The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procoagulant Activity in Platelets

By Joan E.B. Fox, Clifford C. Reynolds, and Cary D. Austin

Although calpain (the Ca$^{2+}$-dependent protease) is widely distributed, its function is poorly understood. One cell in which it becomes activated as a consequence of activation of the cell is the blood platelet. The aim of the present study was to determine whether activation of calpain was responsible for any of the responses of platelets to stimulation. Platelets were incubated with calpeptin, a membrane-penetrating inhibitor of calpain, before being exposed to an agonist. Concentrations of calpeptin that totally inhibited agonist-induced hydrolysis of actin-binding protein (ABP) by calpain had no effect on many other responses associated with platelet activation: phosphorylation of myosin light chain, phosphorylation of P47, platelet shape change, aggregation of platelets, secretion of granule contents, or retraction of fibrin clots. However, these concentrations of inhibitor decreased the agonist-induced generation of procoagulant activity (assayed as the ability of platelets to catalyze the conversion of prothrombin to thrombin in the presence of factor V, and factor X). When thrombin was the agonist, the amount of ABP that was hydrolyzed was small; only a small component of the total agonist-induced procoagulant activity was inhibited by calpeptin. When collagen was the agonist, more ABP was hydrolyzed and the amount of procoagulant activity generated was greater; calpeptin decreased the collagen-induced procoagulant activity to levels comparable with those induced by thrombin in the presence of the inhibitor. We suggest that there are at least two mechanisms by which procoagulant activity is generated on activated platelets and that the agonist-induced activation of calpain mediates one of these mechanisms. These results show that activation of calpain is a component of the stimulus-response pathway in platelets.

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of granule contents, or retraction of clots. However, these concentrations of inhibitor partially inhibit the generation of procoagulant activity by the platelets. We suggest that there are at least two mechanisms by which an agonist induces procoagulant activity on platelets, and conclude that the agonist-induced activation of calpain mediates one of these mechanisms.

MATERIALS AND METHODS

Isolation of platelets. Venous blood was drawn from healthy adult donors, and platelets were isolated from it by centrifugation, as described.24 Platelets were resuspended in a Tyrode's buffer containing 138 mmol/L sodium chloride, 2.9 mmol/L sodium bicarbonate, 0.36 mmol/L sodium phosphate, 5.5 mmol/L glucose, 1.8 mmol/L calcium chloride, and 0.49 mmol/L magnesium chloride, pH 7.4. Platelets were labeled with [32P]phosphate as described elsewhere.25

Incubation of platelets with agonists. Platelets were incubated at 37°C in the absence or presence of the calpain inhibitors leupeptin or calpeptin. Leupeptin (Vega Biochemicals, Tucson, AZ) was added in saline; calpeptin was added in dimethyl sulfoxide (DMSO) (J.T. Baker Chemical Co, Phillipsburg, NJ). The final concentration of the DMSO was 0.2%. Calpeptin (2-Leu-Nle-H) was a generous gift from Dr Toshimasa Tsujinaka (Osaka University Medical School, Osaka, Japan). Platelets were subsequently incubated with 20 μg of collagen/mL (Horme, Munich, West Germany), 1.0 National Institutes of Health (NIH) unit of thrombin/mL (kindly provided by Dr J. W. Fenton II of the New York Department of Health, Albany), or a combination of the two agonists. In some experiments, platelets were incubated with the nonphysiologic agonist, ionophore A23187 (0.4 μmol/L) (Sigma Chemical Co, St Louis, MO), which was added in a final concentration of 0.2% (vol/vol) DMSO. Unless indicated otherwise, all incubations were performed in the presence of stirring.

Assessment of platelet aggregation and secretion. Luciferin-Luciferase reagent (Chronolog Corporation, Haverton, PA) was added to the platelet suspension (3 x 10⁹ platelets/mL), which was then stirred with an agonist in a dual-channel lumiaggregometer (Chronolog). The agonist-induced aggregation of platelets was assessed by a change in transmittance of light through the suspension, as detected in one channel of the lumiaggregometer. The secretion of adenosine triphosphate (ATP) from platelet granules was assessed by the change in luminescence of the Luciferin-Luciferase reagent as measured in the second channel of the lumiaggregometer. The amount of secreted ATP was calibrated by the subsequent addition of known amounts of ATP.

Assessment of procoagulant activity. Procoagulant activity was assayed by a modification of the assay described by Bevers et al.26 Platelets were stirred with various agents at a concentration of 0.1 x 10⁹ to 1.0 x 10⁹ platelets/mL. Platelets were then diluted to a final concentration of 0.02 to 0.25 x 10⁹ platelets/mL with a Tyrode's solution that contained 0.6 mmol/L factor X (Sigma), 1.1 mmol/L factor V (Enzyme Research Laboratories, South Bend, IN), and 1.3 μmol/L prothrombin (Sigma). The amount of thrombin formed was determined by removing 20 to 50 μL of the incubation, transferring the sample into 1 mL of the chromogenic substrate S2238 (150 μmol/L) (Helena Laboratories, Beaumont, TX), and determining the rate of change of absorbance at 405 nm. A standard curve was obtained using purified thrombin. When activation of platelets was induced with thrombin, control assays that contained the same concentration of thrombin (in the absence of platelets) were included.

Assessment of clot retraction. Platelet suspensions (1 x 10⁹ platelets/mL) were incubated for 5 minutes in the presence or absence of calpain inhibitors or a synthetic peptide consisting of the sequence Arg-Gly-Asp-Ser (RGDS). This RGDS peptide was a generous gift of Dr David Phillips (COR Therapeutics, Inc, South San Francisco, CA); it was added in saline to a final concentration of 300 μmol/L. Aliquots of the suspension (400 μL) were then transferred to siliconized aggregometer tubes that contained 120 μg of fibrinogen (Kabi Vitrum, Stockholm, Sweden). Thrombin was added to a final concentration of 1.0 NIH U/mL, and the subsequent retraction of clots was recorded photographically at intervals.

Analytical procedures. Platelet suspensions (3 x 10⁹ platelets/mL) were solubilized in the presence of a reducing agent and analyzed on one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli27 using 3% acrylamide in the stacking gel and a 5% to 20% exponential gradient of acrylamide in the resolving gel. Proteins were stained with Coomassie Brilliant Blue. The distribution of 32P in polyptides was determined by autoradiography of dried gels. Western blotting was performed by the method of Towbin et al28 as described previously.4 Affinity-purified ABP and talin antibodies were raised and characterized as reported previously.4

The concentration of platelets in suspensions of washed platelets was determined with a Coulter counter (Coulter Corp, Hialeah, FL).

RESULTS

To determine the concentration of calpeptin needed to inhibit agonist-induced activation of calpain, platelets were incubated with various concentrations of the inhibitor for 5 minutes before the addition of an agonist. Preincubation of platelets with 10 μg of calpeptin/mL almost totally abolished the subsequent collagen-induced hydrolysis of ABP (Fig 1A) by calpain. In contrast, higher concentrations of calpeptin were required to inhibit the collagen-induced hydrolysis of talin. Figure 1B shows the result of a typical experiment, in which it can be seen that even when the calpeptin concentration was 50 μg/mL, almost 50% of the talin was still hydrolyzed; and even at a calpeptin concentration of 200 μg/mL, hydrolysis of talin was still not totally inhibited. The concentrations of calpeptin required to inhibit the thrombin-induced hydrolysis of ABP or talin were similar to those required to inhibit the collagen-induced hydrolytic events (data not shown). Because calpeptin is a hydrophobic molecule, it appears likely that it would preferentially partition into the plasma membrane. Thus, the preferential inhibition of ABP hydrolysis may occur because this membrane-bound protein29-32 is hydrolyzed by a membrane-associated form of calpain while talin, which appears to be a soluble protein,33 is hydrolyzed by a soluble form of the protease.

Leupeptin, a calpain inhibitor that does not readily cross cell membranes, totally inhibited the hydrolysis of ABP and talin induced by thrombin (data not shown), presumably because it inhibited the proteolytic action of thrombin.16-19 However, leupeptin had little inhibitory effect on the collagen-induced hydrolysis of ABP or talin (data not shown) unless it was preincubated with platelets for 30 minutes at 37°C in the presence of 0.2% DMSO and at a concentration of 1 mg of leupeptin/mL. Under these extreme conditions, a partial inhibition of hydrolysis was observed (Fig 1A and B), presumably because some leupeptin penetrated the membrane.

Experiments were performed to determine whether any of the agonist-induced intracellular changes or functional responses were inhibited if the agonist-induced activation of
calpain was inhibited. One of the intracellular events that occurs after platelet stimulation is activation of PKC. This enzyme induces the phosphorylation of an unidentified cytoplasmic protein of mol wt 47,000, termed P47. Concentrations of calpeptin (Fig 1) that totally inhibited detectable hydrolysis of ABP had no obvious effect on the collagen-induced (Fig 2A) or thrombin-induced (Fig 2B) phosphorylation of P47.

Another intracellular event that occurs when platelets are activated is phosphorylation of the 20,000 mol wt light chain...
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of myosin. This phosphorylation reaction is mediated in large part by the Ca\(^{2+}\)-dependent activation of MLC kinase.\(^8\) Neither the collagen-induced phosphorylation (Fig 2A) nor the thrombin-induced phosphorylation (Fig 2B) was detectably inhibited by calpeptin at a concentration (50 µg/mL) that totally inhibited detectable hydrolysis of ABP. Quantitation of the data presented in Fig 2 confirmed that calpeptin did not inhibit the agonist-induced phosphorylation of P47 or MLC (Table 1).

Agonist-induced intracellular changes mediate several functional responses, including a change in platelet shape and the aggregation of platelets. The shape-change response can be detected as a small decrease in transmittance of light through a platelet suspension and is followed immediately by an increased transmittance as the platelets aggregate. Concentrations of calpeptin that totally inhibited detectable hydrolysis of ABP (50 µg of calpeptin/mL) had virtually no effect on platelet shape change or aggregation as assessed by the changes of light transmittance through a suspension of platelets aggregating in response to thrombin (Fig 3A), collagen (Fig 3B), or a combination of the two agonists (Fig 3C). The lack of an inhibitory effect on the shape-change response was confirmed by fixing the platelet suspensions and examining them under the electron microscope (data not shown). Similarly, these concentrations of inhibitor had no effect on the thrombin-induced (Fig 4A) or collagen-induced (Fig 4B) secretion of the contents of dense granules. In some experiments, higher concentrations of calpeptin (100 to 200 µg/mL) inhibited both aggregation (Fig 3) and ATP secretion (Fig 4). However, the extent of this inhibition varied from experiment to experiment and was overcome by higher concentrations of agonists (Fig 3C). Leupeptin, even under conditions in which it induced partial inhibition of the collagen-induced hydrolysis of ABP and talin (Fig 1A and B) had no effect on collagen-induced aggregation (data not shown) or ATP secretion (Fig 4B). However, as reported by others, leupeptin completely inhibited thrombin-induced aggregation (data not shown) and ATP secretion (Fig 4B), presumably because it inhibited the amidolytic action of thrombin.\(^6\)\(^-\)\(^9\)

Another functional response of platelets to stimulation is the retraction of fibrin clots. Because several of the proteins hydrolyzed by calpain (ABP, talin, and spectrin) can associate with the cytoskeleton,\(^4\) it has been suggested that activation of calpain might regulate the contractile activity that is responsible for the retraction of clots by platelet aggregates. To assess this possibility, platelet suspensions were incubated with various concentrations of calpeptin (or other agents) for 5 minutes before the addition of fibrinogen and thrombin. The subsequent retraction of the fibrin clot by the platelets was recorded photographically. Consistent with the known inhibitory action of leupeptin on the amidolytic action of thrombin, leupeptin inhibited clot retraction (Fig 5A, lane 2). Similarly, as shown by others,\(^3\) the synthetic peptide RGDS, which inhibits binding of fibrinogen to the glycoprotein IIb-IIIa complex on the platelet surface, inhibited the retraction of clots in the assay system used (Fig 5A, lane 3). In contrast, calpeptin, even at concentrations of 200 µg/mL, had no detectable effect on the process (Fig 5B).

Yet another functional response of platelets is the generation of procoagulant activity on their surface. One way that activated platelets accelerate coagulation is by providing a binding site for the prothrombinase complex. This complex, which consists of factor Va and factor Xa, catalyzes the conversion of prothrombin to thrombin. Verhallen et al\(^9\) have noted that the platelet-induced acceleration of prothrombinase activity correlates with the extent of activation of calpain within the platelets. They have suggested that there might be a causal relationship between the two events. We also have observed a relationship between these two events. As demonstrated by the generation of hydrolytic fragments of ABP (Fig 6), thrombin does not induce activation of calpain unless the platelet suspensions are stirred (Fig 6, compare lanes 1 and 2 with lanes 3 and 5). When suspensions are stirred, thrombin induces a small amount of activation of the protease (as detected by a small amount of the 200-Kd

<table>
<thead>
<tr>
<th>Agonist</th>
<th>P47 Minus Calpeptin</th>
<th>P47 Plus Calpeptin</th>
<th>MLC Minus Calpeptin</th>
<th>MLC Plus Calpeptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, 0 time</td>
<td>22,562</td>
<td>26,806</td>
<td>37,302</td>
<td>48,444</td>
</tr>
<tr>
<td>Collagen, 10 s</td>
<td>26,103</td>
<td>40,060</td>
<td>39,306</td>
<td>65,996</td>
</tr>
<tr>
<td>Collagen, 30 s</td>
<td>54,543</td>
<td>61,822</td>
<td>84,365</td>
<td>60,517</td>
</tr>
<tr>
<td>Collagen, 60 s</td>
<td>60,431</td>
<td>63,520</td>
<td>75,421</td>
<td>74,070</td>
</tr>
<tr>
<td>Collagen, 5 min</td>
<td>54,005</td>
<td>60,818</td>
<td>73,566</td>
<td>83,346</td>
</tr>
<tr>
<td>Collagen, 10 min</td>
<td>49,636</td>
<td>56,221</td>
<td>70,375</td>
<td>64,075</td>
</tr>
<tr>
<td>Collagen, 15 min</td>
<td>45,916</td>
<td>56,837</td>
<td>58,413</td>
<td>53,593</td>
</tr>
<tr>
<td>Thrombin, 0 time</td>
<td>16,072</td>
<td>15,280</td>
<td>25,587</td>
<td>31,637</td>
</tr>
<tr>
<td>Thrombin, 10 s</td>
<td>51,200</td>
<td>52,292</td>
<td>29,682</td>
<td>29,927</td>
</tr>
<tr>
<td>Thrombin, 30 s</td>
<td>53,811</td>
<td>54,161</td>
<td>30,487</td>
<td>31,064</td>
</tr>
<tr>
<td>Thrombin, 60 s</td>
<td>55,937</td>
<td>56,633</td>
<td>33,518</td>
<td>30,849</td>
</tr>
<tr>
<td>Thrombin, 5 min</td>
<td>57,695</td>
<td>57,029</td>
<td>36,373</td>
<td>37,693</td>
</tr>
<tr>
<td>Thrombin, 10 min</td>
<td>53,079</td>
<td>54,837</td>
<td>35,756</td>
<td>33,311</td>
</tr>
<tr>
<td>Thrombin, 15 min</td>
<td>50,298</td>
<td>48,896</td>
<td>32,638</td>
<td>34,287</td>
</tr>
</tbody>
</table>

The phosphorylation of P47 or MLC was quantitated by densitometry of the autoradiograms shown in Fig. 2. The numbers given represent arbitrary densitometry units.
hydrolytic fragment) (Fig 6, lane 3). As noted previously, the amount of ABP that is hydrolyzed is very small and can be detected only by the appearance of hydrolytic products on two-dimensional gels or on immunoblots (Fig 6). We find that collagen routinely induces a greater degree of activation of calpain than does thrombin (eg, Fig 6, lane 4), whereas a combination of collagen and thrombin induces sufficient activation to result in hydrolysis of amounts of ABP that can be readily detected as a decreased amount of intact protein (Fig 6, lane 5) (as can the hydrolysis induced by the nonphysiologic Ca²⁺ ionophore, A23187 [Fig 6, lane 6]). Like Verhallen et al.,⁹ we observed that the generation of procoagulant activity in a platelet suspension (Table 2) was greatest in response to the agonists that caused the greatest extent of hydrolysis of ABP. However, there was not a direct correlation between hydrolysis of ABP and generation of procoagulant activity; as reported by others,⁶,⁷ some procoagulant activity (Table 2 and Fig 7) was always generated in suspensions of platelets that were incubated with thrombin under conditions in which no detectable hydrolysis of ABP occurred (ie, in the absence of stirring [Fig 6]).

To determine whether activation of calpain was responsible for generating any of the procoagulant activity induced by agonists, platelets were incubated with 50 μg of calpeptin/mL. This concentration of inhibitor had little or no effect on the procoagulant activity in control platelet suspensions (Fig 7). Similarly, it did not inhibit the generation of procoagulant activity in a suspension of platelets that was incubated with thrombin but was not stirred (Fig 7). As shown in Table
Experiments were performed to eliminate the possibility that the inhibitory effect of calpeptin resulted from an inhibition of the assembly or activity of the prothrombinase complex. Platelets were incubated with collagen or dibucaine, two agents that have been shown to induce the shedding of microvesicles from the platelet plasma membrane. These microvesicles contain binding sites for the prothrombinase complex. As shown in Table 3, calpeptin had no effect on the amount of thrombin generated when these microvesicles provided the binding site for the prothrombinase complex.

**DISCUSSION**

Despite the fact that calpain is present in a wide variety of cells, a physiologic role for this protease has not previously been elucidated. The only cell in which activation of calpain has been shown to occur as a consequence of cellular activation is the platelet. However, the potential role that...
Assessed by the appearance of the hydrolytic fragments (mol wt = 200,000, 100,000, and 91,000) on immunoblots, the extent of calpain plays in mediating the responses of platelets has been a matter of controversy. Because only small amounts of calpain substrates are hydrolyzed in thrombin-activated platelets, some investigators have suggested that activation of calpain has no physiologic consequence. Others have suggested that hydrolysis of small pools of cytoskeletal proteins may be sufficient to induce changes in the function of the cytoskeleton in aggregated platelets. Until recently, investigation of the consequence of activation of calpain in platelets was hindered by the absence of inhibitors of calpain that could enter the cell. Although leupeptin is a fairly specific inhibitor of calpain, it crosses membranes poorly and it inhibits the amidolytic activity of thrombin, an activity that is required for thrombin to initiate its effects on platelets. Thus, studies using this inhibitor to evaluate the involvement of calpain in mediating the responses of thrombin are difficult to interpret.

In the present study, we have used calpeptin, an inhibitor of calpain that has a specificity similar to that of leupeptin but readily penetrates the membrane and does not inhibit the

Table 2. Generation of Procoagulant Activity by Agents That Activate Calpain

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Relative Extent of Hydrolysis of ABP</th>
<th>Procoagulant Activity (mol thrombin/min/10^10 platelets) x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>5.7 ± 0.8</td>
</tr>
<tr>
<td>Thrombin, stirred</td>
<td>+</td>
<td>12.5 ± 2.3</td>
</tr>
<tr>
<td>Collagen</td>
<td>++</td>
<td>28.9 ± 0.6</td>
</tr>
<tr>
<td>Collagen + thrombin</td>
<td>+++</td>
<td>39.3 ± 0.8</td>
</tr>
<tr>
<td>A23187</td>
<td>++++</td>
<td>40.3 ± 0.6</td>
</tr>
<tr>
<td>Thrombin, unstirred</td>
<td>None</td>
<td>7.7 ± 0.3</td>
</tr>
</tbody>
</table>

Incubations from the experiment shown in Fig 4 were terminated by solubilization of the suspensions in an SDS-containing buffer, and the extent of hydrolysis of ABP by the Ca^{2+}-dependent protease was assessed by the appearance of the hydrolytic fragments (mol wt = 200,000, 100,000, and 91,000) on immunoblots (see Fig 6) and is indicated on an increasing scale of + to ++++. Parallel incubations were diluted to 1 x 10^9 platelets/mL with buffers containing factor X, factor X_δ, and prothrombin. The amount of thrombin generated in a subsequent 5-minute incubation was determined with a chromogenic substrate as described in Materials and Methods. Values shown are the mean ± SE obtained from three different incubations.

Fig 7. Effect of calpeptin on the agonist-induced generation of procoagulant activity in platelet suspensions. Platelet suspensions (0.25 x 10^9 platelets/mL) were incubated for 5 minutes in the presence of calpeptin at the concentrations shown. Suspensions were subsequently stirred in the presence of 20 μg of collagen/mL (○—○) or 1.0 NIH U of thrombin/mL (■—■) for 10 minutes. The procoagulant activity generated in the platelet suspension was determined as described under Materials and Methods. The open triangle, indicated by an arrow, indicates the procoagulant activity present in a suspension of unstimulated control platelets.

Fig 8. Effect of calpeptin on the agonist-induced generation of procoagulant activity in platelet suspensions. Platelet suspensions (0.25 x 10^9 platelets/mL) were incubated for 5 minutes in the presence of calpeptin at the concentrations shown. Suspensions were subsequently stirred in the presence of 20 μg of collagen/mL (○—○) or 1.0 NIH U of thrombin/mL (■—■) for 10 minutes. The procoagulant activity generated in the platelet suspension was determined as described under Materials and Methods. The open triangle, indicated by an arrow, indicates the procoagulant activity present in a suspension of unstimulated control platelets.

Table 3. Effect of Calpeptin on Microvesicle-Induced Procoagulant Activity

<table>
<thead>
<tr>
<th>Microvesicles</th>
<th>Minus Calpeptin</th>
<th>Plus Calpeptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mol thrombin/15 min/mL of microvesicles x 10^6)</td>
<td></td>
</tr>
<tr>
<td>Microvesicles 1</td>
<td>0.22 ± 0.01</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Microvesicles 2</td>
<td>1.50 ± 0.05</td>
<td>1.50 ± 0.02</td>
</tr>
<tr>
<td>Microvesicles 3</td>
<td>1.97 ± 0.05</td>
<td>1.84 ± 0.03</td>
</tr>
</tbody>
</table>

Platelet suspensions (1 x 10^8 platelets/mL) were stirred for 15 minutes alone (Microvesicles 1), with 20 μg of collagen/mL (Microvesicles 2), or with 0.5 mmol/L dibucaine (Microvesicles 3). Intact platelets were removed by centrifugation at 15,600 g for 1 minute. The resultant microvesicle-containing supernatant was preincubated for 10 minutes in the presence of 0.2% DMSO or 20 μg of calpeptin/mL and 0.2% DMSO. Factor X_δ, factor X_δ, and prothrombin were then added, and the thrombin generated was measured. Values given are the mean ± SE from three different incubations.
The use of this inhibitor has enabled us to demonstrate that when the agonist-induced hydrolysis of ABP was totally inhibited, most of the platelet responses to collagen or thrombin proceeded normally. However, the generation of procoagulant activity was inhibited. This finding demonstrates that activation of calpain mediates one of the responses of platelets to activation.

The expression of procoagulant activity is an important functional response of platelets. It is critical for efficient hemostasis because it accelerates the formation of fibrin polymers throughout a platelet aggregate, converting the loose aggregate into a tight clot that is not easily disrupted by the shear forces that characterize circulating blood. The importance of platelet procoagulant activity is underscored by the bleeding disorder in a patient in whom the expression of this activity is decreased. One of the ways that platelets activate the coagulation "cascade" is by accelerating the final reaction, the conversion of prothrombin to thrombin by the prothrombinase complex.

The prothrombinase complex consists of factor Va and Xa and is only active when the factors are brought together on a phospholipid surface such as is provided by the membrane of activated platelets. There has been some controversy as to the mechanism by which activation of platelets can increase the activity of this complex. One group of investigators has reported that thrombin-activated platelets have the same number of binding sites for factor Va as control platelets. They suggest, therefore, that factor Va is bound to the surface of unstimulated platelets and that the increased prothrombinase activity on suspensions of activated platelets results from an activation-induced change in the conformation of the prebound factor Va, which allows an increased binding of factor Xa. Others have detected an activation-induced increase in the number of factor Va binding sites.

The reason for the conflicting results is not clear. The work described in the present study suggests that there are two distinct mechanisms by which procoagulant activity is generated and that activation of calpain is responsible for inducing one of them. Because calpain is activated only when platelets aggregate, the calpain-induced component only occurs in suspensions of platelets that are stirred. Because the previous experiments that detected increased binding sites for factor Va were performed under conditions in which calpain was activated, while the experiments that failed to detect increased binding were performed in unstirred platelet suspensions, it appears likely that activation of calpain is responsible for activating the prothrombinase complex by the mechanism that involves an increased number of binding sites for factor Va on the platelet surface.

The present study suggests a mechanism by which activation of calpain could exert its effects. Calpain induces the hydrolysis of several proteins in platelets. The two major substrates are ABP and talin. (Hydrolysis of other minor components, including spectrin, calmodulin-dependent phosphatase, and an unidentified protein of mol wt 87,000 that may represent caldesmon, has also been detected on two-dimensional gels, immunoblots, or calmodulin overlays.) In the present study we assessed the effect of calpain inhibitors by their ability to inhibit the hydrolysis of ABP and talin. Interestingly, the calpain-induced hydrolysis of ABP was totally inhibited by 10 to 20 μg of calpeptin/mL, while the hydrolysis of talin was not completely inhibited even at 200 μg of calpeptin/mL. The differential effect of calpeptin on the hydrolysis of different substrates may result from the fact that the calpeptin is a hydrophobic molecule and therefore partitions into the plasma membrane. Thus, the inhibitory effects that we observed at 10 to 20 μg of calpeptin/mL may result from an inhibition of membrane-associated calpain, whereas the inhibitory effects that were observed only at higher concentrations of calpeptin could result from an inhibition of soluble calpain. If this interpretation is correct, it indicates that the response that is inhibited by the lower concentration of calpeptin (ie, the generation of procoagulant activity) results from the calpain-induced hydrolysis of a membrane-associated protein.

The two known calpain substrates that are associated with the membrane in platelets are ABP and spectrin. Both proteins are components of the membrane skeleton that lines the lipid bilayer. Comfurius et al noted a correlation between the hydrolysis of ABP within the platelet and the externalization of negatively charged phospholipids. This correlation led them to suggest that the platelet cytoskeleton was responsible for maintaining the asymmetry of the phospholipids, and that hydrolysis of the cytoskeleton during platelet activation allowed the phospholipids to "flip" to the outer bilayer, where they could provide new binding sites for the prothrombinase complex. Although we have not determined whether expression of procoagulant activity results from externalization of phospholipids, our data provide evidence that procoagulant activity is generated as a consequence of an action of calpain and indicate that hydrolysis of a membrane-bound cytoskeletal protein may indeed be the critical event. Work is under way to test the hypothesis that procoagulant activity is generated as a consequence of the calpain-induced disruption of the platelet membrane skeleton.

Other investigators have suggested that activation of calpain mediates thrombin-induced aggregation or secretion of granule contents. Their conclusions have been based on the inhibitory action of leupeptin on the responses of platelets to thrombin. Because leupeptin had no effect on collagen-induced responses, it appears likely that the inhibitory action of leupeptin resulted solely from an action of the inhibitor on the amidolytic action of thrombin. The present study supports this conclusion. Leupeptin had little effect on the collagen-induced hydrolysis of ABP or talin; we conclude that leupeptin does not readily enter platelets and that any inhibitory action it has on platelet function must result from inhibition of an extracellular event. Further support for the conclusion that leupeptin does not inhibit thrombin-induced aggregation and secretion as a consequence of inhibiting intracellular calpain came from the observation that we could effect partial entry of leupeptin into platelets by incubating them with 1 mg of leupeptin/mL in the presence of DMSO. Because these conditions were much more extreme than those used by the workers who concluded that calpain mediates thrombin-induced aggregation and secretion, we assume that we were inhibiting intracellular...
calpain to a greater extent than in those studies that reported a functional consequence. Under these extreme conditions, the thrombin-induced hydrolysis of ABP and talin was partially inhibited, but concentrations of calpeptin that caused comparable levels of inhibition had no effect on thrombin-induced aggregation or secretion. Therefore, we suggest that the previously reported actions of leupeptin did not result from inhibition of intracellular calpain.

Although our study provides evidence that the previously reported actions of leupeptin on thrombin-induced aggregation and secretion resulted from inhibiting the ability of thrombin to initiate stimulus-response coupling, it does not exclude the possibility that activation of calpain plays some role in regulating aggregation or secretion. For example, in our experiments inhibitory actions of calpeptin on aggregation and secretion were observed at concentrations at which the calpain-induced hydrolysis of talin was inhibited. Because talin is thought to be a soluble protein in unstimulated platelets, this finding suggests that the calpain-induced hydrolysis of soluble proteins may play some role in regulating aggregation and secretion. Because these responses are well under way before hydrolysis of any of the known calpain substrates can be detected, it appears unlikely that calpain is responsible for initiating the responses. However, it is possible that the calpain-induced hydrolysis of soluble platelet proteins may modulate them. Because calpain can hydrolyze MLC kinase, it is conceivable that activation of calpain could modulate secretion; the report by Tsujinaka et al. that calpeptin inhibited the thrombin- and collagen-induced phosphorylation of myosin is consistent with this possibility. Further, aggregation may be modulated as a consequence of the calpain-induced regulation of thromboxane synthetase activity, a possibility that is consistent with the observation in the present study that higher concentrations of calpeptin had variable effects on platelet aggregation and secretion but appeared to be more inhibitory when collagen was used as the agonist.

One response that was not inhibited, even at high concentrations of calpeptin, was clot retraction. Because talin appears to be involved in linking integrins to stress fibers, it is possible that in activated platelets it is involved in linking the cytoskeleton to the glycoprotein IIb-IIIa complex and thus to the externally bound fibrin clot. Therefore, calpain-mediated hydrolysis of talin has been considered a possible mechanism by which retraction of fibrin clots might be regulated. The present experiments suggest that this is not the case. It is conceivable that the assay used to assess clot retraction was not sensitive enough to detect an inhibitory action of calpeptin. However, this seems unlikely because inhibition of clot retraction was detected when platelets were incubated with leupeptin, which inhibits the amidolytic action of thrombin, or RGDS, which inhibits the association of the glycoprotein IIb-IIIa complex with fibrinogen. Future studies will be required to investigate the functional consequences of the calpain-induced hydrolysis of talin in platelets.

In summary, the present study shows for the first time that activation of calpain is involved in stimulus-response coupling in platelets. The selective inhibition of the calpain-induced hydrolysis of ABP and the generation of procoagulant activity by a membrane-penetrating calpain inhibitor are consistent with the hypothesis that calpain generates procoagulant activity as a consequence of hydrolysis of the membrane skeleton. Because concentrations of calpeptin that inhibit the generation of platelet procoagulant activity do not inhibit the amidolytic activity of thrombin and do not inhibit any of the other functional responses of platelets, membrane-penetrating calpain inhibitors may provide a novel therapeutic method of decreasing the incidence of thrombotic events without causing the hemorrhagic complications that are associated with current anticoagulant therapy.

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