Platelet Activation and Subsequent Inhibition by Plasmin and Recombinant Tissue-Type Plasminogen Activator

By William F. Penny and J. Anthony Ware

The success of plasminogen activators in recanalizing occluded coronary arteries may be influenced by their effect on blood platelets; however, some previous studies have shown platelet activation by plasmin and thrombolytic agents while others have shown an inhibitory effect. Moreover, it has not been determined whether these effects reflect an alteration of intracellular signal transduction, fibrinogenolysis, degradation of adhesive protein receptors, or a combination of these events. To distinguish among these possibilities, the increase of cytoplasmic calcium ([Ca\(^{2+}\)]\(_i\)) ([Ca\(^{2+}\)]\(_{ii}\)), which is an intracellular marker of platelet activation that precedes fibrinogen binding to the surface of activated platelets, was measured along with aggregation and release of 5-hydroxtryptamine (5-HT) in washed human platelets incubated with plasmin or recombinant tissue-type plasminogen activator (rt-PA). Plasmin (0.1 to 1.0 CU/mL) induced a prompt, concentration-dependent [Ca\(^{2+}\)]\(_i\) increase when added to platelets, but subsequently inhibited the [Ca\(^{2+}\)]\(_i\) increase in response to thrombin or the endoperoxide analog U44069. Platelet aggregation accompanied the [Ca\(^{2+}\)]\(_i\) increase if the platelets were stirred, while the aggregation of platelets unstirred during plasmin incubation was inhibited upon agonist addition and resumption of stirring. The release of 5-HT paralleled the [Ca\(^{2+}\)]\(_i\) increase induced by plasmin and was also inhibited after the subsequent addition of a second agonist. The effects of rt-PA, added with plasminogen (100 \(\mu\)g/mL), were similar to those of plasmin, and could be accounted for by the concentration of plasmin generated. The ADP scavengers apyrase and CP/CK each prevented the [Ca\(^{2+}\)]\(_i\) increase, and aggregation caused by plasmin or rt-PA, and also prevented their inhibitory effects on thrombin-induced activation. Thus, plasmin and rt-PA initially activate platelets, inducing a [Ca\(^{2+}\)]\(_i\) increase, and, if the platelets are stirred, aggregation. Such activation is followed by subsequent inhibition of cellular activation by a second agonist; the inhibitory effect is in proportion to the degree of initial activation, and ADP is an important cofactor in both processes. These platelet effects occur at rt-PA concentrations achievable clinically, and may affect the success of therapy with thrombolytic and adjunctive agents.

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Despite the success of thrombolytic agents in recanalizing occluded coronary arteries, significant problems may limit their effectiveness, including failure to achieve initial reperfusion, reocclusion after initially successful thrombolysis, and a high incidence of hemorrhage. Studies in vivo have shown marked platelet activation after the administration of thrombolytic agents. Moreover, a critical role for platelet activation in the pathogenesis of reocclusion is suggested by recent reports in which inhibition of platelet aggregation by monoclonal antibodies (MoAbs) directed against the platelet glycoprotein (GP) IIb-IIIa complex enhanced the efficacy of thrombolytic agents. In apparent contrast, the template bleeding time is prolonged in patients and experimental animals who have received both aspirin and thrombolytic agents, suggesting that platelet dysfunction can account for the hemorrhagic tendency in these patients as well. In parallel with these clinical observations, some studies have shown that plasmin can aggregate platelets in vitro, while others have shown inhibition of the aggregation induced by subsequent addition of an agonist, while platelet aggregation induced by plasmin 1.0 CU/mL was associated with an increase in intracellular calcium and phosphoinositol turnover by Schafer et al., while Schafer and Adelman described an inhibitory effect of plasmin at the concentration range of 0.05 to 0.5 CU/mL. The addition of recombinant tissue-type plasminogen activator (rt-PA) to platelets suspended in whole blood, platelet-rich plasma (PRP), and buffer prevents aggregation and disaggregates platelets after stimulation with ADP and other agonists. Whether platelet inhibition derives from plasmin-mediated fibrinogenolysis or degradation of the surface GPs that bind adhesive proteins or an effect on intracellular signal transduction, as is the case for plasmin-mediated platelet activation, is unknown.

To investigate the mechanism for these disparate actions of plasmin and rt-PA on platelet function, the concentration of cytoplasmic calcium ([Ca\(^{2+}\)]\(_{ii}\)) in washed platelets was measured as a marker of activation that, unlike aggregation, can be distinguished from an effect on adhesive proteins or their GP receptors. The possibility that rt-PA may affect platelet function by a mechanism other than plasmin generation is also considered.

**MATERIALS AND METHODS**

**Platelet preparation.** Human platelets were obtained from normal healthy donors who had not ingested aspirin for 7 days or nonsteroidal anti-inflammatory agents for 48 hours. Blood was collected via antecubital vein phlebotomy into 3.8% sodium citrate (1:10) and then centrifuged at 2,000g to form PRP. Prostaglandin E\(_1\) (PGE\(_1\); 1 \(\mu\)mol/L) was added and the PRP was spun at 430g to form a soft pellet. The pellet was washed by resuspension in HEPES-Tyrode's buffer, pH 7.4, with EGTA 10 mmol/L and PGE\(_1\), 1 \(\mu\)mol/L. The suspension was centrifuged at 11,730g for 8 seconds and resuspended in buffer without EGTA, then layered onto Sepharose 2B (10 mL bed volume) equilibrated with HEPES-Tyrode's buffer containing 1 mmol/L Ca\(^{2+}\). The platelets were

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eluted into this buffer and the cell count was adjusted to 1.6 × 10^6/mL for [Ca^{2+}]i determinations and to 2.5 × 10^6/mL for all other experiments.

**Platelet aggregation.** Samples of gel-filtered platelets were incubated with or without stirring for 1 to 10 minutes at 37°C in the presence of plasmin (0.01 to 1.0 CU/mL) and then stimulated with thrombin (0.1 U/mL), the endoperoxide analog U44069 (200 nmol/L), or the Ca^{2+} ionophore A23187 (100 nmol/L). Control studies were performed substituting NaCl for plasmin or the subsequent agonists, except for A23187, in which a dimethyl sulfoxide (DMSO) control was used. Aggregation was recorded for at least 2 minutes with an aggregometer (Sienco, Inc, Morrison, CO) after agonist addition, and was quantified by measuring the percentage change between the gel-filtered platelet sample and buffer. In separate experiments, washed platelets were incubated with rt-PA (0.1 to 125 pmol/L) and/or human plasminogen (100 pg/mL) for 1 to 10 minutes at 37°C, before stimulation with thrombin 0.1 U/mL. In some experiments, platelets were treated with apyrase (0.2 U/mL) or CP/CK (creatine phosphate, 10 μmol/L; creatine phosphokinase, 80 U/mL) before the addition of plasmin or rt-PA/plasminogen.

**Platelet [Ca^{2+}]i determination.** The concentration of cytoplasmic Ca^{2+} ([Ca^{2+}]i) was measured by incubating the Ca^{2+}-sensitive fluorophore fura-2 acetoxyethyl ester in PRP (final concentration, 1 μmol/L) for 30 minutes. The platelets were then washed, gel-filtered, and eluted into HEPES-Tyrode’s buffer with the final cell count adjusted to 1.6 × 10^6/mL. Fluorescent signals were recorded as the ratio of excitation wavelengths 340 nm/380 nm at the emission wavelength 505 nm with a spectrofluorimeter (SPEX Fluorolog-2; SPEX Industries, Edison, NJ). [Ca^{2+}]i is expressed as this ratio, which can be converted to absolute concentrations as described elsewhere. In some experiments, fura-2 was loaded into washed platelets resuspended in HEPES-Tyrode’s buffer containing 0.38% sodium citrate, hirudin 0.1 U/mL, and apyrase 0.2 U/mL. After incubation at 37°C for 15 minutes, PGE was added (1 μmol/L) and the platelets were spun to a pellet and resuspended in HEPES-Tyrode’s buffer at a concentration of 1.6 × 10^6/mL, followed by the addition of CaCl₂ to a final concentration of 1 mmol/L. Plasmin and rt-PA incubations and agonist stimulation were performed as described in aggregation experiments, and streptokinase (1 to 100 U/mL) with plasminogen (100 μg/mL) was also tested. In some experiments, platelets were treated with apyrase (0.2 U/mL), aspirin (200 mmol/L), hirudin (0.25 U/mL), or CP/CK (creatine phosphate, 10 μmol/L; creatine phosphokinase, 80 U/mL) before the addition of plasmin or rt-PA/plasminogen.

**Release of 5-hydroxytryptamine (5-HT) and ADP.** PRP was incubated with ^14C-5-HT (1 μCi) for 30 minutes at room temperature. The platelets were then gel-filtered as described above and suspended in HEPES-Tyrode’s buffer containing Ca^{2+}. Aliquots of 100 to 200 μL were removed at the times indicated in the experiments and immediately mixed with an equal volume of iced EDTA/formaldehyde 200 mmol/L. The samples were centrifuged at 11,730g for 3 minutes and the supernatant was counted. Release is expressed as the percentage of total counts. In separate experiments, the release of ADP was measured by luciferin/luciferase in a lumigaggrometer (Chronolog Corp, Havertown, PA), as previously described. Plasmin was determined by a colorimetric method using the substrate S-2251 as described.

**Materials.** Plasmin and plasminogen were purchased from Kabi (Stockholm, Sweden) and were reconstituted with water and stored at -70°C. rt-PA was generously provided by Genentech (South San Francisco, CA). Fura-2 was purchased from Molecular Probes (Eugene, OR).

**Data analysis.** Values are expressed as mean ± SD unless otherwise noted. The Student’s two-tailed, paired t-test was used for statistical comparisons in which a P value is reported.

**RESULTS**

Aggregation of gel-filtered platelets induced by thrombin and the endoperoxide analog U44069 was inhibited by prior incubation with plasmin (0.5 CU/mL) for 10 minutes; however, aggregation induced by the Ca^{2+}-ionophore A23187 was not affected by plasmin (Fig 1). These effects on aggregation were accompanied by similar changes in peak [Ca^{2+}]i after stimulation, with inhibition of thrombin and U44069-stimulated [Ca^{2+}]i increase after incubation with plasmin (0.5 CU/mL) and no significant effect on the response to A23187. Thus, plasmin-mediated inhibition of platelet activation, as manifested by decreased [Ca^{2+}]i mobilization, accompanies its inhibition of aggregation.

In the next set of experiments, plasmin was added to stirred fura-2–loaded platelets and the [Ca^{2+}]i was measured continuously. As shown in Fig 2, plasmin added to washed platelets at concentrations as low as 0.1 CU/mL caused a detectable increase in [Ca^{2+}]i, and this response was concentration-dependent in the range tested. The attenuation of [Ca^{2+}]i increase after the subsequent addition of thrombin (0.1 U/mL) was also concentration-dependent, and was correlated with the magnitude of the initial [Ca^{2+}]i increase seen with plasmin alone (Fig 3A). Thus, plasmin inhibits platelet [Ca^{2+}]i increase and activation, but only after inducing an initial stimulatory response itself.

To determine whether plasminogen activators show a similar biphasic response of activation and subsequent inhibition, platelets were incubated with either rt-PA or
PLATELET EFFECTS OF PLASMIN AND rt-PA

Figure 2: Time course of plasmin-induced intracellular [Ca"++] increase in fura-2-loaded platelets, stirred continuously. Plasmin at the concentrations shown (in CU/mL) was added at 0 seconds. The change in [Ca"++] is expressed as the ratio of 340/380 nm fluorescence, minus baseline. Results are expressed as mean ± SD, with some bars removed for clarity; n = 8 to 11 except for plasmin 1.0 (n = 3). Streptokinase with plasminogen added at a half-physiologic level of 100 µg/mL. As shown in Fig. 3B, rt-PA caused an increase in peak [Ca"++] and, as seen with plasmin, this initial increase in [Ca"++] was followed by an inhibited [Ca"++] response to subsequent stimulation with thrombin. Similar results were seen with streptokinase (Fig. 3C). Both an initial [Ca"++] increase and inhibition upon subsequent stimulation by thrombin were seen at rt-PA concentrations as low as 1.0 µg/mL, and at streptokinase concentrations of 10 U/mL. At these concentrations, both rt-PA and streptokinase generated plasmin (Fig. 4A), and the amount of plasmin generated could account for the observed effects on platelets (Fig. 3). To determine whether rt-PA could inhibit platelets by an additional mechanism, separate from that attributable to plasmin, platelets were incubated with rt-PA without added plasminogen (Fig. 4B). Despite the high rt-PA concentrations, only negligible amounts of plasmin could be detected by the chromogenic substrate S-2251 (<0.004 CU/mL plasmin at [rt-PA] of 125 µg/mL). The inhibition of the thrombin-induced [Ca"++] increase was shown at high (75 µg/mL) concentrations of rt-PA; however, the preparation of rt-PA commercially available from Genentech also included L-arginine (3.4 g/100 mg rt-PA) in concentrations sufficient to inhibit thrombin-induced [Ca"++] increase itself. Thus, the effect of rt-PA at high concentrations on platelet activation was related to an inhibition caused by the coexisting arginine.

To investigate the mechanism by which plasmin activates platelets, the effects of inhibitors of various pathways to platelet activation were tested. The addition of apyrase (0.2 U/mL) prevented the initial [Ca"++] increase induced by plasmin, and also prevented plasmin from inhibiting thrombin-induced stimulation of [Ca"++] (Fig. 5). The possibility that apyrase directly interferes with plasmin, rather than with its effect on platelet activation, was considered; incubation of plasmin and apyrase at 0.2 U/mL did not alter the amidolytic effect of plasmin, as assayed using the chromogenic substrate S-2251 (plasmin 0.5 CU/mL: Abs \(_{405}\) 0.54 ± 0.16; plasmin + apyrase: Abs \(_{405}\) 0.60 ± 0.09, P = not significant). Coincubation of plasmin with another ADP scavenger, CP/CPK, similarly reduced the initial [Ca"++] increase and limited the inhibitory effect after thrombin (Fig. 6). Apyrase (200 mmol/L) also attenuated the initial plasmin response, but to a lesser extent than apyrase, as shown in Fig. 6 (compare the second, fourth, and fifth solid bars from the left). Apolipoprotein also moderately reduces the peak [Ca"++] induced by thrombin alone (data not shown); however, apyrase’s reduction of the peak [Ca"++] induced by plasmin was associated with a peak [Ca"++] after a subsequent addition of thrombin that was greater than that seen in the samples treated with plasmin but without apyrase (Fig. 6, compare the second and fifth dotted bars). Thus, the initial [Ca"++] increase caused by plasmin can be related to the subsequent inhibition of both aggregation and [Ca"++] increase induced by a second agonist. The combination of apyrase and apyrase reduced the initial plasmin-induced [Ca"++] increase to control (unstimulated) levels and maintained the inhibition seen by plasmin alone after thrombin (Fig. 6, sixth bar). The combination of plasmin and the thrombin inhibitor hirudin (0.25 U/mL) had no effect on plasmin-induced [Ca"++] increase. As expected, the response to the subsequent addition of thrombin was inhib-
the presence of washed platelets. Platelets were incubated for 10 minutes at 37°C with plasminogen (100 μg/mL) and either rt-PA or streptokinase, with plasmin activity assayed using S-2251. Values are expressed as mean ± SD; n = 3 to 4.

plasminogen on washed platelet 


correspond to those included in the rt-PA preparation (3.4 g L-arginine/100 mg rt-PA). Values are expressed as a percentage of control response to thrombin (0.025 U/mL) after 1 minute of incubation at 37°C (mean ± SD; n = 3 to 9).

[Ca']i increases induced by thrombin and plasmin can be assessed by comparing the cumulative height of the bars in Fig 6. The plasmin-treated sample without inhibitors induced an initial [Ca']i increase, but the inhibition of the thrombin-induced [Ca']i resulted in a total [Ca']i increase near control levels. As noted above, apyrase addition reduced the plasmin-induced [Ca']i increase and restored the thrombin response to normal, and thus the total [Ca']i increase was not affected. Samples containing CP/CPK or aspirin caused a small reduction in the total [Ca']i increase that was not different from plasmin and thrombin alone, without aspirin. The addition of aspirin and apyrase, by reducing both the plasmin and thrombin response, and hirudin, by markedly inhibiting thrombin while not affecting plasmin, resulted in a significant inhibition of the total [Ca']i increase.

The next set of experiments examined the influence of plasmin and rt-PA on platelet aggregation. When platelets were stirred constantly, plasmin at concentrations as low as 0.1 CU/mL caused platelet aggregation, with an increased rate of aggregation after increased plasmin concentrations (Table 1). The addition of thrombin to these platelets after 10 minutes of incubation with plasmin caused further aggregation inversely proportional to the prior aggregation by plasmin. Furthermore, plasmin’s ability to cause aggregation and also to inhibit subsequent aggregation was reversed by apyrase, with complete aggregation induced by thrombin in the apyrase-treated sample despite the presence of plasmin at a concentration of 0.5 CU/mL (Fig 7A).

rt-PA (with plasminogen, 100 μg/mL) showed effects similar to those of plasmin (Table 1), as was the case with [Ca']i increase (Fig 3). Continuous stirring was essential for the initial aggregatory response to plasmin or rt-PA. If stirring was omitted after initial mixing for 2 seconds, only a slight aggregation resulted. The addition of thrombin after 10 minutes of unstirred incubation and resumption of stirring resulted in inhibited aggregation (Table 1 and Fig
Table 1. Effect of Plasmin and of rt-PA on Thrombin-Induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Aggregation Before Thrombin (%AOD)</th>
<th>Aggregation After Thrombin (%AOD)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1 ± 2 (13)</td>
</tr>
<tr>
<td>Plasmin 0.01 CU/mL</td>
<td>0 ± 1 (3)</td>
</tr>
<tr>
<td>Plasmin 0.1 CU/mL</td>
<td>32 ± 21 (4)</td>
</tr>
<tr>
<td>Plasmin 0.5 CU/mL</td>
<td>49 ± 19 (14)</td>
</tr>
<tr>
<td>Plasmin 0.5 CU/mL + apyrase</td>
<td>3 ± 2 (3)</td>
</tr>
<tr>
<td>Plasmin 0.5 CU/mL + CP/CK</td>
<td>0 ± 0 (3)</td>
</tr>
<tr>
<td>Control + plasminogen, no stirring</td>
<td>1 ± 1 (8)</td>
</tr>
<tr>
<td>rt-PA 1 µg/mL + plasminogen</td>
<td>0 ± 0 (5)</td>
</tr>
<tr>
<td>rt-PA 5 µg/mL + plasminogen</td>
<td>16 ± 27 (4)</td>
</tr>
<tr>
<td>rt-PA 10 µg/mL + plasminogen</td>
<td>49 ± 13 (5)</td>
</tr>
<tr>
<td>Control + plasminogen, no stirring</td>
<td>1 ± 1 (3)</td>
</tr>
<tr>
<td>rt-PA 10 µg/mL + plasminogen, no</td>
<td>7 ± 9 (3)</td>
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<td>stirring</td>
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Initial aggregation of washed platelets incubated for 10 minutes with the agents listed is displayed in the first column, and is expressed as the percent change in optical density between platelet-containing buffer and plain buffer (%AOD). The second column displays the subsequent aggregation that occurred 2 minutes after the addition of thrombin (0.1 U/mL) to the same sample. Results are expressed as mean ± SD, with the number of experiments in each group shown in parentheses.

DISCUSSION

The characterization of the effect of plasmin on blood platelets in vitro has been complicated by an apparent discrepancy: evidence of platelet activation in some studies.
The incubation of washed platelets with plasmin concentrations as low as 0.1 CU/mL is accompanied by a prompt increase in fura-2–indicated $[Ca^{2+}]_i$ that increases in a concentration-dependent fashion, in agreement with previous studies using aequorin-loaded platelets; these plasmin concentrations are comparable to those that induce detectable aggregation. After this platelet activation by incubation with plasmin, the $[Ca^{2+}]_i$ increase, aggregation, and release of granular contents in response to stimulation by a second agonist, such as thrombin, are all diminished. The effect on the increase in $[Ca^{2+}]_i$, which accompanies or precedes the earliest stages of platelet activation including shape change, release of granular contents, and aggregation, suggests that plasmin-induced inhibition affects the intracellular activation of the platelet, rather than exerting only an extracellular effect such as degradation of fibrinogen or prevention of its binding to the surface of an activated platelet. Previous studies have shown that proteolytic enzymes such as chymotrypsin and pronase induce platelet aggregation through a proposed mechanism involving direct fibrinogen receptor exposure on the platelet surface. This aggregatory effect was not affected by ADP inhibitors and has been described as independent of intracellular metabolic processes, although subsequent work showing an increase in $[Ca^{2+}]_i$ suggests plasmin-induced platelet aggregation does involve cellular activation. The inhibition of platelet aggregation after incubation with proteolytic enzymes has likewise been ascribed to the degradation of platelet-associated fibrinogen, surface receptors for platelet agonists, GPIb, or the GPIIb/IIIa complex. However, the effects of plasmin seen under the conditions of the present study appear to involve another, perhaps additional, process. The inhibition of fibrinogen binding does not prevent the peak $[Ca^{2+}]_i$ increase after platelet stimulation, and thus plasmin’s reduction of the $[Ca^{2+}]_i$ increase after thrombin cannot be attributed to its effect on aggregation; rather, an effect on the intracellular metabolism of the platelet is suggested. All plasmin-mediated effects were prevented by apyrase at concentrations that did not affect the amidolytic activity of plasmin, suggesting that platelet inhibition and activation are not mediated solely through proteolysis of surface proteins. Moreover, the combination of apyrase with plasmin concentrations that had inhibited thrombin-induced platelet aggregation restored this aggregation to control levels, suggesting that under the short incubation times used in this study the degradative effect on fibrinogen or its receptor, the GPIIb/IIIa complex, was functionally negligible. In addition, $[Ca^{2+}]_i$ increase and aggregation induced by the Ca$^{2+}$ ionophore A23187, which activates platelets through a pathway not mediated by a receptor, were not inhibited by plasmin under these experimental conditions, implying that plasmin interferes with mechanisms by which agonist-receptor binding leads to mobilization of $[Ca^{2+}]_i$. Therefore, plasmin-induced inhibition of response to a subsequent agonist as shown in this study appears to involve an alteration of platelet intracellular metabolism rather than an effect on fibrinogen or its adhesive protein receptors on the platelet surface. A strong effect was seen at a plasmin concentration of 0.5 CU/mL, which is within the range (0.05 to 0.5 CU/mL) in which plasmin-induced platelet inhibition has been described.

In this study, all instances of plasmin-induced inhibition of the $[Ca^{2+}]_i$ increase after a second agonist were preceded by an increase in $[Ca^{2+}]_i$ produced by plasmin alone, and the magnitude of this secondary inhibition was directly proportional to the initial plasmin-induced $[Ca^{2+}]_i$ increase. Furthermore, when the plasmin-induced $[Ca^{2+}]_i$ increase was reversed by apyrase, its ability to inhibit $[Ca^{2+}]_i$ increase, aggregation, and granular release induced by subsequent addition of thrombin was also prevented. The result of this relationship was an increase in the response to thrombin if the plasmin-induced response was decreased by incubation with apyrase or aspirin, and led to a relatively constant total $[Ca^{2+}]_i$ increase after the two stimuli. Thus, plasmin induces a graded intracellular $[Ca^{2+}]_i$ increase at concentrations of 0.1 CU/mL and greater; similarly, the response to a second agonist can be linked to the initial plasmin-induced $[Ca^{2+}]_i$ increase, suggesting that the stimulatory and inhibitory effects of plasmin are closely related. Significant inhibition of overall platelet activation after stimulation by both plasmin and thrombin required a more aggressive use of adjunctive antiplatelet agents, as in the combination of aspirin and apyrase, which limited both plasmin- and thrombin-induced response. The addition of hirudin could also limit total activation by very strong
inhibition of the thrombin response, but did not affect the initial activation stimulated by plasmin.

It is possible that the plasmin-induced [Ca\(^{2+}\)] increase is required for its ability to inhibit subsequent platelet activation, as we have previously suggested for agonists that directly activate protein kinase C.\(^{25}\) This is also activated by plasmin.\(^{10}\) It seems unlikely, however, that the [Ca\(^{2+}\)] increase is the proximate cause of inhibition, because most agonists that cause a [Ca\(^{2+}\)] increase enhance subsequent [Ca\(^{2+}\)] increase, aggregation, and granular release by additional agonists.\(^{24}\) Although inhibition might be explained by prior aggregation, interference with aggregation by discontinuing stirring did not prevent inhibition of aggregation or the [Ca\(^{2+}\)] increase induced by subsequent agonists; thus, these events could be dissociated.

The mechanism of plasmin-mediated stimulation is uncertain; one possibility is that plasmin might induce an initial release of ADP from the platelet that in turn causes a large net [Ca\(^{2+}\)] increase, aggregation, and 5-HT release. This model could explain the effects of ADP scavengers, which would interrupt the cycle by hydrolyzing the ADP initially released. However, the initial calcium-independent secretion that this mechanism would require could not be documented by direct assay of ADP, nor was there evidence of an initial secretion of 5-HT, which is stored along with ADP in dense granules. The failure of ADP release to be detected with low concentrations of plasmin does not entirely discount this putative mechanism, however, because it is possible that ADP is released in quantities not detected by the luciferin/luciferase assay used in these studies. An alternative mechanism to explain our findings is that plasmin may directly affect the ADP receptor, and thus increase the sensitivity of platelets to the (normal subthreshold) levels of ADP found surrounding washed, gel-filtered platelets. In this model, the initial effect of plasmin would not require a substantial net increase in [Ca\(^{2+}\)], although it is possible that a localized [Ca\(^{2+}\)] increase, which may be detectable only with luminescent Ca\(^{2+}\) indicators, could be associated with this effect, as is seen with epinephrine, phorbol esters, and collagen stimulation of aspirin-treated platelets.\(^{38,39}\) An initial stimulation with ADP could then be amplified by a large net [Ca\(^{2+}\)] increase, dense granule release, and aggregation. Of note is that plasmin-induced stimulation cannot be attributed entirely to the effect of released ADP, as the peak [Ca\(^{2+}\)] levels, degree of aggregation in the absence of added fibrinogen, and 5-HT release are all much greater than that seen even with maximally activating concentrations of ADP.\(^{38}\) The inhibition by ADP scavengers, however, suggests that ADP is a necessary cofactor for all three events. An attractive aspect of this latter hypothesis is that it may explain the apparent discrepancy between our results and those of Puri et al.,\(^{40}\) published while this report was under review. Those investigators also found that plasmin strongly activated platelets and that it altered aggregin, a putative ADP receptor. The addition of 5'-P-fluorosulfonylbenzoyl adenosine (FSBA), which is thought to directly bind to a site on the ADP receptor and block ADP-induced activation, did not reduce platelet activation or release of dense granular contents, however. Taken together, these studies suggest that plasmin may alter the ADP receptor to expose a new site, presumably not blocked by FSBA, by which ADP can bind and enhance platelet activation. This mechanism may have clinical relevance as well, as it is possible that ADP in concentrations sufficient to activate platelets can be released from either platelets or erythrocytes subjected to rheologic stresses (such as those seen in a partly occluded artery).

The inhibition of subsequent response to ADP-dependent agonists in proportion to prior activation by plasmin could be explained on the basis of refractoriness after preactivation by ADP,\(^{26}\) which also mobilizes [Ca\(^{2+}\)].\(^{18}\) However, the aggregatory response to thrombin, which is strongly inhibited by plasmin, is not inhibited by ADP-induced preactivation,\(^{37}\) and our attempts to duplicate the inhibitory effect of plasmin on thrombin-stimulated aggregation by preactivating platelets with other agents were unsuccessful. Inhibition of ADP-independent stimulation by thrombin may thus involve a separate mechanism that is initiated upon ADP binding by the platelet in the presence of plasmin.

The effect of rt-PA and streptokinase in vitro on platelets incubated with plasminogen can be attributed to the plasmin generated under these conditions. rt-PA in the concentration range of 1 to 10 μg/mL, which is similar to that measured in the plasma when rt-PA is administered as a thrombolytic agent to patients with myocardial infarction (3 to 5 μg/mL),\(^{28,29}\) together with only a half-physiologic concentration of plasminogen generated sufficient plasmin to activate platelets as indicated by intracellular [Ca\(^{2+}\)] increase, dense granule release as indicated by \(^{38}\)C-5-HT secretion, and platelet aggregation. These effects can be reproduced by plasmin alone in concentrations similar to those generated by rt-PA and plasminogen in this system. As was the case with plasmin, this activation by rt-PA and plasminogen leads to an inhibited response to subsequent stimulation by a second agonist, with both effects prevented by apyrase.

Thus, plasmin generated by the therapeutic administration of rt-PA may have significant effects on platelets. While free plasmin is rapidly bound by circulating α2-antiplasmin and α-macroglobulin, these plasmin inhibitors may be eventually depleted with continued generation of plasmin.\(^{38}\) rt-PA has been shown to bind to platelets\(^{38}\) and to enhance the rate of plasminogen activation in vitro in the presence of platelets,\(^{32,33}\) and may generate the highest levels of plasmin around a thrombus, where fibrin-bound plasminogen and platelets are located. Enhanced platelet-to-platelet contact led to predominance of the proaggregatory effect relative to the inhibitory effect of plasmin in our experimental system; such contact may be promoted by shear stress associated with a severe coronary stenosis, which is thought to promote reocclusion by inducing platelet activation.\(^{44}\) Although findings from our in vitro study should be applied to the clinical setting cautiously, one can speculate that a coronary stenosis with superimposed thrombus may present ideal conditions for plasmin-induced platelet aggregation, and this effect may promote vascular reocclusion and failure to achieve reperfusion after the administration of thrombolytic agents.
REFERENCES


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Platelet activation and subsequent inhibition by plasmin and recombinant tissue-type plasminogen activator

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