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Selective Outside–Inside Translocation of Aminophospholipids in Human Platelets†

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ABSTRACT: Spin-labeled analogues of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin were added to human platelet suspensions. Due to the partial water solubility of these spin-labeled lipids which possess a relatively short β-chain (C_5), they incorporate rapidly in membranes. The orientation of the spin-labels within the platelet plasma membrane was assessed by following the spontaneous reduction at 37 and 4 °C due to endogenous reducing agents present in the cytosol. The rate of spontaneous reduction showed unambiguously that the labels incorporated initially in the outer leaflet of the plasma membrane and that the rate of outside–inside translocation of the aminophospholipids was faster than that of the choline derivatives. For example, at 37 °C, the half-time for the transverse diffusion of a phosphatidylcholine analogue was found to be of the order of 40 min, while it was less than 7 min for the phosphatidylserine analogue. At low temperatures, a fraction of the labels gave rise to a strongly immobilized ESR component. This fraction, which corresponded to 20–30% of the initial spin-label concentration, was found resistant to chemical reduction from the inner side of the membrane and also to externally added reducing agents such as ascorbate. Presumably these immobilized lipids are trapped in a gel phase formed in the outer leaflet at 4 °C. Cell aging, which depletes the cells of ATP, resulted in the progressive inhibition of the fast transport of the aminophospholipids from the outer to inner leaflet. Treatment of the cells with iodoacetamide completely blocked the transverse diffusion of the spin-labels. These experiments suggest the existence in platelets, as in erythrocytes, of an ATP-driven translocator of aminophospholipids, which would be responsible for maintaining the lipid asymmetry of the platelet plasma membrane, under normal physiological conditions.

Since the pioneer work of M. Bretscher in 1972, phospholipid transverse asymmetry had been well documented in erythrocytes and in platelet plasma membranes. The compositional asymmetry was assayed by chemical derivatization and enzymatic hydrolysis [see the reviews by Etemadi (1980), Van Deenen (1981), and Op den Kamp et al. (1985)]. In erythrocytes, the choline derivatives (phosphatidylcholine and sphingomyelin) are found principally on the outer monolayer, while the aminophospholipids (phosphatidylethanolamine and phosphatidylserine) are found principally on the inner monolayer (Verkleij et al., 1973). Although similar in overall lipid

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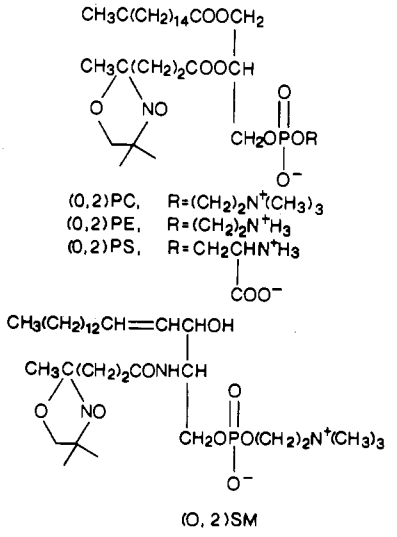
composition and lipid asymmetry, significant differences have been reported between erythrocytes and the platelet plasma membrane. In particular, phosphatidylcholine seems more evenly distributed between both layers in platelets than in erythrocytes (Chap et al., 1977). There are several biological functions in platelets that might be related, at least speculatively, to the plasma membrane lipid asymmetry. In particular, lipid manipulation can modify the platelet cell shape (Daleke & Huestis, 1985). Also, several investigators have shown a correlation between thrombin activation and a redistribution of phosphatidylserine between inner and outer leaflets (Schick et al., 1976; Chap et al., 1977; Perret et al., 1979; Zwaal & Bevers, 1982). Finally, the appearance of phosphatidylserine on the outer leaflet could enhance platelet–clotting factors interactions (Zwaal, 1978; Bevers et al., 1982). Thus the understanding of the mechanisms underlaying the stable asymmetry of phospholipids in platelets is an important issue.

The passive transverse diffusion of phospholipids is a slow process. In red cell ghosts, the half-time for phosphatidylcholine diffusion ranges between 2 and 26 h at 37 °C. The scattering of the results is not due to differences in experimental protocols but rather to the dependence of the transverse diffusion rates on the nature of the acyl chains. Recent results on erythrocytes can be found in Van Meer and Op den Kamp (1982), Middelkoop et al. (1986), and Zachowski et al. (1985). The transverse diffusion rates in platelets are likely to be comparable. Considering the long lifetime of cells such as erythrocytes (120 days) and platelets (20 days), the stable distribution of phospholipids cannot be explained solely by the slow transverse diffusion rate of phospholipids in a lipid bilayer. Previous experiments with spin-labeled phospholipids showed the existence of an ATP-dependent transverse of aminophospholipids in the red cell membrane, which can account for the stability of lipid asymmetry in erythrocytes (Seigneuret & Devaux, 1984). This phenomenon was confirmed later by techniques that did not involve spin-labels (Daleke & Huestis, 1985; Tilley et al., 1986). In addition, by preliminary analysis of cell shape changes consecutive to the addition of exogenous phospholipids, Daleke and Huestis (1985) have suggested that a similar active transport of aminophospholipids might take place in platelets as well.

This paper is devoted to the investigation of the transverse diffusion rates of spin-labeled phospholipids incorporated in the plasma membrane of human platelets. The technique employed is an extension of the method originally introduced by Kornberg and Mc Connell in 1971. The following spin-labeled phospholipids have been used:[1]



A significant advantage of these molecules is a partial water solubility, which facilitates their easy incorporation in biological membranes. The nitroxide is not on the polar head group and thus does not perturb the head group specificity. Although the paramagnetic group is on one of the acyl chains, it can be easily destroyed by a nonpermeant reducing agent, that is, providing the spin-label is on the monolayer exposed to the reducing agent (Seigneuret & Devaux, 1984). The rapid reduction of (0,2) spin-labels is surprising and suggests that the short chain tends to point toward the aqueous phase unlike long-chain fatty acids. The results obtained with platelets, in this study, allow us to conclude that the aminophospholipid analogues flip rapidly to the inner leaflet while the phosphatidylcholine and sphingomyelin analogues stay much longer in the outer leaflet. Evidence of the existence of an active aminophospholipid carrier in platelets is given.

Materials and Methods

Biological. Platelets were prepared at room temperature from fresh human blood anticoagulated with 0.15 volume of ACD (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid). Blood donors had not received any medication in the previous 3 weeks. Blood was centrifuged at 100*g* for 15 min. The platelet-rich plasma was carefully removed in order to avoid any contamination from the buffy coat and the red blood cells and then centrifuged for 20 min at 900*g*. The pellet containing the platelets was suspended in a modified Tyrode–Hepes buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 0.55 mM dextrose, 5 mM Hepes, and 0.15 volume of ACD at pH 6.4). Platelets were counted and centrifuged at 900*g* for 20 min. Finally, the pellet was suspended in the same buffer at 2×10^9 platelets/mL. Aging of platelets was performed by incubating the cells at room temperature in the same medium for 24–48 h, with continuous but mild shaking.

Iodoacetamide Treatment. Platelets (2×10^9 cells/mL) were incubated with iodoacetamide (6 mM) for 1 h at 37 °C.

Determination of the Cellular ATP Level. The ATP concentration in fresh or aged platelet suspension was assayed with a firefly luciferase method (Bohringer, Mannheim) in a LKB-Wallac luminometer.

Spin-Labeling and ESR Experiments. The spin-labeled phospholipids have been synthesized as already described (Seigneuret & Devaux, 1984; Zachowski et al., 1985). The final amount of ethanol was less than 0.5% (v/v). The spin-label concentration (10–20 μM) corresponded, after incorporation, to less than 1% of the endogenous phospholipids. For the reduction experiments at 4 °C, buffered sodium ascorbate was added to the labeled sample at a final concentration of 10 mM from a concentrated stock solution freshly prepared. ESR experiments were carried out on a Bruker ER 200D spectrometer, with a variable-temperature accessory and connected to an Apple II^+ microcomputer. The concentration of nitroxides was determined by comparison with a standard, after subtraction of the base line and double integration of the signal. The reduction of the ESR signal by ascorbate was monitored either by double integration or by following a specific peak intensity (e.g., the medium-field line).

[1] Abbreviations: (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; (0,2)SM, [*N*-(4-doxylpentanoyl)-*trans*-sphingen-1-yl]phosphocholine; (*m*,*n*), general nomenclature of spin-labeled chains, *m* and *n* being respectively the number of methylene groups after and before the labeled position on the acyl chain; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

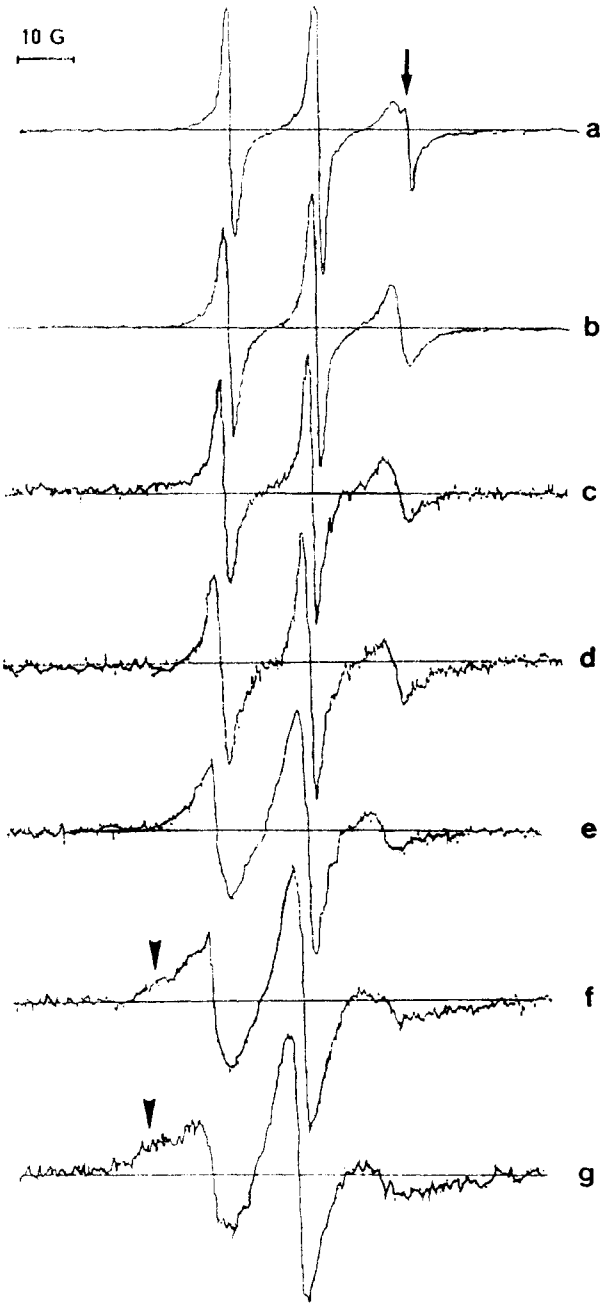


FIGURE 1: ESR spectra of (0,2)PC in the presence of platelets. (a) Experimental spectrum recorded at 37 °C. The arrow in the high-field region indicates a peak associated with rapidly tumbling probes which correspond to hydrolyzed spin-labels. (b) Same spectrum corrected for hydrolysis. (c–g) Spectra corresponding to different temperatures, after correction for hydrolysis, respectively: 31, 26, 12, 6, and 4 °C. Arrows on the low-temperature spectra indicate peaks associated with the strongly immobilized component.

Scanning Electron Microscopy. Samples of platelets in Hepes–Tyrode buffer were incubated for 10 min at 37 °C and then fixed by the addition of an equal volume of 2.5% glutaraldehyde (Sigma), buffered at pH 7 with phosphate. Incubation with the fixative was continued for at least 30 min at 20 °C, after which the fixed sample was treated with phosphate buffer containing 1% osmium tetroxide, dehydrated, through a graded alcohol series, and then dried from carbon dioxide by the critical point method. The dried samples were sputter coated with gold and observed at 20 kV in a Jeol JSM 35 scanning microscope.

RESULTS

Spin-Labeling: The Various Spectral Components. All four spin-labeled lipids incorporated within a few minutes in the platelet membrane as infered from the overall line shapes of the ESR spectra. Spectrum a in Figure 1 corresponds to (0,2)PC, recorded at 37 °C 30 min after addition to a platelet suspension. A very narrow component is seen superimposed on the typical spectrum of a membrane-bound spin-labeled lipid. The height of the narrow component (which is best seen in the high-field region) increased slowly with time. Spectrum b shows the "pure membrane-bound component", which can be obtained by computer subtraction of the narrow triplet. Other spectra corresponding to different temperature are displayed in spectra c–g of Figure 1. The spectra at low temperatures (4 and 6 °C) clearly reveal the existence of two membrane-bound components, which we call respectively a "weakly immobilized component" and a "strongly immobilized component". The same type of spectra were obtained with the three other spin-labeled phospholipids, including the sphingomyelin derivative.

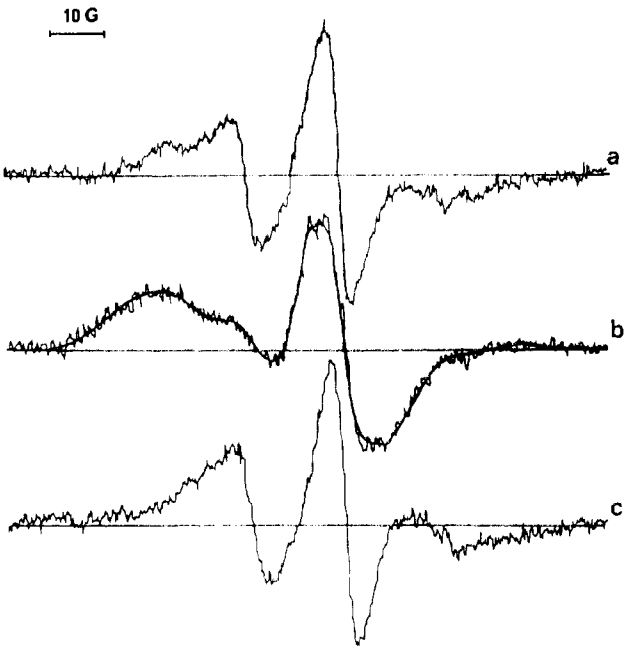


FIGURE 2: Spectra of (0,2)PE at 4 °C in the presence of platelets: (a) hydrolysis corrected spectrum; (b) spectrum obtained with the same sample after addition of 10 mM ascorbate. The smooth curve seen superimposed is a theoretical spectrum obtained by computer simulation of a strongly immobilized spectrum ($T'_{\parallel}$ = 32 G; $T'_{\perp}$ = 9 G; ΔH = 6 G; LW(−1) = 9 G; LW(0) = 6 G; LW(+1) = 6 G). (c) Weakly immobilized component obtained by subtraction of spectrum b from spectrum a.

When ascorbate was added at 4 °C, the weakly immobilized component disappeared shortly after the addition of the reducing agent on the external side of the membranes. Figure 2 shows the spectra of (0,2)PE recorded before (a) and after (b) addition of ascorbate at low temperature. Spectrum b can be simulated as a powder-type spectrum: Figure 2b is, in fact, the superposition of an experimental spectrum (which contains noise) and a calculated spectrum, shown as a smooth curve. Computer subtraction of spectrum b from spectrum a leads to spectrum c. This is a typical weakly immobilized spectrum in a membraneous environment. The strongly immobilized fraction, nonreducible by ascorbate, corresponded to approximately 20% of the signal for (0,2)PC, -PS, and -SM and slightly more (about 30%) for (0,2)PE.

Spontaneous Reduction of the ESR Signal at 37 °C. When the samples were maintained at 37 °C, the amplitude of the ESR spectra decreased with time, with a kinetics that depended upon the nature of the head group of the phospholipid

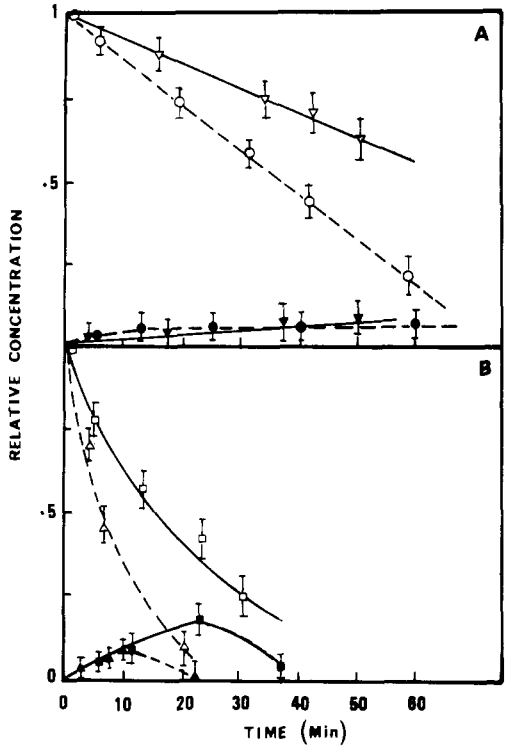


FIGURE 3: Variation at 37 °C of the concentration of probes in the membranes (open symbols) and in water (dark symbols): (0,2)SM (▽, ▼); (0,2)PC (○, ●); (0,2)PE (□, ■): (0,2)PS (△, ▲). Error bars indicate the maximum deviation from four different experiments. The evolution of the concentration of probes in the water reflects the competition between a continuous increase of hydrolyzed spin-labeled fatty acid and a fast chemical reduction of the probes by the interior of the cells.

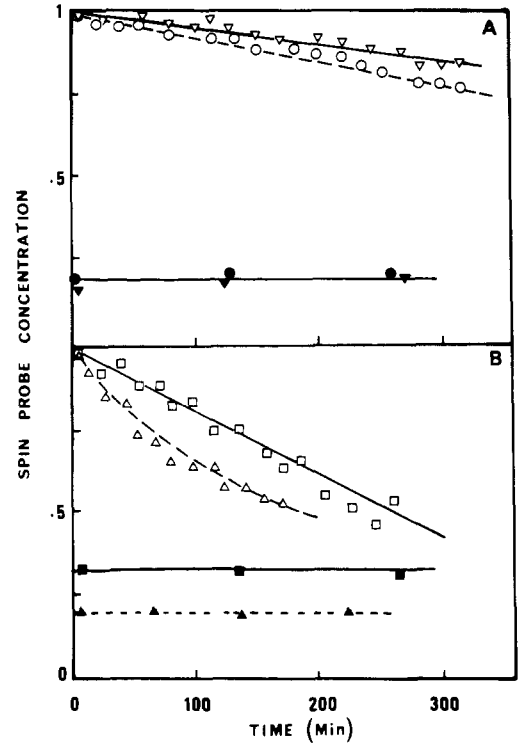


FIGURE 4: Spontaneous reduction at 4 °C. Open symbols correspond to the total spin concentration; the dark symbols correspond to the fraction of immobilized component as determined by use of the ESR spectrum obtained after incubation with 10 mM ascorbate as a reference immobilized spectrum: (0,2)SM (▽, ▼); (0,2)PC (○, ●); (0,2)PE (□, ■); (0,2)PS (△, ▲).

analogues. Figure 3 shows the reduction rates of the four spin-labels after incorporation in platelets. In this figure we have separated the evolution of the membrane-bound components from that of the aqueous components. The reduction rates of the four spin-labels are very different. $\tau_{1/2}$ values are respectively >60, 40, 15, and 7 min for (0,2)SM, (0,2)PC, (0,2)PE, and (0,2)PS. Each experiment was repeated at least 5 times: the uncertainly on τ values was found to be approximately 5%. When the membranes were submitted to sonication to break open the cells, all four spin-labels were rapidly reduced with a $\tau_{1/2}$ at 37 °C of 40 min (not shown). Since the internal content of the cells was diluted by a factor of 20–50 after sonication, one can calculate an effective $\tau_{1/2}$ of $\simeq$2–7 min at 37 °C within the cells. Indeed, $\tau_{1/2} \approx 5$ min for (0,2)PC when higher concentrations of platelets (5×10^{10} cells/mL) were used. Thus, the kinetics of the reduction of the spin-labels in the intact cells give a good indication of the time scale of passage from the outer leaflet of the plasma membrane to the inner leaflet. However, the value of 7 min obtained for (0,2)PS must be considered as an upper limit.

Low Temperature: Spontaneous Reduction. The spectra obtained with the (0,2) phospholipids in platelets are modified simply by their incubation at low temperature. Figure 4 shows that a significant reduction of the ESR signal takes place at 4 °C. Considering that the reduction rate of (0,2)PC by the cytosol is fast, even at 4 °C (data not shown), the reduction in signal intensity of the membrane-bound component must be again grossly indicative of the outside–inside diffusion of the spin-labeled lipids at low temperature. A complication arises from the fact that a fraction of the signal, corresponding precisely to the strongly immobilized component previously observed by addition of ascorbate at low temperature, seems rather stable. It is associated with 20–30% of the initial total

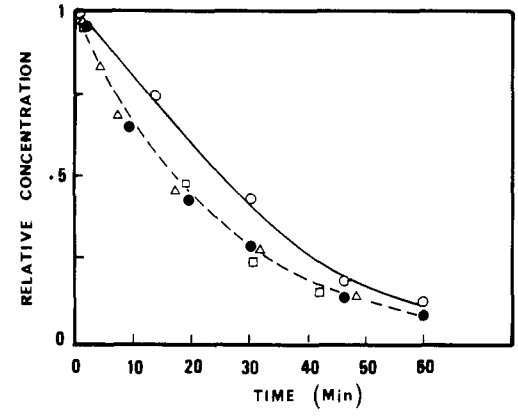


FIGURE 5: Spontaneous reduction at 37 °C of (0,2)PE following various incubation periods at 4 °C: (●) 0 h; (△) 1 h; (□) 4 h; (○) 18 h. The initial amplitudes are normalized so as to emphasize the constant characteristic decay time that is indicative of the outside–inside translocation at 37 °C. The actual amplitude of the signal decreased during the incubation at low temperature.

concentration. Figure 5 shows an experiment that was designed to tentatively localize the sideness of the strongly immobilized component. Samples that had been incubated with (0,2)PE at 4 °C were brought rapidly to 37 °C. Afterward, the spontaneous decrease of the concentration of nitroxide was monitored by ESR at that high temperature. In all instances, as shown in Figure 5, the signal decreased to zero, with a $\tau_{1/2}$ value between 15 and 20 min. Thus (0,2)PE, which forms two pools in the membrane at 4 °C, appears at high temperature to form a single pool of lipids, which have to diffuse from the outer to the inner layer (with a $\tau_{1/2}$ of 15–20 min) in order to be reduced by the cytosol.

ATP Depletion. A possible involvement of ATP in the fast aminophospholipid translocation in platelets was investigated

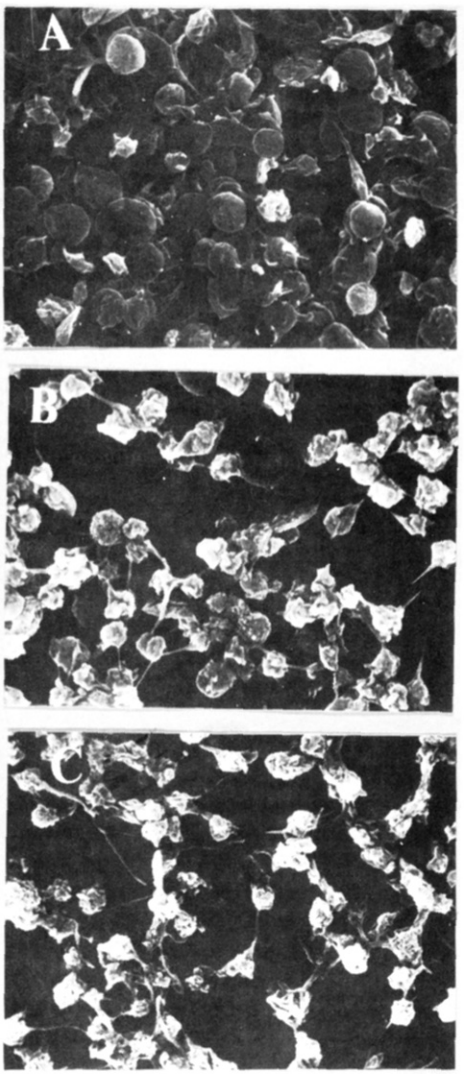


FIGURE 6: Electron micrographs of platelet suspensions after 0 (A), 24 (B), and 48 h (C) of incubation at room temperature. The corresponding intracellular ATP levels were 1.9, 0.3, and 0.15 mM, respectively. Magnification is 2100× in panels A–C.

through the following experiments: platelets were aged at room temperature, in a low-energy medium; after 0, 24, and 48 h, the reduction rates of the four lipids were assayed, the cell shapes were examined by electron microscopy, and the internal ATP concentration was determined. As shown in Figure 6, aging provoked a large shape change of platelets while the ATP content decreased dramatically from 1.9 mM to 0.3 and 0.15 mM after 24 and 48 h, respectively. Viability of the cells was tested by their ability to take up the dye trypan blue. After 12, 24, and 48 h, the precentage of "dead" cells was respectively $\leq 1\%$, $\approx 5\%$, and $\approx 10\%$ as counted with an optical microscope. During this time, the reduction rates of the four lipid probes varied quite differently. While the (0,2)PC rate remained essentially constant,[2] the (0,2)PE and (0,2)PS rates decreased. Finally, the (0,2)SM rate increased slightly. Eventually the four phospholipids seemed to diffuse transversely at the same slow rate ($\tau_{1/2} \simeq 40$ min) (Figure 7).

Iodoacetamide Treatment. Incubation of platelets with 6 mM Iodoacetamide at 37 °C for 1 h decreased the ATP concentration by a factor of $\simeq 2$, as determined by the firefly luciferase test. Iodoacetamide certainly reacts also in a non-

[2] The fact that the (0,2)PC spontaneous reduction rate remained constant after 0-, 12-, or 48-h incubation is proof that the majority of the cells remained sealed during that period.

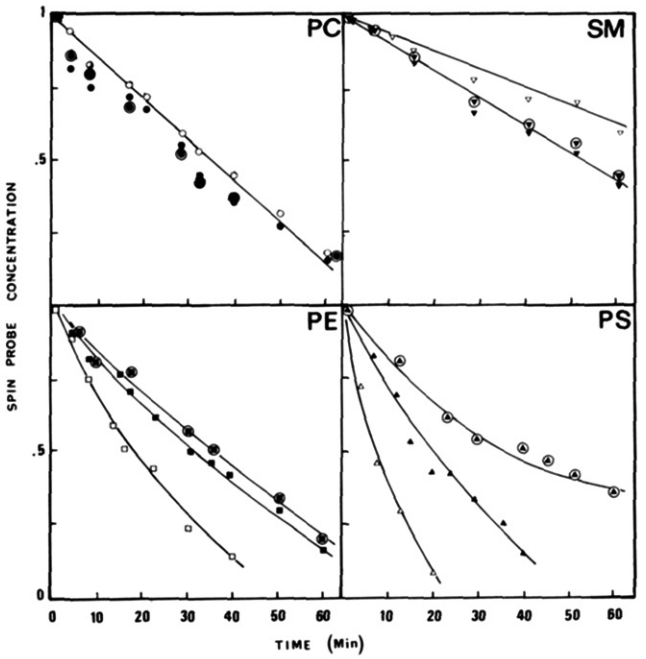


FIGURE 7: Effect of platelets' aging on the spontaneous reduction kinetics of spin-labeled phospholipids in platelets maintained at 37 °C. Preincubation periods prior to the addition of spin-labels are respectively 0 (empty symbols), 24 (dark symbols), and 48 h circled symbols).

specific manner with several proteins and free SH groups. In parallel, we found that the reducing power of the cytosol was abolished. Ascorbate was then added at low temperature (4 °C); this resulted in the complete disappearance of the ESR signal of (0,2)PC and also of (0,2)PS, even after 2-h incubation at 37 °C of iodoacetamide-treated cells. Control experiments were carried out with radioactive ascorbate to show that the cells were not leaky to ascorbate after the iodoacetamide treatment. Thus the combined effect of ATP depletion and protein modification, which are the results of iodoacetamide treatment, blocks the phospholipid transport efficiently.

Discussion

We have used a previously described spin-label technique to investigate the outside–inside translocation rates of spin-labeled phospholipids added to the human platelet plasma membrane. In former publications, we have shown that spin-labeled phospholipids with a short β-chain (C_5) bearing a doxyl group at the fourth carbon position can be used to assess the transverse orientation of the phospholipids within a biological membrane (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Zachowski et al., 1985, 1986). In erythrocytes, spontaneous reduction at the inner leaflet takes place only at high temperature (37 °C) and selectively destroys the labels on the inner monolayer. At low temperature (4 °C), ascorbate can be added extracellularly to chemically reduce the labels included in the outer monolayer. Thus the two types of experiment give complementary results. In platelets a significant reduction of the labels takes place spontaneously at 37 °C and also at 4 °C, making the experiment in principle simpler. Indeed, the data displayed in Figures 3 and 4 show unambiguously that at both temperatures the transferse diffusion of the four spin-labeled phospholipids tested is not identical. The rates vary in the order

$$PS > PE > PC > SM$$

Since the four phospholipids were shown to diffuse transversely in pure lipid vesicles with the same slow rate (Zachowski et al., 1985), it must be concluded that the transport

of aminophospholipids is facilitated in the platelet plasma membrane as it is in erythrocytes. Because of the specificity of the phospholipid translocation and because the same phenomenon can be observed at low temperatures, it does not seem likely that endocytosis is involved. On the other hand, the inhibition of this process by protein-reacting compounds such as iodoacetamide suggests that one or several proteins are involved. In addition, aging experiments reported here show that there is a concomitant decrease in the efficiency of the aminophospholipid transport and in the concentration of ATP within the cell. Thus a phospholipid carrier, similar to one existing in human erythrocytes (Seigneuret & Devaux, 1984), is likely to exist also in platelets as formerly suggested by Daleke and Huestis (1985).

A more detailed quantitative analysis of the phospholipids transverse diffusion rates in platelets is hampered by several difficulties. First, spin-label hydrolysis, which manifests itself by the appearance of a very narrow growing component, is more pronounced in platelets than in erythrocytes. A small hydrolysis of (0,2)SM is observed in platelets while this lipid is stable in erythrocytes. In actual fact, the hydrolysis of the spin-labeled lipids is an obstacle to the determination of outside–inside translocation only provided the endogenous phospholipase is externally exposed. In such a case, the "membrane component" of the ESR spectrum would underestimate the amount of probes still on the outer layer. But since the hydrolysis of the amino derivatives seems significantly larger than that of the PC analogue (see Figure 3), it appears reasonable to assume that the phospholipase is on the inner leaflet (or within the cell), hence that translocation is required before hydrolysis. This would signify that the decrease in concentration of the membrane bound component is a good representation of the rate of phospholipid outside–inside translocation.

Another limitation in the quantitative determination of phospholipid diffusion comes from the finite reduction rate by the cytosol. In fact as for erythrocytes, the rate-limiting step for PS reduction at 37 °C is the cytosol reducing power. Thus not only should the $\tau_{1/2}$ values of 7 min, which we found in platelets at 37 °C, be considered only as an upper limit but also values measured for PE and PC derivatives are approximate.

At 4 °C, the time course of spontaneous reduction is still fast, and the translocation of the spin-labeled phospholipids can still be deduced from the reduction in signal intensity. However, the change in line shape of the ESR spectra that accompanies the decrease in spin concentration introduces a new complication. This is aggravated by the fact that addition of ascorbate externally is ineffective for reducing the "immobilized component", which therefore appears equally protected from the outer and inner sides. We can nevertheless infer that the strongly immobilized component is likely to correspond to spin-labels in the outer monolayer. This comes from the following two observations: (i) the strongly immobilized component is present immediately after incorporation of the probes in the membrane; (ii) the kinetic of reduction at 37 °C following a limited incubation at 4 °C indicates only one pool of lipid located on the outer monolayer. The immobilized lipids would reflect the presence at 4 °C of a gel phase in the outer monolayer of the platelets' plasma membrane. If this is the correct interpretation, one might conclude that, as in erythrocytes, the asymmetry of the phospholipid distribution in the platelet plasma membrane is accompanied by an asymmetry of the viscosity of the two leaflets (Seigneuret et al., 1984).

Yet another difficulty in the study of the platelets is the fact that because of the spontaneous reduction taking place in the cytosol, even at low temperature, only rates of translocation can be studied and not plateau levels. There is another and fundamental reason for not reaching a plateau. Platelets, as opposed to erythrocytes, have inner membranes: thus once a molecule with a nonnegligible water solubility has flipped to the inner monolayer, it is exposed to other membranes in which it should distribute. This phenomenon certainly displaces the actual equilibrium distribution of spin-labeled lipids in the plasma membrane. However, this is due to the water/lipid partition ability of the probes being used and is not generalizable to endogenous phospholipids [the critical micellar concentration of (0,2)PC is of the order of 0.5 μM (Bette-Bobillo & Bienvenüe, 1982)]. The initial rate of diffusion from the outer to the inner leaflet of the plasma membrane, as represented by the initial reduction rates, is certainly not governed by the partition coefficient of the probes.

Finally, this work is based on the use of phospholipids bearing a nitroxide on one of the acyl chains. An implicit assumption is that these spin-labels are reliable reporters of the phenomena involving endogenous phospholipids. In favor of this hypothesis is the fact that such spin-labeled phospholipids have been shown to behave in human erythrocyte as endogenous phospholipids: not only do they spontaneously distribute between inner and outer layer as naturally occurring lipids, but they also reflect the influence of ATP on the transport of aminophospholipids in erythrocytes. We therefore believe that the present data obtained with spin-labeled phospholipids reveal to some extent the behavior of naturally occurring phospholipids in human platelets and that at least semiquantitative conclusions can be drawn.

We have found very significant differences between the translocation rates of the various spin-labeled phospholipids in platelets. Although the absolute rates can be influenced by the short β-chain and/or the nitroxide moiety, the relative rates must be determined by the head groups. Thus, in the platelet plasma membrane, as in the erythrocyte membrane, the transverse diffusion rates of aminophospholipids are faster than those of the choline head group derivatives. However, the transverse rates of all phospholipids are faster in platelets than in erythrocytes. For example, at 37 °C, $\tau_{1/2}$ values for (0,2)PC are $\approx$30 min in platelets but $\approx$120 min in erythrocytes (Seigneuret et al., 1984). Similarly, while the transverse diffusion of (0,2)SM is not measurable at 4 °C in erythrocytes (Zachowski et al., 1985), a significant uptake of (0,2)SM is seen at that temperature in platelets (see Figure 4). Note that the difference between the aminophospholipids and the choline derivatives, which is very marked in erythrocytes, is more subtle in platelets. In fact, one can barely distinguish two classes of lipids in platelets; rather, there is a progressive change from (0,2)SM to (0,2)PS. To some extent, this observation is consistent with the known distribution of lipids at equilibrium in the platelet plasma membrane as determined by the phospholipase technique by Chap et al. (1977). In particular, while phosphatidylcholine is found predominantly in the outer leaflet of erythrocytes, it seems almost evenly distributed in the platelet plasma membrane. The relatively fast transferse diffusion of non-aminophospholipid in platelets could be a constitutive property of that membrane and may not require a specific carrier. A spin-labeled analogue of PAF acether was recently described by some of us (Bette-Bobillo et al., 1986); this molecule, which ressembles the PC analogue used in this study, was found to have a flip-flop rate at 37 °C close to that of (0,2)PC. The fast lipid translocation in

platelets may be required by, and is consistent with, the relatively high lipid metabolism in this cell [for a review on lipid metabolism in platelets, see Holub (1984)].

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Protein Redistribution in Model Membranes: Clearing of M13 Coat Protein from Calcium-Induced Gel-Phase Regions in Phosphatidylserine/Phosphatidylcholine Multilamellar Vesicles†

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ABSTRACT: A model system of M13 bacteriophage coat protein reconstituted into multilamellar vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) is used to examine protein redistribution in membranes in the presence of Ca^{2+}. The reconstitution procedure is analyzed by using protease digestion and gel permeation chromatography of radioactively labeled coat protein and is found to incorporate coat protein into lipid vesicles predominantly in the in vivo orientation and without aggregation. Quenching of protein tryptophanyl fluorescence by spin-labeled PC is used to determine the local lipid environment of the coat protein in binary lipid mixtures. The distribution of coat protein between fluid liquid-crystal (LC) and Ca^{2+}-induced gel (G) phases in PS/PC multilayers, expressed as a concentration ratio $R_{LC/G}$, is found to be 25 ± 5 in favor of the fluid phase, indicating significant clearing of membrane protein from Ca^{2+}-induced gel-phase regions.

Protein-free membrane contact regions in fusing biological membranes have been observed by electron microscopy by use of fixed and stained thin sections and cryoprotected freeze–fracture (Lawson et al., 1977; Orci et al., 1977; Peixoto de Menezes & Pinto da Silva, 1978; Chi et al., 1979; Kalderon & Gilula, 1979). This finding of extensive, protein-free membrane areas is controversial, being disputed by some workers using rapidly frozen membrane samples without cryoprotectant (Chandler & Heuser, 1979, 1980; Ornberg & Reese, 1981) but verified by others (Morris et al., 1983). Freeze–fracture studies of reconstituted model membranes containing Ca^{2+}-ATPase from sarcoplasmic reticulum (Kleeman & McConnell, 1976) or glycophorin (Grant & McConnell, 1974) showed particle-free regions thought to correspond to patches of thermotropic gel-phase lipid. Portis et al. (1979) have suggested that a rigid complex of Ca^{2+} with acidic phospholipids could form a membrane region that excludes proteins. Numerous studies have confirmed that Ca^{2+} can induce phase separation in mixed lipid systems containing negatively charged phospholipids [see Düzgünes (1985) for a recent review]. A question remains as to the behavior of

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