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Effect of Transbilayer Phospholipid Distribution on Erythrocyte Fusion

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Phospholipid packing has been suggested as a relevant variable in the control of membrane fusion events. To test this possibility in a model system, a comparison was made of the fusability of erythrocytes with a normal asymmetric transbilayer distribution of plasma membrane phospholipids (tightly packed exterior lipids) and erythrocytes with a symmetric transbilayer distribution of phospholipids (more loosely packed exterior lipids), using polyethylene glycol as fusogen. Not only were lipid-symmetric cells more readily fused, but fusions of mixtures of lipid-symmetric and lipid-asymmetric cells indicated that both fusing partners must have a symmetric distribution for fusion to be enhanced. Lipid-symmetric cells may fuse more readily because loose packing of the exterior lipids enhances hydrophobic interactions between cells. Alternatively, enhanced membrane fluidity may facilitate intramembranous particle clustering, previously implicated as a potentiator of fusion. Finally, exposure of phosphatidylserine on the surface of lipid-symmetric erythrocytes may be responsible for their enhanced fusion.

KEY WORDS: cell fusion; erythrocyte membrane; phospholipid asymmetry; phospholipid packing; phosphatidylserine; polyethylene glycol.

INTRODUCTION

In normal, active animal cells, fusion at the plasma membrane occurs continuously as an integral part of endocytosis and exocytosis. Recently, experiments in which endocytosing cells were stained with the fluorescent dye merocyanine 540 (MC540) suggested that these fusion events may be controlled in part by the packing of phospholipids in the fusing membranes (Schlegel and Williamson, 1988). The dye, which binds preferentially to loosely packed lipid bilayers (Williamson *et al.*, 1983), was preferentially incorporated into the membrane of internalized vesicles, leading to he hypothesis that loose packing of lipids facilitates fusion.

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This hypothesis is testable using erythrocytes in which the packing of phospholipids in the outer leaflet of the plasma membrane can be experimentally manipulated. The two leaflets of the normal erythrocyte bilayer differ in lipid packing, with the outer leaflet being more tightly packed (Williamson *et al.*, 1982), more ordered (Tanaka and Ohnishi, 1976; Seigneuret and Devaux, 1984), and less fluid (Morrot *et al.*, 1986) than the inner leaflet, presumably as a result of higher levels of unsaturated fatty acids in the amino phospholipids which are concentrated in the inner leaflet (Williams *et al.*, 1966; Op den Kamp, 1979). However, erythrocytes can be lysed and resealed under conditions where this asymmetric distribution of phospholipids is lost (Williamson *et al.*, 1985). This randomization of inner and outer leaflet phospholipids results in a more loosely packed outer leaflet, as judged by a large increase in the amount of MC540 bound. Alternatively, erythrocytes can be lysed and resealed under conditions where membrane asymmetry and the normal low affinity for MC540 is maintained (McEvoy *et al.*, 1986). This pair of erythrocyte models, with either tightly or loosely packed exterior phospholipids, provides a system for assessing the role of lipid distribution in fusion of erythrocytes with one another.

MATERIALS AND METHODS

Materials

Equivalent results were obtained using polyethylene glycol (PEG) purchased from either *Fisher Scientific* (Carbowax 8000) or *Koch-Light Laboratories Ltd.* (MW = 6000). Solutions of PEG were prepared fresh daily in Hanks' balanced saline solution (HBSS; 128.5 mM NaCl, 4.2 mM KCl, 1.0 mM Na_2HPO_4, 0.65 mM KH_2PO_4, 15.1 mM $NaHCO_3$, 0.2 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% dextrose, pH 7.35). Stock solutions of MC540 (*Eastman Kodak*) were prepared at a concentration of 1 mg/ml in 50% ethanol and kept at 4°C for no longer than one month.

Preparation of erthrocytes

Freshly drawn human blood was centrifuged, and the serum and buffy coat were removed. Erythrocytes were washed three times with phosphate-buffered saline (PBS; 122 mM NaCl, 3.0 mM KCl, 15.0 mM Na_2HPO_4, 5.0 mM KH_2PO_4, 0.1% dextrose, pH 7.35) containing 0.1% bovine serum albumin (BSA), resuspended to 50% hematocrit in PBS and used within 24 h. Lipid-symmetric erythrocytes and lipid-asymmetric control erythrocytes were prepared using the preswell lysis and resealing method (Williamson *et al.*, 1985; Schlegel and McEvoy, 1987). Briefly, to 0.1 ml of packed preswelled erythrocytes was added 0.4 ml of a hypotonic lysing solution containing 10 mM Tris, 1.0 mM $MgCl_2$, 0.1 mM EGTA, 0.1% BSA, with (lipid-symmetric) or without (lipid-asymmetric) 1.0 mM $CaCl_2$, pH 7.3 at 4°C. After vortex mixing and 2 minutes on ice, 40 μl of buffer containing 1.22 M NaCl, 30 mM KCl, 150 mM Na_2HPO_4, 50 mM KH_2PO_4,

1.0 mM $MgCl_2$, with (lipid-symmetric) or without (lipid-asymmetric) 1.0 mM $CaCl_2$, pH 7.3 was added to restore isotonicity. The suspension was then incubated at 37°C for 40 minutes and the cells washed before use. Packing of the exterior leaflet of lysed and resealed cells was verified by staining with MC540 and assessing dye binding by fluorescence microsopy as previously described (Schlegel *et al.*, 1987).

Fusion

General procedures for PEG-induced fusion of cells in monolayer have been described (Schlegel and McEvoy, 1987). Glass coverslips (22 mm × 22 mm) were cationized by placing in a boiling 1% solution of alcian blue for 5 minutes, rinsed several times in distilled water, and placed into 150 mm petri dishes. Forty ml of erythrocytes suspended at a concentration of 5×10^7 cells/ml in HBSS were added to each dish. The cells were allowed to settle and attach for 30 minutes at room temperature, and then each coverslip was placed into a 35 mm petri dish. Two ml of HBSS was added to each dish, the dish swirled and the supernatant aspirated, to remove nonadherent cells. After residual buffer was removed from coverslips by aspirating along their four edges, they were allowed to stand for one minute before being overlayed with 1.0 ml of PEG solution at various w/w concentrations to induce fusion. Following a 5 minute incubation at room temperature, 3 ml of HBSS was slowly added down the side of the petri dish to dilute the PEG. After repipeting the solution twice to insure complete mixing, the diluted PEG was removed by aspiration. Two mls of HBSS containing 50 μM La^{3+}, to help maintain membrane integrity (Ahkong and Lucy, 1986), was added; and the cultures were placed at 37°C in a CO_2 incubator. After 1 h, the solution was aspirated and a 2% solution of gluteraldehyde in PBS at 4°C was added to fix the cells. After 30 minutes at 4°C the coverslips were rinsed in HBSS, placed face down on a microscope slide in a drop of 90% glycerol and examined by phase contrast microscopy using a 63X objective. To calculate the fusion index, the number of fused cells in ten random fields per coverslip was counted and divided by the total number of cells in those fields, fused or unfused (approximately 2000).

RESULTS

The fusability of erythrocytes was assessed by their susceptibility to fusion induced by polyethylene glycol (PEG). Erythrocytes were attached to coverslips at a density such that a monolayer with numerous cell-cell contacts was formed (Fig. 1a). Cells were then exposed to PEG for five minutes, washed, incubated at 37°C, fixed and examined for fusion.

Figure 1b shows the result obtained following exposure of cells to 50% PEG: erythrocytes attached only by their edge to the coverslip (visible in Fig. 1a) were removed during PEG treatment and washing, so that fewer cells were present. While the majority of the attached cells still appeared as single cells, membranes

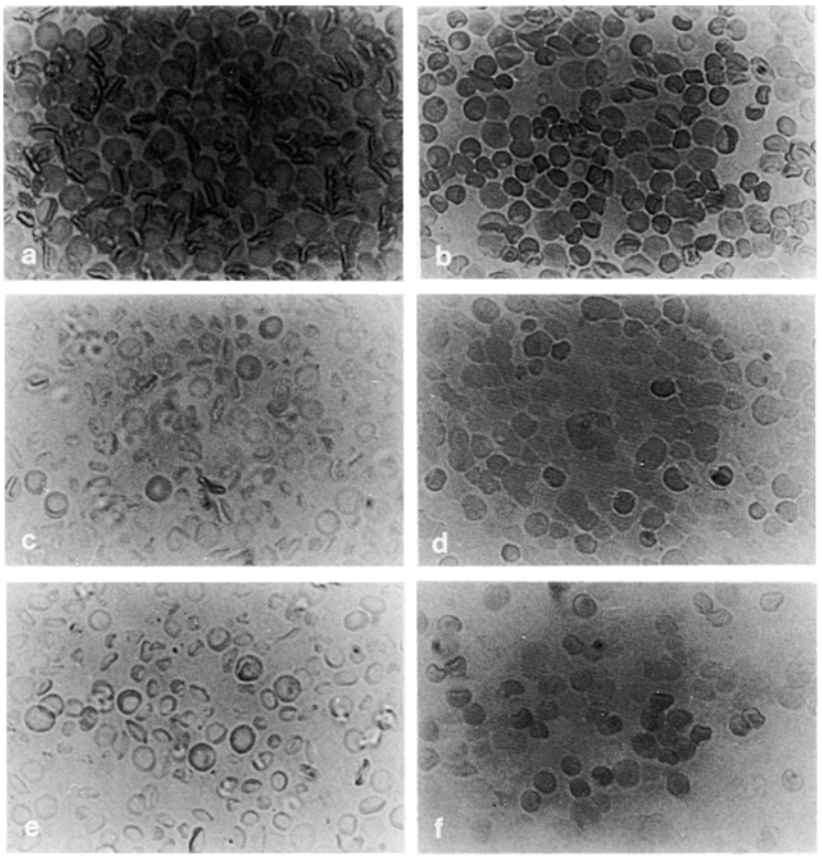

Fig. 1. Fusion of erythrocytes by 50% PEG. Erythrocytes attached to cationized coverslips were treated with a 50% solution of PEG for 5 minutes. After rinsing and a 1 h incubation at 37°C, cells were fixed and the coverslips mounted on microscope slides for analysis by phase contrast microscopy. (a, b), normal erythrocytes; (c, d), lipid-symmetric erythrocytes; (e, f), lipid-asymmetric control erythrocytes; (a, c, e), prior to PEG; (b, d, f), following PEG.

were no longer visible between some of them at points of cell–cell contact, indicative of cell–cell fusion.

When lysed and resealed erythrocytes were plated at the same density as the normal erythrocytes in Fig. 1a, the cells formed a continuous monolayer that was similar to that seen with normal erythrocytes, but more difficult to visualize because of their reduced hemoglobin content. When lipid-symmetric cells (Fig. 1c) were treated with 50% PEG, fusion occurred (Fig. 1d) at a level much higher than that seen with normal erythrocytes with asymmetric membranes. This extensive fusion is not a consequence of lysis and resealing *per se* since lysed and resealed erythrocytes, prepared under conditions where asymmetry was maintained, were no more fused than normal erythrocytes when exposed to 50% PEG (Fig. 1f).

To ensure that the enhanced susceptibility of lipid-symmetric erythrocytes to

fusion was not peculiar to the choice of 50% as the PEG concentration, fusability was measured at other fusogen levels. Following exposure of cells to different concentrations of PEG, fusion was quantified by dividing the number of cells which fused by the total number of cells, both fused and unfused. A representative experiment is shown in Fig. 2. At a PEG concentration of 35%, the lowest concentration at which fusion was induced, the level of fusion of lipid-symmetric erythrocytes was measurable (>2%), while fusion of normal erythrocytes and lipid-asymmetric control cells was not. Exposure to 40% PEG raised fusion of lipid-symmetric cells to 6%, whereas the level of fusion of normal erythrocytes and lipid-asymmetric controls was only 1% or less. At 45% and 50% PEG, the highest concentration which did not induce cell lysis, the level of fusion of lipid-symmetric cells was much higher than lipid-asymmetric controls or normal erythocytes. Similar behavior was observed in six other experiments.

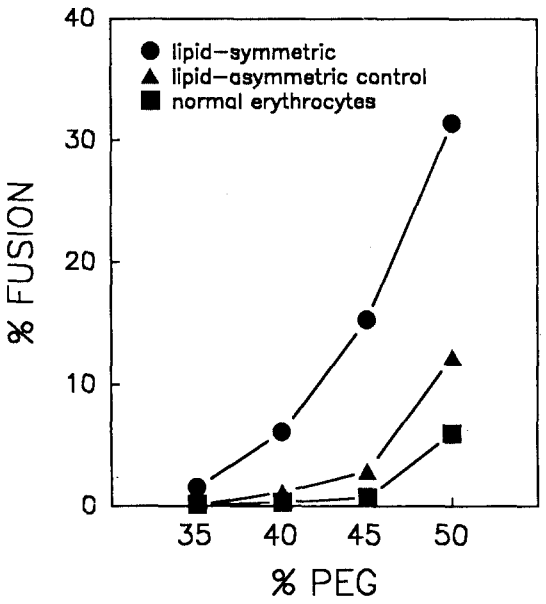

Fig. 2. Effect of phospholipid packing on erythrocyte fusion. Erythrocytes were treated with varying concentrations of PEG, and the percent fusion was quantified.

Since Ca^{2+} is required for PEG-induced fusion of hen erythrocytes (Ahkong *et al.*, 1973, 1975; Maggio *et al.*, 1976), the requirement for Ca^{2+} in the present fusion system was investigated. As shown in Table 1, in the absence of Ca^{2+} and in the presence of EGTA to chelate residual Ca^{2+}, the fusion of both lipid-symmetric and lipid-asymmetric control cells, as well as normal erythrocytes, was reduced to around 1%. Similar results were observed in three other experiments. When Ca^{2+} was not present during any one of the three steps of the fusion procedure (cell attachment, PEG treatment or post-fusion incubation), fusion frequencies were similarly reduced (data not shown), indicating that Ca^{2+} must be present throughout the procedure for fusion to occur.

Table 1. Effect of Ca^{2+} on erythrocyte fusion

	Fusion (%)		
Erythrocytes	$+Ca^{2+}$	$-Ca^{2+}$; EGTA	$-Ca^{2+}$
normal erythrocytes	1.9	1.1	—
lipid-symmetric	20.6	0.8	—
lipid-asymmetric controls	4.0	0.7	—
normal erythrocytes	1.1	—	0.1
lipid-symmetric	15.4	—	0.3
lipid-asymmetric controls	1.9	—	0.2

Since lipid-symmetric erythrocytes are prepared in the presence of Ca^{2+}, whereas lipid-asymmetric controls are not, the Ca^{2+} requirement for fusion raises the possibility that lipid-symmetric cells might leak Ca^{2+}, and thereby enhance fusion by increasing local Ca^{2+} concentrations. To test this possibility, fusions were performed in the absence of both added Ca^{2+} and chelator so that any effects of endogeneously derived Ca^{2+} would be magnified. As Table 1 shows, fusion of lipid-symmetric erythrocytes was not enhanced above lipid-asymmetric controls or normal erythrocytes under these conditions, making it unlikely that release of Ca^{2+} from lipid-symmetric cells is responsible for their higher levels of fusion. Similar behavior was observed in four other experiments.

To determine whether a symmetric distribution of only one partner of a fusing pair is sufficient to enhance fusion, mixtures of lipid-symmetric and lipid-asymmetric control cells were treated with PEG and fusion was measured. Assuming that cells sort into fusing pairs at random, the fusion index of a mixture (I_M) is equal to the sum of the fraction of each type of pair (F) in the mixture multiplied by the fusion index of each pair (I), or:

$$I_M = [(F_S)(F_S)(I_{S-S})] + [2(F_S)] + [2(F_S)(F_A)(I_{S-A})] + [(F_A)(F_A)(I_{A-A})]$$

where the subscript S refers to lipid-symmetric cells and the subscript A to lipid-asymmetric cells. If one lipid-symmetric cell is sufficient to maximally enhance fusion of a pair, then $I_{S-A} = I_{S-S}$. If it has no effect, then $I_{S-A} = I_{A-A}$. On the other hand, if each cell in a pair exerts an effect on fusion, I_{S-A} would equal either $0.5(I_{S-S} + I_{A-A})$ or $[(I_{S-S})(I_{A-A})]^{1/2}$ depending on whether the index was the arithmetic average or the geometric average of the two individual indices.

The values predicted by these possibilities were calculated for various mixtures using for I_{S-S} and I_{A-A} the experimental values from fusions of lipid-symmetric and lipid-asymmetric control cells along, normalized to lipid-symmetric cells and averaged for three experiments. These values as well as the experimental values for mixtures are plotted in Fig. 3. The experimental curve was most closely approximated by the curve produced if the fusion efficiency of a lipid-symmetric/lipid-asymmetric pair is not enhanced over the efficiency of a lipid-asymmetric/lipid-asymmetric pair, indicating that a single lipid-symmetric cell is not sufficient to enhance fusion of a pair. In addition, these results clearly rule out the possibility that trans-acting factors, such as Ca^{2+} released from lipid-symmetric cells, may be enhancing fusion. If positive trans-acting factors

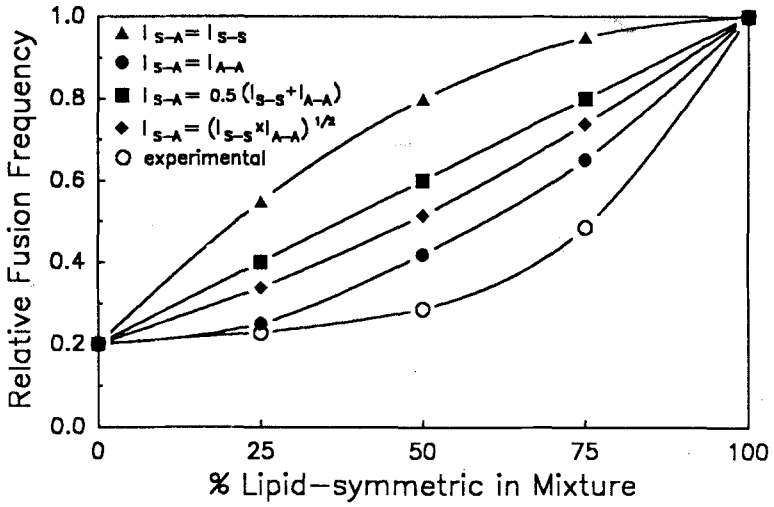

Fig. 3. Theoretical vs experimental fusion frequencies of mixtures of lipid-symmetric erythrocytes and lipid-asymmetric erythrocytes. Mixtures of lipid-symmetric and lipid-asymmetric cells consisting of 100%, 75%, 50%, 25%, and 0% lipid-symmetric cells were prepared, then fused with a 50% solution of PEG. Calculated (see text) and experimental values were normalized to 100% lipid-symmetric cells, averaged for three experiments and plotted as a function of % lipid-symmetric cells.

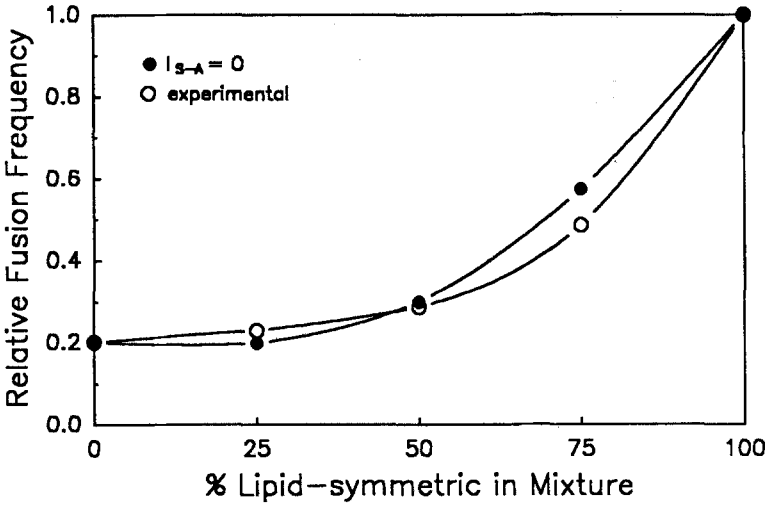

Fig. 4. Theoretical vs experimental fusion frequencies of lipid-symmetric and lipid-asymmetric erythrocyte mixtures. The fusion frequencies calculated assuming $I_{S-A} = 0$ were plotted along with the experimentally obtained values in Fig. 3.

were being released from lipid-symmetric cells, fusion of lipid-asymmetric to lipid-symmetric cells (or to lipid asymmetric cells) would have been enhanced rather than depressed as was observed.

Even so, the experimental values for all mixtures were actually below those predicted if lipid-symmetric-lipid-asymmetric pairs behaved as lipid-asymmetric/lipid-asymmetric pairs. An alternative proposition is that fusion does not occur at all in lipid-symmetric-lipid-asymmetric pairs. Values calculated for a model in which $I_{S-A} = 0$ are plotted in Fig. 4 along with the actual experimental values from Fig. 3. This predicted set of data very closely approximates the actual experimental data, suggesting that lipid-symmetric/lipid-asymmetric pairs do not fuse at all.

DISCUSSION

The results presented here show that PEG-induced fusion of lipid-symmetric erythrocytes with loosely packed exterior leaflets is 5-fold more efficient than fusion of lipid-asymmetric erythrocytes with tightly packed exterior leaflets. Exclusion of trans-acting factors in this enhancement points to the lipids themselves being either directly or indirectly involved in the mechanism by which fusion is enhanced.

The loose packing of the exterior leaflet in lipid-symmetric cells might enhance fusion indirectly by affecting the arrangement of proteins on the cell surface. The inner leaflet of the erythrocyte membrane is more fluid than the outer leaflet (Morrot *et al.*, 1986). Just as randomization of phospholipids in lipid-symmetric cells loosens the packing of the exterior leaflet, so too might the introduction of lipids from the inner leaflet be expected to increase the fluidity of the outer leaflet. A more fluid outer leaflet in turn might increase the lateral mobility of membrane proteins, enhancing the clustering of intramembranous particles (IMPs). Other investigators have found a correlation between clustering of IMPs and the fusion of human erythrocytes (Knutton, 1979; Hui *et al.*, 1985; Huang and Hui, 1986). In the latter study, all chemical pretreatments which enhanced fusion in a system similar to the one used here also resulted in clustering of IMPs in the presence of PEG. Should this be the mechanism by which the fusion of lipid-symmetric cells is facilitated, freeze-fracture analysis should reveal a clustered arrangement of IMP in lipid-symmetric cells in the presence of PEG.

Indirect evidence that this mechanism may be responsible for enhanced fusion comes from studies of erythrocytes from patients with chronic myelogeneous leukemia (CML). Both phospholipase digestion (Kumar and Gupta, 1983) and staining with MC540 (Reed *et al.*, 1985, 1987; Kumar *et al.*, 1987) indicate that phospholipid asymmetry in CML erythrocytes is perturbed, and recently these cells were found to be much more readily agglutinated by concanavalin A than were normal erythrocytes (Basu *et al.*, 1988). Since agglutinability and receptor mobility are probably linked (Nicolson, 1976), these observations suggest that integral membrane proteins in CML erythrocytes may be more

mobile and more easily clustered in the presence of PEG than in erythrocytes with a normal distribution of phospholipids. Experiments are underway to examine the lateral mobility of membrane receptors in both lipid-symmetric erythrocytes and CML erythrocytes.

On the other hand, a looser packing of phospholipids may directly enhance fusion. Cell partitioning in a two-phase aqueous polymer system suggests that the surface of lipid-symmetric erythrocytes is more hydrophobic than the surface of lipid-asymmetric cells (McEvoy *et al.*, 1986), possibly as a result of an increase in headgroup spacing and exposure of the hydrophobic core of the bilayer. Since very close apposition of membranes is a requisite for fusion (Lucy and Ahkong, 1986; Knutton and Pasternak, 1979; Wojcieszyn *et al.*, 1983), increased hydrophobicity may enhance fusion by facilitating this approach.

Finally, the change in the composition of the outer leaflet itself, rather that an alteration in its biophysical properties, might be responsible for enhanced fusion. In the normal erythrocyte, phosphatidylserine (PS) is restricted to the inner leaflet of the membrane. Exposure of PS on the outer surface is therefore a distinguishing property of lipid-symmetric cells. At least with artificial phospholipid vesicles, considerable evidence indicates that Ca^{2+} ions can induce fusion of bilayers when they contain PS (Wilshut *et al.*, 1979, 1980, 1981; Ohki, 1982; Ohki *et al.*, 1984). The introduction of PS into the external leaflet of the erythrocyte plasma membrane may thereby render the bilayer more fusable in the presence of Ca^{2+}. The Ca^{2+} dependence of the fusion of lipid-symmetric cells is consistent with this possibility, although Ca^{2+} was required for fusion of lipid-asymmetric cells as well, with no external PS.

All of these mechanisms are consistent with the finding that enhanced fusion requires that both partners be lipid-symmetric. Not as easily explained is the finding that the experimental data are best fitted by a model in which fusion between lipid-symmetric/lipid-asymmetric partners does not take place. At present it can only be concluded that the physical properties of opposing membranes must be the same before fusion can be induced; why incompatibility would prevent fusion is not known. Regardless, the present studies strongly implicate distribution of phospholipids as a controlling factor for cell fusability in the presence of PEG. If preferential staining of endocytosing membrane by MC540 has the same implications as it does for the membranes of erythrocytes, lipid distribution may play a similar role in natural cell fusion events. Local loss of lipid asymmetry, revealed by MC540 binding, in the presence of normal extracellular Ca^{2+} concentrations could facilitate membrane fusion at the external face, resulting in the formation of endocytic vesicles.

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