Non-bilayer structures in membrane fusion

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Abstract. Membrane fusion is an ubiquitous event in cell biology. One can distinguish two types of fusion: (i) outside/outside fusion, for example endocytosis, and (ii) inside/inside fusion, for example exocytosis. In spite of this difference in types of fusion in relation to membrane asymmetry and in spite of the large variety of lipid compositions encountered in biological membranes, a universal mechanism can be postulated for the role of lipids in membrane fusion. In this concept the lipids leave the bilayer configuration temporarily and locally. This notion, and the fact that any biological membrane contains a substantial amount of lipids which prefer the non-bilayer hexagonal II phase in physiological conditions, has led to the hypothesis that such hexagonal II phase lipids play a crucial role in membrane fusion. This proposition is strongly supported by model membrane experiments in which it has been demonstrated that factors such as Ca^{2+} and temperature, which trigger the transition from bilayer to hexagonal II phase, in fact induce membrane fusion.

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Membrane fusion is an extremely important phenomenon in biology. During this process two membranes, which can be two different membranes or two sites on one membrane, come in close contact, join and subsequently fuse, resulting in an intermixing of the lipids and proteins of the two membranes. Moreover, aqueous compartments, which were separated before the fusion, will intermix. If fusion is stopped at the stage of joining and the two membranes stay connected, one can call it arrested fusion.

Among the important biological phenomena in which membrane fusion is involved are: (i) fusion of the sperm and the egg membrane which leads to fertilization; (ii) the secretion of neurotransmitters, insulin and other hormones, and digestive enzymes from their respective storage vesicles inside the gland cells, referred to as exocytosis; and (iii) the uptake of particles or bacteria (phagocytosis), and the uptake of viruses and removal of receptors from the surface (receptor-mediated endocytosis).

In fact, every biological membrane has the potentiality to fuse, but this potentiality may be revealed more in one membrane than in another. In most types of intracellular membrane, such as the endoplasmic reticulum, lysosomes and the Golgi system, fusion events take place continuously.

Many studies have been undertaken with the aim of understanding the fusion process itself and the factors involved in and/or actively modulating this process. In recent years this basic interest in membrane fusion has been further stimulated. It has been realized that the application of artificially induced fusion is a powerful tool for the hybridization of cells for the production of monoclonal antibodies, for introducing membrane components into a cell membrane, and also in relation to the introduction of drugs into cells (targeting).

Many effectors are known to trigger membrane fusion (Schramm et al 1982), including Ca^{2+} in exocytosis, antibodies and hormones in receptor-mediated endocytosis, and pH during the fusion of endocytic vesicles and lysosomes. Moreover, the involvement of many other substances, such as ATP, cyclic AMP, GTP, drugs and Ca^{2+}-binding proteins, including calmodulin and synexin, has been reported. Also, membrane proteins appear to be involved in the fusion (Schramm et al 1982); and the state of the cytoskeleton, the extent of glycosylation and the distribution of membrane-spanning proteins all affect the process. With respect to the latter aspect, it was assumed that the membrane proteins are cleared from the fusion site before actual fusion can start. This was based on freeze-fracture experiments which revealed smooth patches of bilayer without intramembranous particles (IMP). It has since been shown that this clearance can be attributed to the use of cryoprotectants and/or chemical fixation. No particle clearance has been found using fast-freezing methods (Chandler & Heuser 1980), so there is no requirement for a visible lateral reorganization of intrinsic proteins before membrane fusion. In fact, a small area of lipid bilayer appears to be sufficient for fusion.

Although many factors are thought to play a role in membrane fusion, it is lipids that actually fuse. The lipids of the bilayer have to come into close apposition, which requires a reduction in electrostatic repulsion and in the hydration forces of the lipids. Subsequently, the lipids of the two bilayers have to join, which implies that they temporarily leave the bilayer configuration at the fusion point. Finally, the bilayers fuse with each other, which includes bilayer restabilization.

A major question is whether one general mechanism is involved in the fusion, with respect to the lipids. From model studies a variety of possible factors have been proposed, including the actions of fusogens such as lysolecithin (Lucy 1970) and the presence of solid and liquid domains which can be triggered isothermally in mixtures of phosphatidylserine and neutral

phospholipid (Papahadjopoulos et al 1978). Although lysolecithin or phosphatidylserine may play a role in membrane fusion, they can apply only to certain distinct fusion processes.

Biological fusions can be divided into two types. Firstly, there is fusion in which the outer (exoplasmic) monolayer of the membrane is involved, as in endocytosis and cell fusion. Secondly, the opposite situation is encountered in exocytosis, in fusion between intracellular organelles, and in cell division, where the inner (cytoplasmic) monolayer is primarily involved (Fig. 1). The

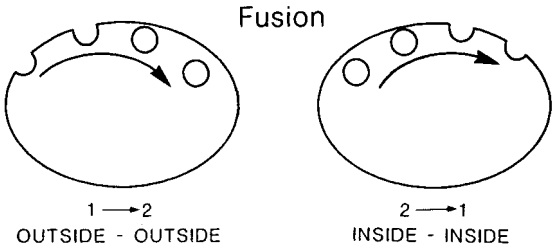

FIG. 1. Diagram showing fusion in which the outer (exoplasmic) monolayer of the membrane is involved, as in endocytosis and cell fusion, and fusion between intracellular organelles and cell division in which the inner (cytoplasmic) monolayer is primarily involved.

involvement of for instance phosphatidylserine can therefore be relevant only in exocytosis of eukaryotic cells, since this phospholipid is present in sufficient quantity only in the cytoplasmic monolayer of the plasma membrane and exocytic vesicles, as a result of phospholipid asymmetry in these membranes (Verkleij et al 1973, Op den Kamp 1979, Altstiel & Branton 1983). Furthermore, the fact that the phospholipid compositions of biomembranes in nature may vary widely—compare the lipid composition of the membranes of Gram-negative bacteria, the plasma membrane of eukaryotic cells and chloroplast membranes—makes it clear that membrane fusion does not require the presence of a special lipid.

Hexagonal II lipids

It has been proposed that lipids which prefer the *hexagonal II phase* on isolation from the membrane are involved in membrane fusion (Cullis & Hope 1978, Verkleij et al 1979, Cullis & De Kruijff 1979). This type of lipid is found in almost every biological membrane. In this conformation, the lipids are organized in hexagonally arranged cylinders in which the polar head groups of the lipid molecules surround a narrow aqueous channel. The

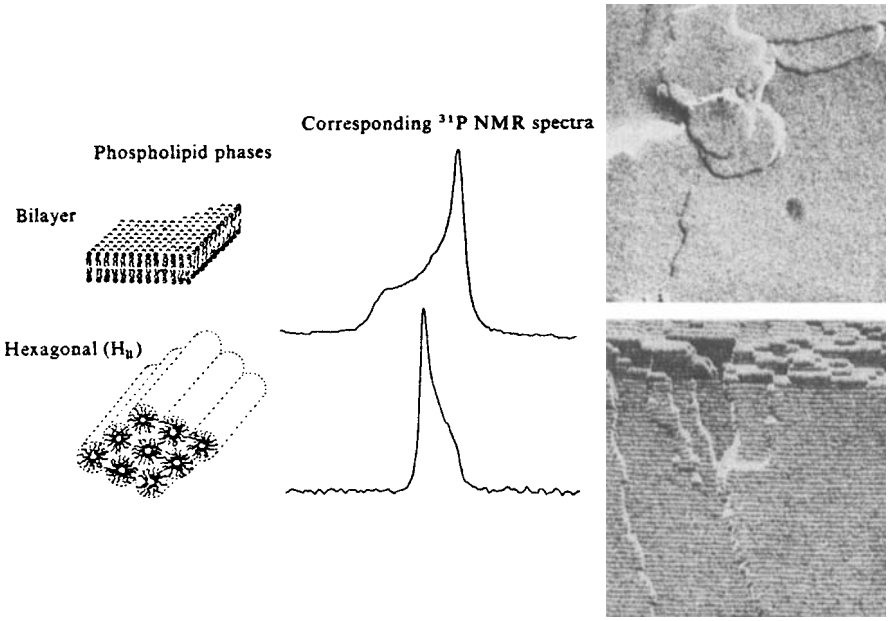

FIG. 2. Molecular arrangements of phospholipids in the bilayer and hexagonal II phases, with their characteristic freeze-fracture morphology and ^{31}P NMR spectra. Micrographs ×50 000.

organization of lipids in the hexagonal II phase can be detected with X-ray diffraction (Luzzati & Reiss-Husson 1962, Luzzati et al 1968, Luzzati & Tardieu 1974), freeze-fracturing (Deamer et al 1970, Verkleij & De Gier 1981) and ^{31}P nuclear magnetic resonance (^{31}P NMR; Cullis & De Kruijff 1979). Figure 2 shows the characteristic ^{31}P NMR spectra and freeze-fracture morphology of the bilayer and the hexagonal II phase.

Examples of lipids that form the hexagonal II phase are unsaturated phosphatidylethanolamines (Reis-Husson 1967, Rand et al 1971, Cullis et al 1978a), monogalactosyldiglycerides (Shipley 1973) and monoglucosyldiglycerides (Wieslander et al 1978, De Kruijff et al 1979). The lipid cylinders in the hexagonal II structures have diameters of about 5–7 nm (Luzzati et al 1968, Rand & Sengupta 1972). Other lipids forming the hexagonal II phase are the negatively charged phospholipids cardiolipin (Deamer et al 1970, Rand & Sengupta 1972, Cullis et al 1978b) and phosphatidic acid at neutral pH (Papahadjopoulos et al 1976, Verkleij et al 1982), which can be converted isothermally from the bilayer structure to the hexagonal II phase by adding Ca^{2+} and Mg^{2+} ions, respectively. For cardiolipin and phosphatidic acid, the local anaesthetic dibucaine and the tranquillizer chlorpromazine can produce

a similar phase change (Cullis et al 1978a, Verkleij et al 1982). Recently, hydrophobic peptides like gramicidin have been found to induce the hexagonal II phase in phosphatidylcholine bilayers (van Echteld et al 1981).

The transition from bilayer to hexagonal II phase in systems of purified unsaturated phosphatidylethanolamines is remarkably abrupt and occurs within a temperature range of only a few degrees. Differential scanning calorimetric studies show that the enthalpy change involved in this structural rearrangement is very small compared to the heat uptake needed to melt the solid bilayer phase. Since the polymorphic transition occurs above the gel–liquid crystalline transition of the bilayers, it is likely that the hexagonal II phase is in the liquid-crystalline state (Cullis & De Kruijff 1979).

Fusion

The hypothesis that lipids preferring the hexagonal II phase play a crucial role in membrane fusion has been strongly supported by experiments with model membrane systems. It was first demonstrated with vesicles composed of an equimolar mixture of bovine heart cardiolipin and egg lecithin, which fuse when Ca^{2+} is added (Fig. 3). Recent experiments have shown that the lipid vesicles formed after a small number of fusion rounds are in fact non-leaky (Wilschut et al 1982).

More relevant for biological membranes are mixtures of phosphatidylethanolamine and negatively charged phospholipids. Ca^{2+}, which can promote biological fusion (Poste & Allison 1973), triggers bilayer to hexagonal II transitions isothermally in these mixtures (Cullis et al 1983). An increase in temperature also triggers such a transition. It is therefore logical to suppose that the presence of Ca^{2+} or an increase in temperature allows the non-bilayer tendency of endogenous lipid to be expressed, thus promoting the fusion event. These predictions hold for a variety of mixtures when Ca^{2+} is added, such as phosphatidylethanolamine/phosphatidylserine, phosphatidylethanolamine/phosphatidylinositol, phosphatidylethanolamine/phosphatidylglycerol, phosphatidylethanolamine/phosphatidic acid, phosphatidylethanolamine/diphosphatidylglycerol (Cullis et al 1983), phosphatidylethanolamine/phosphatidylcholine/cholesterol/phosphatidylserine (A. J. Verkleij, unpublished), and when the temperature is raised in phosphatidylethanolamine/phosphatidylcholine/cholesterol (Verkleij et al 1980).

In all these cases the incubations result in the formation of larger structures, showing that net fusion has occurred. Moreover, fusion of these vesicles is associated with the observation of lipidic particles, sometimes localized in regions corresponding to the fusion interface. These lipidic particles have been interpreted as being inverted micelles at the nexus of two

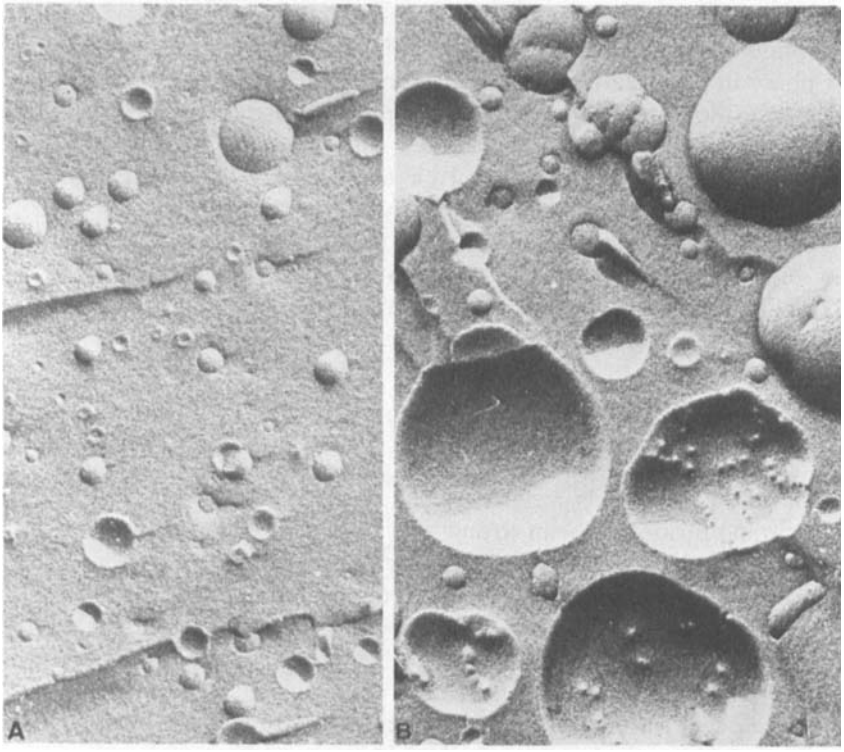

FIG. 3. Freeze-fracture preparations of lipid vesicles composed of an equimolar mixture of cardiolipin and lecithin before (A) and after (B) addition of Ca^{2+}.

fusion membranes (Verkleij et al 1979). However, other interpretations have been put forward by Hui & Stewart (1981). Our interpretation has led to the suggestion that fusion proceeds via the formation of intermediary inverted micellar structures, a mechanism proposed earlier (Lau & Chan 1975, Pinto da Silva & Nogueira 1977). A possible model is presented in Fig. 4.

Recent kinetic experiments (Wilschut et al 1982) have demonstrated that the fusion of vesicles made of an equimolar mixture of cardiolipin and lecithin by Ca^{2+} is extremely fast (on the time scale of seconds). We have therefore repeated our initial fusion experiments using the fast-freezing method of spray-freezing (J. Wilschut & A. J. Verkleij, unpublished). Vesicles composed of cardiolipin were frozen within seconds of the addition of Ca^{2+}. Figure 5 shows that larger vesicles are formed in this time. However, lipidic particles have never been found at the points of fusion between vesicles. Similar results have been obtained by Bearer et al (1982).

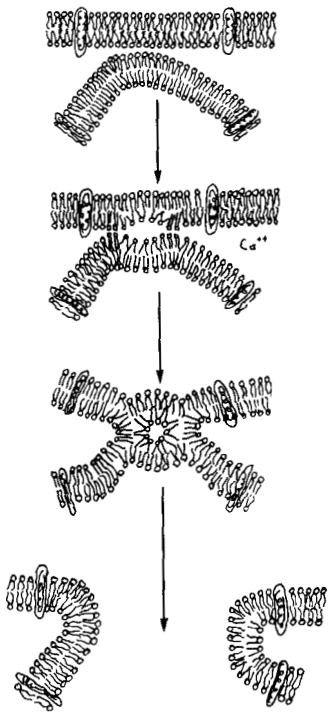

FIG. 4. Tentative model of membrane fusion, including the inverted micelle as intermediary.

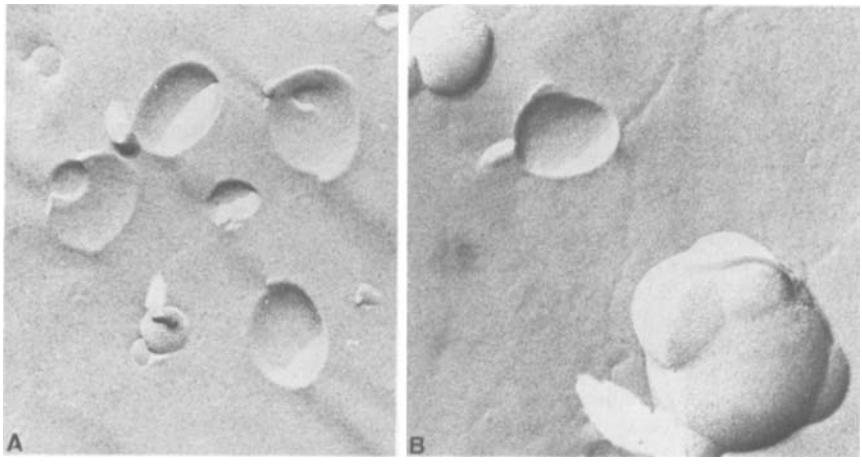

FIG. 5. Freeze-fracture preparation of lipid vesicles composed of an equimolar mixture of cardiolipin and egg lecithin made by reverse-phase evaporation (Wilschut et al 1982), quenched by spray-freezing. (A) Without Ca^{2+}, (B) 2 s after adding Ca^{2+} in concentrated form (20 mM), resulting in a final Ca^{2+} concentration of 5 mM.

In other mixtures too, such as phosphatidylethanolamine/phosphatidylcholine/cholesterol/phosphatidylserine, we could not identify lipidic particles, one second after adding Ca^{2+}, using fast-freezing methods. Lipidic particles appear after several rounds of fusion. This suggests that these particles are intermediates which are stabilized (arrested), for unknown reasons. In view of the very high rate of vesicle fusion (half times less than 1 s), the possibility cannot be excluded that inverted micelles, formed transiently during membrane fusion, escape detection by freeze-fracture electron microscopy.

In summary, vesicles composed of a lipid mixture in which one of the lipids prefers the hexagonal II phase have a strong tendency to fuse. This fusion is extremely fast and, as has been shown for one mixture, non-leaky (Wilschut et al 1982). Whether inverted micelles are the intermediate structures during the fusion is still not proven. This proposed fusion mechanism, which includes an essential role of lipids that prefer the hexagonal II conformation, is a very attractive one, since almost any biological membrane contains such lipids in physiological conditions, or can be induced to show them by external factors. Given the pivotal role of the hexagonal II-preferring lipids for membrane fusion, and knowing the modulating factors which trigger the transition from the lamellar to the hexagonal II phase, including temperature, pH, Ca^{2+}, local anaesthetics, proteins, cholesterol and unsaturated fatty acids, we may be able to understand fusion in biological membranes. A practical application of this knowledge may lie in the design of appropriate lipid vesicles for drug delivery.

REFERENCES

Altstiel L, Branton D 1983 Fusion of coated vesicles with lysosomes: measurement with a fluorescence assay. Cell 32:921-929

Bearer EL, Duzgunes N, Friend DS, Papahadjopoulos D 1982 Fusion of phospholipid vesicles arrested by quick-freezing. The question of lipidic particles as intermediates in membrane fusion. Biochim Biophys Acta 693:93-100

Chandler DE, Heuser JE 1980 Arrest of membrane fusion events in mast cells by quick freezing. J Cell Biol 86:666-674

Cullis PR, de Kruijff B 1979 Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim Biophys Acta 559:399-420

Cullis PR, Hope MJ 1978 Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion. Nature (Lond) 271:672-674

Cullis PR, Verkleij AJ, Ververgaert PH 1978a Polymorphic phase behavior of cardiolipin as detected by ^{31}P NMR and freeze-fracture techniques. Effects of calcium, dibucaine and chlorpromazine. Biochim Biophys Acta 513:11-20

Cullis PR, van Dijck PWM, de Kruijff B, de Gier J 1978b Effects of cholesterol on the properties of equimolar mixture of synthetic phosphatidylethanolamine and phosphatidylcholine. A ^{31}P NMR and differential scanning calorimetry study. Biochim Biophys Acta 513:21-30

Cullis PR, de Kruijff B, Hope MJ, Verkleij AJ, Nayar R, Farren SB, Tilcock C, Madden TD, Bally MB 1983 Structural properties of lipids and their functional roles in biological membranes. In: Aloia RC (ed) Membrane fluidity in biology. Academic Press, New York, vol 1:39-81

Deamer DW, Leonard R, Tardieu A, Branton D 1970 Lamellar and hexagonal lipid phases visualized by freeze etching. Biochim Biophys Acta 219:47-60

de Kruijff B, Verkleij AJ, van Echteld CJA, Gerritsen WJ, Mombers C, Noordam PC, de Gier J 1979 The occurrence of lipidic particles in lipid bilayers as seen by ^{31}P NMR and freeze-fracture electron microscopy. Biochim Biophys Acta 555:200-209

Hui S, Stewart T 1981 Lipidic particles are intermembrane attachment sites. Nature (Lond) 290:427

Lau ALY, Chan SJ 1975 Alamethicin-mediated fusion of lecithin vesicles. Proc Natl Acad Sci USA 72:2170-2174

Lucy LA 1970 The fusion of biological membranes. Nature (Lond) 227:814-817

Luzzati V, Reiss-Husson F 1962 The structure of the liquid crystalline phases of lipid-water systems. J Cell Biol 12:207-219

Luzzati V, Tardieu A 1974 Lipid phases: structure and structural transitions. Annu Rev Phys Chem 25:79-94

Luzzati V, Gulik-Krzywicki T, Tardieu A 1968 Polymorphism of lecithins. Nature (Lond) 218:1031-1034

Op den Kamp JAF 1979 Lipid asymmetry in membranes. Annu Rev Biochem 48:47-71

Papahadjopoulos D, Vail WJ, Pangborn W, Poste G 1976 Studies on membrane fusion. II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. Biochim Biophys Acta 448:265-283

Papahadjopoulos D, Portis A, Pangborn W 1978 Calcium-induced lipid phase transitions and membrane fusion. Ann N Y Acad Sci 308:50-66

Pinto da Silva P, Nogueira MLJ 1977 Membrane fusion during secretion. A hypothesis based on EM observation of Phytophora palmivora zoospores during encystment. J Cell Biol 73:166-181

Poste G, Allison AC 1973 Membrane fusion. Biochim Biophys Acta 300:421-465

Rand RP, Sengupta S 1972 Cardiolipin forms hexagonal structures with divalent cations. Biochim Biophys Acta 255:484-492

Rand RP, Tinker DO, Fast FG 1971 Polymorphism of phosphatidylethanolamines from two natural sources. Chem Phys Lipids 6:333-342

Reiss-Husson F 1967 Structures des phases liquide-cristallines de differents phospholipides, monoglycerides, sphingolipides, anhydres ou en presence d'eau. J Mol Biol 25:363-382

Schramm M, Oates J, Papahadjopoulos D, Loyter A 1982 Fusion and implantation in biological membranes. Trends Pharmacol Sci 3:221-229

Shipley GG 1973 Recent X-ray diffraction studies of biological membranes and membrane components. In: Chapman D, Wallace DFH (eds) Biological membranes. Academic Press, London, New York, vol 2:1-89

van Echteld CJA, van Stigt R, de Kruijff B, Leunissen-Bijvelt J, Verkleij AJ, de Gier J 1981 Gramicidin promotes formation of the hexagonal II phase in aqueous dispersions of PE and PC. Biochim Biophys Acta 648:287-291

Verkleij AJ, de Gier J 1981 Freeze-fracture studies on aqueous dispersions of membrane lipids. In: Knight CG (ed) Liposomes: from physical structure to therapeutic applications. Elsevier, Amsterdam, vol 4:83-103

Verkleij AJ, Zwaal RFA, Roelofsen B, Comfurius P, Kastelijn P, van Deenen LLM 1973 The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipase and freeze-etch electron microscopy. Biochim Biophys Acta 323:178-193

Verkleij AJ, Mombers C, Gerritsen WJ, Leunissen-Bijvelt J, Cullis PR 1979 Fusion of

phospholipid vesicles in association with the appearance of lipidic particles as visualized by freeze fracturing. Biochim Biophys Acta 555:358-361

Verkleij AJ, van Echteld CJA, Gerritsen WJ, Cullis PR, de Kruijff B 1980 The lipidic particle as an intermediate structure in membrane fusion processes and bilayer to hexagonal H_{II} transitions. Biochim Biophys Acta 600:620-624

Verkleij AJ, de Maagd R, Leunissen-Bijvelt J, de Kruijff B 1982 Divalent cations and chlorpromazine can induce non-bilayer structures in phosphatidic acid containing model membranes. Biochim Biophys Acta 684:255-262

Wieslander A, Ulmius J, Lindblom G, Fontell K 1978 Water binding and phase structures for different Acholeplasma laidlawii membrane lipids studied by deuteron NMR and X-ray diffraction. Biochim Biophys Acta 512:241-253

Wilschut J, Holsappel M, Jansen R 1982 Ca^{2+}-induced fusion of cardiolipin/phosphatidylcholine vesicles monitored by mixing of aqueous contents. Biochim Biophys Acta 690:297-301

DISCUSSION

Gingell: Dr Verkleij, you showed freeze-fracture pictures of vesicles which were touching but with a dividing septum between them, and others have shown similar images. In freeze-fracture pictures where you can't see such a partition, the interpretation of other workers has been that one has fortuitously caught the initial result of vesicle-to-vesicle fusion. But I don't know how one can maintain the 'neck' shape without having a force pulling in the plane of contact. What could that force be unless there is a lamella at this plane?

Verkleij: There is still a lamella between the two fusing vesicles. These micrographs represent the 'joining' stage where there is no contact between the two aqueous compartments of the vesicles. Fractures perpendicular to the fusion clearly show a cross fracture of the bilayer, whereas fracturing parallel to or through the fusion zone gives particles and pits.

Gingell: Others seem to be making a different point from these images. They claim that when you see that kind of image, it implies that there is no lamella between the vesicles, but I think it is obvious that something has to be pulling in the contact plane to give the 'neck' between the two. This can be clearly seen with pairs of soap bubbles, which always have a planar contacting membrane.

Verkleij: The image indeed represents an arrested fusion or 'joining' stage, which is expected to occur during fusion. In model systems this 'joining' stage appears to have a rather long life-time.

Schlegel: In a recent report by your colleagues (Hope et al 1983), small, unilamellar artificial vesicles were said to fuse *prior to* hexagonal II phase formation. Both they and you have suggested that an inverted micellular structure may be the critical intermediate in fusion. What then does the hexagonal II phase have to do with the mechanism of fusion?

Verkleij: The H_{II} phase itself is not involved in fusion, only lipids which prefer the H_{II} phase. During the local adhesion of two bilayers, such lipids form an inverted micelle or small hexagonal II tube at the connection. The life-time of such structures is very short during fusion. On the other hand, such a 'joining' intermediate may form a stable intermediate, like that suggested for tight junctions (Kachar & Reese 1982). In both the H_{II} tube and the inverted micelle, the lipids have the same orientation, with the polar head group in the core of the structure.

Shapiro: I gather that you have no evidence that either of these structures (H_{II} or inverted micelle) is on the pathway of membrane fusion. They are simply two structures that are seen when the membrane goes through some transition that allows it to fuse. So there could be yet another structure that has nothing to do with these, and is in fact the fusion intermediate. In a system that is moving so rapidly, where you might be accumulating stable end-products, how do you envisage identifying the true intermediate?

Verkleij: This will be very difficult and may in fact be impossible. However, this model is rather attractive, since all membranes contain lipid which can adopt the H_{II} phase in physiological conditions. The lipids in the H_{II} orientation allow adhesion since they are less hydrated, and these lipids have the ability to form non-bilayer structures, which is essential during fusion. That we see the inverted micelles in model systems can be explained as follows. During the initial fusion events, fusion may proceed very rapidly, because of the difference in ionic composition between inside and outside. Later on, when the separated aqueous compartments are connected, the fusion intermediates that we see during the 'joining' stage may have a longer life-time. We don't know why. So it is probably purely a matter of kinetics. In any case, and more important, in all lipid mixtures in which one of the lipid components prefers the H_{II} phase orientation, the membranes will fuse.

Bangham: I do not entirely understand the topography of the inverted micelle. When two isolated bilayers, each encompassing a discrete aqueous phase, come together, where does the aqueous phase within the inverted micelle come from?

Verkleij: It comes from the external fluid by definition, in the model.

Bangham: And what happens to it when fusion is complete?

Verkleij: According to the model, this aqueous compartment will mix with the aqueous compartments of the two fusing vesicles.

Bangham: So the aqueous region is momentarily trapped?

Verkleij: Yes. During fusion this aqueous compartment is trapped for a very short period, whereas in tight junctions and in the lipidic particles (inverted micelles) encountered in the model systems, these compartments are trapped for longer periods.

Allan: It has been suggested that interactions between spectrin and possibly phosphatidylserine on the internal surface of the red cell membrane maintain the normal lipid asymmetry and that when spectrin is detached from the membrane, transmembrane migration of phospholipids may be much faster (Haest 1982). What is your view of that suggestion, and how does it relate to the formation of H_{II} structures?

Verkleij: I don't know whether the phosphatidylserine 'flip-flop' (movement from the inner to outer monolayer of the membrane) would tie in with the non-bilayer structures. Detachment of the cytoskeleton may lead to destabilization of the inner monolayer, especially if Ca^{2+}, even at very low concentrations, is present. Phosphatidylethanolamine will now induce non-bilayer structures, since phosphatidylserine is no longer stabilizing phosphatidylethanolamine in the bilayer configuration.

Willecke: You mentioned that tight junctions show the H_{II} structure. In this case, one would have to say that the H_{II} configuration is stabilized, which you suggested to be an intermediate in the cell fusion process. What do you envisage as stabilizing such a structure between cells? I have difficulty in understanding what kind of additional factor may be involved.

Verkleij: Studies with peptides such as gramicidin and proteins such as cytochrome *c* are relevant here. Such peptides and proteins can induce an H_{II} phase in cardiolipin, as mentioned in my paper. We can therefore envisage the tight junction as a structure where protein can induce the formation of an H_{II} tube between two cells. Thus it is possible that protein stabilizes an H_{II} cylinder between two cells. This seems to be the best model, because it explains why there is no permeability for ions between the cells, because you have a lipid bilayer, in a way, between them. This is also consistent with the fact that proteins do not move from the apical zone to the basolateral zone of the cell membrane and vice versa (Boulan & Sabatini 1978), because such an inverted structure will not allow an intrinsic protein, which spans the whole cell membrane, to cross the tight junctional area.

Willecke: So you are thinking of a negative regulation, with an inhibitor present in tight junctions which prevents the H_{II} structures from leading to cell–cell fusion? In the absence of the inhibitor, as in a model system, you get fusion. The inhibitor is hypothetical at present, of course.

Verkleij: This is what I think.

Schlegel: If erythrocyte ghosts are prepared in the absence of Ca^{2+}, they maintain their normal, transbilayer phospholipid asymmetry. If, however, micromolar Ca^{2+} is included during the preparation, phospholipid asymmetry is lost (P. Williamson et al, unpublished work). Could an inverted micellar structure be involved in the redistribution of phospholipids rather than a flip-flop mechanism?

Verkleij: There are two hypotheses. The first is that glycophorins or other

proteins (band 3) mediate a flip-flop mechanism. The other is the inverted micelle. We do not know which operates. In model systems where lipid particles are present, there is 'flip-flop' of lipids. But in model systems where glycoprotein is present, there is also an increased 'flip-flop' of lipids. So both models are still possible.

Pethica: When human erythrocyte ghosts are put in a calorimeter, one does not see a (thermal) phase-transition over the 0–50 °C temperature range. If the membrane is in a liquid state, this implies that the transition is at lower temperature than 0 °C. Yet if the same membranes are examined by NMR, you do not find a high resolution spectrum for the envelope protons. There is a broad line spectrum with a spin–spin relaxation time of 10^{-5} s, almost constant from 0 °C to 60 °C. This is unambiguous evidence that the lipid chains are not liquid. This conclusion is one reason why I do not regard the bilayer model as generally valid. How does one get round this objection?

Nicolau: In the NMR measurements of relaxation times (t_2), there is an envelope from the CH_2 resonances with so much non-homogeneous broadening that one cannot claim that this is a measure of the lipid mobility. So I don't know how reliable such measurements are for deciding whether the lipids are in a liquid or gel state.

Pethica: Presumably half the protons in the membrane are in the lipid. If they were 'liquid' at all, they would show a high resolution spectrum, but they do not. Whatever the reason, the lipids do not seem to move, in the human erythrocyte at least.

Verkleij: I can bring in another result shown by Professor Lucy, and which we also found (Gerritsen et al 1979). If you 'take away' the interaction of the cytoskeletal proteins with the membrane, the protein particles can move. I can only interpret this aggregation of the particles in terms of a transition of the lipids from liquid to solid state, which results in lateral phase separation, as found in *Escherichia coli* membranes and other membranes (Verkleij et al 1975). Because there is such a lot of cholesterol in the erythrocyte membrane, this transition seems impossible. However, cholesterol is probably preferentially located in the outer monolayer of the bilayer, so the inner monolayer, where cholesterol is hardly present, could then undergo a transition from the fluid to the solid phase, as a result of either lowering the temperature or adding Ca^{2+}, and induce a lateral phase separation visible as particle aggregation. This indicates that the lipids in the erythrocyte membrane are in the liquid-crystalline state at physiological temperatures.

Maggio: As I understand it, when you look at the NMR or electron spin resonance, you are looking at the mobility of the group involved. If that group is constrained by a strong local molecule-to-molecule interaction, you won't see any movement. Yet the ensemble as a whole can be fluid. The rate

of exchange or tumbling of a particular molecular group can still be very high, even if, at any one moment, that group may be immobilized by interactions.

Verkleij: In studies of phosphatidylethanolamine and glycophorin, the glycophorin keeps the phosphatidylethanolamine in the lamellar phase, rather than in the hexagonal II phase which it prefers (Tarashi et al 1982). So the influence of the protein on the mobility of the lipid is about 200 lipid molecules for one glycophorin molecule. I have no idea how many lipid molecules can be perturbed by the intrinsic proteins in the erythrocyte membrane.

Parsegian: My problem with Dr Verkleij's hypothesis is that a very loose set of connections is being made. There seems to be a rather vague assay of fusion, and some hunch that things that allow lipids to form H_{II} phases might have something to do with allowing them to come together in certain hypothetical configurations that might allow the bridging and the rearrangement of phospholipids. You want to make this correlation, Dr Verkleij, yet you say that none of your diagrams should be taken as specific models. Your assay of fusion is a compilation of almost anecdotal events. Is your point that if you can get H_{II} configurations formed, then that might be the mode of fusion?

Verkleij: As I said earlier, vesicles in which one of the lipids prefers the H_{II} fusion, fuse, and all membranes contain such H_{II}-preferring lipids. This is biologically more important than to know what is the exact intermediate structure. On the other hand, the inverted micelle is rather attractive since, according to that concept, fusion is non-leaky.

Pethica: My own feeling is that to the extent that the lipids can do tricks such as subtle phase transitions, the property that governs the 'trick' must be related to the activity of the lipids in the biological membrane. The H_{II} phase need not be recognizably present in the membrane for one to obtain correlations between the ability to form such phases, and fusion or other membrane events.

REFERENCES

Boulan ER, Sabatini DD 1978 Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc Natl Acad Sci USA 75:5071-5075

Gerritsen WJ, Verkleij AJ, van Deenen LLM 1979 The lateral distribution of intramembrane particles in the erythrocyte membrane and recombinant vesicle. Biochim Biophys Acta 555:26-41

Haest CWM 1982 Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. Biochim Biophys Acta 694:331-352

Hope MJ, Walker DC, Cullis PR 1983 Ca^{2+} and pH induced fusion of small unilamellar vesicles consisting of phosphatidylethanolamine and negatively charged phospholipids: a freeze fracture study. Biochem Biophys Res Commun 110:15-22

Kachar B, Reese TS 1982 Evidence for the lipidic nature of tight junction strands. Nature (Lond) 296:464-466

Tarashi TF, Van der Steen ATM, De Kruijff B, Tellier C, Verkleij AJ 1982 Lectin–receptor interactions in liposomes: evidence that binding of wheat germ agglutinin to glycoprotein–phosphatidylethanolamide vesicles induces non-bilayer structures. Biochemistry 21:5756-5764

Verkleij AJ, Ververgaert PHJTh 1975 Annu Rev Phys Chem 26:101-122