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FUSION OF MAMMALIAN CELLS BY UNILAMELLAR LIPID VESICLES: INFLUENCE OF LIPID SURFACE CHARGE, FLUIDITY AND CHOLESTEROL

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SUMMARY

The fusion of mammalian cells cultured *in vitro* by unilamellar vesicles prepared from individual or mixed phospholipid species is described. Maximum cell fusion in both monolayer and suspension cell cultures occurred at a dose of approx. 10^4 vesicles per cell. No significant cell damage was observed in the treated cells. Variation of the chemical composition of the vesicles has permitted an evaluation of the importance of such membrane parameters as surface charge, fluidity and the presence of cholesterol. The results were as follows.

1. Extensive cell fusion occurred in cell cultures treated with vesicles prepared from negatively charged phosphatidylserine molecules or a mixture of 10% phosphatidylserine and 90% phosphatidylcholine. Neutral phosphatidylcholine vesicles did not induce cell fusion. Incorporation of lysophosphatidylcholine into phosphatidylserine or mixed phosphatidylserine–phosphatidylcholine vesicles produced a small increase in their cell fusion capacity but was accompanied by marked cytotoxicity and cytolysis.

2. The treatment of cells at 37 °C with vesicles containing lipids that were in the liquid–crystalline state at 37 °C (10% phosphatidylglycerol–90% phosphatidylcholine) or were in an intermediate fluid condition at this temperature (10% dipalmitoylphosphatidylglycerol–90% dipalmitoylphosphatidylcholine) induced significantly greater cell fusion than vesicles composed of lipids that were in the solid phase at 37 °C (10% distearylphosphatidylglycerol–90%distearoylphosphatidylcholine).

3. The incorporation of equimolar amounts of cholesterol into dipalmitoylphosphatidylglycerol–dipalmitoylphosphatidylcholine vesicles reduced significantly their ability to fuse cells but similar amounts of cholesterol had no significant effect on the cell fusion potential of phosphatidylserine–phosphatidylcholine vesicles.

4. The influence of surface charge, "fluidity" and the presence of cholesterol on the cell fusion capacity of vesicles is discussed with reference to possible events occurring in the fusion of natural membranes.

INTRODUCTION

The fusion of mammalian cells cultured *in vitro* is now an established technique

Abbreviations: MES, 2-(*N*-morpholino) ethanesulfonic acid; MOPS, morpholinopropane sulfinic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfinic acid; Tricine, *N*-tris-(hydroxymethyl)-methylglycine; TES, *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

in the experimental investigation of cellular control mechanisms[1,2], gene linkage analysis[3] and the interaction between viruses and cells[4,5]. A large number of DNA- and RNA-containing viruses can induce cell fusion[2] and the use of ultraviolet or β-propiolactone-inactivated Sendai virus has become the most common method for fusing cells. However, a number of problems are recognized in the use of viruses to induce cell fusion. Firstly, the viral components responsible for cell fusion are unidentified and the "fusion potential" of virus preparations cannot therefore be assayed accurately before use. Secondly, it has been found that the introduction of inactivated viruses can induce metabolic alteration in the cells[6–11] and stimulate interferon production[12–14] despite complete elimination of viral infectivity. These findings have prompted the search for new methods for fusing cells.

Following the observation that treatment of cells with lysolecithin could induce fusion[15], the efficiency of lysolecithin[16–18] and other lipolytic agents[19] as methods for inducing cell fusion *in vitro* has been investigated in several laboratories. Although the cell fusion potential of lysolecithin has been confirmed[16,17] the lytic effect of this agent on membranes has dictated that despite attempts to limit its cytotoxic properties[16,18], the use of lysolecithin to fuse cells is accompanied by considerable cell damage[17,20] and the number of viable cells recovered after fusion is significantly lower than in cultures treated with inactivated virus[16]. Consequently, there is still a need for a simple procedure for cell fusion which avoids the problems accompanying fusion induced by lysolecithin or inactivated viruses.

In this paper, we describe the fusion of cells by unilamellar vesicles (liposomes) prepared from certain single or mixed phospholipid species. In view of the documented similarities between the properties of lipid vesicles and those of natural membranes[21,22] it is considered that the differences described here in the ability of vesicles of different phospholipid species to induce fusion may also provide useful information on the behavior of similar lipid molecules in the fusion of natural membranes. We report here the effect of surface charge and "fluidity" of different lipid species on their ability to fuse cells and alterations in fusion capacity when mixed with cholesterol.

MATERIALS AND METHODS

Lipids

Most of the phospholipids used in this study were synthesized and characterized in this laboratory using the isolation procedures described in detail elsewhere[23,24]. All lipids were chromatographically pure and the fatty acid ester content was similar to that in previous studies[23]. Phosphatidylcholine was isolated from egg yolk and phosphatidylserine was obtained by extraction from beef brain. Phosphatidylglycerol was prepared enzymatically from egg phosphatidylcholine by the method of Dawson[25]. Dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine were synthesized by the method of Robles and Van Den Berg[25] and purified on a silicic acid column. Dipalmitoylphosphatidylglycerol and distearoylphosphatidylglycerol were synthesized from dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine, respectively, by a minor modification of the method of Dawson[26] as described elsewhere[24]. The fatty acid content of the purified dipalmitoylphosphatidylglycerol as determined by gas–liquid chromatography after methanolysis was 93.4% palmitic, 3.8% myristic and 2.8% stearic acid. A similar analysis of distearoylphosphatidylcholine indicated the presence of 95.8% stearic and 4.2% palmitic acid.

Sheep erythrocytes were washed three times by centrifugation with 0.9% NaCl. The packed cells were then extracted twice with chloroform–methanol (1:1, v/v) at room temperature. The combined filtrates were evaporated to a small volume (5% of original) "*in vacuo*" and subsequently extracted three times with chloroform. The combined chloroform extracts were evaporated to dryness *in vacuo* and the residual lipids washed according to Folch *et al.*[27]. The organic phase was again evaporated to dryness, and the washed lipids dissolved in chloroform and stored in sealed ampoules under N_2 at −50 °C.

Cholesterol (99% pure) was obtained from the Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from methanol. Lysophosphatidylcholine was purchased from Applied Science Labs (State College, Pa.).

Lipids were stored under N_2 in sealed ampoules at −50 °C at a concentration of approximately 10–20 μmoles of phosphate in 1 ml of chloroform. A fresh preparation of lipids from a new ampoule was used for each experiment.

Preparation of phospholipid vesicles

Phospholipid vesicles were made as previously described[28] by sonication of the multilamellar vesicles (liposomes) described by Bangham *et al.*[29]. The chloroform solution containing purified lipid from a newly opened ampoule was transferred to a glass tube which had been flushed with high purity N_2 (Linde, H. P., Dry 99.996%). The chloroform was then evaporated under vacuum. The dry lipids were suspended in water containing 100 mM NaCl, 2 mM histidine, 2 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) and 0.1 mM EDTA adjusted to pH 7.4, by mechanical shaking on a Vortex mixer at 42 °C for 10 min. The tube containing the suspension was then sonicated for 1 h in a bath-type sonicator (Heat systems, Model 5X5, 40 KHz) under a stream of N_2. The temperature of the sonicating bath was controlled to approximately 22 °C for most lipids, except for dipalmitoylphosphatidylglycerol/dipalmitoylphosphatidylocholine which were sonicated at 42 °C and disterearoylphosphatidylglycerol/distearoylphosphaticylcholine at 60 °C The preparation of small unilamellar vesicles obtained by sonication[30] was left to equilibrate at room temperature for 1 h in a N_2 atmosphere before use. The concentration of phosphoplipid in the final preparation was approximately 7 μmoles/ml, as measured by phosphate assay with molybdate and ELON, following perchloric acid digestion. Serial dilutions were made with phosphate-buffered saline (PBS). Serial dilutions were made with phosphate-buffered saline (PBS).

Measurement of lipid transition temperatures

The transition temperature (T_c) of the phospholipids in the vesicles used in cell fusion experiments was determined with a Differential Scanning Calorimeter (Perkin-Elmer DSC-2). The lipid samples contained 0.4–0.7 μmoles of phosphate in 15 μl; they were prepared and sonicated in 100 mM NaCl buffer as described for the preparation of vesicles. The heating rate for the calorimeter runs was 5 °C/min.

Cells

The BHK21 hamster cell line and the 3T3 mouse cell line were cultured in Dulbecco's fortified Eagles medium supplemented with 10% and 4% fetal bovine serum for growth and maintenance. The L929 mouse cell line was grown in Eagles Basal

Medium supplemented with 7% and 4% calf serum for growth and maintenance, respectively, as described elsewhere[31].

Cell counts and viability

Cells were counted in a Fuchs–Rosenthal haemocytometer chamber and viability was estimated by the trypan blue dye exclusion method.

Sendai virus

Sendai virus for use in cell fusion experiments was prepared in embryonated hens eggs and inactivated with β-propiolactone as described elsewhere[32]. Virus titers are expressed as the number of viral haemagglutination units per ml of purified allantoic fluid, and the cell fusion experiments were done at a dose of 2000 heamagglutination units/ml.

Cell fusion

The capacity of vesicles prepared from different phospholipid species to induce cell fusion was investigated using both suspension and monolayer cell cultures. For fusion of cells in suspension the technique described by Harris *et al.*[33] for virus-induced cell fusion was followed except that the vesicles were added instead of virus. Except where stated otherwise, the cells were incubated with the vesicle preparation for 1 h at 37 °C and then 1.0 ml of fresh maintenance medium was added to the cells. After further incubation for 1 h with shaking at regular intervals, the cell suspension was inoculated on to coverslips and the extent of cell fusion measured 24 h later.

To assess the extent of cell fusion occurring after short periods of incubation with the vesicles the cell suspension was centrifuged onto glass slides in a cytocentrifuge (Shandon Instruments, London), fixed and stained with May–Grunwald and Giemsa and counts made of the number of fused cells.

To induce fusion of cells in monolayer cultures the method of virus-induced cell fusion described previously[34,35] was used except that phospholipid vesicles were used instead of virus. In the first method, 1.0 ml of the vesicle preparation was added to confluent monolayer cell cultures on coverslips that had been prewashed with warm phosphate-buffered saline. After incubation for 2 h at 37 °C, fresh maintenance medium was added. The cultures were then incubated for various intervals and finally fixed and stained to determine the extent of cell fusion.

In the case of vesicles containing lysolecithin the methods described above were used to induce cell fusion, but the cells were only exposed to the liposomes for 10 min at 37 °C. The cells were then washed three times with prewarmed phosphate-buffered saline to remove the remaining vesicles. Fusion of cells by lysolectihin alone was done in suspension cell cultures which were first centrifuged at 200 × *g* for 5 min at room temperature to form a loose pellet and then treated with 0.1 ml of a solution of lysolecithin in 1% ethanol, containing 200 μg/ml lysolecithin and 5 mg/ml bovine serum albumin (Pentax, Fraction V) for 1 min at 37 °C as described elsewhere[16]. A further 0.5 ml of heat-inactivated fetal bovine serum was then added to the cells to limit the cytolytic activity of lysolecithin. The cell pellet was resuspended and inoculated onto coverslips and the extent of cell fusion measured 24 h later.

Fusion of cells in suspension and monolayer cultures by β-propiolactone-inactivated Sendai was done by the methods described previously[32,34].

Except where stated otherwise, all cell fusion experiments were done in fluids or culture medium buffered to pH 8.0 which has been shown to increase the frequency of cell fusion induced by viruses[36].

Experiments on the effect of pH on the efficiency of cell fusion by lipid vesicles were done in solutions supplemented with a 15- or 10-mM concentration of the following organic buffers: 2-(*N*-morpholino)-ethanesulfonic acid (MES) (p*K* 6.15); morpholinopropanesulfinic acid (MOPS) (p*K* 7.2); *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfinic acid (HEPES) (p*K* 7.55) and *N*-tris-(hydroxymethyl)-methylglycine (Tricine) (p*K* 8.15). All buffers were obtained from Calbiochem, La Jolla, Calif. Solutions were adjusted to the required pH by titration with 1M NaOH or 1M HCl starting from the normal pH of approx. 7.3 of most cell culture solutions.

Measurement of the extent of cell fusion

Coverslip cell cultures were fixed with methanol both before and at intervals after treatment with lipid vesicles or inactivated Sendai virus and stained with May-Grunwald and Giemsa at pH 6.8. The extent of cell fusion, expressed as the percentage polykaryocytosis, was measured as described previously[35] by counting the number of nuclei present in multinucleate cells (polykaryocytes) and expressing this as a percentage of the total number of nuclei present in the same microscope field. At least 20 microscope fields were counted from each coverslip and four replicate coverslips were examined for each sample.

Other chemicals

L-Histidine·HCl (Sigma Grade) and TES were obtained from the Sigma Chemical Company. All other chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus.

RESULTS

A series of screening experiments were first done to investigate whether unilamellar vesicles prepared from different lipid species could induce cell fusion. These early experiments revealed that vesicles prepared from phosphatidylserine, 10% phosphatidylserine–90% phosphatidylcholine, 10% dipalmitoylphosphatidylglycerol –90% dipalmitoylphosphatidylcholine and 10%phosphatidylglycerol–90% phosphatidylcholine were able to induce extensive cell fusion in both monolayer and suspension cultures of mouse L929 and 3T3 cells. In contrast, 10% distearoylphosphatidylglycerol–90% distearoylphosphatidylcholine vesicles induced little cell fusion and phosphatidylcholine vesicles failed completely to induce fusion. Detailed results of the cell fusion properties of these different phospholipid vesicles will be described later after a description of certain factors affecting cell fusion by lipid vesicles in general.

The ratio of lipid vesicles to cells required to induce cell fusion

To establish the number of vesicles necessary to induce cell fusion replicate 3T3, L929 and BHK21 cell cultures were treated with an undiluted suspension of lipid vesicles and a series of 10-fold dilutions from the original preparation. The number of vesicles added to the cell cultures at each dilution was calculated and expressed on the basis of the number of vesicles per cell. The number of cells in the cultures was obtained by microscopic counts before addition of the vesicles. Chemical

assay of the phosphate content of the undiluted vesicle preparation was used to calculate the number of lipid molecules present. The estimate of the number of vesicles present was based on a knowledge of the number of lipid molecules in a single vesicle, which was estimated from the average particle weight of the vesicles. The mean diameter of the unilamellar vesicles composed of lipids similar to those used in the present experiments was 250 Å[37,38]. An example of these calculations is given in the following: Total phosphate content of the vesicle preparation = 7 μmoles per ml; 1 μmole phosphate = $6 \cdot 10^{17}$ molecules; each ml of the vesicle preparation therefore contains $4.2 \cdot 10^{18}$ molecules; a vesicle of mean diameter approx. 250 Å and average particle weight of $2.1 \cdot 10^6$ contains approx. 3000 molecules; each ml of the vesicle preparation therefore contains approx. $1.4 \cdot 10^{15}$ vesicles.

Cell cultures were grown either on coverslips ($1.5 \cdot 10^6$ cells per coverslip) or maintained in suspension ($5 \cdot 10^6$ cells per ml) and treated with the undiluted preparation or serial dilutions of the original preparation as described in Materials and Methods. The extent of cell fusion induced by different concentrations of vesicles was estimated 24 h after treatment. The results revealed that cell fusion occurred with as little as 10^2 vesicles per cell, but maximum cell fusion required $0.5 \cdot 10^3$–$1 \cdot 10^4$ vesicles per cell. The use of a larger vesicle to cell ratio did not significantly increase the extent of cell fusion. This dose of 10^4 vesicles per cell required for maximum cell fusion was constant, and significant variation in the vesicle to cell ratio producing maximum cell fusion was not found either between different cell types or between vesicles prepared from different lipid species. The latter observation is important since it enables direct comparison of the efficiency of different types of lipid vesicle to induce cell fusion at a uniform vesicle to cell ratio. Thus, the differences described in subsequent sections on the cell fusion properties of different types of lipid vesicle can be attributed to inherent differences in the lipids themselves rather than a failure to provide the optimum technical conditions for cell fusion by a particular type of vesicle.

Experiments on the effect of pH over the range 6.0–8.5 on vesicle-induced cell fusion revealed that maximum cell fusion occurred at pH 8.0 irrespective of the type or number of vesicles used. It is likely, however, that this is a reflection of a more general pH optimum for the cell fusion process, since similar results showing maximum fusion at pH 8.0 have been found elsewhere for both spontaneous[39] and virus-induced cell fusion[36,40].

The effect of surface charge on the cell fusion capacity of lipid vesicles

Net negatively charged unilamellar vesicles were prepared from phosphatidylserine or 10% phosphatidylserine in 90% phosphatidylcholine and neutral vesicles from egg phosphatidylcholine. These were then tested for their capacity to induce fusion of 3T3, L929 and BHK21 cells. The results shown in Table I indicate that the vesicles prepared from neutral phosphatidylcholine molecules did not induce cell fusion but negatively charged phosphatidylserine or 10% phosphatidylserine–90% phosphatidylcholine vesicles produced significant fusion of all three cell types. The finding that the inclusion of 10% phosphatidylserine in phosphatidylcholine vesicles enables cell fusion to occur suggests that a charge interaction between the surface of the vesicle and the cell surface may be necessary for fusion to take place.

No significant change in the viability of cells exposed to the vesicles was found over a 24-h period.

TABLE I

FUSION OF MOUSE 3T3 AND L929 CELLS AND HAMSTER BHK21 CELLS IN MONOLAYER AND SUSPENSION CULTURES BY UNILAMELLAR LIPID VESICLES OF DIFFERING SURFACE CHARGE

Values are mean values derived from three separate experiments and counts on a minimum of 10000 cells.

Phospholipid Vesicle*	*Mean electrophoretic** Mobility of vesicle (± S.E.)* ($\mu \cdot sec^{-1} \cdot V^{-1} \cdot cm$)	*Mean per cent polykaryocytosis (± S.E.)*					
		3T3		*L929*		*BHK21*	
		Monolayer	*Suspension*	*Monolayer*	*Suspension*	*Monolayer*	*Suspension*
Untreated control	—	2.7 ± 1.3	1.8 ± 0.7	3.5 ± 1.5	3.2 ± 1.7	3.2 ± 2.5	3.9 ± 2.1
100% phosphatidylcholine	Isoelectric	1.6 ± 0.9	2.3 ± 1.2	2.7 ± 1.1	3.8 ± 1.4	3.6 ± 1.9	2.9 ± 1.5
10% phosphatidylserine–90% phosphatidylcholine	−0.677 ± 0.015	21.7 ± 7.8	24.3 ± 11.3	26.3 ± 9.3	18.8 ± 9.1	17.4 ± 5.9	24.8 ± 8.3
100% phosphatidylserine	−3.926 ± 0.041	15.9 ± 6.4	26.7 ± 12.6	19.5 ± 11.8	26.2 ± 7.3	26.5 ± 13.2	31.5 ± 14.3

* Input multiplicity of 10^4 vesicles per cell at pH 8.0

** Data from Papahadjopoulos and Weiss[83].

TABLE II

FUSION OF MOUSE 3T3 AND L929 CELLS BY UNILAMELLAR VESICLES OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE CONTAINING LYSOLECITHIN AND BY LYSOLECITHIN ADSORBED TO BOVINE SERUM ALBUMIN

Values are mean values derived from three separate experiments and counts on a minimum of 10000 cells.

*Treatment**	*Mean percent polykaryocytosis (± S.E.)*			
	3T3		*L929*	
	Monolayer	*Suspension*	*Monolayer*	*Suspension*
Untreated control	3.1 ± 1.8	2.2 ± 0.9	2.7 ± 1.3	3.5 ± 1.8
10% phosphatidylserine–90% phosphatidylcholine	23.4 ± 9.2	18.2 ± 12.2	22.5 ± 10.5	20.7 ± 11.3
10% phosphatidylserine–90% phosphatidylcholine/3% lysolecithin	32.6 ± 14.8	30.7 ± 9.6	39.7 ± 16.2	35.8 ± 15.5
10% phosphatidylserine–90% phosphatidylcholine/10% lysolecithin	41.3 ± 17.9	38.5 ± 14.5	41.3 ± 13.8	37.5 ± 16.2
200 μg lysolecithin + 5 mg bovine serum albumin	19.4 ± 6.5	15.8 ± 9.3	21.4 ± 10.1	14.9 ± 7.4
1% ethanol	2.7 ± 0.9	2.3 ± 1.1	1.9 ± 0.7	3.2 ± 1.6

* Input multiplicity of 10^4 vesicles per cell at pH 8.0.

TABLE III

THE VIABILITY OF MOUSE 3T3 AND L929 CELLS AT INTERVALS AFTER TREATMENT WITH LIPID VESICLES PREPARED FROM PHOSPHATIDYLSERINE, PHOSPHATIDYLCHOLINE AND LYSOLECITHIN AND AFTER TREATMENT WITH LYSOLECITHIN ADSORBED TO BOVINE SERUM ALBUMIN.

Values are mean values, derived from counts on a minimum of 500 cells obtained from three separate aliquots.

Treatment *	*Mean per cent cell viability*							
	3T3				*L929*			
	1 h	*2 h*	*4 h*	*24 h*	*1 h*	*2 h*	*4 h*	*24 h*
Untreated control	99	99	95	98	98	97	96	97
10% phosphatidylserine–90% phosphatidylcholine	95	98	98	96	95	93	96	97
10% phosphatidylserine–90% phosphatidylcholine/3% lysolecithin	81	53	47	32	86	72	48	37
10% phosphatidylserine–90% phosphatidylcholine/10% lysolecithin	72	44	35	38	82	61	35	28
200 nm lysolecithin + 5 mg bovine serum albumin	86	59	48	27	90	64	59	35

* Input multiplicity of 10^4 vesicles per cell.

The effect of lysolecithin incorporation into lipid vesicles on their cell fusion properties

As pointed out in the introduction, lysolecithin in aqueous media[15], attached to protein[16] and in microdroplets of glycerol dioleate[18] can induce cell fusion *in vitro*. To investigate the effect of lysolecithin on the fusion potential of unilamellar lipid vesicles, cultures of 3T3 and L929 cells were treated with 10% phosphatidylserine–90% phosphatidylcholine vesicles containing 3% or 10% moles lysolecithin per total phosphate and the degree of cell fusion measured.

The results shown in Table II indicate that lysolecithin increased the cell fusion capacity of phosphatidylserine–phosphatidylcholine vesicles for both 3T3 and L929 cell cultures. The effect of lysolecithin on the cell fusion capacity of the phosphatidylserine–phosphatidylcholine vesicles appeared to be additive since lysolecithin used alone did not induce significantly greater cell fusion than phosphatidylserine–phosphatidylcholine vesicles without lysolecithin (Table II). Control experiments indicated that the concentration of ethanol (1%) present in the lysolecithin solution was not responsible for inducing cell fusion (Table II).

Despite attempts to limit the cytotoxic and cytolytic properties of lysolecithin, a marked decrease in cell viability was found in cultures exposed to vesicles containing lysolecithin or lysolecithin alone (Table III).

These experiments, together with those reported in other sections of this paper, show that lipid vesicles are equally as effective as lysolecithin in inducing the fusion of mammalian cells cultured *in vitro*. Importantly, lipid vesicles do not produce the extensive cell damage found in lysolecithin-treated cell cultures[15–18,20,41].

The cell fusion properties of vesicles prepared from phospholipids of differing "fluidity"

Studies made in many laboratories in the past few years have demonstrated that lipids in natural membranes can undergo a first-order thermal phase change from a gel (solid) to a liquid–crystalline state. The subject has been reviewed recently[42,43]. The transition from the solid to the liquid–crystalline phase involves an increase in the "fluidity" of the hydrocarbon chains of the phospholipid molecules. Below the transition temperature (T_c), lipids exist in a more solid-like state and their hydrocarbon chains are relatively ordered. Above the T_c, the hydrocarbon chains are more disordered, have greater motional freedom and are more "fluid". The increased motional freedom of the hydrocarbon chains in the "fluid" state has important implications for several aspects of membrane function[44–51] and it has been proposed that the lipids in natural membranes may need to be in a "fluid" condition to undergo fusion[52].

To investigate the effect of the "fluidity" of phospholipid molecules in unilamellar vesicles on their ability to fuse cells, vesicles were prepared from phospholipid species that were either above or below their T_c or at their T_c when used to treat cell cultures at 37 °C or 40 °C.

The values for the T_c of the phospholipids used in the cell fusion experiments were determined by differential scanning calorimetry (Fig. 1). The calorimetric data shown in Fig. 1 demonstrates that the T_c of vesicles prepared from brain phosphatidylserine (13.0 °C) and egg phosphatidylcholine (<0 °C) is below the experimental temperature of 37 °C. Vesicles composed of these lipids or mixtures of these (including egg phosphatidylglycerol which is prepared from egg phosphatidylcholine) are in a liquid–crystalline state during the cell fusion experiments. Fig. 2 shows the T_c for the other phospholipids used in this study: dipalmitoylphosphatidylglycerol (40.0 °C);

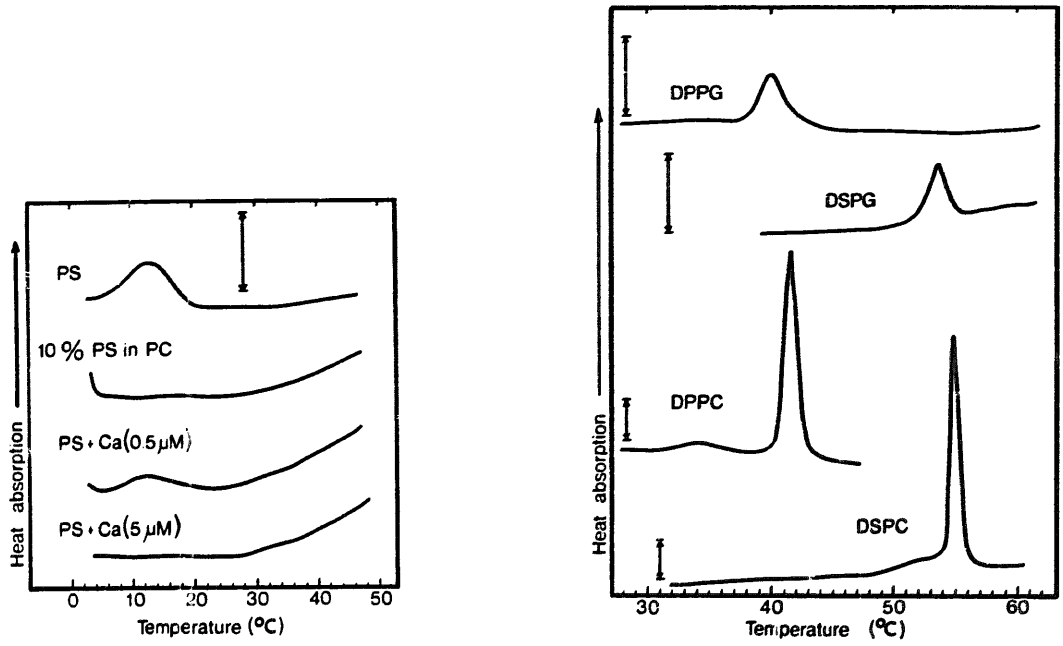

Fig. 1. Differential scanning calorimetry curves for vesicles composed of natural phospholipids. Each experiment was performed with approx. 1.0 μmole of phospholipid in excess buffer solution. Scanning rate, 2.5 °C/min. Marker: 50 μcal/s. The temperatures given in the text for T_c represent the midpoint of the major endothermic peak obtained with each lipid preparation. PS: phosphatidylserine from bovine brain. 10% phosphatidylserine in phosphatidylcholine:percentage of phosphatidylserine in phosphatidylcholine from egg yolk. Phosphatidylserine + Ca^{2+} (0.5 μM): 1 μmole of phosphatidylserine in the presence of 0.5 μmole of Ca^{2+}. Phosphatidylserine + Ca^{2+} (5 μM): 1 μmole of phosphatidylserine in the presence of 5 μmoles of Ca^{2+}. For the last two experiments, the calculated amount of Ca^{2+} was added in a small volume as $CaCl_2$ to the calorimeter pan. The solution was then evaporated to dryness in a current of warm air, and then the preformed phosphatidylserine vesicles were added in 15 μl volume. The addition of Ca^{2+} tends to broaden the peak, and above a certain concentration shifts the peak to higher temperatures. Similar behaviour was observed when Ca^{2+} was added to dipalmitoylphosphatidylglycerol vesicles. With the higher concentrations of Ca^{2+}, no transition was observed within the temperature range of the experiment (lower curve). Since the phospholipid vesicles were added to the cells in the absence of Ca^{2+}, we consider that the phosphatidylglycerol and phosphatidylserine vesicles are still in the liquid-crystalline state (above the T_c) at the experimental conditions used. It is to be noted, however, that both phosphatidylserine and phosphatidylglycerol vesicles would tend to be in the solid state (below the T_c) at high concentrations of Ca^{2+}.

Fig. 2. Differential scanning calorimetry curves for vesicles composed of synthetic phospholipids. Each experiment was performed with approximately 0.7 μmole of phospholipid in excess buffer solution. Scanning rate, 5 °C/min. Marker: 50 μcal/s. The temperature given in the text for T_c represent the midpoint of the major endothermic peak obtained with each lipid preparation. DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol, DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine. In each case the vesicles were formed in 100 mM NaCl at pH 7.4 as described in Materials and Methods.

dipalmitoylphosphatidylcholine (41.5 °C); distearoylphosphatidylglycerol (53.7 °C) and distearoylphosphatidylcholine (55.0 °C). Thus, 10% dipalmitoylphosphatidylglycerol–90% dipalmitoylphosphatidylcholine vesicles are in the region of the solid-to-liquid crystalline transition at the experimental temperature of 40 °C. The 10% distearoylphosphatidylglycerol–90% distearoylphosphatidylcholine vesicles are well below the transition region and would therefore be in a solid-like state at the experimental temperature of 37°.

TABLE IV

FUSION OF MONOLAYER CULTURES OF MOUSE 3T3 AND L929 CELLS AND HAMSTER BHK21 CELLS BY UNILAMELLAR VESICLES PREPARED FROM DISTEAROYLPHOSPHATIDYLGLYCEROL, DISTEAROYLPHOSPHATIDYLCHOLINE, DIPALMITOYL PHOSPHATIDYLCHOLINE, DIPALMITOYLPHOSPHATIDYLGLYCEROL, PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLGLYCEROL AT 37 °C or 40 °C

Values are mean values derived as described in Table I.

Treatment *	*Incubation temperature (°C)*	*Mean percent polykaryocytosis (± S.E.)*		
		3T3	*L929*	*BHK21*
Untreated control	37	1.9 ± 0.7	2.5 ± 1.2	3.6 ± 2.0
10% distearoylphosphatidylglycerol–90% distearoylphosphatidylcholine	37	5.8 ± 3.7	6.2 ± 3.5	5.9 ± 3.2
10% dipalmitoylphosphatidylglycerol–90% dipalmitoylphosphatidylcholine	40**	17.6 ± 10.3	22.5 ± 9.9	21.4 ± 11.4
10% phosphatidylglycerol–90% phosphatidylserine	37	26.4 ± 9.7	31.5 ± 12.7	29.4 ± 106

* Input multiplicity of 10^4 vesicles per cell at pH 8.0.

** For first 2 h only after which cell cultures were incubated at 37 °C.

TABLE V

THE EFFECT OF INCORPORATION OF EQUIMOLAR AMOUNTS OF CHOLESTEROL ON THE CELL FUSION PROPERTIES OF UNILAMELLAR VESICLES PREPARED FROM DIPALMITOYLPHOSPHATIDYLGLYCEROL, DIPALMITOYLPHOSPHATIDYLCHOLINE, PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE

Values are mean values derived as described in Table I.

Treatment	*Incubation** temperature (°C)*	*Mean percent polykaryocytosis (±S.E.)*		
		3T3	*L929*	*BHK21*
Untreated control	37	2.6±1.5	2.6±1.4	3.5±2.2
10% phosphatidylserine–90% phosphatidylcholine	37	19.8±8.3	24.7±12.2	18.5±9.6
10% phosphatidylserine–90% phosphatidylcholine/cholesterol	37	23.8±10.7	20.3±9.3	16.9±8.2
10% dipalmitoylphosphatidylglycerol–90% dipalmitoylphosphatidylcholine	37	16.3±7.1	25.2±8.7	20.8±9.3
10% dipalmitoylphosphatidylglycerol–90%dipalmitoylphosphatidylcholine/ cholesterol	37	5.3±3.8	7.6±3.9	6.5±3.2

* Input, multiplicity of 10^4 vesicles per cell at pH 8 0.

** Maintained for entire experiment.

The ability of vesicles prepared from lipids having different "fluidities" at 37 °C or 40 °C to induce cell fusion at these temperatures is shown in Table IV. The results indicate that vesicles prepared from distearoylphosphatidyiglycerol–distearoylphosphatidylcholine that were below their gel to liquid–crystalline transition temperature produced significantly less cell fusion than similar numbers of vesicles containing lipids that were either at or above their T_c under the experimental conditions. As in the previous experiments, no significant cytotoxicity or cytolysis was found in cell cultures treated with the lipid vesicles.

The effect of cholesterol on the cell fusion capacity of lipid vesicles

Studies with monomolecular films and unilamellar vesicles of single or mixed phospholipid species have established that the presence of cholesterol modifies the packing of the phospholipid molecules and exerts a significant effect on the motional freedom of the phospholipid molecules. The addition of cholesterol to lipids in a liquid–crystalline state produces a "condensing effect" but increases the "fluidity" of phospholipids that are in a gel condition[53]. Consequently, the presence of cholesterol produces a mixed membrane which is in an intermediate condition in that it is more condensed when compared with phospholipid membranes composed of a single lipid that is above its T_c, and more fluid when compared with pure phospholipids that are below their T_c.

In view of the differences in cell fusion properties detected in the previous section between vesicles containing lipids above, at, or below their T_c, it was considered pertinent to examine the effect of cholesterol on the cell fusion capacity of vesicles prepared from the same lipids.

Vesicles of 10% phosphatidylserine–90% phosphatidylcholine and 10% dipalmitoylphosphatidylglycerol–90% dipalmitoylphosphatidylcholine were prepared as before in the presence of an equimolar amount of cholesterol and their cell fusion properties compared with that of vesicles of the same type without cholesterol. The results, shown in Table V, indicate that inclusion of cholesterol produced a significant decrease in the cell fusion capacity of dipalmitoylphosphatidylglycerol–dipalmitoylphosphatidylcholine vesicles but had no significant effect on the ability of phosphatidylserine–phosphatidylcholine vesicles to induce cell fusion. That the significant reduction in the number of fused cells in cultures treated with dipalmitoylphosphatidylglycerol–dipalmitoylphosphatidylcholine–cholesterol vesicles was not due to cell damage was confirmed by measurement of cell viability which showed that the treated cells remained highly viable.

The comparative efficiency of cell fusion by lipid vesicles and inactivated Sendai virus

The efficiency of the various types of lipid vesicle used in the preceding sections to induce cell fusion compared with inactivated Sendai virus is summarized in Figs 3 and 4. The results indicate that inactivated virus induces more extensive cell fusion but certain lipid vesicles induce significant cell fusion and could be used to fuse cells in experiments in which the use of virus may create complications by inducing metabolic alterations[6–11], interferon production[12–14], chromosomal alterations[54,55] or cell surface modifications[2,33] in the fused cells. We have now successfully fused mouse and human cells by lipid vesicles and isolated viable interspecific hybrid cells. These results will be described in a subsequent communication.

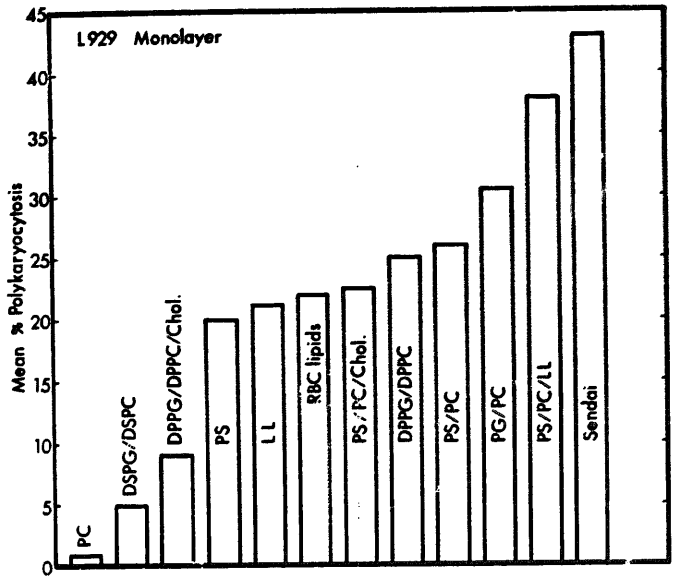

Fig. 3. Fusion of mouse L929 cells in monolayer cultures by lipid vesicles, lysolecithin and inactivated Sendai virus. The extent of the cell fusion, expressed as the mean per cent polykaryocytosis, was measured as described in Materials and Methods. PC, egg yolk phosphatidylcholine vesicles; PS, phosphatidylserine vesicles; PS/PC, 10% phosphatidylserine in phosphatidylcholine vesicles; PS/PC/LL, 10% phosphatidylserine in phosphatidylcholine plus 3% lysolecithin; PS/PC/Chol., 10% phosphatidylserine in phosphatidylcholine plus an equimolar amount of cholesterol; PG/PC, 10% phosphatidylglycerol in phosphatidylcholine; DSPG/DSPC, 10% distearoylphosphatidylglycerol in distearoylphosphatidylcholine; DPPG/DPPC, 10% dipalmitoylphosphatidylglycerol in dipalmitoylphosphatidylcholine; DPPG/DPPC/Chol., 10% dipalmitoylphosphatidylglycerol in dipalmitoylphosphatidylcholine plus an equimolar amount of cholesterol; RBC lipids, extracted sheep red blood cell lipids; LL, 200 μg lysolecithin; Sendai, β-propiolactone-inactivated Sendai virus.

The cell fusion properties of unilamellar vesicles prepared from extracted sheep red blood cell lipids

The results described so far have been obtained using vesicles prepared from highly purified phospholipids synthesized in our laboratory. However, the procedures required for the preparation of phospholipids at the purity limits described here are not available routinely in all laboratories that might wish to employ lipid vesicles to induce cell fusion as an alternative to virus-induced cell fusion. It was considered valuable therefore to investigate the cell fusion potential of vesicles prepared from mixed lipids extracted from sheep red blood cells which can be obtained more easily.

Monolayer and suspension cell cultures were treated with sheep blood cell lipid vesicles as described in Materials and Methods and the extent of cell fusion and the effect of the lipids on cell viability were measured.

The results shown in Figs 3 and 4 indicate that the net negatively charged vesicles were able to induce extensive cell fusion without causing significant cytotoxicity or cytolysis. The extent of cell fusion induced by sheep blood cell lipids is comparable to that produced by vesicles prepared from purified lipids such as phosphatidylserine –phosphatidylcholine, phosphatidylserine and dipalmitoylphosphatidylglycerol–dipalmitoylphosphatidylcholine (Figs 3 and 4) but less than induced by Sendai virus. No signficant cytotoxicity was observed in cell cultures treated with the vesicles.

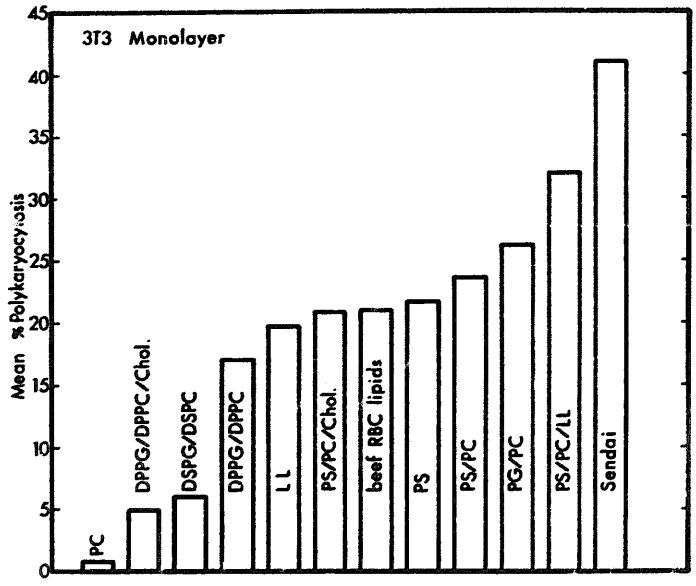

Fig. 4. Fusion of mouse 3T3 cells in monolayer cultures by lipid vesicles, lysolecithin and inactivated Sendai virus. The extent of the cell fusion, expressed as the mean percent polykaryocytosis, was measured as described in Materials and Methods. PC, egg yolk phosphatidylcholine vesicles; PS, phosphatidylserine vesicles; PS/PC, 10% phosphatidylserine in phosphatidylcholine vesicles; PS/PC/LL, 10% phosphatidylserine in phosphatidylcholine plus 3% lysolecithin; PS/PC/Chol., 10% phosphatidylserine in phosphatidylcholine plus an equimolar amount of cholesterol; PG/PC, 10% phosphatidylglycerol in phosphatidylcholine; DSPG/DSPC, 10% distearoylphosphatidylglycerol in distearoylphosphatidylcholine; DPPG/DPPC, 10% dipalmitoylphosphatidylglycerol in dipalmitoylphosphatidylcholine; DPPG/DPPC/Chol., 10% dipalmitoylphosphatidylglycerol in dipalmitoylphosphatidylcholine plus an equimolar amount of cholesterol; beef RBC lipids, extracted mixed beef red blood cell lipids; LL, 200 μg lysolecithin; Sendai, β-propiolactone-inactivated Sendai virus.

DISCUSSION

The present results indicate that mammalian somatic cells cultured *in vitro* can be fused by treatment with unilamellar vesicles prepared from certain phospholipid species. Importantly, the extent of the cell fusion induced by the vesicles examined here compares favourably with that induced by inactivated Sendai virus and is significantly higher than that produced by treating cells with lysolecithin even when lysolecithin was attached to protein to limit cell damage. These results, together with the absence of significant cytotoxicity or cytolysis in cell populations exposed to the lipid vesicles, suggest that vesicles of the type described here may be of value as a method for inducing cell fusion in situations where the use of viruses for this purpose is undesirable. Although the results described here relate only to the fusion of cells of the same type, recent work in our laboratory has established that lipid vesicles can also be used to induce fusion of different cell types and viable mouse–human hybrid cells have been isolated recently following cell fusion by this method (Post, G., Papahadjopoulos, D., Shows, T. and Schaeffer, B., unpublished).

The mechanism by which lipid vesicles cause cell fusion is not clear from the present experiments. The most obvious possibility is that the vesicles penetrate the area between the surfaces of two adjacent cells and fusion occurs between the surface of the vesicle at points where it is in contact with the plasma membranes of the two cells thereby creating a "bridge" between the cells which subsequently enlarges leading

to complete fusion of the cells. In this sense, cell fusion induced by vesicles is considered as being analogous to the way in which lipid-enveloped viruses such as Sendai virus are believed to induce cell fusion by acting as similar "bridges" between apposed cells[2,56–59].

It is considered likely that the small size (approx. 250 Å) of the vesicles used here would facilitate penetration into small intercellular spaces and would increase the opportunities for fusion along the lines suggested above.

The incorporation of labelled lipids from vesicles into cells has been reported[60–63] However, the data in these studies does not allow a clear distinction to be made between uptake of phospholipid molecules by simple exchange or by the incorporation of entire vesicles by fusion or endocytosis. Suggestive evidence for fusion between lipid vesicles and cellular membranes has been obtained in an electron microscope study of the interaction of lipid vesicles with isolated leukocyte lysosomes[60] and it is also pertinent to note that Ahkong *et al.*[18] found large numbers of lipid droplets in direct contact with the plasma membrane of cells undergoing fusion after treatment with lysolecithin incorporated into microdroplets of glycerol dioleate.

Apart from the value of lipid vesicles as an experimental method for inducing cell fusion *in vitro*, it is considered that the differences described here in the ability of different phospholipids to induce fusion might also provide information on the behavior and importance of lipid molecules in the fusion of natural membranes. The present results suggest that parameters such as the extent and nature of the charge on the phospholipid molecules, their "fluidity" and the presence of cholesterol might influence the susceptibility of membranes to fusion.

The observation that negatively charged lipid vesicles induce cell fusion, while neutral vesicles do not, suggests that a charge interaction is required to enable attachment and close contact between the vesicles and the cell surface to take place as a prerequisite to the actual fusion process proposed above. At first sight, it appears surprising that negatively charged vesicles could achieve this type of close contact with a negatively charged cell surface. However, recent evidence indicates that the distribution of negatively charged groups on mammalian cell surfaces is not homogeneous[64,65] and it is not difficult to envisage that contact of vesicles with the cell surface might occur preferentially at areas with a lower than average surface charge [2,52,66,67]. Also, the small size of the lipid vesicles would tend to reduce the potential energy barrier opposing their close contact with the cell surface (see refs 2, 66 and 67). Also, requirement for Ca^{2+} in order for cell fusion to take place suggests that Ca^{2+} bridges between negatively charged groups on the cell surface and the vesicle might play some part in the attachment of vesicles to the cell surface.

The interaction of negatively charged vesicles with cell surfaces resembles more closely the "physiological" situation of contact interaction between cell surfaces bearing net negative charges[66] and the contact and fusion of negatively charged lipid-enveloped viruses with cellular membranes[68] including virus-induced cell fusion[59,69]. The demonstration of free positively charged groups in the cell periphery has proved to be extremely difficult[70–72] and such groups, if present, are considered to play only a comparatively minor role in the contact interactions between cell surfaces. For these reasons, we have not attempted to induce cell fusion using positively charged vesicles since this would appear to bear less resemblance to the interaction and fusion of membranes under natural conditions.

The present observations on the capacity of vesicles composed of phospholipids with different gel to liquid–crystalline transition temperatures to induce cell fusion at 37 °C and 40 °C have established that a significant degree of cell fusion occurs when the lipid molecules in the vesicles are either at or above their transition temperature when used at these temperatures. These results suggest that it might also be necessary for lipids in natural membranes to be in a "fluid" condition for fusion to occur. A similar tentative proposal for the need for a "fluid" membrane during fusion has been advanced on the basis of the kinetics of fusion of hen erythrocytes by glycerol monooleate at different temperatures[73].

Additional support for the importance of a "fluid" membrane in the fusion process is provided by the present findings on the effects of cholesterol on the cell fusion properties of lipid vesicles. The introduction of equimolar amounts of cholesterol into vesicles composed of phospholipids (dipalmitoylphosphatidylglycerol–dipalmitoylphosphatidylcholine) which are near their gel to liquid–crystalline transition at the experimental temperature of 37 °C, resulted in a significant decrease in the capacity of the vesicles to fuse cells. This is in accord with the well-known effects of cholesterol on the physical properties of phospholipid membranes, reviewed recently in detail[45,53]. These studies have shown that cholesterol tends to inhibit molecular motion along the first 10 carbon atoms of the phospholipid acyl chains, producing a condensed membrane which is much less fluid as compared to phospholipid bilayers above their T_c.

The lack of effect of cholesterol on the cell fusion properties of phosphatidylserine–phosphatidylcholine vesicles was somewhat unexpected in view of the fact that its presence reduces the permeability of such vesicles to various solutes[24,74] indicating a condensation effect. An explanation for this apparent discrepancy could be found in studies on the effect of cholesterol in different phospholipid monolayers. These studies have indicated that the "condensing effect" of cholesterol is related to the "fluidity" of the interacting phospholipids, and it is maximal when the phsopholipids are close to their transition temperature[75–77]. A recent study on the effects of different lipids on the reactivation of a delipidated preparation of $(Na^+ + K^+)$-ATPase has revealed a similar apparent discrepancy on the role of cholesterol[50]. Thus, it was noted that addition of cholesterol to brain phosphatidylserine or egg phosphatidylglycerol was only partly inhibitory, while when cholesterol was added to dipalmitoylphosphatidylglycerol, the inhibition of the $(Na^+ + K^+)$-ATPase activity was total (95%).

Quantitative experimental data is not yet available to permit a detailed evaluation of the role of membrane "fluidity" in the fusion of natural membranes. Theoretically membrane "fluidity" might contribute to the fusion process in several ways[52]. Firstly, the molecules in a "fluid" lipid domain would have greater motional freedom than in membranes in the solid phase. This, together with the superimposed increase in the motional freedom and membrane "disorder" resulting from the displacement of Ca^{2+} from membranes during fusion (see refs 2, 52, 67 and 78) would increase the opportunity for interaction between molecules in apposed membranes leading to stable linkages between the membranes. Secondly, a "fluid" membrane would favour the rapid intermixing of components in apposed membranes and this would promote rapid reconstruction and stabilization of a single membrane immediately after fusion[52]. The ability of intrinsic membrane proteins to diffuse laterally within "fluid" membranes might also affect the fusion process. Poste and Allison[52]

have suggested recently that clustering of intrinsic membrane proteins may play a role in the fusion of natural membranes. They suggested that areas of clustered proteins in adjacent membranes were able to interdigitate to initiate fusion which would then spread laterally to involve the lipid bilayer regions of the membranes. Recently, clustering of intrinsic membrane proteins similar to the above theoretical proposals have been identified at sites of membrane fusion in freeze–etching studies of exocytosis[79,80] and fusion of lipid-envelope viruses with erythrocyte membranes[81,82]. Although further experimental work is required before any generalizations can be made there is at least suggestive evidence that membrane fluidity may be an important determinant in the membrane fusion reaction.

It remains to be established how far the cell fusion properties of lipid vesicles described here can be used as a model system for the behaviour of similar lipid species during the fusion of natural membranes, and to study how variations in lipid composition might alter the susceptibility of membranes to fusion. It is suggested by the studies presented here that lipid vesicles prepared from highly purified lipids lend themselves well to the experimental study of these problems. Experiments are also currently in progress in our laboratory on the fusion of lipid vesicles with each other rather than with cells. Using this system it is hoped that it will be possible to define more accurately the factors that can influence the fusion process and provide some insight into the changes occurring during the fusion of more complex natural membranes.

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