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Interaction between phosphatidylserine and the isolated cytoskeleton of human blood platelets

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Binding experiments were performed to demonstrate a direct interaction between cytoskeletons from human blood platelets and phosphatidylserine. A centrifugation technique using radiolabeled phosphatidylserine-vesicles and Triton X-100 insoluble residues from unstimulated human platelets was used to assess the binding. Interaction between cytoskeleton and phospholipid is demonstrated to be specific for phosphatidylserine. No binding was observed for phosphatidylcholine. The binding of phosphatidylserine was saturable and dependent on the concentration of cytoskeleton used. The interaction between phosphatidylserine and the cytoskeleton appeared to be completely reversible. The existence of a reversible and specific interaction between phosphatidylserine and the cytoskeleton of unstimulated platelets would suggest a role for the cytoskeleton in the maintenance of the asymmetric distribution of this lipid in the plasma membrane. We have previously shown (Comfurius et al. (1985) Biochim. Biophys. Acta 815, 143–148) that in activated platelets a strong correlation exists between degradation of platelet cytoskeletal proteins by the endogenous calcium-dependent proteinase (calpain) and exposure of phosphatidylserine at their outer surface. Nevertheless, hydrolysis of the isolated cytoskeleton by calpain did not result in a change in the parameters of the binding between phosphatidylserine and cytoskeleton. Also, sulfhydryl oxidation of the cytoskeleton by diamide did not affect its binding properties for phosphatidylserine, in spite of the fact that diamide treatment of platelets results in exposure of phosphatidylserine at the outer surface. Exposition of phosphatidylserine upon activation of platelets cannot be directly ascribed to a change in affinity or number of binding sites of the modified cytoskeleton as measured in model systems. However, it cannot be excluded that topological rearrangements of the cytoskeleton as occur within the cell during platelet activation lead to a decreased contact between cytoskeleton and lipid, irrespective of the binding parameters.

Introduction

The existence of transbilayer asymmetry of phospholipids in the plasma membrane of different cells is well established (for a review, see Ref. 1). This asymmetric distribution leads to an outer monolayer of the plasma membrane enriched in phosphatidylcholine (PC) and sphingomyelin and an inner monolayer containing the majority of phosphatidylethanolamine and virtually all phosphatidylserine (PS). The ability of platelets to expose PS at their outer surface upon activation, is related to their function in blood coagulation [2]. In particular, the rates of two sequential enzymatic reactions of the coagulation cascade (the conversion of factor X into Xa and the formation of thrombin from its precursor prothrombin) are greatly enhanced by the availability of a negatively charged phospholipid surface containing PS [3].

With respect to the mechanisms responsible for maintaining the asymmetric lipid distribution, attention has been focussed on the possibility of a direct interaction between phospholipids and proteins present in the cell interior. In the cytoplasm of cells a protein network is present, referred to as the cytoskeleton, part of which is located near the plasma membrane (for recent reviews, see Refs. 4–7). Since 1977 several studies on human erythrocytes have indicated the existence of an interaction between phospholipids and components of the cytoskeleton [8–15]. Furthermore, Haest et al. [16] and Franck et al. [17] showed that treatment of erythrocytes with the SH-oxidizing agent diamide, which causes extensive crosslinking of cytoskeletal proteins, leads to an enhanced transbilayer movement of phospholipids.

Abbreviation: PRP, platelet-rich plasma.

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In erythrocytes from patients with sickle cell anaemia, polymerization of haemoglobin during deoxygenation is thought to induce a mechanical decoupling between membrane and cytoskeleton [18], resulting in an enhanced rate of flip-flop of phosphatidylcholine [19] as well as exposure of PS at their outer surface. The latter is most manifest in isolated membrane vesicles that are pinched off from protrusions of the cell body during reversible sickling [18]. Also after treatment of platelets with dilauroylphosphatidylcholine, formation of right-side out membrane vesicles is observed, exposing PS at their outer surface [20]. These vesicles appeared to be essentially devoid of high molecular weight cytoskeletal proteins, spectrin for erythrocyte- and myosin for platelet-derived vesicles, respectively. For intact platelets we have found a striking correlation between modification of the cytoskeleton and exposure of PS at the outer surface of the plasma membrane. Transbilayer asymmetry as measured using phospholipases, is rapidly lost upon stimulation by certain platelet agonists [21]. This reorganization of phospholipids is strictly correlated with the breakdown of cytoskeletal proteins by the endogenous Ca^{2+}-dependent proteinase (calpain) [22–24].

The aim of the present study was to investigate whether direct interactions between phosphatidylserine and the cytoskeleton of unstimulated platelets can be demonstrated and to what extent modifications of the cytoskeleton as induced by calpain or diamide can be held responsible for a reorganization of the lipids in the plasma membrane upon platelet activation.

Materials and Methods

1,2-Dioleoylphosphatidyl[^{14}C]serine (1.11 GBq/mmol) and 1,2-dipalmitoylphosphatidyl[^{14}C]choline (3.7 GBq/mmol) were obtained from Amersham International, U.K. Phosphatidylserine was purified from brain extract type III (Sigma) using CM-cellulose column chromatography as described before [25]. Egg yolk phosphatidylcholine (Sigma) was used without further purification. Platelet calpain (calcium-dependent proteinase, EC 3.4.22.17) was partially purified according to Fox [26]. Ficoll was from Pharmacia Fine Chemicals. Diamide (azodicarboxylic acid bis[dimethylamide]) and Hepes(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were obtained from Sigma Chemical Co. All other reagents were of the highest grade commercially available.

Isolation of platelets

Blood was collected from healthy volunteers. ACD was used as anticoagulant (0.052 M citric acid, 0.08 M trisodium citrate, 0.183 M glucose, 1 part ACD for 5 parts of blood). Platelet-rich plasma (PRP) was obtained by centrifugation at $150 \times g$ for 15 min. After addition of 2% (v/v) of ACD to the PRP, platelets were sedimented by centrifugation at $750 \times g$ for 15 min. The platelet pellet was washed twice in Hepes buffer pH 6.6 (136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose and 0.5 mg/ml fatty acid free human serum albumin). Before each centrifugation 5% (v/v) ACD was added to prevent aggregation during sedimentation. Finally the platelets were resuspended in Hepes buffer pH 7.4 and the count was adjusted to $2.5 \cdot 10^9$/ml using a Coulter counter.

Preparation of cytoskeletons

Platelet cytoskeletons were essentially prepared as described by Fox [26]. Briefly, washed platelets were cooled on ice and made 10 mM in EDTA. After addition of Triton X-100 to a final concentration of 1% (w/v) the suspension was stirred on ice for 30 min. Cytoskeletons were collected by ultracentrifugation (4° C, 30 min, $100\,000 \times g$). Pellets were washed once with 1% (w/v) Triton in buffer A (136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 1 mM EDTA, pH 7.4). Another two washes with buffer A followed to remove the Triton. Finally the preparation was resuspended in buffer A in half the volume of the original platelet suspension. The preparation was sonicated until homogeneous by visual inspection. This suspension is arbitrarily set to contain $5 \cdot 10^9$ cytoskeletons per ml based on the assumption that one cytoskeleton is isolated from each platelet.

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by direct probe sonication at room temperature in buffer A at a concentration of 500 μM. Labeled species were added before sonication in a concentration of 37 kBq per μmole of total lipid. After sonication the suspension was centrifuged for 30 min at $100\,000 \times g$ (room temperature). Lipid concentration in the supernatant was determined as phosphorus [27].

Binding assay

It appeared that after preparing phospholipid vesicles by sonication, it was not possible to obtain a non-sedimentable fraction by taking the supernatant of a precentrifuged sample. Also from that supernatant a substantial fraction (20–40%) was sedimented under the experimental conditions. Centrifugation of sonicated vesicles for 30 min at $100\,000 \times g$ in buffer A induces a phospholipid gradient in the tube, resulting in about 80% of the lipid being present in the bottom 20% of the tube. Increasing the density of the medium to 1.035 g/ml (10% w/v Ficoll) results in a completely even distribution of the vesicles over the length of the tube. Centrifugation of cytoskeleton in 10% Ficoll resulted in essentially complete sedimentation (more than 95%) of the cytoskeletal proteins.

Binding experiments were carried out using a Beckman TL-100 ultracentrifuge equipped with a rotor for 200 μl tubes. In capped incubation vessels the desired dilutions of phospholipid and cytoskeletons were mixed by vortexing with a 25% solution (w/v) of Ficoll in buffer A, sufficient to reach a final concentration of Ficoll of 10% (w/v). After incubation for 30 min at room temperature 200 μl of the mixture is transferred to a centrifuge tube and spun for 30 min at 100000 $\times$ g. To assess the unbound fraction of phospholipid 100 μl of the supernatant is transferred to a vial for liquid scintillation counting.

Results and Discussion

Cytoskeleton of unstimulated platelets is able to bind PS in a concentration-dependent manner. The fraction of bound lipid as a function of the concentration of cytoskeleton is shown in Fig. 1. Binding is specific for PS compared to PC-vesicles which do not bind over the whole range of cytoskeleton concentrations tested.

The binding curves obtained using two concentrations of cytoskeleton and variable amounts of phospholipid as shown in Fig. 2, demonstrate that binding is saturable, which strongly suggests that aspecific sedimentation of lipid is negligible. When ^{14}C-PS in PS-vesicles is replaced by ^{14}C-PC the same binding curve is obtained. Replacing ^{14}C-PC in PC-vesicles by ^{14}C-PS also does not affect the binding, i.e., no binding is observed. This indicates that binding involves intact vesicles, rather than monomeric lipid. Since the cytoskeletal preparation consists of a mixture of proteins and since it is unclear how many lipid molecules of a vesicle actually participate in binding, no attempt was made to translate the data into an apparent K_d or number of binding sites.

The reversibility of the binding of PS is shown in Fig. 3. When 10 μM labeled phospholipid is preincubated with cytoskeleton ($5 \cdot 10^7$/ml) for 30 min, about 65% of the labeled lipid can be sedimented. Addition of increasing amounts of non-labeled PS to this mixture results in progressive displacement of label from the cytoskeleton. However, if non-labeled PC is used, no change in the amount of bound PS is observed (not shown). Since addition of 10 μM of lipid is not saturating for this amount of cytoskeleton (see Fig. 2), addition of extra non-labeled PS will, apart from displacing labeled PS, also occupy residual free binding sites. Taking this into account, one can calculate the amount of label which should be replaced by non-labeled species, assuming complete equilibration of labeled and non-labeled pools. Comparing the two lines in Fig. 3 (the dashed line representing the calculated displacement of label) shows that virtually all labeled PS equilibrates with added non-labeled PS; demonstrating the complete reversibility of the binding.

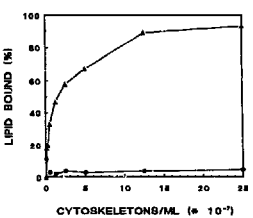

Fig. 1. Phospholipid vesicles (25 μM) were incubated with increasing amounts of cytoskeleton. Bound lipid was determined by measuring the radioactivity remaining in the supernatant after centrifugation for 30 min at 100000 $\times$ g. ▲, Phosphatidylserine; ●, phosphatidylcholine.

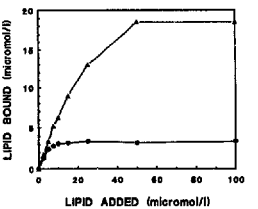

Fig. 2. Cytoskeleton in two concentrations was incubated with different concentrations of phosphatidylserine vesicles. The concentration of bound lipid was measured as described in the legend to Fig. 1. ●, $5 \cdot 10^6$ cytoskeletons/ml; ▲, $5 \cdot 10^7$ cytoskeletons/ml.

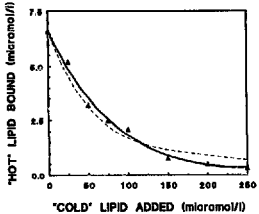

Fig. 3. Supersedition of labeled phospholipid (PS) by excess non-labeled PS. Cytoskeletons ($5 \cdot 10^7$/ml) were incubated for 30 min with 10 μM labeled PS-vesicles. Subsequently non-labeled PS-vesicles were added in increasing concentration. Incubation was carried on for another 30 min. The amount of bound labeled PS was determined as described in the legend to Fig. 1. The dashed line represents the calculated concentration of bound labeled PS, taking into account that binding is not saturated at 10 μM phospholipid and assuming a complete equilibration of the labeled and the non-labeled pools.

Previously we have shown that PS-exposure at the outer surface of activated platelets is strictly correlated with modification of the cytoskeleton [20,22–24]. Conditions leading to exposure of PS appeared to involve degradation of cytoskeletal proteins by endogenous calpain, which becomes activated by a considerable rise in intracellular Ca^{2+}-concentration as for instance effected by Ca^{2+} ionophore or stimulation by the combined action of collagen plus thrombin. Moreover, when platelets are made permeable for leupeptin, a specific inhibitor of calpain, activity of this proteinase was diminished during platelet activation in parallel with a lower amount of PS becoming exposed at the platelet outer surface [24]. Furthermore, intracellular modification of cytoskeleton by means of the sulfhydryl oxidizing compound diamide also results in exposure of PS at the platelet outer surface [21].

Isolated cytoskeleton from unstimulated human platelets was treated with calpain or diamide to study the effect of these treatments on the PS binding properties of the cytoskeleton. To enable detection of either a change in affinity or a decrease in the number of binding sites, a lipid titration was carried out at a fixed concentration of cytoskeleton. However, though the protein degradation by calpain as well as the cross-linking by diamide could be confirmed by gel electrophoresis (not shown), no change in binding characteristics of PS could be observed (Fig. 4). This indicates that flip-flop of PS during stimulation of the platelet cannot be the consequence of a loss of PS-binding capacity of the cytoskeletor after modification, as measured in model systems. However, the present results do not exclude a possible role of the cytoskeleton in the regulation of PS asymmetry within the cell. At least two possibilities remain:

(i) Loss of affinity for PS only occurs during modification of the cytoskeleton. Although this cannot be approached by the present technique, experiments described recently by Verhallen [28] seem to support this notion. Based on observations using the fluorescent membrane probe trimethylammonium diphenylhexatriene (TMA-DPH) it was shown that the occurrence of fast flip-flop is restricted to the timeperiod during which calpain is active and cytoskeletal proteins are in the process of being degraded.

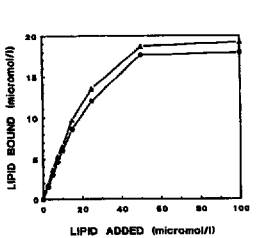

Fig. 4. Titration of modified cytoskeleton with PS-vesicles. $5 \cdot 10^7$ cytoskeletons per ml were used after modification with either calpain (▲) or diamide (●). The conditions for the calpain were chosen to produce 50% degradation of myosin as judged by gel electrophoresis. Diamide treatment was for 15 min with 5 mM diamide. The amount of bound lipid was determined as described in the legend to Fig. 1.

(ii) Modification of the cytoskeleton in intact platelets induces a change in its three-dimensional structure resulting in a mechanical decoupling of membrane and cytoskeleton, irrespective of a potential affinity for PS. Such a phenomenon was demonstrated by Spangenberg et al. [29] who showed that upon treatment of platelets with diamide, crosslinking of proteins leads to a contraction of the cytoskeleton, thereby becoming concentrated in the center of the cell.

Another possible mechanism involved in maintaining the asymmetric distrubution of PS is the presence of a transporting system (translocase), specific for aminophospholipids, as was demonstrated in erythrocytes by Seigneuret and Devaux [30]. The existence of a phospholipid translocase in erythrocytes was later confirmed [31–35] and was also shown to be present in other cells, including platelets [36–38]. This mechanism, thought to consist of a protein, is capable of transporting exogenously added aminophospholipids from the outer to the inner monolayer of a plasma membrane. The process was shown to be ATP-dependent and sensitive to sulfhydryl oxidizing agents. Recently we obtained evidence [39] that the translocase present in human platelet plasma membranes is also capable of transporting endogenous PS-exposed at the platelet outer surface as a result of an activation procedure-back to the inner leaflet of the membrane.

Both the experiments showing the existence of an amino-phospholipid translocating entity and the present observation that an interaction between PS and cytoskeletal proteins could be present in intact platelets, are consistent with the model proposed by Williamson et al. [40]. On theoretical grounds they reach the conclusion that maintenance of the asymmetric distribution of phospholipids can best be explained by the action of a translocase, in conjunction with a direct interaction between PS and cytoskeleton, reducing the amount of free lipid being available for spontaneous transbilayer movement.

However, the rapid transbilayer movement of phosphatidylserine as occurs during platelet activation is not directly related to a change in the binding parameters of the PS–cytoskeleton interaction after modification of the cytoskeleton. This transbilayer movement is not restricted to PS alone, but involves the other phospholipids as well [21]. Therefore, it cannot be excluded that this process is caused by gross structural rearrangements of both membrane and cytoskeleton, in which

potential interactions between PS and cytoskeletal proteins are only of secondary importance.

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