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MINI-REVIEW

Molecular Mechanisms of Calcium-Induced Membrane Fusion

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Abstract

We have reviewed studies on calcium-induced fusion of lipid bilayer membranes and the role of synexin and other calcium-binding proteins (annexins) in membrane fusion. We have also discussed the roles of other cations, lipid phase transitions, long chain fatty acids and other fusogenic molecules. Finally, we have presented a simple molecular model for the mechanism of lipid membrane fusion, consistent with the experimental evidence and incorporating various elements proposed previously.

Key Words: Liposomes; annexins; membrane-fusion; model-membranes; calcium; lipid-bilayer; calcium-binding-proteins.

Introduction

Calcium is known to be an essential requirement in many biological membrane fusion events. The mechanism of its action, however, is not well understood. Calcium may directly mediate the fusion reaction by interacting with phospholipids, or it may induce a conformational change in a fusogenic protein. Calcium may also activate particular enzymes which trigger the formation of the fusogenic state, or it may initiate a cascade of reactions which eventually mediate fusion (Papahadjopoulos *et al.*, 1988).

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It was first reported in the early seventies that membrane vesicles composed of pure phospholipids (liposomes) can undergo fusion in the presence of Ca^{2+} (Papahadjopoulos *et al.*, 1974). This raised the intriguing possibility that Ca^{2+} could mediate membrane fusion by interacting with negatively charged phospholipids in biological membranes (Papahadjopoulos *et al.*, 1976, 1977). Since phosphatidylserine is the most abundant negatively charged lipid found in cell membranes, the fusion behavior of liposomes composed of pure phosphatidylserine has attracted much attention (Papahadjopoulos *et al.*, 1979; Nir *et al.*, 1983a; Düzgünes, 1985). The fusion characteristics of membranes composed of zwitterionic phospholipids in conjunction with phosphatidylserine, as well as other negatively charged phospholipids, such as phosphatidate (phosphatidic acid), phosphatidylglycerol, phosphatidylinositol, and cardiolipin, have been studied in detail (Sundler, 1984; Wilschut and Hoekstra, 1984; Düzgünes *et al.*, 1985; Prestegard and O'Brien, 1987). The specificity of Ca^{2+} over Mg^{2+} in the fusion of pure phospholipid vesicles (Papahadjopoulos *et al.*, 1974; Wilschut *et al.*, 1981), and the modulation of fusion by low-molecular-weight molecules including phosphate (Fraley *et al.*, 1980) and polyamines (Hong *et al.*, 1983; Schuber *et al.*, 1983; Meers *et al.*, 1986) have pointed to the relevance of such simple systems for understanding the control of membrane fusion at the intracellular and intercellular level (Papahadjopoulos *et al.*, 1988). Liposomes are very simple models of biological membranes, and because of that, they provide a convenient system to study the role of individual membrane components in a very complex biological phenomenon such as membrane fusion. The development of sensitive assays to study the kinetics of membrane fusion have also relied on the well-defined and reproducible fusion characteristics of liposomes (Wilschut *et al.*, 1980; Uster and Deamer, 1981; Struck *et al.*, 1981; Ellens *et al.*, 1985; Düzgünes and Bentz, 1988).

The liposome fusion system can be used to study the role of other molecules such as cytoplasmic and membrane proteins (Düzgünes, 1985; Hong *et al.*, 1987; Ohnishi, 1988). The discovery that a cytoplasmic protein, synexin, could mediate the aggregation of isolated secretory granules in the presence of low Ca^{2+} concentrations (Creutz *et al.*, 1978), and could also facilitate the Ca^{2+}-induced fusion of phospholipid vesicles (Hong *et al.*, 1981), suggested that such proteins could be molecular targets of Ca^{2+} in the cytoplasm. Other phospholipid- and calcium-binding proteins have since been identified (Klee, 1988) and the term *annexins* has been proposed for these proteins (Geisow *et al.*, 1987). These include the chromobindins that bind to chromaffin granule membranes in the presence of Ca^{2+} (Creutz *et al.*, 1983), the calelectrins and calpactins, which aggregate secretory vesicles (Südhof *et al.*, 1982; Drust and Creutz, 1988), the endonexins and lipocortins with substantial similarities in molecular structure (Geisow, 1986; Südhof

et al., 1988; Crompton *et al.*, 1988), and synexin-like proteins from human polymorphonuclear leukocytes (Meers *et al.*, 1987).

Calcium-Induced Fusion of Phospholipid Vesicles

Following the first report that liposomes could be used as a model for membrane permeability studies (Bangham *et al.*, 1965), it was observed that the addition of Ca^{2+} could induce aggregation and release of contents of phosphatidylserine liposomes (Papahadjopoulos and Bangham, 1966), although the reaction was not identified as fusion until several years later (Papahadjopoulos *et al.*, 1974). Phosphatidylserine is probably the most extensively studied lipid with respect to interactions with divalent cations and membrane fusion (Papahadjopoulos *et al.*, 1979; Düzgünes and Papahadjopoulos, 1983; Nir *et al.*, 1983a).

Divalent cation binding to the membrane surface appears to be one of the critical determinants of fusion (Nir *et al.*, 1980a; Düzgünes *et al.*, 1980, 1981a). Under conditions where aggregation is rate limiting, for example in the case of small unilamellar vesicles (SUV)[5] composed of phosphatidylserine in 100 mM Na^+, the bulk concentration of divalent cations which induce fusion at a threshold rate of 10% maximal Tb fluorescence/minute increases in the sequence $Ba^{2+} < Ca^{2+} < Sr^{2+} < Mg^{2+}$ (Bentz *et al.*, 1983b). This sequence is the same found for the aggregation of these vesicles (Ohki *et al.*, 1982). Increasing the monovalent salt concentration renders the aggregation step very rapid compared to the fusion step, allowing for the direct determination of the rate of membrane fusion *per se* (Bentz *et al.*, 1983b; Nir *et al.*, 1983b; Braun *et al.*, 1985). Under these conditions Ca^{2+} is more effective than Ba^{2+}; i.e., less Ca^{2+} is bound per phosphatidylserine molecule than Ba^{2+} at the fusion threshold.

Large unilamellar vesicles (LUV) composed of different acidic phospholipids aggregate and fuse in the presence of different threshold concentrations of divalent cations (Table I). There is an absolute specificity for Ca^{2+} over Mg^{2+} in inducing the fusion of LUV composed of phosphatidylserine (Wilschut *et al.*, 1981; Table I). This specificity probably arises during interbilayer contact, since Ca^{2+} can form an anhydrous complex with phosphatidylserine only if the two interacting membranes are allowed to come into close contact (Portis *et al.*, 1979). Ca^{2+} has a much higher affinity for phosphatidylserine in this "*trans*" (intermembrane) binding mode (Portis *et al.*, 1979; Rehfeld *et al.*, 1981; Ekerdt and Papahadjopoulos, 1982; Nir,

[5] Abbreviations: LUV, large unilamellar vesicles; PMN, polymorphonuclear leukocytes; SUV, small unilamellar vesicles.

Table I. Threshold Concentrations (mM) of Ca^{2+} and Mg^{2+} for Inducing the Aggregation and Fusion of LUV Composed of Pure Phospholipids at pH 7.4 and 25°C (adapted from Düzgünes *et al.*, 1985 and Papahadjopoulos, *et al.*, 1988)

Phospholipid	Aggregation		Fusion[a]	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
Phosphatidylinositol	3	6	—	—
Phosphatidylglycerol[b]	5	20	15	—
Phosphatidylserine	2	5[c]	2[d]	—
Phosphatidate	0.2	0.4	0.2	0.4

[a]The dashes indicate that no fusion is observed. For smaller vesicles, the threshold concentrations are significantly lower.
[b]Data from Sundler (1984), Rosenberg *et al.* (1983), and N. Düzgünes, unpublished.
[c]Aggregation in a secondary minimum can occur in the presence of 2.5 mM Mg^{2+} and 200 mM Na^{+} (Nir *et al.*, 1981).
[d]This approximate value for the threshold concentration is for bovine brain phosphatidylserine. For dioleoylphosphatidylserine the threshold concentration is below 1.5 mM (D. Alford, N. Düzgünes, and S. Nir, unpublished).

1984; Feigenson, 1986). The specificity of Ca^{2+} over Mg^{2+} is altered when phosphatidylethanolamine or cholesterol are present in the vesicle membrane; such vesicles undergo fusion in the presence of Mg^{2+}, although at a slower rate than with Ca^{2+} (Düzgünes *et al.*, 1981b; Bental *et al.*, 1987; Düzgünes, 1988; Shavnin *et al.*, 1988). The biophysical basis for this Ca^{2+}/Mg^{2+} specificity has been discussed previously in detail (Düzgünes *et al.*, 1981b, 1985; Sundler *et al.*, 1981; Wilschut *et al.*, 1981; Düzgünes and Papahadjopoulos, 1983). The ability of Mg^{2+} to cause aggregation of phosphatidylserine LUV without fusion provides an experimental system where the fusion reaction *per se*, induced by other agents, is rate-limiting (Bentz and Düzgünes, 1985). This system has revealed that the amount of bound Ca^{2+} per phosphatidylserine molecule at the "threshold" of fusion is about 0.15. Surprisingly, this number is the same for SUV and LUV (Bentz and Düzgünes, 1985). The fusion rate constant of SUV, however, increases more rapidly with the amount of bound Ca^{2+} than that of LUV (Düzgünes *et al.*, 1987a).

A variety of phospholipids have been shown to undergo a transition from the lamellar to the inverted hexagonal (H_{II}) phase (Luzzati and Tardieu, 1974; Cullis and de Kruijff, 1979; Verkleij, 1984; Gruner *et al.*, 1985; Siegel, 1984; Siegel *et al.*, 1989; Das and Rand, 1986). The bilayer–hexagonal transition has been proposed to be the driving force for membrane fusion, since this transition has been observed under conditions which also induce the fusion of certain membranes (Cullis and Hope, 1978; Cullis and Verkleij, 1979). Considerable evidence has accumulated against the hypothesis that the bilayer–hexagonal phase transition is involved as an intermediate in divalent

cation-induced fusion of phospholipid and other lipid vesicles (Düzgünes *et al.*, 1987a; Ellens *et al.*, 1986; Rupert *et al.*, 1987). Recent work with phosphatidylethanolamine-containing vesicles has increased our understanding of the role of aggregation in the leakage of aqueous contents of the vesicles (Ellens *et al.*, 1986; Allen *et al.*, 1990), and the possible involvement of other nonbilayer lipid structures, such as the cubic phase, in membrane fusion (Gruner *et al.*, 1988; Ellens *et al.*, 1989). This phase is observed as a stable structure with some N-methylated phosphatidylethanolamines at temperatures below the bilayer–hexagonal transition (Gruner *et al.*, 1988) and is accompanied by nonleaky fusion (Ellens *et al.*, 1989), unlike the hexagonal phase which is characterized by lipid mixing without any mixing of contents (Ellens *et al.*, 1986). Whether a cubic phase intermediate is involved in the fusion of natural (nonmethylated) phosphatidylethanolamines when they aggregate and fuse below the hexagonal transition temperature is not known at present (Allen *et al.*, 1990).

It has been proposed that lipidic particles could be intermediate structures in the fusion of liposomes of some compositions (Verkleij *et al.*, 1979, 1980; Hope *et al.*, 1983; Verkleij, 1984). When LUV composed of cardiolipin/phosphatidylcholine or phosphatidylserine/phosphatidylethanolamine (1 : 1) are quick-frozen immediately after stimulation with Ca^{2+} and examined by freeze-fracture electron microscopy, either smooth elongated bridges or a tight lip structure between fusing liposomes are observed (Bearer *et al.*, 1982). Lipidic particles are visible only after prolonged incubation of the vesicles with Ca^{2+}, or if glycerol is present as a cryoprotectant (Bearer *et al.*, 1982). These observations have been corroborated by subsequent studies (Verkleij *et al.*, 1984; Hui *et al.*, 1988). Lipidic particles, as defined by their morphology in freeze-fracture electron microscopy, thus do not appear to be involved in the membrane fusion reaction, even in membrane systems in which they are observed after long periods of incubation. Nonbilayer structures probably occur at the sites of fusion at rates too fast to be visualized by morphological studies, or may be confined to a small area in the contact zone between fusing membranes (Bearer *et al.*, 1982; Düzgünes *et al.*, 1985a; Siegel, 1984). The membrane fusion "intermediate" could be a local defect in membrane structure that allows the destabilization and fusion of the apposed membranes (Papahadjopoulos *et al.*, 1977; Hui *et al.*, 1981; Düzgünes *et al.*, 1984). We have proposed that these nonbilayer intermediates could transform in time to more stable structures, such as lipidic particles, the hexagonal phase, or the crystalline bilayer (Bearer *et al.*, 1982). It should be noted, however, that in the presence of large Ca^{2+} concentrations, the times required for the actual fusion process are rather short, of the order of milliseconds. This follows from the magnitude of the fusion rate constants determined (Nir *et al.*, 1982, 1983a; Bentz *et al.*, 1983a, 1985; Wilschut *et al.*, 1985a), and from the

observations of Miller and Dahl (1982). Thus, while it may be hard to observe a fast fusion event, it may be even harder to record intermediate stages of fusion events.

Quantitative analysis of the rate of membrane fusion using fluorescence assays has indicated that for many of the liposome types studied, the rates of lipid mixing and aqueous contents mixing are faster than the leakage of the contents into the medium during fusion (Wilschut *et al.*, 1980, 1983, 1985a,b; Nir *et al.*, 1980b, 1982; Bentz *et al.*, 1983a,b; Düzgünes *et al.*, 1981a,b, 1987b; Bental *et al.*, 1987). Certain types of vesicles can fuse without releasing any aqueous contents (Wilschut *et al.*, 1983; Bentz *et al.*, 1988; Hui *et al.*, 1988), while some leak their contents extremely rapidly when fusion is induced (Sundler and Papahadjopoulos, 1981). Such biophysical evidence has been crucial in understanding whether the vesicles fuse in a biologically relevant manner, with intermixing of aqueous contents and no lysis, or lysis without intermixing of aqueous contents (Düzgünes and Bentz, 1988; Bentz and Ellens, 1988).

Large multilamellar vesicles (1–10 μm diameter) composed of or containing phosphatidylserine have been shown by light and electron microscopy to adhere to and deform each other in the presence of Ca^{2+} (Rand *et al.*, 1985; Kachar *et al.*, 1986). The resulting bilayer stress has been proposed to cause the rupture of the membranes, resulting in the lysis of some vesicles and the fusion of others. Based on these observations, Rand *et al.* (1985) have concluded that the mechanism of fusion between such vesicles may be fundamentally different from cellular fusion events. Kachar *et al.* (1986) have argued that such stochastic processes could not be involved in controlled fusion phenomena in cellular systems. Citing extensive data, Wilschut (1988) has pointed out, however, that the vesicle membranes preferentially break at the site of the intermembrane diaphragm, due to the presence of local defects in lipid packing, and that the degree of bilayer destabilization at this site would determine to what extent the vesicle would have to be deformed before fusion occurs. If the lipid perturbations are minimal, then some strain on the bilayer, such as osmotic strain (Ohki, 1984; Akabas *et al.*, 1984), would be required to induce fusion (Wilschut, 1988). We have indicated previously (Düzgünes and Bentz, 1988) that the microscopic studies mentioned above (Rand *et al.*, 1985; Kachar *et al.*, 1986) were performed with vesicles in the presence of very low ionic strength medium (only 2 mM TES buffer), which would increase the amount of Ca^{2+} bound per phosphatidylserine severalfold higher above the threshold required for fusion (Düzgünes, 1985). The high concentration of vesicles and of Ca^{2+}, and the low ionic strength may thus drive the fusion reaction to the state of vesicle collapse. On the contrary, such collapse is reached only subsequent to contents mixing and fusion in fluorescence experiments utilizing 100 mM Na^+

and relatively low vesicle concentrations (Wilschut *et al.*, 1980; 1981). The latter conditions allow fusion to proceed in a more controlled manner, where the fusion of vesicle dimers and trimers predominates before higher-order aggregates form (Düzgünes and Bentz, 1988). We do not wish to imply that the fusion of cellular membranes takes place via reactions identical to those of pure phosphatidylserine vesicles, since cytoplasmic and membrane proteins are most likely involved in these events. We want to emphasize, however, that both fluorescence assays and freeze-fracture electron microscopy do reveal that the fusion of phospholipid vesicles of various compositions occurs in a controlled manner and is probably physiologically relevant.

Under certain conditions, lipid mixing can occur in the absence of contents mixing (Rosenberg *et al.*, 1983; Ellens *et al.*, 1985). For LUV composed of cardiolipin/phosphatidylcholine (1 : 1), lipid mixing was found in most cases to occur at the same rate as contents mixing (Wilschut *et al.*, 1985a). Lipid mixing proceeds at a faster rate than contents mixing in the case of phosphatidylserine LUV (Wilschut *et al.*, 1985b; Düzgünes *et al.*, 1987b, 1988). The latter observation suggests that the destabilization process leading to the mixing of the lipids in the outer monolayers of the vesicles and that leading to complete mixing of the bilayers and the aqueous contents may be different processes (Düzgünes *et al.*, 1987b, 1988). It is also possible that lipids exchange during the reversible aggregation of the vesicles before the fusion reaction (Wilschut *et al.*, 1985b).

Role of Proteins in Calcium-Induced Membrane Fusion

A comparison of liposome fusion with the fusion of certain biological membranes indicates a considerable difference in the concentration of Ca^{2+} required (Gratzl *et al.*, 1980; Ekerdt *et al.*, 1981). Micromolar free Ca^{2+} concentrations in the cytoplasm are sufficient to induce exocytosis in a variety of cellular systems (Baker *et al.*, 1980; Baker, 1988; Baker and Knight, 1984; Dunn and Holz, 1983; Wilson and Kirshner, 1983). The fusion of isolated secretory granules in the presence of micromolar concentrations of Ca^{2+} is thought to be mediated by membrane glycoproteins (Dahl *et al.*, 1979; Gratzl *et al.*, 1980). The fusion of liposomes, on the other hand, requires millimolar Ca^{2+} concentrations (Table I). Possible sources of this difference are the necessity to overcome the electrostatic, hydration, and steric repulsion between liposomes, and the relatively low concentration of liposomes that must be used in most fusion experiments, compared to the concentration of secretory vesicles at the sites of exocytosis (Papahadjopoulos *et al.*, 1988). Establishment of close contact between negatively charged

phospholipid–glycolipid membranes by means of lectins results in the reduction of the threshold Ca^{2+} concentration required for fusion, particularly when the membrane contains phosphatidic acid (Sundler and Wijkander, 1983; Düzgünes *et al.*, 1984b; Hoekstra and Düzgünes, 1986; Düzgünes and Hoekstra, 1986). The threshold Ca^{2+} concentration can be lowered by the presence of cytoplasmic components including Mg^{2+} and spermine (Portis *et al.*, 1979; Düzgünes *et al.*, 1981b; Schuber *et al.*, 1983). The fusion of cellular membranes at Ca^{2+} concentrations several orders of magnitude lower than that required for the fusion of liposomes composed of similar phospholipids is likely to be mediated by a host of cytoplasmic and membrane-bound molecules.

Ca^{2+}-binding proteins and other cytoplasmic components may be involved in mediating the response to Ca^{2+}. Synexin may be one such protein. It is a Ca^{2+}-dependent membrane-binding protein originally isolated from bovine adrenal medulla (Creutz *et al.*, 1978, 1979). Synexin is one of the "chromobindins," a set of cytoplasmic proteins that bind to chromaffin granule membranes in the presence of Ca^{2+} (Creutz *et al.*, 1983), and elutes from the membranes at 4 μM free Ca^{2+} (Creutz and Sterner, 1983). Chromaffin granules aggregate in the presence of synexin when the Ca^{2+} concentration exceeds 6 μM (Creutz *et al.*, 1978), and they undergo fusion when *cis*-unsaturated free fatty acids are added (Creutz, 1981). Synexin induces the fusion of granule ghost membranes when the pH is lowered to 6, even when the Ca^{2+} concentration is 0.1 μM (Nir *et al.*, 1987).

Calcium-induced aggregation and fusion of negatively charged liposomes is facilitated by synexin, and the threshold Ca^{2+} concentration for the fusion of certain types of liposomes is reduced to 10 μM (Hong *et al.*, 1981, 1982a,b, 1987; Düzgünes *et al.*, 1980). The rate of Ca^{2+}-induced fusion of liposomes containing phosphatidylserine and/or phosphatidic acid is enhanced by synexin (Hong *et al.*, 1981, 1982a,b). With phosphatidate/phosphatidylethanolamine liposomes the initial rate of Ca^{2+}-induced fusion increases by three orders of magnitude (Hong *et al.*, 1982a). Ca^{2+}-induced fusion of phosphatidylinositol-containing liposomes, however, is inhibited by synexin (Hong *et al.*, 1982a). The facilitation of fusion by synexin is drastically reduced by the presence of phosphatidylcholine in the membrane, although membrane aggregation is still enhanced (Hong *et al.*, 1982b). Pure phosphatidylcholine vesicles do not aggregate in the presence of synexin and Ca^{2+}.

Facilitation of membrane fusion by synexin could be effected either by an enhancement of the rate of aggregation of liposomes, or by an action as a fusogenic protein thus affecting the rate of fusion itself. The overall membrane fusion reaction between two vesicles has been modeled kinetically as a two-step process involving aggregation followed by the fusion step (Nir *et al.*, 1980b, 1982, 1983a; Bentz *et al.*, 1983a). The slower of the two steps becomes

rate-limiting and controls the rate of the overall process. It is possible to convert liposome fusion systems that are largely rate-limited by aggregation into systems that are rate-limited by the fusion step, by altering the phospholipid composition and the ionic environment (Bentz *et al.*, 1983b; Bentz and Düzgünes, 1985; Meers *et al.*, 1988b). When fusion is rate-limiting, synexin exhibits either an inhibitory effect, or no effect at all. When aggregation rate-limiting conditions are used, synexin enhances the overall fusion rate, indicating that the protein functions by increasing the aggregation rate. The synexin-mediated increase in the aggregation rate can also be modeled quantitatively using mass action kinetics (Meers *et al.*, 1988a).

Membrane-bound products of phosphatidylinositol turnover that accompany exocytosis (Michell, 1975; Michell *et al.*, 1981) may also be involved in the facilitation of the fusion of intracellular membranes by synexin (Creutz, 1981). Arachidonic acid, one of the products of this turnover, has a synergistic effect on the action of synexin, producing an enhancement of the overall rate of fusion of phospholipid vesicles in the presence of synexin, under experimental conditions where it has no effect by itself (Meers *et al.*, 1988b). Arachidonic acid as well as other fatty acids do enhance the rate of fusion in the absence of synexin. When present together, fatty acids and synexin could act synergistically, synexin enhancing the rate of aggregation and fatty acids enhancing the rate of fusion. The effect of the fatty acids on the overall fusion rate becomes apparent only because synexin, by increasing the aggregation rate, renders the fusion reaction relatively slower and more rate-limiting, at which point the fatty acid effect appears. The observation that this effect is nonspecific in terms of the fatty acid species also suggests that it is not a result of a direct interaction of fatty acids with synexin. Furthermore, the fatty acid synergism is observed with other promoters of the aggregation rate of liposomes besides synexin, such as spermine and Mg^{2+}. While the fatty acid effect is not specific for synexin, it is an example of one way in which phospholipid metabolites could play a role in fusion, along with synexin (Papahadjopoulos *et al.*, 1988).

The above properties of synexin are not shared by several other Ca^{2+}-binding proteins, such as calmodulin, prothrombin, or parvalbumin, which are either inhibitory or have no effect on the rate of fusion of liposomes (Hong *et al.*, 1981, 1982a). Two other proteins that have synexin-like characteristics are the 67-kD and 32-kD calelectrins from bovine liver (Südhof *et al.*, 1984). These proteins bind to phospholipid membranes at approximately 5 μM Ca^{2+}. The 32-kD calelectrin increases the rate of Ca^{2+}-induced fusion of liposomes composed of phosphatidate–phosphatidylethanolamine, but unlike synexin, inhibits the fusion of phosphatidylserine liposomes (P. Meers, K. Hong, and D. Papahadjopoulos, unpublished data). The Ca^{2+}-induced fusion of both types of liposomes is inhibited by the 67-kD calelectrin, which may be identical with synhibin (Pollard and Scott, 1982; Creutz *et al.*, 1987).

The mechanism by which synexin-like proteins bind to membranes is still not well understood. While it is clear that the binding is Ca^{2+}-dependent and at least partially reversible (Creutz *et al.*, 1983; Meers *et al.*, 1987a), it is not known what the requirements for binding are and how they relate to the function of the proteins. Synexin most likely binds to the phospholipid segment of chromaffin granule membranes (Morris *et al.*, 1982). Synexin and the calelectrins self-aggregate in the presence of Ca^{2+} (Creutz *et al.*, 1979; Südhof *et al.*, 1982), suggesting that they are bipolar molecules that can interact with and link two membranes. However, there is no clear evidence that the self-association relates to membrane binding. Self-association of synexin is actually inhibitory to the enhancement by monomeric synexin of the overall fusion rate (Hong *et al.*, 1987; Meers *et al.*, 1988a). Calcium-dependent binding of synexin to phospholipid membranes has been measured by differential centrifugation of liposomes rendered dense by encapsulation of metrizamide, and assaying for synexin activity remaining in the supernatant (Meers *et al.*, 1987b). Synexin binds preferentially to negatively charged phospholipids, and to phosphatidylethanolamine, but does not bind to phosphatidylcholine. It has not been determined as yet whether synexin penetrates into the phospholipid bilayer. Our own data do not indicate extensive hydrophobic interactions during synexin's binding (P. Meers, K. Hong, and D. Papahadjopoulos, unpublished results). However, a recent report from Pollard's laboratory (Burns *et al.*, 1989) indicates that human synexin can produce voltage-sensitive calcium channel activity in phosphatidylserine bilayers. The same report gives information on the sequence of synexin, indicating the presence of a unique, highly hydrophobic N-terminal domain, and a C-terminal domain with pronounced homology to the "consensus sequence" of other calcium-dependent membrane-binding proteins (annexins). It has been suggested that synexin can undergo a conformational change which makes it possible to insert completely into the lipid bilayer (Burns *et al.*, 1989). If this is the case, it should be possible to identify such membrane-embedded domains by specific labeling with lipid-soluble photoaffinity labels.

Recent work has demonstrated the presence of Ca^{2+}-dependent phospholipid-binding proteins in the cytosol of polymorphonuclear leukocytes (PMN) (Ernst *et al.*, 1986; Meers *et al.*, 1987a). These synexin-like proteins were purified using an affinity column with covalently coupled liposomes composed of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine. Three major proteins were obtained with molecular masses of approximately 67, 47 and 28 kD, as determined by polyacrylamide gel electrophoresis. The 47-kD band bound rabbit antiserum to bovine liver synexin, and only a 47-kD band appeared in Western blots of whole PMN cytosol. Two-dimensional electrophoresis showed that the 47-kD protein has an

isoelectric point of approximately 7.0, as does bovine liver synexin (Ernst *et al.*, 1986).

The functional verification of synexin-like proteins in human PMN was further established by testing their activities in granule aggregation and liposome fusion. Both the PMN proteins and bovine liver synexin promoted the Ca^{2+}-induced fusion of liposomes composed of phosphatidate/phosphatidylethanolamine as well as phosphatidylserine/phosphatidylethanolamine. Another measure of synexin activity was the Ca^{2+}-dependent aggregation of specific granules isolated from human PMN. In the absence of synexin, no aggregation of granules was observed up to 10 mM Ca^{2+}. By contrast, 0.4 mM free Ca^{2+} was sufficient to aggregate the granules in the presence of bovine synexin or the PMN proteins. The aggregation was also inhibited by 70% in the presence of 5 μM trifluoperazine (Ernst *et al.*, 1986). The ability of synexin to mediate the fusion of specific granules with liposomes was also tested, using fluorescent lipid probes incorporated into either the liposome (Struck *et al.*, 1981) or granule membranes (Hoekstra *et al.*, 1984). Dilution of these probes into unlabeled membranes upon fusion results in an increase in fluorescence intensity. In the presence of synexin and arachidonic acid, phosphatidate/phosphatidylethanolamine liposomes fused with specific granules upon addition of Ca^{2+} (Meers *et al.*, 1987a). Similarly, octadecylrhodamine-labeled granules fused with unlabeled liposomes. No fusion was apparent if either one of Ca^{2+}, synexin, or arachidonic acid was omitted. The arachidonic acid requirement was particularly interesting in light of the fact that arachidonate is produced upon stimulation of PMN to degranulate (Stenson and Parker, 1979; Waite *et al.*, 1979; Walsh *et al.*, 1981). These results suggest that the fusion of PMN granules with phagosomes or the plasma membrane during degranulation may be mediated by synexin and synexin-like proteins.

Molecular Mechanisms

The structural transitions of phospholipid bilayers during membrane fusion are expected to occur locally at the area of intermembrane contact, thus precluding the formation of a long-range order that can be detected by microscopic or even spectroscopic techniques. These transitions would also be too rapid to be detected by observations on the time scale of seconds to minutes. The putative intermediate structures during membrane fusion have therefore been surmised from equilibrium measurements of the phase behavior of phospholipid membranes.

The earliest studies on the fusion of phosphatidylserine vesicles in the presence of Ca^{2+} suggested that structural defects, domain boundaries, or

regions of transient hydrocarbon–water contact are the points at which membrane intermixing between adhering vesicles takes place (Papahadjopoulos *et al.*, 1977). Point defects have been observed by freeze-fracture electron microscopy in membranes induced to fuse by freezing and thawing (Hui *et al.*, 1981). Membrane fusion has been associated with an increased surface hydrophobicity or increased surface energy (Ohki and Düzgünes, 1979; Ohki, 1982), which translates in molecular terms to the exposure of the hydrocarbon surface upon divalent cation binding to the phospholipid headgroups (Ohki and Ohshima, 1984), consistent with the concept of point defects. The short-range steric and hydration repulsive forces between membranes (Rand, 1981; Marra and Israelachvili, 1985; McIntosh *et al.*, 1987) may be overcome by such alterations in the local structure of the membrane (Horn, 1984; Wilschut, 1988). These local alterations in membrane structure may be governed by both *intra*bilayer and *inter*bilayer forces (Portis *et al.*, 1979; Düzgünes and Papahadjopoulos, 1983; Marra and Israelachvili, 1985; Markin *et al.*, 1984; Leikin *et al.*, 1987; Chernomordik *et al.*, 1987; Helm *et al.*, 1989). In some phospholipid vesicle systems, the thermal fluctuations of the phospholipid molecules are thought to result in a local rupture of the two monolayers in contact and in the formation of a stalk between the membranes; this stalk then enlarges to form a single bilayer diaphragm (Markin *et al.*, 1984; Chernomordik *et al.*, 1987). The local rupture, or outward bending, of the monolayer has been proposed to depend on the shape (headgroup/acyl chain ratio) of the phospholipid molecules (Chernomordik *et al.*, 1985). Vesicles composed of phospholipids that can revert to an inverted isotropic phase (Gruner *et al.*, 1985) undergo fusion at the temperature of this transition, either at low pH or in the presence of Ca^{2+} or Mg^{2+}, suggesting that fusion involves such inverted micellar structures (Ellens *et al.*, 1986, 1989; Siegel *et al.*, 1988; Bentz and Ellens, 1988). Other phase transitions, such as the gel–liquid crystalline transition, also appear to control the fusion susceptibility of phospholipid membranes (Düzgünes *et al.*, 1984a; Wilschut *et al.*, 1985c; Wilschut, 1988).

The schemes presented in Fig. 1 are hypothetical stages in the molecular rearrangements during fusion of two lipid bilayer membranes composed of various phospholipids. We present three alternative pathways which share the same initial and final stages: contact-induced defects (CID, stage 3) and curved bilayer annulus (CBA, stage 5). Some of the intermediates shown in stages 3 and 4 would be the preferred ones depending on the lipid molecule (phosphatidylserine vs. phosphatidylethanolamine vs. phosphatidylcholine) and the agent promoting fusion (Ca^{2+} vs. other cations vs. poly(ethylene glycol). Since the intermediates described in stages 3, 4, and 5 are conceptually related, although conformationally distinguishable, we present them collectively as a "consensus" model. The scheme incorporates ideas and

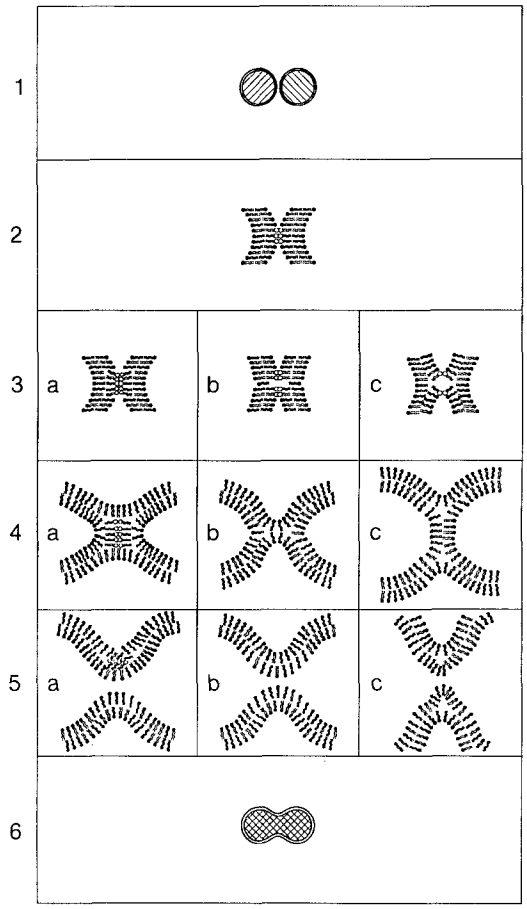

Fig. 1. Hypothetical molecular rearrangements during fusion of two lipid bilayer membranes composed of various phospholipids (see text for details).

suggestions presented earlier both by us and by others (Papahadjopoulos *et al.*, 1977; Hui *et al.*, 1981; Ohki, 1982, 1984; Düzgünes *et al.*, 1987a; Leikin *et al.*, 1987; Wilschut, 1988; Hoekstra and Wilschut, 1989). One distinguishing feature is the absence of intermediates based on equilibrium lipid structures such as "inverted micelles," hexagonal phase," "cubic phase," or "anhydrous gel phase." Our intermediates are much simpler configurations that may incorporate some features from the above structures but are not recognizable unit cells of any specific lipid phase.

Specific intermediate stages during fusion, shown in Figure 1:

1. Two intact liposomes which are not yet at surface contact. In this case Ca^{2+} is bound to the head groups at a relatively low binding mode (Portis

et al., 1979; Ekerdt and Papahadjopoulos, 1982) (Ca/phosphatidylserine binding coefficient of 30 to 35 M^{-1}; Nir, 1984). The contents of the vesicles are distinguishable (shaded areas) and are retained within the aqueous interior.

2. Two intact membranes contacting each other within a short distance ($\leqslant 15$ Å) with partial or complete dehydration at the point of contact. As vesicles form close contact at a point (or at a flattened area), the free energy can be significantly lowered by high-affinity binding of Ca^{2+} to phosphatidylserine molecules (Portis *et al.*, 1979; Ekerdt and Papahadjopoulos, 1982) (Ca/phosphatidylserine binding coefficient of several orders of magnitude; Nir, 1984; see also Feigenson, 1986). In this mode Ca^{2+} is thought to interact with phosphatidylserine molecules in both apposed membranes and is accompanied by local dehydration (Portis *et al.*, 1979). Other situations leading to partial (or complete) dehydration at the point of contact between two membranes are exemplified by the following: phosphatidylethanolamine bilayers which are not well hydrated (compared to phosphatidylcholine) and tend to aggregate at pH 7.4 and physiological ionic strength (Jendrasiak and Hasty, 1974; Stollery and Vail, 1977; Hauser *et al.*, 1981; Ellens *et al.*, 1986b); phosphatidylcholine bilayers at close contact as a result of out-of-plane thermal fluctuations (Leikin *et al.*, 1987); phosphatidylcholine and other bilayers in the presence of poly(ethylene glycol) (Ahkong *et al.*, 1973; Boni *et al.*, 1984; MacDonald, 1985; Parente and Lentz, 1986) or freezing and thawing (MacDonald and MacDonald, 1983) and dielectrophoresis (Pohl, 1978; Zimmermann, 1982).

3. This stage, with three distinguishable intermediates discussed below, is unified conceptually by the coincidence of contact-induced defects (CID) resulting from local dehydration and other intermembrane interactions. These structures cannot exist in the thermodynamical sense since parts of the acyl chains are exposed to water. The destabilizing effect of the membranes contacting each other at close distance increases the probability of coincidence of defects at the point of contact.

3a. The phosphatidylserine–Ca^{2+} interactions can be maximized if molecules in close vicinity to the Ca^{2+} "bridge" are pulled forward, thus exposing parts of their acyl chains to water. This stage amounts to the formation of a local defect or membrane destabilization induced by the molecular interactions at the point of contact (Papahadjopoulos *et al.*, 1979; Düzgünes *et al.*, 1984a; Wilschut *et al.*, 1985b). It should be realized that the vesicles may still have a certain probability of separating and returning to stage 1 (Wilschut *et al.*, 1985b; Düzgünes *et al.*, 1987b). Furthermore, around this local defect a hole is created in the membrane, possibly leading to a certain degree of leakage.

3b. As a consequence of local dehydration (induced by either Ca^{2+} as above, or by other agents, see stage 2), fluctuations in molecular packing

density may occur, with clusters of molecules more densely packed and areas with "point defects" where the packing density is less than average. When two such local defects coincide across the two membranes, there is an increased possibility for a "hydrophobic contact" between the two membranes, discussed in 4b below.

3c. This intermediate has been proposed as a result of the repulsion of the apposed polar heads of lipid molecules inducing the rupture of the interacting monolayers at the point of contact (Leikin *et al.*, 1987). This intermediate may be the preferred conformation for molecules that tend to form hexagonal or cubic phases, such as phosphatidylethanolamine or diglycerides, or cardiolipin or phosphatidate in the presence of Ca^{2+} or Mg^{2+}. (Rand, 1981; Bentz and Ellens, 1988).

4. We consider this as the first "committed" intermediate toward fusion of the two membranes which produces the first possibly irreversible hydrophobic contact between the two initially distant (inner) monolayers (hydrophobic contact intermediate, HCI). It also provides for mixing of lipid molecules between the outer monolayers of the two vesicles, without allowing mixing of contents. It is obvious that the orientation of the lipid molecules at this point may be quite different depending on the energies of bending, and the specific association between headgroups at the point of contact. Such an intermediate would be unstable, because of the high curvature points, and would be short-lived except in special cases such as lipids that favor the formation of lipid particles, the cubic phase, etc. (Gruner *et al.*, 1985).

4a. Since the intermediates in stage 3 cannot correspond to a stable structure, the possibility arises that phosphatidylserine molecules which have partially exposed acyl chains will further move forward to minimize hydrocarbon–water contacts. The structure thus formed in between the vesicles has some features similar to an inverted micelle. There is still a certain possibility that the apposed vesicles will separate and eventually revert to stage 1 with some lipid mixing accompanied by some leakage of contents. This possibility is reduced if larger aggregates have been formed. Indeed, the mass action kinetic analysis (Nir *et al.*, 1983a; Bentz *et al.*, 1983a) has indicated that while the rate of leakage increases with particle concentration, the percent leakage per fusion is reduced.

4b. As a consequence of the coincidence of two local defects at the point of contact, the lipid molecules adjacent to this area will undergo further conformational changes, which will produce areas of high curvature for both bilayers. The two outer monolayers would tend to collapse toward the point of contact, while some of the molecules of the outer monolayers around the defect will orient tangentially, to fill the gap. If a separation of the vesicles occurs from stage 4b back to stage 1, then no volume mixing occurs, whereas partial leakage and membrane mixing would occur.

4c. Monolayer fusion has been obtained as a stable structure connecting two membranes that have fused only by their outer monolayers (Chernomordik *et al.*, 1987).

5. The next step following the short-lived high-curvature intermediate is the formation of a water-filled channel between the two vesicles, at the point of the earlier hydrophobic contact (curved bilayer annulus, CBA). This conformational change would tend to alleviate some of the packing strain involved in the earlier intermediate, and would allow for the mixing of aqueous contents of the vesicles. It is the first point where true fusion has been achieved involving mixing of membrane lipids and vesicle contents.

5a. This intermediate still incorporates within the bilayer the remnants of the anhydrous Ca^{2+} complex. Some additional reorientation of lipid molecules (flip-flop) is expected to occur at this point to equilibrate packing differences between the outer and inner monolayers.

5b. A lipid bridge is formed between two vesicles. This structure (as also in 5a and 5c) can be energetically reduced if the "8" shape (observed by Bearer *et al.*, 1982 and Miller and Dahl, 1982) transforms into a more symmetrical spherical shape of a vesicle in which the contents of the vesicles mix by volume diffusion and the membrane components mix by lateral diffusion (stage 6).

5c. This structure derives from the "monolayer fusion" intermediate shown as 4c, after the formation of an aqueous channel somewhere, either in the periphery or the area of the monolayer itself (Chernomordik *et al.*, 1987). Additional flip-flop of molecules across the bilayer is expected to equilibrate the packing differences.

6. Following the above step, the aqueous channel connecting the vesicles is enlarged, thus diminishing further some of the high-curvature areas around the annulus and thus reducing surface tension and packing discontinuities. The two original vesicles are now one larger vesicle.

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