J. Membrane Biol. 141, 139–148 (1994)

The Journal of
Membrane
Biology

Modeling Degranulation with Liposomes: Effect of Lipid Composition on Membrane Fusion

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Received: 14 December 1993/Revised: 5 April 1994

Abstract. Degranulation involves the regulated fusion of granule membrane with plasma membrane. To study the role of lipid composition in degranulation, large unilamellar vesicles (LUVs) of increasing complexity in lipid compositions were constructed and tested for Ca^{2+}-mediated lipid and contents mixing. Lipid-mixing rates of LUVs composed of phosphatidylethanolamine (PE) and phosphatidylserine (PS) were strongly decreased by the addition of either phosphatidylcholine (PC) or sphingomyelin (SM), while phosphatidylinositol (PI) had little effect. "Complex" LUVs of PC:PE:SM:PI:PS (24:27:20:16:13, designed to emulate neutrophil plasma membranes) also showed very low rates of both lipid mixing and contents mixing. The addition of cholesterol significantly lowered the Ca^{2+} threshold for contents mixing and increased the maximum rates of both lipid and contents mixing in a dose-dependent manner. Membrane remodeling, which occurs in neutrophil plasma membranes upon stimulation, was simulated by incorporating low levels of phosphatidic acid (PA) or a diacylglycerol (DAG) into complex LUVs containing 50% cholesterol. The addition of PA both lowered the Ca^{2+} threshold and increased the rate of contents mixing in a dose-dependent manner, while the DAG had no significant effect. The interaction of dissimilar LUVs was also examined. Contents-mixing rates of LUVs of two different cholesterol contents were intermediate between the rates observed for the LUVs of identical composition. Thus, cholesterol needed to be present in only one fusing partner to enhance fusion. However, for PA to stimulate fusion, it had to be present in both sets of LUVs. These results suggest that the rate of degranulation may be increased by a rise in the cholesterol level of either the inner face of the plasma membrane or the outer face of the granule membrane. Further, the production of PA can promote fusion, and hence degranulation, whereas the subsequent conversion of PA to DAG may reverse this promotional effect.

Key words: Calcium — Cholesterol — Liposomes — Membrane fusion — Phosphatidic acid

Introduction

Degranulation involves the fusion of granule membrane to plasma membrane. Fusion, and hence degranulation, does not occur in resting neutrophils but proceeds rapidly following the appropriate stimulus. It is unclear what role, if any, lipid composition plays in controlling fusion during degranulation. However, membrane remodeling (e.g., through the action of various phospholipases) is known to be an integral part of a number of signaling pathways that lead to exocytosis (Smolen & Shohet, 1974; Serhan et al., 1982; Agwu et al., 1989; Santini et al., 1990; Cockcroft, 1992). It is not known how membrane remodeling might mediate membrane fusion during degranulation.

The use of model membrane systems using one to three phospholipids has proven to be a powerful approach for studying the effect of individual lipids on fusion. However, few studies have examined the fusion properties of liposomes prepared with complex assemblages of phospholipids, as are found in biological membranes. Furthermore, the effect of cholesterol on the fusion of complex assemblages is unclear. In simpler model systems, cholesterol can inhibit (Papahadjopoulos et al., 1974; Breisblatt & Ohki, 1976; Chaudhury & Ohki, 1981; Connor, Yatvin & Huang, 1984), enhance (Evans & Needham, 1986; Rand & Parsegian,

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1986) or be required (Kielian & Helenius, 1984; Nieva, Goni & Alonso, 1989) for fusion.

The present study details the fusion characteristics of liposomes designed to emulate the plasma membranes of neutrophils in composition. Following available analyses (Smolen & Shohet, 1974; Cockcroft, 1984; Diez, Balsinde & Mollinedo, 1990), complex liposomes contained predominantly phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with significant amounts of sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). Membrane remodeling is known to occur in neutrophils (Smolen & Shohet, 1974; Diez et al., 1990; Cockcroft, 1992), and so the effects of low levels of phosphatidic acid (PA) and a diacylglycerol (DAG) on fusion have been tested. Furthermore, increasing cytoplasmic Ca^{2+} is central to neutrophil degranulation (Smolen & Sandborg, 1990; Smolen, 1992). Consequently, we have examined fusion as induced by Ca^{2+}, although membrane fusion during degranulation undoubtedly involves more factors than Ca^{2+} alone (Düzgünes et al., 1987; Papahadjopoulos, Nir & Düzgünes, 1990).

Materials and Methods

Materials

Egg PC, PA from egg PC, egg PE, liver PI, bovine brain PS, and egg SM were purchased from Avanti Polar Lipids (Birmingham, AL). Octadecyl rhodamine (R18), *p*-xylene-*bis*-pyridinium bromide (DPX), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) were purchased from Molecular Probes (Eugene, OR). 1,2-dioctanoyl-*sn*-glycerol (DiC_8) was purchased from both Avanti and Sigma. All other chemicals were from Sigma.

Liposome Preparation

Liposomes were composed of various combinations of PC, PE, SM, PI, and PS and cholesterol. These combinations were systematically related to achieving an ultimate ratio of 24:27:20:16:13 PC:PE:SM:PI:PS. Any liposomes prepared with these five phospholipids in this ratio were designated "complex" liposomes, for brevity. LUVs were used for all assays and were prepared as described previously (Francis et al., 1992). Briefly, vesicle components were prepared and mixed in chloroform, dried in a near-vacuum at 37°C under argon, then resuspended in buffer. Buffers used were (in mM): KHEN (130 KCl, 30 HEPES, 1 EGTA, 10 NaCl, pH 7.0), 25 ANTS (in 19.5 KCl, 30 HEPES, pH 7.0) or 117 DPX (in 30 HEPES, pH 7.0). Samples were frozen (−70°C)/thawed (40°C) seven times, then extruded seven times through two 100 nm polycarbonate filters, using an Extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada). Unincorporated materials were removed by gel filtration with Sephadex G-75. The concentration of liposomes in the pooled opaque fractions was evaluated by phosphorus determination (Morrison, 1964).

Diacylglycerol Micelle Preparation

DiC_8 in chloroform was dried under a stream of nitrogen, resuspended in dimethylsulfoxide and diluted 20-fold in KHEN buffer. The resulting opaque solution was sonicated for 30 sec to completely disperse the diacylglycerol.

Liposome Aggregation Assay

Aggregation was assayed according to Meers et al. (1987), with the following modifications. LUVs (370 μM lipid) were suspended in KHEN buffer with constant stirring. Changes in absorbance at 450 nm were continuously recorded by spectrophotometer (model DU-8, Beckman Instruments).

Lipid-Mixing Assay

The transfer of phospholipid between LUVs was assayed by recording the increase in rhodamine (R18) fluorescence (relief of self-quenching) due to R18 dilution (Francis et al., 1990). LUVs labeled with 2 mol % R18 were stirred with unlabeled LUVs at a ratio of 1:4 (100 μM total lipid) at 37°C. Lipid mixing was evaluated as the initial rate of change in fluorescence, occurring over the first 15 sec, and recalculated as the percent maximum change per minute. Maximum change was defined as the change in fluorescence produced by the addition of 0.1% Triton X-100 to R18-labeled LUVs.

Contents-Mixing Assay

The mixing of aqueous contents between LUVs was evaluated as the decrease in fluorescence from ANTS due to quenching by DPX, as described previously (Francis et al., 1992). ANTS-containing LUVs were stirred with DPX-containing LUVs at a ratio of 1:1 (370 μM total lipid) at 37°C. Contents mixing was evaluated as the initial rate of change in fluorescence, occurring over the first 10 sec, and recalculated as the percent maximum change per minute. Contents leakage was also evaluated by assaying the quenching of ANTS fluorescence by 4.5 mM free DPX in parallel treatments. Maximum mixing was defined as the change in fluorescence produced by the addition of 4.5 mM free DPX and 0.1% Triton X-100 to ANTS-containing LUVs.

Ca^{2+} Threshold Determination

The Ca^{2+} threshold for a given response, defined as the Ca^{2+} concentration above which a positive response would be attained, was determined by curve-fitting the three lowest positive response values, using Sigmaplot Scientific Graphing System, version 5.00, and then calculating the Ca^{2+} value corresponding with a response value of zero. Results are presented as the means ± SEM of at least three separate experiments.

Aggregation, lipid mixing and contents mixing were initiated by the addition of various concentrations of $CaCl_2$. Free Ca^{2+} concentration was determined by the method of Bers (1982). Fluorescence was measured by spectrofluorimeter (model 650-10S, Perkins-Elmer, Norwalk, CT) equipped with a temperature-regulated cell holder and a magnetic stirring device.

Abbreviations

ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DiC_8, 1,2-dioctanoyl-*sn*-glycerol; DPX, *p*-xylene-*bis*-pyridinium bromide; LUV, large unilamellar vesicle; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; R18, octadecyl rhodamine; SM, sphingomyelin.

Results

Fusion of "Simple" Liposome Mixtures

In most membranes, the most abundant fusogenic phospholipids are PE and PS. LUVs composed solely of PE/PS (molar ratio of 27:13) were found to fuse readily, with a Ca^{2+} threshold for lipid mixing of approximately 2 mM Ca^{2+} and a maximum initial rate of 18%/min achieved at a Ca^{2+} concentration of 4–5 mM (Fig. 1). Other major phospholipids in cell membranes include PI, PC and SM. Liposomes composed of PE/PS/PI (27:13:16) showed comparable, although somewhat reduced, fusion properties to PE/PS LUVs. However, LUVs of either PE/PS/PC (27:13:24) or PE/PS/SM (27:13:20) showed greatly reduced lipid-mixing capacities, with thresholds of 7.5–8 mM Ca^{2+} and maximum fusion rates of <1%/min (Fig. 1). Hence, PI appeared to have a neutral effect on fusion, while PC and SM were potent inhibitors.

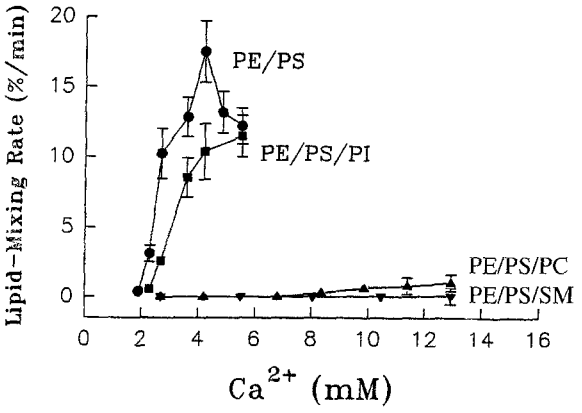

Fig. 1. The effects of PI, PC and SM incorporation on the initial rate of Ca^{2+}-induced lipid mixing of PE/PS LUVs. Large unilamellar liposomes were prepared with PE/PS (27:13 molar ratio), PE/PS/PI (27:13:16), PE/PS/PC (27:13:24) or PE/PS/SM (27:13:20), with or without 2 mol % rhodamine. Labeled (20 μM) and unlabeled (80 μM) liposomes were stirred at 37°C, Ca^{2+} was added as a bolus to the desired concentration, and the initial rate of fusion was calculated from the resulting increase in fluorescence. Values are means ± SEM. Representative experiment; $n = 3$.

Aggregation and Fusion of "Complex" Liposome Mixtures

To emulate the phospholipid composition of neutrophil plasma membranes, LUVs were prepared with the five major phospholipids found in these membranes: PC, PE, SM, PI and PS (molar ratios for PC:PE:SM:PI:PS of 24:27:20:16:13, after Diez et al. (1990)). Liposomes containing these five phospholipids at these ratios were designated "complex" LUVs. The first step in the fusion process should be the apposition and aggregation of the participating liposomes. Using light scatter as a measure of aggregation, we found that the least amount of Ca^{2+} required to obtain a signal was approximately 5.8 mM (Fig. 2). If aggregation is an initial required step in fusion, then we would expect the fusion process to require the same or greater levels of Ca^{2+}. As expected, the Ca^{2+} threshold for lipid mixing of complex LUVs was 9.3 ± 0.3 mM Ca^{2+} (Fig. 3). Maximum initial rate of lipid mixing was 1.1 ± 0.8%/min, attained at approximately 14 mM Ca^{2+}. Contents mixing for complex LUVs required more Ca^{2+}, with a threshold of 10.0 ± 0.2 mM Ca^{2+}, and also reached a maximal rate of only 1.3 ± 0.7%/min (Fig. 4).

To further clarify the interactions between the liposomes, the reversibility of Ca^{2+}-induced aggregation by EGTA was assessed. Changes in light scatter (presumably due to aggregation (Meers et al., 1987)) of complex LUVs were found to occur at 6.8 ± 0.3 mM Ca^{2+} (Fig. 5). When aggregation was initiated with 7.5 mM Ca^{2+} (i.e., below the level required for measurable contents mixing), the subsequent addition of EGTA reversed the light scattering signal, indicating aggregation without fusion. When aggregation was induced with 11.3 mM Ca^{2+}, the subsequent addition of EGTA did not reverse the light scattering signal, although it did prevent further aggregation. Therefore, these complex LUVs showed aggregation at 6–7 mM Ca^{2+}, whereas fusion occurred at higher Ca^{2+} levels.

Cholesterol

Cholesterol contents can differ greatly within different membranes from the same cell. For example, in neutrophils, cholesterol concentrations range from 27–33 mol % for granule membranes to 40–50 mol % for plasma membranes (Woodin & Wieneke, 1966; Mason, Stossel & Vaughan, 1972; Nachman, Hirsch & Bagglioni, 1972; Werb & Cohn, 1972). Furthermore, asymmetric distribution may result in cholesterol levels of as much as 90% in the inner face of the plasma membrane (Schroeder & Nemecz, 1990). To determine the effect of cholesterol on fusion, complex LUVs were prepared with different concentrations of cholesterol. Both the threshold for aggregation and the initial rate of aggregation were independent of cholesterol level, remaining at 6–6.5 mM Ca^{2+} for all cholesterol concentrations (Fig. 2). However, the threshold for lipid mixing was reduced from 10 mM Ca^{2+} with no cholesterol to 8.5 ± 0.4 mM Ca^{2+} with 50% cholesterol (Fig. 3). Also, increasing cholesterol concentrations increased the maximum rate of lipid mixing in a dose-dependent manner, from 1–2%/min for cholesterol-free LUVs to 12 ± 1.7%/min for 50% cholesterol preparations. Contents mixing was also enhanced by cholesterol: increasing cholesterol to 50 mol % resulted in a decrease of the

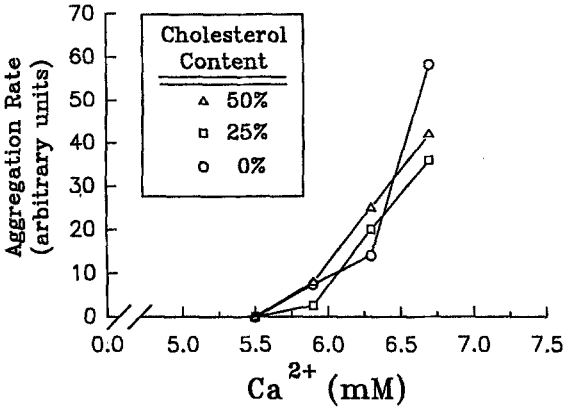

Fig. 2. The effect of cholesterol concentration on the initial rate of aggregation of complex LUVs. LUVs (PC:PE:SM:PI:PS of 24:27:20:16:13) were prepared with the indicated mol % cholesterol, with the phospholipid ratios held constant. Aggregation of LUVs was assayed continuously at room temperature with continuous stirring. Representative experiment; $n = 3$; standard errors were less than 20% of the mean.

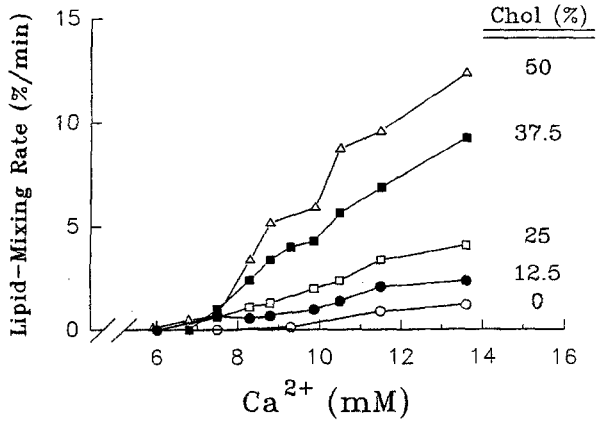

Fig. 3. The effect of cholesterol concentration on the initial rate of lipid mixing of complex LUVs. LUVs (PC:PE:SM:PI:PS of 24:27:20:16:13) were prepared with the indicated mol % cholesterol, with the phospholipid ratios held constant. The initial rate of lipid mixing was assayed at 37°C. Representative experiment; $n = 5$; standard errors were less than 20% of the mean.

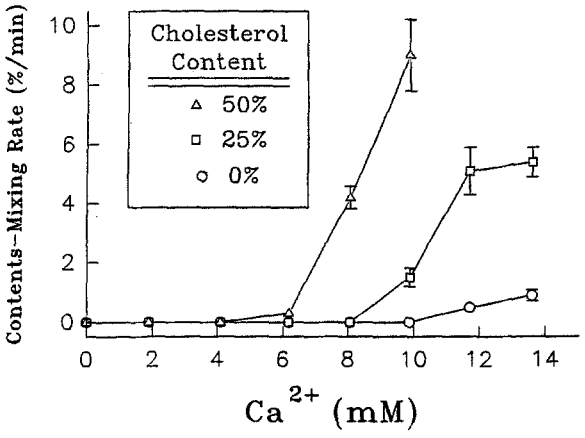

Fig. 4. The effect of cholesterol concentration on the initial rate of contents mixing of complex LUVs. The contents mixing of LUVs, prepared with the indicated mol % cholesterol, was evaluated by the ANTS/DPX assay, using a 1:1 ratio of ANTS- to DPX-containing LUVs (370 μM total) at 37°C. Results indicate the initial rate of contents mixing following the addition of a bolus of Ca^{2+}. Values are means ± SEM. Representative experiment; $n = 3$.

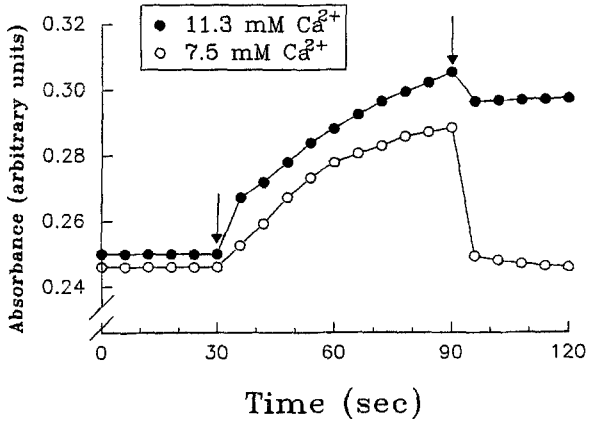

Fig. 5. Irreversible light scatter changes of complex LUVs lacking cholesterol. The absorbance of LUVs (370 μM) at 450 nm was measured continuously after the addition of Ca^{2+} (first arrow; indicated concentration) and then the subsequent addition of 20 mM EGTA 1 min later (second arrow). Representative experiment; $n = 5$.

threshold to 8.6 ± 0.8 mM Ca^{2+}. Much more strikingly, cholesterol increased the maximum rate of contents mixing, to 9.0 ± 1.2%/min at 9.9 mM Ca^{2+} (Fig. 4). Higher levels of Ca^{2+} further increased the rate of contents mixing of 50% cholesterol liposomes, reaching 53 ± 1.7%/min at 13.6 mM Ca^{2+}, but leakage also increased proportionately.

Because intracellular and extracellular concentrations of Mg^{2+} reach the low millimolar range, it was important to see what effect this divalent cation had on fusion of complex liposomes. We therefore determined the extent to which Ca^{2+} thresholds for aggregation and fusion were altered by increasing concentrations of Mg^{2+}. For 50% cholesterol liposomes, the addition of millimolar levels of Mg^{2+} lowered the threshold for contents mixing, and Mg^{2+} alone at 14 mM could produce contents mixing (Table). Aggregation was even more sensitive, with Mg^{2+} being able to replace Ca^{2+} at concentrations of 10 mM and above. Thus, these two divalent cations cooperate in inducing aggregation and fusion of complex liposomes.

Phosphatidic Acid and DiC_8

PA and diacylglycerols appear transiently and locally in a variety of membranes, including neutrophil plasma

Table. Effect of Mg^{2+} on Ca^{2+} thresholds for aggregation and fusion of complex liposomes

Mg^{2+} (mM)	Ca^{2+} thresholds (mM)	
	Aggregation	Contents Mixing
0	9.3, 8.1, 6.8	8.1, 10, 6.8
1.2	5.5, 6.8	ND
2.5	5.5, 6.8	6.8
5.0	4.1, 5.5	5.5
7.5	2.6, 4.1	4.1
10	0	2.5, 1.0
14	ND	0

Complex liposomes containing 50% cholesterol were prepared and Ca^{2+} thresholds for aggregation and contents-mixing fusion were determined as outlined in Materials and Methods and legends to Figs. 2 and 4. These thresholds were determined in the presence of the indicated concentrations of Mg^{2+}. From one to three experiments were performed for each condition, with each threshold determination being given above.

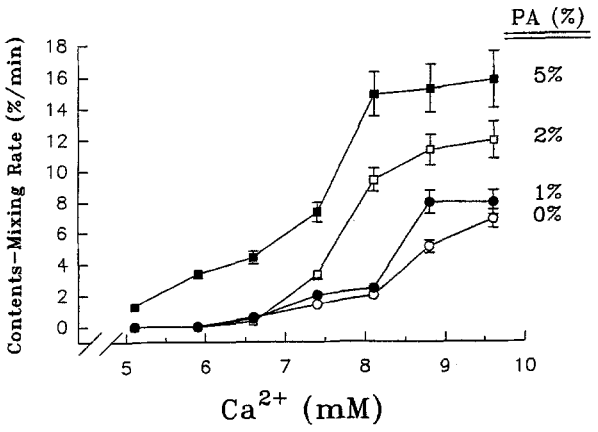

Fig. 6. The effect of PA incorporation on the initial rate of contents mixing of 50% cholesterol complex LUVs. Sets of liposomes were prepared with the indicated concentrations of PA, with either ANTS or DPX, and Ca^{2+}-induced contents mixing was evaluated at 37°C. Values are means ± SEM. Representative experiment; $n = 5$.

membranes, by a variety of mechanisms. To assess the effects of PA and diacylglycerol formation on fusion in membrane formulations, complex LUVs containing 50% cholesterol were prepared with 0–5 mol % PA or 0–5 mol % DiC_8. Increasing the concentration of PA produced a decrease in the threshold for contents mixing, from 8.6 mM Ca^{2+} for PA-lacking LUVs to 5.8 ± 0.6 mM Ca^{2+} for 5% PA preparations (Fig. 6). Furthermore, incorporation of 5 mol % PA also increased the maximal rate of fusion.

In contrast, the incorporation of up to 5% DiC_8 did not significantly change the contents-mixing characteristics (Fig. 7*a*). This result was obtained with DiC_8 from two different suppliers. Similarly, the addition of 20 μM DiC_8 micelles to 100 μM 50% cholesterol complex LUVs, prior to the addition of Ca^{2+}, did not significantly enhance Ca^{2+}-mediated LUV fusion (Fig. 7*b*).

"Heterologous" Fusion

Membrane fusion during degranulation involves two membranes that differ in composition. Consequently, we wished to see how fusion between liposomes of differing compositions compared with those of homologous composition. When two sets of LUVs differing in composition were tested for their requirements for fusing with each other (heterologous fusion), the results were typically intermediate between those for the corresponding identical partner pairings (homologous fusion). For example, when 12.5% cholesterol complex LUVs were tested with 50% cholesterol complex LUVs, the initial rates of contents mixing were typically less than those observed in 50%/50% homologous pairings and greater than those for 12.5%/12.5% pairings (Fig. 8). Similar patterns were found for several other heterologous pairings (*data not shown*).

However, not all cases of heterologous pairings gave results that were intermediate between the corresponding homologous pairings. Most notably, if 2% PA was incorporated into only one partner of a 25%/50% pairing, then fusion proceeded as though neither fusion partner contained PA (Fig. 9). In contrast, when both partners contained 2% PA, there were noticeable changes in both the threshold and the maximal rate of contents mixing.

Discussion

"Simple" Liposomes

The fusion of cell membranes, as in neutrophil degranulation, involves the interaction of two heterogeneous molecular ensembles. Our "complex" LUVs represented a very simplified membrane system that allowed a better understanding of the role of specific membrane components in the fusion process. One immediate conclusion is that both PC and SM drive the Ca^{2+} threshold up dramatically, whereas PI does not. Both PC and SM also act to significantly decrease the maximal rate of fusion. Previous studies have also shown that liposomes containing PC and SM are less fusogenic than liposomes constructed without these phospholipids (Düzgünes et al., 1981; Uster & Deamer, 1981; Stamatotos & Silvius, 1987). However, whereas PI incorporation is relatively neutral in effect on the fusion of PE/PS liposomes, it is apparent that either PC or SM incorporation strongly inhibits Ca^{2+}-mediated fusion.

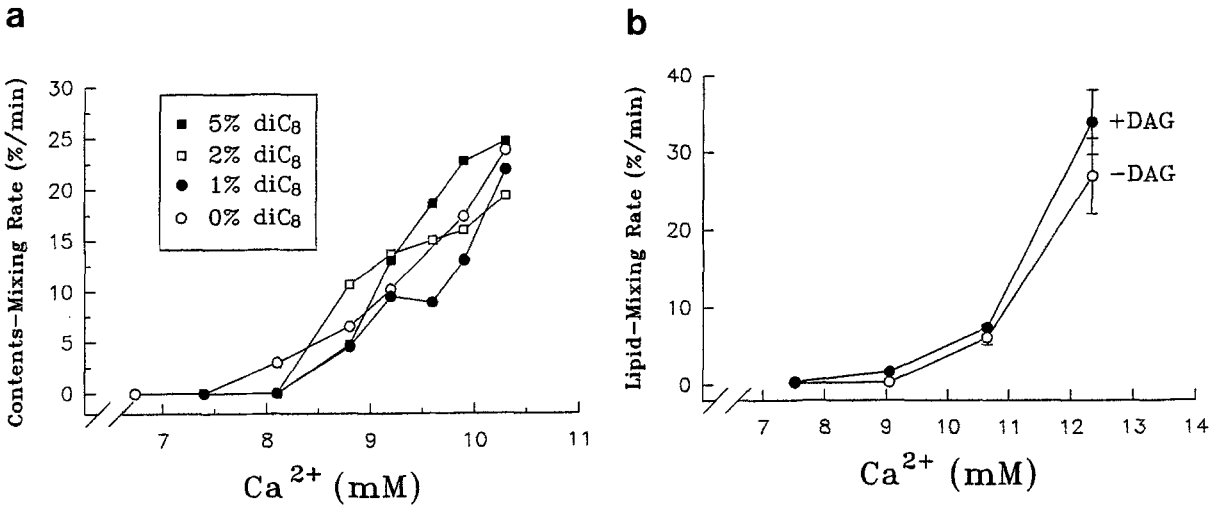

Fig. 7. The effect of DiC_8 on the fusion of 50% cholesterol complex LUVs. (*a*) The effect of DiC_8 incorporation on contents mixing. LUVs were prepared with DiC_8 at the indicated concentrations and assayed for contents mixing by the ANTS/DPX assay at 37°C. Representative experiment; $n = 4$; standard errors were less than 20% of the mean. (*b*) The effect of exogenous DiC_8 on lipid mixing. LUVs were prepared without DiC_8. LUVs (100 μM) were then stirred for 1 min with DiC_8 micelles (20 μM), and Ca^{2+} was added to induce fusion. Values are means ± SEM. Representative experiment; $n = 3$.

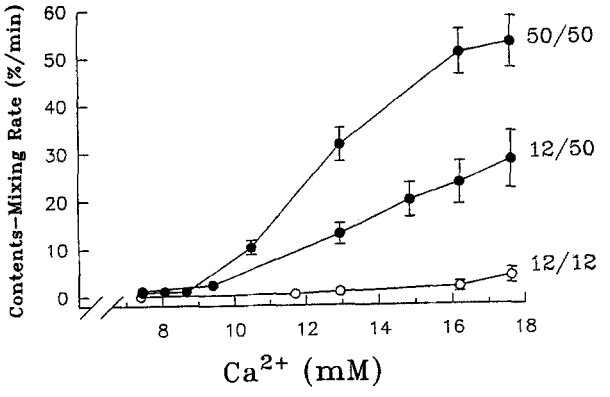

Fig. 8. A comparison of the characteristics of fusion between LUVs of difference *vs.* identical cholesterol content. Complex LUVs were prepared with either 12.5 or 50% cholesterol and assayed for contents mixing by the ANTS/DPX assay. Values are means ± SEM. Representative experiment; $n = 3$.

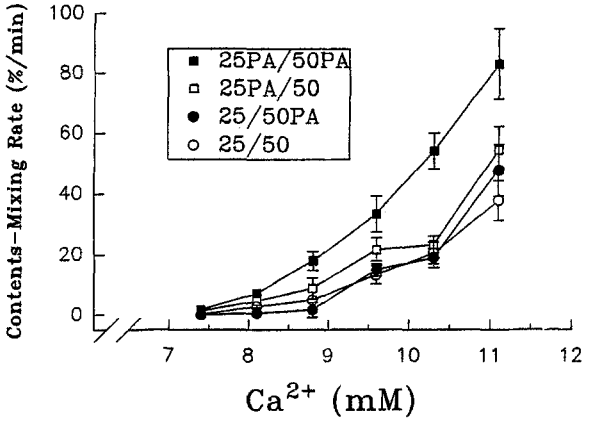

Fig. 9. The effect of 2 mol % PA incorporation into neither, one or both partners in a heterologous fusion assay. Complex LUVs were prepared with either 25 or 50% cholesterol, with or without PA, and assayed for contents mixing by the ANTS/DPX assay. Values are means ± SEM. Representative experiment; $n = 3$.

Similar findings were reported earlier by Sundler, Düzgünes and Papahadjopoulos (1981). Variation in the amount of PC or SM in cell membranes may be one effective way that cells could regulate the fusion capacity of that membrane.

"Complex" Liposomes

We ultimately wished to use liposomes that could serve as models for neutrophil membranes. Published analyses of neutrophil plasma membrane lipids (Smolen & Shohet, 1974; Cockcroft, 1984) suggest PC/PE/SM/PI/PS ratios of approximately 40/35/13/4/7, with substantial variations. However, biological membranes are asymmetric with the cytoplasmic surfaces being much richer in PE and acidic phospholipids (Etemadi, 1980; Sessions & Horwitz, 1983; Rand & Parsegian, 1986; Verhoven, Schlegel & Williamson, 1992). With this in mind, we enriched PE relative to PC and enhanced PI and PS concentrations. Our ultimate phospholipid ratios, designed to approximate the cytoplasmic surfaces of neutrophil membranes were 24:27:20:16:13. We chose as our model the results of Diez et al. (1990), who measured phospholipid contents in subcellular fractions (*see* Fig. 3 of that paper), including plasma membrane.

These compositions admittedly provide only crude models for the endogenous neutrophil lipids:

(i) The fatty acid compositions have not been directly modeled. Our phospholipids were of natural origin, containing a spectrum of fatty acids, in the hope of partially addressing those concerns. Only perfect knowledge of the fatty acid composition of each phospholipid would allow us to address this adequately. The other alternative, making liposomes from extracted neutrophil lipids, was impractical due to yield.

(ii) Neutrophils contain a high content 1-*O*-alkyl linked phospholipids (Mueller et al., 1984), which we have not modeled. Unfortunately, we do not have practical sources for these types of lipids to adequately address this concern.

(iii) "Trace" components, such as polyphosphoinositides, may be crucial in fusion and degranulation (Eberhard et al., 1990; Horkovics-Kovats & Traub, 1990). However, the composition of neutrophil membranes is not sufficiently known to account for such materials.

(iv) As noted above, biological membranes are asymmetric in composition (Etemadi, 1980; Sessions & Horwitz, 1983; Rand & Parsegian, 1986; Verhoven et al., 1992), and this asymmetry may be important in fusion (Eastman et al., 1992; Devaux et al., 1993). Creating asymmetry in model membranes is very difficult and such manipulations produce only crude changes at best (Eastman et al., 1992). Furthermore, the extent of asymmetry in neutrophil membranes is not known. To partially address this problem, we used a liposome preparation that was enriched in PE and acidic phospholipids.

(v) With these complex formulations, it was impossible to ensure that all the liposomes were identical in composition and properties.

These five limitations prevent us from modeling neutrophil membrane lipids with complete fidelity. These limitations must be remembered when interpreting our results. However, we are one of the few groups of investigators who have attempted to use "complex" lipid mixtures to more closely emulate biological compositions.

Cholesterol

In complex (PE/PC/SM/PI/PS) liposomes, cholesterol had clear effects: increasing cholesterol content increased the maximal rates of lipid and contents mixing, and it also decreased the Ca^{2+} requirement for contents mixing. In other studies, the effects of cholesterol on fusion have been inconsistent. One explanation, as suggested by Stamatotos and Silvius (1987), may be that cholesterol enables fusion in nonfusing liposome constructs, enhances fusion in poorly fusing ones (as it did here), and diminishes the fusion of rapidly fusing liposomes. Also, the positive effect of cholesterol on fusion may be masked by a delaying effect on aggregation, in certain types of liposomes. This pattern has been observed in PS-rich liposomes (Braun, Lelkes & Nir, 1985; Bental et al., 1987), but does not apply to complex liposomes.

We also found that high concentrations of Mg^{2+} could assist Ca^{2+} for both aggregation and contents-mixing fusion (Table) when complex liposomes were used. Mg^{2+} ions are typically present in millimolar levels in the cytosol (Grubbs & Maguire, 1987), and peaks of Mg^{2+} distribution are associated with the plasma membrane and granules in neutrophils (Raja et al., 1982). Millimolar levels of Mg^{2+} will significantly reduce the Ca^{2+} requirements for fusion in many systems (Düzgünes et al., 1987), including this one. If Mg^{2+} was raised sufficiently, this divalent cation alone was adequate to induce aggregation (10 mM Mg^{2+}) or fusion (14 mM Mg^{2+}).

PA and DiC_8

As pointed out earlier, membrane remodeling may be an important step in enabling or enhancing fusion. That is, rapid restructuring may convert membranes from unwilling to willing fusion partners. On the basis of earlier studies, a primary candidate for a highly fusogenic lipid was PA (Sundler & Papahadjopoulos, 1981). Here, we report that liposomes containing 5 mol % PA fuse at lower Ca^{2+} levels and at a higher rate than PA-deficient liposomes. It should be noted that sensitivity of fusion to Ca^{2+} was directly related to the content of acidic phospholipids, such as PA. This could be due to enhanced anionic surface charge, which would increase the local concentrations of Ca^{2+} (Bentz, Düzgünes & Nir, 1983). Also, PA could be working by enhancing phase transitions in the membranes, as it has been reported that transitions from bilayer to hexagonal H_{II} phases are critical to the fusion process (Hope, Walker & Cullis, 1983; Cullis et al., 1985).

PA is produced in response to secretagogues in neutrophils (Serhan et al., 1982; Cockcroft, 1984; Agwu et al., 1989; Billah et al., 1989). The PA rise is both rapid and transient, consistent with a role for this type of membrane remodeling in fusion. In neutrophils, PA appears to result primarily from phospholipase D action on PC (Cockcroft, 1984; Tou et al., 1991). Also, treatment of PC-rich liposomes with phospholipase D generates PA and enhances fusion (Park, Lee & Kim, 1992). Hence, a strong fusion inhibitor is converted to a strong promoter in a single process. PA may also be produced from other sources, including diacylglycerol (Cockcroft, Bennett & Gomperts, 1980), but these pathways do not appear to be involved in secretion or exocytosis.

Remodeling to produce PA from PC may be an important mechanism for controlling the timing and location of fusion events.

In contrast to PA, DiC_8 had little effect on Ca^{2+}-induced fusion of liposomes. DiC_8 was selected as our model diglyceride because of its powerful ability to stimulate neutrophils (Boonen, De Koster & Elferink, 1993; Rosenthal et al., 1993). However, we recognize that a short-chain DAG, while physiologically potent, might not be as powerful a fusogen for liposomes as longer chain varieties. Low concentrations of added DiC_8 will enhance annexin-mediated fusion of PA/PE liposomes (Francis et al., 1992). Also, incorporated diacylglycerols can enhance fusion of liposomes composed of either PS/PC (Gomez-Fernandez et al., 1989) or monomethylated-PE (Siegel et al., 1989), although it dramatically increases concomitant contents leakage in either system. However, DiC_8 did not enhance the fusion of 50% cholesterol complex liposomes, whether the DiC_8 was incorporated or exogenously supplied. Like cholesterol, the effect of diacylglycerol seemed to depend on membrane composition.

In neutrophils, the major pathway leading to diacylglycerol production is through the hydrolysis of PA by phosphatidate phosphohydrolase (Billah et al., 1989; Della Bianca et al., 1991), rather than through the hydrolysis of phospholipids by phospholipase C. In terms of fusion, this suggests that the "window of opportunity" for fusion, provided by the production of PA from PC, is subsequently closed by the conversion of PA to diacylglycerol.

"Heterologous" Fusion

Cell fusion typically involves the interaction of membranes from two different sources, of differing composition, and therefore having different characteristics. It was unclear whether fusion would be severely restricted by the least fusogenic membrane composition, or if fusion would proceed with the most fusogenic. Using the contents-mixing assay to measure interactions between heterologous partners, we commonly found that the fusion characteristics of heterologous fusion partners were intermediate between those of the homologous pairings. However, this was not the case when 2% PA was incorporated into only one fusion partner. For a significant PA-induced shift in fusion, 2% PA had to be present in both fusing membranes. This implies that for this form of membrane remodeling to be effective in enhancing fusion, either the PA production must occur in both interacting membranes, or else extensive remodeling must occur in one.

In this study, we evaluated only Ca^{2+}-induced fusion, and the Ca^{2+} requirements were high. Other cofactors undoubtedly are involved in cell fusion (*see, e.g.,* Düzgünes et al., 1987; Papahadjopoulos et al., 1990). For example, proteins such as annexins reduce the Ca^{2+} requirement for fusion (Zaks & Creutz, 1990), interact preferentially with PA (Blackwood & Ernst, 1990) and may play a role in cell fusion (Creutz, 1992). Also, changes in Ca^{2+} may be localized to a membrane region within the cell (Sawyer, Sullivan & Mandel, 1985; Jaconi et al., 1991). Such localized concentrations of Ca^{2+}, as well as asymmetric distributions of PA (Eastman et al., 1992) or cholesterol (Schroeder & Nemecz, 1990) may be effective in driving cell fusion. Finally, many proteins have been implicated in fusion in other cell systems. Fusion in the Golgi system requires a protein that is sensitive to *N*-ethyl maleimide (designated "NEM-sensitive fusion protein" or NSF), a Soluble NSF-Attachment Protein (SNAP), ATP, acetyl-CoA, "Factor B," and several "Sec" (secretion) factors, as well as specific membrane docking proteins (Clary, Griff & Rothman, 1990; Rothman & Orci, 1990; Bennett & Scheller, 1993; De Camilli, 1993). In vitro secretion has been reported to be modulated by rab3A (Edwardson, MacLean & Law, 1993; MacLean, Law & Edwardson, 1993). Also, it has been demonstrated that similar or identical proteins, particularly SNAP's or SNAP receptors, are involved in fusion over a broad range of species and cell types (Barinaga, 1993; Bennett & Scheller, 1993; De Camilli, 1993; Söllner et al., 1993). We expect that both changes in lipid composition and association with protein fusogens will ultimately be found to be involved in in vivo fusion. The complex liposomes that we have described here can be used in studies of fusion with these proteins in the future.

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