Inhibition of Thrombin-Induced Platelet Aggregation by a Derivative of Wheat Germ Agglutinin. Evidence for a Physiologic Receptor of Thrombin in Human Platelets

By Pankaj Ganguly and Nancy G. Fossett

We have prepared a nonagglutinating derivative of wheat germ agglutinin by cyanogen bromide treatment of the lectin and used it as a probe to study the thrombin-platelet interaction. The lectin derivative inhibited platelet aggregation by thrombin while aggregation induced by collagen, ristocetin, adenosine diphosphate, wheat germ agglutinin, or trypsin was not significantly affected. Under similar conditions, the secretion of serotonin by thrombin was blocked by the derivative. The inhibitory action of the derivative on thrombin-induced platelet aggregation could be overcome by increasing the thrombin concentration. A Schild plot of these data yielded a slope of 1.0 and an apparent dissociation constant of 1.0 μM. Thus, the inhibition of thrombin-induced platelet aggregation by the derivative fits a model of competitive inhibition. Control experiments showed that the lectin derivative acted on platelets and not on thrombin. However, the binding of [125I]thrombin to platelets was not affected. Isolated platelet membranes were solubilized and passed through a column of the lectin derivative coupled to Sepharose. After extensive washing, the material bound to the column was eluted with N-acetyl-D-glucosamine. This membrane isolate inhibited platelet aggregation by thrombin while aggregation induced by adenosine diphosphate, trypsin or ristocetin was not significantly affected. It also blocked thrombin-induced serotonin secretion from platelets. Gel electrophoresis followed by autoradiography of the membrane isolate revealed a prominent glycoprotein of apparent molecular weight of 74,000 that contained inhibitory properties. The electrophoretic mobility or the intensity of this band was not significantly affected following incubation of the glycoprotein isolate with physiologic concentrations of thrombin. The glycoprotein also retained its inhibitory activity on thrombin-induced platelet aggregation following incubation with 20 μg/ml of trypsin or chymotrypsin for 10 min. These results suggest that (A) the thrombin receptor in human platelets is different from the ristocetin or ristocetin-von Willebrand factor receptor and (B) the 74,000 dalton glycoprotein may be a physiologic receptor of thrombin in human platelets.

THROMBIN, a serine protease that is intimately involved in blood coagulation, is also capable of activating platelets. The enzyme stimulates platelets at concentrations that are generated physiologically and is thought to be important in the initiation of platelet thrombus formation in response to vascular injury. Thrombin exhibits some unique features in its action on platelets in that it does not require the formation of prostaglandin endoperoxides and release of adenosine diphosphate. Moreover, thrombin may stimulate platelets by a mechanism resembling hormone activation of target cells, rather than simple proteolysis of isolated proteins such as fibrinogen and fibrin-stabilizing factor. The first step in the interaction of thrombin with platelets appears to be equilibrium binding of thrombin to the cell surface. This binding is specific, saturable, and correlates well with the extent of platelet secretion. However, binding of thrombin in itself is not sufficient for platelet secretion and, at least one additional step (effector step) is involved. This is indicated by the fact that thrombin inactivated at the serine center binds in an identical manner as unmodified thrombin but it does not induce platelet aggregation or secretion.

Wheat germ agglutinin is a lectin with specificity for N-acetyl-D-glucosamine and lower precipitation potencies for sialic acid and N-acetyl-D-galactosamine. The lectin is rich in half cystine (15–16 disulfide bonds per monomer) and contains two residues of methionine that have been tentatively assigned to positions 10 and 67 (personal communication, Dr. C. S. Wright, Princeton University). Several lines of evidence suggests that the platelet component to which this lectin preferentially binds is the glycoprotein one (GP-I) complex that has also been proposed to be a receptor for thrombin. Thus, wheat germ agglutinin provides a potential probe to explore the molecular details of thrombin-platelet interaction. However, binding of this lectin leads to platelet aggregation and secretion that precludes its use as a probe. To circumvent this problem, we have prepared a derivative of wheat germ agglutinin by treating the lectin with cyanogen bromide that cleaves methionine residues in a protein. This derivative is free of platelet stimulating activity under the experimental conditions but retains many properties of the parent lectin. We show that the lectin derivative, as well as a...
glycoprotein isolated from platelet membranes by affinity chromatography utilizing the derivative, specifically inhibit platelet aggregation by thrombin.

MATERIALS AND METHODS

Platelets

Blood was collected from healthy volunteers in plastic syringes or bags containing 0.1 volume of 3.8% sodium citrate as the anticoagulant. The red cells were removed by low speed centrifugation. When desired, platelets were washed with oxalate-EDTA and resuspended in Tyrode's solution, pH 7.4 that contained 0.1% glucose and 0.1% bovine serum albumin but was free of Ca++. For the determination of thrombin binding, platelets were suspended in 0.125 M NaCl and 0.025 M phosphate buffer, pH 7.2 containing 0.1% albumin. These platelets routinely aggregated with thrombin or wheat germ agglutinin.

Lectin

Wheat germ agglutinin, purified by affinity chromatography, was purchased from U.S. Biochemicals. It showed a single band in SDS gel electrophoresis. The wheat germ agglutinin derivative was prepared by treating the lectin (1 mg/ml) in 70% formic acid with 500 mg of cyanogen bromide (Kodak) for 20 hr at room temperature. The solution was dried under vacuum, redissolved in water and lyophilized. The dry powder was dissolved in phosphate-buffered saline, pH 7.2 and dialyzed against the same buffer before use. The derivative had a molecular weight similar to the native lectin and retained the capacity to precipitate an antibody to wheat germ agglutinin. Amino acid analysis data confirmed cleavage of a specific methionine residue due to chemical modification of the lectin.

Platelet Aggregation

These experiments were routinely carried out in a dual channel aggregometer. Usually, 50 μl of the aggregating agent was added to 450 μl of a platelet suspension containing 2.5 to 3.5 x 10^8 cells/ml. Platelets were processed and experiments conducted within 2-3 hr after blood collection when the response of platelets, as determined from control experiments, did not significantly change. In a typical experiment, platelets were incubated with the derivative for 10 min at room temperature in the aggregometer cuvette. Then the cuvette was transferred to the aggregometer, held for about 2 min and the stimulating agent was added. The change in light transmission was recorded with time. Secretion was measured with platelets preloaded with [14C]serotonin. The aggregation response of these platelets was first recorded. Five minutes after the addition of thrombin, 0.1 ml of 10% formaldehyde was added to platelets and the cuvettes were centrifuged. The radioactivity in 100 μl of the supernatants was measured and expressed as percent of that added. A minimum of ten sets of aggregation experiments utilizing different preparations of platelets and derivative were carried out. The response of platelets to thrombin and the derivative varied from day to day depending on the donor but each experiment was internally consistent.

Thrombin Binding

Bovine thrombin was purified by ion exchange chromatography and had a minimum specific activity of 2500 NIH U/mg. Thrombin was labeled with 125I by the chloramine T method and the free label was removed by gel filtration. Platelets (2 to 3 x 10^8/ml) in phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma, Fraction V) were incubated with different amounts of the derivative for 15 min at room temperature. A constant amount of thrombin was then added to each tube in a total volume of 1 ml and the incubation was continued for another 15 or 30 min. After the incubation period, 4 ml of buffer without albumin was added to each tube and the platelets were collected by filtration through albumin-coated millipore filters. The tubes and the filters were washed with another 5-ml aliquot of buffer. The filters were removed and the associated radioactivity was measured. Nonspecific binding was determined by including excess inactive thrombin in the incubation mixtures and usually ranged between 10% to 20% of the gross binding of thrombin to platelets. At a constant concentration of thrombin, nonspecific binding to platelets was not affected by the derivative. Details of these procedures have been published. Since the filtration method of determining thrombin binding to platelets has been criticized for the possible nonequilibrium conditions, these measurements were repeated by the oil centrifugation method as described except that Versilub (General Electric) was used as the centrifugation medium. The results were the same.

Affinity Chromatography

Platelets were isolated from 2 units of platelet concentrates, approximately 18 hr old, and resuspended in phosphate-buffered saline, pH 7.2 containing 10 mM EDTA, 0.4 mM N-CBZ-glutamyl-L-tyrosine, 0.4 mM phenylmethylsulfonyl fluoride and 100 KIU/ml of Trasylol. The platelet suspension was sonicated (Branson, setting 5) twice for 3 min each on ice and unbroken cells were removed by low speed centrifugation. The platelet membrane fraction was isolated by sucrose density gradient centrifugation, diluted with buffer and then pelleted by centrifugation at 105,000 g for 1 hr. The membrane pellets were resuspended in PBS containing the protease inhibitors by gentle sonication and then solubilized with 1% SDS. The solubilized membranes were diluted to 0.05% SDS with 0.25 M NaCl-0.025 M PO4, pH 7.2 and then applied to an affinity column of the lectin derivative coupled to activated Sepharose. The column was extensively washed with the above buffer containing 0.05% SDS and the protease inhibitors. The glycoproteins bound to the column were eluted with the same buffer but including 0.1 M N-acetyl-D-glucosamine and 1.5 times the column volume was collected. Two procedures were then followed for further processing of the eluted material. One approach was to dialyze the sample against distilled water for 2 days with several changes. It was then lyophilized, reconstituted in PBS and dialyzed overnight against the same buffer before use. To avoid any possible degradation, in the other approach, the sample volume was reduced to about 10 ml by rapid ultrafiltration through a PM-30 membrane (Amicon). Approximately 90 ml of PBS was added and the volume was again reduced to 10 ml. This process of dilution-concentration was repeated 3 times in approximately 2 hr. The properties of the samples processed by the two procedures were similar. A theoretical concentration of 5 x 10^-6% SDS did not affect thrombin-induced platelet aggregation under the conditions of the experiments. Protein concentration was measured with the Bio-Rad protein assay kit using albumin as standard. The values shown are approximate and indicate total protein.

Electrophoresis

Samples obtained by affinity chromatography were labeled with 125I by the chloramine T method and the free label was removed by dialysis against PBS. With some samples, the dialysis step was omitted and the labeled protein was mixed with the sample buffer (0.05 M Tris-Cl, pH 6.8 with 1% SDS and 1% glycerol) and analyzed in 7.5% acrylamide slab gels. Platelet aggregating capacity or the fibrinogen clotting time of thrombin exposed to a mixture of labeling reagents for 10 min remained unaltered, showing that
the activity of thrombin was not significantly modified by these reagents. When desired, the samples were reduced with 1% β-mercaptoethanol. Molecular weight standards were phosphorylase (94,000), serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000). Occasionally, γ-globulin (150,000) was also included. The standards were run on the same gel and processed in the same way as the samples. Autoradiographs were obtained by exposing the dried gels to Kodak x-ray films for 3–7 days at –70°C.

**RESULTS**

**Effect of Lectin Derivative on Platelet Aggregation**

Addition of small amounts of thrombin to platelets caused a rapid increase in light transmission indicating cell aggregation (Fig. 1). The lectin derivative in μg amounts inhibited the aggregation of platelets by thrombin while aggregation induced by collagen, ristocetin, wheat germ agglutinin, or ADP was not significantly affected (Table 1). The inhibition was equally evident whether the platelets were suspended in plasma or in buffer showing that the action of the lectin derivative is not mediated through a plasma glycoprotein. Similarly, platelet aggregation induced by another serine protease, trypsin, was not significantly influenced by the lectin derivative (Table 1).

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Lectin Derivative (μg/0.5 ml)</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Thrombin, 80 mU</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>ADP, 10⁻⁴M</td>
<td>0</td>
<td>70</td>
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<td>60</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Collagen, 100 μg</td>
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<tr>
<td></td>
<td>20</td>
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</tr>
<tr>
<td></td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td>Ristocetin, 450 μg</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>60</td>
</tr>
<tr>
<td>Trypsin, 20 μg</td>
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<td>80</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>74</td>
</tr>
<tr>
<td>Wheat germ agglutinin, 20 μg</td>
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</tr>
<tr>
<td></td>
<td>75</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>45</td>
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</table>

The effect of the derivative on platelet aggregation, or the lack of it, was observed at different concentrations of the inducers. The derivative even at 200 μg/ml did not aggregate platelets. Thus, the effect of the derivative appears to be specific for thrombin and is not due to a general perturbation of the platelet membrane due to the binding of the derivative.

Platelets incubated with an inhibitory concentration of the derivative in the presence of 50 mM N-acetyl-D-glucosamine upon the addition of thrombin aggregated similarly to the control. The results were the same when the sugar was added after preincubation of platelets with the derivative indicating that binding of the derivative does not irreversibly change the platelets (Fig. 2). N-acetyl-D-glucosamine at this concentration did not affect platelet aggregation by thrombin. These observations suggest that the derivative retains the main saccharide specificity of the lectin and that binding of the derivative is necessary for its inhibitory effect on the thrombin-induced platelet aggregation.

The data presented above could be explained if the lectin derivative blocks a target of thrombin on platelets or if it interacts with thrombin to form an inactive complex. The latter possibility had to be entertained because thrombin is a glycoprotein and con A has been reported to bind and inhibit the activity of this enzyme. To distinguish between these alternatives, platelet aggregation experiments were carried out as in Fig. 1 except that the thrombin solution, instead of platelets, was preincubated with the lectin derivative.
for 10 min before addition to platelets. Platelet aggregation produced by this thrombin-lectin mixture was not significantly different from that caused by thrombin alone (Fig. 2). The derivative neither blocked platelet aggregation when added simultaneously with thrombin nor reversed platelet aggregation when added after thrombin. Inhibition of thrombin-induced aggregation was observed only when the platelets were preincubated with the derivative. These results clearly show that the effect of the derivative is on platelets and not on thrombin.

Platelets rapidly released serotonin when exposed to thrombin. In control experiments with 80 mU of thrombin, the amount of serotonin released ranged between 60% to 80%. This secretion of serotonin was blocked by preincubation of platelets with the lectin derivative (Fig. 3). Studies at different concentrations of the derivative indicated complete inhibition of platelet secretion at about 30 to 50 μg/ml.

**Effect of Thrombin on Aggregation Inhibition**

The effect of different concentrations of derivative on the aggregation response of platelets as a function of thrombin concentration is shown in Fig. 4. Increasing the thrombin concentration overcomes the inhibitory action of the derivative on platelets. In the absence of the derivative, increasing concentrations of thrombin caused progressively greater aggregation of platelets that eventually reached a plateau. The derivative shifted the response curves, which were of the same shape, to a higher concentration of thrombin where the inhibition was overcome. Compared to the control, the shift of the thrombin-induced aggregation curves of platelets was more prominent and readily quantitated at low concentrations of the derivative. This behavior of the derivative on platelet aggregation has been considered as presumptive evidence of competitive inhibition.

To explore this possibility further, we constructed a Schild plot from the platelet response curves to thrombin at different concentrations of the lectin derivative (Fig. 5). This approach has been used previously and from a model for competitive inhibition, the following relationship was derived: 

\[
\frac{T - T'}{T} = \frac{1}{K_d}
\]

where \( T \) is the thrombin concentration in the absence of the inhibitor to elicit a given platelet response, \( T' \) is the thrombin concentration in the presence of the inhibitor, and \( K_d \) is the dissociation constant of the inhibitor from its receptor.

**Fig. 3.** Inhibition of thrombin-induced serotonin secretion from platelets by the lectin derivative. Platelets in plasma (2.5 × 10^9/ml) were loaded with [3H]serotonin and the uptake was about 80%. These platelets were incubated with different amounts of the derivative for 10 min and then their aggregation properties with 80 mU of thrombin was recorded. The reaction was stopped after 5 min with formaldehyde, the cuvettes were centrifuged, and the radioactivity in 100 μl aliquots of the supernatants was measured. The data shown are averages of four different experiments done on different days.

**Fig. 4.** Aggregation response of platelets as a function of thrombin concentration in the presence of various amounts of the lectin derivative. The experiment was conducted as described under Fig. 1 except that the platelets were suspended in buffer, C, control without lectin; 36 μg/ml lectin and 98 μg/ml lectin.
Fig. 5. Schild plot for the inhibition of thrombin-induced platelet aggregation by the lectin derivative constructed from a set of dose-response curves such as in Fig. 4. $T$ is the concentration of thrombin required to produce a platelet aggregation of 40%. $T'$ is the concentration of thrombin to produce the same aggregation response in the presence of the derivative (I). Data from three different experiments utilizing different platelet and lectin derivative preparations are shown. The apparent dissociation constant were calculated from the intercept on the x axis when $y$ is zero.

Concentration that produces the same platelet response in the presence of a certain concentration of the inhibitor I, and $K_d$ is the apparent dissociation constant of the inhibitor. For competitive inhibition, a plot of $\log \left( \frac{T'}{T - 1} \right)$ versus $\log I$ will be linear with a slope of 1 and an intercept on the abscissa of $\log K_d$. This is the case for the inhibition of thrombin-induced platelet aggregation by the derivative; the slope ranged from 0.8 to 1.2 with an average value for $K_d$ of 1.0 $\mu M$.

Effect of Derivative on Thrombin Binding

Thrombin binds to receptors on the platelet surface. It is possible that the lectin derivative competes with thrombin for binding to its receptors. To explore this possibility, we determined the binding of [125I]thrombin to platelets in the presence of different amounts of the derivative. The derivative did not affect the binding of thrombin to platelets (Fig. 6). The results were the same whether the measurements were made by the membrane filtration method or the oil centrifugation method. It is interesting to note that the binding of thrombin to fixed platelets is not blocked by wheat germ agglutinin. Thus, the derivative competes with thrombin for cell stimulation but apparently not for its initial binding to the platelet surface.

Affinity Chromatography

Biochemical studies. Affinity chromatography with the lectin derivative of platelet membranes yielded on an average of 150 $\mu g$ of protein material per unit of platelet concentrate. Since the isolated protein binds to the lectin derivative and elutes with the specific sugar, on this basis, it appears to be a glycoprotein. Gel electrophoresis of this material and staining with Coomassie blue revealed a prominent band that migrated slightly slower than the albumin standard (Fig. 7). Two other faint bands (Mr 82,000 and 55,000) could also been seen in this isolate. At these protein concentrations, staining with periodic acid-Schiff reagent did not show any band. Gel electrophoresis of the [125I]labeled material followed by autoradiography showed one prominent band (Fig. 8). The results were the same when the gel was sliced and the distribution of radioactivity determined. The glycoprotein had an electrophoretic mobility slightly slower than the albumin standard and from a number of experiments we estimated its apparent molecular weight to be 74,000 ± 2000. The position of the band was the same before or after reduction of the sample. In 9 different isolations by affinity chromatography, the 74,000 dalton component was always present, the 55,000 dalton component could be seen in 6, and the 82,000 dalton component in 2 patterns. Glycoprotein one (Mr ~ 150,000) was never observed. In immuno-diffusion studies, the glycoprotein isolate at this concentration (~200 $\mu g/ml$) did not react with antisera to human serum and fibrinogen at different dilutions.

The glycoprotein did not show any significant
change when incubated with 40 mU/ml to 200 mU/ml of thrombin for 5 min at room temperature (Fig. 8). These thrombin concentrations were sufficient to induce 40% to 100% aggregation of platelets in less than 2 min. Again, when incubated with a constant amount of thrombin (100 mU/ml) for 30 sec to 10 min, no systematic alteration of the glycoprotein could be detected. This observation was true in 8 different experiments utilizing 3 different glycoprotein isolates. Since visual examination may not reveal small changes, an autoradiograph was scanned in a densitometer. Any alteration either in the position or peak height of this glycoprotein band observed was within the limits of experimental variation. Thus, thrombin at physiologic concentrations does not appear to hydrolyze this glycoprotein to any significant extent.

**Physiological studies.** The glycoprotein isolate from the lectin derivative column strongly inhibited thrombin-induced aggregation of platelets in plasma (Fig. 9). Inhibitory action of this material on platelet aggregation by thrombin was clearly observed even when the enzyme was incubated with 0.25 μg of the protein in 50 μl or a concentration of 5 μg/ml. Platelet aggregation by thrombin was completely blocked at an
approximate protein concentration of 20 μg/ml. In the above experiments, the isolate was preincubated with thrombin for 10 min at room temperature. Inhibition of aggregation was also noted when the isolate (20 μg/ml) was added to platelets in plasma instead of thrombin. Since plasma contains many proteins, including known inhibitors of thrombin, the above observation again indicates considerable specificity in the interaction of thrombin with the platelet glycoprotein. The inhibition of platelet aggregation by the protein isolate cannot be due to contaminating lectin derivative bleeding out from the column. The lectin derivative when incubated first with thrombin did not block platelet aggregation (Fig. 2D) but the column isolate did. Furthermore, assuming 100% contamination, the amount of the derivative will not be enough for aggregation inhibition under these conditions. The membrane isolate by itself did not cause platelet aggregation. The glycoprotein isolate was run on gel electrophoresis and the area corresponding to the 74,000 dalton component was removed. The protein was eluted, dialyzed, and concentrated by ultrafiltration. In gel electrophoresis, the glycoprotein showed a single band in its original position that again did not change upon exposure to thrombin (Fig. 10). This glycoprotein inhibited thrombin-induced platelet aggregation while the eluates from the remaining top and bottom parts of the same gel processed in the same manner did not show any inhibition. Thus, it appears that the inhibitory activity on thrombin-induced platelet stimulation is associated with the 74,000 dalton glycoprotein. In contrast, aggregation induced by ADP or ristocetin were not affected by the glycoprotein isolate (Table 2). Platelet aggregation by these agents was in fact slightly higher in the presence of the isolate than the corresponding controls. These results show that the column isolate is not a general perturbant of platelets. In addition, the column isolate at concentrations when aggregation by thrombin was completely blocked, had little effect on platelet aggregation by trypsin (Table 2). Furthermore, the glycoprotein preincubated with 20 μg/ml of trypsin or chymotrypsin for 10 min, after which the enzyme was inhibited, retained the capacity to block thrombin-induced platelet aggregation. The control experiment contained all the reagents except that the glycoprotein was replaced by an equal amount of the same buffer. Keeping in mind that small amounts of thrombin or trypsin stimulate platelets in about a minute, it appears unlikely that this glycoprotein is either a general substrate or an inhibitor of serine proteases.

**DISCUSSION**

Thrombin at physiologic concentrations interacts with platelets and several other coagulation factors, e.g., factors I, V, VIII, XIII etc. The enzyme belongs to the general class of serine proteases, yet functional-

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**Table 2. Effect of the Membrane Glycoprotein From Lectin Affinity Chromatography on the Aggregation of Platelets in Plasma by Various Agents**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Glycoprotein (μg)</th>
<th>Aggregation (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
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<td>0</td>
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<tr>
<td>Thrombin, 50 mU</td>
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<td>76</td>
</tr>
<tr>
<td></td>
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<td>56</td>
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<td>68</td>
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<td>5</td>
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<tr>
<td>Ristocetin, 450 μg</td>
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</tr>
<tr>
<td></td>
<td>2.5</td>
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</tr>
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<td>Trypsin, 50 μg</td>
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<td></td>
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</table>

Different amounts of the membrane glycoprotein was incubated in 50 μl of the stimulant for 10 min and then aggregation was initiated by adding 450 μl of the platelet suspension to this mixture. In control experiments, the glycoprotein was replaced with 50 μl of phosphate-buffered saline.
ly, it shows remarkable specificity in its interaction with macromolecular substrates. For example, in fibrinogen (mol wt 330,000) thrombin preferentially cleaves four specific arg-gly bonds to generate fibrinopeptides A and B. However, several aspects of the stimulation of platelets by thrombin observed under the physiologic conditions cannot be explained by a simple proteolytic model. Recent studies have shown that the platelet surface contains specific receptors for thrombin and a close correlation between thrombin binding to platelets and platelet secretion has been reported. Since hirudin blocks the binding of thrombin, it appears that the macromolecular binding site on thrombin is the primary region through which thrombin binds to platelets. However, binding of thrombin in itself is not sufficient for platelet stimulation and other steps are thought to be involved.

The identity of the thrombin receptor in human platelets has remained unclear. Glycocalcin, a glycoprotein easily released from the platelet surface, has been reported to block the binding of thrombin to platelets as well as platelet aggregation by both thrombin and ristocetin. On this basis, glycocalcin has been proposed to be a common receptor for thrombin and ristocetin on platelets. While glycocalcin appears to be related in some way to glycoprotein one (GP-I), its exact identity has remained controversial. We have presented evidence that a platelet glycoprotein of apparent molecular weight of 150,000, presumably GP-I, may be a receptor for thrombin on platelets. It has been reported that GP-I is involved in ristocetin-induced platelet agglutination. However, an antibody preparation that blocked ristocetin-induced platelet agglutination did not inhibit platelet aggregation by thrombin. Since intact platelets contain antithrombin activity, the issue is further compounded by the possible presence of platelet surface components with thrombin inhibitory activity but not necessarily receptor properties involved in the physiologic stimulation of platelets by thrombin. Thus, in this study, our approach to a platelet thrombin receptor has been mainly physiologic. The results of this study show that the lectin derivative inhibited thrombin-induced platelet aggregation, but platelet agglutination by ristocetin was not affected. While these data indicate that the receptor for thrombin and ristocetin (or ristocetin-vWF) on platelets are independent, it may still be argued that the two ligands act on different sites on the same molecule. However, the membrane isolate from the lectin affinity column again inhibited thrombin-induced platelet aggregation but not ristocetin-induced agglutination. These results indicate that the thrombin-receptor and the ristocetin-receptor on platelets may be distinct. If GP-I or glycocalcin is indeed the ristocetin receptor in platelets, then these data make it difficult to maintain that this glycoprotein is also the physiologic receptor for stimulation of platelets by thrombin.

The lectin derivative did not affect the apparent binding of thrombin to platelets. Thus, the binding sites of thrombin and the derivative on platelets may be located on the same molecule or on two different molecules. The derivative exhibited remarkable specificity in its inhibitory action in that only platelet aggregation by thrombin was blocked while aggregation by ADP, collagen, wheat germ agglutinin, ristocetin, or trypsin remained unaffected. These results support the concept that thrombin stimulates platelets through a specific pathway. Since different stimulating agents are thought to interact with different receptors on the platelet surface, the above observation led to the working hypothesis that the lectin derivative may bind to the thrombin receptor. It is unlikely that the derivative binds to a platelet component other than the thrombin receptor and exerts a distal inhibitory action specifically on thrombin-induced platelet aggregation without affecting any of the other stimulating agents tested. In fact, lectin affinity chromatography of platelet membranes led to the isolation of a single glycoprotein of M, ~ 74,000. The observation that the glycoprotein from the affinity column, showing binding to the lectin derivative, specifically inhibited platelet aggregation by thrombin suggest that the derivative and thrombin indeed bind to the same receptor molecule. The binding sites for thrombin and the derivative on this glycoprotein may be independent or may be the same. If the binding sites are different, then it seems that the action of the lectin derivative is in many ways similar to an antibody to the glycoprotein and it may inhibit thrombin-induced platelet aggregation by interfering with the processing of ligand-receptor complexes subsequent to thrombin binding that may be necessary for the generation of a message or a messenger involved in platelet stimulation. This step does not appear to be part of the common pathway for platelet aggregation involving such possible factors as Ca++, or cAMP since platelet aggregation by other inducers was not affected by the derivative. Conceptually, it may correspond to the TR = TR* step in the model proposed by Martin et al. The possibility that the derivative may compete with thrombin for binding to a few functional sites on platelets should also be entertained. Thrombin modified at the active site serine has been reported to bind to platelets similar to unmodified thrombin but it does not compete with active thrombin for platelet stimulation. A straightforward interpretation of these results is that the number of functional thrombin
binding sites on platelets is small and most of the thrombin is bound to nonproductive sites of platelets. Because of this high background value, it may not be possible to detect by conventional binding measurements a slight alteration in thrombin binding to a few functional sites on platelets due to competition by the derivative. The isolated glycoprotein does not appear to be a general nonfunctional inhibitor of thrombin since binding of the lectin derivative specifically blocked thrombin-induced platelet stimulation. In addition, we have shown that cross-linking of the platelet-bound lectin derivatives by immunologic methods leads to serotonin secretion that is insensitive to indomethacin. The results presented support the concept that the initial phase in the interaction of thrombin with platelets may be described on a hormonal model of endocrine tissues.

The binding of a lectin to a cell and the resultant effects are complex phenomena and are determined by a number of factors. The lectin derivative neither inhibited platelet stimulation by wheat germ agglutinin nor had the ability of the native lectin to block ristocetin-induced platelet agglutination. These observations indicated that there are differences in the receptor binding properties of the parent lectin and its derivative. In fact, the derivative bound strongly to a 74,000 dalton membrane glycoprotein while wheat germ agglutinin is known to bind mainly to the GP-I complex although lesser binding to other components may not be ruled out. We have shown that a glycoprotein that migrates slightly slower than the albumin standard is clearly labeled in human platelets by the neuraminidase-galactose oxidase-[3H]borohydride procedure and estimated its apparent molecular weight to be 70,000. The incorporation of radioactivity into this component in intact platelets suggests that this glycoprotein is exposed on the cell surface. There is considerable confusion as to the number of platelet membrane glycoproteins, their nomenclature, and their molecular weights and it is difficult to compare the data from different laboratories. A glycoprotein, designated GP-V, GP 68, SP 85, of uncertain molecular size has been reported to be hydrolyzed by thrombin. This glycoprotein is also hydrolyzed by chymotrypsin as well as trypsin. Chymotrypsin does not aggregate platelets. Moreover, platelets, treated extensively with chymotrypsin to remove surface glycoproteins, aggregate, and secrete serotonin to the same extent as untreated platelets upon subsequent exposure to thrombin. The glycoprotein reported in this study inhibited thrombin-induced platelet stimulation while platelet aggregation by all other agents tested was not significantly affected. At concentrations of thrombin more than sufficient to cause platelet stimulation, there was no evidence of hydrolysis of this glycoprotein. Furthermore, under the conditions of the experiments, the glycoprotein preincubated with trypsin or chymotrypsin retained its capacity to block platelet aggregation by thrombin. Of the platelet glycoproteins known, the one that comes closest to the protein reported in this work is GP 80 (34 nomenclature). It is known that trypsin does not compete with thrombin for binding to platelets and that there are important differences in the kinetics of activation of platelets by thrombin and by trypsin. Neither the lectin derivative nor the isolated glycoprotein had any effect on tryptic-induced platelet aggregation. These observations provide some explanation for the exquisite specificity observed in the stimulation of platelets by thrombin and implicates the 74,000 dalton glycoprotein as a physiologic receptor of thrombin in human platelets.

REFERENCES

12. Wright CS: The crystal structure of wheat germ agglutinin at 2.2 Å resolution. J Mol Biol 111:439, 1977

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Inhibition of thrombin-induced platelet aggregation by a derivative of wheat germ agglutinin. Evidence for a physiologic receptor of thrombin in human platelets

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