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Exposure of Endogenous Phosphatidylserine at the Outer Surface of Stimulated Platelets Is Reversed by Restoration of Aminophospholipid Translocase Activity†

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ABSTRACT: Phosphatidylserine (PS) in the plasma membrane of nonactivated human platelets is almost entirely located on the cytoplasmic side. Stimulation of platelets with the Ca^{2+} ionophore A23187 or combined action of collagen plus thrombin results in a rapid loss of the asymmetric distribution of PS. Also, treatment with the sulfhydryl-reactive compounds diamide and pyridyldithioethylamine (PDA) causes exposure of PS at the platelet outer surface. PS exposure is sensitively measured as the catalytic potential of platelets to enhance the rate of thrombin formation by the enzyme complex factor Xa-factor Va, since this reaction is essentially dependent on the presence of a PS-containing lipid surface. In this paper we demonstrate that endogenous PS, previously exposed at the outer surface during cell activation or sulfhydryl oxidation, can be translocated back to the cytoplasmic leaflet of the membrane by addition of dithiothreitol (DTT) but not by nonpermeable reducing agents like reduced glutathione. Treatment of platelets with trypsin or chymotrypsin, prior to addition of DTT, inhibits the inward transport of exposed PS. Moreover, severe depletion of metabolic ATP, as obtained by platelet stimulation with A23187 in the presence of metabolic inhibitors, though not inhibiting PS exposure at the outer surface, blocks the translocation of endogenous PS to the internal leaflet of the plasma membrane. These results strongly indicate the involvement of a membrane protein in the inward transport of endogenous PS. Recently, an aminophospholipid-specific translocase in the platelet membrane was postulated on the basis of the inward transport of exogenously added PS (analogues) [Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellmann, P., & Devaux, P. F. (1987) *Biochemistry 26*, 2972-2978]. The present data demonstrate for the first time the inward translocation of endogenous PS, most likely supported by the same membrane protein responsible for transport of exogenous PS.

The molecular architecture of biological membranes is highly asymmetric. This phenomenon is not only restricted to the orientation of membrane proteins but also pertains to the lipid distribution with respect to both phospholipid class and fatty acid composition (Op den Kamp, 1979). Detailed information is available on the nonrandom orientation of lipids in the erythrocyte and platelet membrane (Verkley et al., 1973; Gordesky et al., 1975; Zwaal et al., 1975; Chap et al., 1977; Schick et al., 1976): while phosphatidylcholine and phosphatidylethanolamine are present in variable amounts in both membrane leaflets, a most extreme distribution is observed for phosphatidylserine (PS)[1] and sphingomyelin, which are almost exclusively located in the inner and outer leaflets, respectively. In erythrocytes, this highly asymmetric orientation of lipids is essential for normal homeostasis. It has been demonstrated that an increased exposure of PS at the outer surface of the red cell is a signal for sequestration by the reticuloendothelial system (Tanaka & Schroit, 1983; Schwartz

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[1] Abbreviations: PS, phosphatidylserine; DTT, dithiothreitol; diamide, diazinedicarboxylic acid bis(dimethylamide); PDA, pyridyldithioethylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride.

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et al., 1985; Schroit et al., 1985). Also, partial loss of PS asymmetry, as for instance occurs in some pathological red blood cells, may contribute to intravascular coagulation (Franck et al., 1985). In platelets, transbilayer asymmetry as measured with purified phospholipases as probes, is rapidly lost upon stimulation by certain platelet agonists (Bevers et al., 1983; Comfurius et al., 1985; Bevers et al., 1987). This phenomenon fulfills an important physiological function in the hemostatic process, since two sequential reactions of the coagulation cascade are dramatically accelerated in the presence of an anionic phospholipid surface (Rosing et al., 1985). Among the stimuli that can evoke an increased exposure of PS at the outer surface of platelets are SH-oxidizing agents and increase in intracellular Ca^{2+} concentration as for instance occurs by a Ca^{2+} ionophore or the combined action of two physiological stimulators, collagen plus thrombin (Bevers et al., 1983). Considering the mechanism(s) responsible for maintenance of the asymmetric distribution of PS, much attention has been focused on the role of the cytoskeleton (Haest, 1982; Mohandas et al., 1985; Williamson et al., 1987). Direct interactions between several cytoskeletal proteins and PS have been demonstrated for the erythrocyte (Mombers et al., 1979; Wagner et al., 1985; Cohen et al., 1986, 1988; Rybicki et al., 1988) as well as for platelets (Comfurius et al., unpublished results).

In the past few years, several groups have obtained evidence for the existence of another mechanism involved in the maintenance of transbilayer asymmetry. By use of different techniques it was shown that labeled lipids or lipid analogues, introduced in the outer leaflet of the red cell membrane, rapidly migrated to the inner leaflet (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Zachowski et al., 1986; Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit, 1987). The process was shown to be specific for aminophospholipids, ATP dependent and sensitive to SH-oxidizing agents and increased intracellular Ca^{2+} concentration. Moreover, Connor and Schroit (1988) have shown that inhibition of the inward transport following sulfhydryl oxidation by diamide or pyridyldithioethylamine (PDA) can be abolished after the membrane is reduced by dithiothreitol. On the basis of these observations a specific translocase in the plasma membrane was proposed to be responsible for this rapid outward–inward transbilayer movement. Indications for the presence of an aminophospholipid-specific translocase have also been obtained for other cells, such as lymphocytes (Zachowski et al., 1987), fibroblasts (Martin & Pagano, 1987), and platelets (Daleke & Huestis, 1985; Sune et al., 1987).

While outward–inward transport of exogenous aminophospholipids can be inhibited by sulfhydryl-reacting compounds or by raising intracellular Ca^{2+} concentration, the same conditions applied to platelets cause a rapid inward–outward movement of endogenous PS (Bevers et al., 1983). The aim of the present study was to investigate the role of the putative platelet translocase in this process. We demonstrate here that endogenous PS, rendered available at the platelet outer surface by stimulating these cells, can be translocated to the inner leaflet by applying reducing power to the cell interior. This process appears to be ATP dependent and inhibited by proteolytic enzymes.

Experimental Procedures

Materials. Diamide, 2-deoxy-D-glucose, glucono-δ-lactone, fatty acid free human serum albumin, dithiothreitol, trypsin, and chymotrypsin were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium ionophore A23187 was from Calbiochem, and thioredoxin was from IMCO Corp. Ltd. AB (Stockholm, Sweden). Horse tendon collagen (type I) was purchased from Hormon Chemie (München, FRG). Pyridyldithioethylamine (PDA) was a kind gift from Dr. A. J. Schroit (Houston, TX). Coagulation factors thrombin, prothrombin, factor Xa, and factor Va were purified from bovine blood as described elsewhere (Rosing et al., 1985). Thrombin-specific chromogenic substrate S2238 was obtained from AB Kabi Diagnostica (Stockholm, Sweden). All other reagents were of the highest grade commercially available.

Isolation of Platelets. Washed human platelets were obtained from freshly drawn blood by differential centrifugation as described before (Bevers et al., 1983). Platelets were finally resuspended in a Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, and 1 mg·mL^{-1} human serum albumin, adjusted to pH 7.4. Platelets were incubated at a concentration of 10^8 mL^{-1} at 37 °C, unless otherwise stated.

Measurement of Prothrombinase Activity. The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa–factor Va has been shown to be a convenient and sensitive method to monitor in a semiquantitative way the extent of PS in the platelet outer surface (Bevers et al., 1983; Comfurius et al., 1985; Connor et al., 1989). Briefly, platelets at a concentration of 5×10^6 mL^{-1} were incubated with factor Xa and factor Va (final concentrations 3 and 6 nM, respectively) for 2 min in the presence of 3 mM $CaCl_2$. Subsequently, thrombin formation was started by adding prothrombin (final concentration 4 μM) and additional $CaCl_2$ (final concentration 6 mM). Exactly 1 min after addition of prothrombin, an aliquot of the incubation mixture was transferred to a cuvette containing a buffer composed of 50 mM Tris-HCl, 100 mM NaCl, and 2 mM EDTA, pH 7.9. Thrombin activity was determined spectrophotometrically at 405 nm after addition of the chromogenic substrate S2238 (final concentration 150 μM). The amount of thrombin formed was calculated from the change in absorbance per minute, using a calibration curve made with active-site-titrated thrombin.

Measurement of ATP Content of Platelets. ATP was measured by a firefly luminescence test using a test kit (ATP bioluminescence CLS) purchased from Boehringer (Mannheim). Samples from an incubation of platelets (10^8 mL^{-1}) were precipitated by trichloroacetic acid (5% final concentration) and kept for 10 min on ice. After centrifugation aliquots of the supernatant were 100-fold diluted in Hepes buffer before addition of the ATP reagent. The peak flash height emission was detected by a photomultiplier and recorded. ATP levels were determined by comparison to a standard curve prepared with known amounts of ATP. The values obtained represent the total ATP content of the cells, consisting of metabolic and storage pool ATP (ATP present in dense granules). Storage pool ATP was found after maximal stimulation of the platelets with A23187 (1 μM) for 10 min. Samples taken from a 12000*g* supernatant of this incubation were used to determine the ATP content as described above. Metabolic ATP was found by subtracting storage pool ATP from total ATP.

Measurement of Platelet Lysis. Lactate dehydrogenase present in a 12000*g* supernatant of a platelet incubation was used as a parameter for lysis and was determined according to Wroblewski and La Due (1955).

Results

When platelets are treated with sulfhydryl-reactive compounds such as diamide or PDA, increasing amounts of PS become exposed at the outer surface of the plasma membrane,

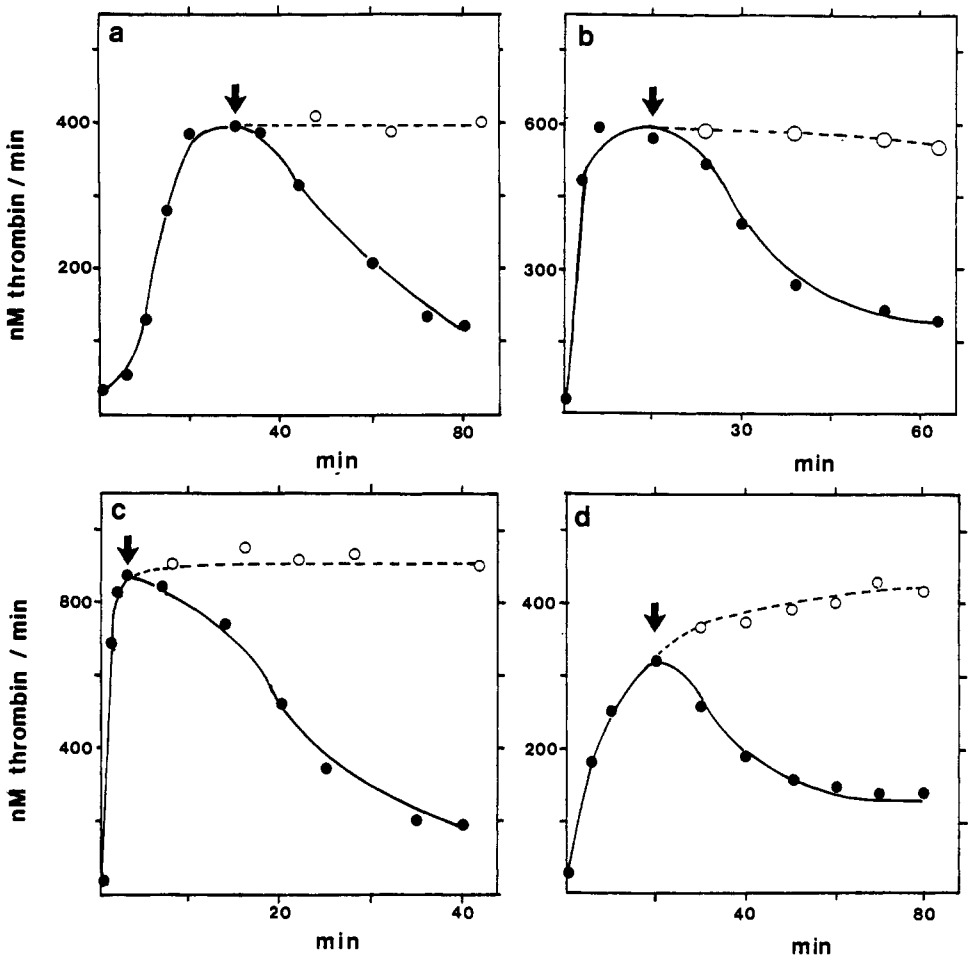

FIGURE 1: Dithiothreitol-induced reversibility of the catalytic potential of platelets in the conversion of prothrombin to thrombin by a complex of factors Xa and Va. The rate of thrombin formation, expressed as nanomolar thrombin formed per minute, is directly correlated with the amount of PS exposed at the outer surface of the platelet. Platelets are incubated at a concentration of 10^8 mL^{-1} with (a) diamide (5 mM), (b) PDA (1 mM), (c) A23187 (1 μM), and (d) collagen (10 $\mu g \cdot mL^{-1}$) plus thrombin (4 nM). At the time indicated by the arrow, the incubation is divided into one part supplied with 5 mM DTT [10 mM in (a)] (●) and another part as a control without DTT (○). At each time point a sample is taken from the incubation mixture and diluted to a concentration of 5×10^6 platelets·mL^{-1} at which the prothrombinase activity is measured as described under Experimental Procedures.

as can be judged from the rise in prothrombin converting activity (Figure 1). Since these reagents react with free SH groups in a reversible manner, it was of interest to see whether reducing agents could lead to a restoration of the original distribution of PS. Addition of DTT to diamide- or PDA-treated platelets results in a progressive decline of the platelets' ability to stimulate prothrombinase activity as can be seen in Figure 1a,b. The effect of DTT cannot be ascribed to inhibition of the prothrombin conversion by factors Xa/Va as such, because no decrease in prothrombinase activity could be observed up to 10 mM DTT when sonicated platelets were used as a procoagulant surface. Since the rate of thrombin formation is critically dependent on the presence of PS in the outer surface (Comfurius et al., 1985), a decrease in prothrombinase activity indicates that addition of DTT causes a partial restoration of the asymmetric distribution of PS. However, generation of a procoagulant platelet surface as induced by the irreversible alkylating agent *N*-ethylmaleimide cannot be reversed by addition of DTT (data not shown). It should be emphasized that the total PS content of the platelets remains constant throughout all treatments. This excludes degradation or loss of PS from the membrane as a possible cause of the decline in prothrombinase activity after addition of DTT. In contrast to human platelets, treatment of human red cells with sulfhydryl-oxidizing agents does not result in exposure of PS at the outer surface.

Other conditions leading to a significant loss of PS asymmetry in the platelet membrane are stimulation by the combined action of collagen plus thrombin or treatment with Ca^{2+} ionophore A23187 in the presence of extracellular Ca^{2+}. Although the effect of these platelet activators is not known to be directly related to modification of free SH groups, subsequent addition of DTT also causes a progressive decline in prothrombinase activity (Figure 1c,d), indicating translocation of PS to the cytoplasmic leaflet of the plasma membrane. The extent of reversibility appears to be dependent on the experimental conditions: at low platelet concentrations (5×10^6 mL^{-1}) not more than 10% reduction of the maximal prothrombinase activity was found after prolonged incubation with DTT, in contrast to 75% or more reduction in procoagulant activity at 10^8 platelets·mL^{-1}. This difference was most pronounced when A23187 was used to evoke PS exposure. The fraction of prothrombinase activity that could not be reversed by DTT was not due to platelet lysis (platelet lysis remained below 5% during all treatments). It was found that A23187, in particular at low platelet concentrations, caused the formation of microparticles, pinched off from the main cell body. The prothrombinase activity of these microparticles could not be reversed by DTT (data not shown).

Several other reducing agents have been studied for their ability to cause outward–inward transport of PS after its exposure at the outer surface. The results are summarized in

Table I: Effect of Reducing Agents on the Prothrombin Converting Activity of Human Blood Platelets[a]

reducing agent	prothrombin converting activity (nM thrombin/min) A23187 (1 μM)	PDA (1 mM)	lysed platelets
none	829	625	1480
dithiothreitol (5 mM)	282	277	1465
β-mercaptoethanol (10 mM)	825	574	1483
glutathione (5 mM)	821	593	1335
thioredoxin reductase[b]	817	619	1184

[a] Platelets were activated at a concentration of 10^8 mL^{-1}. After 10-min activation, the reducing agent was added at a final concentration indicated in the table. Thirty minutes later, samples were taken and diluted to a concentration of 5×10^6 mL^{-1} for assaying prothrombin converting activity. Lysed platelets were used as a control for the effects of reducing agents on the prothrombinase assay. [b] Thioredoxin reductase consists of a mixture of 120 μM thioredoxin, 4 μM reductase, 4 mM NADPH, and 5 μM DTT (final concentrations).

Table II: Effect of Proteolytic Digestion on DTT-Induced Decrease in Platelet Prothrombin Converting Activity[a]

treatment before stimulation with A23187	prothrombin converting activity (nM throbmin/min) without DTT	with DTT
none	825	297
trypsin (5 μg/mL)	828	835
chymotrypsin (5 μg/mL)	832	829
thrombin (5 μg/mL)	829	304

[a] Platelets (10^8 mL^{-1}) were incubated with 1 μM A23187 for 5 min before addition of DTT (5 mM). Five minutes before addition of A23187, proteolytic enzymes were added at the concentrations indicated. Samples were taken 35 min after addition of A23187 and diluted 20-fold for measuring prothrombin converting activity. Proteolytic enzymes at this dilution did not influence the assay. Platelet lysis remained below 5%.

Table I. Neither reduced glutathione nor the powerful reducing system thioredoxin reductase is effective. Since both these reductors are membrane impermeable, this indicates that the sulfhydryl groups critical to the inward transport of PS are buried in the core of the lipid bilayer or present on the cytoplasmic side of the plasma membrane. Although diffusion of β-mercaptoethanol across the membrane is expected to be at least comparable to DTT, this compound does not lead to disappearance of PS from the outer surface. This is most likely due to the fact that β-mercaptoethanol is a much less effective reducing compound than DTT.

Proteolytic treatment of platelets by trypsin or chymotrypsin did not prevent surface exposure of PS induced by ionophore but inhibited DTT-induced outward–inward transport of exposed PS (Table II). Preliminary experiments have shown that papain, thermolysin, subtilisin, and plasmin also can inhibit the inward transport of PS. In contrast, the specific proteolytic enzyme thrombin, which has a high affinity for platelets and very efficiently degrades membrane glycoprotein V, is without effect on the inward transport of PS. Also, proteolysis of diamide-treated platelets did prevent reversal of PS exposure.

Outward–inward transport of exogenously added PS in erythrocytes and platelets was demonstrated to be dependent on intracellular ATP (Seigneuret et al., 1984; Tilley et al., 1986; Sune et al., 1987). The inward transport of previously exposed endogenous PS was also ATP dependent, as illustrated in Figure 2. Platelets were stimulated with 1 μM A23187 for 5 min in the presence or absence of a cocktail of 2-deoxyglucose, glucono-δ-lactone, and KCN and were subsequently supplied with 5 mM DTT. Loss of PS in the outer leaflet was only observed in the absence of metabolic inhibitors.

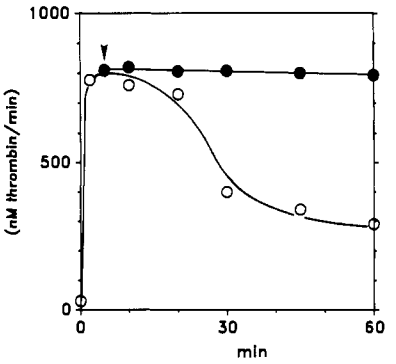

FIGURE 2: ATP dependence of DTT-induced reversibility of the platelet prothrombin converting activity generated by Ca^{2+} ionophore A23187. Platelets are stimulated with A23187 (1 μM) at a concentration of 10^8 mL^{-1}. Five minutes prior to stimulation, a cocktail of metabolic inhibitors is added, composed of 10 mM glucono-δ-lactone, 45 mM 2-deoxyglucose, and 1 mM KCN. Dithiothreitol (5 mM) is added at the time point indicated by the arrow. Samples for measuring the rate of thrombin formation are taken from the incubation with (●) and without (○) metabolic inhibitors at the time points indicated and measured at a concentration of 5×10^6 mL^{-1} (Experimental Procedures).

Measurement of metabolic ATP demonstrated that activation with A23187 in the presence of metabolic inhibitors causes a drop in the level of metabolic ATP below 2% within 5 min. Similar results regarding outward–inward translocation of PS were obtained when platelets were ATP depleted by preincubation with 10 mM sodium fluoride for 1 h prior to activation with the ionophore (data not shown). The presence of metabolic inhibitors did not affect the exposure of PS induced by A23187. The effect of ATP depletion could not be studied on platelets activated by collagen plus thrombin, since in the presence of metabolic inhibitors the activation process is hampered, most likely due to a disturbance of the stimulus–response coupling.

Discussion

Transport of exogenous PS or PS analogues in red blood cells and in platelets has been shown to be dependent on the maintenance of membrane protein sulfhydryls in the reduced state (Zachowski et al., 1986; Daleke & Huestis, 1985; Connor & Schroit, 1987). The present data show that the asymmetric distribution of PS in the platelet membrane, which is lost as a result of thiol–disulfide exchange by diamide or PDA, can be partially restored by addition of a powerful permeable reducing agent. Likewise, exposure of PS during activation of platelets by A23187 or the combined action of collagen plus thrombin can be reversed by subsequent incubation with DTT. Apparently, these activation procedures are accompanied by thiol–disulfide exchange, inhibiting the outward–inward translocation of PS. Indeed, preliminary experiments have indicated that treatment with A23187, as well as the combined action of collagen and thrombin, results in a significant reduction of the total free sulfhydryl content of the cell. Although inward transport of exogenous PS in red blood cells is inhibited when the cytosolic Ca^{2+} concentration rises above 1 μM (Zachowski et al., 1986), a high intracellular Ca^{2+} concentration, as expected upon activation by A23187 in the presence of 3 mM extracellular Ca^{2+}, does not prevent the DTT-induced inward transport of endogenous PS in platelets.

Transbilayer movement of phospholipids during platelet activation is not restricted to PS alone (Bevers et al., 1983). The activation procedures described above tend to randomize all phospholipids in the membrane bilayer, which results in at least half of the PS becoming exposed to exogenously added

phospholipases. Spontaneous movement of phospholipids across the lipid bilayer is considered to be an extremely slow process. The rapid transport of lipids in the platelet membrane upon activation therefore suggests the existence of a facilitating mechanism. A disturbance of the bilayer structure involving interconnection between the two membrane leaflets would dramatically decrease the activation energy for transbilayer movement, allowing rapid randomization of the lipids over inner and outer monolayers. Such a process does not necessarily involve membrane proteins (Cullis & de Kruijff, 1979). Support for the existence of nonspecific flip sites in platelets has been obtained before (Comfurius et al., 1983; Verhallen, 1988). Although it cannot be excluded that a (specific) protein, similar to or identical with the inward-directed translocase, facilitates outward transport of PS, the finding that this transport is not affected by proteolytic enzymes makes such a mechanism less likely.

The rate of exposure of PS, i.e., the rate of outward transport of endogenous PS, depends on the trigger used to induce PS exposure. While outward transport of PS is completed within a few minutes when Ca^{2+} ionophore or PDA is used, a significantly longer period is required with collagen plus thrombin or diamide as a trigger. However, the reverse process after addition of DTT seems to have comparable rates for all four triggers used to evoke PS exposure. This suggests that in all cases a similar process is responsible for the disappearance of PS from the platelet outer surface. In contrast to the translocation of NBD-labeled aminophospholipids in fibroblasts (Martin & Pagano, 1987), inward transport of endogenous PS in platelets is sensitive to proteolytic digestion. This observation strongly supports the idea that the inward transport of PS is driven by a membrane protein. It is most likely that this protein is identical with the aminophospholipid-specific translocase, which is responsible for the observed transport of exogenous PS as proposed by Sune et al. (1987). This is further supported by the ATP dependence of the inward transport of PS.

Previously, we have demonstrated that the platelet cytoskeleton is critically involved in the maintenance of membrane lipid asymmetry (Comfurius et al., 1985; Verhallen et al., 1987, 1988). An inextricable correlation was found between appearance of PS at the outer surface of the platelet and modification of the cytoskeleton by activation of an endogenous Ca^{2+}-dependent protease. Moreover, modification of the cytoskeleton by the membrane-permeable sulfhydryl-reactive compound diamide, as shown by Spangenberg et al. (1987), also results in exposure of PS at the platelet outer surface (Bevers et al., 1983). From these data it was suggested that alterations in cytoskeletal organization may lead to a detachment of the cytoskeleton from the inner leaflet of the lipid bilayer, allowing phospholipid redistribution. At present, it is not clear to what extent the cytoskeleton is involved in (partial) restoration of the PS asymmetry by DTT. It could be argued that most of the conditions previously used to inhibit inward transport of exogenous PS in platelets as well as red blood cells, such as sulfhydryl oxidation, ATP depletion, or increase in cytosolic Ca^{2+}, also affect the cytoskeletal organization. On the other hand, recent experiments from our laboratory, using isolated cytoskeletons from nonactivated platelets, have indicated that calpain-degraded or diamide-treated cytoskeletons have virtually the same binding affinity for PS as nonaffected cytoskeletons (Comfurius et al., unpublished results). However, it cannot be excluded that the interaction of cytoskeletal proteins with the inner leaflet of the plasma membrane is only disturbed *during* and not after modification of the cytoskeleton. In support of this is the observation that flip-flop of phospholipids with progressive loss of membrane asymmetry appears to be a temporary event, occurring in a time span of minutes directly after platelet activation and coinciding with the relatively short period during which calpain exerts its action (Verhallen, 1988). Therefore, it is still possible that even in platelets activated by A23187, the modified cytoskeleton contributes to the redistribution of PS induced by DTT, by fixing PS at the inside of the membrane.

Physiologically, the presence of an aminophospholipid-specific translocase in the membrane could be visualized as a protective mechanism to prevent accumulation of PS in the outer surface of the membrane. Interaction of PS with the cytoskeleton contributes to this process in minimizing spontaneous inward–outward movement of the lipid, hence reducing ATP consumption by the action of the translocase. When increased exposure of PS is required, as in the hemostatic process, both translocase activity and cytoskeleton–PS interactions are rapidly (if only temporary) disturbed as a result of platelet activation.

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Heteronuclear Three-Dimensional NMR Spectroscopy of the Inflammatory Protein C5a

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ABSTRACT: The utility of three-dimensional heteronuclear NMR spectroscopy for the assignment of ^{1}H and ^{15}N resonances of the inflammatory protein C5a (MW 8500), uniformly labeled with ^{15}N, is demonstrated at a protein concentration of 0.7 mM. It is shown that dramatic simplification of the 2D nuclear Overhauser effect spectrum (NOESY) is obtained by editing with respect to the frequency of the ^{15}N heteronucleus in a third dimension. The improved resolution in the 3D experiment largely facilitates the assignment of protein NMR spectra and allows for the determination of distance constraints from otherwise overlapping NOE cross peaks for purposes of 3D structure determination. The results show that ^{15}N heteronuclear 3D NMR can facilitate the structure determination of small proteins and promises to be a useful tool for the study of larger systems that cannot be studied by conventional 2D NMR techniques.

The determination of three-dimensional structures of small proteins in solution by high-resolution NMR spectroscopy has become a well established technique. The method relies on the identification of a large number of internuclear distance constraints obtained from nuclear Overhauser effects (NOEs),[1] which are used to calculate an ensemble of tertiary structures [for a review, see Wüthrich (1986)]. Thus far, 3D structure determinations by NMR have been limited to relatively small proteins of a molecular weight smaller than 10000. This size limitation is due to the difficulty in assigning the NOE cross peaks in two-dimensional (2D) NOE spectroscopy data (NOESY) to specific proton pairs in larger proteins because of the vast number of overlapping signals. In order to determine the structures of larger proteins by NMR, novel NMR techniques need to be developed to resolve this problem of spectral overlap.

Recently, we and others (Fesik & Zuiderweg, 1988; Bax et al., 1988) have proposed the use of heteronuclear three-dimensional (3D) NMR spectroscopy for the simplification of NMR spectra of larger systems. The 3D experiments are combinations of homonuclear 2D NMR experiments [for a review, see Ernst et al. (1987)] such as NOESY and COSY and heteronuclear multiple quantum correlation (HMQC) (Müller, 1979; Bax et al., 1984) techniques. Heteronuclear 3D NMR has the advantage over homonuclear 3D NMR (Vuister & Boelens, 1987; Griesinger et al., 1987a,b; Oschkinat et al., 1988; Vuister et al., 1988) in that one of the coherence transfer steps involves the heteronuclear scalar coupling which is large compared to the ^{1}H line width, allowing high sensitivity even when these experiments are applied to large molecules.

[1] Abbreviations: NOE, nuclear Overhauser effect; 1D, one dimensional; 2D, two dimensional; 3D, three dimensional; NOESY, 2D NOE spectroscopy; HMQC, 2D heteronuclear multiple quantum correlation.

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