REVIEW ARTICLE

Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement

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INTRODUCTION

The function attributed to the lipid bilayer, found in all membranes, has varied over the years. It is a barrier between the extracellular space and the cytoplasm (plasma membrane) or between the cytoplasm and the intra-organellar medium (internal membranes). It is a barrier primarily for strict hydrophilic solutes since the bilayer core is essentially hydrophobic. On the other hand, the lipid phase is a solvent for the proteins required to promote the exchange of hydrophilic solutes between the two compartments adjoining the membrane (carriers and transporters), or to recognize external signals (receptors). These are rather passive roles and it seems that almost any lipid molecule could fulfil this function. More recently, membrane lipids have been seen to play an active role, as a specificity of certain molecular species for given cellular requirements has been established. A typical example of this is the utilization by specific phospholipases of phosphoinositides, but also of phosphatidylcholine and phosphatidylethanolamine, to produce second messengers (such as diacylglycerol and arachidonic acid) following hormonal stimulus. An abundant literature exists concerning this topic and it will not be developed below. Some phospholipids are also crucial for the activity of certain enzymes, such as protein kinase C in the cytoplasm or prothrombinase at the outer surface of circulating cells. Recent studies have shown that, at the macrophage surface, phosphatidylserine receptors exist which are important for the recognition of cells to be cleared from the bloodstream. For phospholipid interaction with enzymes or receptors occurring at one side of a membrane, the distribution of the lipids between the two membrane leaflets is a key parameter. I will describe this distribution, showing that its symmetrical or asymmetrical nature results from transmembrane movements and that it can be perturbed (transiently or permanently) by a variety of events.

LIPID TRANSMEMBRANE DISTRIBUTION

Methods used to determine lipid asymmetry

None of the methods utilized is 'perfect'. Each one has its own drawbacks and advantages. One must also keep in mind that the membrane which is studied may be perturbed with respect to its cellular state: for instance, evaluation of lipid distribution in an intracellular membrane requires cell lysis and isolation of the organelles. We will see that, in some cases, ATP plays a pivotal role in membrane asymmetry. Since during the isolation procedure, the membrane is separated from cytoplasmic ATP, a new lipid repartition may occur, different from that *in situ*. If one is interested in the plasma membrane of nucleated cells, one should add that membrane rupture during cell lysis induces an important perturbation of lipid transverse distribution (Connor et al., 1990; Schrier et al., 1992b).

To determine the transmembrane distribution of endogenous lipids, several methods have been used (Etemadi, 1980; Op den Kamp, 1979; van Deenen, 1981). The principle of all these

techniques is to modify the exposed lipids of the outer leaflet and to quantify them in comparison with the unmodified molecules from the cryptic inner leaflet. This assumption that only the outer molecules are modified will be fulfilled if no appreciable transmembrane movement of lipids occurs during the assay. If this is the case, the kinetics of appearance of modified lipids can be fitted by a single exponential whose plateau corresponds to the outer leaflet composition. If it is not the case, the kinetics can be fitted by the sum of two exponentials, indicating that the membrane lipids are distributed in two pools (Figure 1, and Bloj and Zilversmit, 1976). When the limiting step is the transposition of lipid molecules from the inner to the outer monolayer ($k_a \gg k_{io}$, Figure 1), pool sizes and exchange constants can be derived from a model analysis. When the rate constant of transmembrane relocation is close to or higher than the rate constant of the assay reaction ($k_{io} \geq k_a$) it is not possible to deduce the lipid disposition within the membrane.

A common assay employs hydrolysis of the membrane phospholipids by phospholipases (A_2 or C) and sphingomyelinase. Reaction time with these enzymes is rather long (30–60 min), and when a fast transmembrane redistribution of phospholipids occurs, such as after diamide treatment of human erythrocytes, the result leads to an erroneous description of the asymmetry (Franck et al., 1986; Haest et al., 1978). This drawback can be avoided if one uses palmitoylated phospholipases, which are able to hydrolyse the exposed phospholipids within 5 min. However, the reaction products are lysophospholipids and free fatty acids (phospholipase A_2) or diacylglycerol (phospholipase C) or ceramide (sphingomyelinase), which may also perturb membrane organization. Perturbations

Figure 1 Principle of the two-pool model which has to be considered in the assay of phospholipid asymmetry (adapted from Bloj and Zilversmit, 1976)

When $k_a \gg k_{io}$ (k_{oi}), the kinetics of product formation (exchanged phospholipid or hydrolysed compounds) can be fitted by a single exponential. If not, the experimental points will be fitted by a sum of two exponentials, whose characteristics are a function of the three kinetic parameters.

Figure 2 Asymmetrical distribution of phospholipids in the human erythrocyte membrane

Top: distribution is given as a percentage of each phospholipid; bottom: distribution is given as a percentage of total phospholipids. Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid. Data are collected from: Bretscher (1971a,b), Butikofer et al. (1990), Gascard et al. (1991), Gordesky et al. (1972, 1975), van Meer et al. (1981) and Verkleij et al. (1973).

can be minimized by shortening the reaction time, thus degrading only a small fraction of the lipids, but one can ask whether results are truly representative of the whole outer leaflet. Cholesterol distribution is often studied using cholesterol oxidase; here again, reaction products could destabilize the bilayer. Moreover, the membrane has to be modified (addition of lysoderivatives or detergent) before the reaction can proceed.

Chemical labelling of phospholipids by specific group-reagents is often a fast reaction; however, it cannot be applied to all lipids, and the modification concerns only a fraction of the potentially reactive molecules. Moreover, one has to find experimental conditions where the reagent does not cross the bilayer and label the inner layer phospholipids (Gordesky et al., 1975).

The use of exogenous phospholipid exchange proteins may appear to be minimally perturbing for the bilayer, but the exchange of membrane phospholipids with exogenous counterparts is slow and can interfere with transmembrane movement (Bloj and Zilversmit, 1976). Exchange efficiency also depends on the lipid species (Geldwerth et al., 1991).

Use of antibodies specific to given phospholipids might be a promising tool, but, so far, this approach has been used qualitatively (to prove the presence of a lipid) rather than quantitatively (Gascard et al., 1991; Maneta-Peyret et al., 1989).

Transmembrane distribution can also be determined with exogenous lipid probes. The molecule must carry a reporter

group (e.g. fluorescent or paramagnetic) and the assumption is made that it is a 'good' analogue of the endogenous counterpart (which in fact remains to be demonstrated, especially when a bulky reporter group is involved). The probe is introduced (addition or exchange) in trace amounts into the membrane (to minimally perturb the bilayer), and its final distribution is determined, for instance, by selective quenching or extraction of the molecules on one side of the membrane only. As this last step can be performed at low temperature, the rate constant of transbilayer diffusion will be slower than the time required for the assay and there is no contribution from the other monolayer components. A drawback of the method is the chemical nature of the analogues used: in order to allow easy incorporation into the membrane from the external aqueous phase by a simple phase partition (without requiring a phospholipid exchange protein), these probes either are lysoderivatives, which may not be representative of the diacyl molecules, or possess a β -acyl chain short enough to provide some aqueous solubility. The latter probes resemble nicked, peroxidized lipids and the cell repair system (de-acylase, re-acylase?) can recognize and metabolize them (Bishop and Bell, 1985; Seigneuret et al., 1984; Sune et al., 1987).

Asymmetry in various membranes

Plasma membranes

The plasma membrane of the human erythrocyte is a system in which all the transmembrane asymmetry determinations have given broadly similar results: 65–75 % of the phosphatidylcholine and > 85 % of the sphingomyelin are in the outer leaflet, while the inner one contains 80–85 % of the phosphatidylethanolamine and > 96 % of the phosphatidylserine (Figure 2). More recently, the distribution of the phosphoinositides was established (Bütikofer et al., 1990; Gascard et al., 1991): 100 % of the phosphatidylinositol 4-phosphate, and 80 % of the phosphatidylinositol and of its 4,5-bisphosphate derivative are located in the cytoplasmic leaflet, as is 80 % of the phosphatidic acid. In fact, this distribution, with an outer surface composed principally of the choline-containing phospholipids phosphatidylcholine and sphingomyelin, seems to be a general trend for the plasma membrane of animal cells (see Table 1 and reviews by Devaux, 1992; Op den Kamp, 1979; Zachowski and Devaux, 1990). This outer monolayer is often poor in aminophospholipids, phosphatidylethanolamine and phosphatidylserine, but some plasma membranes can contain large amounts of them. For example, intestinal brush border or hepatocyte contiguous surface membranes have at least 20 % of their phosphatidylserine located in the extracellular leaflet. This distribution has to be considered in the light of the fact that these membranes were purified from a cell homogenate: cell membrane rupture occurring during lysis can induce important redistributions (up to 20–25 %) of all the phospholipids in the bilayer, as tested in the case of human erythrocyte membranes (Schrier et al., 1992b; Shukla et al., 1978). The values of exposed phosphatidylserine might thus not be entirely representative of the distribution *in vivo*. In the case of the myoblast plasma membrane, the large quantity of both phosphatidylserine and phosphatidylethanolamine located in the extracellular leaflet was supposed to reflect the ability of these cells to fuse into a myotube. Indeed, a surface rich in aminophospholipids is fusion-competent (see below).

Besides phospholipids, plasma membranes contain a high amount of cholesterol, but the distribution of this lipid is still a subject of debate: some reports are in favour of an almost symmetrical repartition in the two leaflets (Blau and Bittmann, 1978; Lange and Slayton, 1982), while others have determined

Table 1 Percentage of each main phospholipid class present in the outer leaflet of various animal plasma membranes

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Cell	Sphingomyelin	PC	PE	PS	Reference
Human erythrocyte	80	77	20	< 4	See Figure 2
Mouse erythrocyte	85	50	20	0	Rawlyer et al., 1985
Rat erythrocyte	100	62	20	6	Renooij et al., 1976
	100	63	0		Crain and Zilversmit, 1980
Monkey erythrocyte	82	67	13	0	van der Schaft et al., 1987
Human platelet	93	45	20	9	Perret et al., 1979
Pig platelet	91	40	34	6	Chap et al., 1977
Mouse erythroleukaemic cell	80	45	50	15	Rawlyer et al., 1985
LM cell		48	24		Sandra and Pagano, 1978
Mouse synaptosome			10–15	20	Fontaine et al., 1980
Rabbit intestinal brush border	63	32	34	44	Lipka et al., 1991
		30	28		Barsukov et al., 1986
Rabbit kidney brush border	80	35	23	15	Venien and Le Grimmelc, 1988
Trout intestinal brush border					
Middle			46	32	Pelletier et al., 1987
Posterior			36	31	
Rat cardiac sarcolemma	93	43	25	0	Post et al., 1988
Krebs ascites cell	47	52	45	19	Record et al., 1984
Rat hepatocyte					
Bile canalicular surface	65	85	50	0	Higgins and Evans, 1978
Contiguous surface		80	0	20	
Sinusoidal surface	65	85	55	0	
Chick embryo fibroblast			35	20	Sessions and Horwitz, 1983
Chick embryo myoblast			65	45	Sessions and Horwitz, 1983
Quail embryo myoblast			73	44	Sessions and Horwitz, 1983

Table 2 Percentage of each main phospholipid class present in the outer (cytoplasmic) leaflet of various animal intracellular membranes

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Cell	Sphingomyelin	Cardiolipin	PC	PE	PS	Reference
Bovine rod outer segment disk			55	50	40	Drenthe et al., 1980
Rabbit muscle sarcoplasmic reticulum	25		48	69	15	Herbette et al., 1984
Pig gastric mucosa vesicle	45		80	85	60	Olaissan et al., 1985
Ray synaptic vesicle				60	40	Deutsch and Kelly, 1981
Rat liver endoplasmic reticulum						
Rough	58		68	40	26	Bollen and Higgins, 1980
Smooth	63		76	40	12	
Chick brain microsomes	60		90	32	0	Dominski et al., 1983
Bovine chromaffin granule	20		65	67	50	Buckland et al., 1978
Inner mitochondrial membrane						
Rat liver		18	54	91		Marinetti et al., 1976
				55		Nilsson and Dallner, 1977
						Crain and Marinetti, 1979
Beef heart		25	70	38		Krebs et al., 1979
Pig heart		10	50	80		Harb et al., 1981

an uneven distribution of cholesterol (Brasaemle et al., 1988; Fisher, 1976; Schroeder et al., 1991).

Intracellular organelles

The architecture of some intracellular membranes is given in Table 2. Not all the reported determinations are listed and I have chosen examples of the various organelles. Differences between these membranes can be explained by the specificity of their cellular function. In certain cases, groups have reported different results with the same membrane, such as the rat liver inner mitochondrial membrane. Possible sources of discrepancies are the isolation procedure (degree of contamination by other

membranes, impermeability of the membrane during the assay) and, as cited above, the competition between the kinetics of the asymmetry assay and of transmembrane diffusion of the phospholipids. For inner mitochondrial membranes, the transbilayer motion of phosphatidylcholine and phosphatidylethanolamine can be rapid ($t_{\frac{1}{2}}$ of the order of 10 min; A. Maftah, personal communication) and even applying the two-pool model, it may be difficult to differentiate clearly between the two layers.

Asymmetric fluidity

An asymmetric distribution of the phospholipids may have important consequences for the physical properties of the mem-

Figure 3 How the erythrocyte shape can help in studying the transmembrane redistribution of phospholipids

The requirement is that shape change depends only on the analogue used, and not on a membrane rearrangement of endogenous molecules induced by the analogue, such as scrambling.

brane. In fact, the nature of the fatty acyl chains varies with the head group as has been well documented for the human erythrocyte membrane (Myher et al., 1989): phosphatidylserine molecules have longer and more unsaturated chains than phosphatidylcholine molecules. As a consequence, the asymmetry of the polar head groups is accompanied by an asymmetry of the fatty acids and one can anticipate different chemophysical properties for each leaflet. Thus lower local microviscosity (Morrot et al., 1989; Seigneuret et al., 1984; Tanaka and Ohnishi, 1976) and a faster lateral diffusion (Morrot et al., 1986; Rimon et al., 1984) have been ascribed to the lipids in the cytoplasmic erythrocyte leaflet. Similar properties exist also in other cells, such as fibroblasts (El-Hage Chahine et al., 1993). Studies with artificial membranes reconstituted from the characteristic phospholipids of each monolayer demonstrated that differences in microviscosity and diffusibility were primarily due to the phospholipid composition of the membrane (Cribier et al., 1990).

TRANSMEMBRANE MOTION

Assays

Kinetic parameters of lipid movements between the two hemi-leaflets can sometimes be inferred from the assay of the lipid distribution. Thus, data analysis using the two-pool model allows

one to determine the characteristic time of diffusion from the inner to the outer leaflet. However, it is not possible to get information on movement in the opposite direction. A complete, detailed analysis requires the use of lipid analogues which are radioactive, fluorescent or paramagnetic. In all cases, the amount of analogue incorporated into the membrane will be low ($< 1\%$ of the endogenous lipids) in order to minimize any perturbation due to the probe. At time zero, these analogues have to be localized in one leaflet, and their redistribution into the other leaflet must be determined as a function of the incubation time. If the analogues are strictly water-insoluble, their insertion into the membrane requires either the use of an exchange protein or the fusion of a liposome (Rousselet et al., 1976a,b; Schrier et al., 1992b; Tilley et al., 1986), and determination of the labelled fraction still exposed in the outer membrane half is made by selective modification of the molecules in this leaflet (degradation by phospholipases, reduction of the paramagnetic group by ascorbate). If the analogues are partially water-soluble, their spontaneous insertion in the membrane corresponds to a partition between the aqueous buffer and the hydrophobic bilayer. Their distribution between the two monolayers with time is determined either by selective destruction of the probe in the outer leaflet (Seigneuret and Devaux, 1984) or by selective extraction of the analogue from the external monolayer using

Table 3 Characteristics of passive diffusion of various lipids in some membrane bilayers

Abbreviations: FA, fatty acid; PL, phospholipid; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Membrane	Lipid	$t_{1/2}$	References
Egg PC	Spin label-PC	6.5 h	Kornberg and McConnell, 1971
	Spin label-FA	5–20 s	Yuann and Morse, 1991
	Bile acids	5–140 ms	Cabral et al., 1987
1-Palmitoyl-2-oleoyl-PC	Pyrene-PL	6–350 h	Homan and Pownall, 1988
Dioleoyl-PC	Dioleoyl-PC	> 11 h	Rothman and Dawidowicz, 1975
Dioleoyl-PC + glycophorin	Dioleoyl-PC	< 1 h	de Kruijff et al., 1978
Dioleoyl-PC/dioleoyl-PA	Neutral PA	25 s	Eastman et al., 1991
	Charged PA	12.6 days	
Influenza virus	PC	> 10 days	Rothman et al., 1976
Human erythrocyte	Diacyl-PC	3–26 h	Daleke and Huestis, 1985; Middelkoop et al., 1986
	Lyso-PC	10–30 h	Bergmann et al., 1984
	Spin label-PC	8–12 h	Bitbol and Devaux, 1988; Morrot et al., 1989
Rat erythrocyte	PC	5–11 h	Crain and Zilversmit, 1980
LM cell membrane	PC	88 h	Sandra and Pagano, 1978
Intestinal brush border	Endogenous PL	8–80 h	Lipka et al., 1991
		15–20 min	Barsukov et al., 1986

back-exchange into acceptor structures such as liposomes or bovine serum albumin (Bergmann et al., 1984; Bütikofer et al., 1990; Calvez et al., 1988; Connor et al., 1990; Daleke and Huestis, 1989). In the case of erythrocytes, whose shape is sensitive to the relative amount of lipid in each of the two membrane leaflets according to the bilayer-couple hypothesis (Deuticke, 1968; Sheetz and Singer, 1974), these changes (Figure 3) have also been used to follow the transmembrane motion of lipids (Daleke and Huestis, 1985; Seigneuret and Devaux, 1984). All the assays have given comparable results, with the exception of those performed with certain fluorescent short-chain phospholipids which proved to be poor analogues of their endogenous counterparts (Colleau et al., 1991). These measurements have allowed three types of transmembrane movements to be described.

Mechanism of transbilayer migration

Passive diffusion

The diffusion rate of a molecule is a function of its concentration gradient between the membrane halves and is not saturable with increasing amounts of substrate. It is typically the movement occurring for lipids in liposomes. However, it can also be encountered for some lipids in biological membranes, the best example being phosphatidylcholine in plasma membranes.

The characteristic time of this diffusion depends both on the lipid molecule and on the host membrane. Table 3 lists some of the systems in which such a movement was found. One can expect that neutral species (i.e. with no ionized residues) will diffuse very rapidly through the membrane, as no polar groups are present. This has been shown for diacylglycerol, which diffuses with a characteristic time of less than 1 min in artificial and biological membranes (Allan et al., 1978; Ganong and Bell, 1984). *A priori*, this should be true for cholesterol since related molecules such as bile salts equilibrate in a bilayer with characteristic times well below 1 s (Cabral et al., 1987). However, in the literature, characteristic times for cholesterol flip-flop varied from seconds to days, even in similar membranes. It is possible that this difference is due to the assay technique: with phosphatidylcholine/cholesterol liposomes, transmembrane movement was found to be slower when assayed by exchange ($t_{1/2} > 6$ days; Poznansky and Lange, 1978) than when assayed by

cholesterol oxidase ($t_{1/2} < 1$ min; Backer and Davidowicz, 1981). Some discrepancies may be due to the probes used to mimic membrane cholesterol: spin-labelled and fluorescent analogues are more polar than cholesterol and some important groups may be lacking, such as the 3-hydroxy group. Experimental conditions, such as the addition of detergent sometimes used in the cholesterol oxidase assay, or buffer composition (Brasaemle et al., 1988), may perturb the integrity of the membrane. One also has to keep in mind that, in membranes, cholesterol has preferential interactions with sphingomyelin (Demel et al., 1979), which may explain the different rates found with artificial and natural membranes.

Considering lipids which are weakly acidic or basic, the fraction which is not ionized should also experience easy transverse diffusion. This is true for fatty acids, phosphatidic acid and phosphatidylglycerol (Table 3). In contrast, even if they can exist as uncharged molecules, phosphatidylinositol and cardiolipin have a slow diffusion across bilayers (Bütikofer et al., 1990; Eastman et al., 1991), probably because they maintain an important hydration shell around their polar head groups, which prevents them from crossing the hydrophobic core of the bilayer.

The permanently charged lipids generally exhibit a slow passive transmembrane diffusion. In artificial membranes, the movement has a characteristic time of hours to days as a function of the membrane composition and its physical state (as illustrated by data on phosphatidylcholine in Table 3). The addition of proteins to artificial membranes may sometimes accelerate lipid diffusion (de Kruijff et al., 1978; van der Steen et al., 1982), but this effect remains unspecific, contrary to other effects which will be described below. In the human erythrocyte membrane, all the reported data agree that phosphatidylcholine undergoes a passive diffusion, with a half-time of several hours. This time depends strongly on the molecular phosphatidylcholine species (Fujii et al., 1986; Middlekoop et al., 1986; van Meer et al., 1982) and on the membrane sphingomyelin and cholesterol contents (Bergmann et al., 1984; Morrot et al., 1989). Similar data have been reported in the plasma membranes of erythrocytes belonging to other species (Connor and Schroit, 1989; Crain and Zilversmit, 1980; Devaux et al., 1988) and with cultured cells (Sandra and Pagano, 1978). A slightly faster phosphatidylcholine diffusion rate is characteristic of lymphocytes (Zachowski et al., 1987), platelets (Sune et al., 1987), renal brush border (Venien and Le

Table 4 Characteristics of transmembrane movements in rat liver endoplasmic reticulum

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Phospholipid	$t_{1/2}$ of reorientation	References
Endogenous PE	< 10 min	Hutson and Higgins, 1982
Endogenous PC	< 5 min	van den Besselaar et al., 1978
Endogenous PC, PE, PI, PS	< 40 min	Zilversmit and Hughes, 1977
Dibutyroyl-PC	5 min	Bishop and Bell, 1985
Lyso-butyroyl-PC	< 10 min	Kawashima and Bell, 1987
Spin-labelled PS, PC, PE, sphingomyelin	20 min	Herrmann et al., 1990

Table 5 Active transport of phosphatidylserine (PS) from the outer to the inner leaflet of various plasma membranes

Cell	PS analogue	$t_{1/2}$ of transport	References
Human erythrocyte	Spin-label	< 5 min	Bitbol and Devaux, 1988; Calvez et al., 1988; Morrot et al., 1989
	Long-chain, diacyl	8–10 min	Daleke and Huestis, 1985, 1989; Tilley et al., 1986
	Fluorescent (NBD)	10–30 min	Colleau et al., 1991; Connor and Schroit, 1987, 1989
	Lyso-derivative	3 h	Bergmann et al., 1984
K 562 erythroblast	Spin-label	< 2 min	Cribier et al., 1993
Erythroleukaemic cells	Long-chain, diacyl	< 15 min	Middelkoop et al., 1989
Platelets	Endogenous PS	3–5 min	Comfurius et al., 1990
	Spin-label	7 min	Sune et al., 1987
	Fluorescent (NBD)	4–8 min	Tilly et al., 1990
	Spin-label	7 min	Sune et al., 1988; Zachowski et al., 1987
Lymphocyte	Spin-label	< 3 min	J. Rosso, personal communication
Macrophage	Spin-label	10 min	K. Müller, personal communication
Spermatozoa	Spin-label	30–40 min	Zachowski and Morot-Gaudry Talarmin, 1990
Nerve endings	Fluorescent (NBD)	5–15 min	El-Hage Chahine et al., 1993; Martin and Pagano, 1987
Hamster fibroblast			

Grimellec, 1988) and nerve endings (Zachowski and Morot-Gaudry Talarmin, 1990). In rabbit small intestinal brush border membranes, contradictory results have been reported concerning the diffusion of the endogenous phospholipids, probed by phospholipase attack: Lipka et al. (1991) found a low re-orientation rate, while Barsukov et al. (1986) reported a very fast movement. In fact, these authors did not use enzymes from the same source and it has been shown that some phospholipases can induce an accelerated reorientation of the lipids (Venien and Le Grimellec, 1988). Passive diffusion also exists in some intracellular membranes, such as bovine chromaffin granules (Zachowski et al., 1989) or inner mitochondrial membranes (Rousselet et al., 1976a).

Facilitated diffusion

As in the case of simple diffusion, lipids will diffuse according to their gradient within the bilayer. Proteins act as channels or carriers, forming a pathway for the transmembrane movement of the lipids. The diffusion rate is diminished after modification of the membrane proteins by group-specific reagents or by proteolytic cleavage and is saturable with increasing substrate concentration. The typical example of such a system is found in the rat liver endoplasmic reticulum. Using a short-chain (dibutyroyl), highly water-soluble phosphatidylcholine analogue, Bishop and Bell (1985) described a movement fulfilling all the requirements of a facilitated diffusion. All the determinations of phospholipid reorientation rates in this membrane have shown that the movement is very fast (Table 4). The involvement of component(s) specific to the endoplasmic reticulum membrane was supported by reconstitution experiments (Backer and

Davidowicz, 1987): the fast diffusion appeared only in a mixture of microsomal lipids and proteins and not in microsomal lipids alone or a mixture of erythrocyte lipids and proteins. Spin-labelled phospholipid probes showed that the transfer rates were equivalent for different molecular species and that these molecules were in competition with one another (Herrmann et al., 1990). This argues in favour of a unique system of 'pores' used by all the phospholipids. Nothing is known concerning the mechanism of action although it has been proposed that the role of proteins would be to induce non-bilayer phases. These have been detected only in endoplasmic reticulum membranes and not in liposomes made from purified lipids (van Duijn et al., 1986). A similar diffusion system may exist in rod outer segments, in which a very fast transmembrane movement of the phospholipids is inhibited by chemical and enzymic modifications of the membrane proteins; this fast reorientation is absent from artificial membranes composed of purified lipids and rhodopsin (Wu and Hubbell, 1992). Nevertheless, it was possible to detect some asymmetrical distribution of the lipids in these membranes.

Active transport by aminophospholipid translocase

In this case, phospholipids are moved between the two membrane halves against a concentration gradient and sometimes against an electrical gradient. The reorientation rate will tend to a maximum velocity at high amounts of substrate. Energy, provided by cytoplasmic ATP, is required to overcome the gradient(s). Such a system was first discovered in human erythrocytes by spin-label experiments (Seigneuret and Devaux, 1984) and later confirmed by a variety of other approaches (Table 5): the two aminophospholipids, phosphatidylserine and phospho-

tidylethanolamine, were rapidly moved from the outer membrane layer into the inner one, on condition that the red cells (or their ghosts) contained Mg-ATP. The same specific movement was later described in plasma membranes from other cells (Table 5). It was also detected in organelles, namely bovine adrenal chromaffin granules (Zachowski et al., 1989). We hypothesized that this transport was dependent on a protein that we named 'aminophospholipid translocase' (Zachowski et al., 1986) and whose properties are summarized in the following paragraph.

The energy required to transport the molecules is provided by the complex Mg-ATP, which is hydrolysed at the cytoplasmic membrane surface with an apparent K_m in the millimolar range (Seigneuret and Devaux, 1984). The aminophospholipids are recognized in the outer leaflet, phosphatidylserine being approximately a 10-fold better substrate than phosphatidylethanolamine (Zachowski et al., 1986). Binding of ATP and of the lipid are random, independent events (Devaux et al., 1988). The stoichiometry of the transport is one lipid moved per ATP hydrolysed (Beleznaï et al., 1993). The reaction is inhibited by vanadate (Seigneuret and Devaux, 1984) and fluoride, which argues in favour of the involvement of a P-type ATPase. Transport is sensitive to chemical reagents such as *N*-ethylmaleimide (Daleke and Huestis, 1985; Zachowski et al., 1986) or pyridyldithioethylamine (Connor and Schroit, 1988) and to proteolytic enzymes (Bevers et al., 1989), and is inhibited by cytoplasmic free calcium in the micromolar range (Bitbol et al., 1987). The phospholipid pattern recognized by the enzyme is rather extended (Morrot et al., 1989). (a) The head group has to carry a dissociable amino group, separated from the phosphate group by two methylenes; a carboxyl group reinforces the association, but is unable to create it. (b) If the glyceride backbone is substituted by a ceramide backbone, the transport efficiency is reduced approximately 1000-fold, even though a serine head-group is present. (c) Part of the chain in the *sn*-2 position of the glycerol is important: lysophosphatidylserine is a poor substrate, but an esterified acetyl group already restores some specificity; saturated and unsaturated species are equally well recognized (Daleke and Huestis, 1985; Tilley et al., 1986). Long-chain lipids (Tilley et al., 1986) and lipids with a short 4-(doxyl)-pentanoyl chain (Morrot et al., 1989) exhibit similar transport kinetics, indicating that the nature of the chain is not very important. However, the presence in *sn*-2 of a (6-NBD)-caproyl chain decreased the affinity for phosphatidylserine by one order of magnitude and prevented phosphatidylethanolamine from being recognized (Colleau et al., 1991). This abnormal behaviour is no longer found when the fluorescent fatty acid is a dodecanoyl one (Connor et al., 1992). (d) The bond of the chain in *sn*-1 is not crucial since the transport of diacylphosphatidylethanolamine and of plasmalogen phosphatidylethanolamine are not distinguishable (P. Fellmann, personal communication).

Aminophospholipid translocase is not yet fully characterized at the molecular level. Morrot et al. (1990) and Daleke et al. (1991) have proposed that an Mg-ATPase of M_r 115000–120000 is the major component of the transport system. A partially purified protein fraction from the erythrocyte membrane possesses an ATPase activity which requires the presence of phosphatidylserine. No other phospholipid can substitute for phosphatidylserine, but phosphatidylethanolamine results in reduced activity. Moreover, the ATPase is inhibited by vanadate, vanadyl, fluoride and calcium ions, as is aminophospholipid translocase. Electrophoresis of the fraction, after incubation with radioactive ATP, shows a major labelled band at 115–120 kDa. Moreover, an ATPase of similar molecular mass is found in chromaffin granule membranes. Connor and Schroit (1987, 1988), on the basis of membrane labelling by inhibitors of

phosphatidylserine translocation, concluded that a polypeptide of 32 kDa is an important component of the transport system. This peptide would belong to the Rh blood group system (Schroit et al., 1990); as Rh protein does not contain ATP-binding sites, this peptide has to be associated with an ATP-utilizing protein (Schroit and Zwaal, 1991). Thus, these two proposals are not mutually exclusive.

Other mechanism(s)

It is also possible that phospholipids are moved through the membrane as they are synthesized. In rat (Renooij et al., 1976) and in human (Andrick et al., 1991) erythrocytes, phosphatidylcholine newly synthesized from lysophosphatidylcholine and fatty acid in the cytoplasmic leaflet is relocated in the outer leaflet with a halftime of translocation of approx. 20 min, i.e. ten times more rapidly than could be expected on the basis of a simple diffusion (see Table 3). Note that no outward translocation of newly synthesized phosphatidylethanolamine can be demonstrated, indicating some specificity for phosphatidylcholine (Andrick et al., 1991). Similarly, methylation of phosphatidylethanolamine into phosphatidylcholine at the inner face is accompanied by transmembrane reorientation of the latter lipid (Hirata and Axelrod, 1978).

RELATION BETWEEN MOVEMENT AND ASYMMETRY

A simple diffusion process alone is unable to create an asymmetrical distribution of lipids in a bilayer. To reach such a situation, there has to be a local environment able to trap a lipid in the leaflet where it is to be accumulated. Cullis's group has published several reports (Eastman et al., 1991; Hope and Cullis, 1987; Redelmeier et al., 1990) showing how to generate an asymmetric distribution of lipids which are weak acids or weak bases. It was noted above that such molecules (phosphatidic acid, for instance) diffuse very quickly through a bilayer when in their neutral form, which leads to a symmetrical distribution of the neutral molecules. As Table 3 shows, charged molecules have a slow diffusion through the bilayer. On each side of the membrane, the neutral species are in equilibrium with the charged ones as a function of the *pK* of the dissociable group and the local pH. Thus, if a pH gradient exists over the membrane, one can achieve an asymmetrical distribution of these lipids. However, this mechanism is applicable solely to minor components of an animal cell (free fatty acids, phosphatidic acid and phosphatidylglycerol) and a high asymmetry requires a strong pH gradient which exists only in few organelles (lysosomes, gastric mucosal vesicles). Since such a large proton gradient reflects the activity of an H^+ -ATPase, the asymmetry of these lipids can be considered as energy-dependent. Hubbell (1990) has proposed a model in the disk membranes, where the electrostatic force trapping the lipids on one side of the membrane would result from an asymmetrical charge distribution on proteins, namely rhodopsin.

Based on the model of the human erythrocyte membrane, it was proposed that phosphatidylserine is maintained in the inner layer by interactions with cytoskeletal proteins, such as spectrin, which would prevent the lipid from diffusing towards the extracellular layer (Haest et al., 1978; Middelkoop et al., 1988). Recent experiments have shown that, if this phenomenon exists, it can only play a minor role in maintaining the asymmetry. The energy of the phosphatidylserine-spectrin interaction, under physiological conditions, is small and in the range of thermal agitation (Maksymiw et al., 1987). If an interaction exists, phosphatidylserine would be distributed in domains detectable by spectroscopic techniques, such as electron spin resonance; no signs of such domains were ever detected in spectra (Seigneuret

et al., 1984). Moreover, it was shown that any lipid which is in the inner leaflet is able to move into the outer one (Bitbol and Devaux, 1988; Connor et al., 1992). In addition, heat-denaturation of spectrin did not modify the membrane asymmetry (Gudi et al., 1990) and spectrin-free erythrocyte microvesicles were claimed to retain a close-to-normal asymmetry (Raval and Allan, 1984). Finally, experiments with erythrocyte vesicles poor in or free from spectrin have clearly demonstrated that it is possible to obtain an asymmetrical distribution of the aminophospholipids as a result of aminophospholipid translocase activity (Beleznaï et al., 1993; Calvez et al., 1988). In fact, phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine are subject to diffusion in both directions (Bitbol and Devaux, 1988; Connor et al., 1992); their steady-state distribution is the result of these movements. Phosphatidylserine (and to a lesser extent phosphatidylethanolamine) accumulates in the cytoplasmic layer because its inward transport is much more efficient than its outward motion. As for phosphatidylcholine, the outward diffusion rate being 2–3 times higher than the inward one, it is more abundant in the outer layer. Sphingomyelin distribution is more puzzling, as no inward motion can be seen in the erythrocyte (Morrot et al., 1989; Zachowski et al., 1985), which is in accordance with the fact that the sphingomyelin populations found in each leaflet differ in their species (Boegheim et al., 1983). Apparently, this absence of diffusion might be explained by sphingomyelin–cholesterol interactions, as depleting the erythrocyte membrane in cholesterol stimulated sphingomyelin transmembrane movement (our unpublished data). This absence of sphingomyelin diffusion was also detected in other cells, namely hamster fibroblasts (El-Hage Chahine et al., 1993).

The inner-to-outer lipid movement is reported to be stimulated by cytoplasmic ATP and is dependent on protein integrity (Bitbol and Devaux, 1988; Connor et al., 1992). This can explain why in systems without ATP such as viruses (Allan and Quinn, 1989) or erythrocyte vesicles (Raval and Allan, 1984), membrane asymmetry is very slowly quenched.

PERTURBING THE TRANSMEMBRANE ASYMMETRY

During cell life, events can occur where asymmetry is partially or completely destroyed after sudden scrambling of the two membrane halves. Some of these events are ‘laboratory perturbations’ and are unlikely to happen physiologically. On the other hand, some are physiologically relevant, and correspond to a stimulated state.

Scrambling by membrane rupture

Lysis of a cell is obviously something which normally implies cell death. However, red cells can be lysed under controlled conditions, then tightly resealed, entrapping defined media (ghosts). Lipid asymmetry in ghosts is less marked than in intact cells (Connor et al., 1990; Schrier et al., 1992b; Shukla et al., 1978). Restoration of the aminophospholipid asymmetry (but not that of phosphatidylcholine or sphingomyelin, which diffuse too slowly) can occur rapidly, provided the ghosts contain Mg-ATP, to allow the aminophospholipid translocase to function. One can hypothesize that the asymmetry perturbation due to cell lysis is not peculiar to erythrocytes and may occur with any other cell. This implies that any determination of lipid disposition in isolated plasma membranes may not reflect the *in situ* asymmetry.

Scrambling by amphiphilic drugs

Cationic amphiphilic compounds, such as chlorpromazine or vinblastine, are known to induce a shape change of erythrocytes,

from discocytes to stomatocytes. According to the bilayer couple hypothesis (Deuticke, 1968; Sheetz and Singer, 1974), this change is due to a greater relative expansion of the cytoplasmic leaflet by accumulation of the compound in question. Surprisingly, this shape change depends on the hydrolysis of cytoplasmic ATP (Schrier et al., 1986), at least when moderate amounts of amphipathic species were used. In fact, the insertion of such molecules into the membrane induced, by an unknown mechanism, the scrambling of a small fraction (10–20%) of the membrane lipids (Schrier et al., 1983, 1992a). When ATP is available, the aminophospholipid translocase will remove phosphatidylserine and phosphatidylethanolamine from the outer leaflet and relocate them in the inner one. This movement is not compensated by an opposite and equal movement of phosphatidylcholine and sphingomyelin, and thus there will be an excess of phospholipids in the cytoplasmic monolayer, resulting in the appearance of stomatocytes. At very high amounts of cationic amphipath, the overpopulation of the internal surface is also due to the molecule itself, which is slightly asymmetrically distributed in the bilayer due to the pH gradient.

Scrambling by cellular activation

Platelets are activated by stimuli which induce an increase of the free cytoplasmic calcium concentration. Among the various cellular modifications which follow activation (for a review, see Siess, 1989), one is directly related to the orientation of phospholipids in the plasma membrane. Activated platelets offer a procoagulant surface which is due to the appearance of phosphatidylserine in the outer layer. The degree of phosphatidylserine exposure strongly depends on the agonist used to activate the platelets (Bevers et al., 1983; Zwaal et al., 1989). Parallel to this outward and sudden movement of phosphatidylserine, and also phosphatidylethanolamine (originally present at > 90% and 80% respectively in the inner leaflet), there is an opposite movement of sphingomyelin (initially present at more than 90% in the outer layer). Since phosphatidylcholine is almost symmetrically exposed, it is not easy to detect its redistribution. Strong agonists, such as a mixture of collagen and thrombin, or calcium ionophore, induce an almost complete randomization of the phospholipids. Other membrane events occur during the activation: weak agonists induce the fusion of intracellular granules with the plasma membrane and the release of their internal contents into the external medium; strong agonists induce, in addition, the budding of the plasma membrane followed by the shedding of vesicles. The liberation of these vesicles, as well as the exocytosis of the cell granules, involve the fusion (or fission) of opposing zones of membrane. At the point of fusion/fission, transient non-bilayer structures will appear (Burger and Verkleij, 1990; Sims et al., 1989), allowing a fast mixing of the components within the bilayers. Once these events have ceased, the aminophospholipid translocase should restore the internal localization of phosphatidylserine and phosphatidylethanolamine. In fact, stimulation by weak agonists causes only a slight increase (from 0.1 to 1 μM) in cytosolic calcium, which remains below the inhibitory concentration for translocase activity, and transport can correct the phosphatidylserine and phosphatidylethanolamine distribution (in fact, aminophospholipid translocase activity is even stimulated under these conditions; Tilly et al., 1990). Strong agonists induce a much higher cytosolic calcium concentration, which completely inhibits the aminophospholipid translocase and thus the scrambled repartition of the phospholipids is maintained. Moreover, in these cells, two other mechanisms also inhibit the transport

system: oxidation of thiol groups and protein degradation by intracellular calpain (Comfurius et al., 1990).

Scrambling of the phospholipids between the two membrane halves can be induced in erythrocytes by increasing the cytosolic free calcium using an ionophore (Comfurius et al., 1990; Verhoven et al., 1992; Williamson et al., 1992). All the phospholipids are affected by this rearrangement, and its extent depends on the calcium concentration (Chandra et al., 1987). Aminophospholipid translocase is inhibited by intracellular calcium (Bitbol et al., 1987) and the normal asymmetry of phosphatidylserine can be restored after removal of calcium and regeneration of the usual ATP concentration (Comfurius et al., 1990). At high calcium load, the shedding of microvesicles from the erythrocyte (Allan and Michell, 1975) could explain the scrambling. Actually, the fusion between opposing faces of membranes, necessary for shedding, induces the formation of temporary non-bilayer structures allowing a rapid redistribution of molecules between the monolayers (Sims et al., 1989). At lower calcium concentrations, there is no such budding and the origin of the fast exchange of phospholipids between the membrane halves may involve phosphatidylinositol 4,5-bisphosphate. Recent experiments (Gascard et al., 1993) have shown that incorporation of 1% phosphatidylinositol 4,5-bisphosphate into the outer leaflet (i.e. an amount equivalent to that in the inner leaflet), followed by the addition of calcium, induces a membrane lipid scrambling whose intensity is a function of the amount of cation.

If we assume that any fusion or fission of membranes will induce a (partial) scrambling of their components, this lipid mixing should occur during the following events: endocytosis, exocytosis, virus fusion with or budding from a cell membrane, budding of vesicles from the endoplasmic reticulum or the Golgi apparatus, and cell division. At least during virus fusion, such a lipid redistribution has been observed (Hoekstra, 1983). There may still be other cellular events where non-bilayer phases occur in membranes, such as the insertion of signal peptides in a bilayer (Killian et al., 1990). It is important to point out that such physiological events would induce moderate mixing of membrane lipids: if a large fraction of phospholipid is exchanged and if the aminophospholipid translocase is still active and restores phosphatidylserine and phosphatidylethanolamine to their original location, slow-diffusing phosphatidylcholine and sphingomyelin will be unable to compensate for this movement, and an important imbalance will exist between the two layers. This may not be compensated by cholesterol. To escape from this mechanical stress, some parts of the membrane have to be extruded, as seen through the appearance of pseudo-endocytic vesicles in erythrocytes (Schrier et al., 1992a). It is understandable that a cell cannot survive such repetitive shocks.

Consequences of scrambling on membrane fluidity

It has been noted above that the fatty acyl composition of phospholipids differs with the polar head group, and that this has consequences for the 'fluidity' of each leaflet in an asymmetric membrane. Thus, any important redistribution of phospholipids within the membrane will affect this parameter. This assumption has been verified experimentally. Thus, when comparing intact red cells and resealed ghosts, where a partial scrambling has occurred (see above), the ghost outer leaflet is more fluid than the erythrocyte outer leaflet, and the converse is true for the inner leaflet, as probed with spin-labelled phospholipids (Schrier et al., 1992b; Tanaka and Ohnishi, 1976). Increasing the free cytoplasmic Ca^{2+} concentration, which induces a (partial) randomization of the membrane phospholipids, minimized the difference

in microviscosity between the membrane halves (Yamada and Ohnishi, 1983). An increase of 'fluidity' in the outer membrane layer of cells can also be detected with merocyanine 540 (MC 540); this fluorescent dye is a probe of lipid packing (Williamson et al., 1983), and it does not stain a normal erythrocyte or an ATP-containing resealed ghost. However, when the asymmetry was perturbed by elevating the internal calcium, the cell became fluorescent (Verhoven et al., 1992; Williamson et al., 1985). When the loss of asymmetry has been counteracted by the action of the aminophospholipid translocase, upon providing ATP to a ghost, the amount of dye bound decreased, as phosphatidylserine and phosphatidylethanolamine were imported back into the internal leaflet (Verhoven et al., 1992). Quiescent platelets did not take up MC 540, but activated ones were stained by the dye (Lupu et al., 1986). The destabilization of the cell membrane during cell fusion, that we previously postulated, is obvious if one looks at the lateral diffusibility of phospholipids: the fusion of Sendai virus with human red cells provoked not only a higher transmembrane diffusion rate (Bashford, 1988) but also a higher diffusion rate in the plane of the outer membrane leaflet (Aroeti et al., 1992) which we can attribute to the appearance of phosphatidylserine and phosphatidylethanolamine in this leaflet. If the cell was deprived of ATP, this change was permanent. In the presence of ATP (i.e. when the aminophospholipid translocase was able to re-establish the concentration of phosphatidylserine and phosphatidylethanolamine in the inner leaflet), the change was transient.

POSSIBLE CELLULAR ROLE FOR LIPID ASYMMETRY

The presence of an asymmetric distribution of phospholipids in certain membranes should be important for cellular life. This section is a partial survey of cellular activities which are dependent on the phosphatidylserine and phosphatidylethanolamine content of the lipid phase. Figure 4 illustrates these membranous events.

Phosphatidylserine and protein kinase C

Protein kinase C plays an important role in the transduction of extracellular signals which induce phospholipid modification. Hydrolysis of phosphatidylinositol 4,5-bisphosphate or phosphatidylcholine by phospholipase C generates diacylglycerol, which activates protein kinase C bound in a Ca^{2+} -dependent manner to phosphatidylserine-containing membranes (Nishizuka, 1986). In fact, protein kinase C can bind to any membrane containing acidic phospholipids, but its activity depends on an interaction with phosphatidylserine (Hannun et al., 1986) and Ca^{2+} and diacylglycerol increase the specific affinity of the protein towards phosphatidylserine (Orr and Newton, 1992a,b). Phosphatidylethanolamine can substitute for phosphatidylserine, but at least $10\ \mu\text{M}$ Ca^{2+} is required to observe some phosphorylation, compared with $0.1\ \mu\text{M}$ or less required in the presence of phosphatidylserine (Bazzi et al., 1992). Binding of the protein and kinase activity showed an identical sigmoidal dependence on the mole fraction of phosphatidylserine in the membrane (Newton and Koshland, 1989; Orr and Newton, 1992a,b), favouring a clustering of phosphatidylserine induced by the protein (Bazzi and Nelsuesten, 1991). Substrate specificity is affected *in vitro* by the phosphatidylserine concentration in membranes (Newton and Koshland, 1990): autophosphorylation is favoured at low and intermediate phosphatidylserine concentrations, while phosphorylation of exogenous substrates (histones) is more important at high phosphatidylserine concentrations. However, histone phosphorylation reaches a maximum

Figure 4 Schematic representation of some aminophospholipid-dependent reactions occurring at the extracellular or cytoplasmic surfaces of a plasma membrane

The polar head group of each phospholipid is represented by its usual abbreviation (see the legend to Figure 2).

at phosphatidylserine concentrations equal to 20 mol%, precluding modulation of the protein kinase C activity by the phosphatidylserine membrane content. Other substrates exhibit a greater possibility of regulation by membrane phosphatidylserine; the microtubule-associated protein τ , for instance, is poorly phosphorylated at phosphatidylserine concentrations lower than 20 mol%, and after that its phosphorylation increases linearly with phosphatidylserine content (Newton and Koshland, 1990). If such behaviour is true for other protein kinase C substrates (e.g. the glucose transporter and the insulin receptor), it may suggest how phosphatidylserine could modulate cellular activities through its regulation of protein kinase C activity.

Phosphatidylserine, phosphatidylethanolamine and membrane fusion

Fusion of two membranes, which occurs during cellular events such as exocytosis, is a very complex phenomenon, where proteins, lipids and ions are involved. It is beyond the scope of this Review to cover all the aspects of membrane fusion; however, I will consider two reactions, among many others, where lipid composition plays a critical role.

Binding of annexins

Annexins belong to a family of eight proteins which bind to membranes in a Ca^{2+} -dependent manner (Burgoyne and Geisow, 1989; Crumpton and Dedman, 1990). Their complete physiological role is as yet unknown, but they can aggregate and mediate the fusion of vesicles (Creutz et al., 1978; Drust and Creutz, 1988) and thus could modulate intracellular fusions. Annexin binding is supported by the presence of phosphatidylserine in the membrane, but other anionic phospholipids (phosphatidic acid or phosphatidylinositol) or even phosphatidylethanolamine can be sufficient (reviewed by Klee, 1988), i.e. the major phospholipids of a cytoplasmic membrane leaflet. The

amount of calcium required for annexin binding to membranes depends on the phospholipid composition and is related to the size and hydrophilicity of the lipid head group, considering that Ca^{2+} has to interact with the phosphoryl residue (Huber et al., 1990). Binding of annexins to the membrane modifies the physical state of the bilayer, as it reduces the lateral diffusion of phospholipids (Meers et al., 1991). However, even if annexins promote the aggregation of membranes, their fusion will require a mixing of the two bilayers and lipid composition modulates this phenomenon.

Creation of fusion-competent surfaces

To fuse, bilayers have first to appose; however, when these two hydrated surfaces are at 2 nm, or less, hydration pressure generates a repulsive barrier (LeNeveu et al., 1977). The fact that the fusion of two membranes requires the presence of anionic phospholipids (such as phosphatidylserine) and of Ca^{2+} (Papahadjopoulos et al., 1976) was interpreted, among other mechanisms, as a Ca^{2+} -induced close apposition of membranes without intervening water (Portis et al., 1979). This explains why lipids with a low hydration level of their head group, such as phosphatidylethanolamine, enhance liposome fusion. Note that the Ca^{2+} doses required for a fusion of two liposomes are in the millimolar range, indicating that, in the cell, the ion will be unable to promote fusion by acting directly at the lipid level. Membrane contact has to be followed by bilayer destabilization in order to fuse the membranes. It has been proposed that, in phosphatidylserine-containing membranes, another role of Ca^{2+} is to provoke an isothermic phase change from a fluid to a crystalline state (Papahadjopoulos et al., 1977), fusion occurring at the domain boundaries. Later, it was shown that fusion proceeds approximately ten times more rapidly than phase separation and that Mg^{2+} , which can induce fusion at high concentrations, does not induce phase separation (Hoekstra,

1982). A more recent hypothesis is that destabilization is generated by non-bilayer structures (Ellens et al., 1989) and that fusion between the two membranes occurs at a local point (Burger and Verkleij, 1990). Amongst lipids which easily form non-bilayer phases is phosphatidylethanolamine, abundant in cytoplasmic membrane leaflets. The nature of the phase responsible for fusion is not well defined. Formation of hexagonal H_{II} structures seems to be excluded as they would generate leaky fusion (Ellens et al., 1985). Some theoretical models favour the involvement of inverted micelles, precursors of H_{II} phases appearing in the physiological temperature range, whose lifetime agrees with the membrane fusion rate (Siegel, 1986). These inverted phases are stabilized by diacylglycerol (Kirk and Gruner, 1985) and the temperature at which they appear is noticeably lowered by the same molecules (Siegel et al., 1989). This would explain how phospholipase C induces the fusion of membranes (Burger et al., 1991).

Endocytic invagination as a consequence of exocytic events

Fusion of an exocytic vesicle with the plasma membrane will produce a redistribution of lipids in the bilayer. Phosphatidylserine sited in the outer leaflet will be relocated to the inner leaflet by aminophospholipid translocase. Movement in the opposite direction of phosphatidylcholine and sphingomyelin is much slower, leading to an excess of lipid present in the inner monolayer. This imbalance in the bilayer couple creates an invagination of the membrane and may even cause the formation of a vesicle. This has been obtained in human erythrocytes as a consequence of bilayer scrambling induced by amphipathic compounds (Schrier et al., 1992a). It is clear that endocytosis requires the action of other cellular components (Brodsky, 1988; Evans and Enrich, 1989), but lipid rearrangement is certainly one of the early steps of this phenomenon. That aminophospholipid translocase is involved in membrane curvature and invagination is supported by experiments with erythrocytes having a high ATP content. Under these conditions, an energy-dependent endocytosis is observed (Ben-Bassat et al., 1972; Penniston, 1972; Penniston and Green, 1968), attributed to the activity of a 'shape change Mg^{2+} -ATPase' (Morris et al., 1992; Patel and Fairbanks, 1986), probably the same protein as the aminophospholipid translocase. The shape change and the invagination are the consequence of an increased accumulation of phosphatidylethanolamine in the cytoplasmic monolayer as the ATP level increases (Calvez et al., 1988).

Consequences of the presence of phosphatidylserine in the outer leaflet of blood cells

Under normal conditions, almost no phosphatidylserine is present in the outer leaflet of circulating blood cells (erythrocytes, platelets, and probably leucocytes). However, this phospholipid is exposed in some pathologies, or after platelet stimulation. I will briefly list the consequences of such a change.

Formation of a procoagulant surface

Following platelet activation, there is a release into the extracellular space of coagulation proteins (such as fibrinogen and factor V), stored in the cellular α -granules, which are then activated by proteolytic degradations. This chain of reactions requires both Ca^{2+} and anionic phospholipids (phosphatidyl-

serine) exposed at the outer surface. Phosphatidylserine acts at different stages of the activation cascade, such as specific binding of activated factor VIIIa to membranes (Gilbert et al., 1992) and lowering the apparent K_m for factor X (van Dieijen et al., 1981) and for prothrombin (Rosing et al., 1980) well below their concentrations in plasma.

Phosphatidylserine and cellular recognition

It is possible to detect an abnormal amount of phosphatidylserine in the membrane outer leaflet in some pathological red cells, such as sickle cells. The phospholipid asymmetry is reduced in the old (dense) cell fraction in any conditions and in the young (light) fraction in a de-oxygenated atmosphere (Lubin et al., 1981; Blumenfeld et al., 1991). The high adherence of the sickled cells to the vascular endothelium (Hebbel et al., 1980; Hoover et al., 1979) is a direct consequence of the appearance of a significant amount of exposed phosphatidylserine: the same behaviour is obtained with ghosts from normal red cells where the asymmetry has been quenched (Schlegel et al., 1985). Erythrocytes with exposed phosphatidylserine, due to either natural (sickled cells) or artificial (lipid-symmetric ghosts and phosphatidylserine-enriched erythrocytes) causes, exhibit an increased adherence to monocytes and macrophages (McEvoy et al., 1986; Schwartz et al., 1985; Tanaka and Schroit, 1983) followed by an accelerated clearance (Schroit et al., 1985). This mechanism of recognition might be responsible for elimination of aged erythrocytes from the bloodstream, provided that the loss of phosphatidylethanolamine asymmetry found in the denser cells (Shukla and Hanahan, 1982; Herrmann and Devaux, 1990) precedes phosphatidylserine appearance on the outer leaflet. Note that artificial lipid vesicles which contain phosphatidylserine are also phagocytosed by macrophages (Schroit and Fidler, 1982), as are apoptotic lymphocytes which expose phosphatidylserine at their surface (Fadok et al., 1992). In a similar way, tumorigenic cells are bound and lysed by activated monocytes or macrophages, as they express much more phosphatidylserine in their membrane outer leaflet than their non-tumorigenic counterparts (Connor et al., 1989; Utsugi et al., 1991).

CONCLUDING REMARKS

Amazingly, research on phospholipid transmembrane asymmetry and movement is now focusing on proteins. So far, two 'flippase' proteins have been functionally characterized. The aminophospholipid translocase is responsible for the relocation into the cytoplasmic leaflet of the plasma membrane of phosphatidylserine and phosphatidylethanolamine molecules which appear in the outer leaflet. This carrier, which is an integral Mg -ATPase, seems to be present in the plasma membrane of all animal cells. The characteristics of the transport reaction are now well established, in particular substrate specificities and stoichiometry. But nothing is known about its possible regulation by cellular components, with the exception of the inhibitory role of cell calcium. Since the enzyme is responsible for establishing and maintaining the asymmetry, it will be interesting to study if it is affected during cellular events, such as stimulation, in which the lipid phase is modified. To determine where the asymmetry is created, attempts have been made to localize the aminophospholipid translocase in cell subfractions. It is present in chromaffin granule membranes and absent from endoplasmic reticulum membranes. In this latter organelle, there exists another protein, functioning like a pore, which allows the fast equilibration of any phospholipid between the two membrane halves without requiring ATP. Transverse lipid diffusion has to be

studied in other subcellular membranes in order to ascertain if one of the two 'flippases', or eventually a third one, is present. Unfortunately, all the currently described quantitative assays require a large amount of membranes which limits this search to particular cells.

More progress will be made when the various proteins have been purified and cloned. Antibodies raised against the 'flippases' will allow their localization within any cell and permit their presumably ubiquitous character to be confirmed. Structural studies of the purified proteins will help to answer the puzzling question of the architecture of a peptide which moves simultaneously the hydrophilic and hydrophobic moieties of a molecule through a lipid bilayer. Molecular biology, irrespective of information on protein sequence, will contribute to the understanding of the real involvement of the 'flippase(s)' in cell life, either by overexpression of the proteins or by selection of defective mutants. We can thus expect that the next few years will bring us a lot of interesting new information on this aspect of cell biology.

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