Kinetics of the Inhibition of Factor Xa and the Tissue Factor–Factor VIIa Complex by the Tissue Factor Pathway Inhibitor in the Presence and Absence of Heparin†

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ABSTRACT: The inhibition by tissue factor pathway inhibitor (TFPI) of its two target enzymes—factor Xa and the tissue factor–factor VIIa complex (TF:VIIa)—has been studied under near-physiological reactant concentrations and conditions. Over a TFPI range of 0–1 nM, the rate of inhibition of factor Xa, in the presence of Ca^{2+} and anionic phospholipid vesicles at 37 °C, was proportional to TFPI concentration, giving an association rate, k_1, of 0.96×10^9 M^{-1} min^{-1}. Factor Xa inhibition did not proceed to completion, the reaction attaining a near-equilibrium that was dependent on the TFPI concentration. The estimated dissociation rate of the TFPI:Xa complex, k_{-1}, was independent of TFPI concentration, with a mean value of 0.02 min^{-1}. The resulting calculated value of K_1, the apparent dissociation constant for the initial step, is 21 pM. Slow decay of the remaining factor Xa in such incubations, detectable after attainment of the rapid initial near-equilibrium, confirmed the two-step mechanism proposed by Huang *et al.* (1993) [*J. Biol. Chem. 268*, 26950–26955], but did not permit determination of a rate constant for the second step. Omission of anionic phospholipid had no significant effect on either k_1 or k_{-1}. A high-molecular-weight fraction of heparin, at saturating levels (≥0.05 unit/mL, ≃25 nM), increased k_1 2-fold, with no detectable effect on k_{-1}. The second stage of TFPI action was studied by preformation of the TFPI:Xa complex, and its incubation with the TF:VIIa complex in the presence of factor X. Analysis of the kinetics of factor Xa generation in this system at varying levels of the TFPI:Xa complex gave a value for the rate constant of TF:VIIa inhibition, corrected for the competitive effect of factor X, of 0.64×10^9 M^{-1} min^{-1}. Similar experiments performed in the presence of varying levels of heparin showed a reduction in the rate of TF:VIIa inhibition at saturating heparin levels (≥0.1 unit/mL, ≃50 nM), of 65%.

The tissue factor pathway inhibitor (TFPI)[1] inhibits two enzymes in the early stages of coagulation: factor Xa and the tissue factor–factor VIIa (TF:VIIa) complex (Sanders *et al.*, 1985; Warn-Cramer *et al.*, 1987, 1988; Broze & Miletich, 1987). TFPI is unique among coagulation inhibitors in having two inhibitory domains, one for each enzyme (Broze *et al.*, 1988; Girard *et al.*, 1989). It also has a third inhibitor-like domain. While this is apparently not inhibitory and can be deleted without abolishing TFPI function (Hamamoto *et al.*, 1993), the carboxyl terminus of the molecule, which contains a densely cationic region, is required for optimal inhibition of factor Xa (Wesselschmidt *et al.*, 1992). The three domains are homologous and are of the Kunitz protease-inhibitor type. Available data suggest that the second Kunitz domain first combines with, and inhibits, factor Xa, forming a TFPI:Xa complex. This complex then inhibits the TF:VIIa complex, in a reaction involving the first Kunitz domain. The final product is presumed to be a quaternary complex of TF:VIIa:TFPI:Xa (Broze *et al.*, 1990).

Concerning the controls of the tissue factor pathway that may operate in plasma, it is known that both factor VIIa and the TF:VIIa complex are inhibited at minuscule rates by antithrombin III at its plasma concentration (Jesty, 1978; Broze & Majerus, 1980). It is also known that factor VIIa is nearly as stable in plasma as its zymogen, factor VII, with a half-life of more than 1 h (Radcliffe *et al.*, 1977; Seligsohn *et al.*, 1979). Only in the presence of high heparin concentrations in a pure system has significant inhibition of factor VIIa (Broze & Majerus, 1980) or TF:VIIa (Lawson *et al.*, 1993) by plasma concentrations of antithrombin III been observed.

Two groups have recently reported kinetic studies of the inhibition of factor Xa by TFPI (Huang *et al.*, 1993; Lindhout *et al.*, 1994). The former study focuses on the reversibility of the initial step and the subsequent slow transition to a more stable form, while the latter describes the kinetics of inhibition in the absence of Ca^{2+}. In this report we focus on the rapid initial stages of factor Xa inhibition by TFPI in the presence of Ca^{2+}, anionic phospholipid, and heparin.

Concerning the second stage of TFPI action—the inhibition of TF:VIIa by the TFPI:Xa complex—it is qualitatively clear from several reports that the reaction is very fast, but the quantitative kinetics are essentially unknown.

The question of the effect of heparin on TFPI action—on both its target enzymes—is an important one. Some studies in plasma-based systems have suggested that heparin's anticoagulant function may in part be due to its potentiating the action of TFPI (*e.g.*, Abildgaard *et al.*, 1991; Abildgaard, 1992; Wun, 1992). However, because TFPI action involves combination with factor Xa, and plasma contains a potent,

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[1] Abbreviations: TFPI, tissue factor pathway inhibitor; FPRck, D-Phe-L-Pro-L-Arg chloromethyl ketone; DnsEGRck, dansyl-L-Glu-Gly-L-Arg chloromethyl ketone; PS/PC, a 30:70 mixed-vesicle preparation of phosphatidylserine and phosphatidylcholine; HBS, Hepes-buffered saline, 0.1 M NaCl in 0.05 M Hepes–NaOH, pH 7.5; TF:VII, the tissue factor–factor VII complex; TF:VIIa, the tissue factor–factor VIIa complex; TFPI:Xa, the TFPI–factor Xa complex.

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heparin-dependent inhibitor of factor Xa—antithrombin III—it is not yet clear whether heparin might not exert its apparent TFPI-dependent effect *via* the action of antithrombin on factor Xa: specifically, in modulating the feedback activation of TF:VII by factor Xa, rather than in modulating the action of TFPI on TF:VIIa. Thus, while Huang *et al.* (1993) have confirmed an effect of heparin on the inhibition of factor Xa at high heparin concentrations, the kinetic details are not clear; and the same group has observed no potentiating effect of heparin on the action of TFPI in inhibiting the TF:VIIa-catalyzed activation of factor X in the absence of antithrombin (Broze *et al.*, 1993).

This report, involving measurement of the kinetics of inhibition of both factor Xa and the TF:VIIa complex by TFPI, and study of the effects of heparin, speaks directly to several of these questions. With the kinetic parameters in hand, the comparative roles in the system of TFPI, heparin, and antithrombin III will be predictable and experimentally testable.

MATERIALS AND METHODS

Materials. Chromozym-TH (tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide) and Chromozym-X (*N*-methoxycarbonyl-D-norleucine-Gly-L-Arg-*p*-nitroanilide) are products of Boehringer-Mannheim, Indianapolis, IN. D-Phe-L-Pro-L-Arg chloromethyl ketone (FPRck) and dansyl-L-Glu-Gly-L-Arg chloromethyl ketone (DnsEGRck) are products of Bachem, Torrance, CA, and Calbiochem, San Diego, CA, respectively. They were stored as 1 mM solutions in 10 mM HCl at −20 °C and were diluted in water before use. Phosphatidylserine (PS), phosphatidylcholine (PC), benzamidine hydrochloride, sodium heparin, and bovine serum albumin (grade V, fatty acid-free) were products of Sigma Chemical Co., St. Louis, MO. For prothrombinase assays of factor Xa, a sonicated vesicle preparation of 30% PS/70% PC (PS/PC) was made weekly in Tris-buffered saline (100 mM NaCl/50 mM Tris-HCl pH 7.5), containing 0.02% NaN_3, and was stored at room temperature. Octyl glucoside was purchased from Pierce Chemical, Rockford, IL. Polybrene (hexadimethrine bromide) is a product of Aldrich Chemical, Milwaukee, WI. DEAE-Sephadex, Sephadex G-100, octyl-Sepharose, and Sepharose 4B are products of Pharmacia, Piscataway, NJ.

Human factor X was prepared from a detergent-treated factor X concentrate, kindly provided by Charles Heldebrant of Alpha Therapeutics, as previously described (Morrison & Jesty, 1984). Factor Xa was prepared from human factor X by the method described for bovine factor Xa (Jesty & Nemerson, 1976). To determine its active-site concentration, it was titrated against a >97%-pure preparation of human antithrombin III, which was known from gel electrophoresis to be fully active in complex formation with human α-thrombin (Jesty, 1979).

Prothrombin was prepared as a byproduct of the factor X preparation after separation from factors IX and X on dextran sulfate–agarose. To minimize factor X(a) contamination, prothrombin was passed through a column of rabbit anti-factor X coupled to agarose.

Recombinant human factor VIIa was generously supplied by Novo Nordisk, Bagsvaerd, Denmark. Recombinant TFPI was expressed in *Escherichia coli* and refolded to a fully active state (Diaz-Collier *et al.*, 1994) and was *ca.* 90% of $M_{r,app} \simeq 38\,000$ by dodecyl sulfate gel electrophoresis in a discontinuous gel system (Jesty, 1979) modified from that of Laemmli (1970). The molecular weight from the TFPI sequence is 32 003. To determine its active concentration, TFPI was titrated against a standard sample of factor Xa, using chromogenic assay of the enzyme.

Recombinant human tissue factor produced in *E. coli* was generously provided by Dr. William Konigsberg of Yale University, New Haven, CT. It was relipidated with varying amounts of PS/PC (30:70) in the presence of 0.5% octyl glucoside and exhaustively dialyzed against HBS to remove the detergent and form vesicles. Lipid/protein ratios were chosen to provide varying concentrations of tissue factor, at a constant 25 μM concentration of phospholipid in all experiments. Lipid/protein ratios vary over the range 2.5×10^7 to 2.5×10^5.

Sodium heparin (500 mg, 160 units/mg) was fractionated to produce a crude high-molecular-weight fraction by gel filtration on a 2 × 70 cm column of Sephadex G-100 in 0.1 M NH_4HCO_3. Low-molecular-weight (*ca.* 50%) and very-high-molecular-weight (*ca.* 10%) fractions were discarded. The remaining pool of high-molecular-weight fractions, estimated M_r range 8–11 K, was lyophilized and further held for 24 h under <20 mTorr vacuum to remove the NH_4HCO_3. The resulting material was weighed, and its activity was determined by measurement of its acceleratory effect on the rate of inhibition of human α-thrombin by antithrombin III. By reference to the stated activity of the starting material, its specific activity was 220 units/mg.

Bovine factor Va, which is required in the prothrombinase assay of factor Xa, was prepared from 1.5 L of citrated bovine plasma (Pel-Freez, Rogers, AR) by a modification of the method of Nesheim *et al.* (1981). Three changes were made in their procedure. (1) DEAE-Sephadex A50 (Pharmacia) was used instead of DEAE-cellulose. (2) After the batch absorption of plasma proteins on DEAE-Sephadex, the resin was packed in a column (5 × 15 cm) and eluted at 150 mL/h with a linear gradient, from 0.1 to 0.35 M NaCl, in 20 mM imidazole hydrochloride, pH 7.5/5 mM $CaCl_2$/5 mM benzamidine hydrochloride. (3) The improved purification provided by gradient elution of the DEAE-Sephadex meant that only one further step—octyl-Sepharose chromatography—was necessary to obtain factor V of more than 90% purity. Chromatography on phenyl-Sepharose was omitted.

The pooled active fractions from the octyl-Sepharose (*ca.* 35 mg of factor V in 20 mL of elution buffer) were treated with 60 nM human α-thrombin for 3 min at 37 °C. The thrombin inhibitor FPRck was then added to a concentration of 1 μM. After 10 min incubation at 37 °C the resulting factor Va was dialyzed once against 1 L of TBS/1 mM $CaCl_2$, and then twice against 500 mL of 50% glycerol in 0.1 M NaCl/0.05 M Tris-HCl, pH 7.5/1 mM $CaCl_2$. The yield was 32 mg of factor Va. The product, which is free of single-chain factor V by gel electrophoresis, and free of both thrombin and FPRck by chromogenic assay, was stored at −20 °C.

Prothrombinase Assay of Factor Xa Inhibition. Rates of factor Xa inhibition by TFPI were determined by discontinuous assay, involving incubation of factor Xa with the inhibitor and removal of timed samples from these incubations for the immediate assay of factor Xa. Because the second-order rate constant for the reaction of TFPI and factor Xa is of the order of 10^9 M^{-1} min^{-1}, low TFPI concentrations were necessary to bring the inhibition rate within a manageable range. In order to maintain pseudo-first-order conditions, TFPI was held in at least 5-fold excess over factor Xa. This restriction required initial factor Xa concentrations as low as 20 pM, which is below the limit of assay by direct chromogenic methods, at least with acceptable rapidity.

Scheme 1

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}I^*$$

The prothrombinase assay of factor Xa involves the activation of prothrombin in the presence of factor Va, anionic phospholipid vesicles, Ca^{2+} ions, and a *p*-nitroanilide chromogenic substrate for thrombin. In order to reduce photometric problems arising from partial wetting of the wall of the microplate well, microplates were treated before use with a solution of 0.1% Tween-20, rinsed copiously with water, and allowed to dry. Each timed factor Xa sample from an inhibition incubation, 20 μL, was added to a microplate well containing the other components of the assay, to attain an assay volume of 200 μL. The final concentrations in each well were 280 nM prothrombin, 3 nM bovine factor Va, 25 μM PS/PC, 5 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.25 mM Chromozym-TH in HBS. Because of its coupled design this assay is particularly sensitive to temperature ($Q_{10} \simeq 4$). The microplate reader (VMax, Molecular Devices, Palo Alto, CA) was therefore maintained at 25 ± 0.3 °C in a thermostated box, and every assay series (typically each time course of factor Xa inhibition) was standardized with duplicate assays of a control factor Xa sample. As would be expected, the chromogenic thrombin substrate present in the microplate well inhibits the factor Xa being assayed, but since the assay is standardized under identical conditions, the effect can be ignored.

Assays of factor Xa in all samples containing heparin were done with the addition of the anti-heparin agent Polybrene, 2.5 μg/mL, to the well buffer. Microplate-well absorbances were read every 10–15 s for 4–10 min, depending on the factor Xa activity range in the series. Absorbance/time data for each factor Xa sample (microplate well), typically 20–50 absorbance readings, were fitted to (i) a quadratic, $Y = A + Bt + Ct^2$, and (ii) a cubic, $Y = A + Bt + Ct^2 + Dt^3$, where Y is the absorbance. The values of reduced χ^2 for each were compared, and the fit with the smaller value was accepted. Two restrictions were made in the fitting procedure: (i) absorbance values that exceeded the initial absorbance of each well by more than 0.3 absorbance unit were discarded; and (ii) positive values of D in the cubic fit were not accepted: thus if a positive value of D was returned by the fitting procedure, the quadratic fit was used. The value of C, which is equal to $^1/_2[d^2Y/dt^2]$ at $t = 0$, is directly proportional to the factor Xa concentration over a range (in the original 20–μL sample) of 0–50 pM. In the absence of factor Xa, the base-line rate of prothrombin activation in the assay corresponds to <0.15 pM factor Xa.

Time Resolution of the Prothrombinase Assay Method. The assay includes no specific component to slow or stop TFPI action on factor Xa at the time that a sample is added to the microplate well. Sampling does, however, include a 10-fold dilution into the microplate well and a reduction in temperature from 37 to 25 °C. It is reasonable to assume the rate of factor Xa inhibition is reduced at least 20-fold. It should be noted that data analysis by the quadratic-cubic method described produces accurate extrapolation of d^2Y/dt^2 to the time of sampling.

Analysis of Factor Xa Inhibition. The standard model for the action of the serpin family of inhibitors involves two reversible steps, one rapid and one slow. This model is shown in Scheme 1, using E to represent the enzyme (here factor Xa), and I the inhibitor (TFPI). It is simplest to consider the case where inhibitor is in large excess, as was the case throughout this study; *i.e.*, [I] ≫ [E], so that the formation of E·I is pseudo-first-order, and $k_{1,obs} = k_1[I]$. The remaining rate constants, k_{-1}, k_2, and k_{-2}, are first-order.

To determine the simplest model that described individual experimental time courses of enzyme inhibition, inhibition time-course data sets were fitted to a series of three models of increasing complexity. The simplest model is that of a single irreversible reaction, where $k_{1,obs} > 0$, and k_2, k_{-1}, $k_{-2} = 0$. Enzyme, E, decays to zero according to a single exponential:

$$[E]_t = [E]_0 e^{-k_{1,obs}t} \quad (1)$$

In this case the rate equals $k_{1,obs}$. The next more complex model involves a single reversible step with EI as the final product, *i.e.*, $k_{1,obs}$, $k_{-1} > 0$ and k_2, $k_{-2} = 0$. Enzyme here decays to a final equilibrium according to a single exponential with the exact solution

$$[E]_t = \frac{[E]_0}{k_{1,obs} + k_{-1}}[k_{-1} + k_1 e^{-(k_{1,obs}+k_{-1})t}] \quad (2)$$

Here the rate is the sum of the forward and reverse rate constants, $k_{1,obs} + k_{-1}$.

If the second reaction exists (Scheme 1: $k_2 > 0$; $k_{-2} \geq 0$), decay is characterized as the sum of two exponentials, involving two amplitudes, $\Delta_1[E]$ and $\Delta_2[E]$, and rates, λ_1 and λ_2:

$$[E]_t = \Delta_1[E]e^{-\lambda_1 t} + \Delta_2[E]e^{-\lambda_2 t} + [E]_\infty \quad (3)$$

The present study confirmed that such a second step likely exists (Huang *et al.*, 1993), but two things prevented full analysis. First, the assay methods are insufficiently accurate at the limits of their sensitivity and time resolution. The second problem is that even though the amplitudes ($\Delta[E]$) and rates (λ) are reasonably well determined from the data, derivation of rate constants from these parameters can be hard. If $k_{-1} \gg k_2$, steady-state conditions apply, and amplitude assignment is relatively easy. However, the results of the present study strongly suggest that $k_{-1} \simeq k_2$, which renders the steady-state assumptions invalid (Bernasconi, 1976; Broze *et al.*, 1993) and amplitude assignment difficult. Although methods do exist for the derivation of $k_{1,obs}$, k_{-1}, and k_2 solely from the dependence of λ_1 and λ_2 on inhibitor concentration (Bernasconi, 1969), the present data, even when summed over replicate experiments, were too noisy to support such analysis. The majority of our conclusions depend on the observation that, at all but the lowest TFPI concentrations, a single exponential (eq 2) suffices for an empirical description.

Inhibition of the Tissue Factor–Factor VIIa Complex. Rates of inhibition of the TF:VIIa complex by the TFPI:Xa complex were measured by (i) preformation of the TFPI:Xa complex, with a small molar excess of factor Xa over TFPI; (ii) addition to the TFPI:Xa of factor X, tissue factor, and factor VIIa; and (iii) determination of the subsequent time course of factor Xa generation by discontinuous chromogenic assay of timed samples. Inhibition of TF:VIIa by the TFPI:Xa complex causes the rate of factor Xa generation (in step ii) to fall exponentially, at a rate equal to the rate of TF:VIIa inhibition. The details of the method follow.

(i) TFPI (varying concentration, 2× the final concentration required) was incubated with a small (10–15%) molar excess of factor Xa in the presence of 25 μM PS/PC and 5 mM Ca^{2+} for 15 min at 37 °C, in a total volume of 100 μL. (ii) Further additions (prewarmed to 37 °C) were made to give a final volume of 200 μL and the following final concentrations: 50

nM factor X; 5 mM Ca^{2+}; tissue factor–PS/PC (10 pM TF, 25 μM PS/PC); 50 pM factor VIIa. Timing was started at the addition of factor VIIa.

Timed samples, 20 μL, were removed for chromogenic factor Xa assay into microplate wells containing 100 μL of 10 mM disodium EDTA, 100 mM NaCl, 50 mM Tricine–NaOH, pH 8.3, and 0.5 mM Chromozym-X at 25 °C. Absorbance/time data for each well were analyzed to determine the initial rate of *p*-nitroaniline generation, dA/dt, at the time of sample addition to the well. This was done by comparison of χ^2 estimates of data fitted to a linear, a quadratic, and a cubic function (cf. the prothrombinase assay, above). Initial rates in this assay are proportional to factor Xa concentration, over the range 0.2–30 nM factor Xa.

As we have previously described, in a kinetically similar study of the first-order kinetics of factor VIIIa decay (Neuenschwander & Jesty, 1992), factor Xa generation in such a system, as long as factor X is minimally consumed during the course of the incubation and inhibition is irreversible, is described by the single exponential

$$[Xa]_t = [Xa]_{max}[1 - e^{-k_{obs}(t-L)}] \quad (4)$$

where $[Xa]_{max}$ is the factor Xa concentration at $t = \infty$, k_{obs} is the observed first-order rate constant of TF:VIIa inhibition, and L is a lag time that is included to handle the short lag phase observed in the data.

Nonlinear Regression. Data were fitted to functions by Marquardt's algorithm, as described by Bevington (1969). Data were not weighted in the fitting routine, with two exceptions: (i) time courses of factor Xa decay, where enzyme concentrations fall to <2% of the initial level (Figure 1), were weighted by the reciprocals of the data values in order to give sufficient weight to small numbers; (ii) data sets with large numbers of replicates (studies of the inhibition of the TF:VIIa complex, Figure 4B) were weighted by the variance of each group of replicates.

RESULTS

This section is divided into three parts. In the first we address the kinetics of factor Xa inhibition by TFPI and the effect of anionic phospholipid and heparin on this reaction. In the second the focus is the second stage of TFPI action: the inhibition of the TF:VIIa complex by the TFPI:Xa complex, and the effect of heparin on this reaction. The third section briefly examines the inhibition of factor VIIa by the TFPI:Xa complex in the absence of tissue factor.

Inhibition of Factor Xa by TFPI. The kinetics of factor Xa inhibition by varying concentrations of TFPI were determined in the presence and absence of anionic phospholipid vesicles (PS/PC), and in the presence and absence of heparin. The focus of the study is the quantitative kinetic description, rather than the mechanistic details, of the reaction.

Validation of Prothrombinase Assay. As described in Materials and Methods, the factor Xa concentrations that were required in this part of the study necessitated the use of a coupled factor Xa assay that relies on measurement of prothrombin activation. Control assays, in which TFPI was added at appropriate concentrations directly to a microplate well (already containing factor Xa and the other components of the assay), showed a measurable reduction in the rate of substrate cleavage over the course of the assay.

Although the method of analysis of assay data minimizes the effect of continuing inhibition during the assay (Materials and Methods), it was necessary to confirm that the inactivation

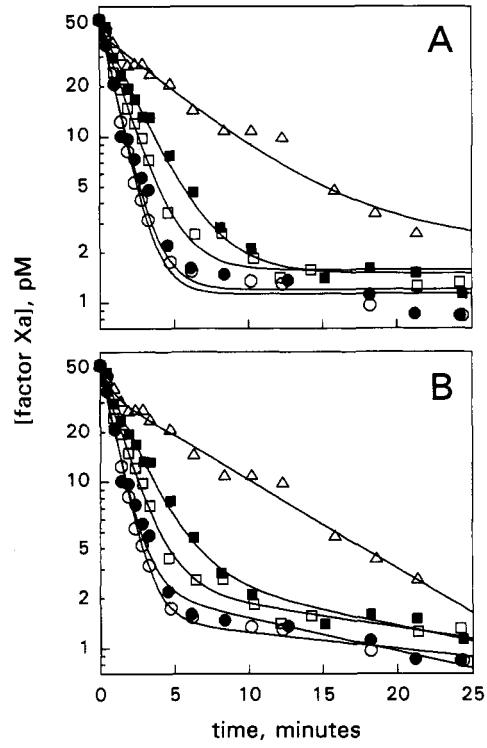

FIGURE 1: The inhibition of factor Xa by TFPI. Factor Xa (20 pM) was incubated at 37 °C with TFPI at 0.2 (△), 0.4 (■), 0.6 (□), 0.8 (●), and 1 (○) nM, in the presence of 25 μM PS/PC and 5 mM Ca^{2+}. Samples were removed at the times indicated and assayed by a prothrombinase assay as described in Materials and Methods. Data sets were fitted to a single exponential (eq 2; lines, panel A) and to a two-exponential model (eq 3; lines, panel B).

kinetics of factor Xa were correctly reported by the prothrombinase method. The method was therefore compared with a standard noncoupled chromogenic assay, using dansyl-Glu-Gly-Arg chloromethyl ketone (DnsEGRck) as a simple factor Xa inhibitor. Since the latter assay includes a high concentration of chromogenic factor Xa substrate in the microplate well ($[S] \gg K_m$), in addition to the 10-fold sample dilution, continuing inhibition of factor Xa during the assay phase may be expected to be minimal.

Factor Xa (10 nM for the chromogenic assay; 50 pM for the prothrombinase assay) was incubated with 0.5 μM DnsEGRck, otherwise under the same conditions as were used in experiments with TFPI (Materials and Methods), and timed samples were removed over the course of 6 min for the assay of factor Xa. The kinetics of inhibition were accurately described by first-order kinetics (eq 1) in both cases. Triplicate determinations using the chromogenic assay and 10 nM factor Xa gave a first-order rate constant for factor Xa inhibition of 1.035 ± 0.038 min^{-1} (SE). Triplicate determinations using the prothrombinase assay and 50 pM factor Xa gave a mean value of 1.062 ± 0.065 min^{-1} (SE). The absence of a significant difference confirms that the prothrombinase assay is suitable for following the inhibition of factor Xa in the picomolar range.

Factor Xa Inhibition by TFPI. Factor Xa (50 pM) was incubated with varying concentrations of TFPI, and timed samples were removed for assay. Figure 1 shows a typical set of factor Xa decay curves in the presence of 25 μM PS/PC and 5 mM Ca^{2+}, at 37 °C. Each set of decay data was first fitted to a simple irreversible decay with an asymptote of zero (eq 1). Comparison of the fits (not shown) with the data showed clearly that inhibition cannot be described by eq 1 at any concentration of TFPI, since inhibition remains incomplete—factor Xa persists—in essentially all incubations. Data

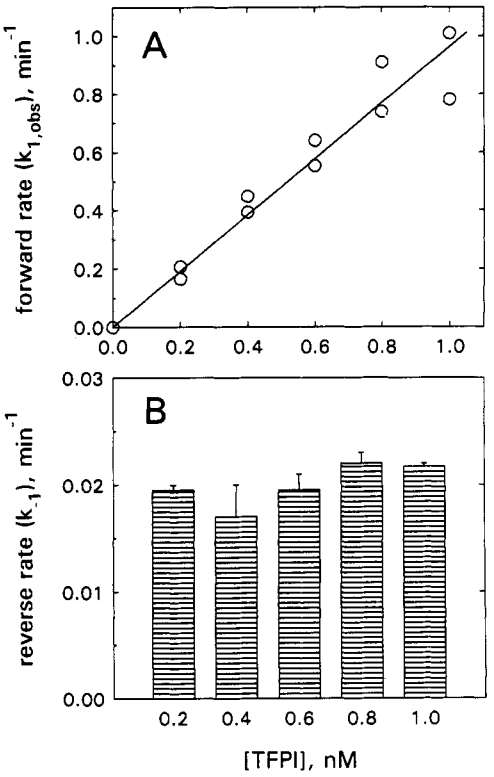

FIGURE 2: Kinetic parameters for the inhibition of factor Xa by TFPI. Values of $k_{1,obs}$ (panel A) and k_{-1} (panel B) were derived by fitting the data of Figure 1, and data from an identical duplicate set, to a single exponential model (eq 2). The data of panel A were fitted to a straight line, yielding a slope (K_1) of 0.96×10^9 M^{-1} min^{-1}.

were therefore next fitted to the most simple reversible model ($k_2, k_{-2} = 0$), which involves a single exponential with a nonzero asymptote, producing estimates for both $k_{1,obs}$ and k_{-1} (eq 2). These fits are shown by the lines in Figure 1, panel *A*, and it is these estimates of $k_{1,obs}$ that are plotted in Figure 2A as a function of [TFPI].

Although fitting the data to a single exponential produces an adequate empirical description of the kinetics of inhibition at all but the lowest TFPI concentration, it nonetheless appears that a second, much slower phase of factor Xa inhibition exists. Panel B of Figure 1 shows the results of fitting the same data sets to a two-exponential function (eq 3), and the fit is measurably better (χ^2 is lower). However, at these low enzyme concentrations the data do not support much more than the general qualitative conclusion that the second step proposed by Huang *et al.* (1993) does exist.

In Figure 2A we show the proportional dependence of the first-order rate of inhibition, $k_{1,obs}$, on TFPI concentration. From the slope, the second-order rate constant of factor Xa inhibition by TFPI was $k_1 = 0.96 \times 10^9$ M^{-1} min^{-1}. It was expected from the simple model that while $k_{1,obs}$ should be proportional to TFPI concentration, k_{-1} should not. As shown in Figure 2B, this is the case. The mean value of k_{-1} determined from duplicate data sets was 0.020 ± 0.001 min^{-1} (SE), although it is likely that the very small error estimate here is fortuitous. The ratio of the two rate constants, k_{-1}/k_1, is the apparent dissociation constant for the initial factor Xa–TFPI interaction, 21 pM.

Anionic Phospholipid. To determine the effect of anionic phospholipid on the reaction between factor Xa and TFPI, duplicate sets of experiments similar to those of Figure 1 were done in the absence of PS/PC (data not shown). The values of k_1 and k_{-1} obtained were respectively [0.86 ± 0.04 (SE)] $\times 10^9$ M^{-1} min^{-1} and [0.040 ± 0.004 (SE)] min^{-1}. The modest differences are probably not significant and demonstrate that anionic phospholipid has no large effect on factor Xa inhibition by TFPI under the conditions studied.

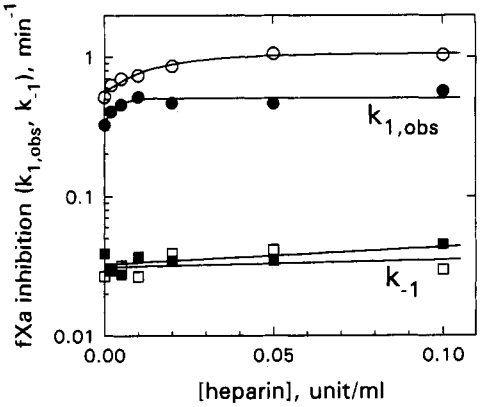

FIGURE 3: The effect of heparin on the kinetics of factor Xa inhibition by TFPI. Experiments identical to those shown in Figure 1 were done in the presence of varying concentrations of a high-molecular-weight fraction of heparin, at TFPI concentrations of 0.5 nM (open symbols) and 0.25 nM (closed symbols). Estimates of $k_{1,obs}$ (circles) and k_{-1} (squares), derived from the single-exponential fitting procedure (eq 2), are plotted against heparin concentration.

Heparin. It has been reported by investigators studying plasma systems that the action of TFPI on the generation of factor Xa by TF:VIIa is significantly potentiated by heparin, and Huang *et al.* (1993) have qualitatively confirmed that there is a significant effect in pure systems. We therefore examined the kinetics of factor Xa inhibition ($k_{1,obs}$ and k_{-1} in the single-step reversible model, eq 2) at varying heparin concentrations, in the presence of PS/PC and Ca^{2+}. The TFPI concentrations were 0.25 and 0.5 nM. The results are shown in Figure 3. Under these conditions heparin accelerates TFPI action on factor Xa approximately 2-fold, with no significant effect on k_{-1}. In order to maintain pseudo-first-order conditions (*i.e.*, [TFPI] $\geq 5 \times$ [Xa]), it was possible to do these experiments only at a very low factor Xa concentration (20 pM), and only over a narrow range of TFPI concentration.

Inhibition of TF:VIIa by TFPI:Xa. The second stage of TFPI action is the inhibition of the TF:VIIa complex by the complex of TFPI and factor Xa. In this study we have isolated this step by preforming the TFPI:Xa complex, and then adding it to factor X in the presence of tissue factor and factor VIIa. While we would have preferred a discontinuous experimental design in which the inhibition of TF:VIIa is separate from its assay, the high rate of the reaction precludes this approach with current techniques.

Figure 4A shows factor Xa generation data obtained at varying concentrations of TFPI:Xa and a single concentration of tissue factor. Although almost all data sets showed a satisfactory fit to a single exponential, significant variation was observed in the derived first-order rate constant, k_{obs}. Seven sets of time-course data were therefore obtained at each of the three tissue factor concentrations, and each time-course set was fitted to eq 4 to determine k_{obs} (Figure 4A, lines). For each tissue factor concentration, k_{obs} values were plotted against the concentration of TFPI:Xa complex, and fitted to a straight line using variance weighting (Figure 4B) to obtain the apparent second-order rate constant for TF:VIIa inhibition, k_{app}.

The inclusion of factor X in these incubations, at a concentration of 50 nM, causes a reduction in the rate of inhibition of TF:VIIa by the TFPI:Xa complex. To determine the extent of competition, similar experiments, using triplicate

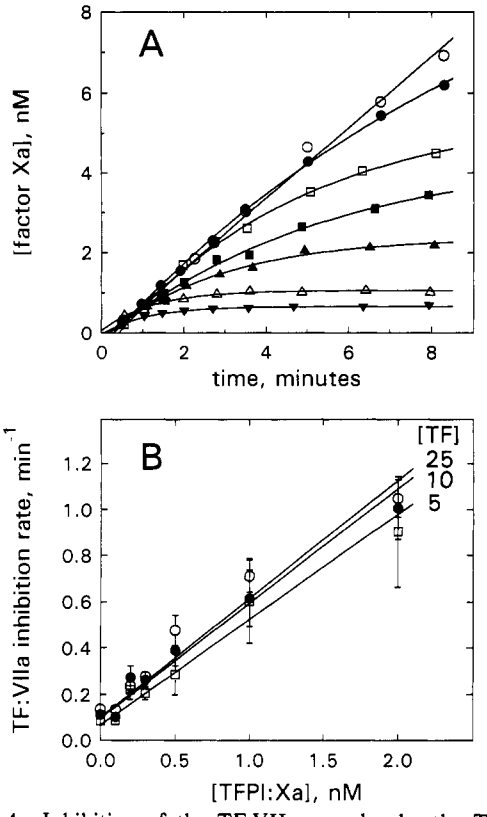

FIGURE 4: Inhibition of the TF:VIIa complex by the TFPI:Xa complex. Panel A: Varying concentrations of TFPI were incubated with a 10% molar excess of factor Xa in the presence of 25 μM PS/PC and 5 mM Ca^{2+} for 15 min. The resulting preparations of TFPI–Xa complex were diluted 2-fold into an incubation to contain (final concentrations) 50 pM factor VIIa, 10 pM tissue factor, 50 nM factor X, and sufficient PS/PC and Ca^{2+} to maintain their respective concentrations. The final concentrations of TFPI:Xa complex were 0 (○), 0.1 (●), 0.2 (□), 0.3 (■), 0.5 (▲), 1 (△), and 2 nM (▼). Timed samples, removed at the times indicated, were assayed for factor Xa by chromogenic assay, and the data were fitted to eq 4 (lines, panel A) to obtain k_{obs}. Panel B: The set of experiments shown in panel A was done seven times at each of three tissue factor concentrations (5, 10, 25 pM). The mean values of k_{obs} under each condition (error bars are ±SE) as a function of TFPI:Xa concentration, and weighted by variance, were fitted to a straight line at each tissue factor concentration (lines) to obtain k_{obs}.

measurements of inhibition rate, were done at factor X concentrations of 100 and 200 nM, at a single concentration of TF:VIIa (10 pM) and a single concentration of TFPI (0.5 nM). Plots of $1/k_{obs}$ against factor X concentration (not shown) yielded an apparent competitive inhibition constant, K_i (for the reduction of TF:VIIa inhibition rate in the presence of factor X), of 180 nM factor X. If we assume the effect of factor X to be competitive, the corrected second-order inhibition rate constant for inhibition of TF:VIIa by TFPI:Xa, k, equals $k_{app}(K_i + [X])/K_i$. Thus for the data obtained at 50 nM factor X (Figure 4B), $k = 1.28 \times k_{app}$. The corrected estimates of the second-order rate constants at each tissue factor concentration, derived from Figure 4B, were as follows: 25 pM tissue factor, 6.5×10^8 M^{-1} min^{-1}; 10 pM tissue factor, 6.4×10^8 M^{-1} min^{-1}; 5 pM tissue factor, 5.8×10^8 M^{-1} min^{-1}.

The intercepts of the plots of Figure 4B are significantly different from zero and show that TF:VIIa activity decays in the absence of TFPI:Xa, at a mean rate (not corrected for factor X competition) of 0.09 min^{-1}. As the TF:VIIa level falls from 25 to 5 pM, a small reduction in this rate is observed—from 0.10 to 0.07 min^{-1}; but given the substantial error estimates in these numbers, its significance is doubtful.

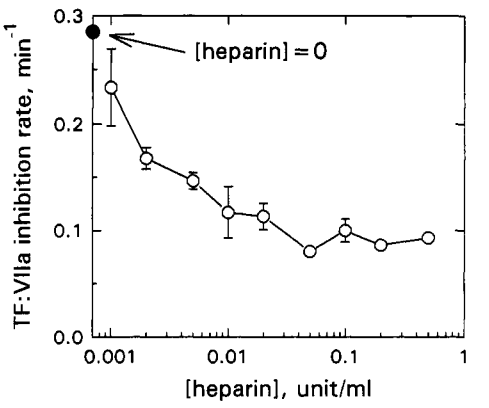

FIGURE 5: The effect of heparin on the inhibition of TF:VIIa by TFPI:Xa. TF:VIIa inhibition was measured as described for Figure 4A, at a tissue factor concentration of 10 pM and a TFPI:Xa concentration of 0.5 nM, at varying concentrations of a high-molecular-weight fraction of heparin. Duplicate experiments were done at each heparin level. Mean values of k_{obs} (error bars show the range), obtained from the factor Xa generation curves (as in Figure 4A), are plotted against heparin concentration.

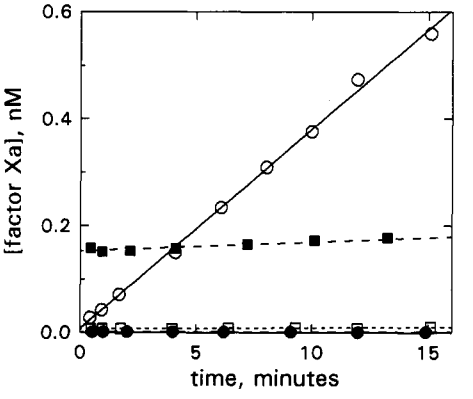

FIGURE 6: The inhibition of factor VIIa by TFPI:Xa in the absence of tissue factor. TFPI (25 nM) and factor Xa (27 nM) were incubated in the presence of PS/PC and Ca^{2+} for 15 min to form the TFPI:Xa complex. To this incubation were then added other components, in the following order, to give the following final concentrations: 10 nM TFPI:Xa; 50 nM factor X; 25 μM PS/PC; 5 mM Ca^{2+}; and 2 nM factor VIIa. Timed samples were removed at the times shown after factor VIIa addition for prothrombinase assay of factor Xa (■). Control incubations lacked TFPI:Xa (○), or factor VIIa (□), or factor X (●).

The Effect of Heparin. In order to address the question of heparin action in the inhibition of TF:VIIa by the TFPI:Xa complex, the experiments of Figure 4 were repeated at 10 pM TF:VIIa and 50 nM factor X, in the presence of varying concentrations of a high-molecular-weight fraction of heparin, over the range 0–0.5 unit/mL. The results, derived from the merged data of three sets of experiments, are shown in Figure 5 as uncorrected values of k_{obs}. Under these conditions heparin, at saturating levels, reduces the rate of TF:VIIa inhibition by about 65%.

Inhibition of Factor VIIa by TFPI:Xa. Factor VIIa is known to activate factor X at low rates in the absence of tissue factor. Since this reaction, though very slow compared with activation by TF:VIIa, is of possible significance in the idling behavior of the clotting system, we examined the inhibition of factor VIIa by TFPI (Figure 6). The TFPI:Xa complex was preformed as before, but at higher concentrations than in the previous experiments, by incubation of TFPI with a small (*ca.* 10%) excess of factor Xa in order to ensure that no significant concentration of free TFPI was present. To this preincubation were added factor X and factor VIIa. Even though a small excess of factor Xa is present initially, a

comparison of the rates of factor Xa generation in the presence and absence of TFPI:Xa shows that the TFPI:Xa complex inhibits factor VIIa in the absence of tissue factor. Given the conditions of these experiments, the kinetics are unknown, but at a concentration of 1 nM, the TFPI:Xa complex inhibits factor VIIa at least 90% under these conditions.

It has been reported that TFPI must combine with (and inhibit) factor Xa before it can inhibit the TF:VIIa complex. However, it has also been reported that TFPI produced by endothelial cells can inhibit TF:VIIa in the absence of factor Xa. We have attempted to examine this possibility, but since the method we use to measure TF:VIIa rapidly generates factor Xa, and since factor Xa reacts extraordinarily rapidly with TFPI, it has been impossible to confirm or refute the suggestion.

DISCUSSION

The study centered on an empirical, but quantitative, description of the inhibition of factor Xa by TFPI, and the subsequent inhibition of the TF:VIIa complex by the TFPI:Xa complex. We have used full-length TFPI produced by recombinant methods in *E. coli*, and although not glycosylated, it includes the highly cationic carboxy-terminal region. It should be emphasized that the results we obtain—particularly those concerning heparin—are specific to this molecule and may be affected by removal of the carboxy-terminal domain. Lindhout *et al.* (1994) have reported, for instance, that k_1 for the truncated variety of TFPI is reduced by 75%, while k_{-1} remains constant.

The goal of the study was to measure the kinetic parameters of both the inhibitory reactions of TFPI, though not necessarily their mechanistic details, under near-physiological conditions. Because both are rapid, there are significant limitations on the conditions under which they can be studied.

TFPI Inhibition of Factor Xa: Limitations of Methods. The primary problem in the design of the study and analysis of the data was the very high rate of inhibition of factor Xa by TFPI, of the order of 10^9 M^{-1} min^{-1}. Since the measurement of factor Xa in multiple timed samples by either chromogenic or prothrombinase assay requires an assay incubation of a minimum of 3–4 min, it is not possible to follow inhibition at rates of much more than 1 min^{-1} without encountering problems caused by the further inhibition of factor Xa during the assay phase. We initially considered using a direct chromogenic assay of factor Xa in the presence of EDTA, which stops further inhibition by TFPI at the moment of sampling into the assay. This approach, however, not only raises significant questions about whether the TFPI:Xa complex might dissociate more rapidly in the presence of EDTA; it also founders on the much poorer sensitivity of the chromogenic assay of factor Xa.

The assay and time-resolution limitations have limited the maximum TFPI concentration in the present study to 1 nM. In addition, we required that TFPI be in substantial molar excess over factor Xa, in order to maintain pseudo-first-order conditions. Although the kinetics of enzyme decay can be analyzed when the concentrations of the two reactants (factor Xa and TFPI in this case) are in the same concentration range, this requires accurate knowledge of active protein concentrations, generally to better than ±5%. Moreover, first-order conditions are absolutely required in the analysis of two-step systems involving more than one exponential.

TFPI Inhibition of Factor Xa. Derivation of the formation and dissociation rates of the TFPI:Xa complex was based on fitting time courses of factor Xa inhibition to a single-step reversible model involving two (first-order or pseudo-first-order) rate constants, $k_{1,obs}$ and k_{-1}. As can be seen from Figure 1, this model gives an acceptable empirical description save at the very lowest TFPI concentrations, of less than 0.4 nM. Since the plasma concentration of TFPI in normal individuals is generally more than 1 nM, we consider this approach acceptable.

Although the single-step model is relatively satisfactory for kinetic analysis when the TFPI concentration is more than 0.4 nM, the experimental data show some evidence for a mechanism of more than one step, as reported by Huang *et al.* (1993): an initial near-equilibrium is attained, followed by the slow conversion of the initial TFPI:Xa complex to a more stable form. Using methods that treat the initial step (Scheme 1) as a rapid equilibrium, in a manner analogous to the analysis of enzyme catalysis by Michaelis and Menten, these authors have estimated that $K_1 = k_{-1}/k_1 = 14.6$ nM and $k_2 = 1.18$ min^{-1} (in the presence of Ca^{2+} and anionic phospholipid at 21 °C). The present study confirms the reversibility of the first step, but gives a calculated K_1 of 21 pM.

That K_1 is indeed lower than 1 nM can be quickly verified from Figure 1A: over the range 0.4–1 nM TFPI, the near-equilibrium factor Xa level, attained soon after the initial rapid inhibition phase ($t \simeq 5$ min), is less than 10% of the starting level, clearly indicating $K_1 < 0.1$ nM.

The differences in parameter estimates for the initial step are cause for concern, but the cause is likely the different methods and different assumptions on which they were based. The method that Huang *et al.* (1993) used is based on steady-state assumptions, and analysis assumes that the first step in Scheme 1 is rapid relative to the second, *i.e.*, $(k_{1,obs}, k_{-1}) \gg (k_2, k_{-2})$. However, the present data suggest that $k_{-1} \simeq k_2$. In this situation, in a manner analogous to Briggs–Haldane analysis of enzyme catalysis, $K_{1,app} \simeq (k_{-1} + k_2)/k_1$. Thus, if $k_2 \gg k_{-1}$, K_1 will be substantially overestimated by steady-state methods. Steady-state methods are, on the other hand, well-suited to the kinetic analysis of later, slower stages. It is also pertinent that the estimate of $K_1 = 21$ pM is in fair agreement with the value of 50 pM obtained by Lindhout *et al.* (1994) in the absence of Ca^{2+}.

The data obtained for the inhibition of factor Xa at varying concentrations of TFPI are summarized in Figure 2B. The forward rate, $k_{1,obs}$, is proportional to TFPI concentration (at least up to 1 nM TFPI), yielding a value for the second-order rate constant of 0.96×10^9 M^{-1} min^{-1}. This is about 3-fold greater than that reported by Lindhout *et al.* (1994), but this difference may reflect the presence of Ca^{2+} in our experiments. By way of comparison with other coagulation inhibitors, the rate of factor Xa inhibition by antithrombin III is nearly 4 orders of magnitude slower, 1.3×10^5 M^{-1} min^{-1} (at 25 °C), which in the presence of saturating heparin increases to 4×10^7 M^{-1} min^{-1} (Craig *et al.*, 1989). If we allow for the difference in temperature and take into account the plasma concentrations of antithrombin and TFPI ($\simeq 4$ μM and 2 nM, respectively), we can predict that, in the absence of heparin, TFPI is probably the major inhibitor of subnanomolar levels of factor Xa in solution in plasma, by a factor of at least 2 over antithrombin. This of course does not include the possible contribution of TFPI bound to the endothelium of other cell membranes in contact with the blood. Although the kinetics of factor Xa inhibition in plasma have previously been studied, the factor Xa concentration used was substantially in excess of the plasma TFPI concentration (TFPI had not then been described), and the presumed initial rapid inhibition of the enzyme by TFPI was not detected (Jesty, 1986).

The Effect of Heparin on the TFPI–Factor Xa Interaction. A number of studies in plasma, with all its added complexity, have suggested that the TFPI-dependent control of factor Xa generation by TF:VIIa is potentiated by heparin (*e.g.*, Abildgaard, 1992; Wun, 1992). Plasma, however, contains antithrombin III, which is itself a major heparin-dependent control of factor Xa. Since factor Xa is required for the generation of the TFPI:Xa complex, which in turn inhibits the TF:VIIa complex, plasma experiments are not well suited to a clear demonstration of the mechanism of the observed effect. With the development of the present methods for measuring factor Xa inhibition by TFPI, it has been possible to isolate the two stages of TFPI action and measure the effect of heparin on each stage of TFPI action directly. Under the conditions we have used, the action of TFPI on factor Xa in a pure system is accelerated by heparin, by a factor of approximately 2 at saturating heparin levels (>0.1 unit/mL, *ca.* 50 nM).

Using heparin at a level of 1 unit/mL, Huang *et al.* (1993) have reported that the initial dissociation constant for the TFPI–Xa interaction, K_1, is reduced approximately 10-fold in the presence of Ca^{2+} + phospholipid, and they have reported a minor effect on k_2. Conversely, in the present study, we report a small effect on k_1 and no effect on k_{-1}, while we have no significant data concerning k_2. Given the difficulties of assigning rate constants to specific steps in this system, we do not consider that these differences are necessarily irreconcilable.

In conclusion, we propose that, at TFPI concentrations in the 1 nM range, an empirically adequate description of the initial stages of TFPI action does not require a two-step model: it is only when factor Xa inhibition is >95–98% complete that the second step, reported by Huang *et al.* (1993), becomes important in the overall kinetics of inhibition. We propose, in other words, that while the second step is significant to questions of the final, near-equilibrium, state, it is less significant in defining the rate of factor Xa inhibition by TFPI.

Inhibition of TF:VIIa by the TFPI:Xa Complex. The primary inhibitor of the TF:VIIa complex is almost certainly the TFPI:Xa complex rather than TFPI (Warn-Cramer *et al.*, 1988; Girard *et al.*, 1990), although it has been reported that TFPI can bind to, and inhibit, TF:VIIa on cell surfaces in the absence of factor Xa (Callander *et al.*, 1992). In this study we have isolated the second step by forming the TFPI:Xa complex (with a small excess of factor Xa), and then adding it to TF:VIIa in the presence of the latter's substrate, factor X. The design ensures that no free TFPI is available during the factor Xa generation phase, and we are able to follow the kinetics of factor Xa generation with no interference from TFPI. It should be noted that, in this system (as opposed to our studies of factor Xa inhibition), samples removed from the inhibition incubation are assayed for factor Xa in the presence of EDTA, which blocks further factor Xa generation at the moment of sampling. The method therefore does not have the time-resolution problems that arise in the study of the interaction of TFPI and factor Xa.

As long as a minimal proportion of the factor X is consumed during the measurement of factor Xa generation, which is true in the present study, it is reasonable to assume that the observed exponential reduction in the rate of factor X activation is due to the inhibition of TF:VIIa. That product inhibition is not involved is shown by the near-linearity of factor Xa generation in the absence of TFPI:Xa (Figure 4A), and by the fact that the rate of reduction of the factor Xa generation rate is a function of the TFPI:Xa concentration. When TFPI:Xa is incubated with TF:VIIa and factor X, the rate of factor Xa generation falls according to a single exponential, indicating first-order kinetics of TF:VIIa inhibition; and the rate constant for TF:VIIa inhibition is obtained directly (Neuenschwander & Jesty, 1992). Because factor X is a substrate of TF:VIIa, it may be expected to compete for TF:VIIa, reducing the rate of inhibition by TFPI:Xa. Experiments at varying factor X levels confirmed this and allowed calculation of an inhibition rate, extrapolated to zero factor X concentration, of 6.2×10^8 M^{-1} min^{-1}.

These experiments also demonstrated decay of the activity of TF:VIIa in the absence of any inhibitors, at a mean rate of approximately 0.1 min^{-1}. This rate was not significantly dependent on the TF:VIIa concentration, but this observation allows no firm conclusions about the mechanism involved.

The Effect of Heparin on the Action of TFPI:Xa on TF:VIIa. The inclusion of heparin in experiments concerning the inhibition of TF:VIIa by the TFPI:Xa complex showed that, under the conditions studied (Ca^{2+} + phospholipid), heparin reduces the rate of TF:VIIa inhibition. At saturating heparin levels (>0.1 unit/mL), an approximately 65% reduction in inhibition rate is observed.

Conclusion. The results of this study suggest that the major effect of heparin in regulating the activation of factor X in the tissue factor pathway is through its potentiation of the inhibition of factor Xa by ATIII. TFPI certainly plays a major role in regulating the activity of the TF:VIIa complex, but the effect of heparin on this inhibitor appears to be relatively minor.

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