Phospholipid flip-flop in activated platelets

PHOSPHOLIPID FLIP-FLOP IN ACTIVATED PLATELETS

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. F.I.M. Bonke,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op vrijdag, 22 september 1989, om 16.00 uur

DOOR

PAUL COMFURIUS

geboren te Utrecht in 1947.

druk: wibro dissertatiedrukkerij, helmond

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Dit werk is gedeeltelijk financieel ondersteund door een programmasubsidie (nr. 900-526-093) van MEDIGON.
Het verschijnen van dit proefschrift werd mede mogelijk dankzij gewaardeerde financiële steun van:
- De Nederlandse Hartstichting
- De Stichting Dr. Ir. J.H.J. van de Laar voor wetenschappelijk biochemisch onderzoek.
- De Jan Dekkersstichting & Dr. Ludgardine Bouwmanstichting
- De Stichting het Scholten-Cordes fonds.

CONTENTS

PREFACE

The articles comprising the contents of this thesis have been published in a time span of ten years. I want to emphasize that discussions as they appear in the different chapters are written in the context of the then existing knowledge. In the general discussion of this thesis an attempt is made to integrate these views into a model describing the present views on regulation of phospholipid asymmetry in platelet plasma membranes.

The first part of the following introduction is meant to provide some relevant background information on platelets, coagulation and the involvement of platelet membranes in coagulation. In the second part an outline is presented of the questions leading to the experiments described in each of the different chapters.

INTRODUCTION

Platelet function and morphology.

Platelets are the smallest cells present in the circulation. They are disk-shaped with a diameter of about 2 microns. In the unstimulated form, platelets are non-adherent to each other or the vessel wall, but capable of sticking to both if properly activated. Platelets play a role in a variety of processes, such as inflammation, wound-healing and immunological reactions. Their main function, however, lies in their pivotal role in the haemostatic process.

Platelets are formed in the bone-marrow as fragments of megakaryocytes. They have no nucleus, thus being virtually unable to synthesize proteins, and have an average lifetime of about 8 days. Their ultrastructure was studied extensively by means of electron microscopy (for a comprehensive review, see Ref. 1). A schematic view of the main morphological features of the platelet is presented in Fig. 1. The cell is surrounded by a plasma membrane, showing several deep invaginations (open canalicular system), which increase the surface area and give the platelet a characteristic spongelike appearance.

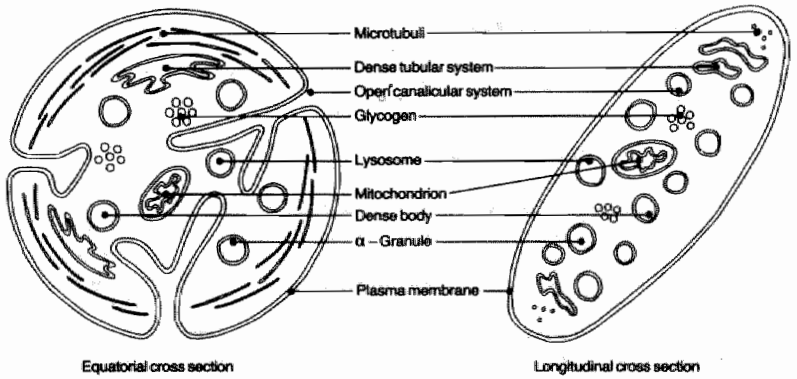

Fig.1: Morphology of the unstimulated discoid platelet.

In the cytosol several kind of storage compartments can be detected. Some of these have a secretory function (dense bodies, α-granules and lysosomes), while others (mitochondria) provide the platelet with the necessary metabolic energy, the source of which is formed by numerous glycogen granules. In the equatorial cross section a ring of microtubuli is visible, which upon activation of the platelets migrates to the center of the cell by means of depolymerization-polymerization cycles

penultimate stage of the cascade. These phenomena, which are strictly surface-related, are stimulatory in nature.- However, also involvement in negative feedback of coagulation is possible. For instance, inactivation of factor Xa by the plasma protein antithrombin III is prevented by binding of factor Xa to the platelet surface [26]. On the other hand, the inactivation of factor Va by activated protein C is enhanced by binding to the platelet surface [27]. Apart from these properties of platelets which are directly related to their surface, a wide variety of compounds involved in coagulation is released from the storage granules upon stimulation [19]. In this thesis, however, attention is focussed on the role of platelets in the catalysis of factor Xa and thrombin formation.

Role of lipid in Xa and thrombin formation.

Both factor Xa and thrombin are formed through limited proteolysis of their zymogens by a complex of an enzyme, a protein cofactor and the substrate assembling at a suitable membrane phospholipid surface. Factor IX, factor X and prothrombin are members of the family of proteins that undergo a posttranslational, vitamin K dependent carboxylation in the liver. Several glutamic acid residues near the N-terminus of these proteins are carboxylated to gamma-carboxyglutamic acid [28]. Binding of these proteins to a lipid surface occurs by formation of calcium bridges between the negatively charged gamma-carboxyglutamic acid residues of the proteins and negative charges present in the lipid surface. In the case of factor Xa generation, the enzyme factor IXa associates with the cofactor VIIIa at a phospholipid surface, forming so-called tenase complex. This complex efficiently brings about the limited proteolysis needed to form the active enzyme Xa from its zymogen, factor X. A similar complex exists for formation of thrombin from its precursor, prothrombin. In this case factor Xa is the enzyme and Va the cofactor, together forming the prothrombinase complex after binding to a lipid surface has taken place. Although the effect of platelets and lipids on the reaction of the prothombinase complex is mainly considered in this thesis, it should be kept in mind that the tenase complex is affected in a similar fashion.

The function of lipids in coagulation can in a first approximation be described as surface-catalysis, involving an increase in the local concentration of proteins by binding to a phospholipid surface and, hence, stepping up the reaction. Another reason for the increase in reaction velocity is the juxtaposing of the molecules, induced by their binding to a surface, which leads to a higher ratio of productive collisions between enzyme and substrate [29,30].

In vitro, this surface can be composed of any mixture

of phospholipids containing net negatively charged polar head groups, although the catalytic efficiency is certainly dependent on the kind of lipid molecule bearing such charge. Comparing the activities of lipids containing different polar headgroups, it was shown [31] that optimal catalytic efficiency can be achieved by the use of phosphatidylserine (PS). Although any lipid surface carrying a negative charge is capable of stimulating thrombin formation, binding affinity to PS-containing surfaces is favoured presumably because a six-coordinated complex with calcium can be formed, involving the phosphate, amino and carboxyl groups of PS on the one hand and the two carboxyl groups of gamma-carboxyglutamic acid plus another electron-donating group of the protein on the other. The resulting strong binding of clotting factors to a PS-containing phospholipid surface explains the potent catalytic effect of phosphatidylserine in blood coagulation, relative to other anionic phospholipids. It should be mentioned that neutral phospholipids can play a modulating role in the procoagulant efficiency of a lipid surface once PS is present [32].

Lipid organization of the platelet plasma membrane.

Platelets, like other cells, are protected from their environment by a plasma membrane in which asymmetry prevails with respect to both proteins and phospholipids. For erythrocytes as well as for platelets it was shown [33-37] that the outer monolayer of the plasma membrane consists primarily of the neutral choline containing lipids phosphatidylcholine (PC) and sphingomyelin (Sph) and part of the phosphatidylethanolamine (PE) which is present on both sides of the membrane. The negatively charged lipids phosphatidylserine (PS) and phosphatidylinositol (PI) are almost exclusively located in the inner leaflet of the membrane. This asymmetry means that the outer surface of the cell is almost completely devoid of negatively charged phospholipids, thus hardly being able to stimulate the enzymatic conversion of factor X and prothrombin.

We have shown that activation of platelets by the combined action of collagen and thrombin, as likely occurs at the site of vascular injury, gives rise to progressive randomization of phospholipids in the membrane bilayer [38]. Thus, the original asymmetric distribution of lipids is lost upon activation, leading to exposure of PS at the platelet outer surface. The activated platelet thus acquires the capability to increase the rate of factor Xa and thrombin formation, thereby also restricting the clotting process to the site of injury, where platelet activation occurs. The question arises which molecular mechanisms in platelets are responsible for the transformation of an inert cell into a body capable of accelerating coagulation by several orders of magnitude.

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CHAPTER 1

THE ENZYMATIC SYNTHESIS OF PHOSPHATIDYLSERINE AND PURIFICATION BY CM-CELLULOSE COLUMN CHROMATOGRAPHY.

Summary.

Phosphatidylserine has been prepared from phosphatidylcholine by a one-step transphosphatidylation reaction catalyzed by phospholipase D in the presence of L-serine. The resulting mixture of phosphatidylserine and phosphatidic acid is easily and rapidly separated by CM-cellulose column chromatography using stepwise elution with solvents containing an increasing percentage of methanol in chloroform. The overall yield of the procedure is 40-50% dependent on the scale of the preparation. CM-cellulose column chromatography proved to be extremely useful in separating phospholipid mixtures obtained by phosphatidyltransferase reactions of phospholipase D and is also suitable for fractionation of other lipid extracts.

Introduction.

As discussed in the introduction of this thesis, phosphatidylserine plays a crucial role in blood coagulation. Since the total chemical synthesis of this phospholipid is a complicated and time consuming task [1-5], its role in a variety of membrane processes has mainly been studied using preparations obtained by tissue extraction. This results in a mixture of phosphatidylserine species with respect to the fatty acid composition. In order to be able to prepare phosphatidylserine with defined fatty acids quikly and easily, we reevaluated its enzymatic synthesis using the base-exchange reaction catalyzed by phospholipase D.

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) hydrolyses the terminal phosphate diester bond of glycerophospholipids with production of phosphatidic acid. It has also been shown to catalyze the transfer of the phosphatidylgroup from phosphatidylcholine to various primary alcohols [6-10]. Until now, however, no significant transferase activity has been observed using L-serine as the acceptor molecule. Upon reinvestigation of the transferase activity of phospholipase D we found that conversion of phosphatidylcholine into phosphatidylserine can occur at elevated temperature using saturated solutions of serine. The resulting mixture of phosphatidylserine and phosphatidic acid is easily and rapidly separated by CM-cellulose column chromatography. This column is eluted step-wise with solvents containing increasing percentages of methanol in chloroform.

The overall yield of the procedure is 40-50%, depending on the scale of the preparation. CM-cellulose chromatography proved to be extremely useful in separating phospholipid mixtures obtained by phosphatidyltransferase reactions of phospholipase D and is also suitable for the fractionation of other lipid extracts.

Materials and methods.

Egg phosphatidylcholine was prepared from egg yolk as described by Singleton et al. [11]. Two synthetic phosphatidylcholines were prepared as described before [12]: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0-phosphatidylcholine) and 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (18:1$_{tr}$/18:1$_{tr}$-phosphatidylcholine).

Phospholipase D was partially purified from the inner, yellowish-white leaves of savoy cabbage by heat treatment and acetone precipitation according to the method of Davidson and Long [13]. After acetone treatment the precipitate was suspended in water (approximately 20 ml per 400 g of cabbage leaves) and immediately lyophilized. The white powder obtained was stored at -20° C.

For routine assays of enzymatic activity, hydrolysis of egg yolk lipoprotein was measured by continuous

titration of the acid produced, using a Radiometer automated titrator with curve recording. The egg yolk lipoprotein substrate was prepared by homogenizing the yolk of one egg in 100 ml destilled water. After centrifugation at 27.000 x g for 30 minutes the supernatant was collected and sodium dodecylsulphate was added to a final concentration of 6.66 mg/ml. The assay was performed using 15 ml of the lipoprotein solution (containing approximately 1 mg/ml lipid phosphorus) to which 1 ml of 1M $CaCl_2$ was added prior to the addition of enzyme. This results in a marked increase in turbidity. The enzymatic reaction was carried out at 37° C for 5 minutes at pH 5.6. The consumption of 0.02N NaOH was usually linear for at least 10 minutes. One unit of phospholipase D is defined as the amount of enzyme which liberates 1 µmol of titratable H^+ per minute at 37° C. In spite of the turbidity of the reaction mixture, the number of units appeared to be comparable with the number of international units (I.U.) found in the substrate-decrease assay as described previously [14]. The phospholipase D preparation from savoy cabbage usually showed an activity of approximately 1 I.U./mg dry weight. The activity decreased by some 20% per month upon storage at -20° C.

The transphosphatidylation reaction catalyzed by phospholipase D was carried out in the presence of L-serine either with egg phosphatidylcholine or with the synthetic lecithins 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine or 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine. The phosphatidylcholines were dissolved in diethylether (distilled from P_2O_5 to remove traces of alcohol) at a concentration of 20 mg/ml. L-serine (Merck) was first lyophilized from a 10% (w/v) aqueous solution to remove traces of methanol and subsequently dissolved at 45° C in different concentrations up to saturation (46% w/v) in 100 mM acetate buffer (pH 5.6) containing 100 mM $CaCl_2$. Phospholipase D was added to the serine solution at 45° C to a final concentration of 1 I.U./ml. An equal volume of the phosphatidylcholine solution in ether was added and the incubation flask was immediately closed, to minimize ether evaporation. Incubation was carrried out at 45° C with stirring to ensure complete mixing of both phases. Usually, additional portions of phospholipase D equal to the starting amount were added after 30 and 60 minutes. Incubation was stopped after 90 minutes by addition of two volumes (with respect to the volume of the acetatebuffer) 100 mM EDTA. Ether was evaporated at room temperature under a stream of nitrogen and the aqueous layer was mixed with 4.3 volumes of chloroform/methanol (5:8, v/v) and stirred for 30 minutes [15]. This single phase mixture was filtered through a glass filter G-2 to remove precipitated serine. The filtrate was stirred for 10 minutes after addition of 1 volume of water and 3.7 volumes of chloroform. In this extraction procedure "volumes" refer to the volume of acetate buffer plus EDTA solution. After centrifugation (10 min., 1000 x g) the lower chloroform

layer was collected and evaporated to dryness under reduced pressure. After removing traces of water by repeatedly adding absolute ethanol and evaporating, the residue was dissolved in chloroform. Similar incubations were carried out at 37° C in which serine was replaced by other primary alcohols. Ethanolamine, glycerol, methanol, or ethanol were used to establish optimal conditions leading to the highest yields of the respective phosphoglycerides.

Preswollen CM-cellulose (CM-52, sodium form, Whatman) was suspended in methanol and decanted several times to remove small fibers and effectively replace water by organic solvent. Columns were packed as described by Rouser et al. [16] for DEAE-cellulose. On top of the bed a glass wool plug and some glass beads were placed in order to prevent disturbance of the adsorbent during solvent changes. Prior to applying the lipids, methanol was removed by eluting the column with at least 10 bedvolumes of chloroform. A rapid separation of phosphatidylserine from phosphatidic acid, phosphatidylmethanol, unconverted phosphatidylcholine and traces of free serine was carried out as follows.

The lipid solution in chloroform was applied to the column in a concentration up to 50 mg/ml with a maximum load of 5 mg total lipid per ml bedvolume. After elution with 3 bedvolumes of chloroform, phosphatidic acid (together with traces phosphatidylmethanol and residual phosphatidylcholine) was eluted with 20 bedvolumes of 20% methanol in chloroform (v/v). Phosphatidylserine was subsequently eluted with 6 bedvolumes of chloroform/methanol (1:1, v/v) and any free serine was removed by elution with 10 bedvolumes pure methanol, resulting in regeneration of the column. The column can be used repeatedly with reproducible performance. Since the cellulose can easily be operated at flowrates of 250 ml/hour without affecting the separation, the procedure is extremely rapid and can usually be performed within 2 days. Stopping elution overnight during the separation procedure has no influence on the final resolution. No differences in separation were observed when the procedure was scaled up to separate 1 gram of the phospholipid mixture.

For analytical purposes, the phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse [17], and quantitated as phosphorus by a modification of the procedure of Fiske and Subbarow [18]. Differential scanning calorimetry was carried out as described by van Dijck et al. [19].

Results and discussion.

In agreement with previous investigations [8,9], 90-95% of phosphatidylcholine can be converted into phosphatidylmethanol, phosphatidylethanol, phosphatidylethanolamine and phosphatidylglycerol at optimal concen-

trations of the acceptor molecule. The remaining 5-10% is converted into phosphatidic acid (Fig.1).

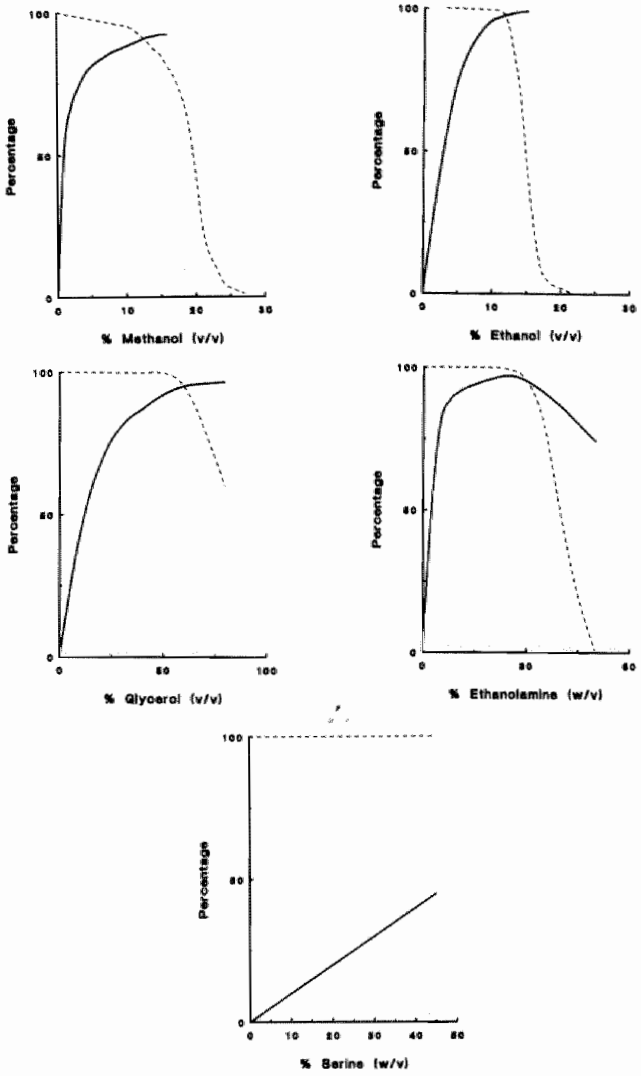

Fig.1: Influence of the acceptor concentration on the conversion of phosphatidylcholine into the respective phosphoglycerides.
_____ , percentage of transphosphatidylation.
_ _ _ _ , percentage conversion of lecithin under the conditions employed. Optimal conditions are reached where both lines are maximal. The reaction in the presence of serine was carried out at 45° C, whereas the reactions using other acceptors were carried out at 37° C.

Higher concentrations of the acceptor lead to (partial) inactivation of phospholipase D and result in an incomplete conversion of phosphatidylcholine under the conditions employed. On the other hand, the percentage conversion of phosphatidylcholine into phosphatidylserine shows a linear increase with the serine concentration up to the saturation point (46%, w/v at 45° C), where some 45% of the phosphatidylcholine is converted into phosphatidylserine, the remainder being hydrolysed to phosphatidic acid. Raising the pH to 8.5 increases the solubility of serine to 55% (w/v). Incubating at this pH with a 10 times higher concentration of phospholipase D results in a conversion of about 55% of phosphatidylcholine into phosphatidylserine. However, due to the large amount of phospholipase D required to produce complete conversion of phosphatidylcholine at pH 8.5, this pH is of no practical value in relatively large scale preparations of phosphatidylserine. No significant change in percentage conversion into phosphatidylserine was found with the three phosphatidylcholine preparations used. Moreover, no differences in fatty acid composition were observed between the phosphatidylcholines and the phosphatidylserines produced after transphosphatidylation. Also the ratio of phosphatidylserine to phosphatidic acid was not found to be dependent on the incubation time.

Separation of phosphatidylserine from the resulting mixture after transphosphatidylation could easily and rapidly be achieved by CM-cellulose column chromatography, using chloroform/methanol mixtures as eluent. Other adsorbents, such as silica gel [20] or DEAE-cellulose [16] were found to be unsatisfactory in this respect. The recovery of phosphatidylserine from the column appeared to be 95-100% and the lipid was pure as judged by two-dimensional thin-layer chromatography. Differential scanning calorimetry of the two phosphatidylserines prepared from *dimyristoyl-lecithin* and *dielaidoyl-lecithin*, was carried out on samples hydrated in a buffer of pH 7.5, containing 40 mM Tris/acetate and 100 mM NaCl. The heating scans revealed sharp phase transitions at 35° C for dimyristoylphosphatidylserine and at 22.5° C for dielaidoylphosphatidylserine, confirming the notion that the phosphatidylserines are essentially pure. It should be mentioned that other investigators [21] have shown that both temperature and enthalpy of the phase transition of synthetic phosphatidylserines are dependent on pH and ionic strength, making a comparison with other phospholipids premature at the present state of knowledge.

By using a stepwise elution with solvents containing increasing percentages of methanol in chloroform, separation of other phospholipid mixtures can be succesfully achieved. The percentage of methanol at which certain lipids start to elute from the column is given in Table I. Small differences in the methanol concentration at which elution starts, still permits substantial separation.

TABLE I

METHANOL CONCENTRATION IN CHLOROFORM REQUIRED TO ELUTE PHOSPHOLIPID FROM CM-CELLULOSE (SODIUM-FORM)

Vol% methanol	Lipids eluted
0	Free fatty acid, cholesterol, di- and tri-acylglycerol
3	Diphosphatidylglycerol
4	Phosphatidylcholine
5	Sphingomyelin, phosphatidyl(m)ethanol, monoglucosyldiacylglycerol, TNBS-PE
9	Phosphatidylethanolamine
12.5	Phosphatidic acid
15	Diglucosyldiacylglycerol
20	Phosphatidylglycerol
23	Lysophosphatidylcholine
30-35	Phosphatidylserine
35-50	Phosphatidylinositol
100 followed by 0	Regeneration

For example, mixtures of equal amounts of phosphatidylcholine and sphingomyelin can be separated to such an extent that approximately 80% of each phospholipid is obtained in pure form. Some sphingomyelin starts to leave the column after prolonged elution with 4% of methanol, where the tail of the phosphatidylcholine fraction elutes. As soon as this occurs, elution is continued with 5% of methanol, first resulting in a mixture of sphingomyelin and the remaining phosphatidylcholine, followed by the appearance of pure sphingomyelin. Although this separation is quantitatively inferior to separation on silicic acid, the procedure is considerably more rapid. Complete separation between two phospholipid classes can easily be obtained when the interval of methanol concentration exceeds 2 vol%, making the method extremely useful for phospholipid mixtures obtained from phospholipase D catalyzed base-exchange reactions. In general, a smaller elution volume is required when lipids are eluted at a higher methanol concentration than strictly necessary.

The basis for lipid separation on CM-cellulose is not completely understood. Separations by ion-exchange chromatography generally proceed through ion-exchange reactions and hydrogen bond equilibria. It is likely that in chloroform, which has a very low dielectric constant, repulsion between negative charges is minimal since little or no dissociation of ion-pairs occurs. This would allow unspecific adsorption, possibly also hydrogen-bonding of phospholipids to CM-cellulose. Increasing the dielectric constant by addition of methanol will also increase the

repulsion of gradually dissociating negative charges, resulting in elution of the lipids when repulsion exceeds counteracting adsorption.

Finally, it is concluded that conversion of phosphatidylcholine into phosphatidylserine by phospholipase D catalyzed transphosphatidylation followed by rapid purification on CM-cellulose columns allows the preparation of a wide variety of phosphatidylserines from synthetic phosphatidylcholine analogs. This enables a systematical investigation on the role of phosphatidylserine in biomembranes and lipoproteins.

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APPENDIX
TO CHAPTER 1

ENZYMATIC SYNTHESIS OF PHOSPHATIDYLSERINE BY USE OF A ONE-PHASE SYSTEM.

Summary.

A modification of the phospholipase D catalyzed synthesis of phosphatidylserine is described, which allows the handling of small quantities of lipid without the need for an ether-water system.

By using octylglucoside to disperse the lipid during the enzymatic conversion it was possible to reduce the volume of the reaction mixture to 50 -100 µl. The amount of lipid that can be handled in such small volumes is in the order of micrograms. This facilitates the synthesis of phosphatidylserine from rare or expensive phosphatidylcholine species.

Introduction.

In 1977 we described [1] the enzymatic conversion of phosphatidylcholine (PC) into phosphatidylserine (PS). The reaction, a base-exchange catalyzed by phospholipase D (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4), is carried out at 45°C in a two phase system composed of a solution of PC in diethylether and a saturated solution of serine in acetatebuffer. The purpose of developing this procedure was to prepare PS species with a defined fatty acid composition, starting from commercially available PC species and avoiding the laborious total chemical synthesis of PS.

Since then, increasing interest has arisen in PS molecules containing groups whose fate can be easily assessed by various physical and chemical methods. For instance, in research aimed at elucidating mechanisms underlying transbilayer movement and the maintenance of phospholipid asymmetry in biological membranes, various labeled PS species are used. Some recent examples are the incorporation of radiolabeled PS in erythrocytes, followed by enzymatic degradation to determine the fraction which remains in the outer monolayer upon prolonged incubation [2]. A similar technique employs a fluorescent analog of PS, where the fraction of label still present in the outer monolayer is assessed by exchange with phospholipid vesicles [3]. Phosphatidylserine containing a spin-label was used in several cells to evaluate their capacity to transport exogenously added aminophospholipids to the inner monolayer of the cells by an energy-dependent translocating system [4-6]. Radio-iodinated, photoactivatable lipid was used in an attempt to identify erythrocyte membrane proteins involved in such a phospholipid transport over the plasma membrane [7,8].

These labeled lipids are in general available only in small amounts and are rather expensive. To prepare PS from the respective phosphatidylcholines by the use of a two-phase system consisting of water and ether layers, incubated at a temperature well above the boiling point of ether, limits the minimal volume that can be handled conveniently. When small amounts of PC are used, the overall yield of the procedure will be lower, mainly because of adsorption problems.

We found that ether could be replaced by detergent as a solute for the lipids, which makes it possible to perform the synthesis in a one-phase system at microscale (volumes less than 1 ml). In such a system the lipids are presented to the phospholipase in the form of a mixed micel of lipid and detergent. Using a proper detergent this micellar structure is such that the phospholipase D can modify the PC molecules at a rate comparable to that found in an etherwater system.

Materials and Methods.

Phosphatidylcholine (from egg yolk), sodiumdodecylsulfate (SDS), sodiumdeoxycholate (DOC), Triton X-100 and octylglucopyranoside were obtained from Sigma. Phospholipase D was partially purified from the inner leaves of Savoy cabbage as described [1]. All other reagents were of the highest grade available.

Incubations were carried out by first drying PC in a glass tube by a stream of nitrogen. The PC was three times solubilized in pure chloroform and dried again, to remove traces of methanol or ethanol which could give rise to unwanted side-products like methyl- or ethyl-phosphatidic acid [1]. To the dry PC a solution is added, containing calcium, acetate, detergent and serine. This solution is prepared at 55°C by mixing 1M $CaCl_2$, 1M Na-acetate, detergent, L-serine and water. Final concentrations are 0.1M Ca^{2+}, 0.1M acetate, 50% w/v serine and 2% w/v detergent. The resulting pH is between 5.4 and 5.8. The reaction is started by adding phospholipase D to the mixture in a ratio of 1 International Unit (I.U.) per 10 mg of lipid (the I.U. is defined as the amount of enzyme which converts 1 µmol of substrate per minute at 37°C). Extraction, purification and analysis by two-dimensional thin-layer chromatography of the resulting lipid mixtures were performed as described before [1]. When PS species are synthesized which are relatively water-soluble, it is preferable to use an one-phase extraction procedure as described by Reed et al. [9] to avoid loss of material by partitioning of lipid over two phases. Briefly, after complexing calcium by EDTA, to the resulting solution five volumes of methanol are added, followed by five volumes of chloroform. After stirring for ten minutes the mixture is centrifuged to remove insoluble material. The supernatant is evaporated and dried by repeated evaporation from absolute ethanol. Finally, the lipid is extracted from the dried material with pure chloroform and purified by CM-cellulose chromatography.

Results and Discussion.

We compared the detergents sodiumdodecylsulfate, sodiumdeoxycholate, Triton X-100 and octylglucoside in their capacity to replace diethylether as a solute for PC in the enzymatic synthesis of PS.

TABLE I

Conversion of PC to PS in the presence of detergents.

Detergent	Inc. time (hours)	PC degradation (%)	PS formation (%)
TX-100	3	-	-
SDS	3	50	25
DOC	1	45	15
	2	87	29
	3	94	21
	4	96	25
Octylglucoside	1	67	25
	2	93	41
	3	95	46
	4	96	45

In all cases a detergent concentration of 2% (w/v) is used. Incubations were carried out for the time indicated in the table. Degradation is expressed as the fraction of lecithin converted. The amount of PS formed is expressed as fraction of the amount of lecithin present at zero time.

Of these detergents, octylglucoside proved to be the most effective, both with respect to the percentage of PC transformed into PS in the reaction and to the reaction velocity (Table I). We found that when 10 mg/ml of PC is used, at least 1% w/v of octylglucoside is needed to allow complete conversion of PC under the conditions employed (Table II). This is in line with the very high critical micellar concentration of octylglucoside, which implies a monomeric concentration of several mg/ml, leaving very little detergent to allow formation of mixed micels of detergent and lipid when less than 10 mg/ml octylglucoside is present. Based on the sudden increase in light scattering which is observed when increasing amounts of PC are dispersed in a solution of octylglucoside, up to 25 mg/ml of PC can be solubilized in the form of mixed micels when 2% w/v octylglucoside is used (data not

shown), before bilayer structures are formed in which PC cannot efficiently be converted by phospholipase D.

TABLE II

Effect of octylglucoside concentration on the efficiency of PC conversion.

Octylglucoside (% w/v)	PC degradation (%)	PS formation (%)
0.5	56	17
1	95	41
2	95	38
5	95	39

Incubations were carried out for three hours. Octylglucoside concentrations are expressed as weight per volume. Degradation and PS formation are expressed in the same way as stated in the legend to Table I.

In conclusion, a method is described for the enzymatic synthesis of PS in a one-phase system, using octylglucoside to diperse the lipids. One advantage of this system is that small quantities of expensive and rare species of PC can be handled in small volumes, thereby reducing losses from adsorption during the procedure. Also it is likely that more polar PC species with a lower solubility in ether, like short chain lecithins or species containing bulky polar groups, will be more efficiently converted to PS using this method compared to the two-phase system.

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CHAPTER 2

ON THE RELATION BETWEEN MEMBRANE ASYMMETRY AND BLOOD COAGULATION.

Summary.

Various mixtures of phospholipids were tested on their ability to shorten the clotting time of plasma in a Stypven assay. Also, different types of sealed or non-sealed erythrocyte membranes were used. It is shown that only lipid mixtures containing PS, and membrane preparations in which the cytoplasmic side is exposed (i.e. lysed cells or insideout sealed vesicles) are able to shorten the clotting time. A physiological relation between membrane phospholipid asymmetry and the regulation of haemostasis is proposed.

Introduction.

Although there is compelling evidence that membrane components are non-randomly distributed between the two surfaces, the physiological significance of membrane asymmetry has so far remained obscure. Since the exterior surface of blood cell membranes is presumably devoid of negatively charged phospholipids which have a regulatory role in blood clotting, a possible function of lipid asymmetry in the coagulation process can be anticipated. We describe here experiments devised to show that the asymmetric distribution of membrane phospholipids in blood cells may serve a biological purpose, by contributing to maintain the delicate balance between regulating hemostasis and avoiding thrombosis.

The main function of phospholipids in blood coagulation is to provide a catalytic surface on which various coagulation factors interact, thus increasing their local concentrations. For example, the final lipid-activated step in the coagulation process is the conversion of prothrombin in thrombin by the prothrombinase complex, which requires at least four components in addition to prothrombin, the proteins factor Xa and Va, calcium-ions and phospholipids [1]. Of these components, only factor Xa is able to convert prothrombin into thrombin by limited proteolysis [1-5], but its individual action is considerably slower than with the complete prothrombinase complex [6].

In situ, the platelet plasma membrane presumably provides the phospholipid-water interface (platelet factor 3), which is required to accelerate the coagulation process [7]. It has been shown [8-10] that the clot-promoting activity of phospholipids in vitro is not attributable to a certain phospholipid class, but to a specific negative charge of the phospholipid surface. In particular, negatively charged phospholipids such as phosphatidylserine and phosphatidylglycerol (when properly diluted with neutral phospholipids like lecithin or phosphatidylethanolamine) exhibit maximal activation of the coagulation process [11,12].

It is generally accepted that membranes are highly asymmetric structures both with respect to their proteins and their lipids [13-16]. It was shown that the non-random distribution of phospholipids between the interior and exterior half of the platelet plasma membrane is similar to that of the erythrocyte membrane [17]. In both membranes, phosphatidylserine is apparently nearly exclusively located in the cytoplasmic surface, whereas the outer monolayer of both membranes consists of neutral phospholipids, particularly sphingomyelin. The other two major phospholipid classes, phosphatidylcholine and phosphatidylethanolamine are present on both membrane sides though not to the same extent. We report here on the possible implications of blood cell membrane asymmetry for the coagulation process. Since methods are readily available

to prepare different types of sealed and non-sealed erythrocyte ghosts, this type of membrane has been used in the present study rather than the platelet surface membrane which is more difficult to manipulate.

Methods.

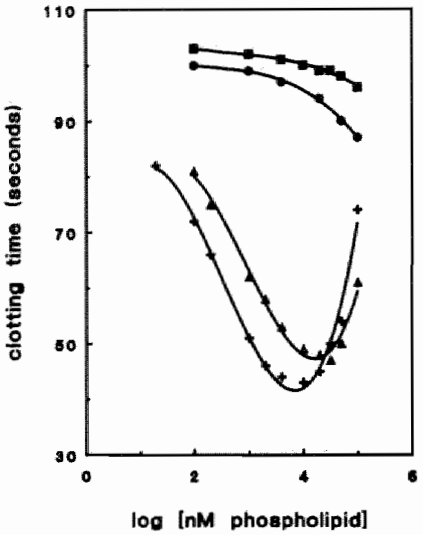
Blood from healthy volunteers was collected in 0.13 mol/liter sodium citrate (9 vol blood + 1 vol citrate). Red cells, non-sealed ghosts and resealed right-side out ghosts were prepared as described before [18]. Inside-out sealed ghost vesicles were prepared according to Steck [19]. Platelet poor plasma was obtained by centrifugation of the blood for 10 minutes at 5000g. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolk [20]. Sphingomyelin was isolated from bovine brain [21]. Phosphatidylserine was isolated from pig brain [22]. Liposomes were prepared by mixing together the proper amounts of lipids as solutions in chloroform. After evaporation of the organic solvent by a stream of nitrogen, a solution of NaCl (0.9% w/v) was added. By vortexing for 5 minutes in the presence of two glass beads, liposomes were formed with a final lipid concentration of 4 mmol/liter. The coagulant activity of these preparations was determined by their ability to shorten the clotting time on recalcification of 'normal' human platelet poor plasma in the presence of Russell's viper venom.

Coagulation assays were performed as follows: 0.1 ml of platelet poor plasma was incubated at 37°C for 30 seconds with 12 ng of Russell's viper venom (RVV). Upon recalcification this amount gave a blank coagulation time of 100 seconds. After incubation with RVV 0.1 ml of various dilutions of the different lipid or cell preparations were added, followed by 0.1 ml $CaCl_2$ (25 mmol/liter). A stopwatch was started simultaneously with the addition of $CaCl_2$. The time required for the formation of a firm fibrin clot was determined by the arrest of a rotating metal wire (2 mm length, cut from a regular paper clip), driven by a magnetic stirrer. This method gave the same clotting times as observed using a traditional Kolle hook, but the standard deviations are smaller and usually below 1%. For the presentation of coagulant activity of membranes and lipids we report directly the clotting times, rather than attempt to convert these to some arbitrary units of activity. It is realized that such clotting times are a complex function of lipid activity, but no real advantage is gained by using conventional log-log calibration curves obtained by serial dilution of plasma. In view of the wide range of final phospholipid concentrations used, the values on the abscissa are plotted logarithmically.

Results and discussion.

When only the outside of the erythrocyte membrane is exposed (either intact cells or resealed right-side out ghosts) no significant reduction of the blank clotting time is observed over a wide range of lipid concentrations (fig.1). On the other hand, when either the membrane interior (inside-out vesicles) or both membrane sides (non-sealed ghosts) are exposed, a two- to threefold reduction of the coagulation time is observed at optimal concentrations.

Fig. 1 Effect of in- and outside of erythrocyte membranes on clotting time of plasma.
PL: phospholipid added as red cells or ghosts.
■, *Erythrocytes*
●, *resealed ghosts*
✚, *inside-out vesicles*
▲, *non-sealed ghosts*



At higher lipid concentrations clotting time increases. This is consistent with the view that excess of lipid will result in a dilution of coagulation factors at the lipid surface [23]. The results suggest that only the phospholipids at the membrane interior have procoagulant activity, also since inside-out ghost vesicles are slightly more active than non-sealed ghosts.

This hypothesis was further investigated by testing liposomes with the same phospholipid composition as present in the outer half and inner half of human red cell membranes and pig platelet plasma membranes (Table 1, data calculated from refs 17 and 24). Handshaken liposomes were used rather than sonicated vesicles, to avoid artificially induced phospholipid asymmetry which is known to occur in mixed vesicles as a result of charge differences and packing properties [25]. It should be noted, however, that using multilamellar liposomes only 10-15% of the lipids

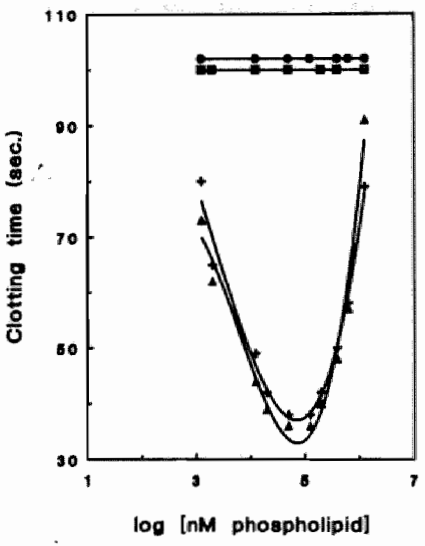
TABLE I

Mole ratios of phospholipids in liposomes simulating outer and inner half of erythrocyte and platelet plasma membrane

	Red cell membrane		Platelet plasmamembrane	
	Outer half	Inner half	Outer half	Inner half
PC	44	15	26	34
Sph	44	10	52	4
PE	12	47	20	28
PS	0	28	2	34

are available at the outer surface [26]. As shown in fig. 2, liposomes having the same composition as present in the outer membrane half of either red cell membranes or platelet plasma membranes do not reduce whatever the blank clotting time.

Fig. 2 Effect of liposomes on the clotting time of plasma. Liposomes simulate inner and outer half of red cell and platelet plasma membranes. PL: phospholipid added as liposomes.
■, *Platelet outer lipid*
✚, *Platelet inner lipid*
●, *Red cell outer lipid*
▲, *Red cell inner lipid*



On the other hand, liposomes with the same phospholipid composition as the inner half of red cell or platelet plasma membranes produce a threefold reduction of the coagulation time at optimal concentrations. The amount of lipid required is higher than with ghosts, since only a fraction of the total lipid is available at the surface of

handshaken liposomes.

It is realized that the measurement of procoagulant activity of the lipids is not performed at one particular transitory stage of the blood clotting process, in spite of the presence of Russell's viper venom which is known to activate both factor X and factor V [27]. Nevertheless, the results strongly suggest that the outer surface of blood cells is devoid of phospholipids which are active in blood coagulation, mainly due to the absence of any significant amount of phosphatidylserine. This phospholipid comprises about 30% of the phospholipids at the membrane interior, representing a concentration which has been shown to be optimal in activating prothrombin by the prothrombinase complex [11].

The presence of procoagulant phospholipids only at cytoplasmic surfaces of blood cells is not only consistent with phospholipid asymmetry in membranes, but might also represent an important mechanism both in avoiding thrombosis and in regulating hemostasis. It could be judged as potentially dangerous to have procoagulant phospholipids at the outer surface of blood cells, which may bring about a 'permanent' condition of hypercoagulability. This fact itself could provide a physiological reason for an asymmetric phospholipid distribution in blood cell membranes. On the other hand, after disruption of the vessel wall the sequence of platelet adhesion, release reaction and ADP-induced platelet aggregation, presumably accounts for the primary arrest of bleeding. Provided that platelet factor 3 activity becomes available during this process, its exposure should come after the release reaction, since no phosphatidylserine can be labelled from the outside during the release reaction [28]. Moreover, it has been demonstrated that only a small fraction of lipid procoagulant activity becomes available during release reaction as compared with the activity obtained upon lysis [29,30].

This raises the question whether during or after formation of the primary hemostatic platelet plug, either (partial) lysis or an as yet unknown mechanism which translocates phosphatidylserine through the plasma membrane is necessary to provide a catalytic surface for interacting coagulation factors.

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CHAPTER 3

THE NATURE OF THE BINDING SITE FOR PROTHROMBINASE AT THE PLATELET SURFACE AS REVEALED BY LIPOLYTIC ENZYMES.

Summary.

The nature of the receptor for the prothrombinase complex at the surface of non-activated platelets was investigated by measuring the platelet prothrombin converting activity with a chromogenic substrate assay, after treatment of the platelets with various phospholipases or three different proteolytic enzymes. Platelet prothrombin converting activity decreased only after treatment with those phospholipases which are able to hydrolyse phospholipids in the intact platelet and also have the capability to degrade negatively charged phospholipids, phosphatidylserine and phosphatidylinositol. Those phospholipases which do hydrolyse phospholipids in the intact platelet but have no activity towards phosphatidylserine (and phosphatidylinositol) produce an increase in the platelet prothrombin converting activity.

Proteolytic treatment of platelets with trypsin, chymotrypsin or papain did not result in a decrease of prothrombin converting activity. It is concluded that negatively charged phosphatidylserine and possibly phosphatidylinositol are involved in the prothrombin converting activity of non-activated platelets. Any involvement of platelet membrane proteins in a receptor for the components of the prothrombinase complex could not be demonstrated.

Introduction.

The conversion of prothrombin into thrombin by factor Xa in the presence of factor Va and Ca^{2+} is known to occur efficiently on negatively charged phospholipid surfaces [1]. Mixtures of phosphatidylserine and phosphatidylcholine have been shown to produce a drop in the apparent K_m for prothrombin from far above to far below the physiological concentration of 2 μM [2]. In platelets, the negatively charged phospholipids, i.e. phosphatidylserine and phosphatidylinositol, are predominantly confined to the inner leaflet of the plasma membrane and to intracellular membranes [3-5]. These lipids under certain conditions become available on the exterior surface after platelet activation, which can explain the higher procoagulant activity of activated cells [6-8].

It was suggested by Miletich et al. [9-11] and Dahlbäck and Stenflo [12] that factor Va serves as the receptor for factor Xa at the platelet surface. Approximately 200-300 binding sites for factor Xa were found at the surface of thrombin-activated platelets that have undergone the release reaction. Moreover, it was demonstrated that the binding sites for the factor Va/factor Xa complex are already present at the outer surface of unstimulated platelets [13]. Activation of platelets by thrombin appears to be required only to release factor Va from the platelets which then serves as the receptor for factor Xa. The nature of the factor Va/factor Xa binding site is at present unknown, but the finding of a patient deficient in these sites has led to the suggestion that some protein component is probably required [14].

In view of the fact that it cannot be excluded that minor amounts of phosphatidylserine are present at the membrane exterior [4-6] and participate in prothrombin conversion, the influence of different phospholipases on the prothrombinactivation sites is of interest. Phospholipases A_2 have been shown by Verheij et al. [15] to exhibit anticoagulant properties in clotting assays that are sensitive to negatively charged phospholipids. This is due to hydrolysis of phospholipids which can only occur with those phospholipases having an appreciable penetration power in highly packed monomolecular lipid films. Moreover, only phospholipases with high penetration power have been shown to be able to attack phospholipids in intact red cells [16,17] and platelets [4].

In this study, we have compared the effect of a number of highly purified phospholipases on the platelet prothrombin converting activity (in the presence of factor Va), using a chromogenic substrate assay avoiding platelet activation by the thrombin formed. Furthermore, the influence of proteolytic treatment of platelets on their prothrombin-converting activity has been tested. The data have implications for the involvement of small amounts of phosphatidylserine in the prothrombin-activation sites at

the outer surface of nonstimulated platelets.

MATERIALS AND METHODS.

Phospholipase preparations.

The following phospholipases were purified according to Zwaal et al. [16]. Phospholipase A_2 from Naja naja venom (Koch Light), phospholipase A_2 from bee venom (Apis mellifica) (Koch Light), phospholipase A_2 from Crotalus adamanteus (Koch Light), phospholipase C from Bacillus cereus, phospholipase C from Clostridium welchii and sphingomyelinase C from Staphylococcus aureus. All phospholipases were diluted to a final concentration of 0.5 I.U./ml in 50 % (v/v) glycerol. The international unit (I.U.) is defined as the as amount of enzyme which degrades 1 µmol of substrate per minute at 37° C.

Phospholipids.

Brain phosphatidylserine and egg phosphatidylcholine were purchased from Sigma. Phosphatidylethanolamine was synthesized from phosphatidylcholine as described before [18]. Sphingomyelin was from Koch Light. All lipids used were chromatographically pure.

Protein preparations.

Bovine prothrombin was prepared according to the method of Owen et al. [19]. Prothrombin concentrations were calculated from the absorbance at 280 nm using an extinction coefficient of 15.5 for a 10 mg/ml solution in a 1 cm cuvet, and 72 kD for the molecular weight of prothrombin [19]. Bovine factor Xa was prepared from factor X_2 by RVV-X as described by Fujikawa et al. [20]. Factor Xa concentrations were calculated after titration of active sites according to Smith [21]. Factor V was isolated as described by Esmon [22] and modified by Lindhout [23]. Factor V (0.3 mg/ml) was activated with RVV-V (1 µg/100 µg factor V) and the specific activity of the activated preparation was 500 units/mg, measured according to Kappeler [24]. One unit is defined as the amount of factor V present in 1 ml normal bovine plasma. The concentration of factor Va was calculated assuming a molecular weight of 330 kD for factor V [25].

Isolation of platelets.

Fresh blood was drawn from healthy male volunteers, who had not taken any medication for at least a week. As anticoagulant acid-citrate-dextrose (0.18 M glucose, 0.08 M trisodium citrate, 0.052 M citric acid) was used in a ratio of one volume for every five volumes of blood. Platelet rich plasma was obtained after centrifugation at 120g for 10 minutes. Platelets were isolated by centrifugation (1400 g, 15 min.) and the pellet was gently resuspended in a calciumfree Hepes buffer pH 6.2 containing 137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM $MgCl_2$, 25 mM glucose and 0.05% fatty-acid free human serum albumin. Cells were washed twice and finally resuspended in the same buffer at pH 7.4. Platelet concentration was deter-

mined with a Coulter Counter and adjusted to a count of 5×10^5 $.ml^{-1}$. All platelet handling was carried out at room temperature.

Measurement of thrombin formation after phospholipase treatment of platelets and lipid vesicles.

Platelet suspensions of 2 ml were incubated in plastic cuvettes at 37° C under stirring in a spectrophotometer.

Prior to the addition of 40 µl phopholipase (final concentration 0.01 I.U./ml) 60 µl 0.1 M $CaCl_2$ was added. At different time intervals during the phospholipase treatment, 35 µl of a mixture containing 30 nM factor Xa and 60 nM factor Va were added to the cuvette. 2 minutes after the addition of the factors Xa and Va, 100 µl chromogenic substrate (S-2238, 5 mM) was added. Thrombin formation was started by adding 40 µl 44 µM prothrombin to the cuvette. The absorbance change was registered as a function of time. Because all phospholipases were dissolved in 50% (v/v) glycerol, measurements of thrombin formation using control platelets, were performed after addition of 40 µl 50% glycerol in stead of the phospholipase solution.

Prothrombin converting activity of pure phospholipid vesicles and the effect of various phospholipase treatments on this activity was determined under the same conditions as used for platelets. Phospholipid vesicles (1 µM phospholipid) were prepared by sonication [26]. They were composed of 26% phosphatidylcholine, 20% phosphatidylethanolamine, 2% phosphatidylserine and 52% sphingomyelin. This composition is comparable to the phospholipid composition of the outer leaflet of the platelet plasme membrane [6].

Results.

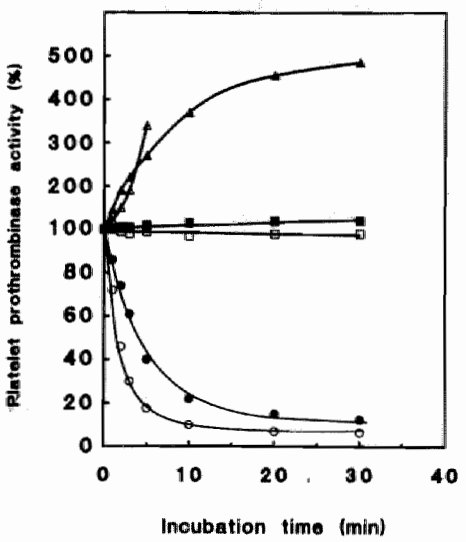
Addition of factor Xa, factor Va and prothrombin to intact platelets in the presence of Ca^{2+}-ions results in the formation of thrombin which in turn can activate platelets to aggregate and release their granule contents. It will be clear that platelet activation by thrombin thus formed, should be avoided. Addition of the chromogenic substrate (specific for thrombin) S-2238 will cause competition between this substrate and platelets for thrombin. Indeed we found that addition of S-2238 to washed human platelets effectively inhibits aggregation and release induced by thrombin (data not shown). The inhibitory effect of S-2238 towards factor Xa is of little importance here, since in all experiments the starting ratio of factor Xa to S-2238 is the same. Moreover, less then 10% of the chromogenic substrate is converted during measurements of prothrombin-converting activity.

The advantage of this competitive inhibitor is that the amount of thrombin formed by the prothrombinase complex can still be measured. In the incubation system described here, the complete prothrombinase complex is

present, together with the substrate used to determine the amount of thrombin formed. In this system the change in absorbance at 405 nm is a linear function of the square of time, because both the formation of thrombin by the prothrombinase complex and the conversion of S-2238 are linear with time. The rate of thrombin formation in these experiments was therefore calculated from a plot of absorbance versus the square of time, and a calibration curve with known amounts of active site titrated thrombin.

The effect of prolonged incubation of six different phospholipases on the prothrombin converting activity of washed human platelets is shown in Fig. 1.

Fig. 1 Time course of platelet prothrombin converting activity during incubation with various phospholipases.
○, PlA_2 from N.Naja
●, from bee venom
□, from C.adamanteus
■, PlC from B.cereus
△, from C.welchii
▲, Sph'ase from S.aureus
Experimental conditions are given in Materials and Methods.
Note the change in dimension on the ordinate of the graph above 100%.



Based on the effect they exert on the prothrombin-converting activity of non-stimulated platelets, these phospholipases can be divided into three groups. There are those phospholipases that lower the platelet procoagulant activity (phospholipase A_2 from bee venom and the same enzyme from Naja naja snake venom). There are phospholipases which do not affect the platelet prothrombin converting activity (phospholipase C from Bacillus cereus and phospholipase A_2 from Crotalus adamanteus). Finally there is the group of phospholipases able to increase the prothrombin-converting activity (sphingomyelinase from Staphylococcus aureus and phospholipase C from Clostridium welchii. Extensive lysis of the platelets occurs upon

treatment with phospholipase C from C. welchii, which is the reason why in this case an incubation period of less then 5 minutes was taken.

In order to obtain further indications concerning the significance of phospholipids in the prothrombin converting activity of non-stimulated platelets, the effect of the above mentioned phospholipases on the prothrombin converting activity of vesicles composed of phospholipids present in the outer monolayer of the platelet plasma membrane was studied. These vesicles which mimick the platelet outer surface as far as phospholipids are concerned, were treated with phospholipases for 30 minutes and the resulting prothrombin converting activity is compared with the activity of platelets after 30 minutes incubation with these phospholipases. The data are given in Table 1. With the exception of phospholipase A_2 from C. adamanteus and phospholipase C from B. cereus, the changes in prothrombin-converting activity of the vesicles produced by the action of the different phospholipases are very similar to the effects observed on intact platelets.

TABLE I

The effect of different phospholipaseson the prothrombin converting activity of platelets and phospholipid vesicles which mimic the outer surface of the platelet membrane. *Results are given for prothrombin converting activity after 30 min phospholipase treatment.*

Phospholipase	Source	Prothrombin converting activity in	
		platelets	vesicles
		%	%
None	-	100	100
Phospholipase A_2	C.adamanteus	96	5
	N.naja	8	3
	bee venom	13	2
Phospholipase C	B.cereus	103	8
	C.welchii	> 300*	250
Sphingomuelinase	S.aureus	380	1000

* Prothrombin converting activity after 5 min phospholipase treatment.

It should be emphasized here that the phospholipid composition of the vesicles may differ from that of the outer monolayer of the platelet, because it is not feasible to determine the amount of phosphatidylserine in the outer leaflet within a range of accuracy of only a few percent [4,5]. Therefore the absolute activities of both preparations cannot be compared. For this reason the

data in Table 1 are expressed as percentages.

To see whether platelet-membrane proteins are also involved in the formation of the prothrombinase complex at the platelet surface, we also compared the prothrombin converting activity of platelets treated with Sepharose-coupled trypsin, chymotrypsin and papain. None of these proteolytic enzymes gave a reduction of the prothrombin converting activity. Although this does not exclude that a membrane protein receptor plays a role in the formation of the prothrombinase complex at the platelet surface, the involvement of such a protein seems to be unlikely unless it is stable to, or protected against, proteolytic attack by these enzymes.

Discussion.

Four out of six phospholipases which were used in this study are able to hydrolyse phospholipids in intact platelets, namely both phospholipases A_2 from Naja naja and bee venom, phospholipase C from Clostridium welchii and sphingomyelinase. The other two phospholipases used, phospholipase A_2 from Crotalus adamanteus and phospholipase C from Bacillus cereus cannot attack phospholipids in intact platelets. Why certain phospholipases are able to hydrolyse phospholipids in the intact cells whereas others are not, was explained by Chap et al. [4] in analogy to the work on the erythrocyte [16,17]. Only those phospholipases which are able to attack monolayers of choline-containing phospholipids with a surface pressure above 310 μN/cm are able to excert their action towards intact platelets. The inability of phospholipase A_2 from C. adamanteus and phospholipase C from B. cereus to hydrolyse platelet phospholipids is reflected in the failure of these enzymes to affect the platelet prothrombin converting activity. On the other hand, of the four phospholipases which can act on the intact platelet, two enzymes produce a decrease in platelet prothrombin converting activity (phospholipases A_2 from N. naja and bee venom), while the other two phospholipases (C.welchii phospholipase C and sphingomyelinase) bring about an increase in the platelet prothrombin converting activity.

The different effects of these phospholipases on the platelet prothrombin converting activity are explained by the difference in substrate specificity of these enzymes towards phospholipids. The glycerophospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol) are all proper substrates for the two phospholipase A_2 enzymes. Only these two enzymes cause a decrease in platelet prothrombin-converting activity. In contrast, sphingomyelinase and phospholipase C from C. welchii have somewhat different substrate specificities. However, these enzymes have in common that they do not hydrolyse negatively charged phosphatidylserine. Sphingomyelinase can only attack sphingomyelin. The

C. welchii enzyme degrades all phospholipids (including sphingomyelin) except phosphatidylserine and very poorly hydrolyses phosphatidylinositol. It is concluded that a decrease in platelet prothrombin converting activity only occurs after treatment with those phospholipases that are able to hydrolyse negatively charged phospholipids in the intact platelet membrane.

The increase in prothrombin converting activity observed after treatment with two of the phospholipases might have several explanations. Significant lysis of the platelets during incubation with the enzyme of C. welchii is partially responsible for the large increase in prothrombin-converting activity. This is due to the exposure of the membrane inner surface in which the majority of negatively charged phospholipids is present [4,5]. Another phenomenon, translocation of phosphatidylserine, leading to enhancement of prothrombin conversion, independent of cell lysis, is described in chapter 6. However, the increased prothrombin-converting activity found as a result of treatment by sphingomyelinase cannot be explained by lysis, since the cytoplasmic enzyme lactate dehydrogenase is not detectable in the supernatant after incubation. From the experiments demonstrating the asymmetric distribution of phospholipids over the platelet plasma membrane [4,5] we conclude that incubation of platelets with sphingomyelinase does not lead to exposure of phosphatidylserine at the outer surface of the cells, as will occur upon incubation with the C. welchii phospholipase C [chapter 6]. Two other possibilities may explain the increased rate of thrombin formation during sphingomyelinase treatment. First, the action of sphingomyelinase leads to the formation of ceramide droplets in the membrane as a result of phase separation between the glycerophospholipids and the product of sphingomyelin degradation as was demonstrated by Verkleij et al. [27]. This leads to an increase in the phosphatidylserine concentration in the segregated phospholipid regions of the platelet outer monolayer which will affect the binding constants of the different components of the prothrombinase complex. A second explanation is that the formation of ceramide results in a decreased packing density of the lipids in the platelet membrane. This may affect the binding properties of especially factor Va with the membrane. It was suggested by Bloom et al. [28], that the interaction of factor Va with a phospholipid is considered to be hydrophobic which requires penetration of factor Va into the phospholipid bilayer. Further investigations have shown that the decrease in sphingomyelin per sé might be the major cause for the increase in platelet prothrombin converting activity after sphingomyelinase treatment. Using sphingomyelin vesicles containing 10% PS (mol/mol), prothrombinase activity was shown to increase 5-8 fold upon replacing Sph by PC [29].

The effects on the prothrombin-converting activity of artificial membranes which mimic the outer surface of the

platelet plasma membrane are essentially the same as those observed for platelets. Only under those conditions where negatively charged phospholipids are hydrolysed, a decrease in prothrombin-converting activity occurs. In contrast to the results seen with platelets, phospholipase A_2 from C. adamanteus as well as the phospholipase C from B. cereus decrease the prothrombin converting activity of these phospholipid vesicles. However, in this case both enzymes are able to degrade phospholipids (including phosphatidylserine) presumably due to the different packing density of the phospholipids caused by the high curvature of the surface of small phospholipid vesicles. The increase in prothrombin converting activity of the phospholipid vesicles caused by incubation with sphingomyelinase or phospholipase C from C. welchii may be explained by a local increase of phosphatidylserine concentration in the segregated phospholipid regions and/or a decrease in packing of the residual phospholipids as a result of the formation of ceramides and diacylglycerol, respectively.

Combining the results obtained with platelets and phospholipid vesicles, we conclude that the negatively charged phospholipid phosphatidylserine (and possibly also phosphatidylinositol) play an essential role in the activation of prothrombin by factors Xa and Va on the surface of non-stimulated platelets. It should be emphasized that the products of hydrolysis of phosphatidylserine by phospholipase A_2 are lysophosphatidylserine and fatty acids. Both these compounds were shown by Verheij et al. [15] to have no procoagulant activity.

It should be mentioned that the loss of platelet prothrombin-converting activity is not the result of a shielding effect caused by the binding of phospholipase A_2 to the surface of the platelet or phospholipid vesicle. Inactivation of phospholipase A_2 (either from N. naja or from bee venom) with p-bromophenacylbromide [30], does not change the prothrombin converting activity of platelets, nor that of phospholipid vesicles (data not shown). p-Bromophenacylbromide destroys the active centre but not the phospholipid binding-site of phospholipase A_2 [31] indicating that the hydrolytic activity rather than the binding of the enzyme to a phospholipid surface is responsible for the loss in prothrombin converting activity. We conclude that negatively charged phospholipids, presumably small amounts of phosphatidylserine localized at the cell outer surface, are responsible for the observed prothrombin converting activity of non-stimulated platelets. Whether the binding of factor Xa and factor Va is exclusively dependent on the presence of these negatively charged phospholipids cannot be concluded from the present data because rates of thrombin formation have been measured which also requires the binding of prothrombin. However, the involvement of platelet membrane proteins in the formation of the prothrombinase complex at the platelet surface could not be demonstrated.

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CHAPTER 4

CHANGES IN MEMBRANE PHOSPHOLIPID DISTRIBUTION DURING PLATELET ACTIVATION.

Summary.

Exposure of phospholipids at the outer surface of activated and control platelets was studied by incubation with a mixture of phospholipase A_2 from Naja naja and bee venom, solely or in combination with sphingomyelinase from Staphylococcus aureus, using conditions under which cell lysis remained below 10%.

Incubation with phospholipase A_2 alone revealed a markedly increased susceptibility of the phospholipids in platelets activated by a mixture of collagen plus thrombin, by the SH-oxidizing agent diamide, or by calcium ionophore A23187, as compared to control platelets or platelets activated separately by collagen or by thrombin.

Collagen plus thrombin, diamide and ionophore treated platelets revealed an increased exposure of phosphatidylserine at the outer surface accompanied by a decreased exposure of sphingomyelin, as could be concluded from incubations with a combination of phospholipase A_2 and sphingomyelinase. These alterations were much less apparent in platelets activated either by thrombin or by collagen alone.

The increased exposure of phosphatidylserine in activated platelets is accompanied by an increased ability of the platelets to enhance the conversion of prothrombin to thrombin by coagulation factor Xa, in the presence of factor Va and calcium.

It is concluded that the altered orientation of the phospholipids in the plasma membrane of platelets activated by collagen plus thrombin, by diamide, or by calcium ionophore, is the result of a transbilayer movement. Moreover, the increased exposure of phosphatidylserine in platelets stimulated by the combined action of collagen and thrombin might be of considerable importance for the hemostatic process.

Introduction.

Phospholipids in the plasma membrane of human platelets are not homogeneously distributed between both halves of the membrane bilayer [1-3]. In a resting, non-activated platelet, the outer surface of the membrane is characterized by the presence of neutral phospholipids, particularly phosphatidylcholine and sphingomyelin. The negatively charged platelet phospholipids, phosphatidylserine and phosphatidylinositol, are almost exclusively present in the inner leaflet of the bilayer. We have previously shown that activation of platelets by simultaneous action of thrombin and collagen changes the distribution of the lipids in the plasma membrane in such a way that a substantial amount of the negatively charged phosphatidylserine becomes exposed at the membrane outer surface [4]. This property of activated platelets is of significant importance for their role in the hemostatic process, since negatively charged phospholipids markedly enhance several reactions of the coagulation cascade. In this study we are dealing with the conversion of prothrombin into thrombin by a complex of factor Xa, factor Va and calciumions. However, the above mentioned stimulatory effect of phospholipids also governs the conversion of factor X into factor Xa by a complex of factor IXa, factor VIIIa and calcium-ions [5-12].

Non-activated platelets have a low capacity to stimulate the formation of factor Xa or thrombin, because of the nearly absolute absence of negatively charged phospholipids at the outer surface of the membrane. Simultaneous activation of platelets by collagen and thrombin results in a large enhancement of the rate of factor Xa or thrombin formation [4,13]. This increased activity has been ascribed to the increased exposure of negatively charged phosphatidylserine, which makes the platelet surface more suitable for the formation of prothrombin and factor Xa activating complexes.

In our previous experiments, exposure of phosphatidylserine at the outer surface of activated platelets was demonstrated using phospholipase A_2 from Naja naja or bee venom [4]. However, only the phospholipids of platelets activated by the combination of collagen and thrombin can be hydrolyzed by these lipolytic enzymes. The phospholipids of unactivated platelets or platelets activated by collagen or thrombin alone are hardly susceptible towards phospholipase A_2. Thus, from incubations with phospholipase A_2 alone it cannot always be concluded whether activated platelets have an increased exposure of negatively charged phospholipids. However, phospholipids of unactivated platelets do become available for hydrolysis by phospholipase A_2 when sphingomyelinase (from Staphylococcus aureus) is also present [1]. Therefore, the accessibility of phospholipids of activated platelets to a combination of phospholipase A_2 and sphingomyelinase was investigated to establish whether a correlation exists

between exposure of phosphatidylserine and platelet prothrombin converting activity after activation of the platelets by collagen, thrombin or a combination of these agents. Since platelets treated with the calcium ionophore A23187 also show a dramatic increase in prothrombin- and factor X-activating capacity [14], it was of interest to investigate whether these platelets also show alterations in the phospholipid distribution across the membrane. Moreover, Haest et al. [15,16] have found that treatment of erythrocytes with the SH-oxidizing agent diamide results in an increased accessibility of the aminophospholipids. Therefore, it was investigated whether this agent is also able to alter the phospholipid arrangement in the platelet membrane and if this is accompanied by an increase of the prothrombin converting activity of these platelets.

Materials and Methods.

Materials.

Fatty acid free human serum albumin, nicotinamide-adenine nucleotide, reduced form (NADH), indomethacin and diamide were obtained from Sigma. Dithiothreitol and o-phenantroline were from Baker. Collagen (type I) was from Hormon Chemie (Munich) and the calcium ionophore A23187 from Calbiochem. Venoms from the snake Naja naja and the bee Apis mellifica were purchased from Koch Light. Chromogenic substrate H-D-phenylalanyl-L-pipecolyl-arginine-p-nitroanilide (S-2238) was from Kabi Diagnostica (Stockholm, Sweden). Methylphosphatidic acid was prepared according to the method described in this thesis [17]. Coagulation factors Xa, Va, prothrombin and thrombin were purified as described elsewhere [4]. Phospholipases A_2 from N. naja and bee venom and sphingomyelinase from Staphylococcus aureus were purified according to Zwaal et al. [18]. The International Unit (I.U.) is defined as the amount of enzyme which hydrolyses one micromole of substrate per minute under optimal conditions.

Isolation of platelets.

Because relatively large amounts of platelets were required, a method was developed to isolate platelets from buffy coats obtained from the local hospital. The buffy coats were prepared from blood of healthy donors which was collected into the anticoagulant acid citrate dextrose (0.052M citric acid, 0.08M trisodium citrate, 0.18M glucose, one part of the anticoagulant ACD for every five parts of blood). For isolation of the platelets these buffy coats were diluted with five volumes of a Hepes buffer pH 6.6, containing 137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM $MgCl_2$, 25 mM glucose and 0.05% (w/v) fatty acid free human serum albumin (HSA). After centrifugation for 15 minutes at 200 g, the plateletrich upper phase was recentrifuged at 1000 g to obtain a platelet pellet. This pellet was resuspended in Hepes buffer pH 6.6, and

one volume of ACD was added to fourteen volumes of the platelet suspension. The platelets were washed twice and finally resuspended in the same Hepes buffer, adjusted to pH 7.5, this time without addition of ACD. Platelet concentration was determined with a Coulter counter. The washed platelet preparations contained less than one percent leukocytes or erythrocytes. All platelet handling was carried out at room temperature.

Phospholipase treatment of platelets.

Stirred platelet suspensions (10 ml), at a concentration of 3 . 10^8/ml, were activated with various stimulators in the presence of 3 mM $CaCl_2$ at 37°C. After 15 minutes, an additional amount of $CaCl_2$ (final concentration 10 mM) as well as o-phenantroline (final concentration 1 µg/ml) were added, followed by a mixture of 36 I.U. phospholipase A_2 from N.Naja and 26 I.U. phospholipase A_2 from bee venom. In some experiments sphingomyelinase (3.5 I.U.) was added 5 minutes after the addition of the phospholipases A_2. At various time intervals, phospholipase activity was inhibited by addition of 1 ml 0.2M EDTA. Prior to addition of EDTA a sample of 1 ml was taken to measure the amount of platelet lysis. It should be noted that for every time point of the phospholipase treatment a seperate incubation was required, since platelet aggregation as a result of the activation procedure makes homogeneous subsampling unreliable.

Platelet lipids were extracted according to the procedure of Bligh and Dyer [19]. Methylphosphatidic acid (250 µg) was added as an external standard, prior to the extraction procedure. Phospholipid analysis was carried out by twodimensional thin layer chromatography followed by phosphorous determination as described earlier [20]. To evaluate phospholipid degradation in intact cells only, corrections were made for phospholipid breakdown in the population of lysed cells, assuming that the phospholipids of the lysed cells are completely degraded. Only those experiments where lysis did not exceed 10% were taken into consideration.

Measurement of platelet lysis.

Lactate dehydrogenase activity in the supernatant was used as a parameter for platelet lysis and was measured according to the method of Wroblewski and La Due [21]. In the samples containing diamide, the measurements were carried out in the presence of excess dithiothreitol (twice the concentration of diamide). The amount of platelet lysis was determined from a calibration curve made with different dilutions of a platelet preparation which was lysed completely by sonication for 3 minutes.

Measurement of platelet prothombin converting activity.

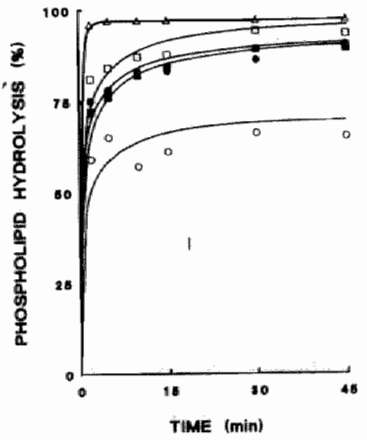
Prothrombin converting activity was measured as described before [4]. Final concentrations in the reaction mixture were: 5 . 10^8 platelets/ml, 15 nM factor Xa, 30 nM factor Va, 4 µM prothrombin and 6 mM $CaCl_2$. Samples (25 µl) for measuring the amount of thrombin formed were taken 30 s and 60 s after addition of prothrombin and assayed in

a cuvette containing 1 ml buffer (50 mM Tris, 120 mM NaCl, 2 mM EDTA, pH 7.5) and 0.25 mM S2238. From the rate of change in absorbance at 405 nm, the amount of thrombin was calculated using a calibration curve made with known amounts of activesite titrated thrombin.

Results.

Exposure of phospholipids at the platelet outer surface was studied using a mixture of two phospholipases A_2 (the enzymes from N. naja and bee venom) solely or in combination with sphingomyelinase from S. aureus. An essential requirement for such a study is that all phospholipids are suitable substrates for the enzymes used and that the amount of enzyme and time of incubation are sufficient to guarantee complete degradation in lysed cells. Therefore, lysed platelets obtained by sonication for 3 minutes were incubated with a mixture of phospholipase A_2 (N. naja and bee venom) and sphingomyelinase, and phospholipid hydrolysis was measured. As shown in Fig. 1, all phospholipid classes with the exception of phosphatidylinositol are almost completely degraded within 5 minutes under the conditions employed.

Fig. 1 Phospholipid hydrolysis in platelet lysate by phospholipase A_2 and sphingomyelinase. At different time intervals part of the incubation was stopped with EDTA and analyzed for phospholipid hydrolysis.
■, *phosphatidylcholine*
△, *phosphatidylethanolamine*
●, *phosphatidylserine*
○, *phosphatidylinositol*
□, *sphingomyelin*



Since hydrolysis of phosphatidylinositol is incomplete, this phospholipid was not considered in further experiments. The results in Fig. 1 can be obtained by incubation of lysed platelets with phospholipase A_2 and sphingomyelinase separately or in combination. In contrast, very little phospholipid hydrolysis occurs when intact, non-activated platelets are treated with phospholipase A_2

alone (Table I). This is also the case when platelets are activated either by thrombin or by collagen; less then 6% of the total phospholipids is hydrolyzed by phospholipase A_2.

TABLE I

Non lytic degradation of phospholipids in activated platelets by exogenous phospholipase A_2 (N.naja plus bee venom)

Platelet activator	% Total phospholipids hydrolyzed
None	2.2 ± 1.4
Thrombin (2 nM)	5.8 ± 2.1
Collagen (10 µg/ml)	4.9 ± 3.0
Thrombin + collagen	20.4 ± 2.1
Diamide (5 mM)	19.2 ± 3.1
A23187 (1 µM)	46.8 ± 3.1

Platelets (3 . 10^8/ml) were activated with various agents for 15 min. At that time phospholipase A_2 was added, and the incubation was continued for another 45 min. Values have been corrected for platelet lysis, which was lower than 8%. Data are the average (mean ± SD) from six experiments. For experimental details see Materials and Methods.

However, when platelets are activated by a mixture of collagen and thrombin, by the SH-oxidizing compound diamide, or by the calcium ionophore A23187, increased amounts of phospholipid become susceptible towards exogenously added phospholipase A_2 without significant lysis of the cells. The increased accessibility of platelet phospholipids towards phospholipase A_2 indicates that changes in the membrane structure have occurred as a result of these activation procedures. Activation of platelets for 15 minutes with the agents listed in Table I, does not cause detectable changes in overall phospholipid composition of the platelets. Platelet release reaction and aggregation occur to a similar extent with all activators except diamide, with which neither release nor aggregation is observed (data not shown). It should be noted that sphingomyelinase treatment of non-activated as well as activated platelets results in all cases in significant degradation of sphingomyelin, without cell lysis. The amount of sphingomyelin degradation is the same whether sphingomyelinase is added alone or in combination with phospholipase A_2 (see below).

In order to obtain more detailed information on the exposure of phospholipids at the outer surface of platelets and to compare the effect of various activating agents on this exposure, a mixture of phospholipase A_2 and sphingomyelinase was used. However, the use of this combination of enzymes increases the risk of platelet

lysis, particularly if the platelets are activated prior to the addition of phospholipases. Therefore, careful examination of platelet lysis during treatment with the combination of phospholipase A_2 and sphingomyelinase is essential. With non-activated platelets and platelets activated either by thrombin, collagen or diamide, breakdown of phospholipid levels off at a moment where platelet lysis is still less then 10%, and neither phospholipid breakdown nor cell-lysis increase upon a further 30 minutes incubation. A different picture was obtained using platelets triggered with collagen plus thrombin, or with the ionophore A23187. Fig. 2 shows the hydrolysis of total phospholipid as a function of time together with the lysis pattern during phospholipase treatment of platelets that have been activated with collagen plus thrombin.

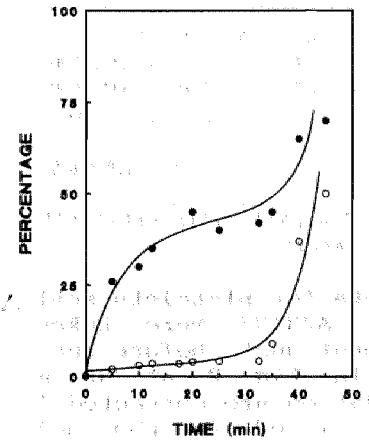


Fig. 2 Total platelet lipid hydrolysis and cell lysis during treatment with phospholipase A_2 and sphingomyelinase. Platelets were activated for 15 min with thrombin (2 nM) plus collagen (10 µg.ml). Incubations were stopped by addition of EDTA.
●, phospholipid degradation
○, platelet lysis.
Further details are given in Materials and Methods.

Phospholipid hydrolysis does not reach a plateau in this incubation. During the first 30 minutes of phospholipase treatment, there is a small increase in cell lysis. At this time, phospholipid hydrolysis approximates 50%. Prolonged incubation gives rise to a sudden increase in cell lysis as well as phospholipid degradation, suggesting that extensive destruction of cell structure occurs. A similar time-course for hydrolysis and lysis was obtained after phospholipase treatment of platelets activated by A23187.

Besides total phospholipid hydrolysis, we also determined the hydrolysis of each phospholipid class upon platelet incubation with a combination of sphingomyelinase and phospholipase A_2. The data are summarized in Table II. For control platelets and platelets activated by collagen, thrombin, or diamide, the hydrolysis of each phospholipid class levelled off after 30 minutes; values at t=45 min

are presented in this table.

TABLE II

Non-lytic degradation of phospholipids by phospholipase A_2 (N.naja and bee venom) and sphingomyelinase (S.aureus) in activated human platelets.

The values are expressed as percentage of total lipid phosphorous (mean ± SD). The numbers in parenthesis refer to the percent degradation of the corresponding lipid class. n= number of experiments. PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; total PL, total phospholipid.

	Phospholipid composition (n=12)	Hydrolysis					
		Control (n=7)	Thrombin (n=9)	Collagen (n=9)	Thrombin + collagen (n=12)	Diamide (n=7)	A23187 (n=7)
PS	10.9 ± 0.7	0.5±0.6(4)	1.6±1.0(15)	2.1±2.0(19)	5.0±0.3(49)	5.3±0.4(49)	8.2±0.7(75)
PC	38.3 ± 1.4	6.5±1.5(17)	12.9±4.6(34)	14.4±4.3(37)	15.1±1.6(41)	15.5±0.5(41)	19.2±1.5(50)
PE	26.8 ± 0.8	2.0±0.9(7)	5.1±3.1(19)	5.9±3.9(22)	16.0±0.9(60)	10.3±0.1(38)	23.3±0.8(87)
SM	19.0 ± 1.4	12.0±1.5(63)	13.2±3.8(69)	12.4±0.6(65)	8.3±1.5(43)	8.6±0.2(45)	14.3±0.6(75)
Total PL	95.0*	21.0 ± 2.4	32.8 ± 6.8	34.8 ± 6.2	44.5 ± 2.3	39.7 ± 0.7	65.0 ± 1.9

* *Phosphatidylinositol (PI) is omitted for reasons explained in Results.*

The data for platelets activated by collagen plus thrombin or by A23187 were taken at a time of phospholipase treatment just before the sudden rise in platelet lysis (e.g. in Fig. 2 at 35 min). The percentages of lipid degradation are corrected for cell lysis assuming complete hydrolysis of the phospholipids in the lysed cell population. Treatment of control cells with a combination of phospholipase A_2 and sphingomyelinase leads to a hydrolysis of 21% of total phospholipid which is in agreement with the findings of Perret et al. [3]. The hydrolyzed phospholipid fraction is mainly composed of the choline containing phospholipids, sphingomyelin and phosphatidylcholine. Activation of platelets by collagen or by thrombin prior to addition of phospholipases results in an increased phospholipid hydrolysis. The amount of sphingomyelin hydrolyzed in these platelets is not different from that in control platelets, but an increased degradation of glycerophospholipids is observed. However, the small quantities of phosphatidylserine and phosphatidylethanolamine that are hydrolyzed together with the large standard deviations make it difficult to discern whether there is indeed an increased susceptibility towards phospholipase A_2. Remarkably deviating patterns of phospholipid hydrolysis are found for platelets activated by collagen plus thrombin, diamide or A23187. Apart from a further increase

in the extent of total phospholipid hydrolysis (even to a maximum of 65% for platelets activated by A23187), the most striking feature is the significant increase in breakdown of phosphatidylserine and phosphatidylethanolamine in these platelets.

A clear picture of the effects of the different platelet stimulators on the exposure of phospholipids at the membrane outer surface is obtained when the compositions of the hydrolyzed phospholipid fractions are compared. These compositions, which are calculated from the data in Table II, are presented in Table III. Compared to control platelets, there is an increase in phosphatidylserine and phosphatidylethanolamine accompanied by a decrease in sphingomyelin in the hydrolyzed phospholipid fraction, when platelets are activated by a mixture of collagen plus thrombin, by diamide, or by A23187.

TABLE III

Composition of phospholipid fractions hydrolyzed by phospholipase A_2 and sphingomyelinase treatment of activated human platelets.

Values are expressed as percentage of the hydrolyzed fraction of total phospholipid (mean ± SD). Data are calculated from Table II by setting the hydrolyzed total phospholipid fraction at 100%. PI is not taken into account for reasons explained in the Results section.

	Control	Thrombin	Collagen	Thrombin+ collagen	Diamide	A23187
PS	2.4±2.4	4.9± 3.2	6.0± 5.8	11.2±0.9	13.4±1.0	12.6±1.1
PC	31.0±8.0	39.3±16.2	41.4±14.4	33.9±3.4	39.0±1.4	29.5±2.5
PE	9.5±4.4	15.5± 9.9	17.0±11.6	36.0±2.0	25.8±0.5	35.8±1.6
SM	57.1±9.7	40.2±14.3	35.6± 6.6	18.7±3.4	21.7±0.6	22.0±1.1

It is not clear why the standard deviations for the phospholipid hydrolysis of thrombin or collagen treated platelets are relatively high. Therefore it remains uncertain whether alterations in phospholipid orientation also occur in these platelets.

The effect of various platelet activators on the ability of platelets to enhance the conversion of prothrombin into thrombin by factor Xa was investigated using a chromogenic substrate assay to measure the rate of thrombin formation. The results are presented in Table IV. It has previously been demonstrated that the rate of thrombin formation is strongly enhanced in the presence of a negatively charged phospholipid surface, in particular one containing phosphatidylserine [5-9]. Therefore, the corresponding values for phosphatidylserine hydrolysis by exogenous phospholipases are also presented in this table, since the rate of thrombin formation may represent a measure of phosphatidylserine exposure at the outer surface. It is obvious from this comparison that an

increased exposure of phosphatidylserine corresponds to an increased rate of thrombin formation.

TABLE IV

Comparison between prothrombin converting activity of activated human platelets and exposure of phosphatidylserine.

Rate of thrombin formation was measured after a 15 min activation period in a system containing 5.8 . 10⁸ platelets/ml, factor Xa (15 nM), factor Va (30 nM) and prothrombin (4 μM) in the presence of 6 mM calciumchloride.

	Rate of thrombin formation (nM/Min)	Percentage of PS exposed[a]
No stimulator	34	4
Thrombin (2-20 nM)	41	15 (p=0.05)
Collagen (10μg/ml)	157[b]	19 (p=0.10)
Thrombin+collagen	352	46 (p<0.001)
Diamide (5mM)	356	49 (p<0.001)
A23187 (1μM)	793	75 (p<0.001)
Lysed platelets	1170	96 (p<0.001)

[a] Differences with respect to non-stimulated platelets were tested by two-sided Student's t-test. p values are given in parenthesis. Consult Table II for corresponding standard deviations.

[b] The increased rate of thrombin formation of collagen activated platelets could be the result of a combined action of the added collagen and the thrombin that is formed during the assay [4].

Discussion.

Platelet activation leads to morphological and metabolical changes. In particular, the enhanced turnover of phosphatidylinositol is one of the primary metabolic events that take place in the membrane during platelet activation [22-27]. However, little evidence is available indicating that the plasma membrane is subject to structural alterations. Using fluorescent probes, Nathan et al. [28] have found indications for changes in microviscosity of phospholipids in the membrane of human platelets after activation by thrombin. Changes in the distribution of membrane phospholipids have been reported by Schick et al. [29], who found increased labeling of phosphatidylethanolamine by trinitrobenzenesulfonic acid (TNBS) after triggering of platelets by thrombin. We have previously shown [4] that activation of platelets simultaneously by collagen and thrombin results in an increased susceptibility of phospholipids towards exogenously added phospholipase A_2. This was not observed for platelets activated

by either one of these activators separately. The increased susceptibility of phospholipids is also apparent following treatment of platelets with diamide or A23187. Differences in availability of phospholipids for phospholipase A_2 in platelets activated by various means may reflect differences in structural organization of the membrane. In this respect, the difference between platelets activated by a combination of collagen and thrombin compared to platelets activated by either of these two is striking, since no differences are observed in the extent of platelet aggregation and release.

The activity of phospholipase A_2 towards platelet membranes is significantly facilitated if sphingomyelin is degraded at the same time by the action of sphingomyelinase [1,3]. When platelet lysis is carefully controlled, this allows the study of the exposure of all individual phospholipid classes at the outer surface of activated platelets in a similar manner as was described for unstimulated platelets by Perret et al. [3]. The amount of phospholipids that can be degraded under non-lytic conditions can be directly influenced by the extent of platelet aggregation and especially release resulting from the activation procedure. Intact unstimulated platelets contain several types of intracellular membranes such as granule membranes, mitochondrial membranes and membranes of the dense tubular system. As was elegantly demonstrated by Perret et al. [3], 57% of the total platelet phospholipids are present in the plasma membrane of unstimulated human platelets. Consequently, complete degradation of the outer leaflet of the plasma membrane would be reflected in 28% hydrolysis of the total phospholipid content of the cell. During the platelet release reaction as a result of platelet activation, granule membranes are thought to fuse with the plasma membrane. This would result in an increased amount of phospholipids that can be hydrolyzed. Assuming that as a result of the release reaction, 60% of the intracellular membranes have fused with the plasma membrane, approximately 80% of the platelet phospholipids would be present in the plasma membrane. Thus, complete hydrolysis of the outer leaflet of the plasma membrane of the activated platelets would result in 40% hydrolysis of total phospholipids. On the other hand, platelet aggregation forms a complicating factor since it may prevent efficient action of phospholipases, restricting the total amount of phospholipid hydrolysis.

The following remarks can be made concerning the data for total phospholipid hydrolysis from Table II. Total phospholipid hydrolysis of intact unstimulated platelets amounts to 21%, which is in close agreement to the 25% reported by Perret et al. [3]. In the absence of aggregation or release this implies a very substantial hydrolysis of the phospholipids present in the outer leaflet. Activation of platelets by thrombin or collagen induces release as well as aggregation. The observed hydrolysis of 32.8% and 34.8%, respectively, are higher than observed with

control platelets as a result of the release reaction, but lower than the expected 40%, presumably due to platelet aggregation. Activation of platelets with collagen plus thrombin results in release and aggregation to a similar extent as is induced by the activators separately, but the phospholipid hydrolysis is higher (44.5%). In the case of diamide treatment of platelets, there is neither aggregation nor release which would predict a phospholipid hydrolysis of at most 28%. The observation that almost 40% of the phospholipids are hydrolyzed in these cells, under non-lytic conditions, indicates that more than half of the plasma membrane phospholipids have been degraded. The greatest amount of hydrolysis is observed with platelets activated by the calcium ionophore A23187; 65% of the total phospholipids are hydrolyzed by phospholipase A_2 and sphingomyelinase under non-lytic conditions. Activation by A23187 leads to substantial release and aggregation, although the size of the aggregates produced by A23187 is much smaller than those induced by collagen or thrombin. Since phospholipid degradation in ionophore treated platelets exceeds the expected 40%, hydrolysis is apparently not restricted to half of the membrane phospholipids. Considering that the data have been corrected for phospholipid degradation in the population of lysed cells and that the plasma membrane is impermeable to phospholipases, the extent of phospholipid degradation observed with platelets that are activated by collagen plus thrombin, diamide or A23187, can be explained if one assumes a transbilayer movement of phospholipids (flip-flop). From the present data, it is difficult to ascertain whether the exposure of phospholipids is significantly changed upon platelet activation by either collagen or thrombin, due to the relatively large standard deviations. Transbilayer movement of phospholipids does apparently not occur in control platelets, which indicates that the action of phospholipases as such, does not necessarily induce flip-flop in these membranes. On the other hand it cannot be excluded that phospholipase treatment induces flip-flop in platelets that have been activated by collagen plus thrombin, by diamide or by ionophore. However, if exposure of phosphatidylserine at the platelet outer surface would be a result of the phospholipase treatment as such, one would expect a delay in the onset of hydrolysis of this phospholipid directly after addition of phospholipase. Since neither phosphatidylserine nor the other phospholipids show any such lag period in hydrolysis, phospholipase induced flip-flop seems unlikely. On the other hand it is very well possible that transbilayer movement of the phospholipids continues during incubation with phospholipase. Therefore it cannot be distinguished whether the hydrolyzed phospholipids are continuously or transiently exposed at the outer surface during the time course of the phospholipase incubation.

As we have shown previously [4], the increased phospholipid hydrolysis in platelets activated by collagen

plus thrombin cannot be explained by the action of endogenous phospholipases. Also platelet treatment with A23187 or diamide does not produce significant phospholipid breakdown on a percentage basis.

Transbilayer movement of phospholipids due to the activation of platelets could explain the increased exposure of phosphatidylserine and phosphatidylethanolamine. To balance this process, sphingomyelin seems to move from the outer to the inner leaflet of the plasma membrane. There is no large change in the orientation of phosphatidylcholine. Particularly after treatment with diamide, the phospholipid composition of the hydrolyzed fraction is very similar to the total phospholipid composition, suggesting a total randomization of the phospholipids over both membrane halves. Transbilayer movement of phospholipids has been reported [15,16] to occur in erythrocytes treated with diamide or tetrathionate. Approximately 50% of the phosphatidylethanolamine and 30% of the phosphatidylserine became accessible to exogenous phospholipase A_2 without hemolysis. The effect was correlated with a significant cross-linking of spectrin which was proposed to play a role in maintaining the asymmetric phospholipid orientation in the erythrocyte membrane. Although treatment of platelets with diamide also results in a significant cross-linking of membrane proteins as was observed by polyacrylamide gel electrophoresis (data not shown), it is not clear whether in all cases exposure of phosphatidylserine at the platelet outer surface is dependent on a similar mechanism, since cross-linking of membrane proteins was not observed following treatment with collagen plus thrombin, or A23187.

The increased exposure of phosphatidylserine at the outer surface of activated platelets may be of considerable importance for the hemostatic process. Unstimulated platelets have little phosphatidylserine exposed at their outer surface. This lack of negatively charged phospholipids is reflected in a relatively poor capacity of resting platelets to stimulate the formation of thrombin from prothrombin by factor Xa, factor Va and calcium. The rate of thrombin formation is critically dependent on the presence of a negatively charged phospholipid surface to which factors Xa, Factor Va and the substrate prothrombin can bind [4-13]. Those platelet activators that induce increased exposure of phosphatidylserine at the outer surface of the membrane as measured by exogenously added phospholipases, also induce the platelets to become more active in enhancing the rate of thrombin formation. Remarkable in this respect is the relationship between the amount of phosphatidylserine exposed and the rate of thrombin formation.

The mechanism by which transbilayer movement of phospholipids in the platelet membrane can take place is not well understood. Although phospholipid flip-flop was demonstrated to be an extremely slow process in vesicles

of pure phosphatidylcholine [30], rapid flip-flop in artificial and natural membranes has been reported (review, ref.31). One possible mechanism for transbilayer movement of phospholipids is the existence or introduction of intrabilayer inverted micelles as described by Cullis and de Kruijff [32,33]. These structures can be formed with those lipids having a conical shape, such as diacylglycerols and phosphatidic acid, that do not adopt bilayer structures. The formation of these lipids has been suggested to play a triggering role for various cellular responses to exogenous stimuli by altering membrane physical states [32,34]. In this respect, it is tempting to speculate that intermediates of the phosphatidylinositol-cycle, which is activated during platelet stimulation [22-27], produce local bilayer disturbances that enable transbilayer movement of phospholipids. Experiments described in chapter 6 have demonstrated that the introduction of diglycerides in the platelet membrane by the action of phospholipase C from Clostridium welchii causes an increased exposure of phosphatidylserine at the outer surface of the plasma membrane. Whether or not differences in the activation of the phosphatidylinositol-cycle exist as a result of different platelet activation procedures remains to be investigated.

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CHAPTER 5

PLATELET PROTHROMBIN CONVERTING ACTIVITY IN HEREDITARY DISORDERS OF PLATELET FUNCTION

Summary.

Prothrombinase activities of platelets have been measured in diluted platelet-rich plasma using a chromogenic substrate assay and purified coagulation factors. No abnormalities in prothrombinase activities were found for platelets from patients with storage pool disease (dense-body deficiency), grey platelet syndrome, and Glanzmann's thrombasthenia. It is concluded that neither release of dense bodies and α-granules nor aggregation of platelets are essential prerequisites for exposure of a procoagulant surface. Platelets from patients with Bernard-Soulier syndrome, however, have approximately 10-fold higher prothrombinase activities in the non-stimulated form than normal non-stimulated platelets. The increased prothrombinase activities cannot be completely ascribed to an increase in platelet size. It is suggested that the increased prothrombinase activity reflects an increased exposure of phosphatidylserine at the outer surface of non-stimulated Bernard-Soulier platelets, earlier described by Perret et al. (Thromb. Res.(1983) 31, 529-537).

Introduction.

Platelet procoagulant activity is expressed in at least two sequential reactions of the coagulation cascade: the activation of factor X by a complex of factors IXa, VIIIa and calcium (intrinsic factor X activation) and the conversion of prothrombin to thrombin by a complex of factors Xa, Va and calcium, also referred to as the prothrombinase complex [1-3]. The essential role of platelets in both reactions is to provide a catalytic surface to which the coagulation factors can bind. This results in an increased local concentration of these proteins, thus leading to an increased rate of factor Xa or thrombin formation [4,5]. The catalytic efficiency of the platelet surface is significantly greater when platelets are stimulated by the combined action of collagen and thrombin. We have demonstrated that an increased exposure of negatively charged phosphatidylserine at the outer surface of the platelet plasma membrane is responsible for the increased rate of factor Xa and thrombin formation [6,7]. Negatively charged phospholipids are essential for a direct interaction of factors Va and VIIIa with the membrane, as well as for the Ca^{2+}-mediated binding of the vitamin-K dependent coagulation factors.

Platelet stimulation by collagen and thrombin induces release of granule contents and platelet aggregation. Since procoagulant activity is expressed particularly as a result of simultaneous action of collagen and thrombin, the possibility cannot be excluded that either release of granule contents or aggregation, or both, form an essential prerequisite for expression of this activity. On the other hand, however, it is known from previous work that the release reaction or aggregation as such is not sufficient, since platelets stimulated by thrombin or by ADP hardly evoke a procoagulant surface [2,6,7]. The present study was undertaken to investigate which platelet functions or structures in particular membrane glycoproteins or different types of granules, are required for the generation of a procoagulant surface. To this end, platelets from patients with different platelet disorders were studied with respect to their ability to enhance the rate of thrombin formation in a system using purified coagulation factors Xa, Va and prothrombin and a chromogenic substrate, specific for thrombin.

Materials and Methods.

Collagen type I was obtained from Hormon Chemie, Munich. Coagulation factors Xa, Va and prothrombin were purified as decribed by Rosing et al. [4]. Thrombin was derived from prothrombin after activation with factor Xa according to Rosing et al. [4]. Thrombin specific chromogenic substrate H-D-phenylalanyl-L-pipecolyl-arginine-p-nitroanilide dihydrochloride (S2238) was obtained from

AB Kabi Diagnostics, Stockholm. Fatty acid free human serum albumin was from Sigma Chemical Co., St Louis.

Platelet handling.

Fresh blood was taken by venipuncture and was collected in 0.1 vol. of sodium citrate (3.8 % w/v). Platelet rich plasma (PRP) was obtained by centrifugation at 120 g for 15 minutes at room temperature. PRP from Bernard-Soulier patients was obtained by allowing whole blood to sediment at 1 g for one hour at room temperature in tubes tilted at an angle of 45°. In order to avoid platelet damage or platelet activation as much as possible, platelets were not washed, but PRP was diluted 50-fold in a buffer containing 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose and 0.05% (w/v) fatty-acid free human serum albumin, pH 7.5. Platelet count was determined by light-microscopy or with a Coulter counter. All platelet handling was carried out at room temperature in siliconized glass or in plastic tubes.

Prothrombinase assay.

To study the influence of platelets on the conversion of prothrombin to thrombin by factors Xa and Va, platelets must be isolated and washed to remove plasma coagulation factors and other components which might interfere with the coagulation assay. This procedure increases the risk of platelet activation and platelet damage which inevitably will affect the procoagulant activity [1,6]. In order to avoid this problem, which might be particularly important for abnormal platelets, the prothrombinase assay was modified using a 50-fold diluted PRP instead of washed platelets. This does not significantly change the procoagulant activities relative to washed platelets at the same platelet count; thus the contribution of the 50-fold diluted plasma coagulation factors may be neglected. Furthermore, this modification has the advantage of saving time-consuming washing procedures which enables a rapid screening of the procoagulant activity. It should be emphasized, however, that using this assy procedure, activation of 50-fold diluted PRP with collagen for 10 minutes in the presence of 3 mM calcium leads to the formation of approximately 5 nM thrombin even before the prothrombinase assay is started. This amount of thrombin makes an estimation of the prothrombinase activity of platelets, stimulated by collagen alone, ambiguous since platelets in diluted PRP will in fact be activated by collagen plus the thrombin that is formed in the diluted plasma.

Measurement of platelet prothrombin converting activity was carried out as described before [1,6]. Briefly, 290 µl 50-fold diluted PRP was activated in the presence of 15 µl 75 mM $CaCl_2$ by 15 µl collagen (250 µg/ml) and 5 µl 200 nM thrombin at 37° C with continuous stirring. 10 minutes after activation, 50 µl of a freshly prepared mixture containing 30 nM Xa and 60 nM Va was added. The enzymatic reaction was started 2 minutes later by the addition of 125 µl 16 µM prothrombin. The solution

of prothrombin contained 14 mM $CaCl_2$ to give a final calcium concentration in the assay of 6 mM. After 1 minute a sample was taken and immediately diluted in a buffer containing 120 mM NaCl, 50 mM Tris and 2 mM EDTA (pH 7.5) to stop the reaction. The amount of thrombin formed was assessed by adding chromogenic substrate to this mixture and measuring the change in absorbance at 405 nm per minute, produced by the action of thrombin on S2238. The concentration of thrombin was calculated from a calibration curve made with known amounts of active-site titrated thrombin. All measurements were done in triplicate (at least) in a standardized time scheme and were completed within one hour after obtaining the plasma from the blood samples.

Because of differences in individual activities within each group of patients, ranges of activity are presented next to mean values and standard deviations. To allow comparison of the data of different patients all prothrombinase activities are calculated for a standard platelet count of 10^5 cells per ml (final concentration in the assay system). Since prothrombinase activity is dependent on the amount of suitable phospholipid surface, maximal prothrombinase activity of a complete lysed platelet preparation (obtained by 3 minutes sonication of the 50-fold diluted PRP) was also measured.

Patients/case histories.

Three unrelated patients with storage pool disease were studied. The diagnosis of storage pool disease relied on the basis of a normal platelet count, a prolonged bleeding time (Simplate II, General Diagnostics), aggregation abnormalities that were indicative for a secretion defect and abnormal levels of total ATP an ADP measured in ethanol extracts with the luciferine-luciferase assay. The platelets of the patients contained a normal amount of ß-thromboglobulin and a normal amounts of ß-N-glucuronidase (E.C. 3.2.1.31) and ß-N-acetylglucosaminidase (E.C. 3.2.1.30) indicating that the patients platelets were only deficient in dense granules. One patient has been diagnosed previously as Hermanski-Pudlak syndrome [8,9].

Three patients with a grey platelet syndrome were investigated. Biological data have already been reported for two patients [10] and were similar for a third patient. Thus, platelet count was decreased. Platelet aggregation and release of ^{14}C-serotonin were markedly reduced after collagen and thrombin stimulation. ADP, arachidonic acid and ionophore A23187 mediated aggregation were apparently normal.

Of the thrombastenic patients we have studied one patient with type I and two patients with type II thrombastenia as defined by Caen [11]. The levels of glycoproteins IIb/IIIa varied from undetectable to 20% of the normal value as reported by Nurden and Caen [12]. In these three patients platelet aggregation in response to ADP, adrenaline, collagen, arachidonic acid, ionophore and

thrombin was absent and bleeding time was considerably prolonged.

Five unrelated patients with Bernard-Soulier syndrome were studied. Two patients were described previously [13,14]. Diagnosis was made on the basis of thrombocytopenia with large platelets, prolonged bleeding time, absence of platelet agglutination with ristocetin despite normal von Willebrand factor related properties. Normal aggregation with collagen, epinephrine and ADP were seen except for two patients who showed subnormal collagen or ADP induced aggregation. Lack of glycoprotein Ib was already described for two patients [15] and was also established for the other three patients by two-dimensional gel electrophoresis of membrane proteins labeled by ^{125}I with Iodogen.

Results.

Table I summarizes the results obtained from patients with storage pool deficiences and Glanzmann's thrombasthenia. The results obtained with Bernard-Soulier platelets are presented in Table II.

Storage pool disease and grey platelet syndrome.

Three patients with α-granule deficiency (grey platelet syndrome) and three patients with dense body deficiency were studied. Basal prothrombinase activities of both types of abnormal platelets were similar to normal platelets.

TABLE I

Prothrombinase activities of pathological platelets.

Numbers in parentheses refer to the range of activities found.

	Prothrombinase activity (nM thrombin / min / 10^8 cells per ml)			
Disorder	Non-stimulated	Thrombin stimulated	Collagen + thrombin stimulated	Lysed platelets
Control (n=15)	5.5±2.0 (2.1-7.0)	12.7±4.0 (8.5-18.7)	70.5±17.1 (52.7-89.3)	521±146 (343-715)
Storage pool disease (n=3)	3.8±1.7 (1.8-4.8)	14.1±1.8 (12.3-15.8)	54.1±15.5 (36.8-66.7)	471±123 (393-613)
Grey platelet syndrome (n=3)	3.1±4.7 (0.1-8.5)	21.7±8.8 (14.4-31.4)	99.4±40.9 (66.9-145.3)	616±242 (416-885)
Glanzmann's thrombasthenia (n=4)	5.6±2.7 (2.8-8.6)	12.1±3.4 (9.5-17.0)	59.5±16.8 (42.7-78.2)	381±87 (264-470)

Stimulation by thrombin resulted in slightly higher activities of the grey platelets compared to dense body deficient platelets, the latter being not significantly different from thrombin stimulated normal platelets. Stimulation of both types of platelets with collagen plus thrombin leads to prothrombinase activities that largely overlap the range found for the corresponding normal platelets, although the range of activity of grey platelets is in the high and the activities of dense body deficient platelets are in the low region of the normal range. Nevertheless, the conclusion seems to be justified that neither release of α-granules, nor release of dense bodies is required for exposure of prothrombinase activity induced by collagen plus thrombin. It should be noted that although both abnormal platelets lack one type of granule and thus a certain amount of granule membrane phospholipids, the expected decrease in phospholipid content is not reflected in the prothrombinase values of lysed platelets.

Glanzmann's thrombasthenia.

Prothrombinase activities of platelets from patients with Glanzmann's thrombasthenia are indistinguishable from those of normal platelets, either in the stimulated or in the non-stimulated form (Table I). Maximal prothrombinase activity obtained after complete lysis was found to be in the same range as observed for control platelets, suggesting that there is no difference in phospholipid content or overall phospholipid composition of thrombastenic platelets. Washed suspensions of thrombastenic platelets were used to check aggregation induced by thrombin or the combined action of collagen plus thrombin. This was confirmed to be seriously impaired. It is concluded that aggregation is not a prerequisite for exposure of phosphatidylserine at the platelet outer surface, leading to an increased prothrombinase activity.

Bernard-Soulier platelets ('giant' platelets)

The results obtained with platelets from patients with Bernard-Soulier syndrome are presented in Table II. The most remarkable feature of the platelets from this group of patients was the high level of the basal prothrombinase activity. In all five patients that were examined, the prothrombinase activity of the non-stimulated platelets was approximately 10-fold higher than that of non-stimulated normal platelets. Because of the large size of Bernard-Soulier platelets, PRP had to be obtained by sedimentation of whole blood for 1 hour at 1g, which could be of influence on the procoagulant activity of the platelets. However, no difference in prothrombinase activity of normal platelets was found, whether PRP was obtained by sedimentation at 1g or by centrifugation at 120g. Furthermore, the possibility that Bernard-Soulier platelets, because of their abnormal size, are more readily activated or lysed, merely as a result of stirring during the prothrombinase assay, was checked by comparing prothrombinase activities after an incubation period of 10

minutes. No significant difference in activity of stirred and non-stirred diluted PRP could be detected (data not shown).

TABLE II

Prothrombinase activities of Bernard-Soulier platelets.

Activities are given as nM thrombin / min / 10^{8} cells per ml.

Patient	Non-stimulated	Thrombin stimulated	Collagen + thrombin stimulated	Lysed platelets
1	38.0±7.6	56.0±16.3	129.5±7.8	670±69
2	21.9±12.9	33.5±10.8	98.1±10.8	1239±88
3	38.0±14.4	93.8±53.3	193.5±53.5	1238±76
4	69.6±18.0	109.8±49.6	161.8±81.3	1473±127
5	46.8±21.1	94.9±16.0	121.8±53.5	1029±58
Mean	42.8±17.4	77.5±31.7	140.9±37.2	1130±302
6**	17.4±3.5	36.6±11.0	80.6±6.4	1027±112
Control	5.5±2.0	12.7±4.0	70.5±17.1	521±146

** *May-Hegglin anomaly.*

Stimulation by thrombin gives a two-fold increase of the procoagulant activity of resting Bernard-Soulier platelets, an increase that is slightly less than seen with normal platelets. An even smaller increase compared with the increase seen with normal platelets is found when stimulation is carried out , by the combined action of collagen plus thrombin. Prothrombinase activity of normal platelets increases from 5.5 to 70.5 nM IIa/min upon stimulation with collagen plus thrombin. In comparison, the activity of Bernard-Soulier platelets increases from 42.8 to 140.9 nM IIa/min upon stimulation (Table II). Finally, a large difference was found between the prothrombinase activities of the lysed platelets, which were on the average two-fold higher for the Bernard-Soulier platelets as compared to the control platelets.

Since procoagulant activity is dependent on the amount of suitable phospholipid surface [2,4,5], the increased prothrombinase activity of Bernard-Soulier platelets could be due to an increased size reported for these platelets [16,17]. On one occasion we had the opportunity to measure the mean platelet volume of preparations from three Bernard-Soulier patients. The values are given in Table III, including the corresponding values of the prothrombinase activities of the nonstimulated platelets. The non-spherical shape and the existence of invaginations make it impossible to calculate a mean platelet surface area from the data in Table III. However, an increase in volume is accompanied by a relatively smaller increase in surface area of the platelet. Thus, the increased prothrombinase activities of non-stimulated Bernard-Soulier

platelets, being some 10-fold higher than with control platelets, cannot be ascribed exclusively to an increased membrane surface area, this area being less than two-fold larger than that of control platelets.

TABLE III
Comparison between mean platelet volume and prothrombinase activity of three Bernard-Soulier patients.

Patient	Prothrombinase activity nM IIa/min/(10^6cells/ml)	Mean platelet volume (μm^3)
Control	5.5±2.0	7.9
1	46.8±21.1	14.8
2	38.0±14.4	13.8
3	69.6±18.0	15.2

From the case histories of the five Bernard-Soulier patients, it appeared that four of them underwent splenectomy. In order to see whether this could be of any influence on the platelet procoagulant activity, the prothrombinase activity of platelets from two healthy persons who were splenectomized after a traffic accident, was investigated. Procoagulant activities of these platelets, either non-stimulated or stimulated by collagen and/or thrombin, fell completely within the range of activity of normal platelets (data not shown).

One patient with May-Hegglin anomaly was investigated since this syndrome was described to be accompanied by the presence of giant platelets [18]. Indeed, the prothrombinase activity of the lysed platelet suspension suggests an increased amount of phospholipid per platelet, similar to the Bernard-Soulier platelets (Table II). The procoagulant activity of the non-activated platelets from this particular patient also appeared to be several times higher than the normal range. The same was found after stimulation by thrombin. The maximal prothrombinase activity of 80.6 nM IIa/min found after stimulation by collagen plus thrombin, however, was not very different from that found for normal platelets (52.7 - 84.3 nM IIa/min).

From several of the patients described above, we have also been able to measure the factor X-activating capacity of the platelets according to van Dieijen et al. [5]. Similar results to those obtained for prothrombinase activities were found in all cases (data not shown).

Discussion.

This paper describes the procoagulant activity of pathological platelets measured as the ability of platelets to enhance the rate of thrombin formation by the action of factors Xa and Va on prothrombin. The assay, which was originally developed for washed platelets, appeared to be equally valid when performed in PRP, diluted sufficiently to allow neglectance of the plasma coagulation factors in comparison with the concentrations of the purified factors Xa, Va and prothrombin that are added to the system.

No large abnormalities were found in prothrombinase activities of storage pool deficient platelets. Thus, exposure of a procoagulant surface does not require release of dense body or α-granule contents. This confirms the observation that stimulation of normal platelets by thrombin alone does not lead to a large increase in prothrombinase activity, which supports the idea that the expression of a procoagulant surface is not a result of the fusion of granule membranes with the plasma membrane occurring during the release reaction [1,6,7].

The major defect of platelets from patients with Glanzmann's thrombasthenia is the lack of aggregation in response to several agonists. The primary cause of this defect is the absence of membrane glycoproteins IIb and IIIa [12,15,19]. The observation that the prothrombinase activity of these platelets does not differ from normal platelets, either in the stimulated or in the non-stimulated form, allows the conclusion that glycoproteins IIb and IIIa are not directly involved in the exposure of this activity. Moreover, it can be concluded that aggregation is not required for the expression of prothrombinase activity. This seems to be in contrast with findings of Hardisty and Hutton [20,21] and Walsh [22] who found a severely impaired platelet factor 3 (PF3) activity in thrombasthenic platelets. Also based on observations with different pathological platelets, it was concluded that PF3 activity was merely a reflection of platelet aggregability [21]. However, the PF3 assay used by these authors is not only dependent on the exposure of procoagulant phospholipids, but is also influenced by other factors as has been discussed previously [1,6]. Therefore, some caution should be exercised when comparing platelet prothrombinase activities obtained by a Stypven assay (PF3) or by the more defined assay used here, which is only dependent on the exposure of a procoagulant surface. In conclusion, the bleeding severity in thrombasthenia is presumably not due to a defect in prothrombinase activity. The most important determinant for this bleeding disorder is the decreased or absent fibrinogen binding to these platelets as described by Lee et al. [23].

Aberrant prothrombinase activities were found for Bernard-Soulier platelets. This was particularly manifested in the prothrombinase activities of non-stimulated

platelets which appeared to be approximately 10-fold higher than found for normal platelets. Bernard-Soulier platelets are predominantly characterized by their large size [16,17] and the absence of membrane glycoproteins Ib, V and IX [14,24,25,26]. Although GP Ib is considered to be the high affinity binding site for thrombin and GP V the surface substrate that is cleaved by thrombin [27], the prothrombinase activity of Bernard-Soulier platelets can still be increased upon treatment with thrombin, though to a somewhat smaller extent than in normal platelets. Maximal prothrombinase activity after stimulation with collagen plus thrombin is only two-fold larger than for normal platelets. This probably reflects the increased size of Bernard-Soulier platelets, which can also be seen from a comparison between the prothrombinase activities of lysed normal platelets and lysed Bernard-Soulier platelets. This is in agreement with the observation of Perret et al. [28] that Bernard-Soulier platelets have an increased phospholipid content. However, the difference in size or phospholipid content is insufficient to explain the increased activity of non-stimulated Bernard-Soulier platelets (see Table II). The most likely explanation for the increased basal prothrombinase activity can be obtained from the work of Perret et al. [28] who found an abnormal phospholipid organization in Bernard-Soulier platelets. Specifically they found an increased exposure of phosphatidylserine (and phosphatidylethanolamine) at the platelet outer surface at the expense of sphingomyelin. We have previously shown that an increased prothrombinase activity is accompanied by an exposure of phosphatidylserine at the platelet outer surface [7].

The altered phospholipid distribution in Bernard-Soulier platelet membranes could have several possible causes. A lack of glycoprotein Ib could be responsible for a change in phospholipid organization. Several investigators [29,30,31] recently showed that glycoprotein Ib is associated with cytoskeletal structures and thus forms a site of interaction between cytoskeleton and plasma membrane. We have found that platelet cytoskeletal proteins might be involved in maintenance of the asymmetric phospholipid distribution [32] as was also demonstrated for the red cell membrane [33]. A defect in membrane-cytoskeletal interaction due to lack of glycoprotein Ib could lead to an altered membrane architecture and hence to a different phospholipid distribution in Bernard-Soulier platelets. Another possible explanation arises from the work of Chevalier et al. [34] who showed that Bernard-Soulier platelets have an abnormal distribution of intramembranous particles which are considered to be integral membrane proteins that are visualized by freeze-fracture electron microscopy. The significantly decreased number of particles in the outer fracture face might indicate an increased protein-free phospholipid surface which could lead to an increase in possible binding sites for the prothrombinase complex. Finally, it

is possible that the altered phospholipid asymmetry reflects a partly activated state of the platelets induced during circulation, due to an increased susceptibility towards activators. This might be caused by the increased platelet size. In this respect, it is of interest to mention that a positive relationship between platelet procoagulant activity and platelet size has been observed in a number of patients with diabetes mellitus [35]. Our observation with the one May-Hegglin patient is not inconsistent with this notion.

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CHAPTER 6

STIMULATION OF PROTHROMBINASE ACTIVITY OF PLATELETS AND ERYTHROCYTES BY SUB-LYTIC TREATMENT WITH PHOSPHOLIPASE C FROM CLOSTRIDIUM WELCHII.

Summary.

Treatment of platelets or red cells with small amounts of phospholipase C from Clostridium welchii enables both cells, prior to the onset of lysis, to stimulate prothrombin conversion by factor Xa and Va in the presence of calcium. Phospholipase C treatment of both cells also exposes significant amounts of phosphatidylserine at the outer surface. The level of phosphatidic acid formed from diglycerides produced by phospholipase C action, is similar to that formed in activated platelets upon triggering the phosphatidylinositol cycle. A possible involvement of this cycle in the activation of platelets to become more procoagulant is discussed.

Introduction.

We have previously shown that stimulation of human blood platelets by the combined action of collagen plus thrombin leads to increased exposure of phosphatidylserine in the outer leaflet of the plasma membrane, presumably resulting from an induced transbilayer movement of phospholipids [1,2]. This activaton procedure enables the platelets to enhance the conversion of prothrombin to thrombin by the coagulation factors Xa and Va in the presence of calcium. This catalytic effect is caused by increased complex formation of coagulation factors and negatively charged polar headgroups in the membrane phospholipid surface [3-5]. Both in platelets and erythrocytes phosphatidylserine is the major negatively charged phospholipid present, but it is virtually absent from the exterior surface of the membrane [6-8]. Its exposure at the outer surface as can occur during platelet activation, may serve to stimulate the coagulation process [9,10].

It has been shown by Mauco et al. [11] for human platelets and by Allan et al. [12] for human erythrocytes that introduction of diglycerides in the membrane by action of phospholipase C from Clostridium welchii results in the formation of phosphatidic acid, which suggests a transbilayer movement of diglycerides to the inner monolayer of the membrane where diglyceride kinase and ATP are available [12,13]. We have previously demonstrated [14] that incubation of platelets with phospholipase C from C. welchii results in an increase of their ability to enhance the conversion of prothrombin to thrombin by the factor Xa-Va complex. At that time we suspected the known lytic effect of phospholipase C from C. welchii [6,11,12] to be responsible for this phenomenon since lysis results in exposure of the inner leaflet of the plasma membrane where phosphatidylserine is located, while this lipid is not degraded by phospholipase C [6]. We now show, however, that before the onset of lysis, phospholipase C treatment of red cells and platelets enables both cells to stimulate prothrombin conversion and that this effect can be ascribed to an increased exposure of negatively charged phospholipid at the cell outer surface.

Materials and methods.

Phospholipase C from Clostridium welchii and phospholipase A_2 from Naja naja were purified according to Zwaal et al. [6]. Blood coagulation factors Va, Xa and prothrombin were prepared as described in Rosing et al. [3].

Blood was drawn from healthy male volunteers and collected in acid-citrate-dextrose (0.052 M citric acid, 0.08 M trisodium citrate, 0.183 M glucose, 1 vol of ACD to 5 vols of blood). Platelet-rich plasma and erythrocytes were separated by centrifugation at 200 g for 15 minutes

at room temperature. Erythrocytes were washed three times in 0.9% NaCl (w/v) by centrifugation at 1000 g for 10 minutes. Platelets were pelleted from platelet-rich plasma by centrifugation at 600 g for 15 minutes. The platelet pellet was carefully resuspended in a buffer containing 136 mM NaCl, 2.7 mM KCl, 2.0 mM $MgCl_2$, 25 mM glucose, 10 mM Hepes and 0.05% (w/v) fatty-acid free human serum albumin, pH 6.6 (Hepes buffer). The platelets were washed twice in this buffer by centrifugation at 600 g for 15 minutes. Before each centrifugation 1 vol of ACD was added to 15 vols of platelet suspension. Finally the platelets were resuspended in Hepes buffer pH 7.5. Before adding phospholipase C, platelets and erythrocytes were diluted in Hepes buffer pH 7.5 to a cell count of $5*10^7$/ml using a Coulter counter. Ca^{2+} was added to a final concentration of 3 mM.

Hemolysis of erythrocytes was measured as the release of hemoglobin, determined by the optical density at 418 nm. Platelet lysis was measured as the leakage of lactate dehydrogenase [15].

Phospholipids were extracted from erythrocytes and platelets according to Bligh and Dyer [16], separated by two-dimensional thin layer chromatography and analyzed as phosphorous as described earlier [6]. Methyl-phosphatidic acid, prepared from egg phosphatidylcholine [17], was used as external standard.

The rate of thrombin formation in the presence of red cells or platelets was measured essentially as described before [1]. Briefly: to 300 µl of a cell suspension, 13 µl 75 mM $CaCl_2$ and 50 µl of a mixture containing 30 nM factor Xa and 60 nM factor Va were added. After a period of 2 minutes to allow equilibration of the clotting factors at the cell surface, the enzymatic reaction was started by addition of 125 µl 16 µM prothrombin. After 1 minute a sample was taken and immediately diluted in a buffer containing 120 mM NaCl, 50 mM Tris and 2 mM EDTA (pH 7.5) to stop the reaction. The amount of thrombin formed was determined by measuring the change in absorbance at 405 nm per unit time, produced by the action of thrombin on the chromogenic substrate S2238 (AB Kabi Diagnostica, Sweden), using a calibration curve made with known amounts of active-site titrated thrombin.

Results and discussion.

Figure 1 shows that upon incubation with phospholipase C from C. welchii (0.01 I.U./ml) both platelets and erythrocytes show an increasing ability to enhance thrombin formation before significant lysis of the cells occurs. To confirm that this activity can be ascribed to the appearance of negatively charged phospholipids at the outer surface of the cells, phospholipase C-treated cells were subsequently subjected to treatment with phospholipase A_2 from N. naja.

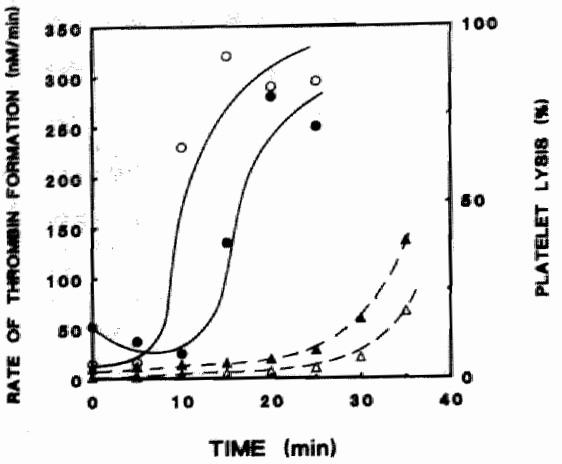

Fig.1: Prothrombinase activity of human red cells (○) and platelets (●) induced by phospholipase C (C. welchii) treatment. Dashed lines represent lysis of erythrocytes (△) and platelets (▲).
0.01 IU/ml phospholipase C is added at t=0 (1 IU is defined as the amount of enzyme able to degrade 1 μmole of substrate per minute at 37°C under optimal conditions [6]).

Since the incubation with phospholipase C alone causes lysis after 30 minutes to both erythrocytes and platelets, cell suspensions (25 ml, $2*10^8$ cells/ml, 10 mM Ca^{2+}) were first incubated during 10 minutes with phospholipase C from C. welchii (0.01 IU/ml). After this preincubation period phospholipase A_2 from N naja (3 IU/ml) was added. Lysis remained below 10% for both cell preparations during 15 minutes following the addition of phospholipase A_2, while the lysis rapidly increased upon further incubation. Samples for lipid analysis were taken 15 minutes after addition of phospholipase A_2. Lipid degradation was stopped by adding EDTA (final concentration 15 mM) and o-phenantroline (final concentration 2 mM). Prior to lipid extraction and analysis methyl-phosphatidic acid was added as external standard.

As shown in Table I, preincubation with phospholipase C allows extensive degradation of phosphatidylserine by subsequently added phospholipase A_2. Some 35 to 40% of the phosphatidylserine in both cells can be hydrolyzed while cell lysis remains below 10%. It should be emphasized that phosphatidylserine is no substrate for phospholipase C from C. welchii and also that this phospholipid is not degraded upon incubation of platelets [1] or red cells [6] with phospholipase A_2 alone. Since in intact erythrocytes and platelets phosphatidylserine is almost exclusively located in the inner leaflet of the plasma membrane [6-8],

the data suggest that substantial amounts of phosphatidylserine become exposed at the exterior surface of platelets and erythrocytes during phospholipase C treatment.

TABLE I
Degradation of phospholipids in human platelets and erythrocytes by the combined action of phospholipase C (C.welchii) and phospholipase A_2 (N.naja)

Phospholipid	Erythrocytes	Platelets
Phosphatidylserine	33-39	35-43
Sphingomyelin	29-40	27-33
Phosphatidylcholine	15-27	25-40
Phosphatidylethanolamine	18-21	16-19

Range of degradation obtained from 4 separate experiments is expressed as percentage of the amount of each lipid class present in non-treated cells. Data are corrected for cell lysis assuming complete phospholipid degradation in the population of the lysed cells. Lysis was always below 10%.
Phosphatidylserine is only degraded by phospholipase A_2, since it is no substrate for C.welchii phospholipase C. Sphingomyelin is no substrate for phospholipase A_2, its degradation being solely caused by phospholipase C. Phosphatidylcholine and phosphatidylethanolamine are attacked by both enzymes.

However, the amount of phosphatidylserine degraded by phospholipase A_2 cannot be directly interpreted as the actual amount of this lipid exposed at the outer surface. Cell lysis, which inevitably occurs, may precede a complete degradation of the outer monolayer. Moreover, appearance of phosphatidylserine in the outer leaflet presumably results from a transbilayer movement and it is likely that this process proceeds during phospholipase A_2 treatment. Also, it cannot be excluded that transbilayer movement is influenced by the phospholipase A_2 treatment per sé.

The increased exposure of negatively charged phosphatidylserine at the outer surface of both cells following phospholipase C treatment can explain their ability to enhance prothrombin conversion. It may also be possible that the formation of phosphatidic acid, resulting from diglyceride kinase action on diglycerides formed by phospholipase C, contributes to this effect [18]. However, under our experimental conditions, treatment of both red cells and platelets with 0.01 IU of phospholipase C for 10 minutes results in the formation of 2-4% of phosphatidic acid (expressed as percentage of total lipid phosphorous). This amount is insufficient to fully explain the enhancement of prothrombinase activity, even assuming that all phosphatidic acid formed in the inner leaflet would be subject to transbilayer movement.

It has been shown that diglyceride formation in

bacterial membranes induces phospholipid flip-flop [19,20]. Our results suggest that transbilayer movement of phospholipids (particularly phosphatidylserine) occurs after formation of diglycerides and phosphatidic acid in blood cell membranes. A possible involvement of ceramides formed from sphingomyelin by phospholipase C action can be ruled out, since treatment of the cells with sphingomyelinase does not lead to an increased exposure of phosphatidylserine towards exogenously added phospholipase A_2 [6,8]. Also, sphingomyelinase treatment of red cells and platelets does not increase their ability to enhance prothrombin conversion to levels comparable to those obtained after phospholipase C treatment [14].

Diglycerides and phosphatidic acid can produce local disturbances in the bilayer structure by the formation of intra-bilayer inverted micelles, which can induce transbilayer movement of phospholipid [21]. Also, changes in physical properties of artificial membranes have been described [22] upon replacing phosphatidylinositol by diglycerides. In situ, diglycerides and phosphatidic acid can be formed in many cells from phosphatidylinositol by the action of phosphatidylinositol-specific phospholipase C and diglyceride kinase [23]. In activated platelets, this can lead to a phosphatidic acid level close to 2% of total lipid phosphorous [24-26], which is very similar to the amount of phosphatidic acid formed during a 10 minutes incubation of the cells with 0.01 IU/ml of phospholipase C. Therefore, diglycerides and phosphatidic acid formed in the PI-cycle of activated platelets may produce perturbations of the bilayer structure which could play a role in evoking transbilayer movement of phospholipids, leading to an increased exposure of phosphatidylserine in the outer leaflet. This process is of physiological importance since it would switch on the platelets to become more procoagulant.

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CHAPTER 7

THE INVOLVEMENT OF CYTOSKELETON IN THE REGULATION OF TRANSBILAYER MOVEMENT OF PHOSPHOLIPIDS IN HUMAN BLOOD PLATELETS.

Summary.

Activation of human platelets by different activators resulted in a different extent of degradation of the cytoskeletal proteins actin-binding protein and myosin, as well as of the non-cytoskeletal protein P235. The highest extent of proteolysis was observed with Ca-ionophore A23187 and decreased on going from A23187 > collagen plus thrombin > collagen > thrombin = ADP. The same order of potency has been found previously (BBA, 736 (1983) 57-66) for the ability of platelet activators to induce exposure of aminophospholipids in the outer leaflet of the platelet plasma membrane, and to stimulate platelets to become procoagulant. Degradation of cytoskeletal proteins as a result of platelet stimulation by collagen plus thrombin was prevented in the presence of dibutyryl cAMP or EDTA but not in the presence of aspirin. This also runs in parallel with platelet procoagulant activity. Moreover, platelets from a patient with a partial deficiency in platelet procoagulant activity revealed a diminished extent of degradation of cytoskeletal proteins upon platelet stimulation by collagen plus thrombin. It is concluded that alterations in cytoskeletal organization upon platelet stimulation may lead to alterations in the orientation of (amino)phospholipids in the plasma membrane, and may therefore play a regulatory role in the expression of platelet procoagulant activity.

Introduction.

The asymmetric distribution of phospholipids between the inner and the outer layer of the platelet plasma membrane is disturbed when platelets are treated with calcium ionophore A23187, SH-oxidizing agent diamide, or when platelets are stimulated by the combined action of collagen and thrombin [1,2]. These treatments result in an increased exposure of aminophospholipids in the outer leaflet of the plasma membrane, resulting from an induced transbilayer movement of phospholipids. Consequently, these platelets become able to enhance the conversion of coagulation factor X to Xa by a complex of coagulation factors IXa and VIIIa, and of prothrombin to thrombin by a complex of coagulation factors Xa and Va [1-3]. Both catalytic effects are caused by complex formation of coagulation factors and negatively charged phospholipid headgroups provided by the increased exposure of phosphatidylserine [4,5].

The non-random orientation of phospholipids in resting platelets resembles that in red cells [6-9]. The origin of this asymmetry and the mechanisms responsible for its regulation are still open to conjecture, but recent studies with red cells have indicated that interactions between the major cytoskeletal protein spectrin and anionic phosphatidylserine may contribute to the maintenance of the orientation of this phospholipid in the inner leaflet of the membrane bilayer [10-13]. Oxidation of spectrin SH-groups by diamide [10], or decoupling of spectrin from the bilayer in spicules from irreversibly sickled erythrocytes [13], results in increased exposure of aminophospholipids (including phosphatidylserine) at the outer surface of the erythrocyte. Also in platelets, treatment with diamide has been shown to produce extensive cross-linking of cytoskeletal proteins (including actin-binding protein, heavy chain myosin and actin) [14,15], and this may result in a dramatic reorientation of aminophospholipids by a mechanism similar to that operating in diamide treated red cells [2,12]. Another distinct possibility to enhance transbilayer movement of phospholipids in platelets is by alteration of the cytoskeleton structure upon activation. In this respect, it is of interest that cytoskeletal organization differs between platelets activated by thrombin or by calcium ionophore [16,17]. Platelets contain a calcium-dependent protease [18,19] that produces almost complete breakdown of actin-binding protein (as well as of the non-cytoskeletal protein P235) when platelets are activated by A23187, whereas activation by thrombin does not result in degradation of cytoskeletal proteins as judged from one-dimensional polyacrylamide gel electrophoresis [16]. In a recent study using two-dimensional polyacrylamide gel electrophoresis, Fox et al. [20] were able to show limited calcium-dependent proteolysis of actin-binding protein upon stimulation of platelets with thrombin.

In view of the finding that the amount of phosphatidylserine exposed at the platelet outer surface depends on the activation procedure [2], we were interested to study whether this would correlate with calcium-dependent proteolysis of cytoskeletal proteins. For this purpose, we used one-dimensional polyacrylamide gel electrophoresis for convenient comparison between the gels. Moreover, two of the known products of calcium-dependent proteolysis (M~, 48 kD and 135 kD) are not found on two-dimensional gels, presumably because they have isoelectric points beyond the range of the Ampholines usually employed [20]. On one-dimensional gels, however, the degradation fragment of M~ 100 kD is usually not observed, perhaps because it comigrates with α-actinin (M~ 105 kD) [19].

Materials and Methods.

Human platelets were isolated from freshly drawn blood and washed by centrifugation as described previously [2]. All steps in the washing procedure were carried out in plastic tubes at room temperature. Prior to the activation procedure, washed platelets were resuspended at a concentration of $2*10^7$ platelets/ml in Hepes buffer (pH 7.5)· containing 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes and 5 mM glucose. Stirred platelets suspensions were made 3 mM in calcium (unless otherwise stated) and activated with different stimulators at 37° C. After a 5 minutes activation period, platelets were spun down at 1000 g for 2 minutes. Platelet pellets were dissolved in 2% SDS (w/v), 5% ß-mercaptoethanol (v/v) and 1 mM EDTA. Electrophoresis was carried out as described by Laemmli [31] using 7.5% polyacrylamide gels with a 4% stacking gel. Gels were stained with Coomassie Brilliant Blue. Cytoskeletal preparations were made essentially as described by Rosenberg et al. [32]. Briefly, to a platelet preparation (control or activated) was added $1/10^{th}$ vol of a solution containing 10% Triton X-100, 100 mM EDTA, pH 7.5. After stirring for 10 minutes in ice, the samples were centrifuged at 1000 g for 10 minutes. The pellets were treated as described above for total platelet preparations.

Results and Discussion.

Fig. 1 shows the protein patterns of control platelets, and platelets activated for 5 minutes with thrombin, collagen, collagen plus thrombin, calcium ionophore A23187, and ADP. On the basis of their apparent molecular weights and by comparison with previous studies [16-20], actin-binding protein, P235, heavy chain myosin, α-actinin and G-actin were identified. In concordance with previous investigators using a similar one-dimensional polyacrylamide gel electrophoresis [16,17], A23187 produced virtual

complete breakdown of actin-binding protein and the protein P235 and the formation of four major degradation products of M- 190 kD, 135 kD, 93 kD and 48 kD (Fig. 1, lane E).

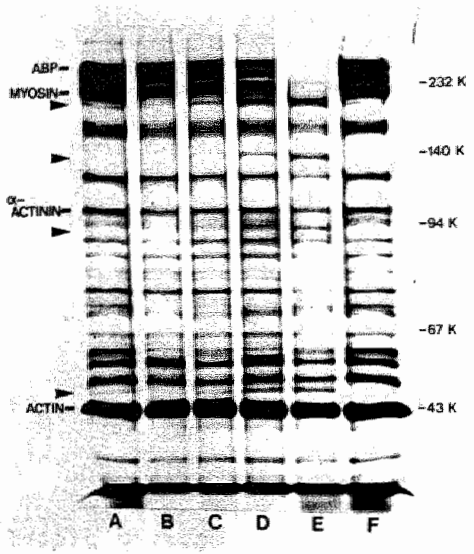

Fig.1: Protein patterns from activated platelets. Lane A, non-stimulated platelets; lane B, stimulated with 2 nM thrombin; lane C, stimulated with 10 µg/ml collagen (Hormon Chemie, Munich); lane D, stimulated with 2 nM thrombin plus 10 µg/ml collagen; lane E, stimulated with 1 µM calcium-ionophore A23187; lane F, stimulated with 10 µM ADP.
Platelet activation, sample preparation and electrophoresis were carried out as described in the text.
The cytoplasmic protein P235 is located between ABP and myosin. The arrowheads designate the four major degradation products. Indicated on the right side are the positions of the molecular weight standards catalase (232 kD), lactate dehydrogenase (140 kD), phosphorylase B (94 kD), bovine serum albumin (67 kD) and ovalbumin (43 kD).

Evidence has been obtained that calcium-dependent proteolysis of actin-binding protein results in the formation of fragments M- 200 kD and 93 kD, while P235 is degraded to fragments of M- 200 kD and 48 kD [21]. Treatment of platelets with A23187 also revealed an essentially complete breakdown of heavy chain myosin, an effect which has not been recognized before. In this respect it is of interest to mention that platelet myosin was shown to be cleaved by endogenous proteases during myosin isolation, to give separable rod (M- 130 kD) and head (M- 100 kD) polypeptides [22]. Therefore, it is possible that the degradation fragment M- 135 kD observed in ionophore treated platelets represents the rod fragment of

myosin.

In contrast to the effect of A23187, platelet activation by thrombin (even at a concentration of 20 nM) produced no visible calcium-dependent proteolysis (Fig. 1, lane B), although limited breakdown has been shown to appear on two-dimensional gels [20]. Stimulation of platelets by collagen seemed to be somewhat more effective than thrombin in that minor formation of the four degradation fragments (M- 190 kD, 135 kD, 93 kD and 48 kD) occurred (Fig. 1, lane C). No change in the extent of degradation was observed when the collagen concentration ranged from 4 to 40 µg/ml or when the activation period varied from 2 to 15 minutes. Simultaneous activation of platelets by collagen plus thrombin produced much more polypeptide degradation (Fig. 1, lane D), than treatment of platelets with either of these stimulants separately. It is evident that activation by collagen plus thrombin resulted in substantial formation of the same four degradation products as observed upon platelet activation by ionophore. Also, a decrease in actin-binding protein, P235 and presumably also in heavy chain myosin is apparent in gels of collagen plus thrombin activated platelets. In general, the degradation patterns were highly reproducible, provided that platelet activation was performed at concentrations below 10^{9} platelets/ml under stirring to produce aggregation. At higher platelet concentrations, the appearance of the degradation products was usually less reproducible, particularly in the preparations activated by collagen or by collagen plus thrombin. It is well known that the higher the platelet concentration, the larger are the aggregates formed. It is therefore conceivable that this will reduce the fraction of platelets in direct contact with collagen, as more platelets will be aggregated by released ADP. Activation of platelets by ADP in the presence of extracellular calcium did not produce any of the proteolysis fragments (Fig. 1, lane F).

Fig. 2 shows that formation of the four degradation fragments induced by activation of the platelets with collagen plus thrombin was essentially the same irrespective of whether platelets were activated for 1 or for 15 minutes (Fig. 2, lanes B and C). However, when platelets were treated first with dibutyryl cAMP (Fig. 2, lane D) or when extracellular calcium is replaced by EDTA (Fig. 2, lane E) none of the degradation fragments were formed. Pretreatment of the platelets with aspirin did not prevent the formation of the four degradation fragments when platelets are subsequently activated by collagen plus thrombin (Fig. 2, lane F). We have previously shown that the appearance of phosphatidylserine at the platelet outer surface as induced after platelet stimulation by the combined action of collagen plus thrombin is abolished in the presence of dibutyryl cAMP or EDTA, but is not prevented in the presence of aspirin [23].

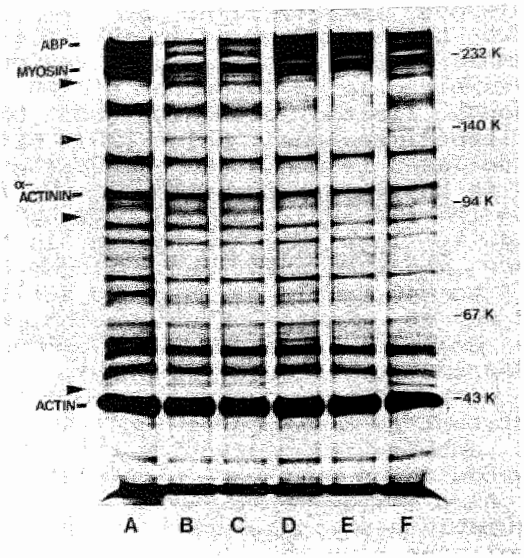

Fig.2: Effect of different platelet function inhibitors on protein patterns from collagen plus thrombin activated platelets. Lane A, non-stimulated platelets; lane B, stimulated with collagen plus thrombin for 1 min; lane C, for 15 min; lanes D,E,F preincubated for 5 min with inhibitors, followed by a stimulation with collagen plus thrombin for 5 min; lane D dibutyryl cAMP (3 mM); lane E, 1 mM EDTA in the absence of calcium; lane F, aspirin (1mg/ml). Further experimental details are described in the legend to Fig.1.

Fig. 3 shows the protein composition of the cytoskeletons, obtained as the Triton-insoluble residues. The main proteins in these preparations are actin-binding protein (M- 250 kD), heavy chain myosin (M- 200 kD), α-actinin (M- 105 kD) and actin (M- 43 kD), which is in agreement with other investigators [22]. In addition it was confirmed that a prominent polypeptide of M- 56 kD is recovered in the cytoskeleton of thrombin-activated platelets [24]. Yields of cytoskeletons were negligible with ionophore treated platelets which may reflect extensive calcium-dependent proteolysis of cytoskeletal proteins. Relative to heavy chain myosin, the largest reduction of actin-binding protein was observed with platelets activated by collagen plus thrombin (Fig. 3, lane E), apart from ionophore treated platelets of which not enough material could be collected. Of the four degradation products observed in protein patterns of the platelet preparations after activation by collagen plus thrombin (Fig. 3, lane F), only the polypeptide of M- 135 kD was (presumably partly) recovered in the corresponding Triton-insoluble residue (Fig. 3, lane E). We did not observe that the highest molecular weight fragment (M- 190 kD) remained associated with the cytoskeleton, as did Truglia and

Stracher [19] after treatment of platelet preparations with highly purified calcium-dependent protease from platelets. The reasons for this discrepancy are not known, but could easily be due to differences in experimental conditions.

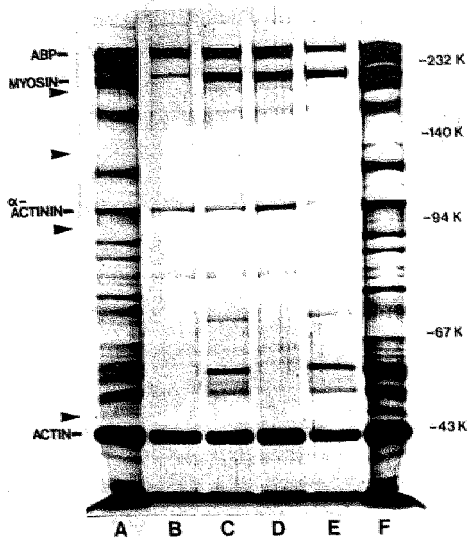

Fig.3: Protein patterns of cytoskeletons from (activated) platelets. Lane A, total protein pattern from non-stimulated platelets; lane B, cytoskeletons of non-stimulated platelets; lane C, cytoskeleton after thrombin (2 nM) stimulation; lane D, cytoskeleton after stimulation with collagen (10 µg/ml); lane E, cytoskeleton after stimulation with collagen plus thrombin; lane F, total protein pattern after stimulation with collagen plus thrombin. Cytoskeletons were prepared as described in the text. For further experimental details see Fig.1.

The results clearly indicate that the extent of calcium-dependent proteolysis upon platelet avtivation depends on the activator: A23187 > collagen plus thrombin > collagen > thrombin = ADP. It is remarkable that both the amount of phosphatidylserine exposed at the outer surface and the concordant ability of platelets to stimulate prothrombinase activity is similarly dependent on the platelet activator (Fig. 4). In addition, we recently studied platelets from a patient earlier described by Weiss et al. [25] to have a deficiency of platelet procoagulant activity. These platelets appeared to have a reduced capability to promote both prothrombin- and factor X-activation after stimulation by collagen plus thrombin, and this was accompanied by reduced exposure of phosphatidylserine at the platelet outer surface [26].

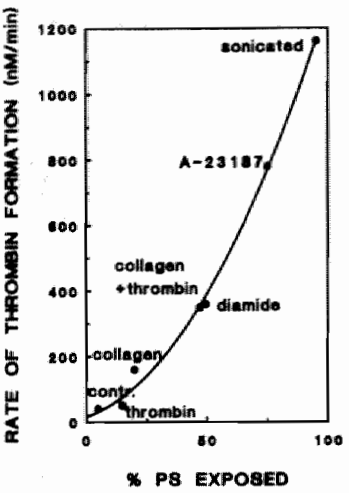

Fig.4: Relation between prothrombin converting activity and exposure of phosphatidylserine in activated human blood platelets. Each point represents a different platelet treatment as indicated. Contr., non-stimulated platelets. This plot is a graphic representation of data from Ref.2.

It is of interest that these platelets also revealed a diminished calcium-dependent proteolysis upon stimulation with collagen plus thrombin (Fig. 5, compare lane D with B).

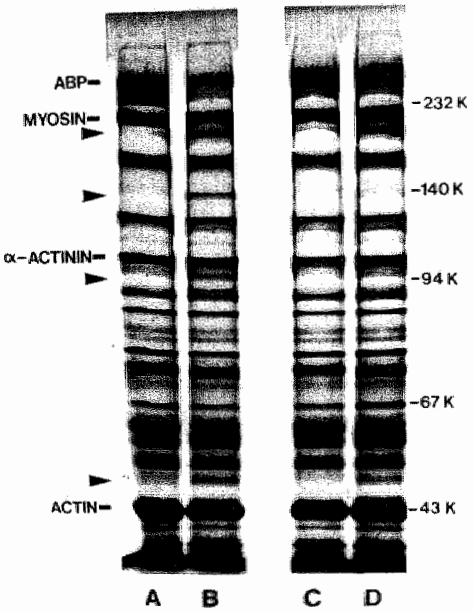

Fig.5: Platelet protein patterns from a patient with a bleeding disorder. Lanes A and B are from a control donor, lanes C and D from the patient. Lanes A and C, non-stimulated platelets, lanes B and D, platelets stimulated with collagen plus thrombin. Further experimental details are given in Fig.1.

In particular, the formation of degradation fragments M- 135 kD and 93 kD was found to be strongly reduced compared to identically activated normal platelets. Treatment of the patients platelets with Ca-ionophore A23187 produced the same extent of proteolysis as found with ionophore-treated normal cells. This strongly

suggests that the patients platelets are not (partly) deficient in calciumdependent protease, but that they have a decreased ability to raise the cytoplasmic calcium concentration upon activation. Also, it is likely that activation of normal platelets by collagen plus thrombin produces a higher increase in cytoplasmic calcium concentration than activation by either of these agonists separately. Dibutyryl cAMP prevents calcium-dependent proteolysis by suppressing cytoplasmic calcium concentrations. The inhibitory effect of EDTA strongly indicates that extracellular calcium has to be taken up in order to attain cytoplasmic calcium levels, sufficiently high to stimulate Ca^{2+}-dependent protease activity.

It is conceivable that degradation of cytoskeletal proteins is accompanied by alterations in cytoskeletal organization which result in a detachment of the cytoskeleton from the interior half of the lipid bilayer membrane. In so far as direct interactions between cytoskeletal proteins and phosphatidylserine exist in platelets (which will be demonstrated in chapter 8 of this thesis), a decoupling might facilitate transbilayer movement of phosphatidylserine similar to that postulated for red cell membranes [12,13]. It should be emphasized, however, that loss of phospholipid asymmetry does not automatically have to occur upon decoupling of cytoskeleton from the membrane. It has been demonstrated that spectrin-free microvesicles obtained from ionophore-treated red cells retain their asymmetric phospholipid distribution, unless incubated for 16 hours at 37°C [27]. It is therefore more likely that decoupling of the cytoskeleton from the lipid bilayer is a prerequisite for the rapid transbilayer movement to occur, the rate of which would be dependent on structural alterations in the membrane bilayer itself to form sites that allow for transbilayer reorientation [28-30].

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CHAPTER 8

INTERACTION BETWEEN PHOSPHATIDYLSERINE AND ISOLATED CYTOSKELETON OF HUMAN BLOOD PLATELETS.

Summary.

Binding experiments were performed to demonstrate a direct interaction between cytoskeletons from human blood platelets and phosphatidylserine. A centrifugation technique employing 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., Biochim. Biophys. Acta 815, 143-148) that in activated platelets a strong correlation exists between degradation of platelet cytoskeletal proteins by the endogenous calciumdependent protease (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. 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 dilauroyllecithin, 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 found a striking correlation between modification of the cytoskeleton and exposure of PS at the outer surface of the plasma membrane. Transbilayer phospholipid 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 protease (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 protease, 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-piperazine ethanesulfonic 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 (0.052M citric acid, 0.08M trisodiumcitrate, 0.183M glucose, 1 part ACD for 5 parts of blood). Platelet rich plasma (PRP) was obtained by centrifugation at 150g for 15 min. After addition of 2% (v/v) of ACD to the PRP, platelets were sedimented by centrifugation at 750g for 15 min. The platelet pellet was washed twice in Hepes buffer pH 6.6 (136mM NaCl, 2.7mM KCl, 2mM $MgCl_2$, 10mM HEPES, 5mM 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 $2.5*10^9$/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 10mM in EDTA. After addition of Triton X-100 to a final concentration of 1% (w/v) the suspension was stirred on ice for 30 minutes. Cytoskeletons were collected by ultracentrifugation (4°C, 30 min., 100,000g). Pellets were washed once with 1% (w/v) Triton in buffer A (136mM NaCl, 2.7mM KCl, 10mM HEPES, 1mM 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 $5*10^9$ 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 100,000g (room temperature). Lipid concentration in the supernatant was determined as phosphorous [27].

Binding assay.

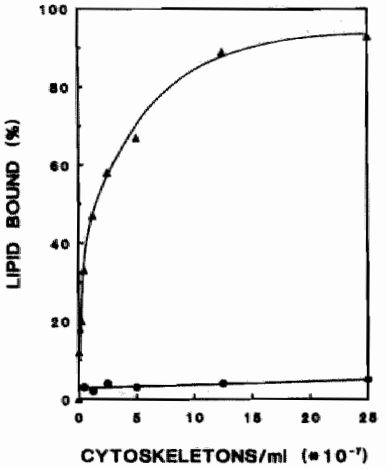
It appeared that after preparing phospholipid vesicles by sonication, it was not possible to obtain a non-sedimentable fraction by taking the supernatant of a pre-centrifuged sample. Also from that supernatant a substantial fraction (20-40%) was sedimented under the experimental conditions. Centrifugation of sonicated vesicles for 30 min. at 100,000g 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 Beckmann 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 was transferred to a centrifuge tube and spun for 30 min. at 100,000g. To assess the unbound fraction of phospholipid 100 μl of the supernatant is transferred to a vial for liquid scintillation counting.

Results and discussion.

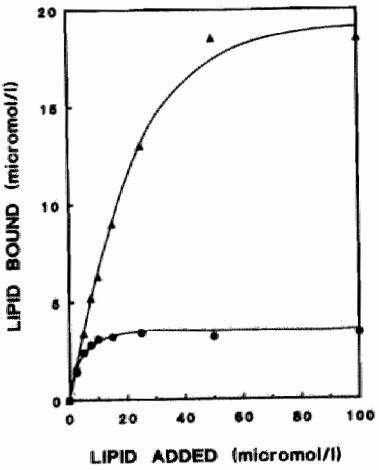
Cytoskeleton of unstimulated platelets is able to bind PS in a concentration dependent manner. The fraction of bound lipid as a function of the concentration of cytoskeleton is shown in Fig. 1.

Fig.1: Phospholipid vesicles (25µM) 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 100.000 g.
▲, phosphatidylserine
●, phosphatidylcholine.



Binding is specific for PS compared to PC-vesicles which do not bind over the whole range of cytoskeleton concentrations tested.

Fig.2: Cytoskeleton in two concentrations was incubated with different concentrations of phosphatidylserine vesicles. The concentration of bound lipid was measured as described in Fig.1.
*●, 5 * 10⁶ cytosk./ml*
*▲, 5 * 10⁷ cytosk./ml.*



The binding curves obtained using two concentrations of cytoskeleton and variable amounts of lipid are shown in Fig. 2, demonstrating 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 Kd 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 ($5*10^7$/ml) for 30', 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.

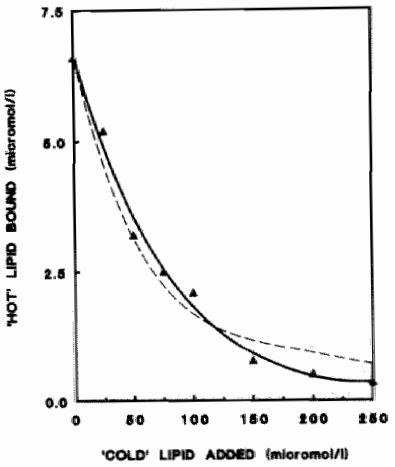


Fig.3: Supersedition of labeled phospholipid (PS) by excess non-labeled PS. Cytoskeletons ($5*10^7$/ml) were incubated for 30 min with 10 µM labeled PS-vesicles. Subsequently non-labeled PS-vesicles were added in increasing concentration. Incubation was carried on for another 30 min. The amount of bound 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 phospholipids and assuming complete equilibration between the labeled and non-labeled pools.

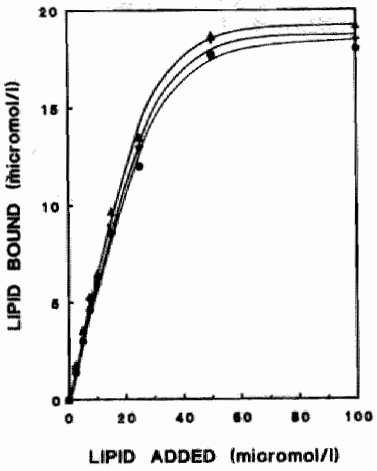
However, if non-labeled PC is used, no change in the amount of bound PS is observed (not shown). Since addition of 10 µM of lipid is not saturating for this amount of cytoskeleton (see Fig. 2), addition of extra non-labeled PS will, apart from displacing labeled PS, also occupy residual free binding 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.

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-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 protease 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.

*Fig.4: Titration of modified cytoskeleton with PS-vesicles. 5*10⁷/ml cytoskeletons were used after modification with calpain (●), diamide (▲) or non-treated (✚). The conditions for the calpain treatment were chosen to produce 50% degradation of myosin as judged by gelelctrophoresis. 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.*



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 crosslinking by diamide could be confirmed by gelelectrophoresis (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 cytoskeleton 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 occurence of fast flip-flop is restricted to the timeperiod during which calpain is active and cytoskeletal proteins are in the process of being degraded.
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 treating 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 disribution 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 aminophospholipid 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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General discussion.

It will be clear from the contents of this thesis that the present ideas about regulation of phospholipid asymmetry have been developed during the last ten years. In 1977, little was known about spontaneous or induced flip-flop in natural and artificial phospholipid membranes. At that time, transbilayer movement of phospholipids was considered to be a very unlikely event, occurring with half-times in the order of several days in lipid vesicles [1,2], to several hours in intact erythrocytes [3-5]. In fact, the very concept that phospholipids have an asymmetric distribution in biological membranes was not postulated until the early seventies, namely by Bretscher [6,7]. Since then, this membrane phospholipid asymmetry has been demonstrated in a wide variety of cells using different techniques whereby one or both membrane sides were reacted with group-specific reagents, lipid transfer proteins or phospholipases (for reviews, see Refs. 8 and 9). Both for erythrocytes [10,11] and platelets [12-14] it was shown that the membrane asymmetry is most pronounced in case of sphingomyelin and PS, which are mainly confined to the outer and inner leaflets of the membrane respectively. The other two major lipids, PC and PE, have a less extreme though still non-random distribution over both halves of the membrane.

A physiological role for the asymmetric distribution of membrane phospholipids was postulated by Zwaal [15,16], based on the catalytic properties of negatively charged phospholipids in blood coagulation. It was proposed that platelets, by exposing a surface containing negatively charged phospholipids during cell activation in reponse to vascular injury, can play a role in the regulation of coagulation. Localization of negatively charged (procoagulant) PS in the inner membrane leaflet of unstimulated platelets thus is essential in preventing untimely acceleration of clotting reactions. Obviously, this applies to all blood cells and cells lining the vessel wall. Indeed, comparing intact cells with completely lysed preparations on their ability to stimulate the rate of thrombin formation, it was shown that not only erythrocytes and platelets, but also intact leukocytes and endothelial cells, are virtually devoid of catalytic potency [17], which implies a similar asymmetric distribution of PS in all these cells.

Apart from the role lipid asymmetry plays in regulating haemostasis, it has also been suggested that it is instrumental in the removal of cells from the circulation. It was shown that enhanced binding of erythrocytes to endothelial cells [18] and recognition by macrophages [19,20], correlates with surface exposure of PS in erythrocytes. These cells are, therefore, rapidly cleared from the circulation [21]. Those erythrocytes which bind to endothelial cells also demonstrate binding of the anionic fluorescent dye Merocyanine 540. This dye was

found to be a reporter of decreased packing density in phospholipid bilayers [22]. It also binds strongly to fluid-phase lipid vesicles, but much less avidly to gel-phase vesicles. Based on the premise that packing density is decreased when membrane asymmetry is lost [23], binding of this probe has been used to test several aspects of phospholipid asymmetry. For instance, it was shown that in lymphocytes the asymmetric distribution of lipids develops during maturation of the cells. Mouse thymocytes were able to bind Merocyanine 540 (MC540), indicating a loosely packed outer monolayer, while mature cells which enter the circulation were unable to bind MC540 [24]. This suggests a reorganization in the lipid distribution over the plasma membrane, leading to a more tightly packed outer monolayer in mature cells (in a state consisting primarily of PC and Sph). Also, erythroid precursor cells and cells transformed to a leukemic phenotype by viral infection, arresting them at the proerythroblast stage, were able to bind MC540 [25]. The suggestion that membrane lipid asymmetry is not yet completely established in immature cells was confirmed by the work of Rawyler et al. using phospholipases [26,27]. They found a partial asymmetry to occur in erythroid precursor cells, with PE and PC still being randomly distributed over the membrane, although PS and Sph are already mainly present in the leaflet to which they are confined in the mature erythrocyte. In addition to these studies showing generation of lipid asymmetry during cellular maturation, also changes in membrane lipid distribution resulting in loss of lipid asymmetry were assessed using MC540 (vide infra).

At present, transbilayer movement of lipids (flip-flop), which for a long time has been considered to be a rare phenomenon, is thought to occur at considerably increased rates whenever the regular bilayer structure is distorted [28]. Among the processes implicated in the acceleration of flip-flop in artificial lipid vesicles are insertion of transmembrane proteins [29], gel to liquid-crystalline phase transition [30], difference in surface pressure between the two monolayers [31] and formation of non-bilayer structures [32]. These non-bilayer structures can be formed when lipids are present that have a conical molecular shape, i.e. a small polar head group relative to the cross-section of the acyl chains, such as diglyceride, phosphatidic acid, cardiolipin and unsaturated PE. Instead of bilayer structures these lipids tend to form hexagonal phases, the smallest unit of such a structure being the intrabilayer inverted micelle [32]. In these inverted micelles, the lipids experience fast isotropic motion, which can readily be detected by ^{31}P NMR. Also by means of freeze-fracture electron microscopy these structures can be visualized as lipidic particles [33]. Inverted micelles are thought to be involved in both enhanced transbilayer movement of lipids and fusion processes as occur in endo- and exocytosis

[28]. For platelets [34] and also for erythrocytes [35], it has been shown that generation of diglycerides in the outer monolayer of the membrane by treatment with exogenously added phospholipase C from C. welchii leads to subsequent formation of phosphatidic acid. Conversion of diglyceride to phosphatidic acid is carried out by the enzyme diglyceride kinase in the presence of ATP. Since both this enzyme and ATP are only present in the cell interior, the formation of phosphatidic acid suggests that flip-flop of diglycerides must have taken place. It has been demonstrated [36] that enhanced flip-flop is not restricted to diglycerides, since both in platelets and in erythrocytes treatment with phospholipase C from C. welchii induces exposure of PS at the outer surface of the plasma membrane as well.

Enhanced flip-flop of phospholipids not directly connected with formation of non-bilayer structures has been observed under several conditions. For erythrocytes it has been suggested that treatment with the SH-oxidizing agent diamide leads to altered membrane asymmetry as detected by availability of the lipids for exogenous phospholipases [37]. However, it has been found [38] that diamide treatment causes destabilization of the bilayer rather than a stationary change in phospholipid distribution. This is indicated by an enhanced flip-flop rate of PC [39,40], as well as by increased binding of MC540 [41], suggesting a more disordered membrane. The observation that membrane destabilization by SH-oxidizing agents is accompanied by sulfhydryl oxidation of the cytoskeletal protein spectrin led to the proposal [42] that the cytoskeleton might be involved in the regulation of membrane phospholipid asymmetry. This concept was supported by the findings of Mombers et al. [43,44] who demonstrated interaction between PS and spectrin by studying the effect of spectrin on the phase transition of lipid vesicles and the penetration of spectrin into lipid monolayers. In ultrastructural studies [45] and a study employing differential scanning calorimetry, monolayers and microfluorescence [46], the interaction between spectrin and PS was confirmed. However, not only spectrin, but also other proteins of cytoskeletal origin were found to interact with phospholipid. The erythrocyte protein band 4.1 was proved to bind to PS vesicles through a centrifugation technique [47,48], an interaction which appeared to be reduced upon phosphorylation of the protein [49]. Also α-actinin [50], profilactin [51] and vinculin [52] appeared to interact directly with phospholipids.

With platelets, further evidence for the possible involvement of the cytoskeleton in regulation of lipid asymmetry can be obtained from experiments showing flip-flop of lipids upon modification of the cytoskeleton [53-55]. For example, activation of platelets by the combined action of collagen plus thrombin or by the calcium-ionophore A23187 gives rise to surface exposure of PS. Concomitant with the flip-flop of PS, degradation of

cytoskeletal proteins by calpain was observed [53]. The same phenomenon occurred when platelets were treated with local anaesthetics (dibucain or tetracain) [54] or with fluoride-ions [55]. In addition, studies with erythrocytes reveal increasing evidence that disturbances of the cytoskeleton can give rise to enhanced flip-flop rates of phospholipids, if not to loss of phospholipid asymmetry [41,56-59]. These observations support the notion that cytoskeleton-lipid interactions play a role in the regulation of membrane lipid asymmetry.

Recently, another mechanism was discovered which supposedly plays an important role in the generation and regulation of membrane lipid asymmetry. In 1984, Seigneuret and Devaux [60] introduced spin-labeled phospholipids in erythrocytes and followed the fate of these molecules by measuring the amount of spin-label which could be reduced by external ascorbate. The non-reducible fraction likely represents the lipids which have been translocated to the inner leaflet of the membrane. The transport of lipid from outer to inner monolayer was also assessed from shape changes occurring in the cells upon incorporation of exogenous lipid. They showed that after incorporation of lipids the erythrocytes crenated immediately. When spin-labeled PS or PE was added, the initially formed echinocytes reversed their shape to discoid and even to stomatocytes, which can be taken to imply transport of material from the outer to the inner leaflet of the bilayer. This process was accompanied by a lower amount of spin-label that could be reduced from the outside. The inward transport of phospholipids inferred from these observations was shown to be rather specific for aminophospholipids (with a preference for PS over PE) and to be dependent on the presence of hydrolyzable ATP.

The spin-labeled phospholipid analogues used in these studies contain a six-carbon acyl chain at the two-position to which a nitroxide group is attached and have the advantage of being readily incorporated in the membrane. A disadvantage may be the structural divergence from the native lipid molecules, inducing the risk of not truely reporting the transport properties of endogenous lipid. However, similar experiments were performed by other groups, using differently labeled phospholipids. Tilley et al. [61] incorporated radio-labeled long-chain phospholipids into erythrocytes by means of a transfer protein and used phospholipases to determine the fraction which remains available for degradation at the exterior surface. The fate of incorporated lysophospholipids was measured by Bergmann et al. [62]. Daleke and Huestis [63] used a range of phospholipids with different acyl chain length and recorded changes in erythrocyte morphology to assess the transport of the incorporated lipids. All these studies essentially confirmed the first observations with spin-labeled phospholipids, i.e. an ATP-dependent transport system for phospholipids is present, which selectively translocates PS and to a lesser extent PE from the outer

to the inner leaflet of the red cell membrane. Furthermore, a transport system with the same characteristics was found in plasma membranes of other cells: lymphocytes [64], cultured fibroblasts [65] and platelets [66]. Apart from its ATP-dependence, this transport was also shown to be inhibited by modification of free SH-groups [63,64,67, 68] or by increase in cytosolic calcium concentration [67,69]. Also, competition between PS and PE for the same translocation site was demonstrated [67], as well as competition between stereoisomers [65]. These observations lead to the conclusion that this outward-inward translocase activity is produced by one or more membrane proteins and functions as a lipid pump in the generation and maintenance of membrane phospholipid asymmetry.

A mechanistic model, adapted from Williamson et al. [70] is depicted in Fig. 1, which in all cells could be responsible for the maintenance of the asymmetric distribution of phospholipids.

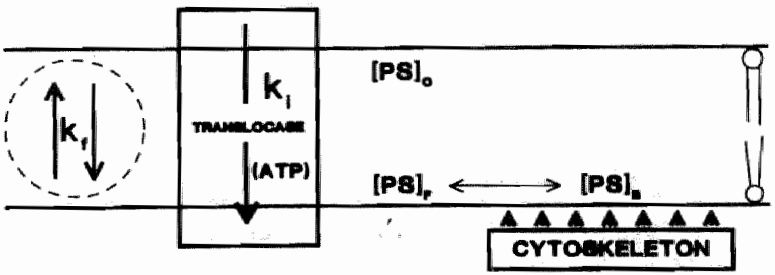

Fig.1: Schematic representation of involvement of translocase and cytoskeleton in the maintenance of PS asymmetry.
From left to right the following processes are depicted. The dashed circle represents spontaneous flip-flop, occurring with a rate constant k_+. Any PS molecule which migrates to the outer monolayer by spontaneous flip-flop is transported back to the inner monolayer through the action of the ATP-dependent translocase. This transport is characterized by a rate constant $k_\perp$. The amount of PS available for spontaneous flip-flop is reduced by binding to the cytoskeleton.
The major difference with the model as proposed by Williamson et al. [70] is that they propose a bidirectional flippase, both inward and outward transport occurring with the same rate constant.

This model takes into account direct interactions between amino-phospholipids and the cytoskeleton, as well as the existence of an amino-phospholipid translocase. The

asymmetric distribution of phospholipids in the plasma membrane of cells is maintained by the action of the translocase. Any PS or, to a lesser extent, PE molecule which migrates by spontaneous flip-flop to the outer leaflet of the membrane is transported back to the inside at the expense of ATP hydrolysis. The amount of lipid experiencing this spontaneous flip-flop is restricted by interaction with cytoskeletal proteins, thereby reducing the amount of ATP needed to maintain asymmetry. The major difference with the theoretical model proposed by Williamson is that we reject the need for outward transport to be ATP-dependent, since any disturbance of the bilayer allowing flip-flop implies (net) transport of asymmetrically distributed lipid species driven by the concentration gradient. It can be envisaged that inward transport of PS and PE is coupled to outward transport of Sph and PC, in order to maintain the balance of material between the two leaflets of the membrane.

Also in resting platelets, the asymmetric distribution of the phospholipids is thought to be maintained by this mechanism. This view is supported by experiments demonstrating the existence of a translocase capable of transporting exogenously incorporated PS and PE to the inner leaflet [66,71]. Also endogenous PS, first exposed at the platelet outer surface as the result of activation, can be transported back to the inner leaflet of the membrane [72]. Moreover, interaction of PS with the platelet cytoskeleton could be proved, in contrast to PC, which does not bind [73].

However, to explain the involvement of platelets in the hemostatic process, these mechanisms are of limited use because they focuss on the maintainance of lipid asymmetry and not on the reverse process that can occur during platelet activation. Platelets contribute to the formation of a clot by becoming procoagulant, which involves exposure of PS at their outer surface [15]. This is inextricably correlated with degradation of (cytoskeletal) proteins by calpain [53-55]. In spite of this correlation, it was not possible to show any influence of either sulfhydryl oxidation or proteolytic digestion of the cytoskeletal proteins on their affinity for PS-vesicles in vitro [73]. This may exclude a possible role for a change in binding parameters in the process of PS-exposure. However, involvement of topological rearrangements of the cytoskeleton occurring within the platelets after activation cannot be excluded to play a role in PS exposure. The concept of involvement of the cytoskeleton other than by a change in the binding to PS is supported by experiments described by Verhallen [74]. Using the fluorescent membrane probe trimethylammonium-diphenylhexatriene (TMA-DPH), Verhallen showed that only during the period of time platelet calpain is active accelerated flip-flop of the probe is observed. In unstimulated platelets, the probe is transported to the inner leaflet of the membrane with an apparent halftime of about 60-90

minutes. However, if the translocation rate of the probe is measured during activation by a calcium-ionophore, the halftime is in the order of seconds rather than minutes, the whole process of fast flip-flop being completed within 30 seconds. This transient burst of flip-flop of the probe is inextricably correlated with the rapid breakdown of cytoskeletal proteins and exposure of PS. In this context, it may be worth mentioning that platelets from a patient suffering from a moderately severe bleeding disorder as described by Weiss et al. [75] showed a diminished breakdown of cytoskeletal protein upon stimulation by the combined action of collagen and thrombin [53], together with a decreased exposure of PS and a reduction of their potency to enhance the formation of factor Xa and of thrombin [76].

Based on the above correlations between modification of the cytoskeleton and the rate at which loss of lipid asymmetry can occur, and taking into account the model previously proposed by Verhallen [74], the following mechanism is postulated for the platelets' ability to present a procoagulant surface at which clotting factors can interact (Fig. 2).

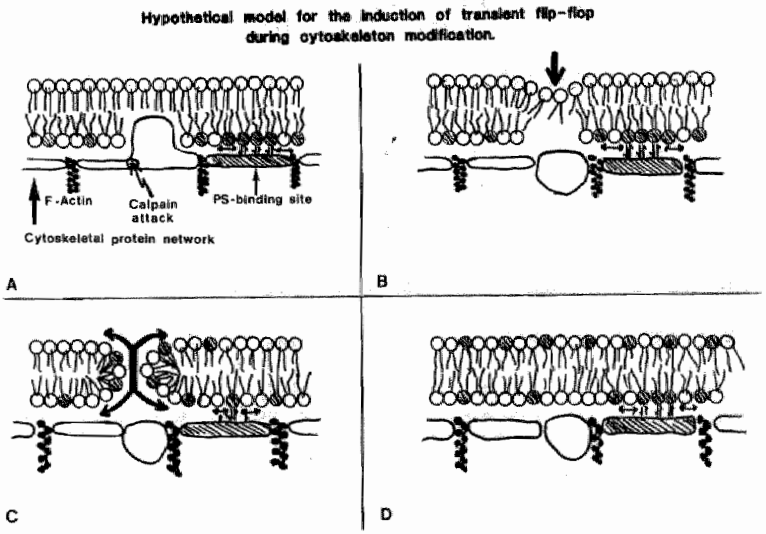

Fig.2: A hypothetical model is depicted of one of several possible mechanisms leading to formation of hydrophilic pore-like structures. Via these pores the asymmetric distribution of PS is lost by diffusion, driven by the concentration gradient. PS molecules are represented by shaded circles. Further details are described in the text.

Intercalated between the lipids of the inner leaflet of

the plasma membrane is a hydrophobic part of a protein of the cytoskeleton (Fig. 2A). Removal of this peptide chain from its position by enzymatic degradation, by oxidative treatment with diamide (shown to concentrate the cytoskeleton in the center of the cell [77]), or by osmotic swelling of the cell membrane, will result in a difference in material present in both monolayers and hence, in a difference in lateral pressure between the two leaflets of the membrane (Fig. 2B). This condition is visualized to favour transbilayer movement, possibly via non-bilayer structures [28,31]. As suggested earlier by Verhallen [74], the pressure difference is annihilated by formation of a pore-like structure (Fig. 2C). Through these pores the available surface for lipid diffusion increases, which favours dissociation of lipids bound to protein. As a result, the lipids will dilute over the total area of surface available, producing a randomization of lipids over the two monolayers before closing of the pores when the pressure difference is terminated by net transport (Fig. 2D).

Although this is a highly speculative model, several aspects are prone to experimental verification. For instance, a protein which is intercalated in the interior leaflet of the membrane should be subject to labeling with hydrophobic photoaffinity reagents, but only before stimulation of the platelet, not afterwards. Also, as already stated by Verhallen [74], the formation of pores in the bilayer during fast flip-flop could be detected as an increased permeability of the cells to uncharged polar solutes or small ions.

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SUMMARY.

Since long it is known that blood cells as well as other cell types possess a plasma membrane which is composed of a bilayer of phospholipids. These phospholipids are non-randomly distributed over the two halves of the bilayer, the monolayers. Choline-containing species are predominantly located in the outer monolayer, while amino-phospholipids mainly reside in the inner monolayer. This asymmetric distribution is correlated with the process of blood coagulation since several reactions of the coagulation cascade are speeded up in the presence of a suitable phospholipid surface and the lipid which is most active in stimulating clotting reactions, phosphatidylserine, is in all cells almost exclusively present in the inner monolayer. Thus, these cells are virtually ineffective in stimulating coagulation (chapter 2). One cell type, namely the blood platelet, is capable of undergoing a massive transport of the procoagulant lipid phosphatidylserine from inner to outer monolayer upon stimulation. Such a stimulation occurs when the endothelial cells, which are lining vessel walls, are damaged, thereby exposing subendothelial structures to the bloodstream. To collagen fibrils, which are present in the subendothelium, blood platelets adhere and become activated. If these platelets are at the same time triggered by the first traces of thrombin, formed as the result of initiation of the coagulation cascade, they become procoagulant. In this form they very efficiently catalyze several reactions of the coagulation cascade, resulting in a strong positive feedback. It is shown that generation of a procoagulant surface is the result of transbilayer movement (flip-flop) of phosphatidylserine (chapter 4). No protein receptors could be demonstrated to play a role in binding of clotting factors to the platelet surface, supporting the notion that the procoagulant nature of the platelet surface is determined solely by phospholipids (chapter 3). Also platelet aggregation and release reaction were shown not to be involved in the generation of procoagulant activity (chapter 5).
To our present knowledge platelets can be activated to become procoagulant in two general ways: on the one hand by an increase in intracellular calcium concentration, on the other by modification of internal free SH-groups.
Both phenomena appear to produce a common result: modification of the intracellular protein matrix, the cytoskeleton. When intracellular calcium increases, as for instance effected by Ca-ionophore, proteolytic digestion by the endogenous Ca-dependent enzyme calpain leads to disruption of the cytoskeletal structure. Incubation of the cells with diamide, which crosslinks free SH-groups, results in changes in the topology of the cytoskeleton. It is shown that a correlation exists between modification of the cytoskeleton and the occurrence of PS flip-flop

(chapter 7) which has been confirmed using various experimental setups.
However, although a direct interaction is demonstrated between isolated cytoskeleton and PS-vesicles in binding experiments, it appeared impossible to show a change in the binding parameters of this interaction after modifying the cytoskeleton with either calpain or diamide (chapter 8). The latter observation excludes the possibility that flip-flop of PS takes place as a mere result of changes in the cytoskeleton leading to a loss in its capacity to interact with this lipid.
This thesis ends with the presentation of a putative model which endeavours to explain the role of the cytoskeleton in both the maintenance of phosphatidylserine asymmetry in cells and the rapid loss of this asymmetric distribution by flip-flop, which is suggested to be a property unique to blood platelets.

SAMENVATTING.

Het is reeds lang bekend dat cellen, waaronder ook de cellen die in het bloed voorkomen, omgeven worden door een plasmamembraan die is opgebouwd uit een dubbellaag van phospholipiden. Deze phospholipiden zijn niet willekeurig verdeeld over de twee helften van de bilaag, de monolagen. Choline-houdende lipiden zijn voornamelijk gelokalizeerd in de buitenste monolaag, terwijl de amino-houdende phospholipiden de meerderheid van de binnenste monolaag uitmaken. Deze asymmetrische verdeling staat rechtstreeks in verband met het bloedstollingsproces aangezien verschillende reakties van de stollingskaskade versneld worden door de aanwezigheid van een geschikt phospholipide-oppervlak en het lipid wat het meest aktief is in het stimuleren van de stollingsreakties, phosphatidylserine, in alle cellen gelokaliseerd is in de binnenste monolaag. Als gevolg hiervan zijn cellen niet aktief in de stolling (hoofstuk 2). Eén celtype, namelijk de bloedplaatjes, is in staat, na aktivatie, een massaal transport te bewerkstelligen van het procoagulante lipid phosphatidylserine van de binnenste naar de buitenste monolaag van de celmembraan. Een dergelijke stimulatie van het bloedplaatje treedt op wanneer de endotheelcellen, die de vaatwand bekleden, beschadigd worden. Bloedplaatjes hechten aan collageenvezels in het onderliggende subendotheel en worden daardoor geaktiveerd. Als deze plaatjes tezelfdertijd worden gestimuleerd door de eerste spoortjes thrombine, die ontstaan als gevolg van het stimuleren van de stolling, worden ze procoagulant. In deze toestand zijn zij in staat zeer efficient verschillende reacties van de stollings cascade te stimuleren, wat resulteert in een sterke positieve terugkoppeling. Er wordt aangetoond dat het ontstaan van een procoagulant oppervlak het gevolg is van transbilaag beweging (flip-flop) van phosphatidylserine (hoofdstuk 4). Er kon geen aanwijzing worden gevonden voor een eventuele betrokkenheid van eiwit receptors in de binding van stolfaktoren aan het oppervlak van bloedplaatjes, wat het idee ondersteund dat het procoagulante karakter van een celoppervlak uitsluitend wordt bepaald door de daarin aanwezige phospholipiden (hoofdstuk 3). Ook van de aggregatie van bloedplaatjes en de releasereaktie werd aangetoond dat deze niet noodzakelijk zijn voor de vorming van een procoagulant oppervlak (hoofdstuk 5).
Voor zover nu bekend is, kunnen plaatjes op twee algemene manieren geaktiveerd worden zodanig dat een procoagulant oppervlak wordt gevormd: enerzijds door verhoging van de interne calciumconcentratie, anderzijds door modificatie van intracellulaire vrije SH-groepen.
Beide fenomenen blijken te leiden tot een gemeenschappelijk resultaat: de intracellulaire eiwitmatrix, het cytoskelet, wordt gemodificeerd. Als de intracellulaire calciumconcentratie toeneemt, zoals b.v. wordt veroorzaakt

door een Ca-ionophoor, zal door aktivatie van het endogene Ca-afhankelijke enzym calpaine proteolytische afbraak van cytoskelet eiwitten leiden tot ontbinding van de structuur van het cytoskelet. Inkubatie van de cellen met diamide, een reagens wat crosslinking van SH-groepen teweegbrengt, resulteert in veranderingen in de ruimtelijke structuur van het cytoskelet. Er wordt aangetoond dat er een korrelatie bestaat tussen modificatie van het cytoskelet en het optreden van PS flip-flop (hoofdstuk 7), wat onder diverse experimentele omstandigheden later bevestigd kon worden.
Toch, hoewel in bindingsexperimenten een direkte interaktie gedemonstreerd wordt tussen geïsoleerd cytoskelet en PS-vesicles (hoofdstuk 8), bleek het onmogelijk een verandering in de eigenschappen van deze interaktie aan te tonen na modificatie van het cytoskelet met hetzij calpaine of diamide. Deze laatste observatie sluit uit dat flip-flop van PS tijdens aktivatie van bloedplaatjes een rechtstreeks gevolg is van een verandering van de capaciteit van het cytoskelet om PS te binden.
Dit proefschrift besluit met het poneren van een model dat poogt de rol te verduidelijken van het cytoskelet in zowel het handhaven van de asymmetrische verdeling van phosphatidylserine als ook het verlies van asymmetrie, wat een fenomeen is dat mogelijk slechts in bloedplaatjes voorkomt.

DANKWOORD

Om te beginnen wil ik mijn gezin roemen, die de laatste twee jaar wel heel erg weinig aan mij hebben gehad. Toch is deze periode dankzij hun steun tot twee jaar beperkt gebleven. Saskia, Marloes, Menno, jullie hebben er goed aan gedaan mij telkens weer aan het werk te zetten, ook wanneer ik niet echt veel zin had. Het heeft wel gewerkt, maar hopelijk komen we als familie nu in wat rustiger vaarwater.
Rob, het zal niet snel nog eens gebeuren dat je een promovendus gedurende bijna twintig jaar kan opleiden voor het wetenschappelijk bedrijf. Wat me in jouw kijk op de wetenschap altijd het meest heeft aangesproken is het streven naar eenvoud, zowel bij het opzetten van experimenten als bij het zoeken naar passende interpretaties. Ik heb de illusie dat ik daarvan in de loop der jaren iets heb opgestoken. Verder wil ik zeker niet onvermeld laten dat zonder jouw initiatief dit proefschrift niet tot stand zou zijn gekomen, waarvoor ik je zeer erkentelijk ben.
Edouard, we hebben de afgelopen jaren prettig en ik geloof ook vruchtbaar samengewerkt. Ik ben blij dat je als mijn co-promotor een grote bijdrage hebt willen leveren aan de uiteindelijke vorm van dit proefschrift.
Als het iemand opvalt dat het gebruikte Engels beter is dan men van mij gewend is, dan heb ik dat te danken aan dhr. P.M.G. Pieters, die mij heeft behoed voor talloze stijl en grammatica fouten.
Mariet, hoewel de uiteindelijke versie van dit proefschrift niet via jouw tekstverwerker is gemaakt, wil ik wel vermelden dat het een geruststellend idee is om altijd bij je te kunnen aankloppen voor de meest uiteenlopende klusjes.
Tenslotte wil ik nog iedereen bedanken met wie ik in de loop der jaren heb samengewerkt, promovendi, analisten en gasten, voor de bijdragen die zij aan dit werk geleverd hebben.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 21 februari 1947 te Utrecht geboren. Na het behalen van het HBS-B diploma aan de Gemeentelijke HBS werd, alvorens de dienstplicht te vervullen, het diploma leerling analist behaald. Na zijn diensttijd werd hij in 1969 als analist aangesteld bij de vakgroep Biochemie van de Subfakulteit scheikunde der Rijksuniversiteit Utrecht. In 1971 legde hij met goed gevolg het staatsexamen Biochemisch Laboratoriumassistent van de S.A.L. af. Hij trouwde in 1974 en werd de trotse vader van een dochter (in mei 1977) en een zoon (november 1978).
Toen zijn huidige promotor in 1977 als hoogleraar werd benoemd aan de Rijksuniversiteit Limburg, is hij op diens verzoek meeverhuisd naar Maastricht, als analist bij de vakgroep Biochemie van de Medische Fakulteit. In die tijd werd de grondslag gelegd voor het werk wat tot dit proefschrift heeft geleid. Inmiddels werd hij bevorderd tot laboratoriumhoofdassistent. In de eerste helft van 1983 volgde hij aan de Katholieke Universiteit de kursus deskundigheid stralingshygiene nivo III.
Teneinde toelating te verkrijgen tot promotie werd in het kursusjaar 87/88 door hem het daartoe benodigde HLO-diploma behaald aan de Hogeschool Heerlen.

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