% PS, while the inclusion of even moderate amounts of PC in such vesicles significantly inhibits the divalent cation-induced mixing of vesicle lipids and aqueous contents. Our results indicate that the inclusion of low mole fractions of PC in PE/PS vesicles has a stronger inhibitory effect on the divalent cation-induced destabilization of the vesicles (reflected in contents-mixing and leakage rates) than on the aggregation of such vesicles (reflected in rates of lipid probe quenching). At PC/PE ratios higher than unity, all of these processes are strongly inhibited.

The inhibitory effects of PC on vesicle fusion have previously been ascribed to two factors. First, it has been shown that the work of dehydration required to bring PC-rich bilayers into contact is considerably larger than that required to bring PE-rich bilayers into contact [32–34]. Second, phosphatidylcholines form non-lamellar structures much less readily than do phosphatidylethanolamines with the same acyl chain compositions, and even fairly low mole fractions of PC can increase substantially the stability of the lamellar phase in lipid mixtures that are rich in PE [35–37]. Both of these effects, which are to some extent interrelated [38], are probably important for the vesicle systems studied here.

The results of our experiments with PS/PE/sphingomyelin vesicles indicate that sphingomyelin influences the divalent cation-mediated interactions of these vesicles in the same general manner that PC does, as might be expected given the similar headgroups of the two species. However, the incorporation of a given proportion of sphingomyelin into PS/PE vesicles inhibits the divalent cation-induced interactions of the vesicles more severely than does incorporation of the same proportion of PC. This result can be correlated with the finding of Cullis and Hope [39] that bovine brain sphingomyelin stabilizes the bilayer organization of an unsaturated PE more effectively than does an equal proportion of egg PC. It is also possible that sphingomyelin resists dehydration of the bilayer surface more strongly than does PC, which could contribute to the differences that we observe in the effects of the two choline phospholipids on vesicle fusion. However, to the best of our knowledge, no quantitative comparison of the relative strengths of hydration of sphingomyelin vs. PC in mixed lipid bilayers has been reported to date.

The incorporation of cholesterol (1 : 2 mol sterol/mol phospholipid) into PS/PE/PC vesicles has only a small effect on the rate of lipid probe quenching between vesicles (which under our conditions is aggregation-limited) in the presence of calcium or magnesium. However, the effects of cholesterol on the subsequent destabilization of such vesicles, leading to contents mixing and leakage of contents, are much more substantial, particularly when the vesicles contain low (but finite) amounts of choline phospholipids. Previous studies [35–37] have shown that cholesterol markedly promotes the formation of the hexagonal II phase in mixtures of unsaturated PE and PC species, and that the amount of cholesterol required to produce this effect increases with increasing mole fractions of PC. Cholesterol has also been shown to promote divalent cation-dependent formation of the hexagonal phase in mixtures of these neutral phospholipids with PS [40]. These findings appear to correlate particularly well with the results of our leakage measurements, which show that the compositional factors which promote ready formation of the hexagonal II phase in bulk lipid dispersions (e.g., a high ratio of cholesterol to choline phospholipids) also enhance strongly the kinetics of contents leakage following divalent cation-induced aggregation of lipid vesicles with the same compositions.

The observed effects of cholesterol on the mixing of contents between PE/PS/(PC or sphingomyelin) vesicles, while they are significant, are less dramatic than the effects observed on contents leakage. We can suggest two possible reasons for this finding. First, as already noted in Results, our data are consistent with the possibility that cholesterol strongly enhances the rates of both contents mixing and subsequent leakage of vesicle contents, with the latter effect partially masking the former [31]. Second, cholesterol may

genuinely enhance the rate of release (relative to that of mixing) of vesicle contents, possibly by promoting the formation of extended nonlamellar structures (e.g., hexagonal II tubes) in place of strictly local nonlamellar ('isotropic') structures [41]. The latter proposal would be consistent with the finding that cholesterol facilitates the formation of the hexagonal II phase, as opposed to 'isotropic' structures, in several PE/PC and PE/PC/PS systems [35–37,39].

A number of studies [42–45] have reported that cholesterol shows a significant preference for interaction with choline vs. amino phospholipids in systems where the two types of phospholipids are segregated into distinct lateral domains or into separate vesicles. Our present results indicate that cholesterol affects the divalent cation-promoted fusion and destabilization of PS/PE/(PC or sphingomyelin) vesicles more strongly than it affects these same processes in vesicles composed purely of PE and PS. These latter findings may appear to support the idea that cholesterol interacts rather differently with choline vs. amino phospholipids even in bilayers where the two types of phospholipids intermix freely. However, it is also possible that cholesterol acts to enhance vesicle fusion and destabilization by modifying some property of the lipid bilayer that is far from optimal (with regard to fusion-supporting potential) for vesicles containing choline phospholipids but which is already near-optimal (i.e., not limiting for fusion) in vesicles composed purely of amino phospholipids. Our results thus demonstrate that the effects of cholesterol on the 'fusion competence ' of a given membrane may vary considerably depending on its neutral phospholipid composition, but we cannot conclude unequivocally that this effect arises from a differential interaction of cholesterol with the different phospholipids found in natural membranes.

The results of a number of studies have indicated that the cytoplasmic surfaces of mammalian cellular membranes are relatively rich in amino phospholipids but also contain significant amounts of choline phospholipids [46–49]. The cholesterol contents of these membranes appear to vary widely, ranging from relatively high levels in the plasma membrane and endo- and phagocytic vesicles derived from it [44,50–53] to much lower levels in many intracellular membranes [50,51]. Our present results suggest that the exact balance between choline phospholipids and cholesterol in these membranes may be a significant determinant of the 'fusion competence' of their lipid bilayers.

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