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Research Article

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Regulation of Factor XIa Activity by Platelets and α_1-Protease Inhibitor

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Abstract

We have studied the complex interrelationships between platelets, Factor XIa, α_1-protease inhibitor and Factor IX activation. Platelets were shown to secrete an inhibitor of Factor XIa, and to protect Factor XIa from inactivation in the presence of α_1-protease inhibitor and the secreted platelet inhibitor. This protection of Factor XIa did not arise from the binding of Factor XIa to platelets, the presence of high molecular weight kininogen, or the inactivation of α_1-protease inhibitor by platelets. The formation of a complex between α_1-protease inhibitor and the active-site–containing light chain of Factor XIa was inhibited by activated platelets and by platelet releasates, but not by high molecular weight kininogen. These results support the hypothesis that platelets can regulate Factor XIa–catalyzed Factor IX activation by secreting an inhibitor of Factor XIa that may act primarily outside the platelet microenvironment and by protecting Factor XIa from inhibition, thereby localizing Factor IX activation to the platelet plug.

Introduction

Several lines of evidence support the view that platelets participate in coagulation reactions leading to Factor IX activation. Studies from our laboratory have demonstrated that platelets, activated by ADP, collagen, or thrombin can promote the proteolytic activation of purified Factor XII by kallikrein in the presence of high molecular weight (HMW)[1] kininogen (1). Thereafter, activated platelets can specifically and tightly bind Factor XI in the presence of HMW kininogen and Zn^{2+} ions (2) and promote the proteolytic activation of Factor XI in the presence of either Factor XIIa or kallikrein in the absence of Factor XII (1). Under the same conditions required for Factor XI binding to platelets (i.e., physiological concentrations of Zn^{2+} and Ca^{2+} ions) HMW kininogen also binds to high affinity, specific, saturable sites on activated platelets (3, 4). Moreover, Factor XIa in the presence of HMW kininogen binds tightly and specifically to a site on the activated platelet surface distinct from the binding site for Factor XI (5). These interactions therefore can be viewed as localizing the activation of Factor XI and the activity of Factor XIa to the platelet surface membrane. Our recent studies indicate that the functional activity and structural integrity of Factor XIa are fully retained when the protein is bound to platelets (5, 6). Kinetic analysis of the Factor XIa–catalyzed activation of Factor IX suggests that this is a physiologically significant reaction (7–9). Since the reaction rates are similar when Factor XIa is bound to platelets and when it is free in solution, we have suggested that Factor IX activation by platelet-bound Factor XIa is also a kinetically favorable reaction (7).

In 1972, we presented evidence that the inactivation rates of both Factors XIa and Xa by plasma proteinase inhibitors were significantly reduced in the presence of isolated platelets activated by ADP or collagen (10). Although the results of these experiments were not definitive because they were carried out with crude preparations of coagulation factors, it was subsequently confirmed by experiments with highly purified proteins that Factor Xa is protected from inactivation by antithrombin III either in the presence of Factor V and phospholipids (11) or when Factor Xa is bound to platelets (12). On the other hand, Soons et al. (9) have reported that activated platelets secrete a protease inhibitor that reversibly inhibits Factor XIa–catalyzed Factor IX activation. Since protection by platelets of coagulation enzymes from inactivation by plasma proteinase inhibitors may be an important mechanism for localization of coagulation to the hemostatic plug, we have investigated the effect of platelets on the inactivation of Factor XIa by α_1-protease inhibitor, which has been demonstrated to be a major Factor XIa inhibitor in plasma (13, 14).

Methods

Materials. All chemicals were the best grade commercially available and were purchased from Sigma Chemical Co., St. Louis, MO; Fisher Chemical Co., Fairlawn, NJ; or J. T. Baker Chemical Co., Phillipsburg, NJ. Plasmas deficient in coagulation factors were purchased from George King Biomedical, Overland Park, KA. Betaphase scintillation fluid was obtained from West Chemical Products Inc., San Diego, CA. Carrier-free $Na^{125}I$ and tritiated sodium borohydride (75 Ci/mmol) were purchased in crystalline form in sealed ampoules from New England Nuclear, Boston, MA. Iodogen (1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril) was obtained from Pierce Chemical Co., Rockford, IL. Ultrol Hepes (*N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid) was from Calbiochem-Behring Corp., La Jolla, CA, and Sepharose 2B was from Pharmacia Fine Chemicals, Piscataway, NJ. The chromogenic substrates pyro-Glu-Pro-Arg-paranitroanilide 2 HCl (S-2366) and H-D-Pro-Phe-Arg-paranitroanilide 2 HCl (S-2302) were purchased from AB KABI Peptide Research, Molndal, Sweden whereas Bz-Arg-*p*-nitroanilide and *N*-succinyl-trialanyl-*p*-nitroanilide were purchased from Sigma Chemical Co. Methyl silicon oil (1.0 DC 200) and Hi Phenyl silicon oil (125 DC 550) were obtained from William F. Nye, Inc., Fairhaven, MA. Acrylamide, SDS, *N,N'*-methylene-bis-acrylamide and *N,N,N',N'*-tetramethyl ethylene diamine were purchased from Bio-Rad Laboratories, Richmond, CA.

Portions of this study were previously reported in abstract form (1986. *Circulation.* 74:93).

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1. *Abbreviations used in this paper:* HMW, high molecular weight; TBS, Tris-buffered saline; TCA, trichloroacetic acid.

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Preparation of platelet suspensions. Platelet rich plasma was obtained from citrated human blood as previously described (5). Platelets were separated from plasma by gel filtration (5) into Ca^{2+}-free, Hepes-buffered Tyrode's solution of the following composition: NaCl, 126 mM; KCl, 2.7 mM; $MgCl_2$ $6H_2O$, 0.98 mM; NaH_2PO_4 H_2O, 0.376 mM; Hepes, 15 mM; dextrose, 5.6 mM; bovine serum albumin, 0.35%; pH 7.4. Alternatively, platelets were washed by the procedure of Mustard et al. (15) and were finally suspended in Ca^{2+}-free, Hepes-buffered Tyrode's solution. Platelets were counted electronically (model ZBI Particle Counter; Coulter Electronics, Inc., Hialeah, FL).

Purification of proteins. Factor XI was purified from 2 liters of human plasma by a modification of the method of Bouma and Griffin (16) as previously described (7). The purified protein appeared homogeneous on polyacrylamide gel electrophoresis in the presence of SDS, as previously published (7), and had a specific activity of 270 U/mg protein. It was stored in 0.2 M sodium acetate, 0.6 M NaCl, pH 5.3 at −70°C. Human HMW kininogen was purified to apparent homogeneity and specific activity of 15 U/mg as previously described by Kerbiriou and Griffin (17). Human Factor IX was purified to a specific activity of 225 U/mg by a modification (7) of methods described by DiScipio et al. (18) and by Miletich et al. (19) and was a single band on SDS gel electrophoresis, as previously published (7). It was stored in 0.012 M Tris, 0.045 M glycine, pH 8.3 at −185°C. Bovine two-chain Factor XIIa was kindly provided by Dr. E. P. Kirby of the Thrombosis Research Center, Temple University School of Medicine, as a homogeneous protein that hydrolyzed 3.78×10^9 mol of the chromogenic substrate S2302/min per μg protein at pH 8.0 and 37°C. Human α-thrombin was a gift from Dr. J. W. Fenton, II, New York State Department of Health, Albany, NY. Human α_1-protease inhibitor was purified from plasma by the method of Glaser et al. (20) and was graciously donated by Dr. Charles P. Glaser (Pacific Medical Center, San Francisco, CA). Another preparation, which gave identical results, was purchased from Calbiochem-Behring Diagnostics, La Jolla, CA. Each preparation appeared as a single band on SDS gel electrophoresis corresponding to a protein of 54,000 M_r. 1 mg of either preparation completely neutralized 0.46 mg of trypsin.

Preparation and characterization of Factor XIa. Purified Factor XI was activated by incubation at 37°C with bovine Factor XIIa, as previously described (7). Maximal Factor XIa coagulant activity appeared at 2 h, at which time 95% of the protein was present in cleavage products of M_r of 48,000 and 32,000 as previously published (7). The Factor XIa was assayed by chromogenic, coagulant, and immunoradiometric assays as previously described (7) and was thereby determined to have a specific activity of 180 U/mg protein.

Radiolabeling of proteins. Factor XI was labeled with ^{125}I utilizing the Iodogen method (21). Most of the free ^{125}I was removed from the labeled protein by passage over a 1-ml G-25 column (22), and the protein was then dialyzed further in the presence of ovalbumin (1 mg/ml) to remove residual ^{125}I. The Factor XI was then converted to Factor XIa as described above, was shown to retain > 85% of its coagulant activity, and had a specific radioactivity of 1117 cpm/ng protein. Factor IX was labeled with tritium by a modification (7) of the method described by Van Lenten and Ashwell (23) and was adapted for bovine Factor X by Silverberg et al. (24). The labeled protein was finally purified by alkaline gel electrophoresis (25), appeared homogeneous as judged by SDS gel electrophoresis and fluorography (26) as previously reported (7), and > 99% of the radioactivity was precipitable in 5% trichloroacetic acid (TCA). The tritiated Factor IX had a specific radioactivity of 475 cpm/ng and retained > 90% of its coagulant activity as compared with unlabeled Factor IX.

Coagulation assays. Factors IX and XI and HMW kininogen were assayed utilizing minor modifications of the kaolin-activated partial thromboplastin time (27) using appropriate congenitally deficient substrate plasmas, and results were quantitated on double logarithmic plots of clotting times vs. concentration of pooled normal plasma. Factor XIa was measured utilizing a similar assay carried out in polystyrene tubes without the addition of kaolin. The quantitation of Factor XIa activity was accomplished by means of a standard curve of the logarithm of clotting time vs. the logarithm of the concentration of purified Factor XIa.

Radiometric assay of Factor IX activation. The release of TCA-soluble ^3H-labeled activation peptide from (sialyl-^3H)-Factor IX was measured as previously described (7). Briefly assays were carried out in Tris (50 mM), NaCl (100 mM), pH 7.5 (Tris-buffered saline [TBS]), containing bovine serum albumin (1 mg/ml) in reaction volumes of 300–500 μl. At given times of incubation of Factor XIa with (sialyl-^3H)-Factor IX in the presence of 5 mM $CaCl_2$ at 37°C, 80 μl samples were removed and added to 240 μl of an ice-cold mixture containing one part TBS and two parts 50 mM EDTA, pH 7.5. To this mixture was added 160 μl of ice-cold 15% TCA or TBS, and after vigorous vortexing for 2 min it was centrifuged at 10,000 *g* for 3 min in a bench-top microfuge (model 3200; Brinkmann Instruments, Inc., Westbury, NY). Aliquots (100 μl) of the supernatants were removed into 10 ml of scintillation fluid and counted in triplicate in a scintillation counter (model LS 8000; Beckman Instruments, Inc., Fullerton, CA).

Binding studies. Determinations of binding of Factor XIa to platelets were carried out as previously described (5). Briefly, 100 μl of gel-filtered platelets (2×10^8–4×10^8/ml) in Ca^{2+}-free, Hepes-buffered Tyrode's solution, pH 7.4, were incubated at 37°C with reactants, including ^{125}I-labeled Factor XIa in a total volume of 115 μl. After specified incubation times, 100 μl aliquots were removed and centrifuged in a microfuge (model B; Beckman Instruments, Inc., Cedar Grove, NJ) through a mixture of silicone oils (5 vol DC 550/1 vol DC 200) in microsediment tubes with narrow bore extended tips (Sarstedt, Inc., Princeton, NJ). The tips containing the sediments were amputated with wire cutters, and the sediments and supernatants were counted separately in a gamma counter (model CG 4000; Intertechnique, Plaisir, France).

Protein analyses. Protein assays were carried out as described by Lowry et al. (28) and were also determined by absorbance at 280 nm employing $E_1^{1\%}{}_{cm}$ (i.e., extinction coefficient for a 1% solution and a 1-cm path length) of 4.36 for α_1-protease inhibitor (29), 13.4 for Factor XI (30), and 7.01 for HMW kininogen (31). Polyacrylamide slab gel electrophoresis in SDS was done by the procedure of Laemmli (32). Gels were stained, dried onto paper, and autoradiograms were prepared from the dried gels using intensifier screens (DuPont Cronex Lightning-Plus screens, mounted in Spectroline Cassettes; Reliance X-Ray Inc., Oreland, PA). Kodak X-Omat AR film was used and developed according to instructions provided with the film. To quantitate percent cleavage of Factor XI, the dried gels were cut into lanes, which in turn were cut into strips. By counting the radioactivity in the individual strips and subtracting background counts, the amount of radioactivity in the 80,000 M_r Factor XI zymogen and the amount in cleavage products (50,000 and 30,000 M_r) representing Factor XI was determined. Percent cleavage was calculated by dividing the amount of radioactivity in cleavage products by the total amount of radioactivity in the lane examined.

Assays of α_1-protease inhibitor. The inhibition of trypsin by α_1-protease inhibitor was determined by an assay described previously (33) with the following modifications. 10 or 20 μl of the sample containing α_1-protease inhibitor was incubated with 20 μg of active-site titrated trypsin in 200 μl of 0.1 M Tris, 5 mM $CaCl_2$, pH 7.6, for 10 min at 22°C. Then 500 μl of 0.1% benzoyl-arginyl-*p*-nitroanilide in H_2O was added with vortexing and the mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of 300 μl of 30% acetic acid. Color development was measured spectrophotometrically at 405 nm. The trypsin inhibitory activity was calculated by comparing the measured activity with the expected activity without inhibitor from a standard curve constructed with differing amounts of trypsin. The α_1-protease inhibitor antigen was determined by radial immunodiffusion (34).

The elastase inhibitory activity of α_1-protease inhibitor was determined as follows: to 1 ml of Tris buffer (0.1 M Tris, 5 mM $CaCl_2$, pH 7.6) in a 1.5-ml cuvette was added 50 μl of porcine pancreatic elastase, 20.9 U/ml (Sigma Chemical Co.), dissolved in the same Tris buffer and

10 or 20 μl of the inhibitor-containing solution. The mixture was allowed to remain at room temperature. After 5 min, 20 μl of *N*-succinyl-trialanyl-*p*-nitroanilide (10 mg/ml) in dimethyl-formamide was added. The cuvette was placed in a spectrophotometer (model 2500, equipped with a recorder; Gilford Instrument Laboratories, Inc., Oberlin, OH) and the optical density at 405 nm was followed for 5–8 min. The elastase activity was calculated from the tangent of the slope of the increasing optical density. The inhibition was calculated from the decreased elastase activity in the presence of inhibitor. A standard curve for elastase was established by measuring the activity of different amounts of elastase. 1 U of elastase was defined as the amount that will solubilize 1 mg of elastin in 20 min at pH 8.8 at 37°C.

Results

Effects of platelets and α_1-protease inhibitor on Factor XIa activity. Initially we determined the effects of platelets or a platelet releasate on Factor XIa activity in the presence or the absence of α_1-protease inhibitor. Thrombin-activated gel-filtered platelets, control buffer, or a platelet releasate was incubated with HMW kininogen and Factor XIa in the presence or absence of α_1-protease inhibitor, and the Factor XIa activity remaining after 15 min was assayed using the Factor IX activation peptide release assay (7). The results (Fig. 1) demonstrate the presence of a Factor XIa inhibitor in the platelet releasate in confirmation of results reported by Soons et al. (9). The extent of Factor XIa inhibition in the presence of platelet releasate (60% in 15 min) was similar to that observed in the presence of α_1-protease inhibitor (59%), but these effects were not additive since only 74% inhibition was observed in the presence of both α_1-protease inhibitor and platelet releasate. When thrombin-activated platelets were present (i.e., platelets and releasate) only 22% inhibition occurred in the absence of α_1-protease inhibitor and 48% in the presence of α_1-protease inhibitor. Therefore, it can be concluded that the presence of platelets partially protects Factor XIa from inactivation in the presence of α_1-protease inhibitor and the secreted platelet inhibitor.

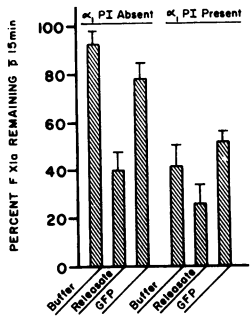

Figure 1. Effect of platelets, platelet releasates, and α_1-protease inhibitor on Factor XIa activity. Gel-filtered platelets (10^8/ml) or Ca^{2+}-free Tyrode's buffer were incubated for 5 min at 37°C with human α-thrombin (0.1 U/ml), and the sample was either left uncentrifuged (designated "GFP") or was centrifuged (10,000 *g* in an Eppendorf Microfuge; Brinkmann Instruments Inc.) for 5 min and the "releasate" was collected. Either buffer, releasate, or activated platelets was then further incubated for 10 min at 37°C with HMW kininogen (1.25 nM). Then either α_1-protease inhibitor (5.8 μM) or Tris (50 mM), NaCl (150 mM), pH 7.4, was added. The samples were then assayed either immediately or after 15 min for Factor XIa activity by adding an aliquot of ^{3}H-labeled Factor IX (350 nM) in the presence of 5 mM $CaCl_2$ and measuring the release of TCA-soluble ^{3}H after 0, 5 or, 10 min of incubation, during which time rates of ^{3}H release were linear. The results were converted to Factor XIa activity by reference to a linear standard curve relating initial rates of ^{3}H release to Factor XIa activity (7). Results represent the means (±SD) of Factor XIa activity remaining after 15 min in six, separate, identical experiments, each performed in duplicate.

Effects of HMW kininogen and platelets on Factor XIa inactivation by α_1-protease inhibitor. The results of the experiment presented in Fig. 1 suggest the possibility that the protection by intact platelets of Factor XIa from inactivation by α_1-protease inhibitor and by the secreted platelet inhibitor may be the consequence of Factor XIa binding to platelets. Since Factor XIa binding to platelets required both platelet activation and the presence of HMW kininogen, we ascertained the effect of HMW kininogen on the inactivation of Factor XIa in the presence and absence of platelets and α_1-protease inhibitor (Table I). Factor XIa was incubated with thrombin in the absence or in the presence of α_1-protease inhibitor, with or without platelets and HMW kininogen, and the amount of Factor XIa remaining after 15 min was determined using the Factor IX peptide release assay. The presence of HMW kininogen had no effect on the amount of Factor XIa inactivated either in the presence or in the absence of platelets or in the presence or absence of α_1-protease inhibitor. Therefore, it appears that the capacity of platelets to protect Factor XIa from inactivation by α_1-protease inhibitor and/or the secreted platelet inhibitor is not a consequence of Factor XIa binding to platelets or of the presence of HMW kininogen.

Effect of platelets on complex formation between Factor XIa and α_1-protease inhibitor. Since activated platelets appear to protect Factor XIa from inactivation in the presence of the secreted platelet inhibitor and α_1-protease inhibitor, we examined the effect of platelets on the interaction between Factor XIa and the inhibitor. First, ^{125}I-labeled Factor XIa was incubated in the presence or absence of α_1-protease inhibitor to determine whether complex formation occurs between the two proteins. As shown in Fig. 2, Factor XIa migrated on an SDS gel under reducing conditions with 63% of the radioactivity in the 30,000-M_r light chain, 25% in the 50,000-M_r heavy chain, and 3% in an 80,000-M_r protein representing residual Factor XI (*A*). In the presence of α_1-protease inhibitor (*B*), the amount of the light chain decreased to 20% and a new radioactive band appeared at a M_r of 85,000 that presumably is a complex between the active-site–containing light chain (M_r of 30,000) and α_1-protease inhibitor (M_r of 54,000). Factor XIa

Table I. Effect of HMW Kininogen and Platelets on Factor XIa Inactivation in the Presence and Absence of α_1-Protease Inhibitor

	Percentage of initial factor XIa activity remaining after 15 min	
Additions	α_1-PI absent	α_1-PI present
Thrombin	96.8 (4.17)	36.0 (4.05)
Thrombin ± HMW Kininogen	97.3 (5.20)	35.3 (5.13)
Thrombin + Platelets	74.3 (6.90)	41.2 (5.38)
Thrombin + HMW Kininogen + Platelets	73.2 (5.74)	42.0 (7.13)

Gel-filtered platelets (10^8/ml) or Ca^{2+}-free, Hepes-buffered Tyrode's solution was incubated for 0 or 15 min at 37°C with human α-thrombin (0.1 U/ml) and Factor XIa (1.25 nM) in the presence or absence of HMW kininogen (125 nM) and α_1-protease inhibitor (4.8 μM). Aliquots were then added to ^{3}H-labeled Factor IX (350 nM) and $CaCl_2$ (5 mM) for assay of TCA-soluble ^{3}H release as described in Methods and in the legend to Fig. 1. The results represent the means (±SD) of Factor XIa activity remaining after 15 min in six identical experiments.

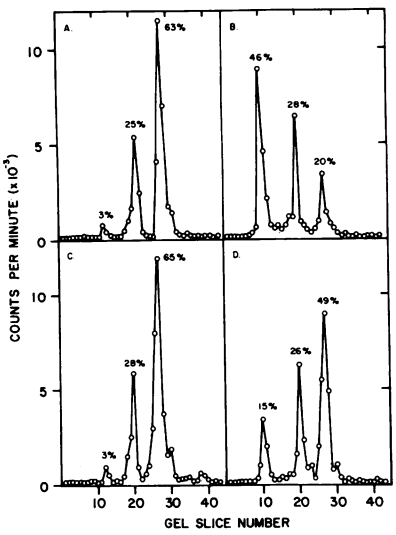

Figure 2. Effect of platelets on complex formation between Factor XIa and α_1-protease inhibitor. Gel-filtered platelets (2.7×10^8/ml) or Ca^{2+}-free, Hepes-buffered Tyrode's solution, pH 7.4, were incubated in polypropylene tubes at 37°C for 30 min with HMW kininogen (5 μg/ml), human α-thrombin (0.25 U/ml), and ^{125}I-labeled Factor XIa (0.8 μg/ml, 2×10^6 cpm/μg) in the presence or absence of α_1-protease inhibitor (320 μg/ml). Reactions were stopped by boiling in SDS sample buffer in the presence of β-mercaptoethanol, and each sample was analyzed by SDS polyacrylamide (10%) gel electrophoresis. The gels were dried, and after autoradiography was carried out, they were cut into 2-mm slices that were counted for ^{125}I. Data are shown for samples containing reactants indicated above, including (*A*) Factor XIa, (*B*) Factor XIa plus α_1-protease inhibitor, (*C*) Factor XIa plus platelets, and (*C*) Factor XIa plus α_1-protease inhibitor plus platelets. The numbers shown above each peak are the percentages of total radioactivity present in each peak.

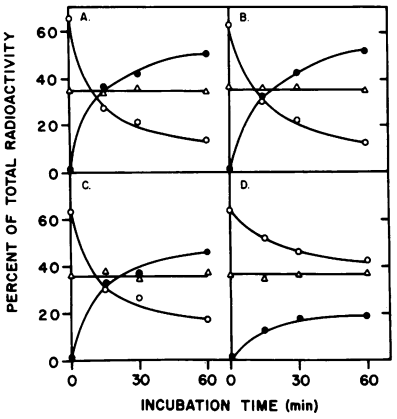

Figure 3. Effects of platelets, HMW kininogen, and thrombin on complex formation between Factor XIa and α_1-protease inhibitor. Gel-filtered platelets (2.05×10^8/ml) or Ca^{2+}-free, Hepes-buffered Tyrode's solution, pH 7.4, were incubated in polypropylene tubes at 37°C for the times indicated with ^{125}I-labeled Factor XIa (0.8 μg/ml, 2×10^6 cpm/μg), HMW kininogen (11 μg/ml) or buffer, and human α-thrombin (0.25 U/ml) or buffer in the presence of α_1-protease inhibitor (320 μg/ml). Reactions were stopped by boiling in SDS sample buffer in the presence of β-mercaptoethanol and samples were analyzed by SDS polyacrylamide (13%) gel electrophoresis. The gels were dried, autoradiography was carried out, and the gels were sliced and counted for ^{125}I. The amount of radioactivity recovered after slicing and counting ranged between 39,800 and 46,200 cpm. Data shown represent the percentage of total counts appearing in the 30,000-M_r light chain (○), in the 50,000-M_r heavy chain (△), and in the 85,000-M_r complex between Factor XIa light chain and α_1-protease inhibitor (●). All incubation mixtures contained ^{125}I-labeled Factor XIa and α_1-protease inhibitor in addition to the following reactants: (*A*) Tyrode's buffer; (*B*) Tyrode's buffer, HMW kininogen, and thrombin; (*C*) platelets; (*D*) platelets, HMW kininogen, and thrombin.

was unaltered in the presence of thrombin-treated platelets and HMW kininogen (*C*). However, when α_1-protease inhibitor, platelets, and Factor XIa were present, the amount of complex was decreased from 46% (*B*) in the absence of platelets to 15% in the presence of platelets (*D*), with a corresponding increase in the amount of free light chain. We conclude from this experiment that thrombin-activated platelets in the presence of HMW kininogen protect Factor XIa from complex formation with α_1-protease inhibitor, which occurred in the presence of thrombin and HMW kininogen without platelets.

To determine the effects of HMW kininogen, thrombin, and platelets on the time course of complex formation, the experiment depicted in Fig. 3 was carried out. In the presence of ^{125}I-labeled Factor XIa and α_1-protease inhibitor, the complex was formed as a function of time with a concomitant decrease in the concentration of free light chain and no effect on the amount of heavy chain in the absence of platelets, HMW kininogen, and thrombin (*A*). The addition of HMW kininogen and thrombin without platelets (*B*) or the presence of platelets alone (*C*) had very little or no effect on the amount of complex formed or the rate of formation. However, in the presence of platelets, HMW kininogen, and thrombin the amount of complex formed was decreased to 19% at 60 min (*D*), compared with 52% in the absence of platelets (*A* and *B*). Additional experiments demonstrated that the effect of platelets on complex formation between Factor XIa and α_1-protease inhibitor requires the presence of thrombin but not HMW kininogen (data not shown). We conclude from these experiments that thrombin-activated platelets can protect Factor XIa from complex formation with α_1-protease inhibitor and that this protective effect of platelets does not require the binding of Factor XIa to platelets since it occurs in the absence of HMW kininogen, which is required for such binding to occur (5).

Effect of platelet releasates on complex formation between Factor XIa and α_1-protease inhibitor. Since the protective effect of platelets in preventing complex formation between Factor XIa and α_1-protease inhibitor required platelet activation with thrombin, but not the binding of Factor XIa to platelets (Fig. 3 and Table I), we postulated that thrombin-activated platelets release substance(s) that inhibit(s) complex formation. To test this hypothesis, the experiments presented in Fig. 4 was carried out. When Factor XIa was incubated with α_1-protease inhibitor, complex formation was apparent between α_1-protease inhibitor and the light chain of Factor XIa (Fig. 4, lane *2*), and the amount of complex formation was unaffected by the presence of the supernatant of unstimulated platelets (Fig. 4, lane *4*), In contrast, the releasate of thrombin-treated platelets, either in the absence or in the presence of HMW kininogen, inhibited complex formation (Fig. 4, lanes *5* and *7*). The SDS gels autoradiographed for Fig. 4 were sliced and counted as described for Figs. 2 and 3. The amounts of radioactivity in each protein band were then expressed as a percentage of total radioactivity in the gel lane as described under Methods. The amount of complex formed in the presence of releasate was 13–14% of total radioactivity as compared with 36–37% in the absence of releasate or in the presence of supernate, and the amount of free light chain was 51–52% in the presence of releasate, compared with 24–29% in its absence and 67% in the absence of α_1-protease inhibitor (Fig. 4). These experiments strongly suggest that material(s) is (are) released from thrombin-treated platelets that inhibit complex forma-

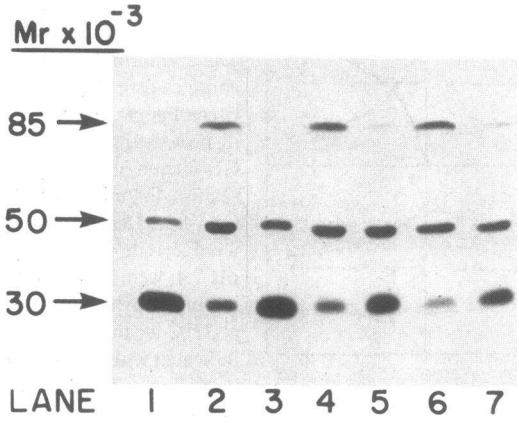

Figure 4. Effect of platelet releasate and HMW kininogen Factor XIa on complex formation between Factor XIa and α_1-protease inhibitor. Gel-filtered platelets (3.9×10^8/ml) were incubated with human α-thrombin (0.25 U/ml) or with TBS for 5 min at 37°C in a polypropylene tube, the sample centrifuged at 10,000 *g* in an Eppendorf Microfuge (Model 3200, Brinkmann Instruments Inc.) for 5 min, and releasate (thrombin-treated sample) or "supernate" (TBS-treated sample) was collected. Then 30 μl of releasate, supernate, or Ca^{2+}-free, Hepes-buffered Tyrode's solution was incubated in a final volume of 50 μl for 15 min at 37°C in a polypropylene tube with HMW kininogen (11 μg/ml) or buffer, ^{125}I-labeled Factor XIa (0.8 μg/ml), and α_1-protease inhibitor (320 μg/ml) or buffer. Reactions were stopped by boiling in SDS sample buffer in the presence of β-mercaptoethanol, and each sample was analyzed by SDS polyacrylamide (13%) gel electrophoresis. The gels were dried and autoradiograms done. Samples shown are as follows: lane *1*, Factor XIa; lane *2*, Factor XIa plus α_1-protease inhibitor; lane *3*, Factor XIa plus platelet supernate; lane *4*, Factor XIa plus α_1-protease inhibitor plus platelet supernate; lane *5*, Factor XIa plus α_1-protease inhibitor plus platelet releasate; lane *6*, Factor XIa plus α_1-protease inhibitor plus HMW kininogen plus platelet supernate; and lane *7*, Factor XIa plus α_1-protease inhibitor plus HMW kininogen plus platelet releasate.

tion between the light chain of Factor XIa and α_1-protease inhibitor.

Effect of platelets on α_1-protease inhibitor. It has been demonstrated by Johnson and Travis (35) and by Beatty et al. (36) that oxidation of a critical methionyl residue at the active center of α_1-protease inhibitor inactivates the inhibitory activity against elastase, whereas more extensive oxidation also abolishes antitrypsin activity. We therefore determined the effect of thrombin-activated platelets on the trypsin-inhibitory activity and the elastase-inhibitory activity of α_1-protease inhibitor (Table II). Mixtures containing α_1-protease inhibitor were incubated in the presence or absence of washed platelets and thrombin for 30 min, and after centrifuging the samples, the supernatants were assayed for α_1-protease inhibitor antigen or for trypsin inhibitory activity and for elastase inhibitory activity. It is apparent from the results that exposure of α_1-protease inhibitor to thrombin-activated platelets had no effect on the trypsin-inhibitory specific activity of the protein. We conclude that the mechanism by which platelets protect Factor XIa from inactivation by α_1-protease inhibitor does not involve inactivation of the inhibitor.

Relationship of Factor XIa binding to platelets and Factor XIa inactivation by α_1-protease inhibitor. To investigate the effect of Factor XIa binding to platelets on the inactivation of the enzyme by α_1-protease inhibitor, the experiment depicted in Fig. 5 was carried out. This experiment examines the rate of inhibition of Factor XIa in the presence (●, ▲, ■) or absence (○, △, □) of α_1-protease inhibitor when the Factor XIa was either free in solution (△, ▲), bound to platelets (○, ●), or incubated in the presence of "treated platelets" (see below), but not bound to them (□, ■). The results plotted represent the means of five separate similar experiments (each done in duplicate) of which one representative experiment was as follows: first washed platelets were incubated for 10 min at 37°C with a mixture of unlabeled and ^{125}I-labeled Factor XIa (3 μg/ml), HMW kininogen (20 μg/ml), and human α-thrombin (0.1 U/ml) in a final volume of 2.5 ml. The platelets with bound Factor XIa were then centrifuged (10,000 *g*, 5 min) through 20% sucrose and resuspended in 2.5 ml of Ca^{2+}-free, Hepes-buffered Tyrode's solution, pH 7.4. The amount of ^{125}I-labeled Factor XIa bound was 8.2 ng/10^8 platelets or 0.14 μg/ml, and it was confirmed that for the duration of the remainder of the experiment > 57% of the Factor XIa remained bound to the platelets. Treated platelets were prepared in exactly the same manner, except that no Factor XIa was added to the incubation mixture before centrifugation through sucrose. After isolation of the platelets with bound Factor XIa and preparation of the treated platelets, an amount (i.e., number of counts per minute) of ^{125}I-labeled Factor XIa corresponding to the

Table II. Effect of Platelets on the Inhibition of Trypsin and Elastase by α_1-Protease Inhibitor

Incubation mixture*	α_1-Protease inhibitor antigen	Trypsin inhibited	Trypsin inhibitory specific activity	Elastase inhibited	Elastase inhibitory specific activity
	μM	μM		*U/ml*	
α_1-Protease inhibitor + platelets + thrombin	3.69	2.94	0.80	28.25	7.66
α_1-Protease inhibitor + platelets	4.0	3.21	0.80	32.25	8.09
α_1-Protease inhibitor + thrombin	3.96	3.28	0.89	31.6	7.89
α_1-Protease inhibitor	4.0	3.15	0.79	26.5	6.63

* In a total incubation volume of 1 ml, gel-filtered platelets (2×10^8/ml), or Ca^{2+}-free, Hepes-buffered Tyrode's solution were incubated at 37°C (30 min with α_1-protease inhibitor, 4.0 μM) in the presence or absence of human α-thrombin (0.1 U/ml). The samples were then centrifuged (10,000 *g* for 5 min), and the supernatants were assayed for α_1-protease inhibitor antigen, for elastase inhibitory activity, and for trypsin inhibitory activity, as described in Methods. The results are means of duplicate assays of incubation mixtures that were also carried out in duplicate.

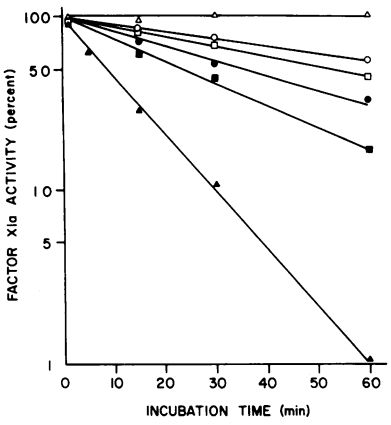

Figure 5. Inactivation of Factor XIa by α_1-protease inhibitor in the presence and absence of platelets. Experimental details are presented in the text. The results represent the means of five similar experiments, each carried out in duplicate, of residual Factor XIa activity measured after incubation of 0.14 μg/ml (0.87 nM) Factor XIa in the presence or absence of 0.5 mg/ml (9.3 μM) α_1-protease inhibitor. Data are shown for Factor XIa alone (△); platelet-bound Factor XIa (○); unbound Factor XIa plus treated platelets (□); platelet-bound Factor XIa plus α_1-protease inhibitor (●); unbound Factor XIa plus treated platelets plus α_1-protease inhibitor (■); and Factor XIa plus α_1-protease inhibitor (▲).

amount of platelet-bound Factor XIa was added to the treated platelets (i.e., 0.14 μg/ml), and it was determined by centrifugation through silicone oil that < 1.5% of the Factor XIa was bound to the treated platelets. Incubations in duplicate at 37°C of 50 μl of α_1-protease inhibitor (1 mg/ml) of buffer with 50 μl of each of these samples or of a sample of ^{125}I-labeled Factor XIa at an equivalent concentration (0.14 μg/ml) were carried out for various time intervals as indicated in Fig. 5. After this incubation, 50 μl of Hepes-buffered, Ca^{2+}-free Tyrode's solution was added to the platelet-containing samples and 50 μl of treated platelets was added to the samples not containing platelets. This addition was immediately followed by additions of 50 μl of Factor XIa–deficient plasma, 25 μl of inosithin, and 25 μl of 100 mM $CaCl_2$ for determination of clotting times as measures of residual Factor XIa activity. These clotting times were converted to Factor XIa concentration by reference to a linear standard curve. The results are expressed as percentages of the initial Factor XIa concentration and represent the means of five similar experiments. This experiment demonstrates that Factor XIa was stable for 1 h in the absence of platelets (△) and that 99% of the Factor XIa activity was inactivated by α_1-protease inhibitor in 60 min (▲). Factor XIa was slowly inactivated in the absence of α_1-protease inhibitor either when bound to platelets (○, 42% in 60 min) or when incubated with treated platelets (□, 54% in 60 min). In the presence of α_1-protease inhibitor, the inactivation rates were 69% in 60 min when Factor XIa was bound to platelets (●) and 83% in 60 min when Factor XIa was incubated with treated platelets (■). It can therefore be concluded that: (*a*) platelets slowly inactivate Factor XIa (○ and □ vs. △); (*b*) platelets protect Factor XIa from inactivation by α_1-protease inhibitor (● and ■ vs. ▲); and (*c*) although inactivation rates appear to be slightly slower when Factor XIa is bound to platelets (●) than when it is incubated with treated platelets (■), the major protective effect arises from the presence of platelets rather than from the binding of Factor XIa to platelets. The presence of platelets appears to decrease four to sixfold the rate of Factor XIa inactivation in the presence of α_1-protease inhibitor.

Discussion

Previously we have postulated that platelets can provide a catalytic and protective surface for coagulation proteins at various stages of intrinsic coagulation, including the "contact phase" reactions that can initiate the coagulation cascade (1, 37, 38). Some of the recent evidence supporting this hypothesis includes the demonstration that activated platelets can bind both Factor XI (2) and HMW kininogen (3, 4), and can promote the proteolytic activation of both Factor XII (1) and XI (1). Activated platelets in the presence of HMW kininogen can also bind Factor XIa in a specific, saturable, and reversible manner to a site distinct from that for Factor XI (5). In attempting to characterize the platelet-bound Factor XIa, we have found that the structure of the bound enzyme is unaltered as compared with the free enzyme (5). Since binding of Factor Xa to platelets in the presence of Factor Va accelerates prothrombin activation ~ 300,000-fold (12, 39), we hypothesized that Factor XIa binding to platelets might accelerate Factor IX activation. However, we have recently demonstrated that the initial rates of Factor IX activation, determined by a highly specific assay that reflects that release of the activation peptide from Factor IX and that correlates well with the generation of Factor IXa activity (7), are no different when Factor XIa is bound to platelets or when it is free in solution (6). We have concluded that the functional activity of Factor XIa as a Factor IX activator is fully retained (i.e., is neither enhanced nor inhibited) when the enzyme is bound to the platelet surface. These results raise a number of interesting questions. Since Factor IX activation by Factor XIa is the only enzyme-substrate reaction in the coagulation cascade for which no specific cofactor has been identified, it is reasonable to ask whether an unidentified cofactor might accelerate the platelet surface-mediated reaction. However, we know of no evidence to support this possibility. Another question arises: what is the function of Factor XIa binding to platelets? Since the kinetics of Factor XIa–catalyzed Factor IX activation suggest that this is a favorable reaction, and since the rate of Factor IX activation is the same when Factor XIa is free in solution and when it is bound to the platelet surface, we suggest that the platelet surface-mediated reaction may also be kinetically favorable. Therefore, the function of Factor XIa binding to platelets may very well be to localize Factor IX activation to platelet membranes, where both Factors IX and IXa are also specifically bound (40) and where the rates of Factor X activation (10) and prothrombin activation (12, 39) are greatly accelerated.

The present studies were initiated to elucidate the complex interrelationships between Factor XIa activity, Factor IX activation, platelets, and plasma proteinase inhibitors. Previous studies from our laboratory examined the rate of inactivation of Factor XIa activity by plasma proteinase inhibitors in the presence and absence of platelets (10). In these previously reported experiments, washed platelets were incubated with a celite eluate of plasma, i.e., "contact product" as a source of Factor XIa activity and heat-inactivated plasma, containing protease inhibitors, but no detectable coagulation factor activities. The rate of inactivation of Factor XIa activity was significantly decreased in the presence of platelets, and it was suggested that platelets can protect Factor XIa from inactivation by plasma proteinase inhibitors (10). A variety of inhibitors

has been shown to inactivate Factor XIa, including antithrombin III (41), C1 inhibitor (42), α_1-protease inhibitor (α_1-antitrypsin) (13, 14), and α_2-antiplasmin (43). A kinetic analysis of the inactivation of Factor XIa by these plasma protease inhibitors provided evidence that α_1-protease inhibitor accounts for 68% of the total plasma inhibitory activity against Factor XIa (14). Therefore, we focused our studies on the effects of platelets on the inactivation of Factor XIa by α_1-protease inhibitor. Initially we found, however, that platelets secrete an inhibitor of Factor XIa, in confirmation of the recent report of Soons et al. (9). Also, our findings demonstrate that the presence of intact platelets partially protects Factor XIa from inactivation by the secreted platelet inhibitor (Figs. 1 and 5). Our results also demonstrate that the presence of platelets partially protects Factor XIa from inactivation in the presence of both α_1-protease inhibitor and the Factor XIa inhibitor secreted from platelets (Figs. 1 and 5). However, as indicated by the experiments depicted in Table I and Fig. 5, this protective effect is independent of Factor XIa binding to platelets.

It has been reported that purified HMW kininogen (25–250 nM) protects Factor XIa from inactivation by α_1-protease inhibitor (14). However, in our experiments the presence of HMW kininogen (125 nM), either in the presence or absence of platelets, had no effect either on the inactivation of Factor XIa by α_1-protease inhibitor (Table I) or on complex formation between Factor XIa and α_1-protease inhibitor (Figs. 2 and 4). Although it has also been reported by Shapira et al. (43, 44) that HMW kininogen can protect kallikrein from fluid-phase inhibition by C1 inhibitor, results from two other independent laboratories (45, 46) indicate that HMW kininogen has no effect on the rate of kallikrein inactivation by C1 inhibitor. Since HMW kininogen forms noncovalent complexes with both Factor XI and prekallikrein in plasma and is required for optimal rates of surface-mediated activation of both proteins (47–49), it is reasonable to determine whether such complex formation results in protection from inactivation by inhibitors. However, the weight of evidence suggests that it does not.

Since even mild oxidizing conditions have been shown to inactivate the inhibitory activity of α_1-protease inhibitor against a variety of serine proteases, including elastase (35, 36), we determined the effects of thrombin-treated platelets on α_1-protease inhibitor functional activity. Both trypsin inhibitory activity and elastase inhibitory activity were determined since it has been demonstrated that oxidation of a critical methionine at the active center of the inhibitor can have differential effects on the capacity of the inhibitor to inactivate these two enzymes (35, 36). However, the results presented in Table II indicate that thrombin-treated platelets had no effect on the specific activities of α_1-protease inhibitor. Therefore, it is unlikely that the capacity of platelets to provide partial protection of Factor XIa from inactivation by α_1-protease inhibitor is a consequence of inactivation of the inhibitor by platelets.

We therefore examined the effects of platelets on the interaction of Factor XIa and α_1-protease inhibitor. Our demonstration that α_1-protease inhibitor (M_r of 54,000) forms a complex (M_r of 85,000) with the active-site–containing light chain (M_r of 30,000) of Factor XIa is the first of which we are aware. The fact that thrombin-activated platelets (Figs. 2 and 3) or releasates derived from them (Fig. 4) prevent such complex formation may provide an explanation for the protection of Factor XIa from inactivation by α_1-protease inhibitor observed in Figs. 1 and 5. The nature of the substance(s) that prevent(s) such complex formation and inactivation of Factor XIa is not known and will be an important question for further study. The possibility that the secretory product that inhibits complex formation between Factor XIa and α_1-protease inhibitor is identical to the platelet Factor XIa inhibitor has not been excluded. Thus, Soons et al. (9) have shown that the secreted platelet material acts reversibly to inhibit Factor XIa, which is consistent with our finding that the electrophoretic mobility of Factor XIa is unaltered in the presence of platelets or releasate. Another question left unanswered by our present studies concerns the mechanisms by which the presence of intact platelets partially protects Factor XIa from inactivation by both the platelet Factor XIa inhibitor and α_1-protease inhibitor. This is even more puzzling since binding of Factor XIa to platelets is apparently not necessary for this protective effect. A possible explanation is that Factor XIa can form a transient, reversible complex with the platelet inhibitor (or with another as yet undescribed component released from platelets) that prevents complex formation with α_1-protease inhibitor. Due to the noncovalent interaction of Factor XIa with the released platelet constituent, subsequent electrophoresis apparently fails to demonstrate complex formation. The retention of Factor XIa activity as a Factor IX activator and its protection from inactivation by α_1-protease inhibitor requires the presence of intact, activated platelets (Figs. 1 and 5). In the experiment presented in Fig. 5 the effects of the released platelet inhibitor are minimized by centrifugation of platelets through sucrose. Under these conditions, the inhibitory effects of α_1-protease inhibitor are partially abrogated, resulting in protection of Factor XIa activity. Therefore, it appears that the platelet constituent resulting in inactivation of Factor XIa is released whereas the component resulting in protection is associated with intact platelets. Further elucidation of these complex interactions will undoubtedly require the purification and characterization of the released platelet inhibitor and detailed studies of its interaction with Factor XIa, with α_1-protease inhibitor, and with intact platelets.

The studies reported here support the hypothesis that platelets provide a protective microenvironment for the assembly of coagulation enzyme-substrate complexes and for the proteolytic activation of coagulation proteins at all stages of intrinsic coagulation (50). Thus, it appears that activated platelets can promote the proteolytic activation of Factors XII and XI (1, 37, 38), localize the activation of Factor XI (1, 2) and the activity of Factor XIa (5, 6) to the platelet membrane, protect both Factors XIa (10) and Xa (10–12) from inactivation by plasma proteinase inhibitors, and greatly accelerate the rates of activation of both Factor X (10) and prothrombin (12, 39). These interactions can be viewed as localizing and regulating the enzymatic reactions of intrinsic coagulation to the platelet plug.

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