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Fluoride-dependent calcium-induced platelet procoagulant activity shows that calpain is involved in increased phospholipid transbilayer movement

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Treatment of platelets with fluoride (10 mM) was found to result in a transient increase in Ca^{2+}-permeability of the platelet plasma membrane. This phenomenon was used to provide supplementary evidence for the suggestions made earlier (Comfurius et al. (1985) Biochim. Biophys. Acta 815, 143; Verhallen et al. (1987) Biochim. Biophys. Acta 903, 206), that cytoskeletal disrupture by calpain is involved in the process leading to transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. This was achieved by relating both calpain activity and exposure of phosphatidylserine with platelet procoagulant activity. It was found that only upon addition of extracellular Ca^{2+} to fluoride-treated platelets, procoagulant activity, expressed as prothrombinase activity, and calpain activity, estimated from protein patterns after gel electrophoresis, were generated. Both Ca^{2+}-inducible prothrombinase activity and calpain activity followed an identical time-course during incubation with fluoride: after a time-lag of about 10 min they sharply increased towards a peak level. Upon further incubation with fluoride, both activities decreased towards a final plateau, still above basal level. The presence of leupeptin during incubation with fluoride was found to inhibit Ca^{2+}-inducible calpain activity and prothrombinase activity in an identical way. Ca^{2+}-inducible exposure of phosphatidylserine, as determined with extracellular phospholipase A_2, showed a similar pattern as Ca^{2+}-inducible calpain activity and prothrombinase activity. From the strict parallelism between prothrombinase activity, calpain activity and exposure of phosphatidylserine, it is concluded that calpain plays an important role in the activation-dependent transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity. It is suggested that degradation of the platelet membrane-skeleton by calpain disturbs the structural organization of the lipid bilayer of the platelet plasma membrane leading to enhanced transbilayer movement of phospholipids and appearance of phosphatidylserine at the platelet outer surface.

Introduction

Blood platelets have a multifaceted function in the hemostatic process. They initiate hemostatic

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plug formation upon vessel wall injury, and they promote fibrin-formation by the coagulation cascade. In order to perform these functions platelets exhibit a wide variety of cellular responses. Primary hemostatic plug formation requires platelet adhesion, aggregation, and exocytosis. Activated platelets accelerate coagulation by increasing the rate of two sequential coagulation reactions: conversion of factor X to Xa by a complex of factors IXa and VIIIa, and the conversion of prothrombin to thrombin by a complex of factors Xa and Va (prothrombinase complex). This property of stimulated platelets, referred to as procoagulant activity, is catalytic in nature, and can be interpreted as an increased number of binding sites for both the factor X activating complex and the prothrombinase complex [1].

In previous papers, we presented extensive evidence that anionic phospholipids present at the outer surface of activated platelets are the major determinant of the binding sites for the prothrombinase complex [2]. Using phospholipid vesicles of various compositions, it was suggested that phosphatidylserine is the physiologically relevant negatively charged phospholipid in both coagulation reactions [1–4]. This notion was further substantiated by the effect of phospholipases on the procoagulant activity of resting, and stimulated platelets. These studies showed that only those phospholipases that can hydrolyse phosphatidylserine will decrease platelet procoagulant activity [1,5], and that stimulation of platelet procoagulant activity by a variety of agonists is related to the amount of phosphatidylserine exposed at the outer surface after platelet activation [6].

The plasma membrane of a resting platelet displays an asymmetric distribution of phospholipids [6–9]. Most extreme is the distribution of phosphatidylserine and sphingomyelin, which are almost exclusively located at the cytoplasmic and extracellular face of the platelet plasma membrane, respectively. To explain the increased exposure of phosphatidylserine after stimulation of platelet procoagulant activity, it is assumed that transbilayer movement of phospholipids (flip-flop) is greatly enhanced during stimulation of platelet procoagulant activity [6]. This flip-flop process is thought to produce randomization of phospholipids in the bilayer membrane, since in the outer leaflet exposure of phosphatidylserine is counterbalanced by a loss of sphingomyelin.

At present, the cellular mechanisms responsible for increased exposure of phosphatidylserine during expression of platelet procoagulant activity are poorly understood. In search of such mechanisms, we observed a striking correlation between the potency of various agonists to induce exposure of phosphatidylserine and to stimulate platelet calpain (endogenous calcium-dependent proteinase, EC 3.4.22.17) resulting in degradation of major cytoskeletal proteins (filamin, talin, and myosin) [10]. The relation between platelet procoagulant activity and calpain activity was further documented by comparing the time-course of their development, their dose-response relationship, and their Ca^{2+} dependence [11].

During studies concerning the long-term effect of metabolic-ATP depletion on transbilayer phospholipid asymmetry in platelets, using sodium fluoride as a metabolic inhibitor, we observed that during incubation with fluoride for several hours, platelets temporarily exhibited an increase in platelet procoagulant activity. This reversible time-course appeared to reflect a transient increase in Ca^{2+}-permeability of the platelet plasma membrane during incubation with fluoride. In this paper, the reversibility of fluoride-dependent Ca^{2+}-inducible platelet procoagulant activity is used to provide supplementary evidence for the suggestions made earlier that calpain activity coincides with exposure of phosphatidylserine. This is achieved by relating both calpain activity and exposure of phosphatidylserine with platelet procoagulant activity expressed as prothrombinase activity. In addition, the effect of leupeptin (an oligopeptide inhibitor of calpain) on the relation between calpain- and prothrombinase-activity was explored.

Materials and Methods

Sodium fluoride was from Merck (Darmstadt, F.R.G.). Leupeptin, (acetyl-L-leucyl-L-leucyl-L-arginal) was from Sigma (St. Louis, MO, U.S.A.). Blood coagulation factors Va, Xa, and prothrombin were isolated and purified from bovine blood according to established procedures described

elsewhere [1]. S2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride), was from Kabi Diagnostica (Stockholm, Sweden). Phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) was purified according to Zwaal et al. [12] from bee venom (*Apis mellifica*) obtained from Koch Light Laboratories (Colnbrook, U.K.). Fura-2/AM was from Molecular Probes Inc (Eugene OR, U.S.A.).

Platelet isolation

Washed human platelets were isolated by differential centrifugation as described previously [5] from fresh blood, drawn from healthy volunteers receiving no medication. Blood was anticoagulated with acid-citrate-dextrose. Washed platelets were resuspended in an isotonic buffer at pH 7.4, containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM glucose and 0.05% fatty acid-free human serum albumin (Hepes buffer). Platelet concentration was inferred from their absorption at 405 nm using a calibration curve determined with a Coulter Counter. Platelet lysis was determined by assaying lactate dehydrogenase (EC 1.1.1.27) in a $12000 \times g$ supernatant according to Wroblewski and La Due [13].

Measurement of platelet procoagulant activity

Platelet procoagulant activity was determined as described earlier [1,11] by assaying prothrombinase activity under conditions at which the procoagulant surface is rate-limiting. The assay was carried out at 37 °C in Hepes buffer under gentle stirring (250 rev./min). To monitor the effect of fluoride on platelet procoagulant activity, platelets ($2 \cdot 10^8$ ml^{-1}) were incubated with 10 mM fluoride at 37 °C, without stirring to prevent aggregation. Immediately before the addition of fluoride, 1 mM EGTA was added to sequester trace amounts of Ca^{2+}. At different time intervals a 15 μl aliquot of the fluoride incubation was diluted 20-fold into Hepes buffer containing 3 mM $CaCl_2$. No change in pH was apparent upon this dilution. After 1 min factor Va (6 nM final concentration) and factor Xa (3 nM final concentration) were added, and 1 min later the prothrombinase reaction was started by addition of prothrombin (final concentration 4 μM, final assay volume 0.5 ml). The amount of thrombin formed 1 min after the addition of prothrombin was enzymatically determined using the thrombin-specific chromogenic substrate S2238.

Occasionally, the time-course of thrombin formation after Ca^{2+} addition was monitored by assaying the thrombin activity in aliquots taken every 10 s after addition of fluoride-treated platelets to the complete prothrombinase mixture.

Determination of Ca^{2+} influx using fura-2

Platelets were loaded with fura-2 according to Rao et al. [14]. Platelets pelleted from platelet rich plasma were resuspended in Hepes buffer to approximately $3 \cdot 10^8$ ml^{-1} and incubated with 1 μM fura-2/AM for 30 min at 37 °C. After loading, platelets were washed as described under platelet isolation. The effect of fluoride on Ca^{2+}-influx into fura-2 loaded platelets was monitored at different time intervals after 10-fold dilution in Hepes-buffer of an aliquot of platelets incubated with fluoride ($2 \cdot 10^8$ platelets/ml, 10 mM fluoride, 1 mM EGTA, 37 °C). 3 mM Ca^{2+} was added under continuous high-speed recording (5 s per inch) of fura-2 fluorescence. The initial rate of change in fluorescence signal after Ca^{2+} addition was taken to reflect quantitatively the rate of Ca^{2+} influx. Fluorescence intensities (F) were recorded at 37 °C under continuous stirring (400 rev./min) with an SLM Aminco SPF-500C fluorimeter equipped with a light-tight injection port and a stirring device (Hellma). Excitation and emission wavelengths were set at 335 and 500 nm with 5 and 20 nm slits, respectively.

Determination of calpain activity by gel electrophoresis

Calpain activity in platelets was estimated from protein patterns after gel electrophoresis, by monitoring degradation of substrates and formation of fragments, as described in detail [11]. Samples for gel electrophoresis were taken simultaneously with samples for the prothrombinase assay, from the same incubation of platelets with fluoride, at various time intervals. They were diluted tenfold in albumin-free Hepes buffer, containing 3 mM Ca^{2+}. After 3 min, calpain activity was arrested by addition of Hepes buffer containing proteinase inhibitors and excess EGTA. Disc electrophoresis

was performed according to Laemmli [15] on 6–12% gradient polyacylamide slab gels. Gels were stained with Coomassie brilliant blue. Quantitative analysis of protein patterns was performed by densitometric scanning of stained gels (LKB 2202 Ultroscan laser densitometer, equipped with an integrator). Calpain activity was expressed as the amount of fragments (at 190, 135 and 95 kDa) relative to total protein. To eliminate differences due to variations in amount of total protein, the amount of actin was taken as an internal standard.

Determination of exposure of phosphatidylserine

The exposure of phosphatidylserine was inferred from its accessibility towards excess extracellular phospholipase A_2 from bee venom. Samples for phospholipase treatment were taken simultaneously with samples for the prothrombinase assay from the same incubation of platelets with fluoride ($5 \cdot 10^8$ platelets/ml, 10 mM fluoride, 1 mM EGTA). After 10-fold dilution into Hepes buffer containing 10 mM Ca^{2+}, phospholipase A_2 was added to a final concentration of 2 IU $\cdot$ ml^{-1}. Every 4 min a subsample (10^9 platelets) from the phospholipase A_2 incubation was taken for examination of phospholipid degradation and determination of platelet lysis. Phospholipase A_2 activity was inhibited by addition of 20 mM EGTA to the subsample, prior to the extraction of lipids according to Bligh and Dyer [16]. Phospholipid analysis was carried out by two-dimensional thin-layer chromatography followed by phosphorus determination as described by Verkleij et al. [17].

Results

Fluoride-dependent Ca^{2+}-induced platelet procoagulant activity

Changes in Ca^{2+} permeability of the platelet plasma membrane during incubation with fluoride were investigated employing fura-2-loaded platelets. The initial change in fura-2 signal after addition of extracellular Ca^{2+} was taken to represent Ca^{2+} influx quantitatively. It was found that the entry of extracellular Ca^{2+} into the platelet cytosol was transiently facilitated during the course of a 2-h incubation of platelets with fluoride (Fig. 1A).

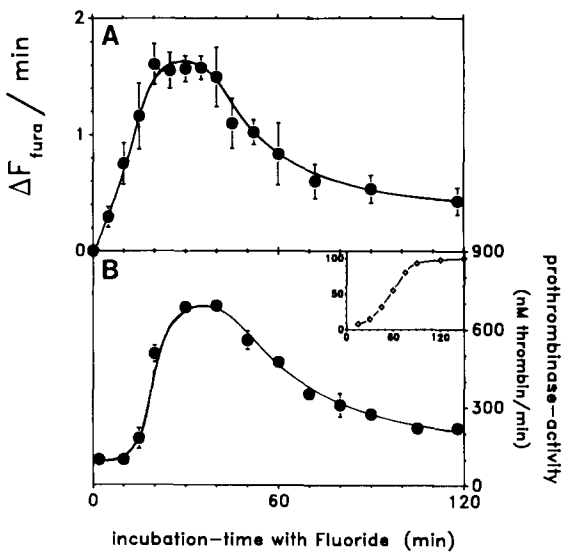

Fig. 1. Time-course of Ca^{2+} influx (A) and prothrombinase activity (B) during incubation of platelets with fluoride. Both parameters were evaluated after addition of 3 mM Ca^{2+} to platelets incubated with 10 mM fluoride, as described under Methods. The curve for Ca^{2+} influx (A), represented by the initial change in fura-2 fluorescence (ΔF_{fura}/min) after Ca^{2+} addition, is the mean ($\pm$ S.E. of five experiments. The curve for prothrombinase activity (B) was constructed for nine experiments in duplo (mean $\pm$ S.E.). In general, the reversible time-course of both effects of fluoride on platelets was highly reproducible, though peak-time and peak-width differed between different platelet preparations. Platelet lysis was negligible during the two hours incubation with fluoride. Inset (B): Generation of prothrombinase activity immediately after addition of Ca^{2+} to platelets incubated with fluoride for 30 min. Ordinate: prothrombinase activity expressed as percentage of maximal activity. Abscissa: time (s) after Ca^{2+} addition. It appeared that throughout the whole incubation of platelets with fluoride, Ca^{2+}-induced generation of prothrombinase activity followed the same time-course (not shown).

The effect of fluoride (10 mM) on platelet procoagulant activity was determined by assaying prothrombinase activity in subsamples taken from an incubation of platelets with fluoride. It was found (Fig. 1B) that during incubation with fluoride prothrombinase activity sharply increased, after a time-lag of 10 to 15 min, to a maximum level of approximately 700 nM thrombin per min. After reaching this maximum, prothrombinase activity gradually decreased upon progressive incubation with fluoride, to level off at about one-third of its peak level. Except for the time-lag, the transient character of Ca^{2+}-inducible pro-

thrombinase activity coincides with the transient increase in Ca^{2+}-permeability during fluoride-treatment. Under the same conditions of fluoride treatment, platelet aggregation and [^{3}H]serotonin release started after a delay of 5 min and levelled off at about 10% and 65%, respectively, within 15 to 20 min after addition of fluoride.

It is emphasized that fluoride treatment is carried out in the absence of extracellular Ca^{2+}, while prothrombinase activity is obligatory measured in the presence of extracellular Ca^{2+}. Considering the importance of extracellular Ca^{2+} for the expression of platelet procoagulant activity [10,11] and the effect of fluoride treatment on Ca^{2+} permeability of the platelet plasma membrane, the question arose whether the observed procoagulant activities were induced during incubation of platelets with fluoride in the absence of Ca^{2+}, or whether they were induced by influx of extracellular Ca^{2+} present during the prothrombinase assay. Therefore, the time-course of thrombin formation during the prothrombinase assay was determined and recalculated as generation of prothrombinase activity [11]. It appeared that the procoagulant activity of fluoride-treated platelets was actually generated by extracellular Ca^{2+} during the prothrombinase assay, reaching its maximum level within 2 min (inset Fig. 1B), i.e. before addition of prothrombin.

Fluoride-dependent Ca^{2+}-induced exposure of phosphatidylserine

Phospholipase A_2 from bee venom was used to determine the amount of phosphatidylserine exposed at the outer surface of fluoride-treated platelets after Ca^{2+} exposure. This approach is based on the observation that under non-lytic conditions only phospholipids present in the outer leaflet of the plasma membrane can be hydrolysed by exogenous phospholipase A_2 [6,8,12]. Fluoride-dependent Ca^{2+}-induced exposure of phosphatidylserine was related with prothrombinase activity at three characteristic time-points during fluoride treatment: at the start, at the peak level and at the lower final level of prothrombinase activity. In order to determine the saturating level of hydrolysis by phospholipase A_2, the time-course of hydrolysis of both phosphatidylserine and total phospholipids was determined. In this way changes

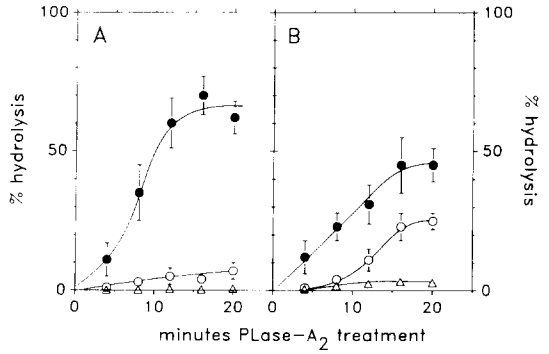

Fig. 2. Hydrolysis of phosphatidylserine (A) and total phospholipids (B) in fluoride-treated platelets, after exposure to Ca^{2+}. Degradation of phospholipids in the outer membrane of fluoride-treated platelets by phospholipase A_2 (Plase A_2) from bee venom was examined 0 min (△), 30 min (●) and 120 min (○) after the addition of fluoride, as outlined in the Methods. The data shown are the mean (±S.E.) of three experiments. During treatment with phospholipase A_2, platelet lysis gradually increased to a maximum of 10%. Data shown were corrected for phospholipid hydrolysis in lysed cells [10]. The presence of residual fluoride during treatment with phospholipase A_2 was found not to affect phospholipase A_2 activity.

in hydrolysis of phosphatidylserine can be compared with changes in hydrolysis of total phospholipids. It was found that at peak levels of prothrombinase activity, accessibility of phosphatidylserine towards phospholipase A_2 was dramatically increased to approximately 65%, compared to less than 1% and 10% at the start and at the end of the incubation with fluoride, respectively (Fig. 2A). In contrast, hydrolysis of total phospholipids was only 2-fold increased at peak level prothrombinase activity in comparison with the hydrolysis at the end of fluoride treatment (Fig. 2B). At the start of the fluoride treatment hardly any hydrolysis of phospholipids was observed, in agreement with earlier observations [8].

Fluoride-dependent Ca^{2+}-induced calpain activity

The relationship between calpain activity and prothrombinase activity was determined by applying a similar Ca^{2+} exposure after fluoride treatment. From the protein patterns (Fig. 3C) and their quantitative analysis (Fig. 3B), it is obvious that calpain activity followed the same reversible time-course as prothrombinase activity: a fast rise after a time-lag, followed by a gradual decline. In

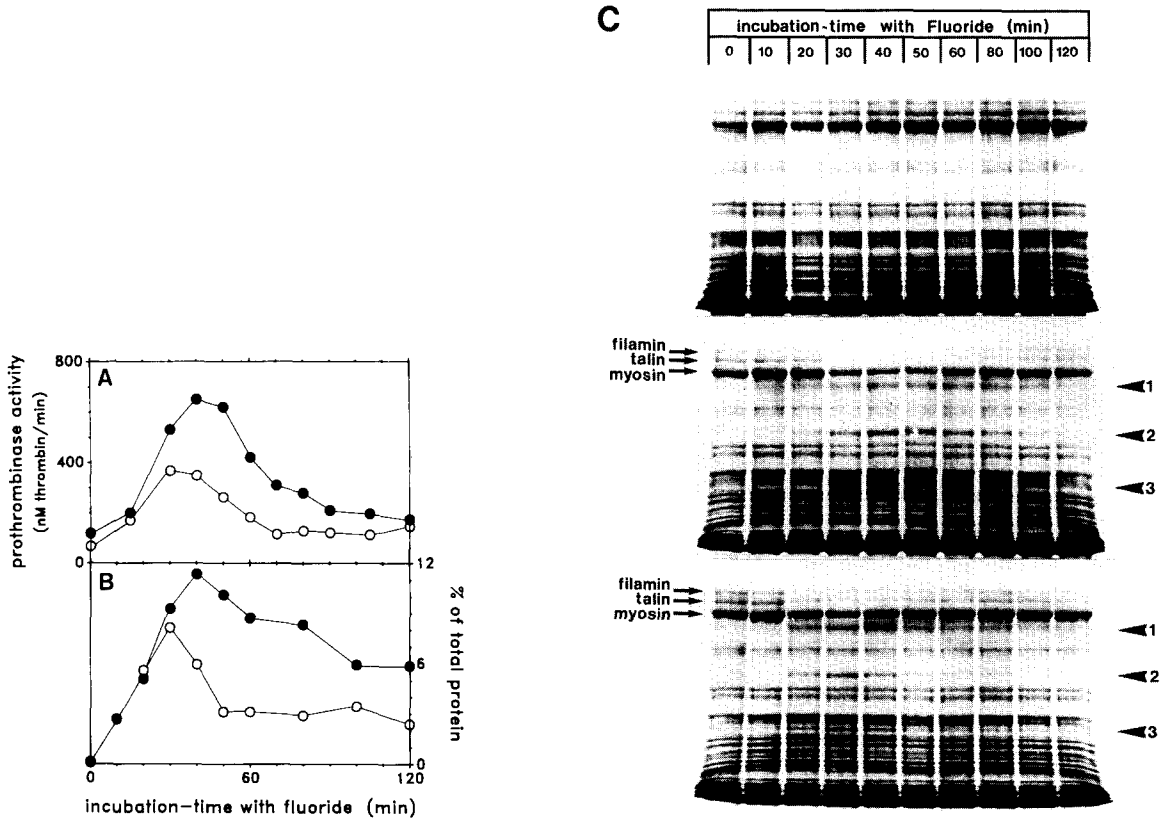

Fig. 3. Relation between Ca^{2+}-induced prothrombinase activity (A) and calpain activity (B,C) of fluoride-treated platelets, and the effect of leupeptin on this relation. Platelets were incubated with 10 mM fluoride in the absence (●) or presence (○) of 0.5 mM leupeptin. (B): Degradation of cytoskeletal proteins by calpain after addition of Ca^{2+} to fluoride-treated platelets, as judged by the formation of their fragments (sum of protein bands indicated at the right side of (C)). Data were quantitated from the gels shown in (C), after densitometric scanning as outlined in the Methods. Changes in the amount of fragments were counterbalanced by changes in the amount of substrates (not shown). (C) Upper panel: demonstration that calpain activity is not expressed in fluoride-treated platelets if no Ca^{2+} is added. Middle and lower panel: time-course of Ca^{2+}-inducible calpain activity during incubation with 10 mM fluoride, in the absence and presence of 0.5 mM leupeptin, respectively. Calpain activity is indicated by the disappearance of its substrates (indicated at the left), and the appearance of their fragments (indicated at the right). Samples for gel-electrophoresis were taken from the same incubation as that of which the Ca^{2+}-induced prothrombinase activity was determined. For experimental details is referred to the Methods. The results shown in this figure are taken from a representative experiment.

addition, when extracellular Ca^{2+} was omitted, no calpain activity could be demonstrated in fluoride-treated platelets (Fig. 3C). Generation of prothrombinase activity in fluoride-treated platelets was similarly dependent on extracellular Ca^{2+} (Fig. 1B).

In search of a direct demonstration of the involvement of calpain in Ca^{2+}-activated platelet procoagulant activity, we investigated the effect of leupeptin (inhibitor of calpain) on the relation between fluoride-dependent Ca^{2+}-induced prothrombinase activity and calpain activity. It has been suggested that leupeptin crosses the platelet plasma membrane [18,19]. The presence of leupeptin (0.5 mM) during incubation of platelets with fluoride resulted in a significantly decreased peak level of calpain activity, and the final low level was reached faster (Fig. 3B, C). An identical effect of leupeptin was observed on prothrombinase activity of platelets sampled from the same incubation with fluoride (Fig. 3A). Considering that leupeptin can inhibit thrombin [20], the

effect of leupeptin on the prothrombinase-assay was investigated. At the concentration used (15 μM in the prothrombinase assay), leupeptin had no effect on the enzymatic activity of the prothrombinase complex in the presence of vesicles composed of 20 mol% phosphatidylserine and 80 mole% phosphatidylcholine (data not shown).

The parallelism between Ca^{2+}-inducible prothrombinase activity and calpain activity was consistently observed ($n = 8$). The effect of leupeptin on both prothrombinase activity and calpain activity was highly variable between different platelet preparations, and frequently difficult to demonstrate. However, on those occasions that leupeptin did exhibit a clear effect ($n = 4$), the time-course of both Ca^{2+}-induced prothrombinase activity and calpain activity during incubation of platelets with fluoride was identically affected.

Discussion

In this paper we used fluoride-treated platelets to extend previous observations suggesting a role for calpain in the stimulus-response coupling of platelet procoagulant activity [10,11]. During incubation with fluoride the Ca^{2+} permeability of the platelet plasma membrane was increased in a reversible manner, causing platelet procoagulant activity, calpain activity, and exposure of phosphatidylserine to be enhanced with a similar time-course.

Fluoride has been reported to activate platelets by activation of G proteins [21–23]. Among others, this results in increased cytosolic Ca^{2+} levels, partly dependent of extracellular Ca^{2+} [22–24]. In addition, it has been suggested that G proteins are involved in the regulation of Ca^{2+} channels [25,26]. Therefore, we assume that the transient facilitation of Ca^{2+}-influx by fluoride is caused by the action of fluoride on G-protein-dependent Ca^{2+} channels in the platelet plasma membrane.

Ca^{2+} permeability increases right from the start of fluoride treatment. However, calpain activity and prothrombinase activity were increased only after a time-lag of 10 to 15 min. Platelet aggregation and release during fluoride treatment were found to level off within 15 to 20 min. This suggests that prothrombinase activity and calpain activity are dependent on higher cytosolic free Ca^{2+} levels than required for aggregation and release, confirming previous findings [11]. In addition, the discrepancy between the development of platelet aggregation and release, and the development of prothrombinase activity and calpain activity upon fluoride treatment, indicates that the cellular mechanisms generally shown to be involved in platelet stimulus-response coupling (phospholipase C, phospholipase A_2 and protein kinase C) are not directly responsible for the generation of increased phospholipid transbilayer movement during the expression of platelet procoagulant activity. These conclusions are in accordance with previous observations that thrombin, being a strong platelet agonist, is hardly able to induce procoagulant activity and calpain activity [10].

Peak level of fluoride-dependent Ca^{2+}-induced prothrombinase activity is accompanied by increased accessibility of phosphatidylserine towards phospholipase A_2 in comparison with unstimulated platelets and in comparison with platelets treated with fluoride for 2 h. This increased accessibility of phosphatidylserine cannot be explained only by increased hydrolysis of total phospholipids. Thus, it is concluded that the ratio phosphatidylserine/total phospholipids in the outer monolayer of the platelet plasma membrane follows a similar reversible time-course as the fluoride-dependent Ca^{2+}-induced prothrombinase activity. It is unlikely that exposure of phosphatidylserine results from fusion of granular membranes with the plasma membrane during the release reaction, since the release preceeds the development of prothrombinase activity in fluoride-treated platelets. Therefore phosphatidylserine must have crossed the lipid bilayer of the platelet plasma membrane considering its location in unstimulated platelets [6–9].

The observation that phospholipase A_2 alone is able to hydrolyse phospholipids in the platelet plasma membrane only under those conditions where an increased prothrombinase activity is seen, confirms earlier findings [6]. The ability of phospholipase A_2 to hydrolyse membrane lipids is greatly dependent on the molecular packing of the lipids, expressed as surface pressure, and may be enhanced by the presence of anionic phospholipids [27]. Increased ability of phospholipase A_2 to

hydrolyse membrane-lipids has been shown to correlate with increased merocyanine-540 uptake of the membrane [28–30], indicating a decreased order of membrane lipids. Therefore, we suggest that the increased hydrolysis of total phospholipids at peak level of fluoride-dependent Ca^{2+}-induced prothrombinase activity reflects both (1) changes in membrane-lipid organization, as they are thought to occur during enhanced transbilayer movement of phospholipids [31], and (2) the presence of anionic phospholipids in the outer monolayer of the platelet plasma membrane.

The observation that fluoride-dependent Ca^{2+}-induced calpain activity follows the same reversible time-course as prothrombinase activity, clearly extends previous observations suggesting a relation between calpain activity and platelet procoagulant activity [10,11]. In particular, the identical effect of leupeptin on both fluoride-dependent calpain activity and prothrombinase activity provides a strong argument for a role of calpain in the transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity, considering that calpain is the major Ca^{2+}-dependent proteinase in platelets [32].

The involvement of calpain-mediated cytoskeletal degradation in the regulation of transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity can be understood by applying the current model of the regulation of phospholipid transbilayer asymmetry in the erythrocyte. In erythrocytes the asymmetric distribution of phospholipids over the plasma membrane is thought to be maintained (1) by interaction of the lipid bilayer with cytoskeletal proteins located underneath the plasma membrane (membrane skeleton) [33,34], and (2) by the ATP-dependent action of an aminophospholipid-specific translocase [35–37]. The loss of phospholipid transbilayer asymmetry in the erythrocyte involves (1) a reorganization of the membrane-skeleton, disabling the interaction with plasma membrane phospholipids [38–40], and (2) an increased transbilayer movement of phospholipids via disturbances of the lipid bilayer structure [41,42].

It is becoming increasingly evident that platelets, besides a phospholipid transbilayer asymmetry similar to erythrocytes [6–9], also contain a membrane-skeleton [43–48], functionally similar to that of the erythrocyte [49]. Therefore, the role of calpain-mediated cytoskeletal degradation in the transbilayer movement of phosphatidylserine during expression of platelet procoagulant activity (i.e. the loss of phospholipid transbilayer asymmetry), is thought to be disabling the interaction between membrane skeleton and membrane lipids, thereby enabling the phospholipids to participate in transbilayer movement. Whether a bidirectionally functioning specific translocase, as proposed by Williamson et al. [50], is involved in the transbilayer movement of phosphatidylserine, is at present unclear. However, it may well be that the loss of interaction between membrane skeleton and plasma membrane, by itself, produces local disturbances in the lipid bilayer (flip sites) via which transbilayer movement of phospholipids can proceed at enhanced rates. This suggestion has been put forward earlier [29,41,42] and is documented by observations that changes in the organization of the membrane skeleton are accompanied by changes in the structural organization of the lipid bilayer of the plasma membrane, in erythrocytes [28–30,51,52] as well as in platelets [53].

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