LIPID MOBILITY AND MICROVISCOSITY

LECTURE

TECHNIQUES TO DETERMINE TRANSBILAYER ORGANIZATION AND DYNAMICS OF MEMBRANE PHOSPHOLIPIDS

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INTRODUCTION

Nowadays, there are no more arguments opposing the view that the structural backbone of a biological membrane is provided by a lipid bilayer, the fatty acyl chains of the phospholipids forming the hydrophobic core of the membrane and their polar head groups facing the aqueous environments at either side of the bilayer. As a consequence of the primary task of a membrane, which is to separate two aqueous compartments from one another in which entirely different processes take place, it is conceivable that the chemical characteristics of one side of a membrane differ considerably from those of the other. Indeed, the absolute asymmetry in both transversal localization and orientation of, respectively the periferal and integral, proteins in a membrane has already been recognized a couple of decades ago. Since the early seventies, it is also known that the different classes of phospholipids may be distributed over both halves of the bilayer in a highly asymmetric fashion, which phenomenon particularly applies to plasma membranes. The first information on this point has been gained from studies on erythrocytes and, still, the membrane of those cells is the best characterized one with regards to the transbilayer organization and dynamics of its lipids [1].

Keywords: Phospholipid asymmetry, Phospholipid flip-flop, Phospholipases, Phospholipid transfer proteins.

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TECHNIQUES FOR PHOSPHOLIPID LOCALIZATION STUDIES

The three types of probes that are most commonly used to assess the transbilayer distribution of phospholipids in a membrane are: (i) group specific chemical reagents, (ii) phospholipases and (iii) phospholipid transfer proteins. The general strategy for their application in such studies is simple and straightforward. Intact, closed membranes are exposed to the action of the probe and the modification of the phospholipids thus obtained is compared with that after treatment of open membranes with the same reagent. The philosophy behind this approach will be obvious. When intact cells are treated with the reagent, only those phospholipids will be modified that are localized in the outer half of the bilayer. Treatment of open membranes serves as an essential control to make sure that, under the conditions applied, the probe can react with all of its potential target molecules when it has access to both sides of the membrane.

This general strategy implies a number of prerequisites which should be fulfilled to achieve conclusive results. These comprise the following: (i) the structural integrity of the membrane should be maintained during the experiment, so that the action of the probe remains restricted to that side of the membrane where it is supposed to explore its action; (ii) this also implies that, preferably, the membrane studied should be impermeable to the reagent; (iii) transbilayer movements of phospholipids should be absent, or at least very slow relative to the time scale of the experiment; and (iv) the treatment as such should not give rise to transbilayer rearrangements of phospholipids.

Each of the three above mentioned types of probes will be briefly discussed below.

Group specific chemical reagents

In the past, quite a number of chemical reagents have been developed which specifically react with free NH_2-groups [1,2], and therefore can provide information as to the localization of the amino-phospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS). A reagent that has been most widely used for this purpose is tri-nitrobenzenesulphonic acid (TNBS). However, the reaction between TNBS and amino-phospholipids may be incomplete, particularly in case of a membrane containing relatively high amounts of these phospholipids, because of sterical hindrance as a consequence of the introduction of the bulky TNB-group, whereas the reactivity of the negatively charged TNBS towards PS will be impaired by electrostatic repulsion between this reagent and the -also negatively charged- polar head group of this phospholipid [1]. Moverover, and in contrast to what has been believed until recently, TNBS can easily permeate through the red cell membrane [3].

Another NH_2-group specific reagent is fluorescamine, which has some major advantages . When applied at pH 8.0 (or higher), it

reacts with free NH_2-groups within a second, the excess of the reagent being destroyed by aqueous hydrolysis within a minute. In contrast to this hydrolysis product, the product formed by reaction with a NH_2-group is a fluorophor, which enables a sensitive quantitative determination of the reaction product. That this probe, despite its ability to permeate through a membrane, can be satisfactorily used to determine the distribution of PE in the plasma membrane of a cell which also contains intracellular membrane systems, is shown in figure 1. Intact Friend

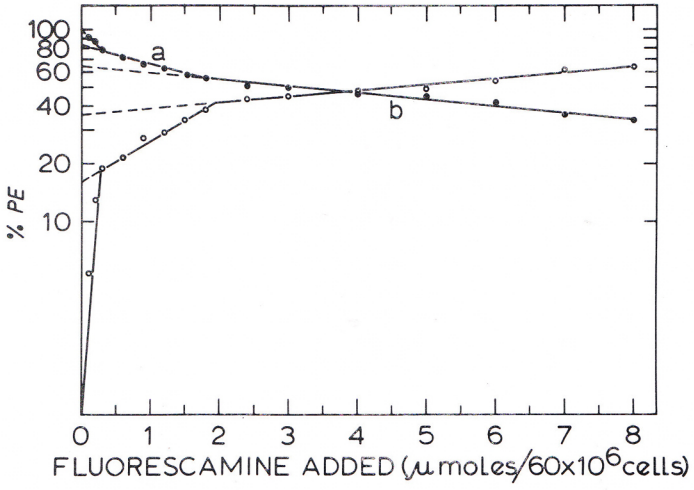

FIGURE 1

Semi-logarithmic plot of the loss in phosphatidylethanolamine (•) and of the appearance of its fluorescamine derivative (o) in Friend cells labeled at 0-4°C with increasing amounts of fluorescamine. Phosphatidylethanolamine pools attributable to the outer and inner plasma membrane monolayer and to the intracellular membranes were derived by extrapolating lines a and b, resp., of the curve representing the loss of phosphatidylethanolamine (o).

erythroleukaemic cells have been exposed to increasing concentrations of fluorescamine. The semi-logarithmic dose-response curves of the labeled, respectively unlabeled, PE in these treated cells, reveals three different pools [4]. The first and fast reacting pool represents the PE in the outer leaflet of the plasma membrane, the second pool represents that fraction of the PE in the inner monolayer of this membrane, and the third and slowly reacting pool reflects the PE located in the subcellular membranes. The distribution of PE in the plasma membrane of these cells, as determined by this technique, could be confirmed by experiments using phospholipases [5].

Phospholipases

Phospholipases represent the most popular group of probes used in phospholipid localization studies, due to the differences in their mode of action and substrate specificity [6].

Phospholipases A_2 (PLA_2), which can be isolated from pancreas, bee venom and a variety of snake venoms, can only attack diacyl *glycero*-phospholipids. These enzymes specifically split the ester bond at the 2-position of the glycerol back bone, thus producing a free fatty acid and a 1-acyl lyso-derivative. It is important to note that both these reaction products remain in the membrane.

Phospholipases C (PLC), which are usually from bacterial origin, cut off the complete polar head group from a phospholipid. This results in the production of diglycerides or ceramides, depending on whether the substrates are diacyl glycero-phospholipids or sphingomyelin (SM), respectively. These digestion products are left behind in the membrane. The polar head groups that are split off are readily water soluble and will, therefore, immediately leave the membrane. Sphingomyelinase C (SMase C) is a genuine phospholipase C, but it has an almost absolute specificity towards SM.

Phospholipases D are from vegetable origin and usually isolated from cabbage or peanuts. Those enzymes convert glycero-phospholipids into the highly negatively charged phosphatidic acid.

The application of phospholipases in phospholipid localization studies requires some special prerequisites [6], which concern: (i) the purity of the enzyme used, because any contamination that disrupts the structural stability of the membrane studied may largely affect the reliability of the results; (ii) a proper and complete inhibition of phospholipase activity after the incubation and prior to lipid extraction, because most of these enzymes will not be inactivated by organic solvents such as methanol and chloroform, which are most commonly used for such extraction procedures; and (iii) the selection of the proper procedure to extract the lipids from the phospholipase treated membranes, taking into account that this treatment may have caused extensive changes in lipid composition of the membranes which, in turn, may have important consequences for their extraction and final recovery.

The possibilities and limitations that are inherent to the use of phospholipases in phospholipid localization studies, can be illustrated by their use on erythrocytes. The strategy of such experiments is depicted in figure 2. In addition to the exposure of intact cells and open ghost membranes to the action of phospholipases, the erythrocyte provides the possibility of performing a third type of experiments (Fig. 2). To that end, a suspension of open ghosts (prepared by hypotonic lysis) is supplemented with a phospholipase in its inactive form, i.e. in the presence of a chelator such as EDTA to remove the divalent cations that are necessary for its activity. Subsequently, ghosts are resealed by restoring isotonicity, thereby trapping the (inactive)

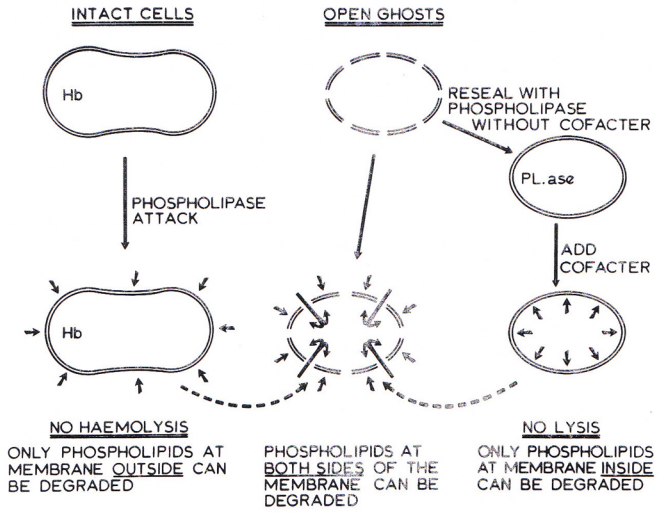

FIGURE 2

Scheme of phospholipase attack on intact red cells and open ghosts, as well as inside resealed ghosts. Hb, haemoglobin; PL.ase, phospholipase.

enzyme in their cytosol. After several washes of the resealed ghosts to remove the phospholipase from the medium, the trapped enzyme is activated by addition of its essential cofactor which, in case of phospholipase A_2, is Ca^{2+}. The enzyme then starts to degrade its substrates present in the inner membrane leaflet. The hydrolysis patterns thus obtained should, at least in the ideal situation, be complementary to the hydrolysis pattern of phospholipids achieved by treatment of the intact cells with the enzyme.

Treatment of intact human erythrocytes under non-lytic conditions with pure *Naja naja* PLA_2 ultimately results in the hydrolysis of 68% of the PC. When after 1 h, SMase C is added to this incubation mixture, upto 82% of the SM is rapidly degraded. It is of considerable interest to note that this degradation of SM in the outer monolayer enables the PLA_2, that is still present, to degrade an additional fraction of the PC as well as 20% of the PE [7]. Even under those conditions, any hydrolysis of PS is still not detectable which indicates that this phospholipid is exclusively located at the cytoplasmic side of the membrane. The degradation of the other three phospholipids comprises 48% of the total phospholipid complement of the membrane (Table I), suggesting that

TABLE I Percentage of nonlytic degradation of phospholipids by phospholipases in human erythrocytes. Incubations were started in the presence of phospholipase A_2 (*N.naja*), followed by the addition of sphingomyelinase C (*S. aureus*) after 1 h.

Incubation time (min)	Enzyme		% degradation				
	PLA_2 (*N.naja*)	SMase C (*S.aureus*)	Total phospho-lipid	SM	PC	PE	PS
0	↓	↓	0	0	0	0	0
60			20	0	68	0	0
120			48	82	76	20	0

indeed all phospholipids in the outer monolayer have been degraded to completion. Hence, it is important to note that a complete picture of the phospholipids constituting the outer membrane leaflet, can only be obtained by using this combination of phospholipases. PLA_2 alone is not able to degrade all of its substrates in the outer monolayer. This is due to the fact that the action of the enzyme ceases as a consequence of an increase in lateral surface pressure in that layer [8]. This, in turn, is due to the production of split products (free fatty acids and lyso-PCs) in that layer, since these two split products occupy a surface area which is slightly larger than that occupied by the original diacyl-PC. In this situation, the enzyme can no longer reach its substrates; in other words, the reaction is self-quenching [9]. Conversily, the removal of the polar head group from SM by SMase C has the opposite effect, namely a decrease in lateral surface pressure in the outer monolayer, which enables the PLA_2 that is still present to complete its job [7].

Figure 3 shows the time course for the hydrolysis of the phospholipids in the inner leaflet, as brought about by pancreatic PLA_2 trapped inside resealed ghosts. Unfortunately this treatment ultimately results in complete lysis of these ghosts. Nevertheless, it is of interest to note that -just before the onset of lysis- the degradation of PC tends to level off at 25%, which is exactly complementery to the 76% of the PC that can be degraded in the outer layer of the intact erythrocyte (Table I). On the other hand, the hydrolysis of PE and PS is already considerable and still in full swing at the moment lysis of the cells starts to take place, indicating that indeed the amino-phospholipids are predominantly located in the inner half of the bilayer [7].

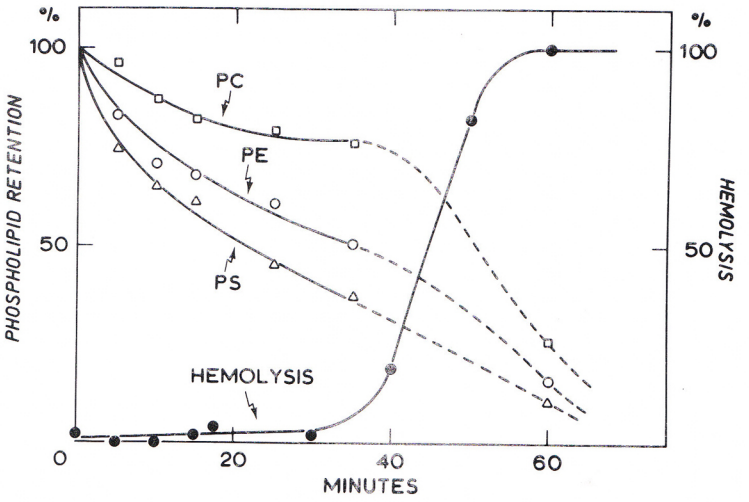

FIGURE 3
Percentage retention of glycerophospholipids after trapping of pancreatic phospholipase A_2 (without Ca^{2+}) inside resealed human erythrocyte ghosts followed by the addition of Ca^{2+} to start enzymatic breakdown at t=0. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Phospholipid transfer proteins

A third type of probes to determine membrane phospholipid distributions has been introduced more recently, namely the phospholipid transfer proteins [10]. Their major advantage is that, at least in principle, they do neither modify the phospholipid composition, nor the structure of the membrane studied.

A transfer protein that has been most widely used so far is the PC-specific one (PC-TP), purified from beef liver. This water soluble protein mediates a one-for-one exchange of PC molecules between two membrane systems [10]. The intact cells to be studied, usually referred to as the "acceptor", are incubated in the presence of the PC-TP together with a suitable "donor" system, i.e. either small unilamellar vesicles or rat liver microsomes containing radiolabeled PC of known specific activity. During the incubation, part of the PC in the acceptor membrane will be replaced by radioactive species. After the incubation and separation of donors and acceptors, the lipids from both systems are extracted, chromatographically separated and the specific

radioactivity of the PC in both fractions determined. By comparing both values, it is quite easy to calculate what fraction of the PC in the acceptor membrane is in equilibrium with that in the donor system.

Figure 4 shows the results of such experiments involving intact erythrocytes from man (panel A) and rat (panel B), respectively. In normal human erythrocytes, 74-76% of the PC appeared to be readily available for PC-TP mediated exchange [11]. This is in perfect agreement with the fraction of PC, namely 76%, that is available in the intact cell for attack by PLA_2 in the presence of SMase C (compare Table I). Obviously, this pool represents the fraction of the PC in the outer membrane leaflet which, as can be deduced from the fact that the exchange profile ultimately reaches a horizontal plateau (Fig. 4, panel A), does not appear to

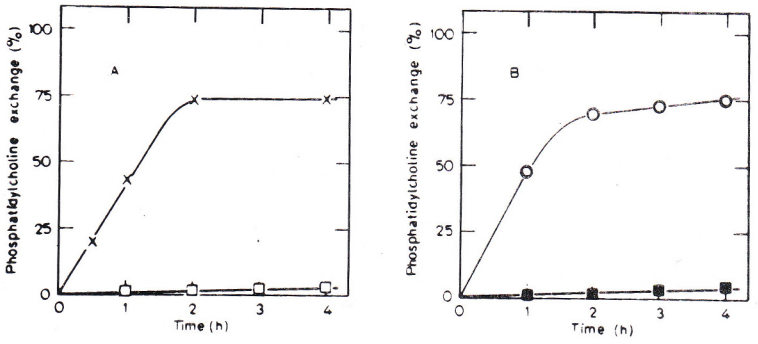

FIGURE 4

Exchange of PC between erythrocytes and rat liver microsomes. The amount of PC exchanged in the absence (□, ■) and presence (X,O) of PC-TP is plotted as a percentage of total erythrocyte PC, for human (A) and rat (B) erythrocytes.

equilibrate with the PC in the inner layer. The latter phenomenon indicates that, relative to the time scale of the experiment, transbilayer movements ("flip-flop") of PC in the human erythrocyte must be very slow.

Rat erythrocytes appeared to behave a bit differently in this respect, in that a fast exchangeable pool, comprising 50-60% of all the PC present, was followed by a slowly exchangeable one (Fig. 4, panel B). From this biphasic curve, it was estimated that PC equilibrates between the two halves of the bilayer with a half-time of 7 h [11]. Both these results are in quite good agreement with data obtained by using a non-specific TP [12] or phospholipases [13], respectively showing 63 and 62% of the PC in the outer monolayer of the rat erythrocyte membrane and half-times for PC flip-flop of 7.2 and 4.5 h.

From the results gained from experiments involving phospholipases, chemical reagents and the PC-TP, it could be concluded that the four major phospholipid classes in the human erythrocyte show a marked asymmetry in their distribution over both halves of the membrane bilayer[1,7]. The two choline containing phospholipids dominate the outer layer, in which 82% of the SM and 76% of the PC is found, whereas the inner membrane leaflet mainly consists of the amino-phospholipids: 80% of the PE and all of the PS.

DETERMINATION OF PHOSPHOLIPID TRANSBILAYER MOBILITY

In the procedure described in the previous section, the first aim of the experiments was to determine the pool sizes of the PC present at either side of a membrane bilayer. In many cases, an estimate can be made of the rates of transbilayer movement of the molecules by analysis of the kinetics of biphasic exchange processes.

To determine the actual flip-flop rates of (even individual molecular species of) PC, a technique has been developed recently [14] in which PC-TP is used to introduce radiolabeled PC into the outer membrane layer of the intact erythrocyte, after which its fate is followed by selectively degrading the outer monolayer by phospholipase treatment (Fig. 5). Intact erythrocytes are incubated

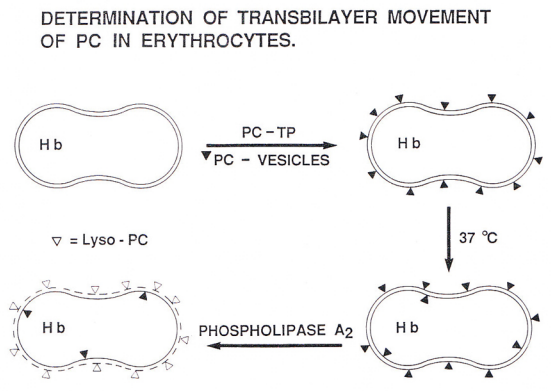

FIGURE 5
Principle of an experiment to determine the rate of transbilayer movement of PC in the erythrocyte membrane. Abbreviations: Hb, heamoglobin; PLEP, phospholipid exchange protein, i.e., PC-TP.

for 1 h in the presence of PC-TP, either with [*methyl*-^{14}C]PC labeled rat liver microsomes or with sonicated vesicles prepared from egg PC mixed with an equimolar amount of cholesterol, PA (5mol%) and trace amounts of [*methyl*-^{14}C]egg PC and [^{14}C]cholesteryloleate or glycerol[^{3}H]trioleate, the latter compounds as non-exchangeable marker. After removal of the PC-TP and the [^{14}C]PC donor system by careful washing, the erythrocytes are subsequently incubated in isotonic buffer to enable the radioactive PC, introduced into the outer monolayer, to equilibrate between the two halves of the membrane bilayer. At timed intervals, samples are taken and the intact cells are subsequently incubated with a suitable phospholipase A_2, thereby selectively converting the PC in the outer monolayer into its lyso-derivative. It should be kept in mind, however, that the use of phospholipase A_2 alone does not result in a complete degradation of the PC in the outer monolayer. This can only be achieved when this enzyme is used in combination with sphingomyelinase C, which should be added after preincubation of the cells with the phospholipase A_2 [6,7]. The lyso-PC thus produced, representing the PC in the outer monolayer, and the residual PC originating from the inner leaflet, can be easily separated from one another, which enables an accurate

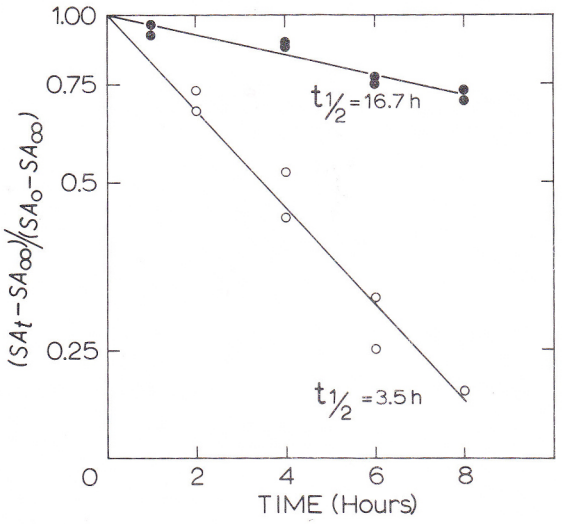

FIGURE 6

Transbilayer movement of 1,2-dioleoyl-PC (●) and 1-palmitoyl-2-linoleoyl-PC (○) in the human erythrocyte during incubation of the cells at 37°C.

determination of their specific radioactivities. The rate of transbilayer movement of the PC and the corresponding half-time for this process can be easily calculated from the time-dependent decrease in specific radioactivity of the lyso-PC, which should be proportional to the increase in that of the PC. In practice, the half-time value of PC flip-flop is determined from the slope of the straight line that is achieved when the changes in relative specific radioactivity of the lyso-PC are plotted semilogarithmically versus the time of incubation. Figure 6 shows the results of a typical experiment, involving 1,2-dioleoyl-PC and 1-palmitoyl-2-linoleoyl-PC, respectively. The half-time values ($t_{1/2}$) for the rate of their transbilayer movements, as indicated in Figure 6, have been calculated from the slopes of the corresponding lines.

The above method has been used to determine the rates of flip-flop of four individual molecular species of PC in the human erythrocyte membrane[15]. The results are presented in Table II.

Table II Flip-flop of individual species of phosphatidylcholine in the human erythrocyte membrane. Figures represent means ± S.D. values.

PC species	Halftime (h)
1,2-Dipalmitoyl	26.3 ± 4.4
1,2-Dioleoyl	14.4 ± 3.5
1-Palmitoyl-2-linoleoyl	2.9 ± 1.7
1-Palmitoyl-2-arachidonoyl	9.7 ± 1.6

In agreement with earlier studies [14], unsaturated PC species experience transbilayer movements in the human red cell membrane which are considerably faster than that of a fully saturated one, such as 1,2-dipalmitoyl-PC (Table II). On the other hand, and in contrast to earlier suggestions [14], there appears to exist no direct proportionality between the rate of flip-flop of a particular PC species and its total degree of unsaturation. 1-Palmitoyl-2-linoleoyl-PC experiences a transbilayer movement which is about four to five times as fast as that of 1,2-dioleoyl-PC (Table II; Fig. 6), although these two species have the same degree of unsaturation. This indicates that not only the total number of double bonds in a PC molecule, but more specifically their distribution over the two acyl chains, is an important determinant of its transbilayer mobility. Possibly even

more surprising is the finding that the flip-flop rate of the higher unsaturated 1-Palmitoyl-2-arachidonoyl-PC is appreciably slower than that of the 1-palmitoyl-2-linoleoyl species (Table II).

At present, one may only speculate whether these differences are a consequence of differences in the molecular geometry of the various PC species which, in turn, may influence their packing in the bilayer as well as their interactions with adjacent membrane constituents. Alternatively, they may represent a reflection of a possible heterogeneity in their lateral distribution in the membrane, i.e., in different domains in which the "facilities" for transbilayer movements may differ from one another. Further studies will be necessary to gain insight into the factors which govern the transbilayer mobility of the PC molecules in the erythrocyte membrane, and, more specifically, that of the individual molecular species.

Using radio-labeled egg-PC, rather than individual molecular species, as probe molecules in this flip-flop assay procedure, one in fact determines the average half-time value of PC flip-flop. This value appears to be 15-20 h in the human erythrocyte membrane, when determined at 37°C [16]. Although this process is relatively slow, it is still fast when compared to the average lifetime of the human erythrocyte, which is 120 days. Hence, it is fascinating to realize that this transbilayer movement has no consequences as to the asymmetric distribution of the different phospholipid classes in the red cell membrane. The mechanisms that are involved in maintaining this marked phospholipid asymmetry will be dealt with in the following chapter.

ABSTRACT

The general strategy to determine the transbilayer distribution of phospholipids in a membrane is based on the selective modification of those phospholipid molecules that are present in the outer membrane leaflet when intact cells are exposed to a suitable probe. Those probes can be sub-divided in the following three main classes, i.e. (i) chemical reagents, usually NH_2 group specific reagents, (ii) highly purified phospholipases, differing in mode of action and substrate specificity and (iii) phospholipid transfer proteins. This strategy implies that the following prerequisites have to be fulfilled in order to achieve conclusive results: (i) The structural integrity of the membrane should be maintained during the experiment, (ii) the probe should not be able to permeate through the membrane, (iii) transbilayer movements of phospholipids should be either absent or very slow relative to the time scale of the experiment, (iv) neither the probe itself, nor the incubation conditions should give rise to transbilayer reorientations of phospholipids, (v) all potential targets present in that half of the bilayer that is exposed to the reagent should show a complete reaction. Since none of the above mentioned types of probes meet all of these prerequisites,

definite conclusions can be drawn only when a great number of different probes and techniques are used, producing results that are in good agreement with each other. Three different techniques have been developed recently to determine the transbilayer mobility of phospholipids in a membrane, all of them being based on the same principle: the introduction of a labeled "reporter" molecule into the outer membrane leaflet of an intact cell, after which its reorientation to the inner monolayer is followed in time. These techniques involve the following types of reporter molecules: (i) radiolabeled lyso-phospholipids, (ii) spin-labeled phospholipid analogues, (iii) radiolabeled species of naturally occuring phospholipids. Only the last type of probe molecules, the insertion of which requires the use of a suitable transfer protein, provides information as to the actual transbilayer mobility of the native phospholipid molecules in a membrane.

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