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Relationship between the Transverse Distribution of Phospholipids in Plasma Membrane and Shape Change of Human Platelets†

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ABSTRACT: ESR spectroscopy was used to investigate the distribution of spin-labeled analogues of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the presence of human platelets. Three rates were determined: hydrolysis of the ester bond at position 2, reduction of labels by cytoplasm, and internalization of labels situated in the outer leaflet of the plasma membrane. We found that the half-time for transverse diffusion of added phospholipids was shorter for aminophospholipids (40 min and less than 10 min for PE and PS, respectively) than for the choline derivatives (>120 min for PC, not measurable for SM). Addition of any of the phospholipids led to a considerable change in the initial platelet shape (assessed by electron microscopy) from a discoid form to a smaller body with very long pseudopods. When aminophospholipids were used, the platelets quickly returned to the initial shape [half-time of 20 min and less than 5 min for (0,2)PE and (0,2)PS, respectively]. Conversely, there was no relaxation after (0,2)PC or (0,2)SM was added. We conclude that there is a relationship between the excess of phospholipids in the outer leaflet of the plasma membrane and cytoskeletal organization presumably via actin polymerization, which is responsible for platelet shape.

It has been accepted for some time that the transverse distribution of the phospholipids in plasma membranes is very dissymmetric (Bretscher, 1972). The clearest example is the erythrocyte. The determination of the percentages of different polar heads in its bilayer is unambiguous because of the presence of a single membrane. The choline head group derivatives are mostly situated outside, whereas the aminophospholipids are preferentially situated inside (Verkleij et al., 1973). Although it has been claimed that dissymmetry applies to other cell types, it has only actually been demonstrated in blood cells, i.e., erythrocytes typical of falciform anemia (Zachowski et al., 1985a), malaria-infected erythrocytes (Van Der Schaft et al., 1987), platelets (Chap et al., 1977), and erythroleukemic Friend cells (Rawyler, 1984). This dissymmetry can only be explained if there is a difference in speed between the outside–inside ("flip") and the inside–outside diffusion ("flop"). Phospholipids move very slowly between the two halves of the bilayer in pure phospholipid systems (Kornberg & McConnell, 1971). In biological membranes, however, it appears that the transfer rate can be high, depending strongly on polar heads of the phospholipids involved (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Sune et al., 1987) and the fatty acid composition (Middelkoop et al., 1986). By use of phospholipids labeled with fluorescent probes or nitroxide groups, it has been demonstrated that aminophospholipids are quicker than choline derivatives in entering different cell types such as normal or pathological erythrocytes (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Zachowski et al., 1985a), human platelets (Sune et al., 1987), and fibroblasts (Martin & Pagano, 1987). The transverse diffusion of these phospholipids may be easier in the presence of ATP-dependent carrier having a higher specificity for PS than for PE (Seigneuret and Devaux, 1984; Seigneuret et al., 1984, Zachowski et al., 1986).

In the case of erythrocytes, for example, the addition of exogenous phospholipids results in a very large modification of cell form (Ferrel et al., 1985), which first becomes echynocytic and then relaxes with kinetics comparable to that of marker internalization. After addition of (0,2)PC[1] and (0,2)SM, the cells stay crenated for several hours. In contrast, erythrocytes return to the discocyte form in less than 1 h at 37 °C after addition of (0,2)PE or (0,2)PS (Seigneuret & Devaux, 1984). The addition of exogenous phospholipids to platelets results in more drastic shape changes, with the appearance of micrometer long pseudopods. These pseudopods may become vesicularized by specifically losing membrane material (Kobayashi, 1984). Daleke and Huestis (1985) have attributed this erythrocyte shape change following alteration of the membrane composition to a physical cause. According

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[1] Abbreviations: (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; (0,2)SM, [*N*-(4-doxylpentanoyl)-*trans*-sphingen-1-yl]phosphocholine; (0,2)FA, 4-doxylpentanoic acid; (*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; BSA, bovine serum albumin.

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to them, the relative increase in outer leaflet area is sufficient to create spicules with curves of very small radii when an excess of phospholipids is fixed to the external surface. The relationship between the kinetics of shape change and that of transverse diffusion has never been studied in platelets, although we have demonstrated that (0,2)PC and (0,2)SM enter more slowly than (0,2)PS and (0,2)PE, as in other cell types (Sune et al., 1987).

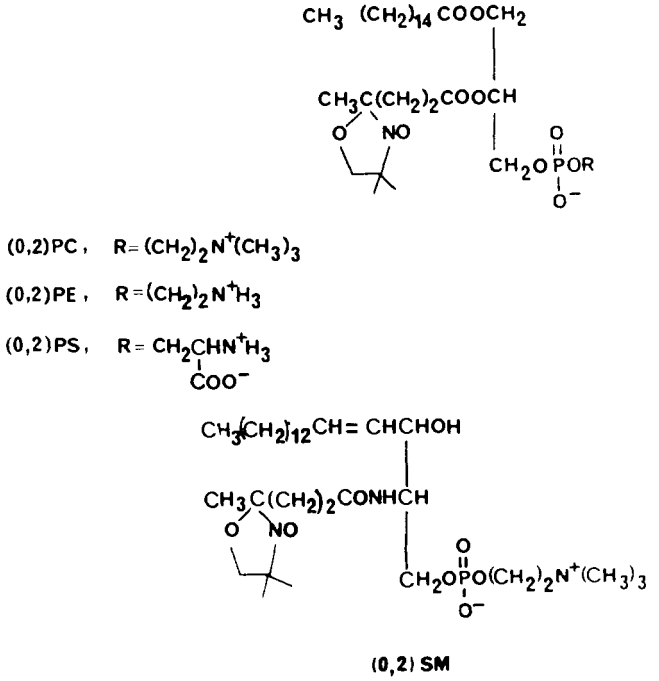
In the present paper, we compare the kinetics of platelet shape relaxation and that of the internalization of exogenous phospholipids. We show that paramagnetic analogues of aminophospholipids, placed outside the plasma membrane, pass inside the platelet more quickly than choline derivatives. The kinetics of platelet discoid shape recovery also depends on the type of polar head. However, the physical effect alone does not appear to explain the phenomenon sufficiently.

Materials and Methods

Biological Methods. Platelets were prepared at room temperature from fresh human blood anticoagulated with 0.15 volume of ACD (85 mM trisodium citrate, 111 mM dextrose, and 71 mM citric acid). Blood donors had not received any medication in the previous three weeks. Blood was centrifuged at 100*g* for 15 min. Platelet-rich plasma was carefully removed to avoid any contamination from the buffy coat and 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.

The red blood cells were washed 4 times by centrifugation at 1000*g* for 10 min with an isotonic solution (150 mM NaCl at pH 7.4), and the concentration was adjusted to 10^{10} erythrocytes/mL.

Spin-Labeling and ESR Experiments. The following spin-labeled phospholipids were used:



They were synthesized as previously described (Seigneuret & Devaux, 1984; Zachowski et al., 1985b). The spin-labels were added to the cells from a concentrated ethanol solution. 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. ESR experiments were carried out on a Bruker ER 200D spectrometer with a variable-temperature accessory and connected to an Apple II+ microcomputer. Each spectrum was digitized by 1024 8-bit values. The nitroxide concentration was determined by comparison with a standard (the same spin-labeled phospholipid in 10 mM egg lecithin at 1/200 mol/mol ratio) after subtraction of the base line and double integration of the signal.

Evaluation of Hydrolysis Rate. After reaction with a membrane enzyme, 4-doxylpentanoic acid, the hydrolysis product of the probe was found in the sample by means of its very typical ESR spectrum. This fatty acid [(0,2)FA] was not bound by BSA but distributed between the extracellular and the intracellular aqueous phase.

After incubation of a known quantity of (0,2)FA with cells for different times (2–30 min), extraction was carried out with the same buffer as the one used for phospholipid extraction (see below). Equilibrium was obtained in less than 10 min. Reoxidation of the signal present in the supernatant gave the amount of (0,2)FA in the extracellular phase. A simple subtraction gave the quantity of (0,2)FA present in the cells. Every value of hydrolysis indicated in this study was corrected for the excess of hydrolysis product not visible in ESR but present in the intracellular medium.

Determination of Labeled Lipid Located in the Outer Leaflet of the Platelet Membrane Lipid Bilayer. The labeled phospholipid molecules located in the outer leaflet, and thus exposed on the outer surface of the platelet membrane, were determined by a selective BSA (fatty acid free, Sigma) extraction method. The labeled phospholipid was added to the cell suspension (2×10^9 cells/mL) at a known concentration (determined by ESR) and incubated at 37 °C. At specific times, aliquots of platelet suspension were mixed with 0.6% BSA and incubated for 5 min at 4 °C. The mixture was then centrifuged for 30 s at 4 °C. The quantity of signal present in the supernatant was determined before and after reoxidation of the probes with 10 mM $K_3Fe(CN)_6$, which represented the amount of labeled phospholipid localized in the outer leaflet.

The following conditions were verified: Incubation of cells with BSA at 4 °C for 5 min was sufficient to extract all external labeled phospholipids. Incubation was extended to 15 min, confirming this result. Although the ferricyanide was paramagnetic, it did not change the ESR spectrum integral of the solubilized phospholipids in the presence of BSA. Ferricyanide completely reoxidized the nitroxide free radical prereduced by platelet cytosol, as measured on sonicated platelets after 2 h of incubation.

Scanning Electron Microscopy. Platelets were prepared as described above and incubated for 10 min at 37 °C before use. Spin-labeled phospholipid was added to the cell suspension and incubated at 37 °C. At defined times, aliquots were transferred to vials containing equal volumes 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 time the fixed samples were treated with phosphate buffer containing 1% osmium tetraoxide, dehydrated through a graded alcohol series, and dried with carbon dioxide by the critical point method. The dried samples were sputter-coated with gold and observed at 15 kV in a JEOL JSM 35 scanning microscope.

Platelet Counting. When platelets were discoid, there was little accumulation of gold and the image lacked contrast.

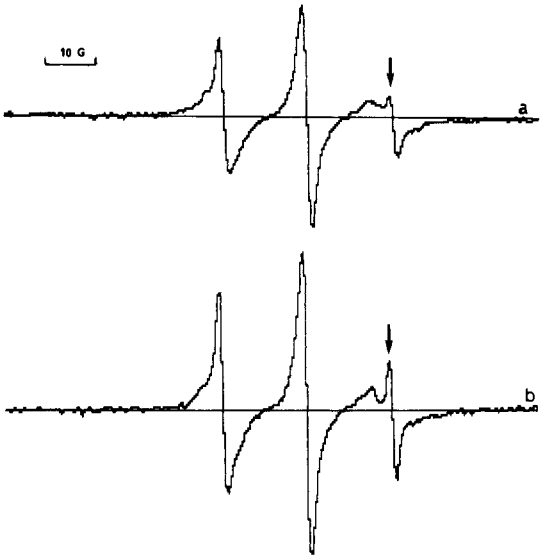


FIGURE 1: ESR spectra (37 °C) of (0,2)PC after incubation for 10 min at 37 °C with platelets then extracted in BSA supernatant, before (a) and after (b) treatment with 10 mM K_3FeCN_6. The peak in the high-field region (arrows) corresponds to hydrolyzed spin-labels.

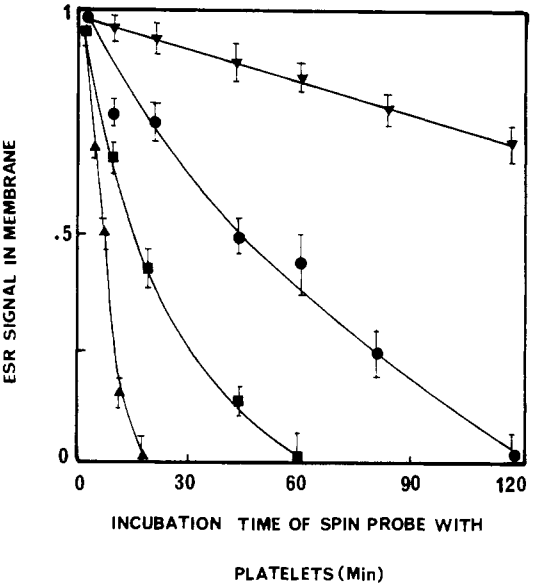


FIGURE 2: Time course of relative ESR signal (hydrolysis corrected) in BSA supernatant after addition of (0,2)PC (●), (0,2)SM (▼), (0,2)PE (■), or (0,2)PS (▲) to platelets at 37 °C. Error bars indicate the maximum deviation in four different experiments.

Conversely, shape-changed platelets effectively retained the gold and appeared more clearly on photographs. About 300 cells were counted on three different photographs selected at random to assure a relative precision in the estimation of form distribution.

Measurement of Serotonin Release. Platelet-rich plasma was incubated with 1 μCi (^{3}H)serotonin/100 mL of plasma for 30 min at 37 °C, and platelets were prepared as described above. Release of (^{3}H)serotonin was determined in the platelet supernatant after centrifugation at 7000*g* for 2 min in an Eppendorf microfuge.

RESULTS

Internalization and Hydrolysis of Phospholipids in the Platelets. Following addition of phospholipid paramagnetic analogues to a suspension of purified human platelets, the ESR signal disappeared because of the reduction of the nitroxide probe by the internal content of the cells (Giotta & Wang, 1972). Concentrations could be measured from the ESR spectra of cellular supernatants after extraction of external phospholipids by BSA (Figure 1). The ESR spectrum consisted of two components. The three thin lines corresponded to 4-doxylpentanoic acid, which is very water soluble; the largest spectrum corresponded to BSA-associated phospholipids. This measurement was done before and after ferricyanide treatment (ferricyanide reoxidized the fraction of the nitroxide free radical that had been reduced in contact with intracellular material). The first measurement gave the ratio of nonreduced markers. The second corresponded to the reduced or nonreduced markers, even those situated on the outer leaflet of the plasma membrane.

Relative amounts of markers extracted from platelets by BSA are shown in Figure 2 before reoxidation for the the four phospholipids [two choline derivatives, (0,2)PC and (0,2)SM, and two aminophospholipids, (0,2)PE and (0,2)PS]. The kinetics are similar to those obtained by simple measurement of signal reduction (Sune et al., 1987); this confirms the presence of the nonreduced markers exclusively in the outer leaflet of the membrane.

After the extracellular medium was treated with ferricyanide, the probe fraction found in the phospholipid form (reduced or not) in the outer leaflet and that found as a hydrolysis product (4-doxylpentanoic acid) are shown in Figure 3. The spin-labeled phospholipid initially situated on the outer leaflet could be completely extracted by BSA, as shown by the results obtained immediately after mixing. It then disappeared more quickly or slowly depending on the polar head of the phospholipid fixed on it. As above, the rate at which phospholipids became inaccessible to BSA decreased in the order PS > PE > PC > SM.

All of the (0,2)SM remained on the outer leaflet. In contrast, the half-time of disappearance was <5, 40, and >120 min for (0,2)PS, (0,2)PE, and (0,2)PC, respectively. At the same time, the paramagnetic probe [except for (0,2)SM] was partially hydrolyzed. The higher resistance of the latter phospholipid to cellular phospholipase functions was not surprising, since there is little sphingomyelinase in platelets, and the degradation product should be a spin-labeled ceramide and no (0,2)FA.

Platelet Shape Change. Scanning electron microscopy made it possible to follow very precisely platelet shape change after the addition of exogenous phospholipids. It should be recalled that the incorporation of phospholipids into platelets is very fast, due to their micellar concentration resulting from the short chain at position 2. Figure 4A gives an example of the discoid shape associated with resting platelets, showing a little orifices typical of the canalicules that penetrate cells. Following addition of any of the paramagnetic phospholipids, the platelet took a shape characterized by two parameters: numerous extensions small in diameter (0.1 μm), of variable length (2–4 μm) and diverse orientations, and a smaller cell-body diameter. All these phenomena suggest that the platelet interior contracts while the membrane creases and crenates. This shape change was not related to platelet activation, because the added phospholipid did not induce the release of any serotonine or cause platelet aggregation at the concentrations used. Parts A and B of Figure 4 show the extreme cases, but intermediate cases, as shown in Figure 4C, also eventually appeared. Although the differences are clear, it is difficult to estimate precisely the ratios of the different shapes. We assessed it on the basis of the differences in photographic contrast described under Materials and Methods. Figure 5A shows that the platelets were completely deformed immediately after mixing. All the phospholipids produced the

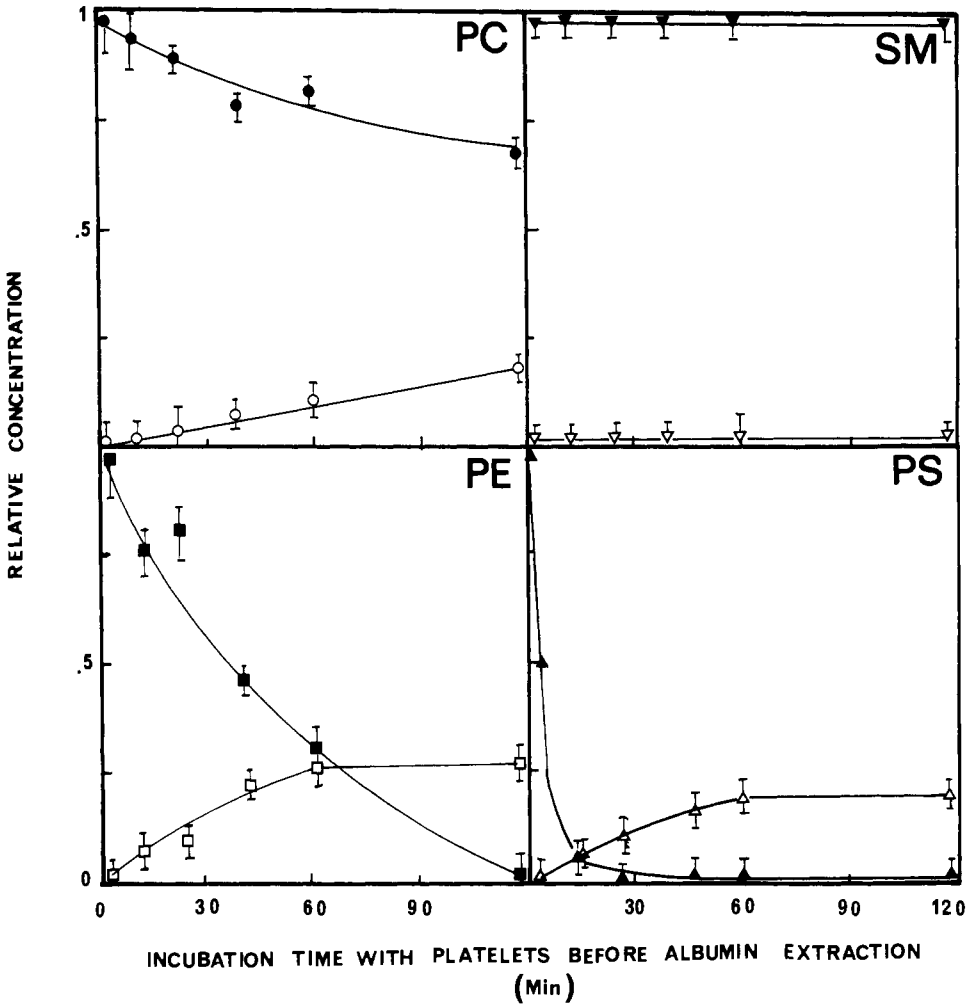


FIGURE 3: Time course of relative ESR signal of phospholipid associated with BSA (filled symbols) and hydrolyzed water soluble probe (open symbols) in ferricyanide-oxidized BSA supernatant after addition of (0,2)PC (●, ○), (0,2)SM (▼, ▽), (0,2)PE (■, □), or (0,2)PS (▲, △) to platelets at 37 °C. Error bars indicate the maximum deviation in four different experiments.

same effect. However (Figure 5B), cells treated with (0,2)PS almost completely recovered their original shape after a short time. This was not the case for choline derivatives (Figure 5C). The results shown in Figure 6 are based on observation of many hundreds of platelets incubated for different times at 37 °C after each of the four phospholipids are added. The kinetics of cell shape relaxation varied considerably (half-time of about 5–20 min) after amino head probes were added, but no relaxation was observed in the case of choline head probes. To study the effect of dissymmetric phospholipid distribution on platelet shape, markers situated on the outer leaflet of cells were extracted by BSA before fixation, and the platelets were observed with an electron microscope (Figure 7). After extraction of phospholipid from platelets that had reacted to the addition of (0,2)PE, their original shape was immediately and totally recovered. Platelets treated with (0,2)PC extraction also showed an easily detectable relaxation of their shape after phospholipid extraction, with much slower kinetics than in the case of (0,2)PE ($T_{1/2}$ = 15–20 min). When red cells were subjected to the same experiment (Figure 7), they recovered their discoid form immediately after extraction of excess of (0,2)PC.

Discussion

Analyzing the ESR spectra of spin labels (Figure 1) in the supernatant of BSA-treated cells determines the amount of phospholipids on the outer leaflet of cells in their initial phospholipid form (Figure 2) or in a form of a free acid chain, the two forms being native or reoxidized by ferricyanide (Figure 3). The other unknown forms, i.e., lyso derivatives and their metabolites, or the nitroxide reduced forms could not be reoxidized with the method used. The lyso derivatives were probably scarce in the outer leaflet, considering that the (0,2)FA ratio during the reaction was always very low (Figure 3) and that the hydrolysis of the chain at position 2 was catalyzed by phospholipase A_2, which is only found on the inner leaflet of the membrane (Van Den Bosch, 1980). If the phospholipase activity was the same with endogenous phospholipid as with the short-chain analogues studied here, then excess lyso derivatives would appear: This is incompatible with the maintenance of membrane structure. Indeed, the paramagnetic derivatives are probably more sensitive than the others, as reported in the case where the chain at position 2 bears a peroxide group (Van Kuijk, 1987).

The time course of the ratio of different phospholipids located on the outer leaflet differed according to the polar head group. The choline probes penetrated very slowly [(0,2)PC] or not at all [(0,2)SM], whereas the aminophospholipids entered the cells with a half-time of 40 min for (0,2)PE and 5 min for (0,2)PS. Once more, there was a clear difference in transverse distribution, depending on the polar head group, as previously described in platelets (Sune et al., 1987) and erythrocytes (Seigneuret & Devaux, 1984; Seigneuret et al., 1984) using the same spin-labels. It should be noted that the probe present at a given time on the outer leaflet was partially reduced, since the measured ratios of the marker were different

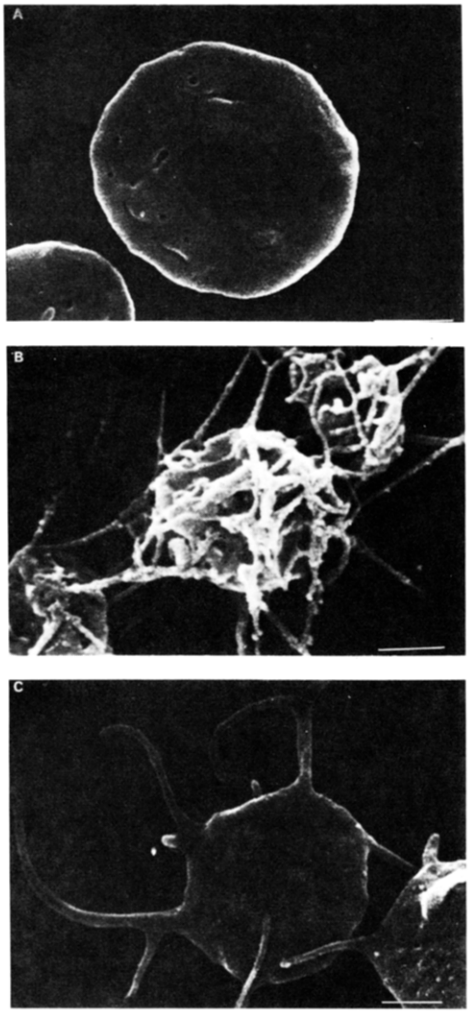


FIGURE 4: Scanning electron micrographs of a typical platelet at 37 °C before (A) and after (B and C) addition of (0,2)PE. Bar represents 1 μm.

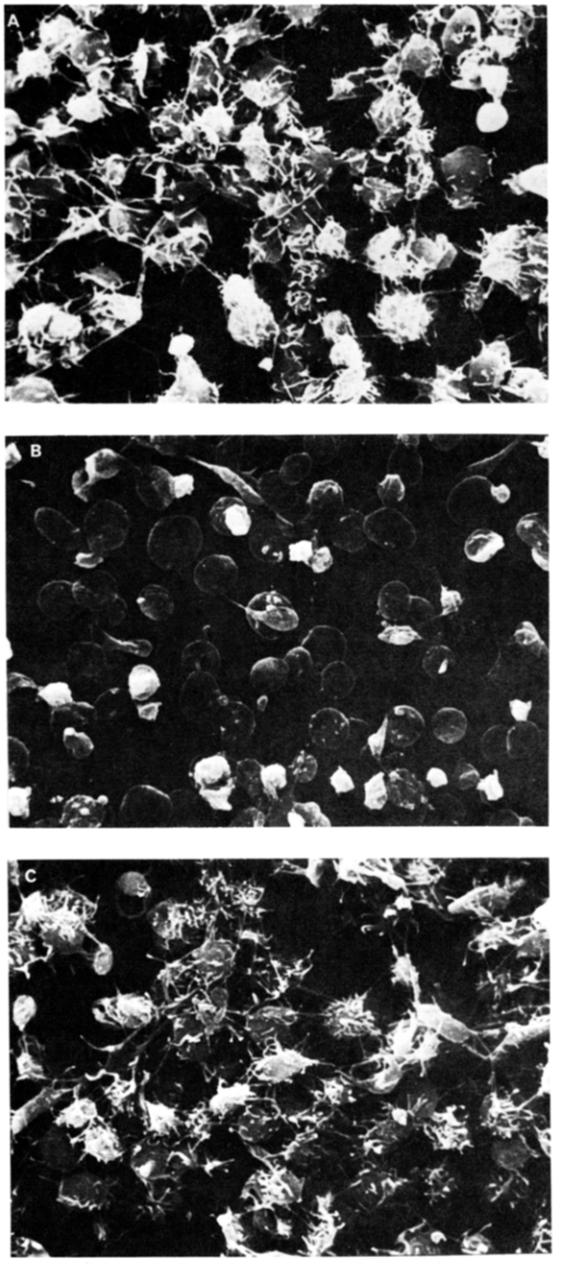


FIGURE 5: Scanning electron micrographs of platelets incubated for 2 (A), 10 (B), and 60 min (C) with (0,2)PC (A and C) and (0,2)PS (B). Magnification is 1800× in each panel.

before and after ferricyanide reoxidation (Figure 3). This reduction could occur by one of the following processes: (i) reaction with cytoplasm followed by a transverse flop movement; (ii) reaction in the outer leaflet by an unknown mechanism. More experiments are needed to clarify this point. In any case, following the outside–inside transition, a stable state appeared to be established (Figure 3) in which the (0,2)SM remained outside the cell, as well as most of the (0,2)PC, whereas all of the (0,2)PE and (0,2)PS was inside. The structure and exact localization of the probes inside the platelets are of course unknown, and the polar head distribution between the two leaflets of the platelet plasma membrane cannot be deduced from these results. It should be noted, however, that the results with platelets resemble those obtained with erythrocytes; i.e., the outer leaflet appears to contain all the SM, mainly PC, whereas the amino heads are completely excluded. Quantitatively, however, the results obtained with (0,2)PE are appreciably different from those obtained with endogenous platelet phospholipid (Chap et al., 1977; Holub, 1984). This difference could be a result of the high hydrophilicity of the probe, which facilitates the exchange with the internal cell membranes.

Concerning the platelet shape, the addition of less than 1% spin-label to the plasma membrane led to a much greater shape change than in red cells (Daleke & Huestis, 1986). Instead of 0.5–1 μm pseudopods, very long (several micrometers) and very thin (0.1 μm) filaments appeared, while the cell body shriveled (Figure 4B). In the presence of 10–100 times as much phospholipid, platelets even lose some membrane material (Kobayashi et al., 1984).

When shape change was produced by a choline derivative, it was almost completely irreversible for 2 h at 37 °C. In contrast, platelets recovered their resting shape after only approximately 20 min for (0,2)PE and 5 min for (0,2)PS. The platelet shape change in the presence of PE appears to be faster than the internalization of the exogenous phospholipid between the two monolayers. This is probably due to the fact that the transverse equilibrium ratio (Chap et al., 1977) is reached before all (0,2)PE entered the cell. It would thus appear to be a cause-and-effect relationship between the excess of phospholipids in the outer leaflet of the plasma membrane and the platelet shape, as previously reported for erythrocytes (Ferrel et al., 1985; Daleke & Huestis, 1985). In the case of platelets, shape change after the addition of an aggregation agonist is often the first step (possibly reversible under appropriate conditions) in a series of other reactions such as

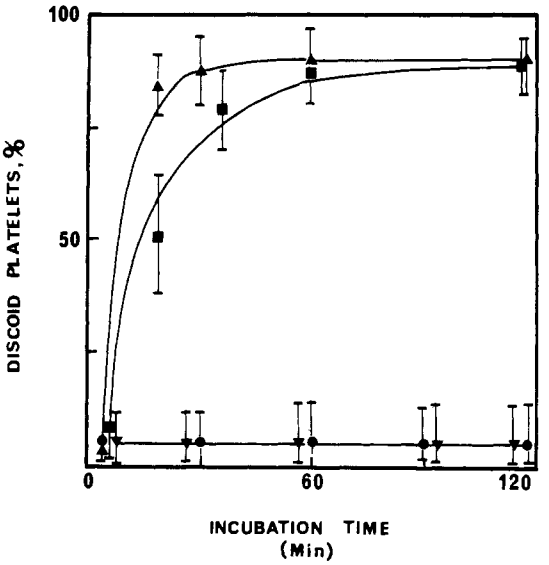


FIGURE 6: Percentage of discoid platelets versus incubation time at 37 °C after labeling with (0,2)PC (●), (0,2)SM (▼), (0,2)PE (■), or (0,2)PS (▲). Percentages are based on separate counts of at least 300 cells distributed over three different photographs selected at random.

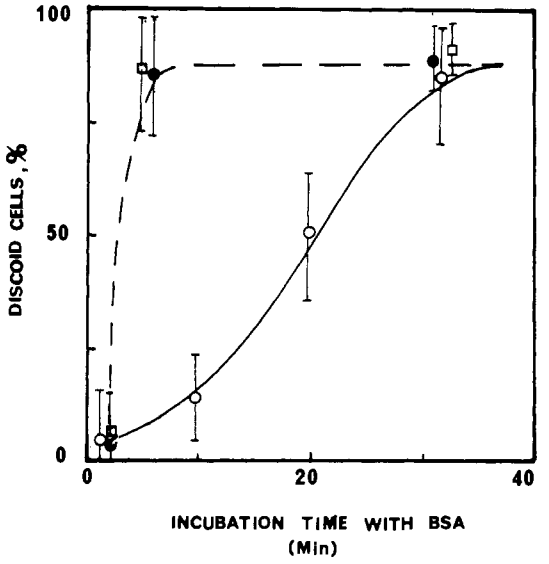


FIGURE 7: Percentage of discoid erythrocytes (filled symbols) or platelets (open symbols) versus incubation time with BSA at 37 °C after labeling with (0,2)PC (●, ○) or (0,2)PE (□).

aggregation and the release of granule content (dense and α) that are involved in blood clot formation [for review, see Zwall and Bevers (1982)]. In any case, shape change is associated with a polymerization of intracellular actin into long filaments, which causes cell deformation, as seen in Figure 4B (Jennings et al., 1981). These microfilament bundles are probably formed by the partial polymerization of soluble, nonfilamentous actin, due to physiological or physical activation (Pribluda & Rotman, 1982). Hence, it would appear that at least in platelets two factors contribute to the adaptation of cell shape to the phospholipid excess in the outer leaflet: (i) the creation of membrane filaments, the frame of which is probably composed of actin; (ii) the very small radius of filament curvature (about 50 nm), which accommodates the phospholipid excess in the outer leaflet, as pseudopods may do in erythrocytes (Ferrel et al., 1984). Nevertheless, there is a fundamental difference between erythrocytes and platelets. In the first case, the possible shape change of the cellular cytoskeleton may only be a deformation caused by the shape change of the membrane, whereas in the second case the platelet cytoskeleton may play a very active role in the phenomenon. The difference is not very surprising since actin organization is subject to numerous and rapid modifications under the influence of many factors such as calcium and various actin-binding proteins (Piazza & Wallace, 1985), whereas spectrin seems to reorganize much more slowly.

This work strongly suggests that cytoskeletal shape change is caused by an excess of phospholipids in the outer leaflet of the plasma membrane. Since specific interactions of the very different polar heads studied here are not probable, it is likely that this shape change is caused by expansion of the outer membrane surface. The link between the two events is not yet known. It is possible that a small initial deformation of the plasma membrane slightly displaces the interaction sites between the cytoskeleton and the membrane (Davies, 1984), which modifies the actin polymerization equilibrium and leads to the formation of the long filaments observed. Conversely, when the added phospholipids have been redistributed, the platelets' shape completely relaxes.

The direct relationship between the excess of the outer surface area of the membrane and polymerization of actin is confirmed by the relaxation of the platelet shape when the excess of (0,2)PE was removed by BSA (Figure 7). However, when the same experiment was done with (0,2)PC, the shape relaxation was slower (Figure 7). This suggests that another more complex physiological phenomenon occurs with this probe.

In conclusion, the direct relationship between the expansion of the outer cell surface and cell shape is clearly demonstrated here. It seems to occur through a modification of the platelet cytoskeletal structure.

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Effect of Phospholipid:Protein Ratio on the State of Aggregation of the $(Ca^{2+}$-$Mg^{2+})$-ATPase†

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ABSTRACT: The organization of the $(Ca^{2+}$-$Mg^{2+})$-ATPase has been studied in reconstituted systems by fluorescence polarization of the ATPase labeled with fluorescein isothiocyanate (FITC) and resonance energy transfer between ATPase labeled with FITC and with eosin isothiocyanate (EITC). The fluorescence polarization of FITC-ATPase was found to decrease with increasing labeling ratio FITC:ATPase, indicating depolarization as a result of resonance energy transfer between ATPase molecules. Fluorescence polarization was, however, independent of the molar ratio of phospholipid to protein above a molar ratio of 50:1. Resonance energy transfer between FITC-ATPase and EITC-ATPase was also found to be independent of phospholipid:protein ratio. It is suggested therefore that the ATPase is not randomly distributed in the plane of the membrane but rather forms ordered clusters (probably rows of monomers or dimers) on the fluorescence time scale (nanoseconds) even in the presence of a large excess of phospholipid. This organization within the membrane is dependent both on the chemical structure of the phospholipid and on its physical phase.

Half the surface area of a typical biological membrane is occupied by protein so that interaction between the protein components in the membrane is likely. In a membrane where the protein diffusion coefficient is high, the rate of collision between membrane proteins will be high because of the small distance of separation of the proteins (Poo & Cone, 1974). Nevertheless, calculations of the expected distribution of phospholipid and protein molecules in a membrane show that, for random mixing of the components, the probability of protein–protein contact at any given instant in time is low at a molar ratio of phospholipid to protein of 100:1, typical of most membranes (East et al., 1985). Of course, in real systems, the mixing of phospholipid and protein molecules is unlikely to be ideal (random) and the distribution of molecules within the membrane will depend on the relative strengths of the phospholipid–phospholipid, phospholipid–protein, and protein–protein interactions. Currently, there is no single method to define such distributions. Rather, it is necessary to employ a variety of techniques, the results of each of which alone may be ambiguous, but which together may allow the drawing of an unambiguous conclusion.

The sarcoplasmic reticulum of skeletal muscle is particularly suited to studies of this kind, because approximately 80% of the protein in the membrane is one protein, the $(Ca^{2+}$-$Mg^{2+})$-ATPase, and the $(Ca^{2+}$-$Mg^{2+})$-ATPase can be purified and reconstituted in an active form into bilayer systems where the ATPase is the only species of protein (Warren et al., 1974). Electron microscopic studies of negatively stained membranes have shown that the ATPase in sarcoplasmic reticulum of rabbit skeletal muscle is present as extended rows of dimeric species in the presence of vanadate (Taylor et al., 1984); the ATPase in the sarcoplasmic reticulum of scallop muscle adopts an analogous structure even in the absence of vanadate (Ferguson et al., 1985). In the presence of Ca^{2+}, the ATPase in rabbit sarcoplasmic reticulum adopts a structure consisting of rows of monomeric species (Dux et al., 1985). These studies, of course, present a picture of the organization of the membrane with all motion frozen. A dynamic picture of the membrane, on a millisecond time scale, has been developed from saturation transfer ESR studies of spin-labeled ATPase (Thomas & Hidalgo, 1978; Lewis & Thomas, 1986; Napier et al., 1987). These studies have shown that the ATPase has considerable rotational freedom within the membrane, even under conditions where electron micrographs show the presence

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