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MONITORING OF PHOSPHOLIPID VESICLE FUSION BY FLUORESCENCE ENERGY TRANSFER BETWEEN MEMBRANE-BOUND DYE LABELS

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Summary

A sensitive method which utilizes fluorescence energy transfer to assay Ca^{2+}- or Mg^{2+}-mediated fusion of phospholipid vesicles is reported. More than 85% quenching results when phosphatidylserine vesicles labelled with dansyl phosphatidylethanolamine (donor) are fused with vesicles labelled with rhodamine phosphatidylethanolamine (acceptor) in the presence of 5 mM $CaCl_2$ or 10 mM $MgCl_2$. Higher concentrations of divalent cations are required to obtain maximal quenching when phosphatidylserine is partially replaced with phosphatidylethanolamine or phosphatidylcholine. The rate of vesicle fusion is dependent upon the concentrations of both cation and vesicles. Maximum quenching occurs within 5 min using phosphatidylserine vesicles and 5 mM Ca^{2+}, but quenching is incomplete even after 20 h with 0.8—2 mM Ca^{2+}. This probably reflects the heterogeneous size distribution of these vesicles, since the extent of fusion was found to correlate with vesicle size. Binding of antibody to membrane-localized phenobarbital hapten effectively blocks Ca^{2+}-mediated vesicle fusion. This effect can be inhibited by preincubation of the antibody with phenobarbital. Leakage of tempocholine from intact vesicles induced by 5 mM Ca^{2+} occurs even when fusion is prevented by bound antibody. This demonstrates that fusion is not a necessary requirement for Ca^{2+}-induced leakage.

Introduction

Fusion of biomembranes is the central event in a variety of cellular and subcellular activities [1—3]. Because of the prominence of the phospholipid

bilayer in the structure of biomembranes, it is evident that fusion of phospholipid bilayers is an integral part of membrane fusion. The tendency of phospholipids to form bilayers in aqueous medium [4] has promoted the use of phospholipid vesicles as model systems for the study of membrane fusion [5—8]. Thus, the formation of large membraneous structures upon incubation of small unilamellar phosphatidylserine vesicles with Ca^{2+} is best accounted for by vesicle fusion [6]. It is of interest that fusion processes such as the exocytotic release of hormones and neurotransmitters also require Ca^{2+} [1,2]. The incorporation of vesicle membranes into cell membranes [9,10] and black lipid membranes [11] are other examples of related Ca^{2+}-dependent fusion processes.

A number of methods including differential scanning calorimetry, electron microscopy, gel filtration, uncoupling of cytochrome oxidase and luminescence [5—8,12,13] have been used to study membrane fusion of phospholipid vesicles. We wish to report here a new and convenient method which utilizes energy transfer quenching of fluorescence. Ca^{2+}-mediated fusion of vesicles containing dansyl-labelled phospholipid (donor) with vesicles containing rhodamine-labelled phospholipid (acceptor) results in dramatic quenching of fluorescence. Using this method to study membrane fusion, we have shown that vesicles containing haptenic sites on the surface are inhibited from fusing in the presence of specific antibody.

Experimental procedures

Materials. Tetramethylrhodamine-5-isothiocyanate was obtained from Baltimore Biological Laboratories. 5-Dimethylaminonaphthalene-1-sulfonyl chloride was purchased from Pierce Chemical Co. Egg phosphatidylcholine, egg phosphatidylethanolamine, bovine brain phosphatidylserine and dipalmitoyl phosphatidylethanolamine were supplied by Sigma Chemical Co. K. Gottwald (Syva, Co., Palo Alto) kindly provided 5-phenyl-5-(1-carboxyl-1-propen-3-yl)barbituric acid and *N,N*-dimethyl-*N*-(2-hydroxyethyl)-*N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxyl)ammonium chloride (tempocholine).

The synthesis of *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoyl phosphatidylethanolamine (dansyl phosphatidylethanolamine) was accomplished as described by Waggoner and Stryer [14]. The adduct of tetramethylrhodamine-5-isothiocyanate and dipalmitoyl phosphatidylethanolamine (rhodamine phosphatidylethanolamine) was prepared by addition of 50 μmol of the isothiocyanate to 5 ml of $CHCl_3/CH_3OH$ (2 : 1), 50 μmol dipalmitoyl phosphatidylethanolamine and 500 μmol triethylamine. This mixture was stirred for 4 h and additional rhodamine-5-isothiocyanate was added (2 μmol amounts) at 2-h intervals until no phosphatidylethanolamine remained as determined by reaction with ninhydrin. Solvents were removed at reduced pressure and after two preparative thin-layer chromatographic separations on silica gel ($CHCl_3/CH_3OH/NH_4OH$ hydroxide, 65 : 25 : 4), the desired product was obtained which migrated as a single spot (R_F 0.76). The ratio of aliphatic to *N*-methyl protons was determined to be 5.7 (expected 5.2) by NMR measurements. The ratio of the dye (based on $\epsilon = 90\,000$ at 553 nm in 0.1% sodium dodecyl sulfate) to total phosphate was 1.05.

The synthesis of *N*-[4-(5-phenyl-5-barbituryl)-2-butenoyl]dipalmitoyl phosphatidylethanolamine (phenobarbital phosphatidylethanolamine) was accomplished in two steps. A mixture of 92 μmol 5-phenyl-5-(1-carboxy-1-propen-3-yl)barbituric acid and 500 μmol oxalyl chloride in 20 ml dry benzene was refluxed for 3.5 h. After removal of volatile materials at reduced pressure, 20 ml $CHCl_3$ (treated with alumina) and 90 μmol dipalmitoyl phosphatidylethanolamine were added and the mixture was refluxed for 2.5 h. The desired product (R_F 0.66) was isolated by thin-layer chromatography on silica gel developed twice with $CHCl_3/CH_3OH/NH_4OH$ (65 : 25 : 4). No phosphatidylethanolamine was detected by ninhydrin. The ratio of aliphatic to aromatic protons was determined to be 12.0 (expected 12.4) using NMR spectroscopy.

Preparation of phospholipid vesicles. Vesicles were prepared containing phosphatidylserine, and where indicated, other phospholipids, either 15 mol% rhodamine phosphatidylethanolamine or 9 mol% dansyl phosphatidylethanolamine, and 3 mol% phenobarbital phosphatidylethanolamine. $CHCl_3$ or $CHCl_3/CH_3OH$ solutions of the components were evaporated in a large culture tube under a stream of argon in such a manner that the lower part of the tube was coated with lipid. Final traces of solvents were removed at reduced pressure and 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.1 mM sodium EDTA was added to give a final concentration of 1 μmol lipid phosphate/ml. The mixtures (usually 5 ml) were mechanically mixed until a uniform suspension was obtained and sonicated for 5—10 min at 32—35°C using a microtip attachment to a Branson 350 power supply. The output was adjusted so that no foaming was evident. Particulate material was removed by centrifugation at 48 000 × *g* for 60 min at 4°C. Gel filtration over Sepharose 4B was carried out only where so indicated.

Sheep were immunized by subcutaneous and intramuscular routes with bovine serum albumin conjugates of phenobarbital. Crude antibody was prepared by addition of saturated $(NH_4)_2SO_4$ to an equal volume of sheep serum. The precipitate was redissolved in 40% of the original volume of 0.055 M Tris (pH 8.1), 0.05% sodium azide and dialyzed against the same buffer. Partially purified IgG was prepared by the method of Levy and Sober [15].

Methods. Double-diffusion immunoprecipitation was carried out using 0.8% agarose in 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl. The center well contained 5 μl of vesicles and the peripheral wells contained the antibody fractions. Plates were read after 16—20 h incubation at ambient temperature. Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as standard. Total phosphate was determined by the method described by Bartlett [17].

In this assay, the donor and acceptor are initially restricted to different vesicles. Fusion of dansyl-labeled vesicles with rhodamine-labeled vesicles results in rapid mixing of the membrane lipids and permits energy transfer quenching of dansyl fluorescence. Assays were carried out by addition of 2.91 ml 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl to 0.06 ml of a 5 : 1 mixture of dansyl-labeled and rhodamine-labeled liposomes. Fusion was initiated by the addition of 30 μl 0.5 M $CaCl_2$ immediately followed by mixing. After 15 min, 80 μl of 0.2 M sodium EDTA (pH 8.0) was added and the fluorescence intensity was measured. Similar incubation mixtures which contained no $CaCl_2$

or to which EDTA was added prior to the addition of $CaCl_2$ provided reference fluorescence intensities under non-fusion conditions. Fluorescence measurements were made on a Perkin-Elmer MPF2A spectrofluorimeter (emission at 500 nm, excitation at 350 nm) using excitation and emission band widths of 8 nm and a 430 nm cut-off filter in the emission beam. Unless otherwise noted, all operations were carried out at ambient temperature.

To assay for fusion of vesicles containing phenobarbital phosphatidylethanolamine, antibody, phenobarbital and vesicles were added sequentially in 10-min intervals to 0.05 M Tris-HCl (pH 8.0)/0.1 M NaCl. The final volume was 0.25 ml. After an additional 10 min 2.75 ml 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 5.45 mM $CaCl_2$ was added. After 15 min 0.08 ml sodium EDTA was added and the fluorescence was measured.

Vesicles containing phosphatidylserine (50 mol%), phosphatidylethanolamine (47 mol%) and phenobarbital phosphatidylethanolamine (3 mol%) were prepared by sonication of the lipids (1 ml, 10 μmol lipid phosphate) in 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.1 M tempocholine chloride. Sonication was carried out at 300 W for 20 min at 30—35° C using an immersion bath horn attachment with a Branson 350 sonifier. Particulate material was removed by centrifugation and 0.5 ml of the supernatant was chromatographed on Sephadex G-50 (0.9 × 53 cm) in order to separate external tempocholine from tempocholine-loaded vesicles.

Results

Changes in fluorescence during fusion

A 5 : 1 mixture of dansyl-labeled to rhodamine-labeled vesicles composed of 50% phosphatidylserine plus phosphatidylethanolamine was prepared, and the fluorescence was determined before and after the addition of $CaCl_2$. The fluorescence of the dansyl moiety was quenched 82% by addition of $CaCl_2$ (Table I). This effect was not due to calcium-induced aggregation of the vesicles, as subsequent addition of EDTA did not affect the fluorescence intensity. However, addition of the detergent Triton X-100 increased the fluorescence intensity to about 1.5 times the original value.

In the presence of Triton X-100, mixed detergent/phospholipid micelles are formed [18] in which the effective donor and acceptor concentrations are reduced. Thus, addition of Triton X-100 to a mixture of dansyl-labeled and rhodamine-labeled vesicles or to dansyl-labeled vesicles alone (no $CaCl_2$) also resulted in a 1.5-fold increase in fluorescence. Since the dansyl absorption spectrum was unaffected by the addition of Triton X-100, the changes in fluorescence intensity are associated with changes in quantum yield, presumably due to modification of the polarity of the dansyl environment. Interestingly, the fluorescence intensity of dansyl-labeled vesicles was also increased slightly by addition of $CaCl_2$ although subsequent addition of EDTA resulted in a return to the initial fluorescence value (Table I).

When dansyl-labeled and rhodamine-labeled vesicles were separately incubated in 5 mM $CaCl_2$ and subsequently mixed, little change in fluorescence was observed. Similarly mixing of rhodamine-labeled vesicles with dansyl-labeled vesicles in the absence of $CaCl_2$ had no effect (Table I). These results suggest

TABLE I

RELATIVE FLUORESCENCE INTENSITIES AT 500 nm OF DANSYL-LABELLED VESICLES (DPE) AND RHODAMINE-LABELLED VESICLES (RPE) CONTAINING 50% PHOSPHATIDYLSERINE

Reagents were added to the vesicle solutions in the order shown with a 10 min incubation following $CaCl_2$ addition. Final concentrations were 5 mM $CaCl_2$, 5 mM EDTA, and 0.1% Triton X-100. 60 μl of DPE or 50 μl of DPE and 10 μl of RPE were employed in a final volume of 3 ml 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.06 mM EDTA. The mixture of fused DPE and fused RPE was prepared by incubating DPE and RPE separately with 5 mM $CaCl_2$, adding EDTA up to 5 mM, mixing the separate solutions, and diluting to the final concentrations.

Vesicle solution	Added reagents				
	None	$CaCl_2$	$CaCl_2$ + EDTA	$CaCl_2$ + EDTA + Triton X-100	Triton X-100
DPE	120	120	106	155	159
DPE + RPE	100	18	16	152	154
Fused DPE + fused RPE	110	110	98	153	

that exchange diffusion does not contribute to the mixing of chromophores, and moreover the Ca^{2+}-modified vesicles are altered such that further fusion or transfer of membrane components does not occur even in the presence of Ca^{2+}.

The emission spectra of fused dansyl-labeled phosphatidylserine vesicles with various concentrations of rhodamine phosphatidylethanolamine are given in Fig. 1A. After correction for a small amount of fluorescent impurity in the phosphatidylserine the efficiencies of energy transfer were calculated using the relationship

$$E = 1 - \frac{F}{F_0}$$

where the donor fluorescence intensity is F_0 in the absence of quencher and F in the presence of quencher [19]. The efficiency of energy transfer increased with increasing concentration of rhodamine phosphatidylethanolamine up to 0.87 at 2.5 mol% (Fig. 1B).

Increase in vesicle size during fusion

Dansyl-labeled or rhodamine-labeled phosphatidylserine vesicles were separately chromatographed over 4% agarose and each was resolved into two peaks [20], one of which, peak I, emerged at the excluded volume. The smaller vesicle fractions in peak II were pooled. The thus prepared vesicles were mixed and rechromatographed on 4% agarose. Incubation of selected fractions with $CaCl_2$ (8.8 mM final concentration) for 30 min followed by addition of EDTA resulted in a nearly constant 84% decrease in fluorescence intensity across the peak. Subsequent addition of detergent produced increases of about 1.5-fold over the original intensities.

When dansyl-labeled and rhodamine-labeled vesicles (peak II) were mixed and incubated with 10 mM $CaCl_2$ prior to chromatography, virtually all of the phospholipid was converted to a form which was excluded from 4% agarose. The material was weakly fluorescent and also showed a large increase in fluo-

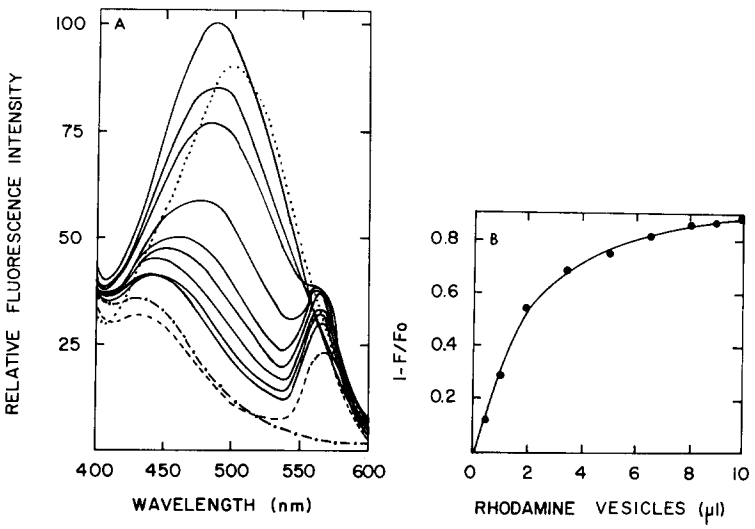

Fig. 1. Effect of $CaCl_2$ on the emission spectra of mixed dansyl and rhodamine-labelled vesicles. Vesicles were prepared from 55 mol% phosphatidylserine and 45 mol% phosphatidylethanolamine. Fusion mixtures (3.0 mol final volume) contained 50 μl dansyl-labelled vesicles and varying volumes of rhodamine-labelled vesicles. The total phospholipid concentration was held constant (20 μM) by inclusion of unlabelled vesicles. Fusion was initiated with 5 mM $CaCl_2$ and stopped after 30 min with EDTA. (A) ———, represent in decending order the emission spectra of fusion mixtures containing 0, 0.5, 1.0, 2.0, 3.5, 5.0, 6.5, 8.0 and 10.0 μl of rhodamine-labelled vesicles, respectively. Spectra are also given for dansyl-labelled vesicles before fusion (· · · · · ·); · — · — ·, unlabelled vesicles after fusion; - - - - -, a mixture of 10 μl of rhodamine-labelled vesicles and unlabelled vesicles after fusion. (B) Transfer efficiencies calculated from the emission intensities at 500 nm and corrected for fluorescence of unlabelled vesicles after fusion.

rescence intensity upon subsequent addition of detergent. These results are consistent with Ca^{2+}-mediated fusion of small vesicles resulting in the formation of larger assemblies with concomitant randomization of the membrane-localized donor and acceptor.

The effects of divalent cation concentration and phospholipid compositions on fusion of unfractionated vesicles are summarized in Fig. 2. Maximal quenching of dansyl-labeled and rhodamine-labeled phosphatidylserine vesicles occurred with 5 mM Ca^{2+} and little if any was observed at Ca^{2+} concentrations less than 0.1 mM. Progressive substitution of phosphatidylserine by phosphatidylethanolamine up to 80 mol% resulted in slight shifts of the 50% quenching point from 1.6 to 3.2 mM Ca^{2+}. Roughly 2-fold higher concentrations of Mg^{2+} were required to effect similar quenching. In contrast, substitution of phosphatidylserine by phosphatidylcholine resulted in much less quenching even with 10 mM Ca^{2+} or Mg^{2+} (Fig. 2).

The rates of quenching were found to be dependent upon the vesicle concentrations. Using 1.5 mM Ca^{2+} and phosphatidylserine vesicles (peak II) the initial rate of quenching increased with increasing concentration (Fig. 3A), but after 4 h the rates fell off sharply and the fluorescence intensities were nearly concentration independent.

The rate of fusion of phosphatidylserine vesicles was also dependent on Ca^{2+} concentration (Fig. 3B). In the presence of 5 mM Ca^{2+}, quenching was 90%

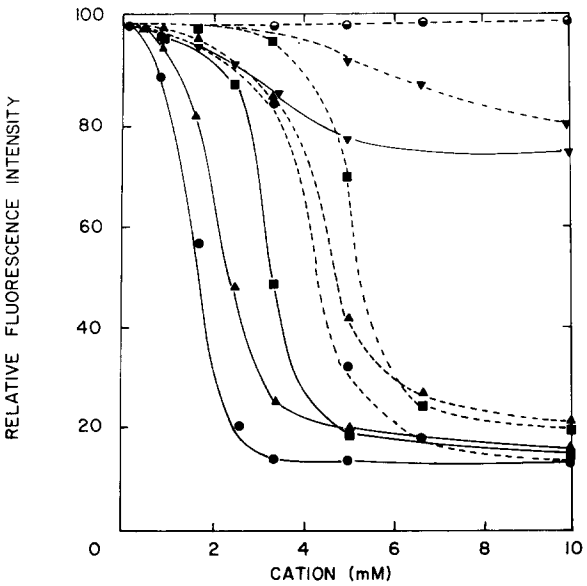

Fig. 2. Effect of cation concentration and vesicle composition on extent of fusion. Dansyl and rhodamine-labelled vesicles were prepared from phosphatidylserine alone (●) or with phosphatidylethanolamine (▲, 40%; ■, 80%) or added phosphatidylcholine (▼, 20%; ⊖, 40%). The percent of the initial fluorescence intensity at 500 nm was recorded after 30 min incubation with $CaCl_2$ (———) or $MgCl_2$ (- - - -) followed by addition with EDTA.

complete in 1 min and no further change was observed after 10 min. At lower Ca^{2+} concentrations the rate of vesicle fusion was slower. Although increased quenching was observed for as long as 20 h the fluorescence intensities appeared to be approaching intermediate values which were dependent on the Ca^{2+} concentrations. These results suggest that a certain population of the vesicles do not fuse at low Ca^{2+} concentrations even after prolonged incubation. In support of this interpretation maximum quenching was obtained upon further addition of $CaCl_2$ to 5 mM final concentration. Thus vesicles which were inert at low Ca^{2+} concentrations still retained their ability to fuse at higher concentrations.

The effect of vesicle size on fusion in the presence of low Ca^{2+} concentrations is illustrated in Fig. 4. A mixture of dansyl-labeled and rhodamine-labeled vesicles was resolved by chromatography over 4% agarose and the degree of quenching of individual fractions was determined after incubation for 20 h in the presence of 0.8 or 1.2 mM Ca^{2+}. In each case quenching increased with decreasing liposome size. However, all fractions were more than 85% quenched upon incubation with 5 mM Ca^{2+}. Thus the observation of incomplete fusion at low concentrations of Ca^{2+} can be attributed to the range of vesicle sizes in these preparations.

Inhibition of fusion by bound protein

Experiments were designed to test whether Ca^{2+}-mediated fusion could be modulated by binding of protein to the vesicles. The extent of $CaCl_2$-mediated

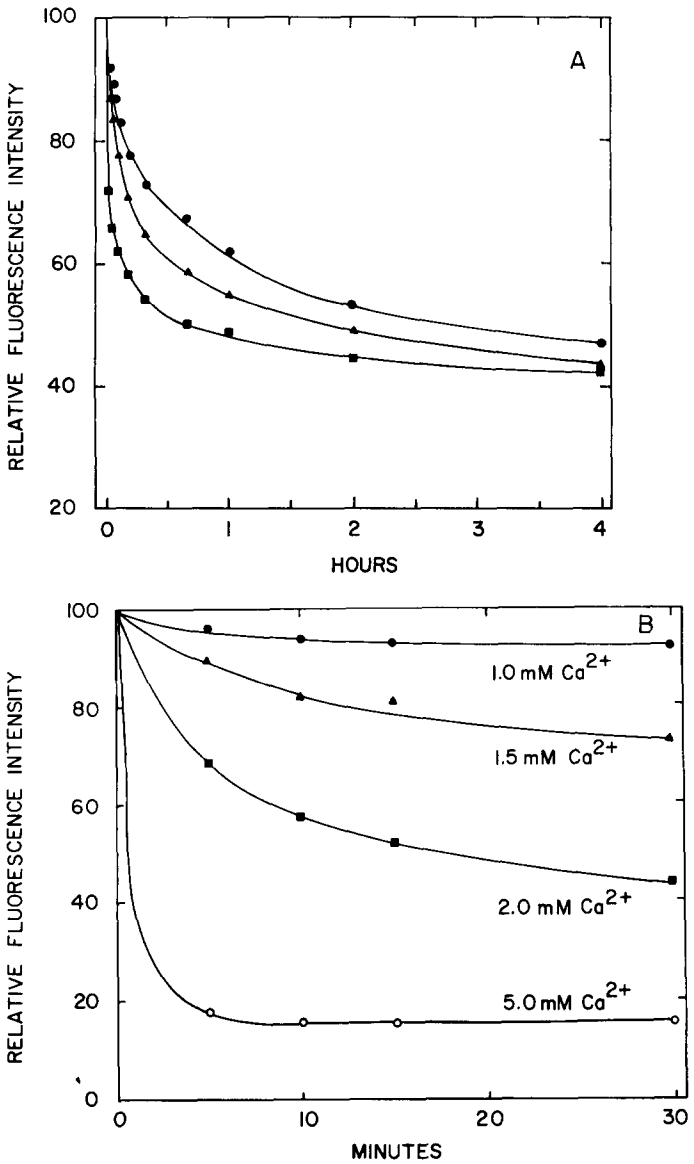

Fig. 3. Dependence of rate of vesicle fusion on vesicle and $CaCl_2$ concentration. Vesicle fusion at 25°C was initiated by $CaCl_2$ and interrupted at various times by addition of EDTA. Fluorescence intensities at 500 nm are given relative to intensities measured prior to fusion. (A) Gel filtered (peak II) mixtures of dansyl and rhodamine-labelled vesicles composed of phosphatidylserine at 20 μM (●), 40 μM (▲) and 200 μM (■) concentrations were fused with a 1.5 mM final concentration of $CaCl_2$. (B) Unfractionated mixtures of 20 μM dansyl and rhodamine-labelled vesicles composed of phosphatidylethanolamine and 50 mol% phosphatidylserine were fused using 1.0 mM (●), 1.5 mM (▲), 2.0 mM (■), or 5.0 mM (○) $CaCl_2$.

quenching of unfractionated phosphatidylserine vesicles containing phenobarbital phosphatidylethanolamine was measured after incubation with various concentrations of purified antiphenobarbital (Fig. 5). The presence of the hapten alone on the vesicle did not significantly affect the quenching. How-

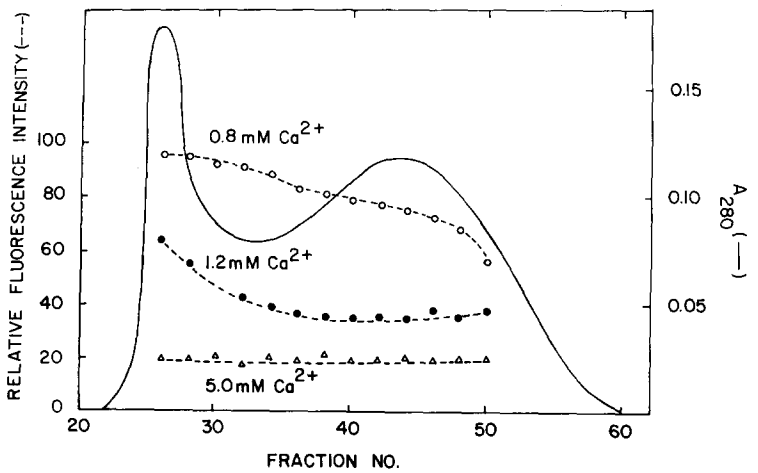

Fig. 4. Effect of vesicle size on extent of fusion. An unchromatographed 5.67 : 1 mixture of dansyl and rhodamine-labelled phosphatidylserine vesicles (8.62 μmol in 1.72 ml) was applied to Sepharose 4B (1.6 X 32 cm), eluted with 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.1 mM sodium EDTA, and the transmittance recorded at 280 nm (———). The fluorescence was measured before and after incubation of selected fractions at 25°C for 20 h in the presence of 0.8 mM (○), 1.2 mM (●) and 5.0 mM (△) $CaCl_2$ followed by addition of EDTA. Fluorescence intensities are given relative to intensities prior to fusion.

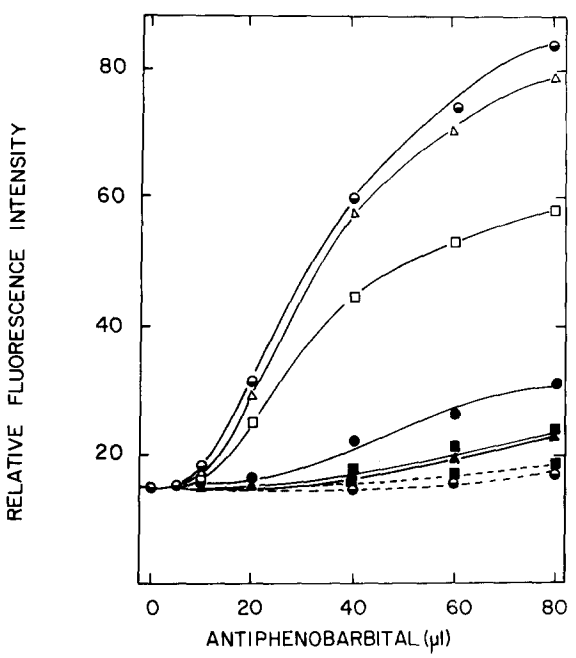

Fig. 5. Antiphenobarbital-induced inhibition of fusion of various phenobarbital-labelled vesicles. Unfractionated phosphatidylserine vesicles containing 3 mol% (⊖), 1.5 mol% (△), 0.7 mol% (□), 0.3 mol% (●), 0.15 mol% (▲) and 0 mol% (■) phenobarbital phosphatidylethanolamine were incubated with antiphenobarbital (———) or antiphenobarbital was preincubated with 0.133 mM phenobarbital (- - - - -). Fusion in 5 mM $CaCl_2$ was terminated with EDTA after a 20 min incubation period. Fluorescence intensities at 500 nm are given relative to the intensities prior to fusion.

ever, quenching was progressively inhibited by increasing concentrations of antiphenobarbital. Inhibition of quenching was also directly dependent on the number of haptens per vesicle, the maximal effect occurring with vesicles having the highest concentration (3 mol%) of phenobarbital phosphatidylethanolamine.

Evidence that vesicles containing phenobarbital phosphatidylethanolamine were immunoreactive was obtained by double diffusion in 0.8% agarose. Vesicles containing the hapten formed a precipitin line with antiphenobarbital but not with nonspecific antibody (antigentamicin).

When the antiphenobarbital was preincubated with excess phenobarbital only a slight inhibition of fusion was observed (Fig. 5). Thus, antibody-induced inhibition of fusion is competitively blocked by the presence of free ligand.

An assay for phenobarbital could be set up using vesicles containing 1.5 mol% phenobarbital phosphatidylethanolamine and antiphenobarbital which had been preincubated with various concentrations of phenobarbital. $CaCl_2$ was added to these mixtures and the degree of fluorescence quenching was measured. Increasing concentrations of phenobarbital correlated with increasing quenching. The assay was sensitive in the range of 0.1—10 μM phenobarbital.

Leakage of vesicle contents during fusion

The use of tempocholine as a spin label for the quantitation of immune lysis of liposomes and erythrocyte ghosts has been described [21]. The amplitude of the ESR signal is initially small due to exchange interactions resulting from a high concentration of tempocholine in the intravesicular cavity. Upon complement-mediated lysis of the membrane the label can leak into the bulk solution resulting in enhanced signal intensity [21]. We have used this technique to investigate the fate of the vesicles contents during fusion. Phenobarbital-labeled vesicles incubated with $CaCl_2$ followed after 20 min by addition of EDTA gave ESR signals that were about 2-fold larger than those obtained when EDTA was added first followed by addition of $CaCl_2$. Moreover only the former signals were susceptible to reduction by added ascorbate. Quantitatively identical results were obtained when the vesicles were preincubated with excess antiphenobarbital antibody. In both cases a slightly larger signal increase was observed when vesicles were lysed in the presence of Triton X-100.

These data require that $CaCl_2$ increases membrane permeability with concomitant release of almost all of the entrapped tempocholine, and that $CaCl_2$-induced leakage of tempocholine can also occur under conditions where fusion is inhibited by antibody bound to the vesicles. Thus binding of Ca^{2+} to the vesicle membrane is itself sufficient to induce membrane permeability. In previous studies Ca^{2+} was shown to promote leakage across phosphatidylserine membranes but the leakage was correlated with the onset of fusion [8].

Discussion

The present studies show that the extent of mixing of membrane phospholipids can be monitored by excitation energy transfer and used to quantitate

Ca^{2+}-induced fusion of phospholipid vesicles. Previously described assays of vesicle fusion have measured mixing of membrane components as determined by differential scanning calorimetry [7,8] or by modulation of enzymatic activity of integral membrane proteins [13]. The quenching of dansyl fluorescence which is observed when dansyl-labeled vesicles fuse with rhodamine-labeled vesicles is best explained by lateral diffusion of the labels within the membrane upon mixing of the phospholipids. This permits the average distance between donor and acceptor to fall within the limits required for energy transfer. The increase in average size of the vesicles which occurs concomitantly with increased quenching, and the absence of spontaneous quenching without $CaCl_2$, suggest that vesicle fusion accounts for most, if not all, of the observed mixing of membrane phospholipids.

Under optimal conditions for fusion, we have observed 80—90% decreases in fluorescence intensity. Since the largest ratio of rhodamine-labeled vesicles to dansyl-labeled vesicles was 1 : 5, on the average several fusion events must occur before a dansyl-labeled vesicle will fuse with a rhodamine-labeled vesicle. Thus even if there were 100% quenching upon fusion, each dansyl-labeled vesicle would have to fuse with ten additional vesicles to effect quenching of 84% of the vesicles. This is consistent with previous observations that Ca^{2+} causes many small vesicles to fuse to form one large vesicle [6].

A number of considerations are involved in the choice of donor and acceptor to be used in studies of membrane fusion. It is important that the chromophores are attached to hydrophobic chains of sufficient length so that the rate of exchange diffusion is negligible relative to the rate of Ca^{2+}-induced fusion. Intervesicle exchange of chromophores covalently attached to phosphatidylethanolamine was not detected. In contrast, the rate of exchange of simple lipid-soluble chromophores such as perylene or *N*-phenyl naphthylamine was found to be too fast to permit their use for studying membrane fusion, as was the exchange of palmitoyl and eicosanoyl derivatives of fluorescein which approached equilibrium in about 1 min and 30 min, respectively (unpublished results). These observations are consistent with the generalization that the energy of transfer of an alkyl group from aqueous medium to the interior of the membrane is proportional to the number of methylene residues [4]. Apparently two hydrocarbon chains per chromophore ensure that the chromophores remain localized in the membrane within the time scale of the present experiments.

The rate of energy transfer and hence the efficiency of fluorescence is critically dependent on the spectral overlap between the fluorescence emission and the absorption of the quenching species as expressed in the Förster equation [22]. In addition, the rate of energy transfer is inversely proportional to the sixth power of the distance between the fluorescer and quencher [22]. Tetramethylrhodamine phosphatidylethanolamine has absorption maxima at 515 nm and 550 nm which are associated with dimeric and monomeric forms of the dye and accordingly are dependent on the dye : phospholipid ratio. At the dye : phospholipid ratios employed, R_0, the distance required for 50% efficient energy transfer, was calculated to be 49 Å. Using fused vesicles containing 2.5% rhodamine phosphatidylethanolamine the observed quenching efficiency was about 88% (cf. Fig. 1B). A similar quenching efficiency was

reported by Fung and Stryer [19] using another pair of dyes incorporated into phosphatidylcholine vesicles which also had a calculated R_0 of 49 Å [19].

A significant finding of the present studies is that binding of protein to the vesicle results in inhibition of Ca^{2+}-mediated fusion. The inhibition was dependent upon the number of antibodies associated with membrane-bound phenobarbital. Assuming a vesicle comprises 2500 phospholipid molecules, 67% of which are in the outer half of the bilayer [23], vesicles containing 0.3 mol% hapten have about five haptens exposed to the outside and available for antibody binding. A slight inhibition of vesicle fusion is apparent with only 0.3 mol% hapten and thus binding of even a small number of proteins to the vesicle is sufficient to cause partial inhibition of fusion. However, maximum inhibition requires at least 25 external haptens (1.5 mol%).

The inhibition of vesicle fusion by bound antibody is almost completely reversed in the presence of competing free ligand. The specific inhibition by antiphenobarbital is therefore clearly distinguishable from nonspecific inhibition of fusion which has been observed at higher protein concentrations. Inhibition of vesicle fusion by antibodies could provide the basis for a novel method for the quantitation of specific antibody or competing ligand concentrations.

We propose that binding of antibody prevents the close opposition of small unilamellar vesicle membranes which is required for fusion. Thus, the mixing and randomization of membrane phospholipids is effectively prevented, resulting in diminished quenching of fluorescence.

Papahadjopoulas et al. [6] have observed the formation of multilamellar cylindrical structures during Ca^{2+}-mediated fusion of vesicles. These structures, called 'cochleate lipid cylinders', are apparently formed as a result of fusion of many unilamellar vesicles (200—500 Å in diameter) and are stabilized by bound Ca^{2+} [6]. Addition of EDTA causes their transformation to large unilamellar vesicles (1000—10 000 Å diameter). The present results are consistent with these earlier observations that large structures are formed that resist further Ca^{2+}-mediated fusion.

Previous work has indicated that Ca^{2+} or Mg^{2+}-mediated fusion of vesicles is accompanied by extensive leakage of small molecules from the occluded vesicle space into the external medium [8]. This would of course be a necessary consequence of the formation of cochleate cylinders. While the present results are consistent with these findings, they suggest that binding of Ca^{2+} to vesicles is sufficient to induce leakage even in the absence of vesicle fusion. Thus incubation of vesicles with $CaCl_2$ under either fusing (no bound antibody) or non-fusing (bound antibody) conditions was equally effective in inducing leakage. It has been proposed [6] that unilamellar vesicles are transformed into flattened disks in the presence of Ca^{2+} and that these disks can aggregate and fuse along the edges. It is possible that bound antibody interferes only at the fusion stage but does not affect disk formation. The formation of disks may nevertheless be accompanied by leakage of the internal contents.

An additional conclusion of the present study is that fusion is dependent on vesicle size and that the larger the vesicle the higher the concentration of Ca^{2+} required to effect fusion. The dependence of fusion on vesicle size may be

related to the strain imposed by different packing of negatively charged phospholipids in the two leaflets of the bilayer [24]. The charge repulsion of the head groups (particularly in the inner leaflet) is balanced by the hydrophobic interactions of the phospholipid tails. Addition of Ca^{2+} to the outer surface results in condensation of the charged phospholipids in the outer leaflet [8,25] and induces destabilization of the membrane. It appears probable that less bound Ca^{2+} would be required to destabilize small vesicles because of the relatively large degree of instability imposed by the difference in phospholipid packing in the two leaflets of the membrane.

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