Mechanisms of thrombin-induced modifications of human platelet cytoskeleton

Fabiola SINIGAGLIA,* Carlo L. BALDUINI,† Antonella BISIO* and Cesare BALDUINI*
*Department of Biochemistry and †Department of Internal Medicine, University of Pavia, Pavia, Italy

INTRODUCTION

Thrombin, a serine proteinase that is intimately involved in blood coagulation, stimulates platelets at concentrations that are generated physiologically. The mechanism of its action on platelets is not understood but appears to embody aspects both of an enzyme-catalysed reaction and of agonist–receptor equilibrium (Martin et al., 1975). Though thrombin-induced release and aggregation require catalytically active thrombin (Martin et al., 1975) which removes a fragment from a membrane glycoprotein (GPV) (Berndt & Phillips, 1981a), some effects strictly related to the binding of the enzyme to specific membrane receptors have been demonstrated. Phosphatidate synthesis as well as acid hydrolase secretion are tightly coupled to thrombin receptor occupancy in thrombin-stimulated platelets (Holmsen et al., 1984). Moreover, when the platelet level of cyclic AMP is increased by prostaglandin E₁, it may be decreased by ADP or adrenaline only if thrombin is bound to its receptors (Zavoico & Feinstein, 1984).

Recently we observed that thrombin significantly increases cell adhesion to collagen interacting with platelets by a non-enzymic mechanism (Sinigaglia et al., 1985); PMSF-inhibited thrombin was equally active in this respect and the phenomenon could be reversed by hirudin, an inhibitor of the macromolecular-binding site of thrombin (Tam et al., 1979).

In order to gain more information about the mechanism by which thrombin influences platelet function, and since it is known that thrombin also modifies cytoskeleton organization, the Triton-insoluble residue was investigated after having treated platelets with PMSF-inhibited thrombin or after having removed active thrombin by hirudin. Moreover, it is known that thrombin interacts with GPI and GPV (Okumura et al., 1978; Ganguly & Gould, 1979; Berndt & Phillips, 1981a,b) and that chymotrypsin is able to promote the breakdown of both these glycoproteins (Okumura & Jamieson, 1976; Detwiler, 1983), platelets were treated with this enzyme and their ability to modify cytoskeleton assembly and collagen adhesiveness after thrombin treatment were investigated.

EXPERIMENTAL

Materials

Human thrombin, calf-skin collagen, hirudin, α-chymotrypsin and human albumin (fraction V) were purchased from Sigma; Na⁺[³²P] and [¹⁴C]serotonin (5-hydroxytryptamine) were obtained from Amersham International; Sepharose 4B and Sephadex G-25 were from Pharmacia; tosyl-glycyl-prolyl-arginine-4-nitroanilide acetate (Chromozym TH) used for thrombin activity detection was from Boehringer Mannheim. All other reagents were analytical grade.

Platelet preparation

A suspension of washed human platelets was prepared as previously described (Balduini et al., 1984) and resuspended at a final concentration of 10⁹ cells/ml in a buffer (pH 7.4) containing Tris/HCl (25 mM), EDTA (0.3 mM), NaCl (140 mM) and glucose (5 mM). Platelet morphology was monitored by phase-contrast microscopy.

Chymotrypsin treatment of human platelets

Chymotrypsin-treated platelets were prepared as described by Tam et al. (1980). Briefly, chymotrypsin, that had been preincubated with tosyl-lysylchloromethane at a 5:1 molar ratio in ice for 30 min to inhibit possible contaminating trypsin, was added to a platelet suspension to give a final concentration of 20 μM. After 10 min of incubation at 23 °C, the platelet suspension was cooled in an ice-bath and diluted with 6 vol. of ice-cold wash solution. The platelets were washed twice and resuspended as previously described in order to determine their adhesion to collagen and cytoskeleton composition.

Adhesion to collagen of chymotrypsin-treated platelets

Platelets proteolytically modified with 20 μM-chymotrypsin were treated with 6.6 nM-thrombin for 10 min at 37 °C and then passed through collagen–Sepharose columns in order to determine their adhesion to collagen by the affinity-chromatography method described by Brass et al. (1976) with some modifications (Balduini et al., 1984).

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulhate.

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Binding of thrombin to chymotrypsin-treated platelets

Thrombin was labelled with $^{125}$I by the Chloramine-T method as described by Tollesen et al. (1974) and its binding to platelets was measured as previously described (Sinigaglia et al., 1985).

Serotonin release detection

When the release reaction had to be determined, washed platelets were preincubated with $[^{14}C]$serotonin for 30 min at room temperature. Then platelets were pelleted by centrifugation in a Fischer Microfuge (12000 g) for 2 min in order to remove the excess of radioactivity and resuspended in the wash solution as previously described (Sinigaglia et al., 1985). The release caused by different treatments of platelets was adjusted by subtraction of the release due to platelet manipulation (i.e. centrifugation) only.

Inhibition of thrombin

Inhibition of thrombin at the active site with PMSF was performed as described by Lundblad (1971). Proteolytic activity was tested using tosyl-glycyl-prolyl-arginine-4-nitroanilide acetate as substrate (Collen et al., 1980); clotting activity was determined as reported by Baughman (1970). Both the tests proved the absence of any detectable residual activity even if a 10-fold higher concentration of the PMSF-thrombin was used. Platelets prepared as previously described were incubated with active thrombin or PMSF-thrombin at a final concentration of 6.6 nM. In order to remove active thrombin from the platelet surface, hirudin was added after 1 min to the thrombin-treated platelets in 1:5 molar ratio with the enzyme and the suspension was incubated for an additional 10 min at 37 °C before cytoskeleton extraction.

Triton-insoluble residue analysis

Platelet cytoskeletons were prepared as described by Phillips et al. (1980). At the desired time, Triton X-100 (10%) in 50 mM-EGTA solution was added to the platelet suspension to a final concentration of 1% Triton. The mixture was stirred for 1 min, cooled in ice for 30 min and the Triton X-100 residue was centrifuged in a Fischer Microfuge (12000 g) for 4 min. The pellet was re-extracted with 0.1% Triton, then dissolved in wash solution containing 20% SDS to give a final concentration of 2% SDS. The relative protein content of Triton-insoluble residue was evaluated by the method of Lowry et al. (1951). Triton interference in the assay was suppressed according to Wang & Smith (1975). Electrophoretic analysis of Triton-insoluble residue was performed according to Laemmli (1970). Each sample contained an amount of protein that could be obtained from the same number of platelets (usually 5 $\times$ 10$^7$ cells), which ranged between 40 and 80 $\mu$g depending on the type of treatment that the platelets had undergone. Samples were dissolved by boiling for 10 min with 2% SDS, reduced with 4% 2-mercaptoethanol and then were electrophoresed by using 3% polyacrylamide for the stacking gel and a 5–15% polyacrylamide exponential gradient for the separating gel. Electrophoresis was performed at constant voltage (25 V/slab) until the Bromphenol Blue marker dye was within 1 cm of the bottom of the gel. $M_r$ standards were routinely coelectrophoresed with samples.

Table 1. Effect of different treatments on the relative protein content of Triton-insoluble residue of human platelets

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Cytoskeletal protein (% w/w)</th>
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<tbody>
<tr>
<td>None</td>
<td>10.5±1.1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>17.6±3.2 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>PMSF-thrombin</td>
<td>15.3±2.1 ($P = 0.05$)</td>
</tr>
<tr>
<td>Hirudin</td>
<td>10.6±1.8</td>
</tr>
<tr>
<td>Thrombin + hirudin</td>
<td>10.5±1.2</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>10.3±2.0</td>
</tr>
<tr>
<td>Chymotrypsin + thrombin</td>
<td>13.3±1.9</td>
</tr>
</tbody>
</table>

The gels were stained with Coomassie Blue for protein pattern visualization.

RESULTS

In our experimental conditions a partial activation of platelets probably occurs as a consequence of washing procedures; phase-contrast microscopy in fact usually showed some signs of shape modification also in untreated platelets. Thrombin treatment, in accordance with the data reported in the literature (Phillips et al., 1980; Rotman, 1984), resulted in the typical cytoskeleton modifications consisting of an increase of protein content of the Triton-insoluble residue (Table 1) and in particular in the increase of actin-binding protein, myosin, $\alpha$-actinin and actin; moreover, additional bands with approximate Mr values of 68000, 55000 and 50000 appeared, corresponding to the $\alpha$, $\beta$ and $\gamma$ chains of fibrinogen (Casella et al., 1983; Tuszinski et al., 1984) (Fig. 1) that in our experimental conditions probably coprecipitated with cytoskeletons. Triton-insoluble residue obtained from platelets incubated with active-site inhibited thrombin (PMSF-thrombin) showed an increased relative protein content similar to that obtained from platelets treated with the active enzyme (Table 1); the electrophoretic protein pattern did not show significant differences with respect to that obtained from the Triton-insoluble residue from thrombin-treated platelets, except that the three fibrinogen bands were obviously absent (Fig. 1). The addition of hirudin, which in our experimental conditions removes most of bound $^{125}$I-thrombin (about 80%) (Sinigaglia et al., 1985), caused a decrease of protein content of the Triton-insoluble residue with respect to thrombin-activated platelets (Table 1) and an apparent concomitant decrease in the intensity of the bands of actin-binding protein, myosin, $\alpha$-actinin and actin with respect to activated platelets; as expected, the three fibrinogen bands were still present since hirudin was added to the platelet suspension 1 min after thrombin addition. Chymotrypsin treatment of
Platelet cytoskeleton modifications

Triton-insoluble residues were prepared and electrophoresed after the following treatment of platelets: lane (a), none; lane (b), 6.6 nm-thrombin; lane (c), 6.6 nm-PMSF-thrombin; lane (d), 6.6 nm-thrombin and then 40 nm-hirudin; lane (e), 20 μM-chymotrypsin; lane (f), 20 μM-chymotrypsin and then 6.6 nm-thrombin. Each sample contained the amount of Triton-insoluble protein that could be obtained from the same number of platelets (5 x 10^6 cells), and ranged between 40 and 80 μg depending on the type of treatment that platelets had undergone.

intact platelets, which is known to result in a decrease of GPI and GPV, did not cause a modification of the amount of membrane-bound [14C]thrombin, in accordance with the data of Tam et al. (1980).

After incubation with 20 μM enzyme, a significant modification of cytoskeleton organization occurred, mainly consisting of the absence of actin-binding protein (Fig. 1). After a further incubation with thrombin no modification of the protein content of Triton-insoluble residue was observed, and the cytoskeletal protein pattern was essentially unchanged (Fig. 1). Chymotrypsin-treated platelets showed a decrease in their adhesion to collagen; however, a further incubation with 6.6 nm-thrombin was still able to increase the adhesiveness to the same extent as for intact platelets (Table 2). Moreover, after 20 μM-chymotrypsin treatment, platelets preincu-

![SDS/polyacrylamide-gel electrophoresis of Triton-insoluble residue from human platelets after different treatments](image)

**Fig. 1.** SDS/polyacrylamide-gel electrophoresis of Triton-insoluble residue from human platelets after different treatments

<table>
<thead>
<tr>
<th>Adhesion (%)</th>
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<tbody>
<tr>
<td>Before</td>
</tr>
<tr>
<td>Untreated platelets</td>
</tr>
<tr>
<td>Platelets + 20 μM-chymotrypsin</td>
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</tbody>
</table>

Table 2. Effect of thrombin on the adhesion to collagen of chymotrypsin-treated platelets

Results are mean ± s.d. for three experiments and are expressed as the percentage of platelets that is retained by a column of Sepharose covalently coupled to microfibrillar type I collagen (Balduini et al., 1984). Experiments were carried out at 4 °C.

**DISCUSSION**

Despite a great number of investigations the mechanisms by which thrombin interacts with and stimulates platelets are not yet completely elucidated. It is known that this enzyme can act both by an enzymic and a receptor-mediated mechanism and that different interaction sites for thrombin are present on platelet membrane (Okumura et al., 1978; Ganguly & Gould, 1979; Berndt & Phillips, 1981a,b).

Recently in our laboratory it was observed that thrombin increases platelet adhesion to collagen by a non-enzymic process (Sinigaglia et al., 1985). The evidence here reported is indicative that also the cytoskeletal modifications induced by thrombin are at least in part independent of its catalytic activity. PMSF-inhibited enzyme, in fact, is able, like active thrombin, though with a lower statistical significance level, to increase the protein content of the Triton-insoluble residue. Moreover the effect of the active enzyme on platelet cytoskeleton can be partially reversed by an additional incubation with hirudin, which is known to remove thrombin from the platelet surface. It seems therefore that the mechanism by which thrombin modifies cytoskeletal assembly depends on its interaction with the platelet surface, but not on its enzymic activity. It must be remarked that a similar mechanism was observed when the effect of thrombin, PMSF-thrombin and hirudin on platelet adhesion to collagen was investigated (Sinigaglia et al., 1985). It can therefore be speculated that the sequence of events by which thrombin triggers cytoskeletal modifications and increases platelet adhesiveness could in some way relate to each other. In an attempt to investigate this problem, platelets were treated with chymotrypsin, which is known to promote the breakdown of both GPIb and GPV, respectively a binding site and the substrate of thrombin on the platelet surface, and that, like other proteinases (Ascarì et al., 1985), reduces in our experimental conditions the adhesion of platelets to collagen by about 50%.

The cytoskeleton of 20 μM-chymotrypsin-treated platelets is quantitatively unmodified with respect to untreated ones (Table 1); nevertheless, electrophoretic analysis

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showed that the component with $M_r$ 250000, corresponding to actin-binding protein, was totally lost. This protein, as well as P235, can be proteolysed by calcium-activated proteinases which are present in human platelets (Tsujinaka et al., 1982; Fox et al., 1985), so that it can be hypothesized that its disappearance after chymotrypsin treatment could result from the increase of intracellular Ca²⁺ concentration with the consequent activation of these proteinases. However, chymotrypsin-treated platelets, though still able to bind thrombin, are unresponsive to this ligand with respect to quantitative cytoskeletal modifications. On the other hand, though less reactive toward collagen, they increase their adhesiveness after thrombin treatment. It can therefore be concluded that the mechanisms by which thrombin stimulates platelets to adhere to collagen and to modify cytoskeletal assembly depend on the binding of the enzyme to the cell surface. However, the transducers of these interactions are probably different, since chymotrypsin digestion abolish thrombin’s effects on the cytoskeleton but preserves its influence on adhesion to collagen. Though the targets of chymotrypsin action are known, it is impossible with our results to identify the molecular modulators of thrombin effects under investigation.

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REFERENCES

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