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Stabilization *Versus* Inhibition of TAFIa by Competitive Inhibitors *in Vitro**

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Two competitive inhibitors of TAFIa (activated thrombin-activable fibrinolysis inhibitor), 2-guanidinoethylmercaptosuccinic acid and potato tuber carboxypeptidase inhibitor, variably affect fibrinolysis of clotted human plasma. Depending on their concentration, the inhibitors shortened, prolonged, or had no effect on lysis *in vitro*. The inhibitor-induced effects were both tissue-type plasminogen activator (tPA) and TAFIa concentration-dependent. Inhibitor-dependent prolongation was favored at lower tPA concentrations. The magnitude of the prolongation increased with TAFIa concentration, and the maximal prolongation observed at each TAFIa concentration increased saturably with respect to TAFIa. A theoretical maximal prolongation of 20-fold was derived from a plot of the maximum prolongation *versus* TAFIa. This represents, for the first time, a measurement of the maximal antifibrinolytic potential of TAFIa *in vitro*.

Because TAFIa spontaneously decays, the stabilization of TAFIa was investigated as a mechanism explaining the inhibitor-dependent prolongation of lysis. Both inhibitors stabilized TAFIa in a concentration-dependent, non-saturable manner. Although their K_I values differed by three orders of magnitude, TAFIa was identically stabilized when the fraction of inhibitor-bound TAFIa was the same. The data fit a model whereby only free TAFIa decays. Therefore, the variable effects of competitive inhibitors of TAFIa on fibrinolysis can be rationalized in terms of free TAFIa and lysis time relative to the half-life of TAFIa.

Thrombin-activable fibrinolysis inhibitor (TAFI)[1] is the plasma zymogen of a carboxypeptidase-B like enzyme, TAFIa (1, 2). TAFIa is generated from TAFI by thrombin produced during coagulation, in complex with thrombomodulin, present both on the endothelial cell surface and in plasma (3–5). TAFIa attenuates fibrinolysis by catalyzing the removal of C-terminal lysine and arginine residues present in plasmin-degraded fibrin and fibrin degradation products (6, 7). Because C-terminal lysine residues facilitate tPA-mediated plasminogen activation, TAFIa likely exerts its antifibrinolytic effect by down-regulating tPA-mediated plasminogen activation (6, 7). Consequently, the inhibition of TAFIa may potentiate thrombolytic therapy by preventing the TAFIa-mediated attenuation of plasminogen activation (8–11).

Although there is no known inhibitor of TAFIa present in plasma, several competitive inhibitors of carboxypeptidase B-like enzymes have been described, including the small molecule inhibitor GEMSA (12), and the peptide inhibitor from the potato tuber, PTCI (13). Physiologically, the only known mechanism for down-regulating TAFIa activity is an autoregulatory event in which TAFIa activity is lost spontaneously (1, 2, 14–17). Because inhibition of TAFIa may prove to be an effective strategy for potentiating thrombolytic therapy (8), we investigated the effectiveness of GEMSA and PTCI as profibrinolytic agents in the lysis of clots formed in normal human plasma. We found that the two inhibitors exhibited complex behavior. High concentrations of the inhibitors shortened lysis times. Unexpectedly, low concentrations of the inhibitors prolonged fibrinolysis by 3-fold (coincidently, an abstract describing this paradoxical behavior was recently presented by Schneider (18)). Therefore, the work described in this report was undertaken to investigate the relationships between competitive TAFIa inhibitors, TAFIa, tPA, and fibrinolysis in an effort to understand the complex behavior exhibited by the competitive inhibitors of TAFIa in an *in vitro* system.

EXPERIMENTAL PROCEDURES

Materials—L-α-Phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), and L-α-phosphatidylserine (PS) were obtained in chloroform from Avanti Polar Lipids (Alabaster, AL). The TAFIa substrates 3-(2-furyl)acryloyl-Ala-Arg-OH (FAAR) and 3-(2-Furyl)acryloyl-Ala-Lys-OH (FAAK) were obtained from Bachem Bioscience (King of Prussia, PA). The TAFIa inhibitor 2-guanidinoethylmercaptosuccinic acid (GEMSA) and the thrombin inhibitor Phe-Pro-Arg-chloromethyl ketone (PPAck) were obtained from Calbiochem (San Diego, CA). The potato tuber carboxypeptidase inhibitor (PTCI) was obtained from Sigma-Aldrich (St. Louis, MO). Rabbit lung thrombomodulin (TM) was obtained from American Diagnostica (Greenwich, CT). Lipid reconstituted, recombinant tissue factor (TF), RecombiPlasTin, was from Instrumentation Laboratory (Lexington, MA). Human thrombin was a generous gift from Dr. M. E. Nesheim (Queen's University, Kingston, Ontario, Canada). TAFI and TAFIa were prepared as previously de-

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[1] The abbreviations used are: TAFI, thrombin-activable fibrinolysis inhibitor; TAFIa, activated TAFI; TAFI*a*, decayed, inactivated TAFIa; tPA, tissue-type plasminogen activator; GEMSA, 2-guanidinoethylmercaptosuccinic acid; PPAck, Phe-Pro-Arg-chloromethyl ketone; PTCI, potato tuber carboxypeptidase inhibitor; NHP, normal human plasma; TdP, TAFI-deficient plasma; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PS, L-α-phosphatidylserine; FAAR, 3-(2-furyl)acryloyl-Ala-Arg-OH; FAAK, 3-(2-furyl)acryloyl-Ala-Lys-OH; TM, rabbit lung thrombomodulin; TF, recombinant tissue factor; CPA, carboxypeptidase A; CPD, carboxypeptidase D; CPD-II, carboxypeptidase D domain II.

scribed (7, 17). The recombinant tissue-type plasminogen activator (tPA), Activase, was a kind gift from Dr. Gordon Vehar at Genentech (South San Francisco, CA). Pooled normal human plasma (NHP) was produced from anticoagulated (1/10 acid/citrate/dextrose) whole blood from nine donors. TAFI-deficient plasma (TdP) was prepared from NHP by immunodepletion of TAFI as previously described (17). PC:PE:PS (60:20:20, v/v) phospholipid vesicles were made using the procedure of Barenholz *et al.* (19).

The Effect of Competitive Inhibitors of TAFIa on the tPA-induced Lysis of Clots Formed in NHP or TdP Supplemented with TAFIa—All lysis assays were carried out at 37 °C in Dynex Immulon 1B Removawell strips (Thermo-Labsystems, Franklin, MA). Assays were initiated by the addition of a 60-μl aliquot, consisting of 25 μl of 0.02 M Hepes/0.15 M NaCl/0.01% Tween 80 (HBST) plus 35 μl of either NHP or TdP, to a 40-μl aliquot containing the variable constituents ($CaCl_2$, tPA, thrombin, TM, PC:PE:PS vesicles, TF, TAFI, TAFIa, GEMSA, or PTCI) of the assay. The effect of the competitive inhibitors GEMSA and PTCI on the tPA-induced lysis of clots made in NHP was assessed as follows. NHP containing either PTCI (0–500 nM) or GEMSA (0–1 mM) was clotted with 3 nM thrombin in the presence of 5 mM $CaCl_2$, 5 nM TM, 1/800 dilution of TF, 15 μM PC:PE:PS vesicles, and 0.02–0.08 μg/ml tPA. The TM, TF, and PC:PE:PS vesicles were included in these assays to promote the rapid and complete activation of TAFI to TAFIa *in situ*. The effect of GEMSA and PTCI on the tPA-induced lysis of clots made in TdP supplemented with TAFIa was assessed as follows. TdP containing GEMSA (0–3 mM) or PTCI (0–1 μM) was clotted with 3 nM thrombin in the presence of 5 mM $CaCl_2$, 5 nM TM, 0.02 μg/ml tPA, and 0–100 nM TAFIa. For all experiments, the plates were sealed with clear tape and the turbidity of the clots at 405 nm was monitored at 37 °C using a SpectraMax Platereader (Molecular Devices, Sunnyvale, CA). The lysis time, the time at which the turbidity equals one-half of the full-scale value, of each clot was determined.

The Effect of GEMSA on the Decay of TAFIa—TAFIa (20 nM) in HBST/5 mM $CaCl_2$ was incubated at 37 °C in the presence of GEMSA (0–200 μM). Samples (220 μl) were taken at 0, 10, 25, 60, and 120 min and placed on wet ice to prevent further decay. The TAFIa activity of each sample was determined at the end of the experiment using the chromolytic TAFIa substrate FAAR. The TAFIa samples (100 μl) were mixed with 100 μl of FAAR (2 mM, in HBST) in the wells of a microtiter plate, and the absorbance (340 nM) of duplicate reactions was monitored continuously over 3 h at 26 °C. The initial rate of absorbance decrease for each reaction was measured, and the data obtained at each concentration of GEMSA were fit to the decay equation,

$$rate_t = rate_{t=0} \times e^{-kt} + C \qquad \text{(Eq. 1)}$$

where $rate_t$ is the rate of decrease of absorbance at 340 nm (FAAR hydrolysis) after incubation at 37 °C for t minutes, $rate_{t=0}$ is the rate of FAAR hydrolysis prior to incubation at 37 °C, k is the decay constant, t is the time in minutes, and C is the intrinsic rate of FAAR hydrolysis. The half-life ($t_{1/2}$) of TAFIa at each concentration of GEMSA was obtained from Equation 1 using the following relationship.

$$t_{1/2} = \frac{\ln(2)}{k} \qquad \text{(Eq. 2)}$$

Modeling the Decay of TAFIa in the Presence of a Large Excess of Competitive Inhibitor—Of the models that could be used to fit the data for the spontaneous decay process or inactivation of TAFIa, the one that fit our data the best was one where the decay of TAFIa requires an empty active site.

$$\begin{array}{l} \text{TAFIa} + I \rightleftarrows \text{TAFIa} \cdot I \\ k \downarrow \\ \text{TAFIa}i \end{array}$$

REACTION 1

In this model, the parameters I, TAFI$a i$, and k represent the inhibitor, the inactive decayed TAFIa, and the decay constant, respectively. Because the decay of TAFIa is spontaneous, the rate of decay is directly proportional to the concentration of TAFIa where k is the decay constant.

$$\frac{d[\text{TAFIa}]}{dt} = -k \times [\text{TAFIa}] \qquad \text{(Eq. 3)}$$

When only unoccupied TAFIa can decay, the rate of decay of TAFIa in the presence of a competitive inhibitor is therefore proportional to $[\text{TAFIa}]_{free}$,

$$\frac{d[\text{TAFIa}]}{dt} = -k \times [\text{TAFIa}]_{free} \qquad \text{(Eq. 4)}$$

or

$$\frac{d[\text{TAFIa}]}{dt} = -k \times f_{\text{TAFIa}} \times [\text{TAFIa}] \qquad \text{(Eq. 5)}$$

where f_{TAFIa} is the fraction of TAFIa free. Solving for [TAFIa] yields,

$$[\text{TAFIa}] = e^{C} e^{-ktf_{\text{TAFIa}}} \qquad \text{(Eq. 6)}$$

where C is a constant of integration. When $t = 0$, $[\text{TAFIa}] = [\text{TAFIa}]_o$, and therefore,

$$[\text{TAFIa}] = [\text{TAFIa}]_0 e^{-ktf_{\text{TAFIa}}}. \qquad \text{(Eq. 7)}$$

The half-life, $t_{1/2}$, of TAFIa in the presence of a competitive inhibitor is, therefore,

$$t_{1/2} = \frac{\ln(2)}{kf_{\text{TAFIa}}} \qquad \text{(Eq. 8)}$$

For a given concentration of TAFIa and inhibitor (I), $[\text{TAFIa}]_{free}$ is related to $[I]_{free}$ and K_I through the following,

$$K_I = \frac{[\text{TAFIa}]_{free} \times [I]_{free}}{[\text{TAFIa} \cdot I]} \qquad \text{(Eq. 9)}$$

or

$$K_I = \frac{[\text{TAFIa}]_{free} \times [I]_{free}}{[\text{TAFIa}]_{bound}}. \qquad \text{(Eq. 10)}$$

Because

$$\text{TAFIa}_{total} = \text{TAFIa}_{free} + \text{TAFIa}_{bound} \qquad \text{(Eq. 11)}$$

then

$$K_I = \frac{[\text{TAFIa}]_{free} \times [I]_{free}}{[\text{TAFIa}]_{total} - [\text{TAFIa}]_{free}}. \qquad \text{(Eq. 12)}$$

When $[I]_{total} \gg [\text{TAFIa}]_{total}$ (*i.e.* in the case of GEMSA), then $[I]_{free} \cong [I]_{total}$. Thus,

$$K_I = \frac{[\text{TAFIa}]_{free} \times [I]_{total}}{[\text{TAFIa}]_{total} - [\text{TAFIa}]_{free}}. \qquad \text{(Eq. 13)}$$

Solving for $[\text{TAFIa}]_{free}$ yields,

$$[\text{TAFIa}]_{free} = \frac{[\text{TAFIa}]_{total} \times K_I}{[I]_{total} + K_I} \qquad \text{(Eq. 14)}$$

and dividing out by $[\text{TAFIa}]_{total}$, we obtain the fraction of TAFIa free (f_{TAFIa}),

$$f_{\text{TAFIa}} = \frac{1}{1 + ([I]_{total}/K_I)}. \qquad \text{(Eq. 15)}$$

Substitution of Equation 15 into Equation 7 yields the exponential decay (Equation 16) and the half-life (Equation 17) of TAFIa in the presence of a competitive inhibitor when $[I] \gg [\text{TAFIa}]$,

$$[\text{TAFIa}] = [\text{TAFIa}]_0 e^{-kt/(1+[I]/K_I)} \qquad \text{(Eq. 16)}$$

$$t_{1/2} = t^0_{1/2} + t^0_{1/2} \frac{[I]}{K_I} \qquad \text{(Eq. 17)}$$

where $t^0_{1/2}$ is the half-life of TAFIa in the absence of inhibitor. A plot of $t_{1/2}$ *versus* the concentration of inhibitor yields a straight line whose intercept equals $t^0_{1/2}$ and whose slope equals $t^0_{1/2}/K_I$. The data from the experiments using GEMSA were fit globally to Equation 16 using the Systat program (SPSS Inc., Chicago, IL).

The Effect of PTCI on the Decay of TAFIa—TAFIa (20 nM) in HBST/5 mM $CaCl_2$ was incubated at 37 °C in the presence of PTCI (0–70 nM). Samples (400 μl) were taken at 0, 5, 10, 25, and 60 min and placed on wet ice to prevent further decay. The TAFIa activity of each sample was determined at the end of the experiment, using the chromolytic TAFIa substrate FAAK, as follows. Duplicate samples (180 μl) were mixed

with 20 μl of FAAK (10 mM, in HBST) in the wells of a microtiter plate, and the absorbance of the reactions at 340 nm was monitored continuously over 3 h at 26 °C. The initial rate of absorbance decrease for each reaction was determined.

Modeling the Decay of TAFIa When the Competitive Inhibitor Is Not in a Large Excess—According to Reaction 1, the decay of TAFIa is dependent on $[TAFIa]_{free}$, which is determined from the equilibrium binding equation (Equation 10). In the case of the experiments using PTCI, however, the assumption that $[I]_{free} \cong [I]_{total}$ is not valid, and therefore, unlike the case where GEMSA was used, the fraction of TAFIa that is $TAFIa_{free}$ changes over time. Thus, our experiments using PTCI were not modeled using Equations 15–17. However, from Equation 4, the rate of decay is simply proportional to $[TAFIa]_{free}$. Using the mass action relationship in Equation 11, Equation 10 can be rewritten as follows.

$$K_I = \frac{([TAFIa]_{total} - [TAFIa]_{bound}) \times ([I]_{total} - [TAFIa]_{bound})}{[TAFIa]_{bound}} \quad \text{(Eq. 18)}$$

Solving for $[TAFIa]_{bound}$ yields a quadratic whose solution is,

$$[TAFIa]_{bound} = 0.5 \times (K_I + [TAFIa]_{total} + [I]_{total} - \sqrt{(K_I + [TAFIa]_{total} + [I]_{total})^2 - 4([TAFIa]_{total} \times [I]_{total})}) \quad \text{(Eq. 19)}$$

substitution into Equation 4 (using Equation 11) yields the generalized rate of TAFIa decay in the presence of a competitive inhibitor.

$$\frac{d[TAFIa]}{dt} = -k \times [[TAFIa]_{total} - 0.5 \times (K_I + [TAFIa]_{total} + [I]_{total} - \sqrt{(K_I + [TAFIa]_{total} + [I]_{total})^2 - 4([TAFIa]_{total} \times [I]_{total})})] \quad \text{(Eq. 20)}$$

The data from the experiments using PTCI were fit iteratively to Equation 20. Briefly, $[TAFIa]_{free}$ was determined at time = t from $[TAFIa]_{total}$, $[I]_{total}$, and K_I; $[TAFIa]_{free}$ then decayed exponentially for dt seconds with decay constant k; $[TAFIa]_{total}$ at time $t + dt$ was determined by subtracting the decayed TAFIa; $[TAFIa]_{free}$ was determined at time = $t + dt$. This cycle was repeated for a total time of 60 min using a time interval (dt) of 0.1 s. The decay constant k (and thus $t^0_{1/2}$ for TAFIa) and the K_I value for PTCI were determined by non-linear regression of the observed data to the iteratively calculated data (varying k and K_I) using the Solver tool in Microsoft Excel (Redmond, WA).

Intrinsic Fluorescence of TAFIa—The intrinsic fluorescence of TAFIa was monitored over time at 30 °C or 37 °C in the presence and absence of both GEMSA (20, 40, 50, and 100 μM) or PTCI (120, 180, and 240 nM). In some experiments, pre-activated TAFIa (4 μM) was added to HSBT/5 mM $CaCl_2$ in a stirred, thermostatted, acrylic cuvette in the presence and absence of GEMSA and PTCI to give a final TAFIa concentration of 100 nM. In other experiments, 100 nM TAFI in HBST/5 mM $CaCl_2$ was activated to TAFIa by 50 nM thrombin, 50 nM TM in the presence and absence of GEMSA and PTCI in a stirred, thermostatted, acrylic cuvette. The activation was initiated by the addition of thrombin. Activation of TAFI to TAFIa under these conditions was essentially complete by 60 s. The thrombin in the reaction was quenched with PPAck (275 nM final) at 90 s. The fluorescence intensity of the samples was monitored in a PerkinElmer Life Sciences LS-50B fluorescence spectrophotometer using λ_{ex} = 280 nm, slit = 5 nm, λ_{em} = 340 nm, slit = 4.5 nm with a 290-nm emission filter in place. Samples (110 μl) were removed at various times from the reactions, added to 110 μl of ice-cold HBST/5 mM $CaCl_2$, placed on ice until the end of the experiment, and then assayed for FAAK activity as described above.

Determination of the K_I for GEMSA and PTCI with TAFIa—TAFIa (10 nM) was mixed with GEMSA (0, 5, 10, 20, 50, and 100 μM) or PTCI (0, 10, 20, 30, 40, and 50 nM), and the initial rate of hydrolysis of FAAR (0.5, 1, 1.5, 2, 2.5, and 3 mM) in the presence of the various concentrations of inhibitor was determined as above. The data was non-linearly regressed to the Michaelis-Menten equation describing competitive inhibition,

$$\text{rate} = \frac{V_{max} \times [FAAR]}{K_M\left(1 + \frac{[I]}{K_I}\right) + [FAAR]} \quad \text{(Eq. 21)}$$

where I is the concentration of the inhibitor, using the Systat program.

RESULTS

The Effect of the Competitive Inhibitors GEMSA and PTCI on the tPA-induced Lysis of Clots Formed from NHP—The lysis of clots formed from NHP in the presence of various concentrations of tPA was studied in the presence and absence of various concentrations of GEMSA and PTCI, two competitive inhibitors of TAFIa. Fig. 1 shows the variable effect of GEMSA (*panel A*) and PTCI (*panel B*) on the lysis of clots in NHP with 0.08 μg/ml tPA. Compared with the lysis profiles seen in the absence of inhibitors (Fig. 1, *circles*), both inhibitors could either prolong (*squares*) or shorten (*triangles*) lysis, depending on the concentration of the inhibitor. The concentration dependence of tPA on inhibitor-mediated prolongation of lysis was investigated. Fig. 2 shows the effect of GEMSA (*panel A*) and PTCI (*panel B*) on the lysis time of clots formed from NHP using 0.02–0.08 μg/ml tPA. In addition, the figure shows the lysis time in the presence of GEMSA (*panel C*) and PTCI (*panel D*) relative to the lysis time in the absence of inhibitor. The figure also shows that both competitive inhibitors exhibit a complex, bell-shaped behavior with respect to plasma clot lysis. Over the concentration ranges of tPA and inhibitor used, both GEMSA (*panel C*) and PTCI (*panel D*) can prolong, shorten, or display no effect on the lysis time. For example, at a PTCI concentration of 100 nM, the lysis times were prolonged 3.1-fold, 2.0-fold, and 1.4-fold at 0.02, 0.04, and 0.08 μg/ml tPA, respectively. However, at 500 nM PTCI, the lysis times were longer at 0.02 μg/ml tPA (1.5-fold) but shorter at 0.04 and 0.08 μg/ml tPA (0.78-fold and 0.48-fold, respectively). The data show that the effect (prolongation or shortening) of a competitive inhibitor on the lysis time of clots formed from NHP is dependent on the concentration of both the inhibitor and tPA, with lower concentrations of tPA promoting the prolongation of lysis at any given inhibitor concentration.

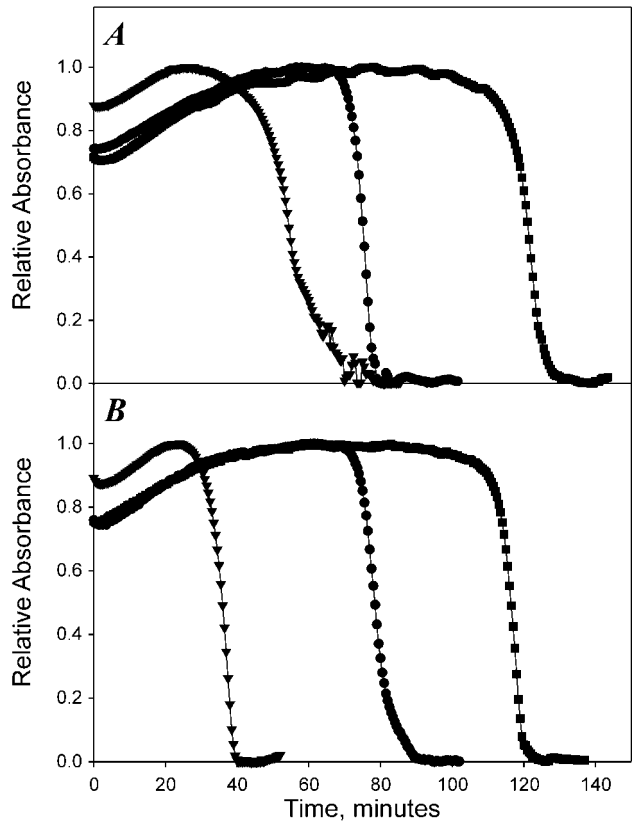

FIG. 1. **Competitive inhibitors of TAFIa can prolong or shorten fibrinolysis.** Representative absorbance profiles of clots formed from NHP in the absence (●) or presence of either GEMSA (*A*, 100 μM (■), 1000 μM (▼)) or PTCI (*B*, 50 nM (■), 500 nM (▼)) at 0.08 μg/ml tPA. The figure shows that, depending on the concentration of inhibitor, both GEMSA and PTCI can prolong or shorten tPA-induced fibrinolysis.

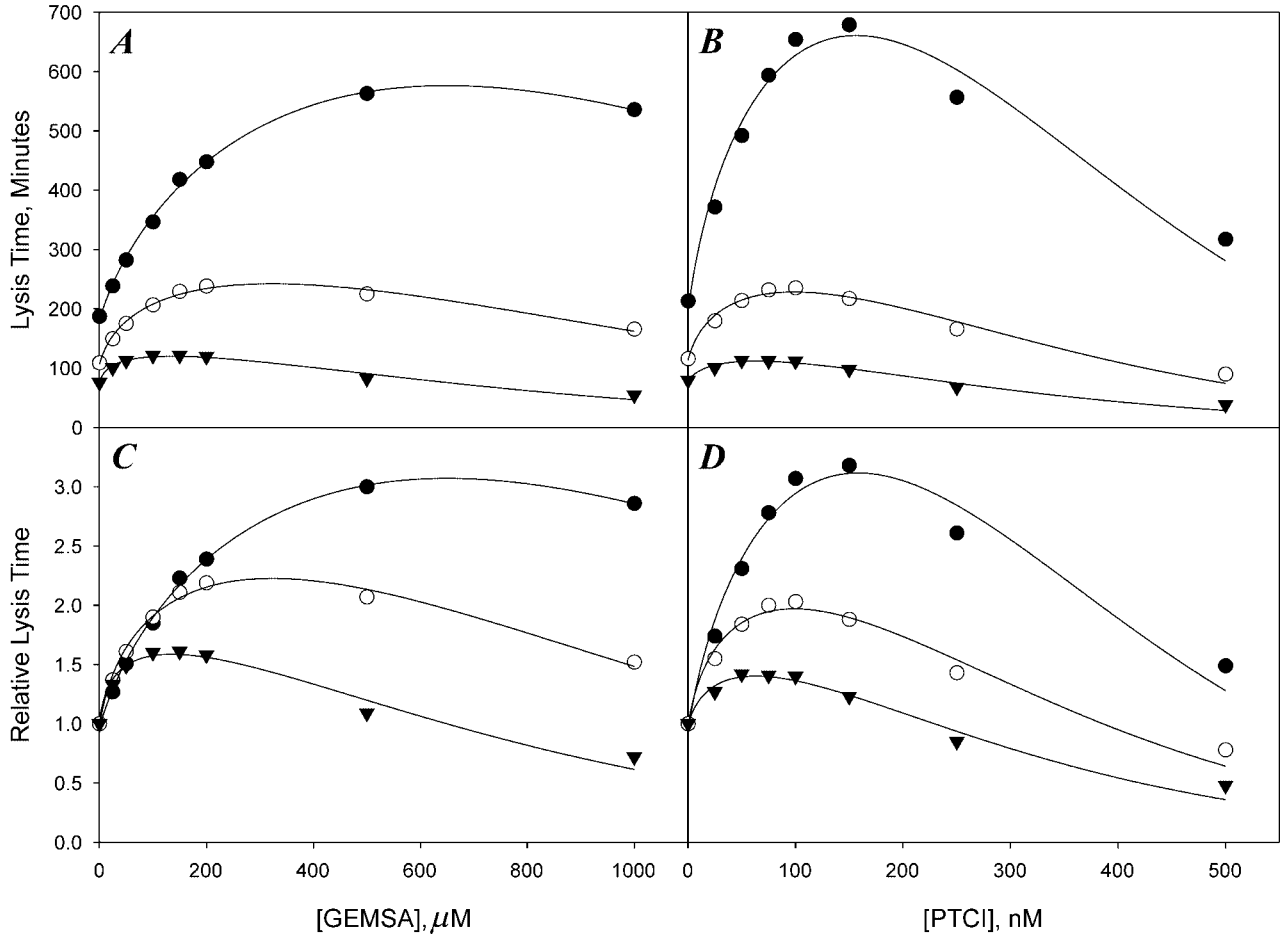

FIG. 2. **The effect of competitive inhibitors on lysis is dependent on both the inhibitor and tPA concentrations.** Clots were formed from NHP in the presence of various concentrations of either GEMSA (*A* and *C*) or PTCI (*B* and *D*) at 0.02 (●), 0.04 (○), and 0.08 (▼) μg/ml tPA. The data (average of duplicates) are presented as the absolute (*A* and *B*) and relative (*C* and *D*) lysis times at each concentration of tPA and inhibitor. The figure shows that both inhibitors display complex behavior over the range of tPA and inhibitor concentrations used and that, for any given inhibitor concentration, decreasing tPA promotes the inhibitor-dependent prolongation of fibrinolysis.

The Inhibitor-induced Prolongation Observed in Plasma Is TAFIa-dependent—In order to verify that the inhibitor-induced prolongation was both specific for TAFIa and not due to differences in the rates of TAFIa formation by thrombin-TM, the lysis times of clots formed from TdP supplemented with 0.02 μg/ml tPA and various concentrations of TAFIa were determined in the absence and presence of either GEMSA or PTCI. Fig. 3 shows that in the absence of GEMSA (*panel A*) and PTCI (*panel B*), the TAFIa-dependent prolongation is saturable, with the half-maximal effect observed below 5 nM. The dependence of lysis time on the inhibitor concentration displayed similar behavior for each TAFIa concentration; as the inhibitor concentration is increased from zero the lysis time increases to some maximum value, the magnitude of which increases with increasing TAFIa concentration. Once the maximum lysis time is attained, any further increase in the inhibitor concentration decreases the lysis time. To understand the behavior of the system with respect to TAFIa concentration, we plotted the maximum prolongation observed, relative to the lysis time seen in the absence of TAFIa, *versus* the input TAFIa concentration. As shown in Fig. 4, the maximum prolongation observed at each TAFIa concentration in the presence of both GEMSA and PTCI appears saturable with respect to TAFIa concentration. Thus, the data from each inhibitor was non-linearly regressed, using the Systat program, to the rectangular hyperbola,

$$P_{[\mathrm{TAFIa}]} = 1 + \frac{P_{\max} \times [\mathrm{TAFIa}]}{T_{\mathrm{HalfMax}} + [\mathrm{TAFIa}]} \qquad \text{(Eq. 22)}$$

where $P_{[\mathrm{TAFIa}]}$ is the maximum relative prolongation observed at a given TAFIa concentration, $P_{\max}$ is the maximal relative prolongation (*i.e.* the lysis time expected at infinite TAFIa concentration relative to the lysis time seen in the absence of TAFIa), and T_{HalfMax} is the TAFIa concentration required to yield a prolongation equal to one-half of $P_{\max}$. The regression yielded $P_{\max}$ values of 23 ± 3-fold and 21 ± 3-fold and T_{HalfMax} values of 85 ± 17 and 110 ± 24 nM for GEMSA and PTCI, respectively. It should be noted that the maximum prolongation observed at any given TAFIa concentration is not dependent on the inhibitor K_I but on the concentration of inhibitor present relative to K_I, *i.e.* the maximum prolongation occurs when the inhibitor is present at some -fold over K_I. Thus, the values of $P_{\max}$ and T_{HalfMax} are also independent of K_I. The regression curves are shown in Fig. 4.

The Effect of GEMSA and PTCI on the Activity and Spontaneous Decay of TAFIa—TAFIa was incubated with various concentrations of either GEMSA or PTCI at 37 °C and timed samples were taken and assayed for TAFIa activity. Fig. 5 shows the TAFIa activity (*panels A* and *B*) and the fraction of initial TAFIa activity (*panels C* and *D*) *versus* the time of incubation at 37 °C in the presence of various concentrations of GEMSA (*panels A* and *C*) and PTCI (*panels B* and *D*). The figure shows the data points (*symbols*) as well as the fits of the data (*lines*) to Equation 16 for GEMSA and, iteratively, to Equation 20 for PTCI. That the iterative approach provided reasonable estimates of $t^0_{1/2}$ and K_I for the experiments using

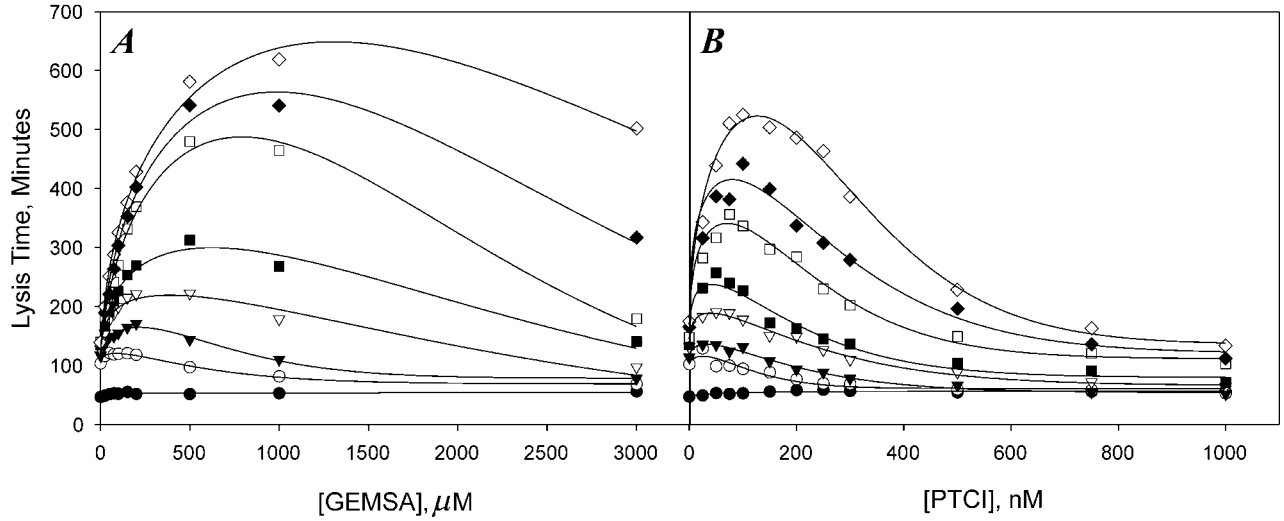

FIG. 3. **The inhibitor-induced prolongation of fibrinolysis observed with both GEMSA and PTCI is TAFIa-dependent.** Clots were formed from TdP supplemented with 0.02 μg/ml tPA and various concentrations of TAFIa (0 nM (●), 5 nM (○) 10 nM (▼), 20 nM (▽), 30 nM (■), 50 nM (□), 70 nM (◆), and 100 nM (◇)), in the presence and absence of various concentrations of either GEMSA (*A*) or PTCI (*B*). The data show that the prolongation of the lysis times for both inhibitors is TAFIa-dependent and that the maximum prolongation is dependent on the TAFIa concentration.

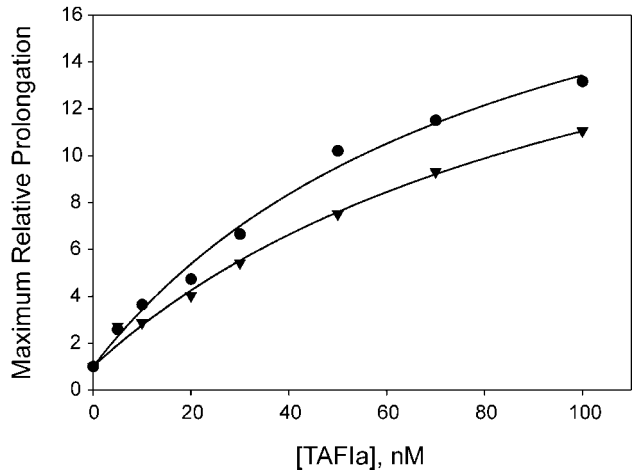

FIG. 4. **The prolongation effect is saturable with respect to TAFIa.** The maximum relative prolongation (the largest -fold increase in lysis time observed in the presence of an inhibitor) at each TAFIa concentration in Fig. 3 was plotted against the input TAFIa concentration for the experiments using GEMSA (●) and PTCI (▼). The figure shows that the maximum relative prolongation is a function of TAFIa concentration and that this effect is saturable. The *lines* show the fit of the data to a rectangular hyperbola (Equation 22) and indicate that the maximal relative prolongation for TAFIa (*i.e.* -fold prolongation at infinite TAFIa) is ~20-fold.

PTCI is supported by the agreement between the estimates obtained for GEMSA using Equation 16 ($t^0_{1/2}$ = 9.2 min, K_I = 36 μM) and those obtained, iteratively, using Equation 20 ($t^0_{1/2}$ = 9.1 min, K_I = 41 μM). The figure shows that the inhibition of TAFIa activity by GEMSA and PTCI (*panels A* and *B*) is accompanied by a stabilization of TAFIa (*panels C* and *D*), resulting in a decreased rate of TAFIa decay. The values for $t^0_{1/2}$ and K_I derived from the decay experiments, as well as the measured K_I values for both inhibitors, are shown in Table I. To determine the relationship between the inhibitor concentration, the inhibitor K_I, and the decay of TAFIa for both GEMSA and PTCI, we determined the apparent half-life of TAFIa ($t^{app}_{1/2}$) at each inhibitor concentration by fitting each decay curve (Fig. 5, *C* and *D*) to an exponential decay (Equation 1) and plotted $t^{app}_{1/2}$ *versus* the fraction of TAFIa bound at each inhibitor concentration. Although the decay of TAFIa activity with PTCI was not truly exponential (see "Experimental Procedures"), the fit curves (Fig. 5*D*, *dashed lines*) show that a single exponential closely approximates the decay, and, therefore, the derivation of $t^{app}_{1/2}$ by this analysis is reasonable. Fig. 6 shows the effect of GEMSA (*circles*) and PTCI (*triangles*) on the apparent half-life of TAFIa *versus* the fraction of TAFIa bound at each of the inhibitor concentrations. The fraction of TAFIa bound was calculated from Equation 19 using K_I values derived from the models (*closed symbols*) or from K_I values determined independently (*open symbols*). The figure shows that, regardless of the method used to determine the K_I, both inhibitors exert the same effect on the decay of TAFIa when the inhibitors are used at concentrations that yield the same fraction of TAFIa bound. Therefore, the extent of stabilization of TAFIa by any given concentration of a competitive inhibitor is directly related to K_I.

DISCUSSION

It is well established that the inhibition of TAFIa, an attenuator of fibrinolysis, results in the potentiation of tPA-induced clot lysis *in vitro* (17, 20, 21). We have observed, however, that two competitive inhibitors of TAFIa, GEMSA and PTCI, can be antifibrinolytic, profibrinolytic, or show no effect on fibrinolysis. Furthermore, for a given TAFIa concentration, the effect observed at any given concentration of the inhibitor is dependent on the tPA concentration, with lower tPA concentrations favoring the antifibrinolytic effect. Of particular note is the observation that the maximal prolongation in lysis time is dependent on the TAFIa concentration for both GEMSA and PTCI. An analysis of the TAFIa concentration dependence on the prolongation of lysis time showed that 1) the concentration of inhibitor at which the prolongation is greatest increases as TAFIa concentration increases and 2) the prolongation of lysis is saturable with respect to TAFIa. By fitting the maximum prolongation observed at each concentration of TAFIa to a rectangular hyperbola, we determined the maximal effect of TAFIa, *i.e.* the prolongation expected at infinite TAFIa, to be a 20-fold prolongation of the lysis time seen in the absence of TAFIa. To our knowledge, this represents the first quantitative estimate of the maximal antifibrinolytic activity of TAFIa in an *in vitro* static system.

Because the phrase "inhibitor-mediated potentiation of enzyme activity" appears contradictory, we sought a mechanism that could account for the paradoxical effects of competitive

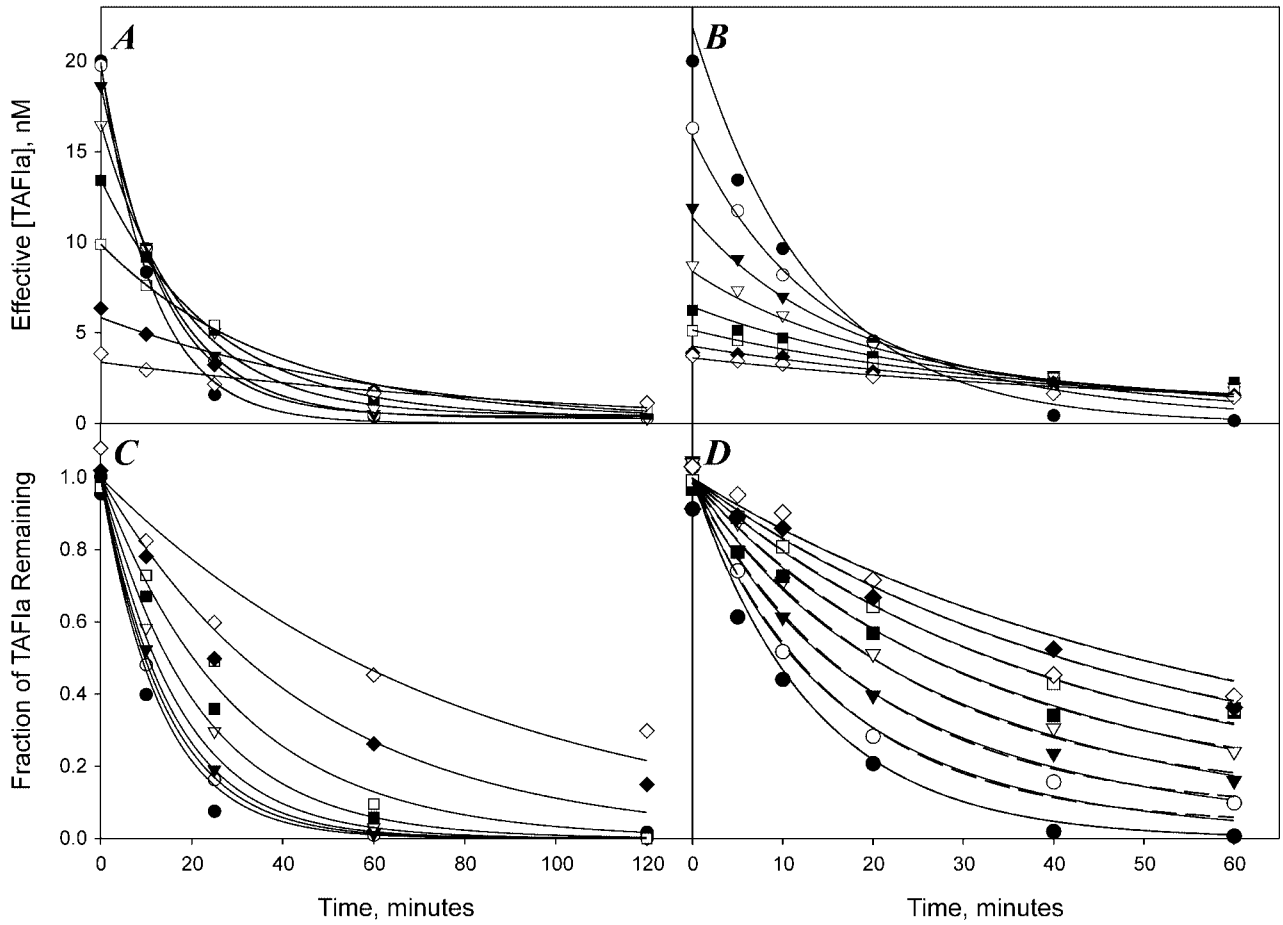

FIG. 5. **The inhibition of TAFIa activity by GEMSA and PTCI is concomitant with a decrease in the rate of TAFIa decay.** TAFIa (20 nM) was incubated with various concentrations of either GEMSA (*A* and *C*; 0 μM (●), 3.1 μM (○), 6.3 μM (▼), 12.5 μM (▽), 25 μM (■), 50 μM (□), 100 μM (♦), and 200 μM (◇)) or PTCI (*B* and *D*; 0 nM (●), 10 nM (○), 20 nM (▼), 30 nM (▽), 40 nM (■), 50 nM (□), 60 nM (♦), and 70 nM (◇)) at 37 °C for various periods of time and then assayed, in duplicate, for TAFIa activity. The *solid lines* in *A* and *B* show the fit of the data to Equation 16 (GEMSA, *A*) or Equation 20 (PTCI, *B*). The *solid lines* in *C* and *D* show the decay of TAFIa in the presence of GEMSA (*C*) and PTCI (*D*) calculated from the fit curves in *A* and *B*. *Panel D* also shows the deviation of the curves derived using Equation 20 (*solid lines*) from a true single exponential decay (*dashed lines*). The figure shows that the concentration-dependent inhibition of TAFIa activity by GEMSA and PTCI is accompanied by a concentration-dependent stabilization of TAFIa.

TABLE I
The half-life of TAFIa and the K_I for GEMSA and PTCI

The table shows the half-life of TAFIa and the K_I value for each inhibitor determined from modeling the data from the experiments to Equation 16 (GEMSA) using the Systat program (±S.E.) or Equation 20 (PTCI) using the Solver program (Microsoft Excel). The values determined experimentally according to Equation 21 using the Systat program (±S.E.) are shown for comparison.

	Calculated		Measured
	$t^0_{1/2}$ of TAFIa	K_I	K_I
	min	*nM*	
GEMSA	9.2 ± 0.2	35,800 ± 1,800	13,700 ± 1,900
PTCI	9.1[a]	10.2[a]	3.7 ± 0.6

[a] The Solver program (Microsoft Excel) used to iteratively calculate the values for PTCI does not return standard error values.

inhibitors of TAFIa on tPA-induced fibrinolysis. TAFIa decays spontaneously, an intrinsic property not observed with pancreatic carboxypeptidase B or plasma carboxypeptidase N, and previous work has shown that GEMSA (14, 22) and ϵ-aminocaproic acid (22–24), both competitive inhibitors of TAFIa, stabilize the activity of TAFIa toward chromogenic substrates when used at saturating concentrations. In addition, natural isoforms of TAFI, which, upon activation, vary in their intrinsic stability have been characterized and show antifibrinolytic effects correlating with their intrinsic stability (25). Therefore, we investigated the possibility that the prolongation of lysis by inhibitors of TAFIa directly results from stabilization of TAFIa activity. Both GEMSA and PTCI decreased the activity (Fig. 5, *A* and *B*) and concurrently increased the stability (Fig. 5, *C* and *D*) of TAFIa activity in a concentration-dependent manner. Because both GEMSA and PTCI inhibit TAFIa activity by occupying the active site (*i.e.* competitively), we therefore attempted to rationalize the stabilization of TAFIa activity according to a model where inhibitor-bound TAFIa is protected from spontaneous decay.

Two models were proposed to account for the stabilization of TAFIa activity. In the first model, only free TAFIa is allowed to decay, and therefore decay is active site-dependent. In the second model, both free TAFIa and inhibitor-bound TAFIa are allowed to decay independently, and therefore decay is both active site-dependent (TAFIa) and active site-independent (inhibitor-bound TAFIa). Applying a model with a single decay event, in which only the free TAFIa can decay, we obtained a good global fit to the data for both GEMSA and PTCI (Fig. 5). The model describes well the observed decrease in TAFIa activity (Fig. 5, *A* and *B*) and the associated stabilization of the enzyme by each inhibitor (Fig. 5, *C* and *D*). The model returned calculated $t^0_{1/2}$ values for TAFIa at 37 °C of 9.2 (GEMSA) and 9.1 (PTCI) min, in good agreement with those reported by others (8.7 (14), 9.1 (22), and 8.0 (25) min), as well as reasonable estimates of the K_I values for both inhibitors (GEMSA = 36 μM, PTCI = 10 nM) when compared with their measured

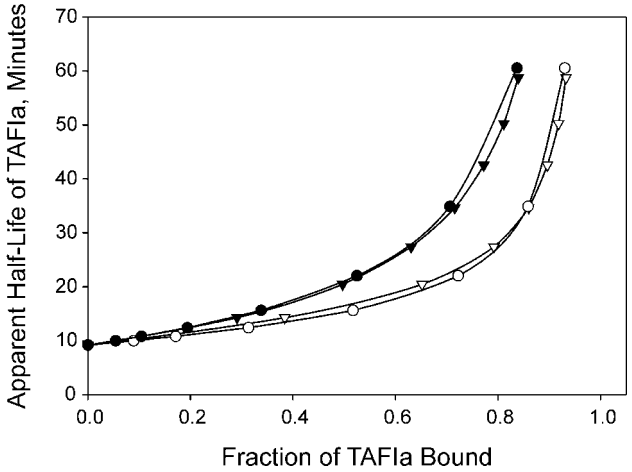

FIG. 6. **The effect of GEMSA and PTCI on the apparent half-life of TAFIa.** The apparent half-life of TAFIa, calculated for each concentration of GEMSA (●, ○) and PTCI (▼, ▽), from the decay curves in Fig. 5 (*C* and *D*), was plotted against the fraction of TAFIa bound, calculated from Equation 19 using the K_I values derived from the models (●, ▼) or determined independently (○, ▽). The figure shows that both GEMSA and PTCI have equivalent effects on the stability of TAFIa when the enzyme is expressed as the fraction bound to the inhibitor.

values (GEMSA = 14 μM, PTCI = 4 nM). When the data from the experiments with both inhibitors were analyzed according to the second model, the best fit was obtained when the rate constant for the decay of inhibitor-bound TAFIa was zero, *i.e.* interpretation of the results using this model suggests that the effect, if any, of the decay of inhibitor-bound TAFIa was inconsequential under our experimental conditions (data not shown). Although our analysis was not exhaustive, we interpreted our data according to the simpler of the two models, because no improvement was found when inhibitor-bound TAFIa was assumed to decay.

Although the derived K_I value for each of the inhibitors studied differs by greater than three orders of magnitude, an analysis of the apparent half-life of TAFIa *versus* inhibitor concentration (Fig. 6) showed that GEMSA and PTCI prolonged the apparent half-life of TAFIa to the same extent when TAFIa was expressed as the fraction bound to the inhibitor. Thus, the degree of stabilization conferred on TAFIa by any given concentration of GEMSA and PTCI is directly related to the K_I of the inhibitor. Furthermore, the stabilization effect seen with both inhibitors is not saturable (Fig. 6), suggesting that inhibitor-bound TAFIa is protected from spontaneous decay as opposed to simply decaying at a slower rate than free TAFIa. Thus, the spontaneous decay of TAFIa in the presence of competitive inhibitors is consistent with a model whereby the conformational change underlying the thermolability of TAFIa requires a free active site and, as a corollary, that active-site bound TAFIa does not decay. Whether or not TAFIa is similarly stabilized in the presence of its primary substrates, plasmin-degraded fibrin and fibrin-degradation products, remains to be investigated.

That TAFIa bound to a competitive inhibitor is protected from decay is consistent with previous findings that showed the decay of the intrinsic fluorescence of TAFIa to be dose-dependently stabilized by GEMSA (14). The model described in the previous study employed a two-step, sequential first-order exponential decay process in which both decay events are stabilized, if not eliminated, by GEMSA (14). In contrast to the fluorescence decay, the authors found that the TAFIa activity decayed as a single exponential and proposed that the activity decayed according to the second, slower transition (14). We performed similar fluorescence decay experiments in the absence and presence of GEMSA and PTCI. In agreement with the previous study, both inhibitors stabilized the intrinsic fluorescence of TAFIa in a dose-dependent manner with an accompanying stabilization of TAFIa activity (data not shown). However, no one single mathematical relationship was found that directly correlated either single or sequential exponential decay of fluorescence with the TAFIa activity stabilization data. In addition, we observed several differences between the two studies. First, we consistently observed only a 10–15% total decrease in intrinsic fluorescence upon activity decay, significantly less than what has been reported (38% (22), 51% (14)). Second, we did not consistently observe the presence of two exponential fluorescence decay events. Because TAFIa activity has consistently been shown to decay according to a single exponential (14, 22, 25), we have described the stabilization of TAFIa activity according to a model with a single decay event. Parenthetically, even though we cannot rule out the two-step model of decay with certainty, due to the apparent rapid rate of the first decay and the relatively small fluorescence change associated with it, following a two-step model would not significantly alter the interpretation of our data. The magnitude of the difference between the rates of the first (0.50 min^{-1}) and second (0.064 min^{-1}) transitions in concert with the greater affinity of the inhibitor (GEMSA) for the second form (K_D = 6 μM) compared with the first form (K_D = 14 μM) of TAFIa would result in the nearly quantitative accumulation of the second form in both the presence and absence of the inhibitor (14). As a consequence, the loss of activity and the stabilization of TAFIa in such a two-step model can effectively be described by a single exponential decay event of the free TAFIa.

It could be argued that the competitive inhibitors stabilize TAFIa by binding at sites distinct from the active site. This scenario is unlikely, because it would be consistent with our data only if 1) the ratio of the K_D values for the exosite(s) for both inhibitors was, fortuitously, the same as the ratio of the K_I values for the active site and 2) the K_D values for the exosite(s) were essentially indistinguishable from the K_I values, because both inhibitors express their effects at concentrations approximating their respective K_I values (Fig. 6). Alternatively, one could envisage a scenario in which the competitive inhibitors stabilize TAFIa by attenuating TAFIa degradation by proteolysis. For example, a recent report (26) suggests that plasmin, in contrast to thrombin (14, 24), directly inactivates TAFIa. However, we found little, if any, significant reduction in the activity of TAFIa incubated in the absence and presence of 100 nM plasmin at either 25 °C or 37 °C: samples taken during a 1-h time course yielded a half-life of 22 *versus* 19 min at 25 °C and 6.9 *versus* 6.1 min at 37 °C in the absence and presence of 100 nM plasmin, respectively. It is unlikely that the steady-state concentration of plasmin would approach even 10 nM during fibrinolysis under the conditions used in our study, due to the low tPA concentrations used and the presence of α_2-antiplasmin in plasma. Furthermore, were plasmin capable of significant proteolytic inactivation of TAFIa, the generation of significant levels of plasmin is not temporally coincident with the period of TAFIa activity (14). Finally, protection from proteolytic inactivation, by itself, would not extend the half-life of TAFIa beyond that imposed by thermal instability. For these reasons, proteolytic inactivation of TAFIa likely represents a minor pathway for inactivation in comparison to thermal instability, consistent with the conclusions of others (14, 24, 25). As a result, we conclude that the inhibitor-mediated stabilization of TAFIa observed in functional assays, both chromogenic and clot lysis experiments, occurs via prevention of the spontaneous decay of TAFIa resulting from intrinsic thermal instability.

Marx *et al.* (27) have recently shown that the catalytic Zn^{2+} ion remains associated with the decayed TAFIa*i*, indicating that loss of Zn^{2+} is not involved in the conformational change underlying the thermal instability of TAFIa. Because proteolytic inactivation of TAFIa is also not required (14, 24), the protective effect of competitive inhibitors is likely to involve direct interactions with residues in the active site. The crystal structures of bovine carboxypeptidase A (CPA) bound to PTCI (28) and of duck carboxypeptidase D (CPD) domain II (CPD-II) in complex with GEMSA (29) show that both inhibitors form extensive contacts with residues at the active site. Both inhibitors directly coordinate the catalytic Zn^{2+} ion and form numerous contacts with residues involved in coordinating the peptide substrate (28, 29). GEMSA remains intact in CPD-II and forms contacts with active site residues involved in substrate binding, both proximal and distal to the scissile bond (29). In contrast, PTCI is cleaved at its C terminus (Val^{38}-Gly^{39}) by CPA, and the free Gly^{39} remains in the active site, at least in the crystal structure (28). Although nearly all residues in the tail region of PTCI are involved in complex formation, the new C-terminal Val^{38} contributes half of the binding energy to the PTCI·CPA complex and directly coordinates the Zn^{2+} ion (28, 30). Thus, whereas GEMSA inhibits CPD-II as a non-hydrolyzable substrate, PTCI inhibits CPA as a tight-binding product. Although TAFIa differs significantly from both CPA and CPD-II, not only in sequence but also in stability, and it is not known whether PTCI is C-terminally processed by TAFIa, it is likely that TAFIa interacts with both PTCI and GEMSA in a manner similar to CPA and CPD-II, respectively. This assertion stems from the comparison of metallocarboxypeptidases from different species with different substrate specificities (CPD (duck and human), carboxypeptidase E (human), CPA (bovine), and carboxypeptidase B (bovine)) showing a highly conserved active site (29), with the differences in substrate specificity primarily attributed to the insertion/deletion of residues in the funnel which leads to the active site (29, 31). The binding energy involved in the inhibitor-TAFIa interactions may exert a stabilizing effect by maintaining the intrinsically unstable positions of critical residues in the active site. It is also possible, however, that stability is conferred by a simple steric effect: spontaneous decay may require the "space" occupied by the inhibitors. In either case, a detailed, structure-based mechanism describing TAFIa stabilization is likely to be provided only when crystal structure of TAFI, TAFIa, and/or TAFIa*i* have been solved.

The tPA dependence of the inhibitor-induced effect can be rationalized on the basis that the inhibition of TAFIa is concomitant with the stabilization of TAFIa. This is best illustrated by considering fibrinolysis in clotted plasma, in the absence of an inhibitor, when the lysis time is very long relative to the half-life of TAFIa, *e.g.* low [tPA], and TAFI activation is rapid and quantitative. Because the concentration of plasminogen activator is low, the intrinsic rate of plasmin generation on intact fibrin is low. Thus, the concentration of TAFIa required to maximally inhibit plasminogen activation ($TAFIa_{max}$), by removing all C-terminal lysines and arginines before they can be used as cofactors, represents a small fraction of the initial TAFIa concentration. However, because the lysis time is very long relative to the half-life of TAFIa, the TAFIa will decay to a concentration less than $TAFIa_{max}$ long before lysis is complete, even if TAFIa is initially present at high concentrations. In other words, TAFIa is not present at a concentration sufficient for complete attenuation of plasminogen activation for the duration of fibrinolysis.

In the presence of a competitive inhibitor the active concentration of TAFIa (*i.e.* $[TAFIa]_{free}$) is decreased but the half-life is increased. Because $TAFIa_{max}$ is only a small fraction of the initial TAFIa concentration, the inhibitor can be present at high concentrations (relative to K_I) and still leave the initial $[TAFIa]_{free}$ at a concentration above $TAFIa_{max}$. Thus, TAFIa activity can be present at an effective level for a longer period of time than is the case when no inhibitor is present. Therefore, for any given TAFIa concentration, the peak of the curve in Fig. 3 represents the concentration of inhibitor at which both the initial $[TAFIa]_{free}$ and the half-life of TAFIa are balanced such that effective TAFIa activity is present for the maximal time. At concentrations of inhibitor less than the peak amount, the TAFIa decays more quickly, reducing the inhibitor-dependent prolongation of lysis, whereas at greater concentrations of the inhibitor, the initial $[TAFIa]_{free}$ is reduced, thereby diminishing the prolongation effect.

Now consider the two effects that occur as the tPA concentration increases, again in the absence of an inhibitor. First, the intrinsic rate of plasmin generation on intact fibrin increases and, therefore, the concentration of TAFIa required to maximally attenuate plasminogen activation also increases, *i.e.* $TAFIa_{max}$ increases. Second, the lysis time is reduced relative to the half-life of TAFIa. Therefore, TAFIa will decay through fewer half-lives before the lysis time is reached. Consequently, the effect of TAFIa becomes more dependent on the initial TAFIa concentration and less dependent on the half-life of TAFIa. In other words, as tPA concentration increases, more TAFIa is required, but for a shorter period of time. This effect can be seen in the curves shown in Fig. 2. At any fixed concentration of inhibitor, the relative lysis time (*panels C* and *D*) decreases as the concentration of tPA increases. This results from the stabilization of TAFIa being dependent on the inhibitor concentration but independent of the tPA concentration (*i.e.* the fraction of TAFIa bound, and therefore $[TAFIa]_{free}$ is the same regardless of the tPA concentration), whereas the intrinsic rate of plasmin generation, and therefore the intrinsic rate of C-terminal lysine formation on fibrin, increases as tPA concentration increases. Thus, as tPA concentration increases, more TAFIa activity is required to maintain the fibrin surface in a non-stimulatory state (*i.e.* $TAFIa_{max}$ increases). Therefore, at any given inhibitor concentration, $[TAFIa]_{free}$ becomes a smaller fraction of $TAFIa_{max}$ as the concentration of tPA increases.

The work described in this report provides a rationale for the complex behavior of TAFIa observed in the presence of competitive inhibitors. These inhibitors stabilize TAFIa to an extent that is proportional to the fraction of TAFIa bound. The results can be described according to a model where TAFIa can spontaneously decay only if it has a free active site, and thus the stabilization of TAFIa is directly related to the concentration of an inhibitor through K_I. The tPA dependence of the pleiotropic behavior of the inhibitors can be rationalized by considering two quantities: 1) $TAFIa_{max}$, or the amount of TAFIa required to maximally attenuate plasminogen activation, and 2) the lysis time in relation the half-life of TAFIa. The *in vitro* experiments described here indicate that the prolongation of lysis is related to the concentration of inhibitor relative to its K_I and is favored under conditions in which both the concentration of TAFIa is high and that of tPA is low. Caution should be exercised in extending these results to the *in vivo* situation, because the local concentrations of TAFIa, tPA, and inhibitor at the site of a thrombus in the complex, non-static *in vivo* milieu are currently unknown. Although the ramifications of the pleiotropic behavior of TAFIa inhibitors on their potential clinical utility should be addressed using appropriate animal models, no antifibrinolytic effects have been reported using PTCI *in vivo* in animal models to date (8–11). With this caveat in mind,

however, it is likely that choosing an inhibitor with a low K_I will minimize any prolongation of fibrinolysis. Finally, we have provided, for the first time, an estimate of the maximal antifibrinolytic potential of TAFIa *in vitro*.

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Stabilization *Versus* Inhibition of TAFIa by Competitive Inhibitors *in Vitro*

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