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Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase

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Influx of calcium in platelets and red cells produces formation of vesicles shed from the plasma membrane. The time course of the shedding process closely correlates with the ability of both cells to stimulate prothrombinase activity when used as a source of phospholipid in the prothrombinase assay. This reflects increased surface exposure of phosphatidylserine, presumably resulting from a loss in membrane asymmetry. Evidence is presented that the shed vesicles have a random phospholipid distribution, while the remnant cells show a progressive loss of membrane phospholipid asymmetry when more shedding occurs. Removal of intracellular calcium produces a decrease of procoagulant activity of the remnant cells but not of that of the shed vesicles. This is consistent with reactivation of aminophospholipid translocase activity, being first inhibited by intracellular calcium and subsequently reactivated upon calcium removal. Involvement of aminophospholipid translocase is further supported by the observation that reversibility of procoagulant activity is also dependent on metabolic ATP and reduced sulfhydryl groups. The finding that this reversibility process is not apparent in shed vesicles may be ascribed to the absence of translocase or to a lack of ATP. These data support and extend the suggestion made by Sims et al. ((1989) J. Biol. Chem. 264, 17049–17057) that membrane fusion, which is required for shedding to occur, produces transient flip-flop sites for membrane phospholipids. Furthermore, the present results indicate that scrambling of membrane phospholipids can only occur provided that aminophospholipid translocase is inactive.

Introduction

There is ample evidence to support the notion that the outer leaflet of the lipid bilayer membrane of most, if not all, eukaryotic cells is rich in choline-phospholipids (phosphatidylcholine and sphingomyelin), whereas aminophospholipids (phosphatidylethanolamine and phosphatidylserine) are abundant in the inner leaflet [1,2]. Although this asymmetric orientation is not absolute, it has been shown that both in red cell membranes and platelet plasma membranes phosphatidylserine is virtually completely absent from the outer leaflet of the membrane lipid bilayer [1]. In erythrocytes, this phenomenon is essential for normal homeostasis. It has been shown that increased surface exposure of phosphatidylserine serves as a signal for sequestration by the reticuloendothelial system [3,4]. Moreover, partial loss of the asymmetric orientation of phosphatidylserine may occur in sickled red cells [5,6]. This leads to the formation of a procoagulant surface which may contribute to intravascular coagulation. In platelets, transbilayer asymmetry is rapidly lost upon activation by certain platelet agonists [7–9]. This phenomenon serves an important function of platelets in the hemostatic process, since two sequential reactions of the coagulation cascade are dramatically accelerated in the presence of a phospholipid surface containing phosphatidylserine [10,11]. Among the agonists that can evoke increased exposure of phosphatidylserine at the outer surface of platelets are Ca^{2+} ionophore, the combined action of collagen and thrombin, and membrane permeable SH-oxidizing agents. All these platelet treatments have in common that they produce a rapid up-

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take of calcium from the extracellular medium, a process which is essential for redistribution of the lipids to occur. Studies using the fluorescent membrane probe trimethylammonium diphenylhexatriene strongly suggest that this process involves a rapid bidirectional transbilayer flip-flop of the phospholipids by which they randomize over both membrane leaflets [12].

During the last decade, two mechanisms have been proposed to be involved in the maintenance of phospholipid asymmetry during the normal life span of the cell. First, it has been suggested by Haest [13] that endofacial proteins of the membrane skeleton by interacting with aminophospholipids, contribute to the maintenance of the inside orientation of these lipids in the cell membrane. This concept is supported by a number of observations that selective interactions between phosphatidylserine and skeletal proteins of red cells and platelets occur in in vitro binding studies [14–17]. Second, Seigneuret and Devaux [18] proposed the existence of an aminophospholipid translocase activity in the plasma membrane, based on the observation that introduction of spin-labelled aminophospholipids in the outer leaflet of the red cell membrane is followed by a rapid transfer to the inner leaflet. This process, which has meanwhile been demonstrated by various laboratories using different techniques [19–23], will undoubtedly contribute to membrane phospholipid asymmetry since it occurs much more slowly with phosphatidylcholine and not with sphingomyelin [24]. Moreover, it is likely that translocase activity is brought about by one or more membrane proteins, since this process is ATP-dependent and inhibited by SH-oxidizing agents and D-isomers of phosphatidylserine and phosphatidylethanolamine. Recent data from the group of Schroit [25] suggest that at least in red cells an integral membrane protein of 32 kDa is involved. Indications for the presence of an aminophospholipid translocase activity have also been obtained for a variety of other cells [26–28], including platelets [19,29].

Surface exposure of phosphatidylserine in activated platelets and to a lesser extent in pathological red cells could, therefore, involve decreased interactions between membrane skeletal proteins and phosphatidylserine and/or inhibition of aminophospholipid translocase activity. Whereas the latter is presumably inhibited by influx of extracellular calcium [30], we have proposed that in platelets the degradation of cytoskeletal proteins by activation of intracellular calpain would undo their interactions with phosphatidylserine [8]. As will be made clear in this paper by using a specific calpain inhibitor, this proposition can no longer be maintained. In a recent study, Sims et al. [31] suggested another mechanism for the loss of membrane phospholipid asymmetry in platelets upon certain activation procedures. Influx of extracellular calcium was shown to produce shedding of vesicles from the platelet plasma membrane, which involves fusion between apposing segments of cytoplasmic membrane surfaces. This process was proposed to cause a local collapse of the asymmetric phospholipid distribution through transient formation of a phase of non-bilayer lipid [32] at the point where the plasma membrane fuses to form the budding vesicle.

In this study we show that in platelets the surface exposure of phosphatidylserine correlates in time with calcium induced shedding of vesicles and that a similar process can be brought about in red cells as well. Moreover, we provide evidence that such a process can only be accompanied by scrambling of membrane phospholipids provided that the aminophospholipid translocase is inhibited.

Materials and Methods

Materials

Calcium ionophore A23187 was obtained from Calbiochem. Horse tendon collagen (type I) was from Hormon Chemie (München, F.R.G.). Dithiothreitol, 2-deoxy-D-glucose, glucono-δ-lactone, tetrapeptide Arg-Gly-Asp-Ser (RGDS), and fatty acid free human serum albumin were from Sigma Chemical Co. (St. Louis, MO). Calpain inhibitor MDL 28170 (carbamic acid, [1-(((1-formyl-2-phenylmethyl)amino)carboxyl)-2-methylpropyl]-, phenylmethyl ester) was a kind gift from Merrell Dow, Research Institute (Cincinnati, OH). Pyridyldithioethylamine (PDA) was prepared as described by Connor and Schroit [23]. Coagulation factors thrombin, prothrombin, factor Xa, and factor Va were purified from bovine blood as described elsewhere [10]. Thrombin-specific chromogenic substrate S2238 was obtained from AB Kabi Diagnostica (Stockholm, Sweden). All other reagents were of the highest grade commercially available.

Isolation of platelets and red cells

Washed human platelets and red cells were obtained from freshly drawn blood by differential centrifugation as described before [7]. Both platelets and red cells were finally resuspended in a Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, and 1 $mg \cdot ml^{-1}$ human serum albumin, adjusted to pH 7.4. For the various incubations, $CaCl_2$ was added to a final concentration of 1 mM. Platelets were incubated at a concentration of 10^8 ml^{-1} and red cells at $2 \cdot 10^8$ ml^{-1}, at 37°C.

Measurement of prothrombinase activity

The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va has been shown to be a convenient, rapid, and sensitive method to monitor in a semiquantitative way the extent of exposure of phosphatidylserine at the outer cell surface [7,8,33]. Briefly, platelets at a concentration of $5 \cdot 10^6$

ml^{-1} and red cells at a concentration of 10^7 ml^{-1} were incubated at 37°C with factor Xa and factor Va (final concentrations 3 and 6 nM, respectively) for 2 min in the presence of 3 mM $CaCl_2$. Subsequently, thrombin formation was started by adding prewarmed prothrombin (final concentration 4 μM) and additional $CaCl_2$ (final concentration 6 mM). Exactly 1 min after addition of prothrombin, an aliquot of the incubation mixture was transferred to a cuvette containing a buffer composed of 50 mM Tris-HCl, 100 mM NaCl, and 2 mM EDTA (pH 7.9). Thrombin activity was determined spectrophotometrically at 405 nm after addition of the chromogenic substrate S2238 (final concentration 150 μM). The rate of thrombin formation was calculated from the change in absorbance per min, using a calibration curve made with active-site-titrated thrombin [10].

Separation of shed vesicles from remnant cells

Shedding of membrane vesicles has been shown to occur upon activation of platelets [31,37] and upon treatment of red cells with Ca^{2+} ionophore [38,39]. For the purpose of the present investigation, it was necessary to develop a rapid procedure to separate shed vesicles from remnant cells. If platelets were activated by collagen plus thrombin or by A23187, and subsequently centrifuged at $850 \times g$, it appeared that both the phospholipid content of the supernatant and its respective ability to catalyze thrombin formation when used as a source of phospholipid in the prothrombinase assay, approached constant values at 20 min centrifugation time. No significant differences in these values were found when the platelets were spun at $11\,500 \times g$ for 1.5 min (i.e., at the same $g \cdot$ min). This latter centrifugation procedure was adopted as a rapid method to produce satisfactory separation between shed vesicles and remnant platelets. For ionophore treated red cells a centrifugation procedure of 1.5 min at $200 \times g$ was adopted, since neither phospholipid content of the supernatant nor its respective prothrombinase activity changed when the red cells were spun for an additional 5 min at $200 \times g$.

Miscellaneous

Platelet samples for gel electrophoresis were prepared as described before [8]. SDS-PAGE was performed according to Laemmli [34], using 7.5% separating gels with a 4% stacking gel. Gels were stained with Coomassie brilliant blue. Phospholipid analysis was carried out after two-dimensional thin-layer chromatography as described before [35]. Lactate dehydrogenase present in a $12\,000 \times g$ supernatant of a platelet incubation was used as a parameter for lysis and was determined according to Wroblewski and La Due [36].

Results

Generation of platelet prothrombinase activity

Activation of platelets by Ca^{2+} ionophore or by collagen plus thrombin has been shown to be effective in enhancing the ability of the cells to stimulate prothrombinase activity [10]. This property arises from the increased exposure of phosphatidylserine at the outer surface [7,8]. Fig. 1 shows time generation curves of platelet prothrombinase activity upon activation by A23187 and by collagen plus thrombin. In ionophore-activated platelets, prothrombinase activity reaches a plateau in approx. 1 min after activation. In collagen plus thrombin activated platelets, prothrombinase activity levels off after 10 min following activation, approaching an activity of approx. half of that found with ionophore-activated platelets. At different time points, supernatants were prepared by centrifugation for 1.5 min at $11\,500 \times g$ to obtain a measure for the prothrombinase activity of shed vesicles (cf. Materials and Methods). As shown in Fig. 1, prothrombinase activity of the shed vesicles from ionophore treated platelets reaches a plateau in approx. 1 min with an activity of about 30% of that of the respective total incubation.

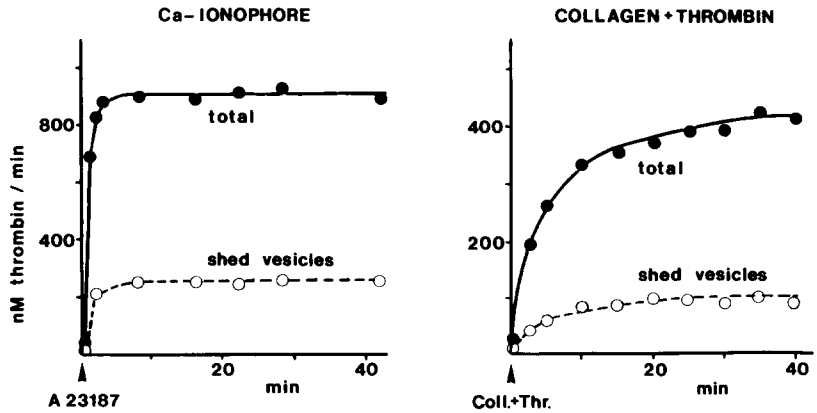

Fig. 1. Development of the catalytic potential of a platelet suspension to stimulate prothrombinase activity as a function of platelet activation time. Left panel: platelets activated by Ca^{2+} ionophore A23187 (1 μM); right panel: platelets activated by collagen (10 μg/ml) and thrombin (2 nM). Total activity (●———●) is expressed per $5 \cdot 10^6$ platelets/ml. Activity of shed vesicles (○———○) was measured with the supernatant obtained from the activated platelet suspension after centrifuging at $11\,500 \times g$ for 1.5 min (see Materials and Methods).

With collagen plus thrombin activated platelets, the shed vesicles showed a similar time generation curve as the respective total incubation with a prothrombinase activity of 20–25% of the total. Sonication of the shed vesicles preparations produced an increase in prothrombinase activity of 1.4–1.5-fold, irrespective of the time point at which the supernatants were prepared.

Phospholipid analyses of supernatants and remnant cells showed no significant differences in phospholipid composition apart from a minor increase in lyso compounds. With ionophore activated platelets, 15–16% of the total phospholipid was found in the supernatant, both after 2 and 40 min of activation. With collagen plus thrombin activated platelets, the percentage of total phospholipid in the supernatant followed a similar increase as the prothrombinase activity reaching a value of $11 \pm 1\%$ after 20 min activation.

Reversibility of prothrombinase activity of ionophore activated platelets

Since it is known from work on red cells that aminophospholipid translocase activity for phosphatidylserine becomes inhibited when intracellular calcium reaches about 0.5 μM concentration [30], we hypothesized that, assuming a similar effect in platelets, removal of intracellular calcium after ionophore stimulation would restore aminophospholipid translocase activity resulting in a decrease of the platelets' ability to stimulate prothrombinase. To test this assumption, platelets were first activated with A23187 in the presence of 1 mM Ca^{2+} after which 2 mM EGTA was added. As shown in Fig. 2 (left panel), addition of EGTA caused no decrease in platelet prothrombinase activity. A different result was obtained when platelets were first preincubated with the specific calpain inhibitor MDL 28170. This inhibitor effectively prevents degradation of the cytoskeletal proteins filamin, talin and myosin by calpain, which is normally observed when

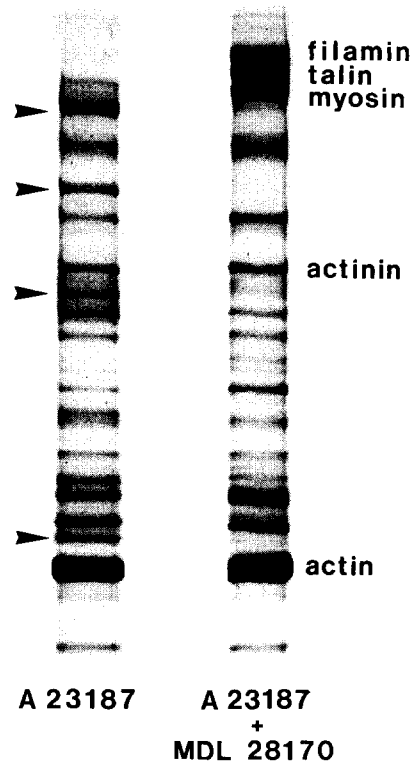

Fig. 3. Protein patterns revealed by SDS-PAGE of Ca^{2+} ionophore activated platelets in the absence or presence of calpain inhibitor MDL 28170 under the experimental conditions of the experiment shown in Fig. 2. Arrowheads indicate the major degradation polypeptides resulting from calpain activity towards filamin, talin and myosin [8].

platelets are treated with Ca^{2+} ionophore (Fig. 3). However, irrespective of whether calpain is inhibited or not, generation of platelet prothrombinase activity by A23187 is not affected. This result convincingly refutes our earlier proposal (Ref. 8) that cytoskeletal degradation by calpain would be required to make platelets procoagulant. As shown in Fig. 2 (right panel), platelets

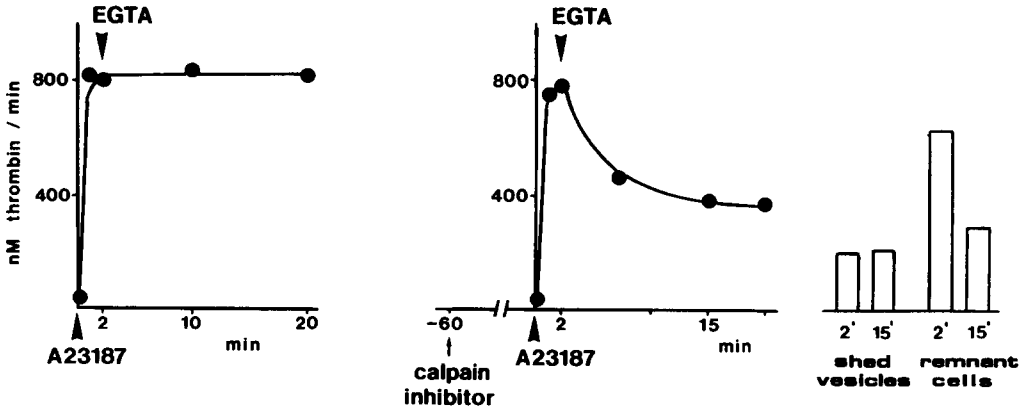

Fig. 2. Catalytic potential of platelets to stimulate prothrombinase activity after activation by Ca^{2+} ionophore A23187 (1 μM) and subsequent addition of EGTA (2 mM) at 2 min after the activation procedure. Prothrombinase activity is expressed per $5 \cdot 10^6$ platelets/ml. Left panel: without calpain inhibitor MDL 28170; Right panel: with MDL 28170 (100 μM) added 60 min prior to activation by Ca^{2+} ionophore. Bars represent prothrombinase activity of shed vesicles and resuspended remnant platelets from the incubation with calpain inhibitor at time points indicated.

with calpain inhibitor become normally procoagulant upon stimulation with A23187. However, in contrast to platelets in which calpain is not inhibited, subsequent addition of EGTA produces a considerable decrease in the platelets' ability to stimulate prothrombinase activity. Separation of the total incubation in shed vesicles and remnant cells revealed that this decrease is not caused by a reduction of prothrombinase activity of the shed vesicles. Careful resuspension of the remnant platelets after centrifugation, allowed to show that only the prothrombinase activity of the remnant cells is reversible upon removal of intracellular calcium (Fig. 2, bars). It should be mentioned that the measured activity of the resuspended remnant cells is higher than expected from the difference between total activity and activity of shed vesicles. This is most likely due to some cell damage occurring during resuspending, since about 5% of the lactate dehydrogenase activity was found to have leaked out of the resuspended remnant platelets.

ATP and sulfhydryl dependence of aminophospholipid translocase in platelets

In order to demonstrate that the result shown in Fig. 2 can most likely be explained by reactivation of aminophospholipid translocase, two experimental protocols were developed to show that reversibility of platelet prothrombinase depends on ATP and reduced sulfhydryls. It is well known that addition of metabolic inhibitors to platelets rapidly blocks ATP synthesis [40,41]. Subsequent activation of these platelets produces a virtual complete exhaustion of metabolic ATP, being consumed in the various activation phenomena. (e.g., shape change, release and aggregation) [42]. However, development of a procoagulant platelet surface by activation with Ca^{2+} ionophore is not dependent on ATP [9]. As shown in Fig. 4 (left panel), reversibility of platelet prothrombinase activity following addition of EGTA to ionophore stimulated platelets, does not occur in the presence of metabolic inhibitors. This strongly suggests this process to be ATP dependent. In this respect, it may be of interest to mention that the decrease in prothrombinase activity following addition of EGTA to ionophore-stimulated (calpain-inhibited) platelets, does not occur when Mg^{2+} is omitted from the Hepes buffer in which the platelets are routinely suspended. Also, EDTA is ineffective irrespective of whether Mg^{2+} is present or not. This suggests that intracellular Mg-ATP is required to reduce the procoagulant activity of stimulated platelets upon removal of intracellular Ca^{2+}.

As was shown previously, treatment of platelets with sulfhydryl oxidizing agents such as pyridyldithioethylamine (PDA) or diamide in the presence of extracellular Ca^{2+} makes the cells procoagulant [9]. This process is also accompanied by production of shed vesicles, containing about 20% of the total procoagulant activity. The requirement for extracellular calcium presumably reflects increased calcium permeability of the platelet plasma membrane as a result of sulfhydryl oxidation. Moreover, both agents have been shown to directly inhibit aminophospholipid translocase activity [9,23]. As shown in Fig. 4 (right panel), treatment of platelets with PDA (and 1 mM Ca^{2+}) considerably enhances the platelets' ability to stimulate prothrombinase activity.

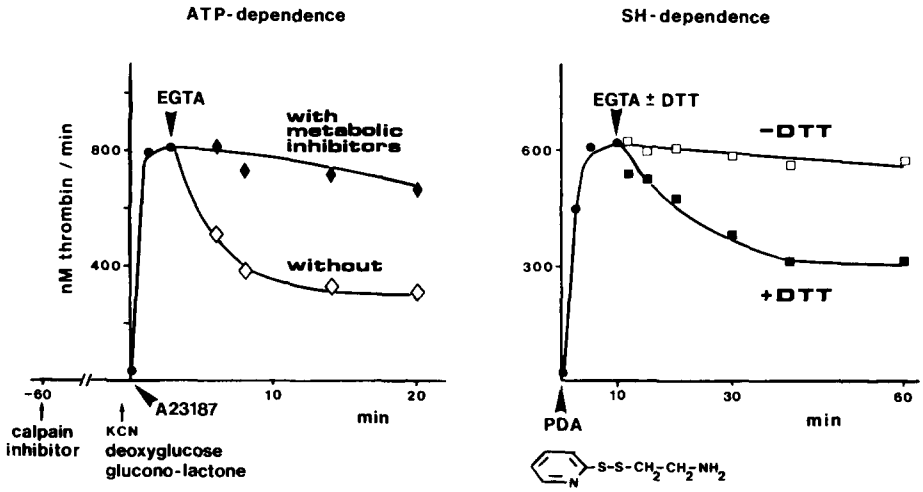

Fig. 4. ATP dependence and sulfhydryl dependence of the reversibility of the platelets' ability to stimulate prothrombinase activity (expressed per $5 \cdot 10^6$ platelets/ml). Left panel: calpain inhibited platelets (see legend Fig. 2) were activated by Ca^{2+} ionophore A23187 (1 μM) in the absence or presence of a mixture of KCN (1 mM), 2-deoxy-D-glucose (45 mM), and glucono-δ-lactone (10 mM) added 5 min prior to the activation procedure. After development of prothrombinase activity (●———●), 2 mM EGTA was added: (◆———◆) prothrombinase activity of metabolically inhibited platelets; (◇———◇) prothrombinase activity of platelets without metabolic inhibitors. Right panel: Platelets were stimulated with 1 mM pyridyldithioethylamine (PDA) (in the presence of 1 mM Ca^{2+}). After development of prothrombinase activity (●———●), 2 mM EGTA plus or minus 5 mM dithiothreitol (DTT) was added: (■———■) prothrombinase activity of platelets reduced by DTT; (□———□) prothrombinase activity of unreduced platelets. Since calpain is inhibited by PDA it was not necessary to add MDL 28170 prior to platelet stimulation. Platelet incubations with DTT were carried out in 1 mM RGDS to avoid DTT-induced aggregation.

Addition of EGTA to remove intracellular calcium does not reduce the procoagulant activity, unless sulfhydryl reduction is carried out at the same time by addition of dithiothreitol (DTT). These experiments were performed in the presence of the aggregation inhibiting peptide RGDS. This was necessary to prevent the tight platelet aggregation which otherwise occurs in the presence of DTT [43] and which may reduce the available surface for the assembly of prothrombinase complexes (see Discussion). Very similar results were obtained when PDA was replaced by diamide (data not shown).

Generation and reversibility of red cell prothrombinase activity

Treatment of red cells with Ca^{2+} ionophore is known to produce influx of extracellular Ca^{2+} and shedding of vesicles from the cell membrane [38,39]. As shown in Fig. 5 (left panel) this treatment gradually makes the cells procoagulant when used as a source of phospholipid in the prothrombinase assay. This activity develops much slower as compared to that observed with ionophore activated platelets (cf. Fig. 1) where maximal activity is attained in about 1 min. At different time points, supernatants (containing shed vesicles) were prepared by centrifugation for 1.5 min at 200 × *g*, while the pellet (containing remnant cells) could be easily resuspended in the original volume. Fig. 5, left panel, shows that at every time point about two-third of the total activity is recovered in the resuspended remnant cells while about one-third is present in the shed vesicles. Moreover, both preparations followed a similar time course of generation of prothrombinase activity as the total incubation.

Phospholipid analyses of supernatants and remnant cells revealed no significant differences in phospholipid composition. Moreover, the percentage of total phospholipid recovered in the supernatant followed a similar increase as the prothrombinase activity, reaching a value of approx. 10% after 60 min incubation.

Addition of EGTA to the ionophore treated red cells produced a considerable decrease in the cells' ability to stimulate prothrombinase activity (Fig. 5, right panel). Separation of the incubation in shed vesicles and remnant cells showed that this decrease is exclusively caused by the remnant cells, since no decrease was found with the supernatants containing the shed vesicles. In contrast to platelets, it is not necessary to preincubate the red cells with calpain inhibitor in order to show reversibility upon removal of intracellular Ca^{2+} by EGTA. This is presumably due to the much lower calpain activity in red cells [44]. However, as also observed for platelets, Mg^{2+} is required for the reversibility process to occur. In the absence of Mg^{2+}, addition of EGTA only slows down the shedding process, accompanied by a levelling off of the development of prothrombinase activity. Subsequent addition of Mg^{2+} results in reduction in prothrombinase activity, again being exclusively produced by the remnant red cells.

Discussion

As has been convincingly demonstrated for platelets by Sims et al. [31], and for red cells by Allan and Michell [38], influx of extracellular calcium can produce shedding of vesicles from the plasma membrane. Release of lipid and protein containing microparticles from activated platelets has been reported before by Sandberg et al. [45], but they incorrectly assumed that these were secreted from intracellular stores. It has now become obvious that these vesicles are shed from the plasma membrane, since they contain most of the plasma

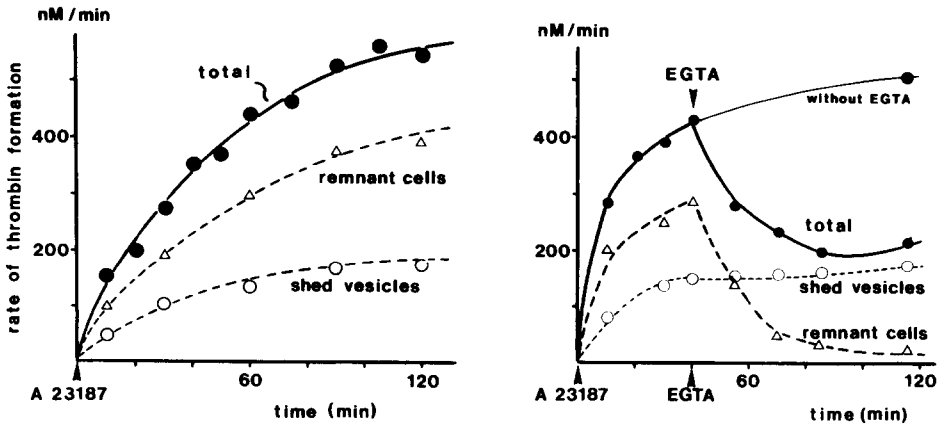

Fig. 5. Left panel: development of the catalytic potential of a red cell suspension to stimulate prothrombinase activity as a function of incubation time with Ca^{2+} ionophore A23187 (1 μM). Total activity (●———●) is expressed per 10^7 red cells/ml. Prothrombinase activity of resuspended remnant cells (△———△) and shed vesicles (○———○) was measured after centrifugation for 1.5 min at 200 × *g* (see Materials and Methods). Right panel: Prothrombinase activity of ionophore stimulated red cells on subsequent addition (after 40 min) of 2 mM EGTA: (●———●) total incubation; (△———△) remnant red cells; (○———○) shed vesicles.

membrane glycoproteins as well as components of the membrane skeleton [31,37].

We have previously demonstrated that the development of a procoagulant membrane surface is most likely caused by loss of membrane phospholipid asymmetry, resulting in a net increase of procoagulant phosphatidylserine in the outer leaflet of the membrane [6–8]. Our present data show that this process correlates in time with the formation of shed vesicles from the plasma membrane. Moreover, this association is not restricted to platelets but is also demonstrated to occur in red cells. This strongly supports the suggestion made by Sims et al. [31], that fusion between apposing segments of cytoplasmic membrane surfaces, which is required for shedding to occur, produces a (temporary) scrambling site through transient formation of non-bilayer lipid structures [32] at the point where fusion occurs. It could be argued, however, that shedding occurs as a result of loss of phospholipid asymmetry. In that case one would expect that vesicles shed in an early phase have smaller amounts of exposed phosphatidylserine and are less procoagulant than vesicles shed in a later phase. Our results show that irrespective of the time point of incubation, sonication of the shed vesicles caused a constant (1.4–1.5-fold) increase in their ability to catalyze prothrombinase activity. The average diameter of shed vesicles both from red cells [38] and platelets [31] has been reported to be 100 nm. Vesicles of this size have equal amounts of phospholipid in both leaflets of the bilayer. Sonication produces smaller vesicles in which more than two-third of the lipid is present in the outer leaflet [46]. The increase in prothrombinase activity upon sonication is, therefore, consistent with the increase in lipid molecules in the outer leaflet. This strongly suggests that all the shed vesicles have already a more or less symmetric phospholipid distribution. Although the remnant cells gradually lose lipid, they become more procoagulant. Apparently, each fusion event contributes to a gradual loss of phospholipid asymmetry through formation of transient fusion-, c.q. flip-flop sites, and the more shedding occurs the more procoagulant the remnant cells become.

As has been reported by Bitbol et al. [30], a rise in intracellular calcium inhibits aminophospholipid translocase activity. Under the activation conditions employed in the present study, translocase activity was found to be inhibited for fluorescently labelled amino phospholipids (manuscript in preparation). Apparent inhibition of translocase is also evident from the observation that decrease in procoagulant activity occurs upon removal of intracellular calcium, a condition which has been shown to reactivate translocase activity, at least in red cells [30]. Considering the rate at which this occurs, it can be concluded that loss of membrane phospholipid asymmetry through formation of transient scrambling sites would not occur to a significant extent if aminophospholipid translocase were active at the same time. The observation that translocase activity can only be restored in remnant cells and not in shed vesicles, may be due to a sorting phenomenon during shedding or to a lack of sufficient ATP in the shed vesicles presumably due to inability to synthesize ATP.

Remarkably, restoration of translocase activity in platelets requires that intracellular calpain is inhibited. Such a precaution is not necessary for red cells, but calpain activity in red cells is much lower than that in platelets [44]. Most likely, the very active calpain I activity in platelets, operating at calcium concentrations in the micromolar range [47], destroys aminophospholipid translocase at the cytoplasmic membrane surface or degrades membrane skeletal proteins possibly required for the translocase to be active [25]. Also, by using the specific calpain inhibitor MDL 28170, it appeared that calpain activity is not required for shedding or for the formation of a procoagulant lipid surface. This implies that our earlier observation [8] that extent of calpain activity closely correlates with generation of platelet prothrombinase activity rests on sheer coincidence. At best, calpain activity can be taken as a sensitive marker for intracellular calcium levels.

We have previously reported that amino phospholipid translocase activity can be restored in activated or sulfhydryl-oxidized platelets upon reduction by DTT [9]. However, addition of DTT to platelets produces extremely dense platelet aggregation [43] (unlike the flocculent aggregation normally observed with platelet activators), which at least in part reduces the available surface for the assembly of prothrombinase complexes used to monitor phosphatidylserine at the outer surface. This aggregation is completely prevented in the presence of the tetrapeptide RGDS, which contains the recognition site of adhesive proteins such as fibronectin [48]. The inclusion of this peptide in the present experiments with sulfhydryl-oxidized platelets, strongly indicates that considerable reactivation of aminophospholipid translocase by DTT only occurs when intracellular calcium is also removed.

It is known that not all platelet activators produce membrane shedding [31,37] and the formation of a procoagulant surface [7,8]. However, all activators induce secretory events in platelets which also involves fusion between apposing cytoplasmic surfaces of plasma membrane and secretory granules. The question remains as to whether the secretory events do not necessarily lead to scrambling of the membrane phospholipids. The answer might be that intracellular calcium concentrations required for secretion are lower than those required for shedding, and below the calcium level at which the aminophospholipid translocase is inhibited. On many occasions, we have observed that inhibition of translocase by sulfhydryl oxidizing agents in red cells and platelets in the absence of extracellular

calcium does not lead to a measurable loss of membrane phospholipid asymmetry during prolonged incubations, which may imply that once a membrane is asymmetric it does not require an active translocase to maintain phospholipid asymmetry. Therefore, it can be speculated that a major function of the aminophospholipid translocase is to maintain phospholipid asymmetry during secretion of secretory cells.

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