Interaction of Type 1 Plasminogen Activator Inhibitor with the Enzymes of the Contact Activation System*

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The interaction between type 1 plasminogen activator inhibitor (PAI-1), a serine protease inhibitor, and the three serine proteases generated during contact activation of plasma was studied using functional and immunologic approaches. Incubation of Factor XIIa, Factor XIa, and plasma kallikrein with either purified PAI-1 or platelet-derived PAI-1 resulted in the formation of stable, sodium dodecyl sulfate-stable complexes as revealed by immunoblotting techniques. Functional assays indicated that Factor XIa and, to a lesser extent, Factor XIIa and plasma kallikrein neutralized the ability of purified PAI-1 to bind to immobilized tissue-type plasminogen activator (t-PA). Immunoblotting demonstrated that these enzymes also neutralized the ability of PAI-1 to form complexes with fluid-phase t-PA. Clot lysis assays employing purified proteins and 125I-fibrinogen were used to investigate the profibrinolytic effect of these contact activation enzymes. At enzyme concentrations that did not result in direct activation of plasminogen, only Factor XIa was capable of stimulating the lysis of clots supplemented with both t-PA and PAI-1. As a consequence of their interactions with PAI-1, the amidolytic activity of Factor XIIa, Factor XIa, and plasma kallikrein was neutralized by this inhibitor in a time-dependent and concentration-dependent manner. Minimum values estimated for the apparent second-order rate constant of inhibition were 1.6 x 10^7, 2.1 x 10^6, and 6.0 x 10^4 M^-1 s^-1 for Factor XIIa, Factor XIa, and plasma kallikrein, respectively. These data define new reactions between coagulation and fibrinolysis proteins and suggest that a major mechanism for stimulation of the intrinsic fibrinolytic pathway may involve neutralization of PAI-1 by Factor XIa.

Abnormal thrombus formation plays a critical role in a number of cardiovascular diseases including atherosclerosis and both hemorrhagic and thrombotic disorders. The fibrinolytic system is pivotal in maintaining the hemostatic balance. Two immunologically distinct classes of plasminogen activator (PA) exist, the tissue-type (t-PA) and the urokinase-like (u-PA). These PAs can initiate fibrinolysis by converting the inactive plasma proenzyme plasminogen into the active proteolytic enzyme plasmin (1-4). A number of other blood enzymes may also play a role in the generation of plasmin. For example, a marked stimulation of the fibrinolytic system is observed when plasma is exposed in vitro to negatively charged surfaces. This phenomenon, known as intrinsic fibrinolysis, is dependent on the activation of the plasma enzymes that participate in the contact phase of blood coagulation (i.e. Factor XII, Factor XI, and plasma prekallikrein) (5). In the presence of an appropriate activating surface and the nonenzymatic cofactor, high molecular weight kinogen, these three plasma zymogens are converted by limited proteolysis to the corresponding serine proteases Factor XIIa (F.XIIa), Factor XIa (F.XIa), and plasma kallikrein (6). The mechanisms responsible for the increased plasmin generation in contact-activated plasma have been only partially elucidated. Thus, F.XIIa (7), F.XIa (8), and plasma kallikrein (9) are able to activate plasminogen, but their activity as PAs are at least 20,000 times lower than that of urokinase (5, 10). Moreover, contact-dependent fibrinolysis requires the presence of molecules immunologically related to urokinase (11, 12), and a new pathway of intrinsic fibrinolysis has recently been proposed based on the observation that plasma kallikrein converts single-chain u-PA into the more active two-chain molecule (13) with an activation rate comparable to that of plasmin. The existence of an additional, distinct pathway of intrinsic fibrinolysis, involving an unidentified Factor XII-dependent proactivator, has also been proposed (5, 14).

It is becoming increasingly apparent that control of the fibrinolytic reactions is achieved in part through the modulatory activity of specific PA inhibitors (PAl). Although four distinct PAl have been described (15, 16), the inhibitor produced by endothelial cells, platelets, and other cells, and designated as type 1 PAI (PAI-1) (16), appears to be a physiologic inhibitor of t-PA. Since PAI-1 is a member of the serine protease inhibitor (serpin) superfamily (17, 18), we have investigated the possible interaction between this inhibitor and the three serine proteases generated during contact activation of plasma. In this report, we present studies demonstrating complex formation and reciprocal inhibition between PAI-1 and each of the enzymes of the contact system (i.e. F.XIIa, F.XIa, and plasma kallikrein). In addition, we

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1 The abbreviations used are: PA, plasminogen activator; t-PA, tissue-type plasminogen activator; u-PA, urokinase-like plasminogen activator; PAI, type 1 plasminogen activator inhibitor; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
suggest that neutralization of PAI-1 activity by F.XIa may represent a relevant mechanism for stimulation of clot lysis during contact activation of plasma.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bovine serum albumin (BSA) and 5,5'-dithiobis-2-nitrobenzamide were obtained from Sigma, Z-Lys-S-Bzl ester from Peninsula Laboratories, oligopeptide substrates (S-2302, H-D-Pro-Phe-Arg-p-nitroanilide; S-2366, proly-Glu-Pro-Phe-Arg-p-nitroanilide) and human fibrinogen (grade L) from Helena Laboratories. All other reagents were of the best grade available and were purchased from Sigma or from Behring Diagnostics.

Protein purification procedures were used to prepare F.XIa (19) and plasma kallikrein (20). The specific activity of the two enzymes employed here was 72.5 and 40.3 mol min⁻¹ mg⁻¹ against S-2366 and S-2302, respectively, when the substrates were used at concentrations of 0.4 mM/liter in 0.09 mol/liter Tris-HCl, 0.09 mol/liter NaCl, 1 mg/ml BSA, pH 8.3, at room temperature. F.XIa was prepared by incubation of purified human Factor XII with 5 mg/ml dextran sulfate at 37°C for 90 min, as previously described (21). The preparation appeared as α-F.XIa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reduced and unreduced samples and had a specific activity of 16.6 mol/min/mg protein (22). The final product by SDS-PAGE and staining with Coomassie Brilliant Blue or periodic acid-Schiff revealed a single band of M, 55,000 was either purchased from Sterling-Winthrop (WinKinsae) or supplied by the National Institute for Biological Standards and Controls (WHO reference 66/46). The molar concentrations of urokinase were determined by titration with p-nitrophenyl-p'-guanidinobenzoate hydrochloride (23). Glu-plasminogen was purified by chromatography on lysine-Sepharose followed by DEAE-Sephadex as described (25).

Human PAI-1 was purified from the media conditioned by a human transformed pulmonary fibroblast cell line (SV40 WI-38 VA13 2RA) according to procedures published previously (23). Analysis of the final product by SDS-PAGE and staining with Coomassie Brilliant Blue or periodic acid-sulfuric acid revealed a single band of M, 50,000. The purification procedures employed (23) result in the isolation of the native, but latent form of PAI-1, which requires activation with denaturants for its inhibitory activity (24). For activation, aliquots (0.2 ml, 10 μg/ml) of purified PAI-1 were treated (1 h, 37°C) with an equal volume of 8 mol/liter guanidine-hydrochloride, and the denaturant was then removed by extensive dialysis against phosphate-buffered saline (0.011 mol/liter, 0.01 mol/liter, 0.02% BSA, pH 7.3) containing 0.01% Tween 80 (23, 24).

**Platelet Releasates**—Platelet releasates were also used as a source of active PAI-1 (25). To prepare platelet releasates, gel-filtered platelets were prepared from normal donors as described (26). The platelet release reaction was induced by incubating platelet suspensions (1×10⁹ platelets/ml) with calcium ionophore A23187 (4 μmol/liter, final concentration) for 10 min at 37°C, followed by centrifugation at 12,000 g for 5 min at room temperature.

**PAI-1 Activity Assays**—PAI-1 activity was routinely quantitated using the t-PA binding assay (27) to determine the amount of PAI-1 available to bind t-PA. Briefly, samples and preparations of purified PAI-1 for standard curves were prepared in IRMA buffer (PBS supplemented with 3% BSA, 5 mM EDTA, 0.1% Tween 80 and 0.02% NaN₃) and incubated (1 h, 37°C) in microtiter wells precoated with purified t-PA (50 μg/ml, pH 8, overnight at 4°C) and blocked as described above. PAI-1 bound to t-PA-coated wells was detected radiometrically after incubation (1.5 h, 37°C) initially with rabbit antiserum to PAI-1 (1:75 dilution, 50 μl/well) and then with 125I-labeled goat anti-rabbit IgG (2.5×10⁶ cpm/well, 1.5 h at 37°C). Purified proteins for the PAI-1 assay were established using the 125I-fibrin plate assay to monitor inhibitor activity as described (28). One unit of PAI-1 activity is defined as the amount required to inhibit the activity of 1 μg of the urokinase by 50%. Purified preparations of guanidine-activated PAI-1 used in these studies for specific activity of 1.2–1.5×10⁶ units/mg.

For kinetic analysis, the concentration of active PAI-1 preparation was determined using a modification of the Z-Lys-S-Bzl assay method of Coleman and Green (29) to directly measure the amount of urokinase inhibitory activity (20). Briefly, two concentrations of human PAI-1 were incubated in microtiter plates with urokinase (final concentration 2.4 pm) as determined by titration with p-nitrophenyl-p'-guanidinobenzoate) for 45 min at 37°C to allow the PAI-1 interaction to go to completion. Glu-plasminogen (5 μl; final concentration between 0.5 and 22 μM) was added, and the samples incubated for 60 min. Finally, 200 μl of Z-Lys-S-Bzl ester plasmin substrate (200 μM Z-Lys-S-Bzl ester, 220 μM 5,5'-dithiobis-2-nitrobenzoic acid in 200 mM phosphate, pH 7.5, 200 mM NaCl, 0.01% Triton X-100) was added and the absorbances were determined every 1 min using a Titerlyte Multiscan with a 414-nm filter. The amount of PAI-1 inhibiting 2.4 pm of urokinase by 50% is the plot of percentage inhibition versus volume of PAI-1 was thus equivalent to 1.2 pm.

**Immunoblotting**—Immunoblotting studies were performed as described previously (30). In brief, 50-μl reaction mixtures containing enzymes and purified or platelet-derived PAI-1 in Tris-buffered saline (0.05 mol/liter Tris-HCl, 0.14 mol/liter NaCl, pH 7.4) containing 1 mg/ml BSA (TBS-BSA) were incubated at 37°C for the indicated periods of time. The reaction was stopped by the addition of an equal volume of sample buffer containing 2.5% SDS, and the mixture was applied to a slab gel (4% polyacrylamide for the stacking gel, 5% polyacrylamide for the resolving gel) prepared according to procedures described by Laemmli (31). After electrophoresis at 5 mA for 14–18 h, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) at 50 V for 2 h. The membrane was exposed to a blocking solution (30) for 1 h at room temperature and then to a 1:100 dilution of rabbit anti-human F.XIa antiserum. The detection of bound rabbit immunoglobulins was then accomplished by incubating the membrane for 1 h with 125I-labeled goat anti-rabbit immunoglobulins (2×10⁶ cpm/ml). After washing, the membrane was exposed to x-ray film at −70°C for 18–24 h. 125I-Labeled purified human Factor XI, plasma prekallikrein, and thrombin were included in each gel as molecular weight standards.

**Clot Lysis Assay**—The measurement of plasmin-induced clot lysis in mixtures containing purified proteins and 125I-fibrinogen was performed as described (32). In brief, mixtures (1 ml final volume) containing human fibrinogen (2 mg), 125I-Fibrinogen (0.5 μg; 2.5×10⁶ cpm) and plasminogen (100 μg) in TBS-BSA were clotted in glass tubes with thrombin (0.5 units) and CaCl₂ (2.5 mM/liter) final concentration) in the presence or absence of PAI-1 (10 ng), t-PA (0.05 ng), and various concentrations of contact enzymes. The mixtures were allowed to clot at 37°C for 10 min, and the clot was freed from the wall of the tube by rimming the clots with a plastic tip. To measure fibrinolysis, 20-μl aliquots were removed from around the clot at intervals and the soluble radioactivity was determined. Results were expressed as the percent release of total radioactivity, applying a correction for the influence of repeated subsampling upon the volume of the supernatant.

**Clot Lysis Assay**—The influence of PAI-1 on the enzymatic activity of F.XIIa, F.XIa, and plasma kallikrein was evaluated by amidolytic assays. Buffer (TBS-BSA) or PAI-1 dilutions in the same buffer (15–60 nmol/liter final concentrations) were incubated at 37°C with each of the enzymes (5 nmol/liter F.XIIa; 4 nmol/liter F.XIa; and 5 nmol/liter plasma kallikrein, final concentrations). At intervals, aliquots were withdrawn and diluted 20-fold in 1 mmol/liter solutions of the appropriate substrate (S-2302 for F.XIIa and plasma kallikrein; S-2366 for F.XIa) in 0.05 mol/liter Tris-HCl, 0.1 mol/liter NaCl, 4 mmol/liter CaCl₂, 1 mg/ml BSA, pH 8.2. Residual enzymatic activity was determined by measuring the change in absorbance at 405 nm and was compared with the activity observed in control solutions without added PAI-1. In some experiments, commercial porcine heparin (5 IU/ml, final concentration) was included in the reaction mixtures.

An approximate estimate of the inhibition rates was obtained from the initial slope of the plots of percent amidolytic activity versus time. The data were fitted to a pseudo-first-order equation: k = 1/τ ln (E₀/E₁), where Kₜ is the pseudo-first-order rate constant, (E₀) is the initial concentration of enzyme and (E₁) is the concentration of the enzyme at time t. The second-rate order constant, k₂, was obtained by dividing k by the concentration inhibited.

**General Methods**—Protein concentrations were measured by the method of Lowry et al. (33) using BSA as a standard. 125I-Labeled proteins were obtained using the chloramine-T method (34). Antiserum to the purified PAI-1 was raised in New Zealand White rabbits by standard procedures according to procedures described previously (35).
RESULTS

Complex Formation between PAI-1 and the Enzymes of the Contact Activation System—Experiments were performed to test whether the enzymes of the contact activation system physically interacted with PAI-1 to form enzyme-inhibitor complexes. Purified guanidine-activated PAI-1 was incubated (20 min, 37 °C) in the absence or presence of either F.XIIa, F.XIa, plasma kallikrein, or t-PA, and the mixtures were analyzed by immunoblotting using polyclonal antibodies against PAI-1 (Fig. 1). PAI-1 incubated in buffer alone was detected by immunoblotting at M₀, 80,000, with a minor proportion of immunoreactive material at M₀, 96,000 (Fig. 1, lane 1). This second faint band was not detected when PAI-1 was immediately processed without incubation at 37 °C and was due to a time-dependent dimerization of purified PAI-1 (32). Fig. 1 also indicates that incubation of PAI-1 with either t-PA or various contact activation enzymes resulted in the formation of high molecular weight enzyme-inhibitor complexes. For example, a band of M₀, 92,000 was detected in mixtures of t-PA and PAI-1 (Fig. 1, lane 3). Treatment of PAI-1 with F.XIa (Fig. 1, lane 4) resulted in the appearance of two major bands with M₀, 220,000 and 200,000 which likely represent complexes of dimeric F.XIa with one or two molecules of inhibitor. Minor bands at M₀, 150,000 and 135,000 were probably due to complexes of small amounts of monomeric F.XIa present in our preparation since these complexes could also be detected using antibodies to Factor XI (data not shown). Treatment of PAI-1 with plasma kallikrein and F.XIIa resulted in the formation of complexes of apparent M₀, 130,000 and 120,000, respectively (lanes 5 and 6). In each case, a new immunoreactive band with M₀, 46,000 was also detected, probably representing a degradation product of PAI-1. As seen here and below in Figs. 2 and 4, PAI-1 in complex with a protease is detected with much greater sensitivity by the anti-PAI-1 antibodies employed.

Since human platelets contain a number of active protease inhibitors including PAI-1 (25), platelet releasates represent a physiologically relevant source of active PAI-1 that can be utilized without the potential problems associated with denaturant-activation of purified PAI-1. Therefore, a series of experiments employing platelet releasates were performed to confirm our observations and establish the effects of these enzymes on PAI-1 that may be released from platelets during coagulation. Platelet-derived PAI-1 (Fig. 2, lane 1) was detected by immunoblotting at M₀, ~48,000. This lower apparent molecular weight for platelet-derived PAI-1 was previously shown to be caused by the presence of high concentrations of other blood proteins that distort the electrophoretic mobility of PAI-1 (36). Platelet releasates also contained a faint PAI-1 immunoreactive band of high M₀ (>200,000) which may represent PAI-1 associated with another molecule. PAI-1 was not detected in supernatants of nonactivated platelets (Fig. 2, lane 2). Immunoblotting analysis of platelet releasates incubated for 20 min at 37 °C with t-PA, F.XIa, F.XIIa, or plasma kallikrein (Fig. 2, lanes 4–7, respectively) showed similar incorporation of PAI-1 into high molecular weight enzyme-inhibitor complexes.

Inhibition of PAI-1 Activity by the Enzymes of the Contact Activation System—The effect of the enzymes of the contact activation system on PAI-1 activity was tested using two different approaches. In a first series of experiments (Fig. 3), PAI-1 was incubated with or without the three enzymes of the contact system at a 1:2 molar ratio of inhibitor/enzyme, and residual PAI-1 activity was monitored at intervals by measuring its ability to bind to immobilized t-PA. As previously observed (37, 38), an spontaneous decrease of PAI-1 activity occurred progressively during incubation of PAI-1 at 37 °C. In the presence of F.XIa more than 80% of PAI-1 activity was lost in 10 min (Fig. 3), and F.XIIa and plasma kallikrein also accelerated the inactivation of PAI-1.

To account for the possibility that the contact activation enzymes may simply alter the ability of PAI-1 to bind to solid-phase t-PA, the effect of these enzymes on the formation of t-PA - PAI-1 complexes in a fluid-phase was also examined (Fig. 4). Platelet-derived PAI-1 was used as a source of active PAI-1. Platelet releasates were incubated first with contact system enzymes for 20 min and then with t-PA for an additional 20 min. Immunoblotting analysis of these incubation mixtures demonstrated that preincubation of F.XIa (160 ng) with platelet releasates prior to t-PA treatment resulted primarily in the formation of high molecular weight F.XIa .
PAI-1 Inhibition of Contact Activation Enzymes

1741

Fig. 3. Inhibition of PAI-1 activity by the enzymes of the contact activation system. Purified guanidine-activated PAI-1 (2 nmol/liter) was incubated at 37°C with TBS-BSA (●—●) or with 4 nM F.XIIa (△—△), F.XIa (○—○), and plasma kallikrein (□—□). At the time indicated, the residual PAI-1 activity was measured by the t-PA binding assay and expressed as percent of the activity at time 0 (=100%). Each point represents the mean of duplicate observations.

Fig. 4. Prevention of complex formation between platelet PAI-1 and t-PA by the enzymes of the contact activation system. Platelet releasates (25 μl from 4.5 x 10^7 platelets) were mixed in a final volume of 50 μl with TBS-BSA (lanes 1 and 2); F.XIa, 160 ng (lane 3) or 800 ng (lane 4); plasma kallikrein, 200 ng (lane 5) or 1 μg (lane 6); F.XIIa, 250 ng (lane 8) or 1 μg (lane 9). After incubation at 37°C for 20 min, 15 μl of t-PA (150 ng) were added to samples analyzed in lanes 2–6 and 8–9, and the mixtures were further incubated for 20 min at 37°C. The samples were then fractionated using 6% SDS-PAGE and immunoblotting with anti-PAI-1 polyclonal antibodies as described under "Experimental Procedures." Lane 7 contained radiolabeled F.XIa, plasma kallikrein, and thrombin as molecular weight reference.

1 complexes and only a small amount of t-PA-PAI-1 complexes (Fig. 4, lane 3). Furthermore, preincubation of platelet releasates with higher concentrations of F.XIa (e.g. 800 ng, Fig. 4, lane 4) completely prevented the subsequent formation of complexes between PAI-1 and t-PA. In contrast, preincubation of platelet lysates with similar concentrations of either plasma kallikrein (Fig. 4, lanes 5 and 6) or F.XIIa (Fig. 4, lanes 8 and 9) only partially prevented the formation of t-PA-PAI-1 complexes. In a parallel series of experiments, when F.XIa and t-PA were mixed simultaneously with PAI-1, both t-PA-PAI-1 complexes and F.XIIa-PAI-1 complexes were detected by immunoblotting (data not shown).

t only t-PA-PAI-1 complexes were detected using immunoblotting when t-PA, PAI-1, and either F.XIIa or plasma kallikrein were mixed simultaneously.

Clot Lysis Assays—The results above indicate that the three contact system enzymes interact with PAI-1 and prevent this inhibitor from forming complexes with t-PA. These studies were extended by utilizing a clot lysis assay system to assess whether inactivation of PAI-1 may be involved in the profibrinolytic effect of these enzymes. The lytic activity of 0.05 ng of t-PA was completely neutralized when 10 ng of PAI-1 was included in clot lysis assay reaction mixtures (32) (Fig. 5). To study the effects of contact activation enzymes, increasing amounts of F.XIIa, F.XIa, and plasma kallikrein were added simultaneously with t-PA to 125I-fibrinogen-plasminogen mixtures containing PAI-1, and following clotting with thrombin, the clot lysis was compared with parallel incubation mixtures containing neither t-PA nor PAI-1. The profibrinolytic activity of F.XIa in the presence of PAI-1 was seen

Fig. 5. Effect of the enzymes of the contact system on clot lysis in the presence or in the absence of t-PA and PAI-1. One ml (final volume) mixtures containing fibrinogen (2 mg), 125I-fibrinogen (0.5 μg; 2 x 10^4 Ci), and plasminogen (100 μg) were clotted by thrombin (0.5 units) and CaCl₂ (2.5 mol/l) at 37°C after the addition of t-PA alone (0.05 ng, C—○) or t-PA (0.05 ng) plus PAI-1 (10 ng, ●—●). Parallel mixtures containing t-PA (0.05 ng) and PAI-1 (10 ng) were supplemented with panel A, F.XIa, 0.25 μg (●—●), 0.5 μg (△—△), or 1.5 μg (●—●); panel B, plasma kallikrein, 1 μg (●—●) or 5 μg (△—△); panel C, F.XIIa, 1 μg (●—●) or 3 μg (△—△). In each panel, the dashed lines indicate control experiments in which the same amount of contact enzyme was added to mixtures containing neither t-PA nor PAI-1. At intervals, the soluble radioactivity was counted and the clot lysis was measured as percent of the total radioactivity added to the tube.

Fig. 6. Effect of PAI-1 on the amidolytic activity of the enzymes of the contact activation system. Purified human F.XIIa (4 nmol/liter, panel A), plasma kallikrein (5 nmol/liter, panel B), or F.XIIa (4 nmol/liter, panel C) were incubated at 37°C with increasing concentrations of purified guanidine-activated PAI-1 (15 nmol/liter, ○—○; 30 nmol/liter, C—○; 60 nmol/liter, ●—●). At the indicated time points, the residual amidolytic activity of the enzymes against the appropriate oligopeptide chromogenic substrate was measured as described under "Experimental Procedures" and compared with that of control solutions without added PAI-1.
during the time course of clot lysis (Fig. 5, panel A). For example, the addition of 0.25–0.5 μg/ml of F.XIa to mixtures containing t-PA and PAI-1 resulted in a progressive reduction in the inhibitory activity of PAI-1 on t-PA-induced clot lysis. Clot lysis was not stimulated when similar concentrations of F.XIa were incubated in tubes not containing t-PA or PAI-1, thus suggesting that F.XIa did not directly activate plasminogen. Direct activation of plasminogen was observed only at higher concentrations of F.XIa (e.g. 1.5 μg/ml) and probably contributed in part to the rapid lysis of clots observed when 1.5 μg/ml of F.XIa was added to mixtures containing t-PA and PAI-1. Fibrinolysis was also accelerated by F.XIIa and plasma kallikrein, but concentrations of 1–5 μg/ml were required and clot lysis in the presence of t-PA and PAI-1 was only slightly faster than that observed in the absence of PAI-1 (Fig. 5, panels B and C), indicating that direct activation of plasminogen was the prominent profibrinolytic mechanism for F.XIIa and kallikrein. In a parallel series of experiments, comparable results were observed when platelet PAI-1 in the platelet releasate obtained from 4.5 × 10^5 platelets replaced the purified PAI-1 (data not shown).

**Inhibition of the Enzymes of the Contact Activation System by PAI-1**—The effect of PAI-1 on the amphotolytic activity of F.XIIa, F.XIa, and plasma kallikrein was studied using oligopeptide and chromogenic substrates. Incubation of PAI-1 with each enzyme resulted in a time-dependent and concentration-dependent inhibition of their amphotolytic activity against the appropriate synthetic oligopeptide chromogenic substrate (Fig. 6). Under the experimental conditions used, nonlinear plots of percent residual amphotolytic activity versus time were observed, suggesting a progressive decline of inhibitor activity due to its spontaneous decay at 37 °C and/or to enzyme-induced inactivation. When the second-order rate constant, k₂, of inhibition was calculated based on the initial slope of the semilog plots of activity versus time, values of k₂ were estimated to be 1.6 × 10⁻⁸, 2.1 × 10⁻⁸, and 6.0 × 10⁻⁸ M⁻¹ s⁻¹ for F.XIIa, F.XIa, and plasma kallikrein, respectively. For the same preparation of PAI-1, the apparent second-order rate constant for t-PA inhibition was 1.3 × 10⁻⁸ M⁻¹ s⁻¹. Heparin (5 IU/ml) did not accelerate the PAI-1-induced inactivation of enzymes.

**DISCUSSION**

The present study provides experimental evidence for the interaction between the enzymes generated in contact-activated plasma and PAI-1, the main physiologic inhibitor of t-PA. Incubation of F.XIIa, F.XIa, and plasma kallikrein with PAI-1 resulted in the formation of SDS-stable enzyme-inhibitor complexes and in the proteolytic degradation and functional inactivation of the inhibitor. Complex formation with subsequent degradation of the inhibitor, a frequent feature of many serine protease-serpin interactions (29), has been observed after incubation of PAI-1 with PAs (23, 40, 41) and with activated protein C (32, 37) while other enzymes, such as thrombin, seems to be able to generate the inactive proteolytic derivative without apparent complex formation with PAI-1 (37). Using immunoblotting techniques for PAI-1, a degraded form of the inhibitor after incubation with t-PA was detected. Dissociation of complexes between t-PA and PAI-1 is believed to occur during SDS-PAGE as a result of nucleophilic attack on the acyl-enzyme complex by the positively-charged Tris ion at high pH (40). Degradation products of PAI-1 were not readily apparent by immunoblotting when either the enzymes of the contact activation system or t-PA was incubated with platelet-derived PAI-1. However, the higher electrophoretic mobility of platelet-derived PAI-1 caused by the presence of other proteins in the M₅₀,₀₀₀–70,₀₀₀ region (25) may have interfered with the separation of the native and degraded inhibitor into two distinct bands during SDS-PAGE.

Of the three enzymes of the contact activation system, F.XIIa reacts with PAI-1 much faster than F.XIa and plasma kallikrein, as suggested by (i) the rapid inactivation of PAI-1 by F.XIIa (Fig. 3), (ii) the ability of F.XIa to compete with t-PA for complex formation with PAI-1 (Fig. 4), and (iii) the estimated second-order rate constants of inhibition of the enzymes by PAI-1 (Fig. 6). This conclusion is also supported by the results of clot lysis assays in which only F.XIa clearly stimulated fibrinolysis through a PAI-1-dependent mechanism. In this context, it is notable that F.XIa was found to neutralize PAI-1 activity at rather low concentrations (e.g. 0.25 μg/ml). In similar clot lysis assay, much higher concentrations (i.e. 3.4 μg/ml) of bovine-activated protein C were required to inactivate PAI-1, and human protein C was even less potent than the bovine enzyme (32).

The physiological significance of PAI-1 inactivation by the enzymes of the contact activation system remains to be established. Neutralization of PAI-1 activity has been proposed as a relevant mechanism for the profibrinolytic effects of other plasma enzymes. For example, the addition of activated protein C to cultured endothelial cells inactivates PAI-1 and results in a marked increase in the fibrinolytic activity of these cells (42, 43). Since F.XIa appears to be more potent than activated protein C in inactivating PAI-1, it is possible that the activation of the contact system at sites of vascular injury could generate F.XIa in amounts that may significantly reduce the local concentration of active PAI-1, thus liberating t-PA from the control of its primary inhibitor. The concomitant generation of kallikrein, an enzyme that converts single-chain u-PA into the two-chain form (13), may further increase the fibrinolytic potential through a separate, independent pathway.

The results here also raise the question of whether PAI-1 plays any significant role in the regulation of the reactions of the contact activation system. The calculated rates for the inhibition of F.XIIa, F.XIa, and kallikrein by PAI-1 are similar to those previously reported for other inhibitors (44), with the possible exception of F.XIa which seems to be inhibited by PAI-1 more rapidly. However, it is unlikely that PAI-1 contributes to the regulation of F.XIIa and the whole contact activation system in plasma because it is normally present in the circulation at very low concentrations (<10 ng/ml). It is possible that during coagulation, PAI-1 released from activated platelets or present in the surrounding environment of endothelial cells and their extracellular matrix (45) may effectively increase the local PAI-1 concentration such that this inhibitor can affect the reactions initiating the intrinsic pathway of blood coagulation and other related defense mechanisms. Therefore, the studies have indicated that PAI-1 should be added to the list of F.XIa inhibitors released by activated platelets, which already includes α₂-antiplasmin (46) and Cl-inactivator (47). Further studies will be necessary to clarify the relationships between PAI-1 and several other recently detected platelet inhibitors of F.XIa (48, 49).

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PAI-1 Inhibition of Contact Activation Enzymes

11743

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