Platelet Plasminogen Activator Inhibitor: Purification and Characterization of Interaction with Plasminogen Activators and Activated Protein C†

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ABSTRACT: Plasminogen activator inhibitor (PAI) was purified in active form from porcine platelets under nondenaturing conditions. The purified inhibitor (M_r 47 000) reacts with tissue-type plasminogen activator (t-PA), urokinase (UK), and activated protein C (APC) to yield both SDS-stable complexes and a modified PAI of slightly reduced molecular weight. The second-order rate constants for the inhibition of t-PA and UK by PAI are 3.5×10^7 and 3.4×10^7 M^{-1} s^{-1}, respectively. Activated protein C reacts with PAI with a second-order rate constant of 1.1×10^4 M^{-1} s^{-1}. This rate is not accelerated by protein S, phospholipid, and calcium, or heparin. It is concluded that (1) PAI can function as both inhibitor and substrate of its target proteases, (2) if APC promotes fibrinolysis via inactivation of PAI, then APC must be present in concentrations several orders of magnitude greater than t-PA, or the interaction of APC and PAI must be accelerated by presently unknown mechanisms, and (3) in the absence of heparin, platelet PAI is the most rapid inhibitor of APC yet described.

An interaction of activated protein C (APC)[1] and plasminogen activator inhibitor has been implicated in the regulation of fibrinolysis. The physical interaction of enzyme and inhibitor has been established by the finding that APC and PAI can form SDS-stable complexes (de Fouw et al., 1987). Activated protein C stimulates fibrinolysis in vitro in a PAI-dependent manner (Sakata et al., 1986), and fibrinolytic activity appears after intravenous infusion of APC into dogs (Comp & Esmon, 1981). The profibrinolytic effect of APC may arise, at least in part, from the inactivation of PAI with consequent increased activity of plasminogen activators. Such a hypothesis presupposes that under certain physiologic conditions APC competes with plasminogen activators for interaction with PAI, either (1) by reacting with PAI in an environment in which the concentration of APC vs plasminogen activator favors interaction of the former with PAI or (2) by reacting in an environment in which the kinetics of interaction of APC and PAI are similar to the very rapid rate (second-order rate constant of approximately 10^7 M^{-1} s^{-1}) of interaction of PAI with plasminogen activators (Kruithof et al., 1986a; Sprengers & Kluft, 1987).

Activated protein C and PAI most likely react at sites of hemostasis and thrombosis. In this environment, platelets are the likely source of PAI, since they contain PAI and release it upon activation (Erickson et al., 1984; Booth et al., 1985; Kruithof et al., 1986b). Platelet PAI has been classified immunochemically as belonging to the first of the three classes of PAI (i.e., PAI-1) so far described (Erickson et al., 1985; Heeb et al., 1987; Sprengers & Kluft, 1987). However, the physiological relevance of the platelet vs the endothelium as a source of PAI has not yet been defined, nor has it been established that platelet PAI and endothelial PAI are structurally and functionally identical.

To characterize platelet PAI and better define the possible role of APC in the regulation of fibrinolysis, we have purified PAI from platelets and quantitatively studied its interaction with APC and plasminogen activators in a purified, reconstituted system.

Experimental Procedures

Materials

Human plasminogen, poly(L-lysine hydrobromide) (M_r 30 000–70 000), bovine albumin (fraction V), and Reactive Blue 2-Sepharose CL-6B were obtained from Sigma (St. Louis, MO); low molecular weight human urokinase (Abbokinase), specific activity 100 000 IU/mg, was from Abbott Laboratories (North Chicago, IL); Ultrogel AcA 34 and Ultrogel AcA 44 were from LKB (Bromma, Sweden); concanavalin A–agarose (Affi-Gel Con A) was from Bio-Rad (Richmond, CA); heparin (porcine intestinal mucosa) was from Eli Lilly (Indianapolis, IN); and Spectrozyme TH (H-D-HHT-L-Ala-L-Arg-pNA·2AcOH), Spectrozyme UK [Cbo-L-(γ)Glu(α-t-BuO)-Gly-Arg-pNA·2AcOH], Spectrozyme t-PA (CH_3SO_2-D-CHT-Gly-Arg-pNA·AcOH), and Spectrozyme PL (H-D-Nle-HHT-Lys-pNa·2AcOH) were from American Diagnostica (New York, NY). Recombinant hirudin was the gift of Robert B. Wallis, CIBA-GEIGY Pharmaceuticals. Dextran sulfate–agarose was prepared as previously described (March et al., 1974).

Methods

Purification of Platelet Plasminogen Activator Inhibitor. Porcine blood was collected at slaughter into 0.1 volume of

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[1] Abbreviations: PAI, plasminogen activator inhibitor; APC, activated protein C; t-PA, tissue-type plasminogen activator; UK, urokinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PRP, platelet-rich plasma; KIU, kallikrein inhibitor unit; SP, sulfopropyl; APTT, activated partial thromboplastin time; α_1-PI, α_1-proteinase inhibitor.

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0.4% citric acid, 1.3% sodium citrate, 1.5% dextrose, and 25 mM EDTA. Platelet-rich plasma was prepared by centrifugation at 200*g* × 15 min. The PRP was aspirated without agitating the buffy coat and then centrifuged at 2500*g* × 15 min. The platelet pellet was washed 5 times with 0.01 M Tris-HCl, 0.15 M NaCl, and 2.5 mM EDTA, pH 7.5. The resultant pellet was resuspended in a minimal volume of washing buffer and frozen at −40 °C until used. The platelet count of the concentrates averaged approximately 3 × 10^{10}/mL. Microscopic examination of a Wright–Giemsa-stained sample from a typical preparation of washed platelets revealed no contamination by erythrocytes or leukocytes. An average purification preparation was started with 80–100 mL of washed platelet concentrate from approximately 25 L of blood. The platelet concentrate was thawed, diluted with 4 volumes of 0.02 M Tris-HCl, 0.05% (v/v) 2-mercaptoethanol, 0.01% (v/v) Tween 80, 0.2 mM EDTA, and 50 KIU/mL aprotinin, pH 8.0, and stirred overnight. This and all subsequent steps were performed at 4 °C, unless otherwise indicated. The suspension was centrifuged at 10000*g* × 20 min. The supernatant was brought to 40% saturation with ammonium sulfate, stirred for 30 min, and then centrifuged at 13000*g* × 20 min. The supernatant was brought to 65% ammonium sulfate saturation, then stirred, and centrifuged as before. The pellet was dissolved in a minimal volume (<15 mL) of 0.02 M Tris-HCl, 0.5 M NaCl, 0.01% Tween 80, 0.05% 2-mercaptoethanol, 0.2 mM EDTA, and 50 KIU/mL aprotinin, pH 8.0 (gel filtration buffer). This solution was made 0.1 M in dithiothreitol, allowed to stand for 2 h, and then centrifuged at 20000*g* × 10 min. The supernatant was fractionated on a column (2.5 × 120 cm) of Ultrogel AcA 44 developed at 15 mL/h with gel filtration buffer. Fractions (7 mL) containing PAI activity were pooled, made 1 mM in $CaCl_2$ and 1 mM in $MnCl_2$, and then applied (3 mL/h) to a column (0.7 × 6 cm) of concanavalin A–agarose equilibrated with 0.02 M Tris-HCl, 0.1 M NaCl, 0.01% Tween 80, 0.05% 2-mercaptoethanol, and 50 KIU/mL aprotinin, pH 8.0. The column was washed with equilibrating buffer until the A_{280} of the eluate was less than 0.05 and then was eluted at 2 mL/h with equilibrating buffer containing 0.5 M methyl α-D-mannopyranoside. Fractions containing PAI activity were pooled and applied to a Mono Q HR 5/5 anion-exchange column (FPLC system, Pharmacia, Uppsala, Sweden) equilibrated with 0.02 M Tris-HCl, 0.1 M NaCl, and 0.01% Tween 80, pH 8.0 at room temperature. The column was washed and then developed with a linear gradient of 0.1–0.8 M NaCl. Fractions containing the purified inhibitor were pooled, aliquoted, and then frozen at −70 °C until use. Shoulder fractions from the PAI peak were dialyzed and rechromatographed to increase yield.

Porcine t-PA. Tissue plasminogen activator was purified from porcine hearts by a modification of the method of Soeda et al. (1986). After gel filtration on Ultrogel AcA 34, fractions containing t-PA activity were pooled, dialyzed against 0.02 M sodium acetate, 0.15 M NaCl, and 0.01% Tween 80, pH 5.5, and then chromatographed on an SP-Sephadex column developed with a linear (0.15–0.6 M) NaCl gradient. Purified t-PA was eluted at 0.3 M NaCl. The purified plasminogen activator was of the two-chain form and yielded a single band when analyzed by SDS–PAGE (Figure 2).

Protein C and Protein S. Proteins C and S were purified from porcine plasma according to the method of Stenflo and Jönsson (1979), with the following modifications: (1) blood was collected into 0.1 volume of 3.2% sodium citrate, 50 mM benzamidine, 10 units/mL heparin, 200 KIU/mL aprotinin, and 1 μg/mL hirudin, and (2) the breakthrough fractions from Blue Sepharose chromatography, which contained protein C and protein S, were chromatographed on dextran sulfate–agarose. Protein S was not retained on the column, whereas protein C was eluted at 0.35 M NaCl. Protein C was activated by incubation overnight with 1/100 weight bovine α-thrombin at 4 °C. Thrombin was removed by adsorption to SP-Sephadex.

Phospholipid suspensions containing 2.0 mg/mL phosphatidylcholine–phosphatidyl-L-serine (5:1 PC:PS ratio) were prepared by sonication for 30 min at 23 °C in a Branson 1200 sonicator.

Polyacrylamide gel electrophoresis in SDS with silver staining was performed with the PhastSystem (Pharmacia, Uppsala, Sweden). Molecular weight standards included phosphorylase *b* (97 400), bovine serum albumin (66 200), glutamate dehydrogenase (55 400), lactate dehydrogenase (36 500), and α-chymotrypsinogen A (27 000).

Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL) with BSA as standard.

Functional Assays. The activity of purified protein S and APC, and of the phospholipid vesicles, was assayed with the one-stage factor Xa clotting time of barium-adsorbed and nonadsorbed plasma as previously described (Comp & Esmon, 1979). Activated partial thromboplastin times were measured with the General Diagnostics Automated APTT reagent (Organon Teknika, Durham, NC).

Plasminogen activator inhibitor activity was determined by measuring the inhibition of amidolytic activity of a reference amount of UK, as follows: Fifty microliters of sample was incubated with 50 μL of 1.0 μg/mL UK in 0.02 M Tris-HCl and 0.1 M NaCl, pH 7.5, for 5 min at 23 °C, then 160 μL of 0.02 M Tris-HCl, 0.02 M imidazole, and 0.3 M NaCl, pH 8.4 (buffer B), containing 300 μM Spectrozyme UK was added, and the change in absorbance at 405 nM (ΔA/min) was measured in an Abbott biochromatic analyzer (Abbott Laboratories, South Pasadena, CA). Plasminogen activator inhibitor concentrations were determined at that dilution of sample which inhibited 50% of UK activity. It was assumed that inhibition of UK by PAI was the result of 1:1 complex formation. Platelet PAI was assayed for latency by treating both the platelet extract (i.e., crude PAI preparation) and purified PAI with 4 M guanidine hydrochloride as previously described (Hekman & Loskutoff, 1985).

Kinetic Assays of Platelet PAI. (A) Inhibition of APC by PAI. In microtiter wells, 150 ng of PAI was incubated with 16 ng of APC in 10 μL of 0.02 M Tris-HCl, 0.1 M NaCl, 0.1% BSA, and 2.5 mM $CaCl_2$, pH 7.5 (buffer A), at 23 °C. At intervals, 100 μL of buffer B containing 110 μM Spectrozyme TH was added, and the absorbance change at 405 nM (ΔA/min) was measured for 5 min in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA).

(B) Inhibition of PAI by APC. Equal volumes of PAI (2.0 μg/mL) and APC (20 μg/mL) in buffer A were mixed and incubated at 23 °C (stage 1). At intervals, 25 μL of the reaction mixture was transferred to 25 μL of buffer A containing 1.2 μg/mL UK and then incubated for 5 min (stage 2). Twenty-five microliters from the stage 2 reaction was added to 25 μL of buffer A containing 200 μg/mL plasminogen and then incubated for 30 min at 23 °C (stage 3). Twenty-five microliters from each stage 3 reaction was added to microtiter wells with 200 μL of buffer B containing 250 μM Spectrozyme PL. The absorbance change at 405 nm was measured as above. When stage 1 was omitted, the amount

of plasmin generated in stage 3 was proportional to the concentration of UK in stage 2 over a range of 0.05–0.6 μg/mL. Residual PAI activity was calculated by subtracting UK activity (as measured by plasmin generated in stage 3) from the UK activity observed when PAI was omitted in stage 1. When APC was omitted in stage 1, the amount of PAI used inhibited approximately 90% of the UK activity in stage 2. The activities of APC, UK, and PAI showed no spontaneous decline during the brief incubations used in the assay.

(*C*) *Inhibition of UK by PAI.* Equal volumes of UK (25 ng/mL) and PAI (180 ng/mL) in buffer A were mixed and incubated at 23 °C (stage 1). At intervals, 100 μL of the reaction mixture was transferred to capped polypropylene tubes containing 10 μL of 1 M acetic acid. The acidified (pH 3.1) mixtures were heated (60 °C) for 60 min, then cooled, and neutralized to pH 8.0 by the addition of 10 μL of 2 M Tris, pH 11.0. Control experiments revealed that (1) 100% of UK activity, as assayed by plasminogen activation, was recovered after acidification, heating, and then neutralization, (2) PAI activity was completely and irreversibly inactivated under the same conditions, and (3) UK activity was recovered quantitatively when first acidified, then mixed with PAI, heated, and neutralized, as above. Twenty-five microliters of the neutralized UK–PAI reaction mixture was assayed for residual UK activity by incubation with 25 μL of buffer A containing 200 μg/mL plasminogen at 23 °C for 16 h (stage 2). No plasmin generation was noted during stage 2 in control tubes lacking UK. Plasmin generated during stage 2 was assayed by adding 25 μL from each reaction mixture to microtiter wells containing 200 μL of buffer B made 250 μM in Spectrozyme PL. In the absence of PAI, the amount of plasmin generated in stage 2 was proportional to the UK concentration over a range of 2.5–12.5 ng/mL.

(*D*) *Inhibition of t-PA by PAI.* The inhibition of t-PA by PAI was studied in essentially the same manner as was UK, except for the following modifications: (1) equal volumes of t-PA (15 ng/mL) and PAI (175 ng/mL) were mixed at 23 °C, and at intervals, aliquots of the reaction mixture were acidified, heated, and then neutralized; (2) in stage 2, 90 μL of the neutralized t-PA–PAI reaction mixture was added to 10 μL of buffer A containing 2.4 mg/mL plasminogen and 1.2 mg/mL poly(L-lysine) and then incubated at 37 °C × 4 h; (3) 25 μL from each stage 2 reaction mixture was added to microtiter wells containing 200 μL of buffer B made 100 μM in Spectrozyme PL. The amount of plasmin generated in stage 2 was proportional to the concentration of t-PA over the range of 1.0–7.5 ng/mL. Heating of acidified porcine t-PA to 60 °C for 60 min resulted in a loss of approximattely 20% of activity, as measured by plasminogen activation. With lower temperatures and shorter incubations, t-PA activity was recovered completely, but PAI was not totally inactivated. Therefore, in each experiment, a standard curve of t-PA not incubated with PAI, but acidified, heated, and neutralized, was included.

Inhibition reactions were performed under conditions which yielded pseudo-first-order kinetics. The pseudo-first-order rate constant was calculated as $(\ln 2)/t_{1/2}$; the observed second-order rate constant was calculated by dividing the pseudo-first-order rate constant by the molar concentration of the reactant in excess.

Results

Purification and Characterization of Platelet PAI. A summary of the purification procedure is presented in Table I. From approximately 25 L of blood, approximately 0.5 mg of PAI was reproducibly obtained. The purified inhibitor

Table I: Summary of Purification of Plasminogen Activator Inhibitor from Porcine Platelets

sample	volume (mL)	total protein (mg)	titration factor[a] (mg of protein/mg of UK)	purification factor (x-fold)	yield (%)
platelet extract	400	2350	1923	1	100
Ultrogel AcA 44	53	143	195	9.9	60
concanavalin A–agarose	18	6.5	12	160	44
Mono Q HR 5/5	2.5	0.53	1.6	1200	27

[a] Mass of protein required to inhibit a unit mass of UK. For assay details, see Methods.

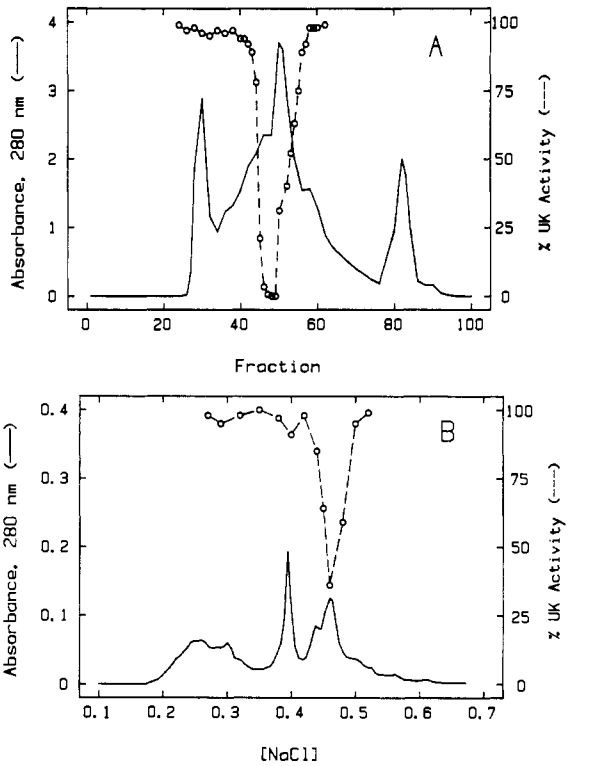

FIGURE 1: Chromatography of PAI. (A) Gel chromatography on Ultrogel AcA 44 (2.5 × 120 cm column) of the ammonium sulfate fractionation. Samples from each fraction were diluted 6-fold and then assayed for PAI activity by incubating with UK and measuring residual UK activity on Spectrozyme UK as described under Methods. The peak in absorance at fraction 82 is due to dithiothreitol. (B) Mono Q anion-exchange chromatography of pooled fractions from concanavalin A–agarose chromatography. Samples from each fraction were diluted 250-fold and then incubated with UK. Residual UK activity was determined as described under Methods.

yielded a single band of M_r 47 000 when analyzed by SDS–PAGE under both reducing and nonreducing conditions (Figure 2). Assuming 1:1 complex formation between PAI and UK and a molecular weight of 47 000 for PAI and 34 000 for low molecular weight UK, titration of purified PAI with UK indicated that 88% of the purified protein was in the active form. Treatment of both crude and purified PAI with 4 M guanidine hydrochloride resulted in no significant (less than a 1.2-fold) increase in specific activity.

Plasminogen activator inhibitor can react with t-PA, UK, and APC to yield not only SDS-stable complexes but also a modified inhibitor of slightly lower molecular weight (Figure 2, lanes 5–7). The PAI–UK complex and modified PAI both formed within 2 s, and the complex underwent no apparent dissociation during incubation at 23 °C for 18 h (Figure 3). When PAI was reacted with ^{125}I-labeled UK and then subjected to SDS–PAGE, radioautography revealed the presence of label only at M_r 34 000 (free UK) and 78 000 (UK–PAI complex). There was no radioactivity detected at the position

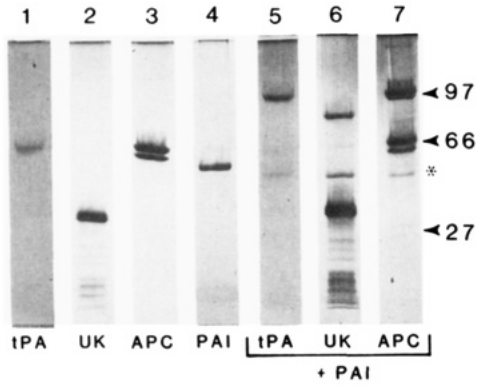

FIGURE 2: Reactions of PAI with t-PA, UK, and APC analyzed by SDS–PAGE. Plasminogen activator inhibitor (lane 4) and t-PA (lane 1) were reacted in equimolar amounts for 10 min at 23 °C in 0.02 M Tris-HCl and 0.2 M NaCl, pH 8.0 (lane 5). Urokinase (lane 2) and APC (lane 3) were reacted in molar excess with PAI for 10 min under the same conditions (lanes 6 and 7). Molecular weight standards are indicated by arrows. The asterisk denotes the electrophoretic mobility of modified PAI.

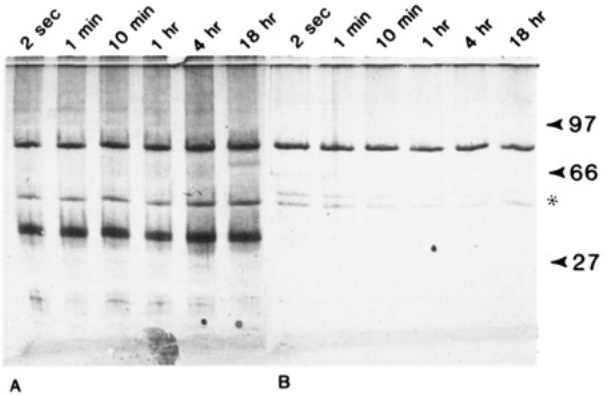

FIGURE 3: Time course of reaction of UK and PAI. (A) Urokinase (3.2 μM) and PAI (1.6 μM) were incubated at 23 °C in 0.02 M Tris-HCl, 0.2 M NaCl, 0.01% Tween 80, and 0.02% sodium azide, pH 8.0. At the time intervals indicated, samples of the reaction mixture were made 1% in SDS and then analyzed by SDS–PAGE. (B) Same as (A), except final concentrations of UK and PAI were 0.8 and 1.6 μM, respectively. Molecular weight standards are indicated by arrows. The asterisk denotes the electrophoretic mobility of modified PAI.

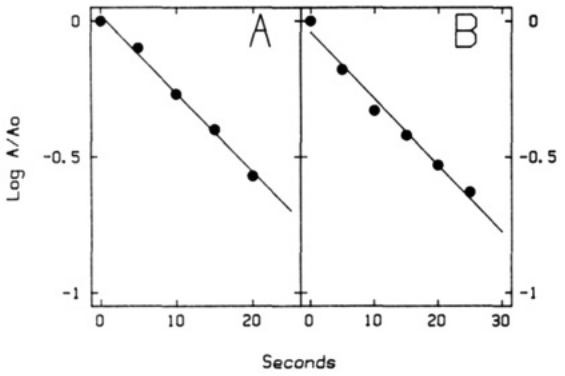

FIGURE 4: Inhibition of plasminogen activators by platelet PAI. (A) Inhibition of t-PA by PAI: Tissue plasminogen activator (7.5 ng/mL) and PAI (90 ng/mL) were incubated at 23 °C. At intervals, samples of the reaction mixture were assayed for residual t-PA activity as described under Methods. (B) Inhibition of UK by PAI: Urokinase (12.5 ng/mL) and PAI (90 ng/mL) were incubated at 23 °C. At intervals, samples of the reaction mixture were assayed for residual UK activity, as described under Methods. Residual activity is expressed as log (A/A_0), where A equals enzyme activity at the indicated intervals and A_0 equals initial enzyme activity.

corresponding to that interpreted as modified inhibitor (data not shown).

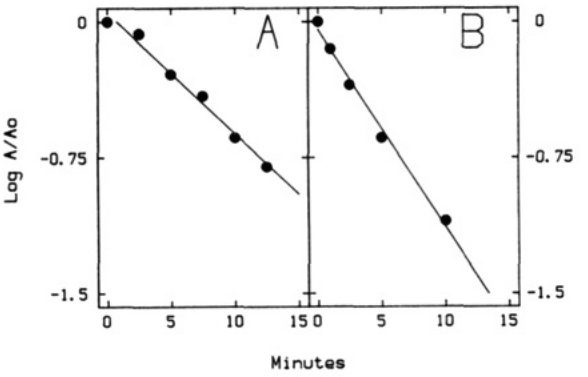

FIGURE 5: Kinetics of interaction of APC and PAI. (A) Inhibition of PAI by APC: Activated protein C (10 μg/mL) and PAI (1.0 μg/mL) were incubated at 23 °C. At intervals, samples of the reaction mixture were assayed for residual PAI activity as described under Methods. (B) Inhibition of APC by PAI: Activated protein C (16 ng) and PAI (150 ng) were incubated for varying time intervals, and the residual APC activity was determined as described under Methods. Data are expressed as in Figure 4. The second-order rate constants for the inhibition of PAI by APC and for the inhibition of APC by PAI were 1.1×10^4 and 1.2×10^4 M^{-1} s^{-1}, respectively. Line A and line B are not parallel because of the different concentrations of inhibitor used in each experiment.

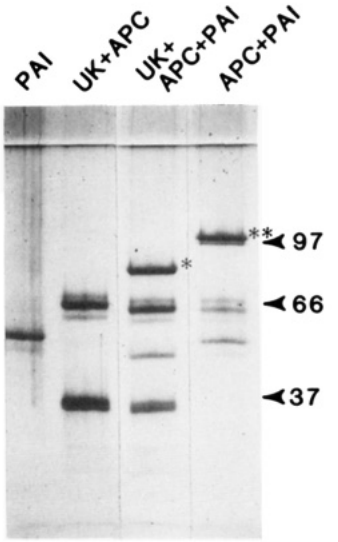

FIGURE 6: Competition of APC and UK for PAI: SDS–PAGE analysis. One microgram of APC and 1 μg of UK were mixed and then added to 0.6 μg of PAI. The reaction was incubated for 5 min at 23 °C in 0.02 M Tris-HCl and 0.1 M NaCl, pH 8.0, and then SDS–PAGE was performed. Lane 1, PAI; lane 2, APC + UK; lane 3, (APC + UK) + PAI; lane 4, APC + PAI. Note APC–PAI complex formation in lane 4, but lack thereof in lane 3, where UK is present. Molecular weight markers are indicated by arrows. The electrophoretic mobilities of UK–PAI and APC–PAI complexes are indicated by the single and double asterisks, respectively.

Kinetics of PAI. The second-order rate constant of inhibition of plasminogen activator by PAI was 3.4×10^7 M^{-1} s^{-1} for UK and 3.5×10^7 M^{-1} s^{-1} for t-PA (Figure 4). The kinetics of interaction of PAI with APC were studied by measuring the inhibition of the activity of each protein. The second-order rate constants for the inhibition of APC by PAI and for the inhibition of PAI by APC were 1.2×10^4 and 1.1×10^4 M^{-1} s^{-1}, respectively (Figure 5).

The 3000-fold difference in rate constants for the inhibition of plasminogen activators vs APC by PAI indicates that under the experimental conditions employed, APC would be unable to compete with equal amounts of UK or t-PA for complex formation with PAI. This was confirmed in experiments in which approximately equimolar UK and APC were incubated

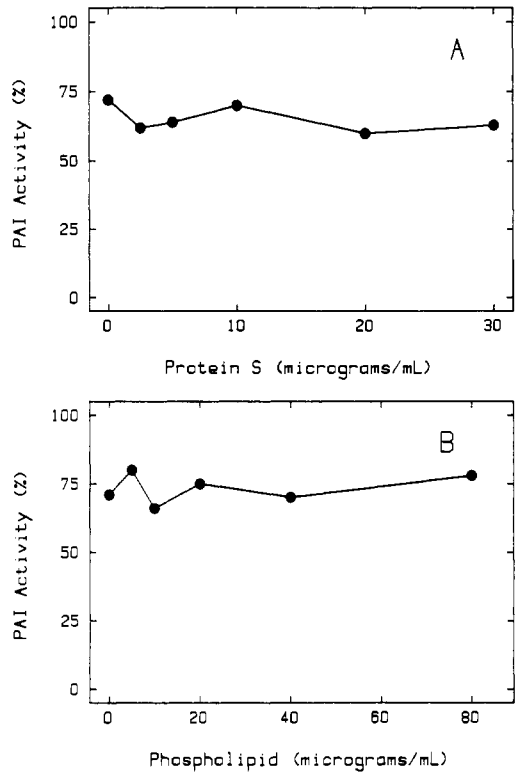

FIGURE 7: Effect of protein S, calcium, and phospholipid on the inhibition of PAI by APC. (A) Protein S effect: Plasminogen activator inhibitor (1 μg/mL) and APC (10 μg/mL) were incubated for 5 min in the presence of 2.5 mM $CaCl_2$ and varying concentrations of protein S, as shown. The residual PAI activity (percent) was determined for each protein S concentration as described under Methods. (B) Effect of protein S and varying phospholipid: Plasminogen activator inhibitor (1 μg/mL) and APC (10 μg/mL) were incubated for 5 min in the presence of 2.5 mM $CaCl_2$, 20 μg/mL protein S, and varying concentrations of phospholipid vesicles, as shown. The residual PAI activity (percent) was determined for each phospholipid concentration as described under Methods.

with PAI, and the amount of UK–PAI complex vs APC–PAI complex formed was evaluated by SDS–PAGE. The lack of any detectable APC–PAI complex formation in the presence of UK is shown in Figure 6.

Because of the magnitude of the difference in rates of interaction of PAI with plasminogen activators as compared to APC, we studied the capacity of protein S, a known cofactor for APC activity, to accelerate the inhibition of PAI by APC. However, protein S, phospholipid vesicles, and $CaCl_2$ had no effect on the inhibition of PAI by APC (Figure 7). The functional activity of the APC, protein S, and phospholipid vesicles used in these experiments was verified by demonstrating their potent effect on the one-stage factor X_a clotting time using barium-adsorbed porcine plasma. The binding of APC to protein S on the surface of phospholipid vesicles was inferred by demonstrating that (1) APC prolonged the clotting time only when protein S was added, and (2) prolongation of the clotting time by APC and protein S was dependent on the presence of phospholipid vesicles (data not shown).

Protein C Inhibitor Activity of Platelet PAI. The second-order rate constant for the inhibition of APC by platelet PAI was comparable to that determined for the protein C inhibitor (Suzuki et al., 1984), thus prompting the further investigation of PAI as an inhibitor of protein C. Heparin, in concentrations as high as 20 units/mL, had no effect on the rate of inactivation of APC by PAI. The capacity of PAI to inhibit the anticoagulant effect of APC in plasma was examined by adding PAI and APC to normal porcine plasma and determining their effect on the APTT. Plasminogen activator inhibitor can significantly reduce the prolongation of the APTT caused by the addition of APC to plasma (Table II).

Table II: Effect of Platelet PAI on the Anticoagulant Effect of APC

sample	APTT (s)
control plasma	32.3 ± 0.9
+2.2 μg of PAI	34.1 ± 1.1
+0.6 μg of APC	76.9 ± 0.9
+2.2 μg of PAI + 0.6 μg of APC	37.8 ± 1.5

[a] Activated protein C and/or PAI as shown was added to 90 μL of porcine plasma. One hundred microliters of APTT reagent was added. Following incubation for 10 min at 37 °C, 100 μL of 0.02 M $CaCl_2$ was added and the APTT measured. Values shown are mean ± 1 standard deviation of three determinations of the APTT.

Discussion

Platelet-derived PAI is of interest foremost because of its possible role in the regulation of fibrinolysis. The storage of PAI within circulating platelets might serve as a mechanism of providing a pool of this inhibitor which could be rapidly released in relatively high concentrations at sites of hemostasis or thrombosis, thus inhibiting fibrinolysis.

The principal source of PAI preparations has been tissue culture conditioned medium. A significant problem with tissue culture sources has been that the inhibitor in conditioned media is predominantly latent—i.e., it does not inhibit plasminogen activators unless activated by treatment with denaturants (Hekman & Loskutoff, 1985). Although the ratio of active to latent PAI present in conditioned media varies with cell lines, greater than 98% of PAI present in endothelial cell conditioned medium at 24 h of incubation is in the latent form (Levin, 1986). The platelet is a readily available source of working quantities of PAI which can be purified in active form by using nondenaturing conditions. Because it entails a small number of conventional procedures, this purification scheme with minor modificiation should be useful for isolation of PAI from the platelets of different species. Whether the activity (i.e., nonlatency) of platelet PAI arises from inherent differences between platelet and secreted endothelial PAI or from species variation was not addressed by our experiments.

The interaction of PAI with UK, t-PA, and APC yields an enzyme–inhibitor complex and a modified inhibitor of slightly reduced molecular weight. Other investigators have noted the generation of a modified PAI of slightly reduced molecular weight in tissue culture conditioned media to which had been added t-PA or APC, but the origin and rate of appearance of this modified inhibitor were unclear (Kooistra et al., 1986; de Fouw et al., 1987). Our results indicate that the generation of modified PAI occurs simultaneously with the generation of a SDS-stable complex, rather than from dissociation of complex during prolonged incubation or after exposure to SDS. This indicates that a portion of the PAI present in the reaction mixture is catalytically inactivated by plasminogen activator or APC. The functional inactivity of the modified PAI can be concluded by noting the apparent lack of any decrease in the amount of modified inhibitor during incubation with excess UK for 18 h (Figure 3A). These products would arise if at some point in the pathway, competing reactions yielded either stable complex or modified inhibitor. We did not quantify the amount of PAI which proceeded to stable complex vs the amount which was enzymatically altered, but the SDS–PAGE pattern suggested that the pathway yielding stable complex predominated. Alternatively, two forms of the inhibitor in the purified PAI, one which preferentially interacts with enzyme to form complex and the other to form modified inhibitor, would yield the same reaction products. An analogous example

is α_1-PI, which exhibits 30% homology with PAI (Ny et al., 1986). Native α_1-PI reacts with elastase to form α_1-PI–elastase complex. However, oxidized α_1-PI, which appears identical with native α_1-PI on SDS–PAGE, reacts with elastase to form a modified inhibitor of slightly reduced molecular weight (Matheson et al., 1981).

The kinetics of interaction of platelet PAI with APC are of relevance to the study of the role of APC in the regulation of fibrinolysis. The 3000-fold difference in the rates of interaction of PAI with APC vs plasminogen activators suggests that if APC is to promote fibrinolysis in vivo via inhibition of PAI, then either the concentration of APC must exceed that of t-PA by several orders of magnitude or other cellular or humoral factors must accelerate the rate of interaction of PAI with APC (or retard the interaction of t-PA with PAI). The relative amounts of plasminogen activators and APC at sites of hemostasis and thrombosis are not known and likely exhibit both spatial and temporal variability due to differing rates of synthesis, metabolism, and adsorption to surfaces. We found no accelerating effect on protein S, phospholipid, and $CaCl_2$ on the inactivation of PAI by APC in a purified, reconstituted system, results similar to those reported by de Fouw et al. (1988). However, de Fouw et al. (1986) have observed protein S to enhance the acceleration of whole human blood clot lysis by APC in vitro. Likewise, D'Angelo et al. (1987) found that protein S accelerates the neutralization of human platelet PAI activity by APC. The differences between the results observed in these two nonpurified systems and in purified systems may be explained by a requirement for additional factor(s) to enhance protein S activity, such as the protein S binding protein (Walker, 1986).

The interaction of APC and PAI has primarily been considered from the viewpoint of neutralization of PAI activity by APC. However, we have found that platelet PAI can function as a protein C inhibitor in plasma. Although present in plasma in concentrations much less than those used in this experiment, it may be anticipated that the concentration of PAI in and around a platelet hemostatic plug or thrombus would be orders of magnitude greater than that in the blood. The second-order rate constant for the inhibition of activated protein C by PAI (1.1×10^4 M^{-1} s^{-1}) is of the same order of magnitude (2.5×10^3 M^{-1} s^{-1} in the absence of heparin; 7.5×10^4 M^{-1} s^{-1} in the presence of 5 units/mL heparin) as that reported for the inhibition of APC by the protein C inhibitor purified and characterized by Suzuki et al. (1983, 1984) and subsequently found to be immunologically identical with PAI-3 (Heeb et al., 1987). There was no effect of heparin on the inhibition of APC by platelet PAI. Heeb et al. (1988) have reported that α_1-antitrypsin (α_1-PI) is a heparin-independent inhibitor of APC with a second-order rate constant of 10 M^{-1} s^{-1}. Thus, in the absence of heparin, platelet PAI is the most rapid inhibitor of APC yet described. In addition, by virtue of its presence in platelets, it is the only protein C inhibitor with a mechanism for targeting to sites of hemostasis. It is possible that PAI released by activated platelets functions to promote clot stability not only by inhibiting fibrinolysis but also by preventing the inactivation of factor V_a and factor $VIII_a$ by APC.

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