Biochimica et Biophysica Acta 903 (1987) 206–217
Elsevier

BBA 73679

Correlation between calpain-mediated cytoskeletal degradation and expression of platelet procoagulant activity. A role for the platelet membrane-skeleton in the regulation of membrane lipid asymmetry?

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(Received 16 April 1987)

Key words: Platelet activation; Cytoskeleton; Prothrombinase activity; Calpain; Calcium dependence; Phospholipid asymmetry; (Human erythrocyte membrane)

The relationship between platelet calpain-activity and platelet procoagulant-activity was investigated by comparison of the time course of their generation after platelet stimulation by calcium ionophore A23187, or by the combined action of collagen and thrombin, or during exposure of platelets to the local anesthetics dibucaine or tetracaine. In addition, the Ca^{2+} dose-response curves of both activities in intact platelets, obtained by stimulation with A23187 in the presence of Ca^{2+}/HEDTA-buffers, were compared. Platelet procoagulant activity was determined by assaying for prothrombinase activity in the presence of saturating concentrations of factors Xa, Va, and prothrombin. Platelet calpain activity was monitored by the degradation of its major substrates (filamin, talin, myosin) and the formation of their fragments as judged from protein patterns after gel electrophoresis. Platelet stimulation by A23187 resulted in a fast increase in prothrombinase activity, reaching its maximum level after about 20 seconds. Filamin and talin were completely hydrolysed within 15 s, and myosin was partly degraded between 15 and 30 s after platelet activation. When platelets were activated by collagen plus thrombin, prothrombinase activity was generated with a sigmoid time course, the steepest increase being observed between 1 and 2 min after platelet activation. Proteolysis of filamin and talin occurred between 0.5 and 1.5 min after platelet activation, while degradation of myosin became visible after 2 to 2.5 min. Dibucaine and tetracaine were both found to be potent stimulators of prothrombinase activity, with half-maximal activities obtained at 0.7 and 2.8 mM, respectively. Using suboptimal concentrations of both local anesthetics, it was found that the generation of prothrombinase activity closely paralleled that of calpain activity over a time course of 1 hour. Ca^{2+} titration of intact platelets using A23187 and Ca^{2+}/HEDTA buffers, revealed half-maximal response at about 15 μM free Ca^{2+} for both calpain and prothrombinase activity. These findings strongly suggest a causal relationship between generation of a procoagulant platelet surface and calpain-mediated degradation of filamin, talin, and myosin. Since an increased procoagulant activity reflects an increased exposure of phosphatidylserine at the platelet outer surface, the present findings suggest that platelet cytoskeletal proteins are involved in the regulation of membrane lipid asymmetry.

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Introduction

One of the responses that platelets display upon activation is the generation of a procoagulant surface at the outside of the plasma membrane [1–4]. This procoagulant surface greatly, enhances the rate of two important sequential reactions in the coagulation cascade: the activation of factor X by a complex of factor IXa and factor VIIIa, and the activation of prothrombin by a complex of factor Xa and factor Va (the prothrombinase complex) [4]. The major determinant of platelet procoagulant activity is the membrane phospholipid phosphatidylserine, which upon activation becomes exposed at the outer surface of the platelet plasma membrane as a result of a transbilayer movement of phospholipids [4–6].

The agonist-pattern of platelet procoagulant activity [6] is different from any other platelet response: collagen is the only single physiological agonist able to significantly increase platelet procoagulant activity. Higher stimulation of platelet procoagulant activity is achieved by the combined action of collagen and thrombin. The most potent stimulator of platelet procoagulant activity, however, is the calcium-ionophore A23187, indicative for the importance of increased cytosolic Ca^{2+} levels in this response. Investigation of the protein pattern from platelets activated by different agonists showed a strong correlation between the extent of expression of platelet procoagulant activity and the extent of proteolysis of cytoskeletal proteins by calpain * [7].

In view of findings that interactions between membrane-skeletal proteins and anionic phospholipids may stabilize membrane lipid asymmetry in erythrocytes [8–12], it has been suggested that calpain-mediated degradation of cytoskeletal proteins may be a prerequisite for the loss of membrane lipid asymmetry which occurs during the expression of platelet procoagulant activity [7].

In order to substantiate this suggestion, the correlation between stimulation of calpain-activity and stimulation of platelet procoagulant activity was further explored by investigation of their time-response relationship after platelet activation by collagen plus thrombin, by A23187, and by exposure of platelets to the local-anesthetics dibucaine or tetracaine, which have been reported to activate platelet calpain [13–15]. Furthermore, the Ca^{2+} response relationship of both activities was investigated, using Ca^{2+} buffers and A23187 to equilibrate extra- and intra-cellular free Ca^{2+}.

Materials and Methods

Blood coagulation factors Va, Xa, prothrombin and thrombin were isolated and purified according to Rosing et al. [16]. Collagen type I from equine tendon was obtained from Hormon Chemie (Munich, F.R.G.). The thrombin-specific chromogenic substrate, S2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-*p* – nitroanilide dihydrochloride), was purchased from Kabi Diagnostica (Stockholm, Sweden). Dibucaine-HCl and tetracaine-HCl were from ICN Pharmaceuticals (New York, U.S.A.) and from Sigma (St. Louis, U.S.A.), respectively. Ionophore A23187 was obtained from Calbiochem-Hoechst (San Diego, U.S.A.). HEDTA (*N*-hydroxyethylenediaminetriacetic acid) was purchased from Sigma (St. Louis, U.S.A.). Other (bio)chemicals were of the highest grade commercially available.

Platelet isolation. Human platelets were isolated from freshly drawn blood as described previously [17]. Washed platelets were resuspended in a calcium-free buffer at pH 7.5, 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 0.1% fatty acid free human serum albumin (Hepes buffer). Platelet concentration was adjusted to approx. 10^8 ml^{-1}, using a Coulter Counter. Platelet lysis was determined by measuring lactate dehydrogenase activity in a $12000 \times g$ supernatant according to Wroblewski and La Due [18].

Assay of platelet procoagulant activity. Platelet procoagulant activity was determined essentially

* The name calpain (EC 3.4.22.17) is used for the ubiquitous Ca^{2+}-dependent neutral thiol-protease [82], originally described in platelets by Phillips and Jakabova [24]. The platelet protein known as P235 is named talin, in accordance with the demonstrated similarity between P235 and talin [27,28]. The name filamin [83] is used for ABP (platelet actin-binding-protein, M_r 250000), considering their identical properties [25] and immunochemical cross-reactivity [84].

as described by Rosing et al. [4], by assaying for prothrombinase activity under conditions at which the procoagulant surface is rate limiting. The assay was carried out at 37°C in Hepes buffer under continuous stirring (300 rev./min). To monitor the generation of prothrombinase activity after stimulation by collagen plus thrombin or A23187, factor Xa, factor Va, and $CaCl_2$ were added to prewarmed Hepes buffer 45 s before the start of the assay. 15 s later A23187 or collagen plus thrombin were added. Prothrombin was added 15 s before the assay was started by addition of platelets. Final concentrations: factor Xa 3 nM, factor Va 6 nM, prothrombin 4 μM, $CaCl_2$ 3 mM, A23187 1 μM, collagen 10 μg/ml, thrombin 4 nM, platelets $5 \cdot 10^6$ ml^{-1}. After various time-intervals, the prothrombin-activation was arrested by diluting an aliquot of the assay mixture into a cuvette containing a buffer composed of 50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA (pH 7.5). Thrombin activity was determined spectrophotometrically at 405 nm, after addition of chromogenic substrate S2238 to a final concentration of 150 μM. The amount of thrombin formed was calculated from the change in absorbance per minute, using a calibration curve made with active-site titrated thrombin.

The time-dependent effect of dibucaine and tetracaine on platelet procoagulant activity was followed by assaying a subsample for prothrombinase activity after dilution to $5 \cdot 10^6$ platelets per ml. Platelets were incubated with local anesthetics at a count of $1 \cdot 10^8$ ml^{-1}. Thrombin formation was measured one minute after the addition of the subsample to the assay mixture containing factor Xa, factor Va, prothrombin, and $CaCl_2$ in Hepes buffer, as described for collagen plus thrombin and A23187.

Calcium titration of prothrombinase activity. All Ca^{2+}-titrations were carried out at 37°C in a magnesium-free HEDTA/Ca^{2+} buffer (10 mM Hepes, 120 mM NaCl, 3 mM KCl, 5 mM glucose, 30 mM HEDTA, various amounts of $CaCl_2$ (pH 7.4)), at a platelet count of $2 \cdot 10^8$ ml^{-1} and under continuous stirring. An apparent K_d for HEDTA/Ca^{2+} of $1.9 \cdot 10^{-6}$ M was determined under the same conditions as used for the Ca^{2+} titrations by computer-fitting of the titration curve, obtained when a Ca^{2+} solution of known titer was titrated with a HEDTA solution, using a Ca^{2+}-selective electrode and a recording automatic titrator (Radiometer, Copenhagen, Sweden; type F2110Ca and RTS-system, respectively). The free Ca^{2+} concentration of the various Ca^{2+} buffers was calculated using the apparent K_d for HEDTA/Ca^{2+}, and was verified with the Ca^{2+}-selective electrode. It was found that the Ca^{2+}-buffering capacity of the employed HEDTA/Ca^{2+} buffer was sufficient to maintain applied extracellular free Ca^{2+} concentrations when platelets were challenged with A23187, over the whole range of extracellular free Ca^{2+}.

The Ca^{2+} dose-response relationship of platelet procoagulant activity was determined as follows: 1 min after addition of 1 vol. platelets (suspended in 50 mM Hepes buffer (pH 7.4) at $2 \cdot 10^9$ ml^{-1}) to 9 vol. HEDTA/Ca^{2+} buffer, A23187 was added from a stock solution in DMSO to a final concentration of 0.3 μM (final DMSO concentration: 0.5%). Platelets were left to equilibrate with extracellular free Ca^{2+} for 5 min at 37°C under continuous stirring. Then formaldehyde was added to a final concentration of 300 mM. After 5 min a sample was taken and diluted into Hepes buffer containing 1% fatty acid-free human serum albumin and 3 mM Ca^{2+} to a platelet count of $5 \cdot 10^6$ ml^{-1}. Prothrombinase activity was assayed by determination of the amount of thrombin formed in one minute, after the addition of factor Xa, factor Va, and prothrombin.

The fixation of platelets after equilibration with the Ca^{2+} buffer is necessary to prevent post-activation of procoagulant activity by A23187-mediated entry of Ca^{2+}, which has to be present at millimolar concentrations during the prothrombinase assay for functional binding of the prothrombinase complex to a procoagulant surface. It was found that residual formaldehyde, present during the prothrombinase assay, inhibited prothrombinase activity by some 50%. This inhibition, however, was independent of the extent of prothrombinase activity itself, and independent of the free Ca^2 concentration of the Ca^{2+} buffer.

Determination of calpain activity by gel electrophoresis. For proper correlation of calpain activity with prothrombinase activity, the same batch of platelets was used for both determinations throughout all experiments. Generation of

calpain-activity after platelet stimulation by A23187 or collagen plus thrombin was monitored at a higher platelet count than used for the prothrombinase assay, for practical reasons. The protein patterns were found not to be affected by the increased platelet count.

At 37°C, under continuous stirring, washed human platelets were added to human serum albumin-free Hepes-buffer containing 3 mM $CaCl_2$ and the stimulators. After various time-intervals calpain-activity was arrested by addition of an equal volume of Hepes buffer (pH 6.6) containing 2 mM mersalylic acid, 2 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM benzamidine and 20 mM EGTA. After collection of platelets by centrifugation at $5000 \times g$ for 5 min in the presence of 15% ACD (acid/citrate/dextrose: 180 mM glucose, 80 mM trisodium citrate, 52 mM citric acid), gel samples were prepared by dissolving the platelet pellet to a concentration of $2 \cdot 10^9$ ml^{-1} in sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 3% mercaptoethanol, 0.005% bromophenol blue, 10 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 mM mersalylic acid). Routinely all samples were boiled for 5 min before application to the gel. Disc electrophoresis was performed according to Laemmli [19], using 6–12% gradient polyacrylamide slab gels with a 3% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue.

Generation of calpain activity during incubation of platelets (10^8 ml^{-1} in Hepes buffer) with dibucaine or tetracaine, was followed by arresting and processing subsamples taken after various time intervals, as described for A23187 and collagen plus thrombin.

The effect of extracellular free Ca^{2+} concentration on platelet calpain activity was determined by equilibration of washed platelets with various HEDTA/Ca^{2+} buffers in the presence of 0.3 μM A23187, as described for the determination of prothrombinase activity. After 10 min platelets were arrested and processed as described for collagen plus thrombin.

Results

Generation of calpain activity and prothrombinase activity by A23187

The Ca^{2+}ionophore A23187, in the presence of extracellular Ca^{2+}, is a very potent stimulator of platelet procoagulant activity: within one minute full activity is already expressed [20]. In order to monitor the generation of prothrombinase activity on such a short time-scale, platelets were activated in the presence of all components of the pro-

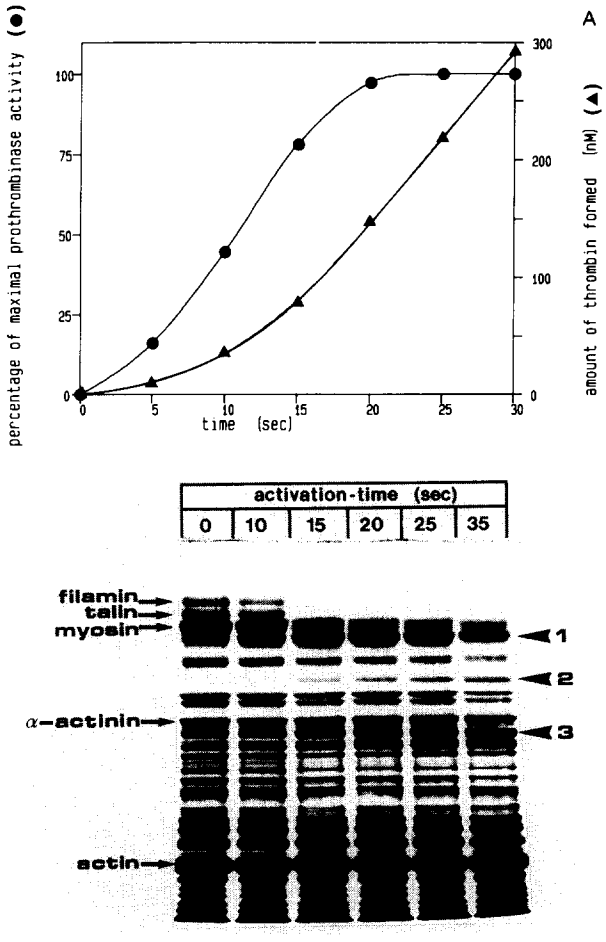


Fig. 1. Generation of prothrombinase activity (A) and calpain activity (B) by A23187. (A) Generation of prothrombinase activity (●) was reconstructed from the time course of thrombin formation (▲) by determination of the slope of its tangent at different time points. Prothrombinase activity was assayed as described in the methods, with the exception that factor Xa and factor Va were present at 15 and 30 nM, respectively. (B) Washed human platelets, at $1 \cdot 10^8$ ml^{-1}, were stimulated by 1 μM A23187. Calpain activity is monitored after gel electrophoresis by the appearance of fragment 1 (from filamin and talin), fragment 2 (from myosin), fragment 3 (from filamin), and fragment 4 (from talin). The fragments are indicated at the right by numbered arrows. At the left the major cytoskeletal proteins are identified. The data shown are taken from a representative experiment.

thrombinase complex, and samples were taken every 5 s to determine the amount of thrombin formed. In this way a time course of thrombin formation was obtained which initially increases exponentially and then gradually develops into a straight line (Fig. 1A), reflecting the generation of prothrombinase activity to a constant level. Since this curve represents the time course of thrombin formation, the actual rate of thrombin formation (prothrombinase activity) at any time is reflected by the tangent of the curve at that time point. In this way the data were recalculated as prothrombinase activity relative to the final level (fig. 1A). It was found that upon activation of platelets with A23187, prothrombinase activity is increased without any obvious delay and almost in a linear fashion, to reach its maximum level after about 20 s.

A complicating factor in the determination of prothrombinase activity on such short time scales, is the non-zero assembly time needed for the components of the prothrombinase complex to form a functional unit on the procoagulant surface. To overcome this problem, high concentrations of factor Xa and factor Va were applied (15 and 30 nM, respectively). It was found that under these conditions the assembly-time was negligible.

Platelet calpain activity was monitored by the degradation of its major substrates and the formation of their fragments as judged from protein patterns obtained after polyacrylamide gel electrophoresis of reduced protein samples. The major substrates of calpain are the cytoskeletal proteins filamin, talin, and myosin [7,13, 21–24]. The major degradation products are found at 190 kDa (fragment 1, derived from filamin [25,26] and talin [25,27–29]), at 135 kDa (fragment 2, originating from myosin [7,30,31]), at 93 kDa (fragment 3, coming from filamin [25,26]), and at 48 kDa (frag-

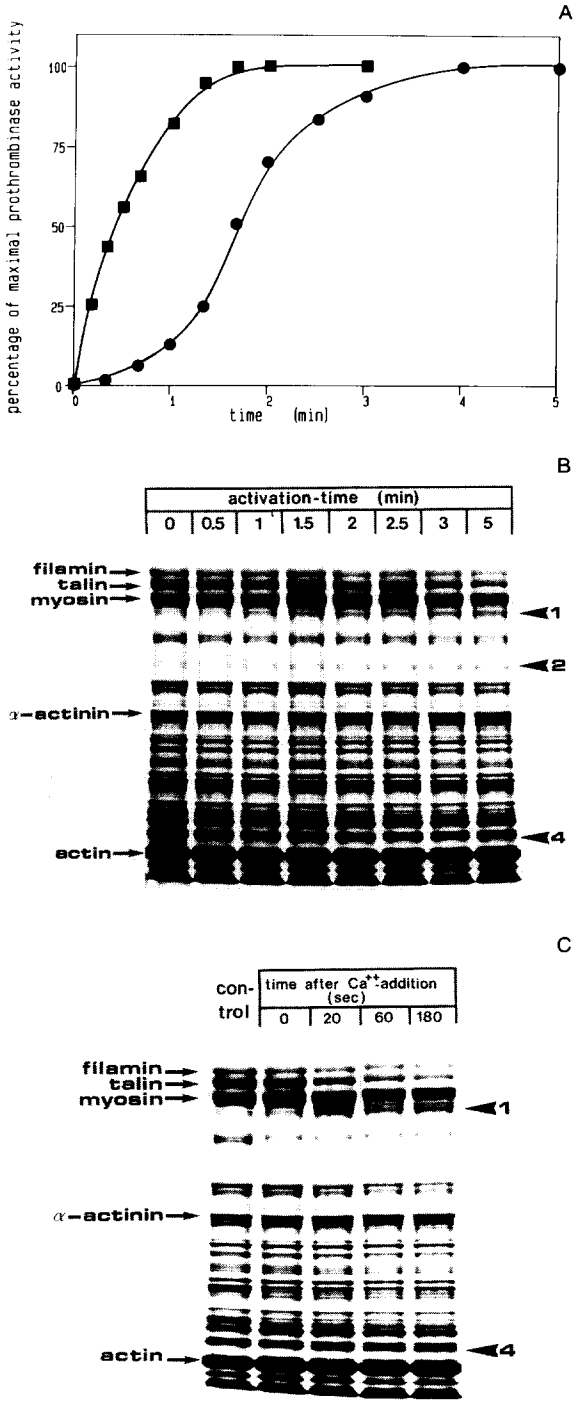


Fig. 2. Generation of prothrombinase activity and calpain activity by the combined action of collagen plus thrombin. Development of prothrombinase activity and calpain activity was followed after platelet activation by collagen (10 μg/ml) plus thrombin (4 nM), either in the presence of 3 mM Ca^{2+} (A, ●; B), or after addition of 4 mM Ca^{2+} to platelets previously activated with collagen plus thrombin for 5 minutes in the presence of 1 mM EGTA (A, ■; C). The lane identified by control (C) refers to untreated platelets. Incubations for gel electrophoresis were performed at a platelet count of $5 \cdot 10^7$ ml^{-1}. Reference arrows aside the protein patterns are explained in Fig. 1. The data shown are taken from a representative experiment. Experimental details are outlined in Materials and Methods.

ment 4, produced from talin [25,27–29]). When platelets are activated by A23187 in the presence of 3mM extracellular Ca^{2+}, calpain activity is already evident after about 10 s (Fig. 1B). Filamin and talin are most rapidly degraded the next 5 s, followed by proteolysis of myosin.

When the generation of platelet procoagulant activity and calpain activity after stimulation by A23187 are compared, it is obvious that filamin and talin are completely degraded when half-maximal prothrombinase activity is reached, while myosin degradation becomes apparent at maximal prothrombinase activity.

Generation of calpain activity and prothrombinase activity by the combined action of collagen plus thrombin

Upon stimulation of washed platelets by both collagen and thrombin in the presence of Ca^{2+} and the factors of the prothrombinase complex, platelet procoagulant activity developed slowly during the first minute, followed by a steep increase between 1 and 2 min after platelet stimulation, and gradually levelling off after 2 to 4 min (Fig. 2A). At this time scale the assembly time of the prothrombinase complex was not rate limiting.

Development of calpain activity was judged by the appearance of fragment 1 and fragment 2 (Fig. 2B). Most of fragment 1 was produced between 0.5 and 1.5 min after platelet stimulation, while fragment 2 appeared only faintly at about 2 min. Clearly, the degradation of filamin and talin by calpain coincides with the initial phase of generation of prothrombinase activity when platelets are stimulated by collagen plus thrombin.

Previously we have shown that when platelets are challenged with collagen plus thrombin in the presence of EDTA, proteolysis of cytoskeletal proteins by calpain does not occur [7]. Based on this observation we investigated the generation of both calpain and procoagulant activity after the addition of millimolar Ca^{2+} to platelets incubated with collagen plus thrombin for 5 min in the presence of 1 mM EGTA (Fig. 2A). It was found that immediately after the addition of Ca^{2+}, the prothrombinase activity steeply increased to its maximum level in 1.5 min, while partial proteolysis of filamin and talin occurred during the first 20 s after the addition of Ca^{2+} (Fig. 2C).

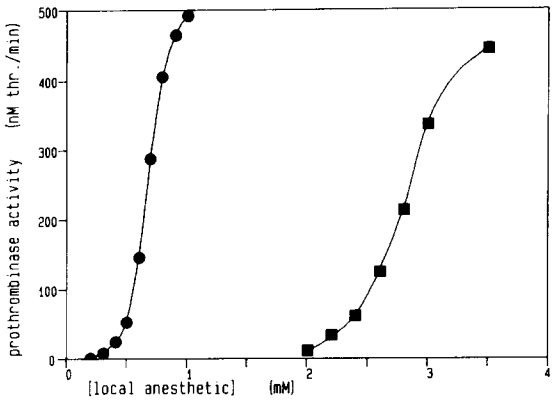


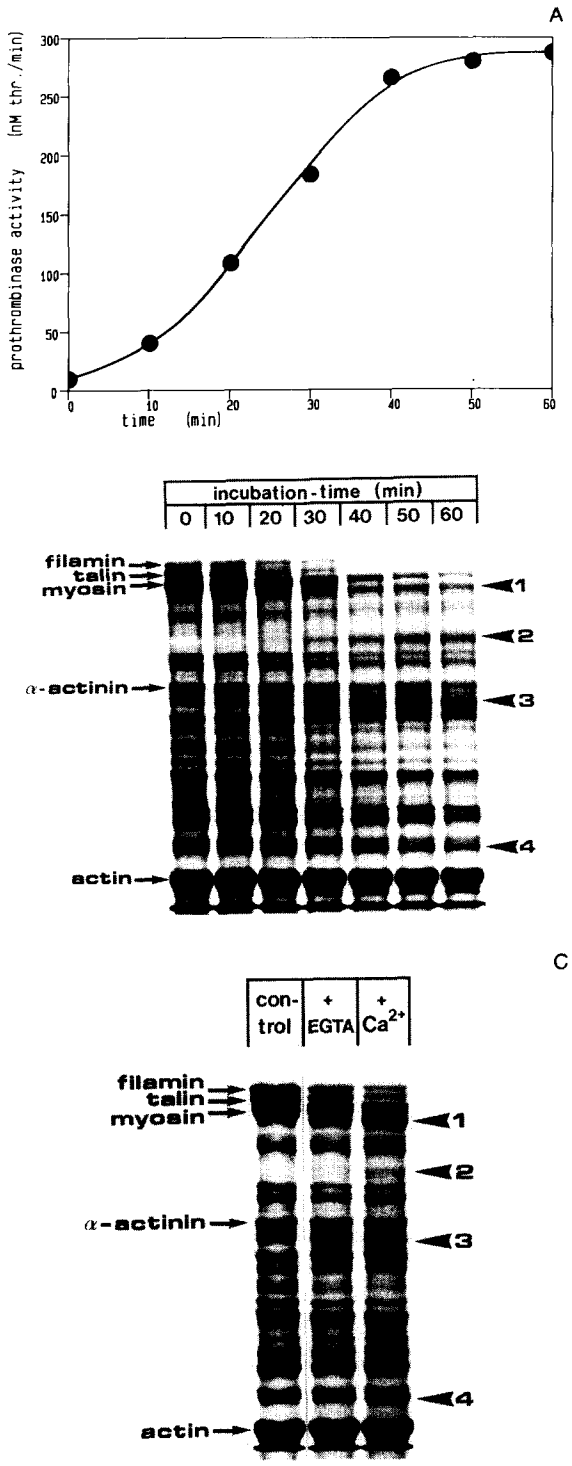
Fig. 3. Dose-dependent stimulation of prothrombinase activity by the local anesthetics dibucaine (●) or tetracaine (■). Platelets ($5 \cdot 10^6$ ml^{-1}) were challenged with the local anesthetics for 10 min before prothrombinase activity was determined 1 min after addition of the components of the prothrombinase complex, as described in Materials and Methods.

Generation of calpain activity and prothrombinase activity by local anesthetics

To further explore the relationship between platelet calpain activity and procoagulant activity the local anesthetics dibucaine and tetracaine were employed. These agents seemed especially attractive, since they were reported to activate calpain [13–15], while they inhibit platelet aggregation and secretion [32]. First, their ability to generate platelet procoagulant activity was investigated at various concentrations. It was found that both dibucaine and tetracaine were potent stimulators of prothrombinase activity (Fig. 3). Dibucaine was 4–5-times more potent than tetracaine. Above 1 mM dibucaine or 3.5 mM tetracaine, measurements became unreliable as a result of considerable platelet lysis. Below these concentrations no significant lysis occurred.

To compare the generation of calpain activity and procoagulant activity by the local anesthetics, platelets were incubated with a suboptimal dose of dibucaine or tetracaine. In the presence of 0.5 mM dibucaine, platelet prothrombin-converting activity was raised to a maximum level within 45 min, with most of the increase after the first 10 min (Fig. 4A). When samples, taken from the same incubation, were investigated for calpain-activity (Fig. 4B), it was found that between 10 and 50 min after the addition of dibucaine, cytoskeletal degradation develops to its maximum level. This

time course coincides with that of the generation of platelet procoagulant activity by dibucaine.



When platelets were incubated with 2 mM tetracaine, a similar correlation as with dibucaine was observed between the generation of calpain activity and prothrombinase activity, on a time scale of one hour (not shown).

Incubation of washed platelets with 1 mM dibucaine for 10 minutes in the presence of 1 mM EGTA did not result in inhibition of prothrombinase activity (not shown), while cytoskeletal degradation by calpain was only moderately inhibited, in comparison to a similar incubation in the presence of 1 mM Ca^{2+} (Fig. 4C). When platelets were lysed by sonication in the presence of EGTA, subsequent addition of 1 mM dibucaine did not produce any degradation of cytoskeletal proteins (not shown). Therefore, activation of platelet calpain by local anesthetics is not dependent on extracellular Ca^{2+}, and is not a result of direct interaction with calpain.

Ca^{2+} dose-response relationship of the stimulation of calpain activity and procoagulant activity

To gain additional support for a causal relationship between calpain activity and procoagulant activity, the response to the level of intracellular free Ca^{2+} of both activities was examined by stimulation of washed platelets with A23187 (0.3 μM), in the presence of Ca^{2+}/HEDTA buffers at various free Ca^{2+} levels.

The dependence on free Ca^{2+} of the generation of platelet procoagulant activity, is shown in Fig. 5A. It was found that prothrombinase activity increased slowly upon raising free Ca^{2+} from sub-

Fig. 4. Generation of prothrombinase activity and calpain activity by dibucaine. The effect of dibucaine (0.5 mM) in the presence of 3 mM Ca^{2+} on the development of prothrombinase activity and calpain activity is shown in panel A and panel B, respectively. Panel C shows the effect of 1 mM EGTA on the stimulation of calpain by 1 mM dibucaine for 10 min (lane: +EGTA), in comparison with a similar stimulation in the presence of 1 mM Ca^{2+} (lane: $+Ca^{2+}$), and in comparison with untreated platelets (control lane). The data shown are taken from a representative experiment. (A) Prothrombinase activity was assayed after dilution to $5 \cdot 10^6$ pl/ml, as described in Materials and Methods. Untreated platelets retained a constant basal level of prothrombinase activity throughout the incubation-period. (B,C) Reference arrows aside the protein patterns are explained in Fig. 1. Samples for gel electrophoresis were prepared as outlined in Materials and Methods.

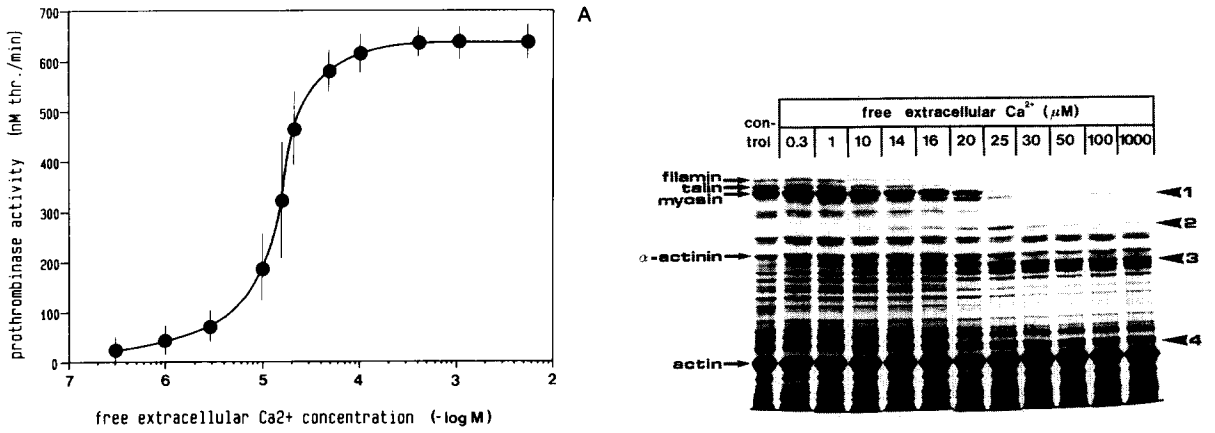


Fig. 5. Ca^{2+} dose-response relationship of platelet prothrombinase activity (A) and calpain activity (B). Washed human platelets, at a concentration of $2 \cdot 10^8$ ml^{-1}, where challenged with 0.3 μM A23187 in the presence of 30 mM HEDTA/Ca^{2+} buffer. Prothrombinase activity was determined after fixation with formaldehyde and dilution to $5 \cdot 10^6$ platelets per ml. Protein samples were prepared after arrest of calpain activity by addition of surplus EGTA and protease inhibitors. The data shown in (A) are the mean of the three experiments. The data shown in (B) are taken from a representative experiment. Experimental details are described in Materials and Methods.

micromolar levels. Between 5 and 30 μM free Ca^{2+}, prothrombinase activity developed with a steep increase. Half-maximal activity was obtained at 15 μM free Ca^{2+}. Beyond 30 μM free Ca^{2+}, prothrombinase activity gradually leveled off to reach maximal activity at 100 μM free Ca^{2+}.

Investigation of calpain activity over the same range of free extracellular Ca^{2+} levels (Fig. 5B), led to the following observations: (i) Hydrolysis of filamin and talin already starts at submicromolar levels of free Ca^{2+} as evidenced by the appearance of fragment(s) 1 and fragment 3. Major degradation of filamin and talin takes place going from 10 to 20 μM free Ca^{2+}, and between 20 to 25 μM free Ca^{2+} all filamin and talin are degraded. (ii) Hydrolysis of heavy-chain myosin rapidly increases just above 10 μM free Ca^{2+}, as judged from the appearance of fragment 2. Between 20 to 30 μM free Ca^{2+} myosin degradation is completed. (iii) In addition to the changes in the protein pattern with regard to the major cytoskeletal proteins filamin, talin, and myosin, several other proteins are affected by Ca^{2+}-dependent proteolysis. Noteworthy is the decrease in intensity of a protein band corresponding to the position of α-actinin (105 kDa), especially when free Ca^{2+} is raised above a level of 20 μM. Other Ca^{2+}-dependent changes in the protein pattern involving unidentified proteins are decreased bands corresponding to 170, 160, 130, 83, 75, 69, and 56 kDa, and new protein fragments at 140, 135, 125, 120, and 90 kDa (Fig. 5B). At all free Ca^{2+} levels the extent of protein degradation obtained after 15 min exposure to A23187 did not differed from those obtained after 5 min treatment with ionophore. Platelet lysis was insignificant at all Ca^{2+} concentrations. Generalizing, the most dramatic increase in Ca^{2+}-dependent proteolysis of platelet proteins is observed between 15 and 25 μM free Ca^{2+}. This level is quite similar to that at which half maximal activity of prothrombinase is observed.

Discussion

The experiments presented in this study demonstrate that platelet calpain activity and procoagulant activity are closely related phenomena with regard to the time course of their development after stimulation of platelets with A23187 or collagen plus thrombin, or after exposure of platelets to the local anesthetics dibucaine or tetracaine. This parallel generation covers a time scale ranging from less than half a minute to one hour. The similarity between the Ca^{2+}-dependence

of prothrombinase activity and calpain activity further strengthens their relationship.

The local anesthetics dibucaine and tetracaine appeared to be convenient agents to investigate the relationship between platelet procoagulant activity and calpain activity, since they were already known to stimulate calpain [13–15]. We found that local anesthetics are also able to stimulate platelet prothrombinase activity, supporting a finding by Peerschke [33] who used a clotting assay. The simultaneous stimulation of platelet prothrombinase activity and calpain activity by the local anesthetics strongly supports the notion that these two platelet processes are causally related.

It is generally observed that local anesthetics are inhibitors of other platelet activation phenomena [32,34–37]. More specifically, it has been shown that they inhibit phospholipase A_2 [38,39] and protein kinase C [40,41]. Therefore, the use of local anesthetics implicates that many processes normally involved in platelet activation (protein phosphorylation, arachidonate metabolism, secretion, aggregation) are not obligatory involved in the expression of platelet procoagulant activity. In fact, these implications have already been deduced from earlier experiments [42,43].

The potent effect of Ca^{2+}-ionophore A23187 both on calpain activity and on prothrombinase activity, suggests that extracellular Ca^{2+} may be required for both activities to develop. The role of extracellular Ca^{2+} is also obvious from the difference between the time course of development of prothrombinase activity of platelets triggered by collagen plus thrombin in the presence of Ca^{2+}, and of platelets to which Ca^{2+} was added after previous activation by collagen plus thrombin in the presence of EGTA. In the second situation prothrombinase activity steeply increased immediately after the addition of Ca^{2+}, while in the first situation such a steep increase is only observed after a lag phase of about 1 min. From these observations it is concluded that the entry of extracellular Ca^{2+} is an obligatory step in the generation of platelet procoagulant activity by collagen plus thrombin, and that the influx of this extracellular Ca^{2+} is enabled only after a delay of approximately 1 min.

In contrast, the stimulatory effect of local anesthetics on calpain activity was independent of the presence of extracellular EGTA, and was not a result of direct activation of calpain. Therefore, Ca^{2+} must have been released from intracellular sources, though influx of extracellular Ca^{2+}, if present, may also occur [33]. Most likely, this intracellular source is membrane-bound Ca^{2+}, or more specifically lipid-bound Ca^{2+} being displaced by dibucaine or tetracaine [44–46].

Quantitative information about the levels of cytosolic free Ca^{2+} can be obtained from the Ca^{2+}-titrations of platelet calpain activity and procoagulant activity, which both display the major increase at 10 to 15 μM free extracellular Ca^{2+}. The reliability of this information is based on the assumption that 0.3 μM A23187 will efficiently and accurately equilibrate cytosolic free Ca^{2+} with extracellular free Ca^{2+}. The validity of that assumption could not be assessed directly by measurements of the cytosolic level of free Ca^{2+} by quin2 or Fura2, since the binding constants for Ca^{2+} of these fluorescent chelators do not allow accurate determination of free Ca^{2+} levels greater than 1 or 2 micromolar [47–49]. Jy and Haynes [50] used a low concentration of A23187 (0.5 μM) and a Ca/EGTA buffer system to manipulate cytosolic free Ca^{2+}. Using quin2 they found that cytosolic free Ca^{2+} reflected extracellular free Ca^{2+} only in the range around the K_d of quin2. In our experiments, accurate equilibration of cytosolic and extracellular free Ca^{2+} is suggested by the abundant transport capacity of A23187 as document by the fast rise of calpain activity observed after addition of A23187 to platelets. In addition, half-maximal activity of calpain is observed at about 15 μM free Ca^{2+}, as estimated from the protein patterns. This value is in excellent agreement with values obtained with purified calpain-I.

Platelets are known to contain two isomers of calpain, calpain-I which is active at micromolar levels of free Ca^{2+} [22,25,51–54], and calpain-II which is active at millimolar levels of free Ca^{2+} [22,26,55]. Halfmaximal activity of calpain-I is obtained at free Ca^{2+} levels ranging from 1 to 25 micromolar. Our experiments with intact platelets revealed Ca^{2+}-dependent cytoskeletal degradation with halfmaximal activity at about 15 μM free Ca^{2+} as estimated from the protein patterns, supporting the notion that Calpain-I is the major

calcium-dependent protease in human platelets [22].

A peculiar finding, especially with regard to the Ca^{2+} titration of calpain activity in intact platelets is that degradation of cytoskeletal proteins stabilized within 5 min. It has been published that, upon stimulation, calpain is subject to autolysis of its heavy subunit [56], also in platelets [25], resulting in a reduction in molecular weight of the catalytic subunit. In addition it was observed that upon exposure to free Ca^{2+}, platelet calpain becomes very labile [53]. Therefore we assume that the termination of calpain activity in intact platelets within 5 min is due to autolysis, rendering calpain inactive. Another explanation could be that activated calpain becomes susceptible to inhibition by its endogenous inhibitor, calpastatin [57], also present in platelets [52].

The Ca^{2+} dependence of prothrombinase activity would suggest that collagen plus thrombin raise cytosolic free Ca^{2+} to 10–15 μM. Activation of platelets by thrombin has been observed to result in cytosolic free Ca^{2+} levels ranging from 1 to 6 μM as reported by quin2 [58–62] and Fura2 [63], or ranging from 7 to 12 μM as reported by aequorin [60,61]. Activation of platelets by collagen has been shown to elevate cytosolic free Ca^{2+} levels to about 2 μM as monitored by quin2 [61], or to levels ranging from 3 to 7 μM as reported by aequorin [61,64]. Studies with electrically permeabilized platelets indicated that Ca^{2+}-stimulated serotonin release is maximal at about 10 μM free Ca^{2+} [65,66]. Taking these summarized data into account, a level of 10 to 15 μM cytosolic free Ca^{2+}, resulting from stimulation of platelets by collagen plus thrombin, seems feasible considering the synergistic action of both potent platelet stimulators.

The close relationship between calpain activity and prothrombinase activity with regard to time course of development as well as Ca^{2+} requirement is consistent with a previous study by Comfurius et al. [7]. They found the same order of potency for various platelet stimulators to evoke procoagulant activity and calpain-induced cytoskeletal breakdown. Extensive studies have demonstrated that prothrombinase activity reflects the presence of phosphatidylserine at the outer surface of the platelet plasma membrane [4,6,43,67]. The present findings may therefore be interpreted as a relationship between exposure of phosphatidylserine and cytoskeletal degradation by calpain.

In erythrocytes a similar relationship between the loss of cytoskeletal integrity and the loss of asymmetric transbilayer distribution of phosphatidylserine is amply documented [8–11,68–70]. The major cytoskeletal protein of erythrocytes, spectrin, is thought to interact with anionic phospholipids [71–73], contributing to the maintenance of their asymmetric transbilayer distribution [12]. In addition, transbilayer reorientation of phosphatidylserine is thought to involve the formation of particular sites in the membrane, at which phosphatidylserine can take part in fast transbilayer movement, once released from binding to spectrin [74,75]. A recent development with regard to the factors involved in regulation of transbilayer orientation of anionic phospholipids in erythrocytes, is the discovery of an ATP-dependent inward transport of these lipids [76–79].

In platelets, the regulation of transbilayer asymmetry of anionic phospholipids, in resting platelets as well as upon platelet activation, is poorly understood. As yet, interactions between the major platelet cytoskeletal proteins and anionic phospholipids have not been demonstrated directly. Bearer et al. [80,81] showed that sub-membrane cytoskeletal structures change their appearance with the same time course as the appearance of anionic lipid at the platelet out surface, supporting the notion that in resting platelets interactions between structural proteins and anionic phospholipids play a role in the maintenance of transbilayer asymmetry of these lipids, analogous to erythrocytes. The experiments presented in this paper and in a previous one [7], show that degradation of cytoskeletal proteins by endogenous calpain could disturb these cytoskeleton-membrane interactions, contributing to a loss of membrane lipid asymmetry. This suggests a role for calpain in the regulation of the mechanisms by which phosphatidylserine becomes exposed at the outer platelet surface thus increasing the procoagulant activity of platelets.

Acknowledgements

The authors thank Mrs. W.M.A. Linskens for her excellent technical assistance. The work pre-

sented in this paper was financially supported by program grant 900-526-093 from Medigon (The Dutch Foundation for Medical- and Health Research).

References

1 Zwaal, R.F.A. and Hemker, H.C. (1982) Haemostatis 11, 12–39
2 Bevers, E.M., Rosing, J. and Zwaal, R.F.A. (1985) Adv. Ex. Med. Biol. 192, 359–371
3 Zwaal, R.F.A. and Bevers, E.M. (1986) in Lipids and Membranes: Past, Present and Future (Op den Kamp, J.A.F., Roelofsen, B. and Wirtz, K.W.A., eds.), pp. 231–257, Elsevier, Amsterdam
4 Rosing, J., Van Rijn, J.L., Bevers, E.M., Van Dieijen, G., Comfurius, P. and Zwaal, R.F.A. (1985) Blood 65, 319–332
5 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122 429–436
6 Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1983) Biochim. Biophys. Acta 736, 57–66
7 Comfurius, P., Bevers, E.M. and Zwaal, R.F.A. (1985) Biochim. Biophys. Acta 815, 143–148
8 Franck, P.F., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T., Op den Kamp, J.A., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190
9 Mohandas, N., Rossi, M., Berstein, S., Ballas, S., Ravindranath, Y., Wyatt, J. and Mentzer, W. (1985) J. Biol. Chem. 260, 14264–14268
10 Schneider, E., Haest, C.W., Plasa, G. and Deuticke, B. (1986) Biochim. Biophys. Acta 855, 325–336
11 Dressler, V., Haest, C.W., Plasa, G., Deuticke, B. and Erusalimsky, J.D. (1984) Biochim. Biophys. Acta 775, 189–196
12 Haest, C.W. (1982) Biochim. Biophys. Acta 694, 331–352
13 Solum, N.O. and Olsen, T.M. (1985) Biochim. Biophys. Acta 817, 249–260
14 Coller, B.S. (1982) Blood 60, 731–743
15 Nachmias, V.T., Sullender, J.S. and Fallon, J.R. (1979) Blood 53, 63–72
16 Rosing, J., Tans, G., Govers-Riemslag, J.W., Zwaal, R.F.A. and Hemker, H.C. (1980) J. Biol. Chem. 255, 274–283
17 Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 81–85
18 Wroblewski, R. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. Med. 90, 210–215
19 Laemmli, U.K. (1970) Nature 227, 680–685
20 Van Rijn, J., Rosing, J. and Van Dieijen, G. (1983) Eur. J. Biochem. 133, 1–10
21 Fox, J.E., Reynolds, C.C. and Phillips, D.R. (1983) J. Biol. Chem. 258, 9973–9981
22 Tsujinaka, T., Sakon, M., Kambayashi, J. and Kosaki, G. (1982) Thromb. Res. 28, 149–156
23 White, G.C., II (1980) Biochim. Biophys. Acta 631, 130–138
24 Phillips, D.R. and Jakabova, M. (1977) J. Biol. Chem. 252, 5602–5605
25 Fox, J.E., Goll, D.Ek., Reynolds, C.C. and Phillips, D.R. (1985) J. Biol. Chem. 260, 1060–1066
26 Truglia, J.A. and Stracher, A. (1981) Biochem. Biophys. Res. Commun. 100, 814–822
27 O'Halloran, T., Beckerle, M.C. and Burridge, K. (1985) Nature 317, 449–451
28 Beckerle, M.C., O'Halloran, T. and Burridge, K. (1986) J. Cell. Biochem. 30, 259–270
29 Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C. and Burridge, K. (1986) Nature 320, 531–533
30 Inomata, M., Hayashi, M., Nakamura, M., Imahori, K. and Kawashima, S. (1985) J. Biochem. 98, 407–416
31 Peleg, I., Muhlrad, A., Eldor, A., Groschel-Stewart, U. and Kahane, I. (1984) Arch. Biochem. Biophys. 234, 442–453
32 Feinstein, M.G., Fiekers, J. and Fraser, C. (1976) J. Pharmacol. Exp. Ther. 197, 215–228
33 Peerschke, E.I. (1986) Blood 68, 463–471
34 Lévy-Toledano, S., Maclouf, J., Bryon, P., Savariau, E., Hardisty, R.M. and Caen, J.P. (1982) Blood 59, 1078–1085
35 Zilberman, Y., Gutman, Y. and Koren, R. (1982) Biochim. Biophys. Acta 691, 106–114
36 Anderson, E.R., Foulks, J.G. and Godin, D.V. (1981) Thromb. Haemost. 45, 18–23
37 Glass, F., Lippton, H. and Kadowitz, P.J. (1980) Prostaglandins Med. 5, 85–92
38 Vanderhoek, J.Y. and Feinstein, M.B. (1979) Mol. Pharmacol. 16, 171–180
39 Feinstein, M.B., Becker, E.L. and Fraser, C. (1977) Prostaglandins 14, 1075–1093
40 Uratsuji, Y., Nakanishi, H., Takeyama, Y., Kishimoto, A. and Nishizuka, Y. (1985) Biochem. Biophys. Res. Commun. 130, 654–661
41 Haslam, R.J. and Lynham, J.A. (1978) Thromb. Res. 12, 619–628
42 Zwaal, R.F.A., Comfurius, P., Hemker, H.C. and Bevers, E.M. (1984) Haemostasis 14, 320–324
43 Bevers, E.M., Comfurius, P., Nieuwenhuis, H.K., Lévy-Toledano, S. Enouf, J., Belluci, S., Caen, J.P. and Zwaal, R.F.A. (1986) Br. J. Haematol. 63, 335–345
44 Kelusky, E.C., Boulanger, Y., Schreier, S. and Smith, I.C.P. (1986) Biochim. Biophys. Acta 856, 85–90
45 Cullis, P.R. and Verkley, A.J. (1979) Biochim. Biophys. Acta 552, 546–551
46 Ritchie, J.M. (1971) in International Encyclopedia of Pharmacology and Therapeutics. Section 8: Local Anesthetics, Vol. 1 (lechat, P., ed.), pp. 131–166
47 Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450
48 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res., 17, 535–541
49 Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell. Biol. 94, 325–334
50 Jy, W. and Haynes, D.H. (1984) Circ. Res. 55, 595–608
51 Kosaki, G., Tsujinaka, T., Kambayashi, J., Morimoto, K., Yamamoto, K., Yamagami, K., Sobue, K. and Kakiuchi, S. (1983) Biochem. Int. 6, 767–775
52 Tsujinaka, T., Shiba, E., Kambayashi, J. and Kosaki, G. (1983) Biochem. Int. 6, 71–80

53 Sakon, M., Kambayashi, J., Ohno, H. and Kosaki, G. (1981) Thromb. Res. 24, 207–214
54 Wang, L.L. and Bryan, J. (1981) Cell 25, 637–649
55 Yoshida, N., Weksler, B. and Nachman, R. (1983) J. Biol. Chem. 258, 7168–7174
56 Pontremoli, S., Melloni, E. and Horecker, B.L. (1985) Curr. Top. Cell. Regul. 27, 293–304
57 Cottin, P., Vidalenc, P.L. and Ducastaing, A. (1981) FEBS Lett. 136, 221–224
58 Rao, G.H., Peller, J.D. and White, J.G. (1985) Biochem. Biophys. Res. Commun. 132, 652–657
59 Rink, T.J., Smith, S.W. and Tsien, R.Y. (1982) FEBS Lett. 148, 21–26
60 Salzman, E.W., Johnson, P.C. and Ware, J.A. (1985) Adv. Exp. Med. Biol. 192, 163–170
61 Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W. (1986) J. Clin. Invest. 77, 878–886
62 Hatayama, K., Kambayashi, J., Kawasaki, T., Morimoto, K., Ohshiro, T. and Mori, T. (1986) Thromb. Res. 41, 761–770
63 Pollock, W.K. and Rink, T.J. (1986) Biochem. Biophys. Res. Commun. 139, 308–314
64 Nakano, T., Terawaki, A. and Arita, H. (1986) J. Biochem. 99, 1285–1288
65 Haslam, R.J. and Davidson, M.M. (1984) Biochem. J. 222, 351–361
66 Knight, D.E. and Scrutton, M.C. (1984) Nature 309, 66–68
67 Zwaal, R.F.A., Bevers, E.M. and Rosing, J. (1986) in Biology and Pathology of Platelet-Vessel Wall Interactions (LeGrand, Y., Jolles, J., Nurden, A., eds.), pp. 247–265, Academic Press, New York
68 Choe, H.R., Williamson, P., Rubin, E. and Schlegel, R.A. (1985) Cell. Biol. Int. Rep. 9, 597–606
69 Kuman, A. and Gupta, C.M. (1983) Nature 303, 632–633
70 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.R. and Schlegel, R.A. (1982) Cell 30, 725–733
71 Cohen, A.M., Liu, S.-C., Derick, L.H. and Palek, J. (1986) Blood 68, 920–926
72 Mombers, C., De Gier, J., Demel, R.A. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 603, 52–62
73 Marinetti, G.V. and Crain, R.C. (1978) J. Supramol. Struct. 8, 191–213
74 Bermann, W.L., Dressler, V., Haest, C.W. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398
75 Raval, P.J. and Allan, D. (1984) Biochim. Biophys. Acta 772, 192–196
76 Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A. and Van Deenen, L.L. (1986) FEBS Lett. 194, 21–27
77 Zachowski, A., Favre, E., Cribier, S., Herve, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590
78 Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416
79 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755
80 Bearer, E.L. and Friend, D.S. (1986) Lab. Invest. 54, 119–121
81 Bearer, E.L. and Friend, D.S. (1982) J. Cell. Biol. 95, 249a
82 Murachi, T. (1985) Biochem. Soc. Trans. 13, 1015–1018
83 Weihing, R.R. (1985) Can. J. Biochem. Cell. Biol. 63, 397–413
84 Okita, J.R., Pidard, D., Newman, P.J., Montgomery, R.R. and Kunicki, T.J. (1985) J. Cell. Biol. 100, 317–321