The Use of the Synthetic Peptide, Gly-Pro-Arg-Pro, in the Preparation of Thrombin-degranulated Rabbit Platelets

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The method for preparing thrombin-degranulated platelets has been modified to avoid the use of plasmin or successive treatments with small amounts of thrombin, while still achieving more than 90% release of platelet amine storage granule contents. It was necessary to prevent the fibrinogen released from the platelets during thrombin treatment from forming an insoluble fibrin mesh that could trap the platelets and hinder their deaggregation. To accomplish this we have treated rabbit platelets with 0.73 U/ml of thrombin for 1 min in the presence of the synthetic peptide, Gly-Pro-Arg-Pro, which prevents the polymerization of fibrin molecules. We have demonstrated that it also prevents 131I, initially added as 131I-fibrinogen, from associating with the platelets in a form that was not removed by centrifuging and washing during the preparation of thrombin-degranulated platelets, and we infer that products formed from the fibrinogen released from the platelets would also be prevented from associating with them. Thrombin-degranulated platelets prepared by this method have lost 92% of their granule contents and they can be washed and resuspended. These platelets aggregate normally upon stimulation with thrombin, adenosine diphosphate (ADP), or arachidonate. Thus, Gly-Pro-Arg-Pro is useful in preparing thrombin-degranulated platelets for studying platelet reactions without the complicating effects of released materials such as ADP and fibrinogen.

It has been established that fibrinogen plays an essential role in ADP-induced platelet aggregation and binds to human or rabbit platelets in a saturable, reversible, and specific manner when they are stimulated with ADP.67 Other aggregating agents, such as thrombin, collagen, or arachidonate, cause the release of platelet granule contents, including ADP and fibrinogen,7 making it difficult to determine whether aggregation and fibrinogen binding are due to the aggregating agent under study or whether the released ADP is responsible. Because platelets that have been treated with thrombin and released approximately 90% of their amine storage granule contents can be deaggregated and recovered as normally functioning platelets, except that they do not have granule contents to release,89 we decided that thrombin-degranulated platelets might be used to study fibrinogen binding during aggregation with agents other than ADP. Besides activating platelets, thrombin causes the conversion of added or released fibrinogen to fibrin, and unless the formation of a fibrin clot is prevented, the platelets become trapped in a fibrin mesh and do not deaggregate readily. In the original method described for the preparation of thrombin-degranulated platelets, the formation of such a fibrin mesh was prevented by the use of three successive applications of a very low concentration of thrombin that aggregated platelets with partial release of granule contents without clotting the fibrin.8 Later, a modified method was developed in which the platelets were exposed to a higher concentration of thrombin, and plasmin was used to degrade the fibrin to soluble products.911 Successive treatments with thrombin are tedious, and we wanted to avoid the use of plasmin, since this enzyme affects platelet membrane glycoproteins12 as well as fibrin. Glycine-L-proline-L-arginine-L-proline (Gly-Pro-Arg-Pro) is a synthetic peptide, shown by Laudano and Doolittle to prevent the polymerization of fibrin molecules into an insoluble clot.1314 In this article we report results obtained using Gly-Pro-Arg-Pro to prevent fibrin clot formation during the thrombin treatment of rabbit platelets.

MATERIALS AND METHODS

Human fibrinogen (grade L) was from AB Kabi, Stockholm, Sweden; in some experiments, factor XIII was removed from the fibrinogen by chromatography on DEAE-cellulose,15 but this did not affect the experimental results. Human α-thrombin (lot H-1) was kindly supplied by Dr. D.L. Aronson, Bureau of Biologics, FDA, Bethesda, Md.; it was dissolved at 75 U/ml in 50% glycerol and stored at −20°C. For early experiments, Gly-Pro-Arg-Pro was kindly supplied by Drs. R.F. Doolittle and G.D. Wilner; for later experiments it was obtained from Vega Biochemicals, Tucson, Ariz. and stored at −20°C as a 20 mM solution in H2O. Hirudin, from Sigma Chemical Co., St. Louis, Mo., was dissolved in 0.15 M NaCl at 100 U/ml and stored at −20°C. Protaglandin E1 (PGE1), a generous gift of the Upjohn Co., Kalamazoo, Mich., was dissolved in 95% ethanol at a concentration of 2.9 mM and stored at −20°C; dilutions were made in 0.15 M NaCl. Bovine albumin (Pentex, fraction V) was from Miles Laboratories, Inc., Elkhart, Ind. Apyrase, prepared by the method of Molnar and Lorand,16 was dissolved in 0.15 M NaCl and stored at −20°C; at a concentration of 9 μl/ml,
it caused 91% conversion of 9 μM ADP to AMP and adenosine in 2 min at 37°C; at concentrations of 1 μM or lower, no degradation of 9 μM ADP was observed in 10 min at 37°C.

Na125I (carrier-free, NEZ 033L) and Na25tCrO4 (specific activity 200–500 Ci/g of Cr, NEZ 030) were from New England Nuclear, Boston, Mass. 14C-serotonin was obtained at 5-hydroxytryptamine-3.14C creatinine sulfate (54 mCi/mmol) from Amersham Corp., Arlington Heights, III. All other chemicals were reagent grade, and all concentrations are expressed as final values after all additions.

Fibrinogen was iodinated with 125I by the ICI method, and the product was characterized as previously described. Suspensions of washed rabbit platelets to be used in the preparation of thrombin-degranulated platelets were prepared essentially as described by Ardle and associates18,19 at a platelet count of approximately 1.2 x 10^10/ml. The final suspending medium was Tyrode solution containing 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, albumin (3.5 mg/ml), and apyrase (1 μl/ml); this solution is designated as Tyrode-albumin.

A stirred platelet suspension, at 37°C, was treated for 1 min with 0.73 U/ml of thrombin, either in the presence or in the absence of Gly-Pro-Arg-Pro. The reaction was stopped by the addition of 2.8 U/ml of hirudin, 0.01 mM PGE₁, was added, and the platelets were allowed to deaggregate with slow stirring for 30 min at 37°C. The final suspension was centrifuged, and the platelets were washed once with calcium-free Tyrode-albumin, pH 6.5, containing hirudin at 0.5 U/ml, and resuspended in Tyrode-albumin. After 1 hr at room temperature, the platelets were again centrifuged and resuspended in Tyrode-albumin solution. For the determination of the release of amine storage granule contents during thrombin treatment, the platelets were labeled during the initial isolation with 14C-serotonin, washed, and resuspended; the proportion of 14C released 3 min after stimulation of the platelets with thrombin was measured as described previously. Similarly, platelet lysis was determined by labeling with Na125I and measuring any 51Cr lost into the platelet suspending medium after thrombin stimulation.21

RESULTS

Rabbit platelets were exposed to 0.73 U/ml of thrombin for 1 min, the minimum exposure time that caused more than 90% release of 14C-serotonin from prelabeled platelets. Release was 92.1% ± 1.8% (mean ± SD of 8 experiments) in the presence of Gly-Pro-Arg-Pro (0.4 μM) and 92.4% ± 2.6% in its absence (5 experiments). Higher concentrations of thrombin caused some loss of 51Cr from prelabeled platelets, and longer exposure times did not increase the percentage of 14C-serotonin released. The use of imipramine (2 μM) to prevent the reuptake of serotonin, or of paraformaldehyde to prevent further release during centrifugation of the platelet suspensions, did not change the values obtained for 14C-serotonin release.

Gly-Pro-Arg-Pro was added to the platelet suspension before thrombin treatment to avoid the formation of a fibrin mesh that would entrap the platelets and hinder deaggregation. In addition to deaggregating rapidly, the platelets resuspended more readily, and higher recoveries of thrombin-degranulated platelets were achieved (an average of 70.9% ± 4.3% when Gly-Pro-Arg-Pro was used compared with 58.0% ± 11.8% when it was not).

When platelets were aggregated with 0.75 U/ml of thrombin in an aggregometer without Gly-Pro-Arg-Pro, wide oscillations in light transmission were observed after 2–3 min, representing large platelet aggregates. Usually a platelet–fibrin aggregate became wound around the stirring bar. When Gly-Pro-Arg-Pro (0.4 μM) was added to the platelet suspension before the thrombin, the height of the aggregation curve was the same, but the oscillations remained moderate for at least 6 min after the addition of thrombin, and the aggregates remained small and evenly dispersed throughout the suspension. With higher concentrations of Gly-Pro-Arg-Pro (up to 1.0 μM), no further inhibitory effects were apparent.

Although we could not determine directly the fate of the fibrinogen released from the platelets by thrombin, we could study the fate of added 125I-fibrinogen. 125I-fibrinogen was added to the platelet suspension and then the thrombin treatment was carried out both without added Gly-Pro-Arg-Pro and with Gly-Pro-Arg-Pro added before the thrombin. Hirudin and PGE₁ were added at 1 min in the usual manner. By determining the radioactivity in the supernatant from the first centrifugation after thrombin-degranulation (i.e., at 30 min) and in the final platelet suspension, we obtained the results shown in Table 1. Without Gly-Pro-Arg-Pro, approximately 79% of the added 125I was associated with the platelets and was not removed by the initial centrifugation. Even after 3 washings, 21% remained with the platelets that were recovered; if all the platelets had been recovered, it would have been apparent that 36% of the added 125I actually remained associated with the platelets. With 0.4 μM Gly-Pro-Arg-Pro, 91% conversion of 9 μM ADP to AMP and adenosine in 2 min at 37°C; at concentrations of 1 μM or lower, no degradation of 9 μM ADP was observed in 10 min at 37°C.

### Table 1. Effect of Gly-Pro-Arg-Pro on Percent of 125I. Added as 125I-Fibrinogen Before Thrombin, That Associated With Thrombin-degranulated Platelets

<table>
<thead>
<tr>
<th>Gly-Pro-Arg-Pro (μM)</th>
<th>Associated With Platelets After Thrombin Treatment</th>
<th>Associated With Platelets After 3 Washings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>79 ± 5</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>0.2</td>
<td>26 ± 8</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>0.4</td>
<td>8 ± 3</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

Results are means ± SD of 4 experiments (2 experiments in the case of 0.2 mM Gly-Pro-Arg-Pro).

Platelets were incubated with 125I-fibrinogen (10.3 ± 4.8 μg/ml) and Gly-Pro-Arg-Pro or saline for 20 min at 37°C before addition of thrombin (0.73 U/ml).

*Determined from cpm in supernatant from first centrifugation.
†Determined from cpm in final platelet suspension. Note that platelet recovery after 3 washings was 58%; if recovery had been 100%, it would have been apparent that 36% of the 125I rather than 21% had remained associated with the washed platelets when no Gly-Pro-Arg-Pro was present during thrombin treatment.
Pro-Arg-Pro in the suspension, only 8% of the \(^{125}\text{I}\) remained associated with the platelets after the initial centrifugation and only 2% with the washed platelets. Gly-Pro-Arg-Pro at a concentration of 0.2 mM had an intermediate effect. We found that 0.4 mM Gly-Pro-Arg-Pro added 1 min after the thrombin, when aggregation had occurred, was as effective as when added before the thrombin, but that it was much less effective when added 20 min after the thrombin, during deaggregation (16% of the \(^{125}\text{I}\) remained associated with the washed platelets when this was done).

Gly-Pro-Arg-Pro (0.4 mM) did not affect the amount of Fbgt-fibrinogen that associated with untreated control platelets during thrombin-induced aggregation. In both the presence and absence of Gly-Pro-Arg-Pro, \(^{125}\text{I}\)-fibrinogen binding was similar to that reported previously.\(^5\)

Figure 1 shows that thrombin-degranulated platelets, prepared using Gly-Pro-Arg-Pro, aggregated in response to thrombin, ADP, or arachidonate and that their responses were similar to those of untreated control platelets. Their ability to deaggregate after ADP-induced aggregation was somewhat impaired.

When stimulated with ADP or arachidonate, the thrombin-degranulated platelets bind \(^{125}\text{I}\)-fibrinogen in a specific, saturable, and reversible manner.\(^23\)

**DISCUSSION**

Thrombin-degranulated platelets have been shown to be useful in studies of platelet reactions with agents that normally cause the release reaction.\(^7\) More than 90% of platelet amine storage granule contents were removed by a 1 min treatment of rabbit platelets with 0.73 U/ml thrombin. Previously developed methods involved successive treatments with low concentrations of thrombin, or the use of plasmin, and although thrombin-degranulated platelets prepared by the former method could be aggregated by stimulation with a high concentration of thrombin,\(^8\) the use of plasmin apparently abolished the susceptibility of the platelets to thrombin.\(^9\) These procedures were not required in the preparation described in this article and the time of exposure of the platelets to thrombin was much shorter.

Fibrin clot formation was avoided by the use of the peptide Gly-Pro-Arg-Pro in the platelet suspension during thrombin treatment. This peptide prevented \(^{125}\text{I}\), added as \(^{125}\text{I}\)-fibrinogen before the thrombin treatment, from associating with the platelets in a form that could not be removed from the deaggregated platelets by centrifugation and washing. It seems likely that Gly-Pro-Arg-Pro would have the same effect on fibrinogen released from the platelets by thrombin. It should be emphasized that Gly-Pro-Arg-Pro probably did not prevent \(^{125}\text{I}\)-fibrinogen binding per se during thrombin treatment, since it does not prevent fibrinogen binding during ADP-induced aggregation. However, bound fibrinogen would be lost during deaggregation\(^5\) and if its conversion to polymerizing fibrin were prevented, \(^{125}\text{I}\) would not remain associated with the platelets. Polymerizing fibrin has been shown to bind to platelets\(^25\); this earlier observation supports the
conclusion that Gly-Pro-Arg-Pro probably prevents fibrin from polymerizing and associating with the platelets during thrombin-degranulated release of platelet granule contents. Thus, Gly-Pro-Arg-Pro can be used to prevent the formation of large stable platelet-fibrin aggregates during the stimulation of platelets with thrombin. Degranulated platelets prepared in this manner are suitable for studies of fibrinogen binding during subsequent stimulation with other agonists, e.g., arachidonate. Thus, thrombin-degranulated platelets, prepared using Gly-Pro-Arg-Pro, are useful in studying platelet reactions in which the complicating effects of the released granule contents, particularly ADP and fibrinogen, are to be avoided.

REFERENCES

13. Laudano AP, Doolittle RF: Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. Proc Natl Acad Sci USA 75:3085, 1978
The use of the synthetic peptide, Gly-Pro-Arg-Pro, in the preparation of thrombin-degranulated rabbit platelets

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