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Tissue factor pathway inhibitor
A possible mechanism of action

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We have analyzed several mathematical models that describe inhibition of the factor VIIa–tissue factor complex (VIIa–TF) by tissue factor pathway inhibitor (TFPI). At the core of these models is a common mechanism of TFPI action suggesting that only the Xa–TFPI complex is the inhibitor of the extrinsic tenase activity. However, the model based on this hypothesis could not explain well all the available experimental data. Here, we show that a good quantitative description of all experimental data could be achieved in a model that contains two more assumptions. The first assumption is based on the hypothesis originally proposed by Baugh *et al.* [Baugh, R.J., Broze, G.J. Jr & Krishnaswamy, S. (1998) *J. Biol. Chem.* **273**, 4378–4386], which suggests that TFPI could inhibit the enzyme–product complex Xa–VIIa–TF. The second assumption proposes an interaction between the X–VIIa–TF complex and the factor Xa–TFPI complex. Experiments to test these hypotheses are suggested.

Keywords: blood coagulation; extrinsic pathway; tissue factor pathway inhibitor; tissue factor; mathematical model.

Blood coagulation is initiated upon contact of the integral membrane glycoprotein tissue factor (TF) with plasma [1,2]. TF is present on membranes of tissue cells that are normally not in contact with blood. After vascular damage, TF is exposed to plasma and binds to circulating factor VIIa, greatly enhancing its proteolytic activity. The VIIa–TF complex activates factors IX and X via limited proteolysis. This initiates a cascade of enzymatic reactions resulting ultimately in fibrin clot formation. The main regulator of the VIIa–TF complex activity is tissue factor pathway inhibitor, TFPI [3,4]. TFPI inhibits VIIa–TF activity towards factors IX and X in a rather complex, factor Xa-dependent way [5,6]. It appears most likely that this complexity provides both termination of the initial stage of blood coagulation and also its regulation depending on plasma state. Therefore elucidation of the details of the TFPI inhibitory mechanism is of great interest.

TFPI is a Kunitz-type inhibitor containing three Kunitz-type domains. The first Kunitz-domain is known to bind factor VIIa, while the second domain binds factor Xa. The function of the third domain is still unknown [7]. Free TFPI binds factor VIIa very slowly in comparison with its binding of factor Xa [5,6], while the Xa–TFPI complex is a potent inhibitor of VIIa–TF. Their interaction results in the formation of a quaternary Xa–TFPI–VIIa–TF inhibitory complex. These data led to the hypothesis [5] of the two-step mechanism of action of TFPI (Scheme 1): first, TFPI binds factor Xa; second, the Xa–TFPI complex binds VIIa–TF, completely blocking its activity.

Recently, it has been shown that this common inhibitory mechanism of TFPI cannot explain experimental data for the kinetics of the VIIa–TF complex inhibition during factor X activation [8]. Baugh *et al.* [8] measured the kinetic constants for the Xa/TFPI and Xa–TFPI/VIIa–TF interactions. On the basis of these data they developed a mathematical model for the process of the inhibition of the factor Xa generation. The model predicted rather slow decrease of the factor Xa generation rate in the presence of TFPI. However, the experiment under the same conditions revealed rapid and complete inhibition of the factor Xa production [8]. As a possible explanation of the contradiction, Baugh *et al.* proposed that the predominant pathway of inhibition involves the inhibition of factor Xa bound to VIIa–TF by TFPI. They suggested that TFPI can bind to factor Xa at the stage of the enzyme–product Xa–VIIa–TF complex (Scheme 2); this reaction is followed by a unimolecular reaction leading to the formation of the final Xa–TFPI–VIIa–TF complex. The scheme proposed, however, has not been investigated in detail. Interestingly, a recent model study [9] confirms the fact that the common two-step pathway of the TFPI inhibitory action should lead to insignificant inhibition of the VIIa–TF complex. The authors of the study speculate that the VIIa–TF complex is efficiently inhibited because of the covering of endothelium with platelets. However, this idea cannot explain the results of Baugh *et al.* [8], which were obtained under conditions with no platelets present in the system.

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Abbreviations: TF, tissue factor; TFPI, tissue factor pathway inhibitor; I, inhibitor; VII, factor VII; VIIa, factor VIIa; VIIa–TF, the complex of factor VIIa and tissue factor; E, enzyme; X, factor X; S, substrate; Xa, factor Xa; P, product; X–VIIa–TF, the complex of X and VIIa–TF; ES, enzyme/substrate complex; Xa–VIIa–TF, the complex of Xa and VIIa–TF; EP, enzyme/product complex; Xa–TFPI, the complex of Xa and TFPI; PI, product/inhibitor complex; Xa–TFPI–VIIa–TF, the final quaternary inhibitory complex of Xa, TFPI, VIIa and TF; PIE, product/inhibitor/enzyme complex; TFPI–Xa–VIIa–TF, the intermediate inhibitory complex in the hypothetical reactions of TFPI pathway; EPI, enzyme/product/inhibitor complex.

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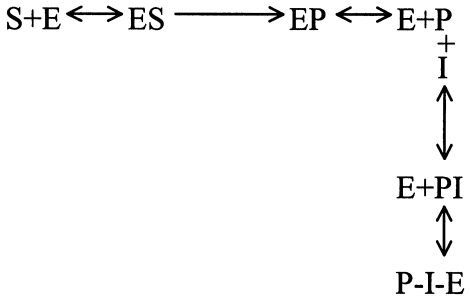
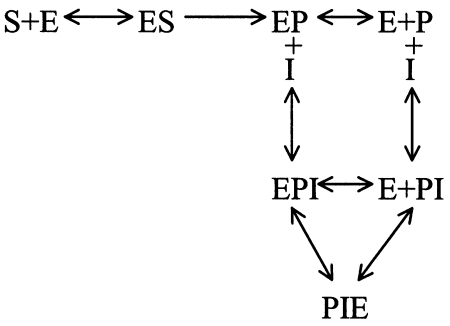
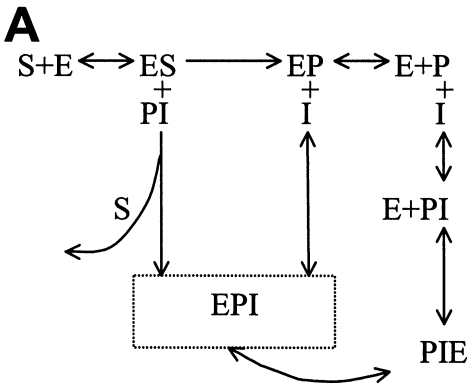
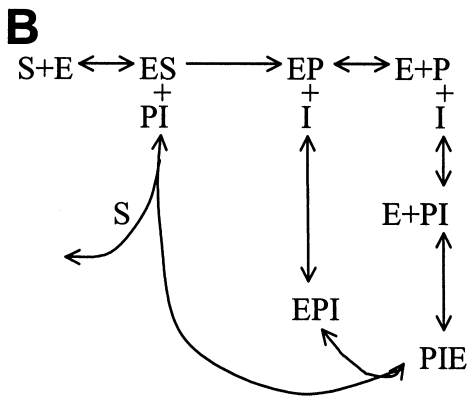


Scheme 1. The common two-step mechanism of action of TFPI (I) during factor X (S) activation by VIIa–TF (E). The first step is binding of TFPI (I) to Xa (P), the second is inhibition of VIIa–TF by Xa–TFPI (PI).



Scheme 2. A modification of Scheme 1 by addition of the inhibition of factor Xa bound to VIIa–TF. The inhibitory mechanism was proposed in [8]. TFPI (I) binds Xa–VIIa–TF (EP) thus directly inhibiting the extrinsic tenase in a one-step fashion. This is followed by unimolecular conversion to yield the final inhibitory complex.





Scheme 3. A development of Scheme 2 by addition of the reaction of the enzyme–substrate X–VIIa–TF (ES) complex inhibition by Xa–TFPI (PI). This reaction was proposed to explain the data of Fig. 2A. In (A), a version of the reaction involving intermediate inhibitory complex formation is shown. This version was used in the calculations of the present study. (B) Another possible version of the reaction (see also Scheme 4C) directly leads to the final inhibitory complex formation.

The objective of our present study was to analyze theoretically the process of inhibition of the VIIa–TF-dependent factor X activation by TFPI. We have compared experimental data obtained by Baugh *et al.* [8] with several mathematical models of the process and have shown that: (a) the mechanism suggested by Baugh *et al.* [8] allows quantitative description of the inhibitory action of TFPI on the kinetics of factor X activation in the absence of factor Xa at the initiation point of the reaction. Yet this mechanism based on the hypothesis of the interaction between TFPI and Xa–VIIa–TF cannot explain factor X activation kinetics in the presence of the preformed Xa–TFPI complex. These kinetic considerations necessarily led us to the hypothesis that the Xa–TFPI complex is capable of inhibiting both free VIIa–TF and some other VIIa–TF-containing species. (b) If the hypothesis of Baugh *et al.* (Scheme 2) is supplemented with another hypothetical reaction of inhibition of the X–VIIa–TF and/or Xa–VIIa–TF complex by the factor Xa–TFPI complex (Scheme 3), it becomes able to quantitatively describe the existing set of experimental data [8]. (c) Existence of all the hypothetical reactions considered in the present study can be tested experimentally. The most direct way to do it is to create conditions under which factor Xa or VIIa–TF would be in excess thus providing a significant amount of the Xa–VIIa–TF complex. The mathematical model has shown that the analysis of the inhibition curves of the corresponding limiting components (VIIa–TF by Xa–TFPI or Xa by TFPI, respectively), can provide the arguments to confirm or disprove these hypotheses.

MATERIALS AND METHODS

Kinetics of the systems shown in Schemes 1–3 were simulated with the help of the ordinary differential equations systems. They were numerically integrated using the embedded Runge–Kutta–Fehlberg method of the second (third) order [10].

Several recent studies concerning reactions, which involve protein–membrane interactions, describe the kinetics of these reactions in detail taking into consideration the interaction between membrane and each reactant involved [11,12]. However, under the saturating concentrations of phospholipids used in the experiments simulated in the present study, the factor Xa production can be described in terms of Michaelis kinetics, though it is clear that the apparent values of k_{cat} and K_m may have a more sophisticated interpretation than the constants in the classical scheme of Michaelis. This approach was used in the present study.

We examined three mechanisms of the VIIa–TF complex inhibition by TFPI: model 1, the common two-step Xa-dependent pathway (Scheme 1); model 2, the mechanism of Baugh *et al.* [8] allowing direct one-step inhibition of the Xa–VIIa–TF complex by TFPI (Scheme 2); and model 3, the mechanism of Baugh *et al.* [8] supplemented with the hypothesis of the enzyme–substrate X–VIIa–TF (or enzyme–product Xa–VIIa–TF) complex inhibition by Xa–TFPI (Scheme 3).

The descriptions of the corresponding mathematical models are presented below.

The model for the two-step mechanism of TFPI inhibitory action (model 1)

A mathematical model simulating two-step action of TFPI has been developed in a previous study [8]: the enzyme (E), VIIa–TF binds its substrate (S) factor X into the enzyme–substrate complex (ES) X–VIIa–TF; then, nonreversible activation of factor X and dissociation of factor Xa from the enzyme follow; the product (P) of the reaction, factor Xa, binds inhibitor (I) TFPI; and the factor Xa–TFPI complex can inhibit the free enzyme, VIIa–TF. Analysis of the model has shown that this scheme cannot explain the effect of TFPI upon factor X activation [8]. To explain the contradiction, the authors suggested that TFPI can directly and efficiently inhibit the enzyme–product Xa–VIIa–TF complex.

To test the ability of the hypothesis to describe these experiments accurately, the enzyme–product stage must be added to the model of the study [8]. Therefore it was included into all the models considered in the present study. Scheme 1 shows the reactions of the two-step mechanism of action of TFPI. It is shown in the Appendix that Scheme 1 is equivalent to Scheme I of [8] within the area of the applicability of the latter. In addition, Scheme 1 allows consideration of the factor Xa influence on the system behavior.

The differential equations for the concentrations of the reactants based on the law of mass action were as follows:

$$\frac{d[VIIa-TF]}{dt} = -k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] + k_d^{X-VIIa-TF}[X-VIIa-TF] - k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa] + k_d^{Xa-VIIa-TF}[Xa-VIIa-TF] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \quad (1)$$

$$\frac{d[X]}{dt} = -k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] + k_d^{X-VIIa-TF}[X-VIIa-TF]; \quad (2)$$

$$\frac{d[X-VIIa-TF]}{dt} = k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] - k_d^{X-VIIa-TF}[X-VIIa-TF] - k_{cat}^{X,VIIa-TF}[X-VIIa-TF]; \quad (3)$$

$$\frac{d[Xa-VIIa-TF]}{dt} = k_{cat}^{X,VIIa-TF}[X-VIIa-TF] + k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa] - k_d^{Xa-VIIa-TF}[Xa-VIIa-TF]; \quad (4)$$

$$\frac{d[Xa]}{dt} = -k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa] + k_d^{Xa-VIIa-TF}[Xa-VIIa-TF] - k_a^{Xa,TFPI}[Xa]\cdot[TFPI] + k_d^{Xa-TFPI}[Xa-TFPI]; \quad (5)$$

$$\frac{d[TFPI]}{dt} = -k_a^{Xa,TFPI}[Xa]\cdot[TFPI] + k_d^{Xa-TFPI}[Xa-TFPI]; \quad (6)$$

$$\frac{d[Xa-TFPI]}{dt} = k_a^{Xa,TFPI}[Xa]\cdot[TFPI] - k_d^{Xa-TFPI}[Xa-TFPI] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \quad (7)$$

$$\frac{d[Xa-TFPI-VIIa-TF]}{dt} = k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] - k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \quad (8)$$

The VIIa–TF complex equilibrium dissociation constant is very low and equals to 7 pM [13]. In all simulated experiments, saturation of TF by VIIa was ensured. Therefore, we considered VIIa–TF to be a single nondissociable enzyme. Its concentration was assumed to be equal to the concentration of the limiting component of the complex, TF.

The criteria for choosing of the values of the kinetic constants

The values of the kinetic constants of those reactions whose existence is well established, are summarized in Table 1. The values of several rate constants are unknown. The discussion of the criteria for choosing of the values of these constants is presented below.

The factor X activation was assumed to involve the formation of the enzyme–substrate X–VIIa–TF complex, the generation of the product and the dissociation of factor Xa from the enzyme. The rate constants of the enzyme–substrate complex formation/dissociation are not known. In the Michaelis scheme if the rate constant of association $k_a^{VIIa-TF,X}$ were known, the dissociation constant could be estimated from the equation $k_d^{X-VIIa-TF} = K_M^{VIIa-TF,X} \cdot k_a^{VIIa-TF,X} - k_{cat}^{VIIa-TF,X}$ using the known values of $k_{cat}^{VIIa-TF,X} = 435\ \mathrm{min}^{-1}$, $K_M^{VIIa-TF,X} = 238\ \mathrm{nM}$ [14]. It follows from the same equation that $k_a^{VIIa-TF,X} = k_{cat}^{VIIa-TF,X} / K_M^{VIIa-TF,X} \approx 2\,\mathrm{nM}^{-1} \cdot \mathrm{min}^{-1}$. The analysis carried out (see Appendix) has shown that during characteristic times of 1 min and more a variation of the $k_a^{VIIa-TF,X}$ value from 2–10 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$ and higher does not affect the kinetics of the system. Therefore we assumed $k_a^{VIIa-TF,X}$ to be equal to the plausible value of 5 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$ [15], which gives $k_d^{X-VIIa-TF} = K_M^{VIIa-TF,X} \cdot k_a^{VIIa-TF,X} - k_{cat}^{VIIa-TF,X} = 770\ \mathrm{min}^{-1}$. However, one should note that if we include the enzyme–product complex stage into our model we shall see that the apparent value of $K_M^{VIIa-TF,X}$ depends on the values of the enzyme–product complex formation/dissociation constants and on $k_{cat}^{VIIa-TF,X}$ (see Eqn. A12). So, the value of $k_d^{X-VIIa-TF}$ obtained in a simple way described above is not precise, though the error is rather small.

The constants of the enzyme–product Xa–VIIa–TF complex formation/dissociation ($k_a^{VIIa-TF,Xa}, k_d^{Xa-VIIa-TF}$) are also unknown. It has been shown, however, that factor Xa inactivated with *p*-amidophenylmethanesulfonyl fluoride binds VIIa–TF with the affinity, which is nearly equal to that of factor X [16]. This provides convincing evidence that the Xa–VIIa–TF complex is very similar to X–VIIa–TF. So we investigated the dependence of the model predictions on the variation of the $k_a^{VIIa-TF,Xa}, k_d^{Xa-VIIa-TF}$ near the values of the corresponding $k_a^{VIIa-TF,X}, k_d^{X-VIIa-TF}$ constants (see Results and Appendix).

Table 1. The values of the constants of the model.

Constant	Value (experimental)	Ref.	Value (model)
$k_a^{X,VIIa-TF}$	No data		5 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$ [a]
$k_d^{X-VIIa-TF}$	No data		770 min^{-1} [b]
$K_M^{X,VIIa-TF}$	238 nM	14	238 nM
$k_{cat}^{X,VIIa-TF}$	420 min^{-1}	14	420 min^{-1}
$k_a^{VIIa-TF,Xa}$	No data		5 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$ [c]
$k_d^{Xa-VIIa-TF}$	No data		770 min^{-1} [d]
$k_a^{Xa,TFPI}$	0.054 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$	8	0.054 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$
$k_d^{Xa-TFPI}$	0.02 min^{-1}	8, 18	0.02 min^{-1}
$k_a^{Xa-TFPI,VIIa-TF}$	0.44 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$	8,	0.44 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$
	0.64 $\mathrm{nM}^{-1}\cdot\mathrm{min}^{-1}$	18	
$k_d^{Xa-TFPI,VIIa-TF}$	0.066 min^{-1}	8	0.066 min^{-1}

[a] Assumed [15]. [b] Calculated from $K_M^{X,VIIa-TF}, k_{cat}^{X,VIIa-TF}$ and $k_a^{X,VIIa-TF}$. [c] Assumed to be equal to $k_a^{X,VIIa-TF}$ on the basis of [16]. [d] Assumed to be equal to $k_d^{X,VIIa-TF}$ on the basis of [16].

We used the constants of the factor Xa–TFPI association reported in the study [8] (see Table 1). This reaction has been established to be two-step [3,8,17]. There is no generally accepted opinion about the values of the kinetic constants for all the steps of this reaction. Which step is the rate-limiting is also under question. However, the comparative analysis has shown that the existence of the second step significantly affects only the description of the experimental results of the study [18]. Therefore we considered this reaction to be two-step when we simulated these experiments (see Results). The constants of the first step were assumed to be equal to those obtained in the study [8] (Table 1). The rate constants of the second step were obtained by variation so as to describe the data of the study [18] (see below). In other cases the binding of factor Xa to TFPI was assumed to be plain bimolecular reaction basing on the data of the study [8].

The model including inhibition of the enzyme–product complex by TFPI (model 2)

When supplemented with the reaction of Xa–VIIa–TF inhibition by TFPI the system (Eqns 1–8) changed to that corresponding to Scheme 2 [8]:

$$\begin{aligned}\frac{d[VIIa-TF]}{dt} = &-k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] + k_d^{X-VIIa-TF}[X-VIIa-TF] - k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa]\\ &+ k_d^{Xa-VIIa-TF}[Xa-VIIa-TF] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF]\\ &+ k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF] - k_{a1}^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF]\\ &+ k_{d1}^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF]; \end{aligned} \quad (1a)$$

$$\begin{aligned}\frac{d[Xa-VIIa-TF]}{dt} = &\ k_{cat}^{X,VIIa-TF}[X-VIIa-TF] + k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa] - k_d^{Xa-VIIa-TF}[Xa-VIIa-TF]\\ &- k_a^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF]\cdot[TFPI] + k_d^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF]; \end{aligned} \quad (4a)$$

$$\frac{d[TFPI]}{dt} = -k_a^{Xa,TFPI}[Xa]\cdot[TFPI] + k_d^{Xa-TFPI}[Xa-TFPI] - k_a^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF]\cdot[TFPI] + k_d^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF]; \quad (6a)$$

$$\frac{d[Xa-TFPI]}{dt} = k_a^{Xa,TFPI}[Xa]\cdot[TFPI] - k_d^{Xa-TFPI}[Xa-TFPI] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF] - k_{a1}^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_{d1}^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF]; \quad (7a)$$

$$\frac{d[Xa-TFPI-VIIa-TF]}{dt} = k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] - k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF] + k_{+1}^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF]$$

$$\frac{d[TFPI-Xa-VIIa-TF]}{dt} = k_a^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF]\cdot[TFPI] - k_d^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF] + k_{a1}^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] - k_{d1}^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF] - k_{+1}^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF] + k_{-1}^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \quad (9a)$$

Eqns 2, 3 and 5 did not change. We varied the values of the rate constants of the following hypothetical reactions so as to describe the results of [8] (see Results): interaction of enzyme–product complex with TFPI ($k_a^{Xa-VIIa-TF,TFPI}$ and $k_d^{TFPI-Xa-VIIa-TF}$), association of Xa–TFPI and VIIa–TF, which results in the intermediate inhibitory complex formation ($k_{a1}^{Xa-TFPI,VIIa-TF}$ and $k_{d1}^{TFPI-Xa-VIIa-TF}$), intramolecular reaction of the inhibitory complex ($k_{+1}^{TFPI-Xa-VIIa-TF}$ and $k_{-1}^{Xa-TFPI-VIIa-TF}$).

The reader should notice that the rate constants of the Xa–TFPI:VIIa–TF interaction, $k_a^{Xa-TFPI,VIIa-TF}$ and $k_d^{Xa-TFPI-VIIa-TF}$, which were obtained from the experiments, are only apparent constants and not real ones. If the hypothetical pathway investigated in this model exists, then these measured values of $k_a^{Xa-TFPI,VIIa-TF}$ and $k_d^{TFPI-Xa-VIIa-TF}$ will depend on the constants of Xa, VIIa–TF and TFPI interaction, $k_a^{Xa-TFPI,VIIa-TF}$, $k_d^{TFPI-Xa-VIIa-TF}$, $k_{a1}^{Xa-TFPI,VIIa-TF}$, $k_{d1}^{TFPI-Xa-VIIa-TF}$, $k_{+1}^{TFPI-Xa-VIIa-TF}$ and $k_{-1}^{Xa-TFPI-VIIa-TF}$, in a complex way. For example, the first approximation gives us $k_{a\ app}^{Xa-TFPI,VIIa-TF} = k_a^{Xa-TFPI,VIIa-TF} + k_{a1}^{Xa-TFPI,VIIa-TF}$. Apparent values of $k_a^{Xa-TFPI,VIIa-TF}$ and $k_d^{TFPI-Xa-VIIa-TF}$ are rather low. So we assumed the true rate constants of the final inhibitory complex formation $k_a^{Xa-TFPI,VIIa-TF}$ and $k_d^{Xa-TFPI-VIIa-TF}$ to be equal to their apparent values and found the values of the hypothetical reactions separately.

The model of the inhibitory action of the Xa–TFPI complex on the enzyme–substrate complex (model 3)

The reaction of X–VIIa–TF inhibition by Xa–TFPI was added as follows. We suggested that Xa–TFPI interacts with the enzyme–substrate complex by displacing the substrate, factor X, and forming the intermediate TFPI–Xa–VIIa–TF inhibitory complex. Equations 1a, 2, 3, 5, 7a and 9a were changed in the accordance to Scheme 3A. The constants of the hypothetical reactions $k_{a1}^{Xa-TFPI,VIIa-TF}$, $k_{d1}^{TFPI-Xa-VIIa-TF}$, $k_{+1}^{TFPI-Xa-VIIa-TF}$ and $k_{-1}^{Xa-TFPI-VIIa-TF}$ were equal to 0 basing on our investigation of model 2 (see Results), so the terms corresponding to these reactions were not included into the following system for the purpose of better presentation.

$$\frac{d[VIIa-TF]}{dt} = -k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] + k_d^{X-VIIa-TF}[X-VIIa-TF] - k_a^{VIIa-TF,Xa}[VIIa-TF]\cdot[Xa] + k_d^{Xa-VIIa-TF}[Xa-VIIa-TF] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \quad (1b)$$

$$\frac{d[X]}{dt} = -k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] + k_d^{X-VIIa-TF}[X-VIIa-TF] + k_{+1}^{X-VIIa-TF,Xa-TFPI}[X-VIIa-TF]\cdot[Xa-TFPI]; \quad (2b)$$

$$\frac{d[X-VIIa-TF]}{dt} = -k_{cat}^{X,VIIa-TF}[X-VIIa-TF] + k_a^{VIIa-TF,X}[VIIa-TF]\cdot[X] - k_d^{X-VIIa-TF}[X-VIIa-TF] - k_{+1}^{X-VIIa-TF,Xa-TFPI}[X-VIIa-TF]\cdot[Xa-TFPI]; \quad (3b)$$

$$\frac{d[Xa]}{dt} = -k_a^{VIIa-TF,Xa}[Xa]\cdot[VIIa-TF] + k_d^{Xa-VIIa-TF}[Xa-VIIa-TF] - k_a^{Xa,TFPI}[Xa]\cdot[TFPI] + k_d^{Xa-TFPI}[Xa-TFPI]; \quad (5b)$$

$$\frac{d[Xa-TFPI]}{dt} = k_a^{VIIa-TF,TFPI}[VIIa-TF]\cdot[TFPI] - k_d^{Xa-TFPI}[Xa-TFPI] - k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot[VIIa-TF] + k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF] - k_{+1}^{X-VIIa-TF,Xa-TFPI}[X-VIIa-TF]\cdot[Xa-TFPI]; \quad (7b)$$

$$\frac{d[TFPI-Xa-VIIa-TF]}{dt} = k_a^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF]\cdot[TFPI] - k_d^{TFPI-Xa-VIIa-TF}[TFPI-Xa-VIIa-TF] + k_{+1}^{X-VIIa-TF,Xa-TFPI}[X-VIIa-TF]\cdot[Xa-TFPI]; \quad (9b)$$

The other equations of the system (Eqns 1b–9b) are identical to those of system (Eqns 1a–9a). The values of the constants $k_a^{Xa-VIIa-TF,TFPI}, k_d^{TFPI-Xa-VIIa-TF}, k_{+1}^{X-VIIa-TF,Xa-TFPI}$ were obtained by variation (see Results).

RESULTS

Model for the Xa-dependent two-step mechanism of TFPI action (model 1)

The model for the two-step mechanism of the TFPI action developed in a previous study [8] has led the authors to the conclusion that two-step mechanism predicts too weak inhibition of the factor Xa activation and cannot describe the experiments of the study. To test the adequacy of our model and the correctness of the values of the unknown constants ($k_a^{VIIa-TF,X}, k_a^{VIIa-TF,Xa}, k_d^{Xa-VIIa-TF}$), we did the calculations of the study [8] anew. In Fig. 1A, experimental data of the study [8] for the factor X activation by VIIa–TF on phospholipids in the presence of TFPI are shown (see [8] for details). The VIIa–TF complex concentration was 1 nM. Factor X and TFPI were present at their mean plasma concentrations, 170 nM and 2.4 nM, respectively. Experiments in the absence of inhibitor revealed rapid and nearly complete activation of factor X. The presence of TFPI caused rapid ($\approx$ 30 s), complete and irreversible suppression of the VIIa–TF activity; factor Xa concentration has ceased its growth.

The activation curve calculated with the help of model 1 (Eqns 1–8) gives us a rather good description of the experiment carried out in the absence of the inhibitor (Fig. 1A, curve 1), with the values of kinetic constants given in Table 1. To simulate this experiment we used the kinetic constants of the enzyme–product complex formation, $k_a^{VIIa-TF,Xa}$ and $k_d^{Xa-VIIa-TF}$, whose real values are unknown. To test their influence we varied $k_d^{Xa-VIIa-TF}$ in the range of 200–2000 min^{-1}, while the equilibrium constant $K_{eq}^{VIIa-TF,Xa}$ was changed in the range of 0–0.05 nM^{-1} (which corresponds to the variation of $k_a^{VIIa-TF,Xa}$ from 0 to 10 $nM^{-1}\cdot min^{-1}$). It turned out that the values of these constants in these ranges do not significantly affect the kinetics of the system (Fig. 1B). Therefore in the following calculations we used fixed values $k_a^{VIIa-TF,Xa} = 5\ nM^{-1}\cdot min^{-1}$ and $k_d^{Xa-VIIa-TF} = 770\ min^{-1}$.

Curve 2 of Fig. 1A shows the results of our simulation of TFPI inhibitory action in this experiment and corresponding experimental data of the study [8]. It can be seen that the model predicts much weaker inhibition than there is in the experiment. These experiments were simulated over the whole range of the VIIa–TF complex concentrations used in [8], 0.032–1.024 nM, and gave similar results (data not shown). To test the two-step mechanism of TFPI action for its ability to describe the experiments in principle, we increased the constant for factor Xa inhibition by TFPI 10-fold (Fig. 1C, curve 2), but no significant increase of inhibition was obtained. The 10-fold increase of the constant of VIIa–TF and Xa–TFPI association produced a larger effect (Fig. 1C, curve 3). Additional increase of inhibitory action was obtained by the 10-fold increase of both constants (Fig. 1C, curve 4). Still, model 1 was not able to describe the experiment. It looks unlikely that a 10-fold error occurred in the measurements of TFPI pathway constants carried out by several independent groups. Therefore, we suggest that our calculations support the conclusion of study [8] that the notion that the Xa–TFPI complex inhibits only free enzyme (VIIa–TF) is not sufficient for the description of the regulation of factor Xa formation.

Further evidence for this conclusion is provided by the analysis of TFPI effect in the reconstituted systems of purified proteins containing factors IX, X, II, V, VIII in their mean plasma concentrations (see [19,20]). The modeling of these experiments (M. A. Panteleev, V. I. Zarnitsina, F. I. Ataullakhanov, unpublished results) shows that in such

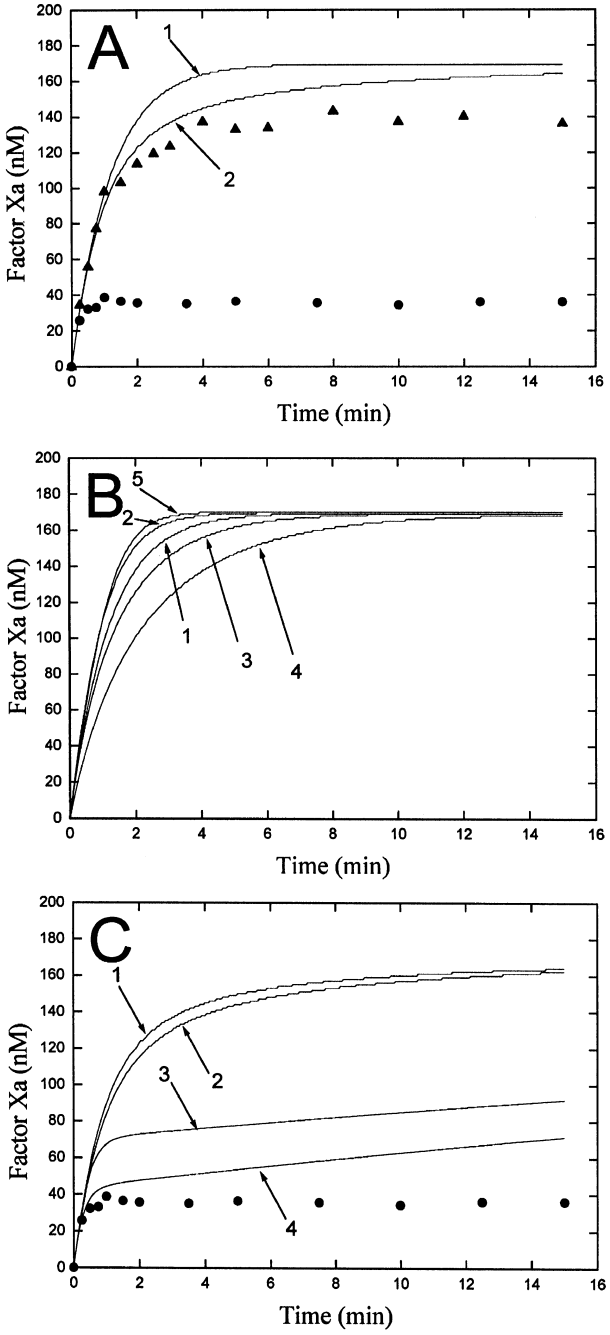


Fig. 1. Factor X activation by VIIa–TF in the presence of TFPI: simulation with the help of model 1. (A) Simulation of an experiment in [8]. Reaction mixture contains 1 nM of VIIa–TF, 170 nM of factor X, in the absence (▲, curve 1) or presence (●, curve 2) of 2.4 nM of TFPI. Progress curves were obtained by numerical simulation of Eqns 1–8 (Scheme 1) using the constants listed in Table 1. (B) The influence of the values of the kinetic constants of the enzyme–product complex formation/dissociation on the behavior of the system. All the curves were drawn according to the initial conditions of curve 1 of (A). Curve 1: all the constants used were those of Table 1. Curve 2: $k_a^{VIIa-TF,Xa} = 0$. Curve 3: $k_a^{VIIa-TF,Xa} = 10$ nM^{-1}·min^{-1}. Curve 4: $k_d^{Xa-VIIa-TF} = 200$ min^{-1}. Curve 5: $k_d^{Xa-VIIa-TF} = 2000$ min^{-1}. (C) The constants of the TFPI pathway were increased [initial conditions correspond to those of curve 2 of (A)]. Curve 1: all the constants used were those of Table 1. Curve 2: $k_a^{Xa,TFPI}$ was increased from 0.054–0.54 nM^{-1}·min^{-1}. Curve 3: $k_a^{Xa-TFPI,VIIa-TF}$ was increased from 0.44–4.4 nM^{-1}·min^{-1}. Curve 4: both constants were increased 10-fold; (●) an experiment from [8]. Experimental data are reproduced from [8] by kind permission of the American Society of Biochemistry and Molecular Biology, Copyright 1998.

reconstituted systems of blood coagulation proteins effect of the two-step mechanism of VIIa–TF inhibition by TFPI would be insignificantly small, which does not correlate with the experiments [19,20]. The 10-fold increase of the kinetic constants of Xa and TFPI, Xa–TFPI and VIIa–TF association cannot affect this observation (data not shown).

Investigation of the model, which involves the enzyme–product complex inhibition by TFPI (model 2)

To explain the discrepancy between the two-step mechanism of inhibition (Scheme 1) and the experiment (Fig. 1A), the authors of the study [8] introduced a hypothesis of inhibitory action of TFPI on the enzyme–product complex as the predominant pathway of TFPI action (Scheme 2). The second step in the development of our model was to include this reaction into our model, investigate it and test its ability to fit the experiments that caused its inclusion.

Baugh *et al.* [8] conducted two series of experiments investigating TFPI effect on factor X activation. In series 1 (see [8]), the kinetics of the factor Xa formation was studied at different concentrations (0.032–1.024 nM) of the VIIa–TF complex in the presence of TFPI. Factor X and TFPI were present at their mean plasma concentrations, 170 nM and 2.4 nM, respectively. In series 2 (see [8]) the effect of the Xa–TFPI complex preformation was studied. To achieve it, the same kinetics was investigated at the same factor X and TFPI concentrations and under the same conditions with one exception: before the start of the experiment TFPI had been preincubated with 0–1 nM of factor Xa for 2 h to allow the Xa–TFPI complex formation. VIIa–TF concentration was fixed and equaled to 0.128 nM. Model 2 (Eqns 1a–9a) allows adequate description of the experimental curves of series 1 (Fig. 2A) at plausible values of the hypothetical reactions constants ($k_a^{Xa-VIIa-TF,TFPI} = 10$ nM^{-1}·min^{-1}, $k_d^{TFPI-Xa-VIIa-TF} = 0$ min^{-1}). The rates of the hypothetical reactions of VIIa–TF:Xa–TFPI binding into the intermediate TFPI–Xa–VIIa–TF inhibitory complex and intramolecular conversion TFPI–Xa–VIIa–TF/Xa–VIIa–TF–TFPI in the ranges of 0–0.02 nM^{-1}·min^{-1} and 0–1 min^{-1}, respectively, did not make a significant effect on the kinetics of the system considered. If they are increased, inhibitory effect decreases because of the dissociation of inhibitory complexes VIIa–TF–Xa–TFPI and Xa–TFPI–VIIa–TF. Therefore to evaluate the maximal inhibitory effect we assumed them to be equal to 0 (also in the following models).

Unlike model 1, the enzyme–product complex inhibition by TFPI does not allow us to consider the kinetics to be independent of the constants of this complex formation/dissociation, $k_a^{VIIa-TF,Xa}$ and $k_d^{Xa-VIIa-TF}$. Therefore the values of $k_a^{Xa-VIIa-TF,TFPI}$ and $k_d^{TFPI-Xa-VIIa-TF}$, which are required to describe the experiment, are also dependent on $k_a^{VIIa-TF,Xa}$ and $k_d^{Xa-VIIa-TF}$. The mathematical model reduction shown

in the Appendix has shown that $k_a^{Xa-VIIa-TF,TFPI}$ and $k_d^{TFPI-Xa-VIIa-TF}$ are practically independent of $k_a^{VIIa-TF,Xa}$ when the latter is changed in the range of 0–10 nM^{-1}·min^{-1}, and change of $k_d^{Xa-VIIa-TF}$ does not influence the behavior of the system if the condition shown in Appendix Eqn A20 is satisfied.

The best descriptions of the experiments of series 1 were obtained at $k_a^{Xa-VIIa-TF,TFPI} = 10$ nM^{-1}·min^{-1} and $k_d^{TFPI-Xa-VIIa-TF} = 0$ (the values of other constants are listed in Table 1) (Fig. 2A). However, the suggestion of the direct inhibition of the enzyme–product Xa–VIIa–TF complex by TFPI (Scheme 2) cannot explain series 2 (Fig. 2B). In the experiment [8], series 2 shows a strict regularity: the more factor Xa is added for preincubation with TFPI, the stronger the inhibition is. Theoretical calculations carried out with the values of the constants which were used to describe series 1 predict a directly opposite result: the more factor Xa is added, the more TFPI is bound into the Xa–TFPI complex, the less TFPI is free and the less is the rate of the enzyme–substrate complex inhibition by TFPI. Thus the inhibition is weaker because the hypothesis of Baugh *et al.* (Scheme 2) suggests that free TFPI is more effective than TFPI bound in the Xa–TFPI complex.

It appears that the hypothesis of the direct inhibition of the enzyme–substrate complex is not sufficient; other reactions must be included to complete it, to explain the existing experimental data.

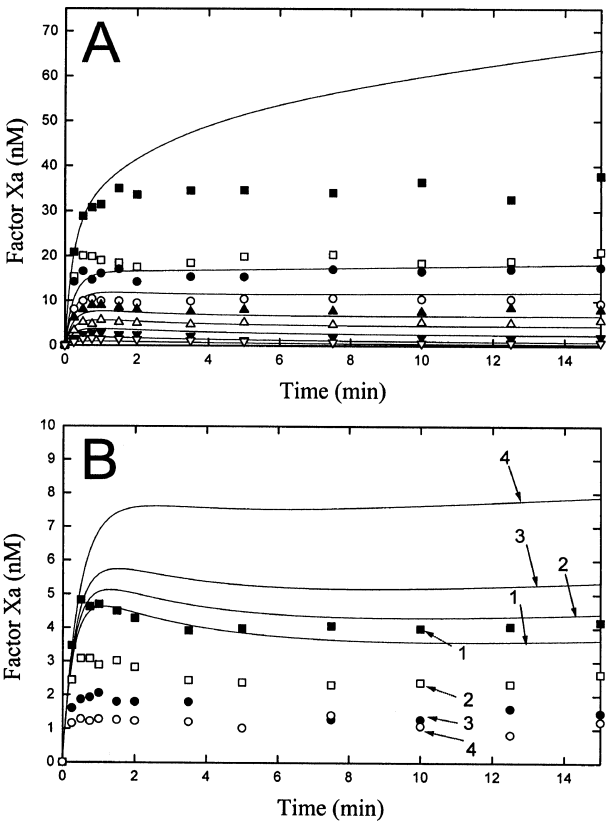


Fig. 2. Computational simulation of the experimental curves for the factor X activation carried out with the help of the hypothesis of Xa–VIIa–TF inhibition by TFPI (model 2). (A) (see [8]) Activation of factor X (170 nM) by VIIa–TF (1024, 512, 384, 256, 192, 128, 64 and 32 pM from top to bottom), in the presence of 2.4 nM TFPI. (B) (see [8]) Activation of factor X (170 nM) by VIIa–TF (128 pM) in the presence of 2.4 nM TFPI preincubated with factor Xa present at concentrations: (1) 0, (2) 0.25, (3) 0.5, and (4) 1 nM. Theoretical curves were obtained by digital integration of Eqns 1a–9a (Scheme 2). The values of the constants for the hypothetical reactions were $k_a^{Xa-VIIa-TF,TFPI} = 10$ nM^{-1}·min^{-1}, $k_d^{TFPI-Xa-VIIa-TF} = 0$ min^{-1}. All other constants are listed in Table 1. Experimental data are reproduced from [8] by kind permission of the American Society of Biochemistry and Molecular Biology, Copyright 1998.

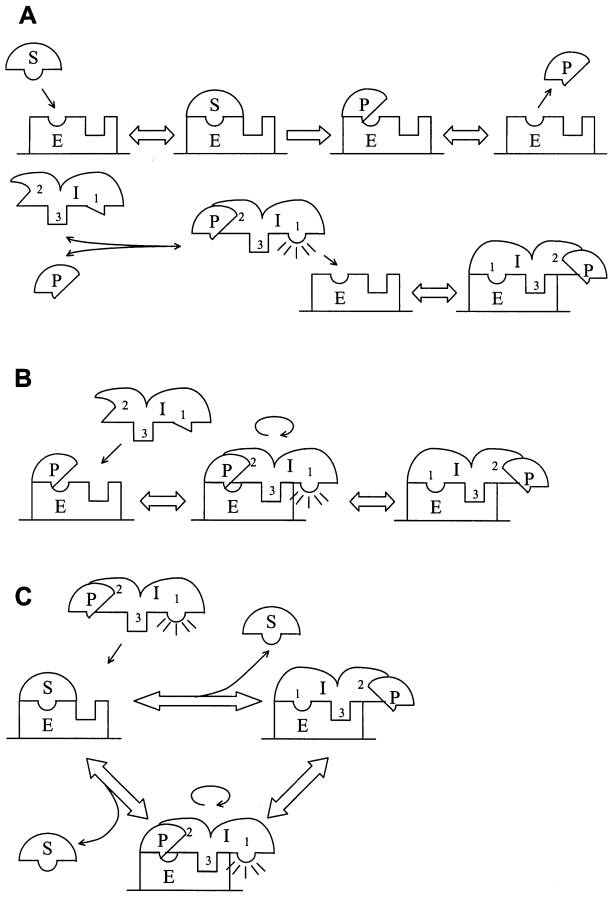
The enzyme–substrate complex inhibition by the Xa–TFPI complex (model 3)

An effective inhibition of VIIa–TF by Xa–TFPI is clearly necessary for the explanation of series 2 (Fig. 2B). All known species and their complexes present in the system are shown in Scheme 1. The constants of the direct binding of Xa–TFPI and VIIa–TF were independently measured by a number of groups [8,18,21]. They are not sufficiently large to explain series 2. The only VIIa–TF-containing species, which could be inhibited are the X–VIIa–TF and Xa–VIIa–TF complexes. It is logical to suppose that Xa–TFPI can interact with one or both of them, which could probably result in the final inhibitory complex Xa–TFPI–VIIa–TF formation after displacement of factor X (or Xa). One can imagine several ways of the specific realization of this pathway (Schemes 3A,B and 4C). The fact that TFPI has the third Kunitz-type domain whose role is not yet clear is a good structural basis for these speculations.

We supposed that the Xa–TFPI complex binds X–VIIa–TF or Xa–VIIa–TF, displacing factor X (or Xa, respectively) and forming the intermediate TFPI–Xa–VIIa–TF complex (Scheme 3A). Preliminary calculations have shown that only the first stage of the reaction, the binding of Xa–TFPI to X–VIIa–TF (or Xa–VIIa–TF), is important for the description of the experiments. The following conversions do not significantly affect the kinetics of the process.

Any of these two pathways (inhibition of X–VIIa–TF or Xa–VIIa–TF by Xa–TFPI) allows quantitative description of the experiments. First, let us consider the version of the X–VIIa–TF complex inhibition (Scheme 3A). The results of the modeling of the experimental series 1 and 2 [8] with the help of this mechanism are shown in Fig. 3A and B, respectively. The description of experimental results in Fig. 3B is qualitatively better than in Fig. 2B. The upper curve of Fig. 3A is also much closer to the experimental curve than that of Fig. 2A. The values of the constants for the hypothetical reactions which give the best description (Fig. 3) are: $k_a^{Xa-VIIa-TF,TFPI} = 6$ nM^{-1}·min^{-1}, $k_d^{TFPI-Xa-VIIa-TF} = 0.02$ min^{-1}, $k_{+1}^{X-VIIa-TF,Xa-TFPI} = 20$ nM^{-1}·min^{-1}, $k_{-1}^{TFPI-Xa-VIIa-TF,X} = 0$ min^{-1}. As in the previous section, the problem is how these values depend on the unknown constants of the enzyme–product complex formation/dissociation. Theoretical analysis shown in the Appendix shows that these values are independent of $k_a^{VIIa-TF,Xa}$ in the range of 0–10 nM^{-1}·min^{-1}, and the change of $k_d^{Xa-VIIa-TF}$ does not affect the kinetics of the system if the condition shown in Appendix Eqn A20 is satisfied.

If we use this hypothesis (Scheme 3) in the model system of purified proteins to describe thrombin formation, we obtain good description of the experiments from



Scheme 4. (A) The common two-step inhibitory mechanism of TFPI (I) (Scheme 1), (B) inhibition of factor Xa bound to enzyme by TFPI (Scheme 2), and (C) possible pathways for the enzyme–substrate complex inhibition by Xa–TFPI (the upper pathway corresponds to Scheme 3A, the lower one corresponds to Scheme 3B). In (A), the 1st, the 2nd and 3rd domains of TFPI are notified with numbers 1,2 and 3, respectively. In (B), a possible role of the 3d domain as an anchor during the structural reorganization is shown.

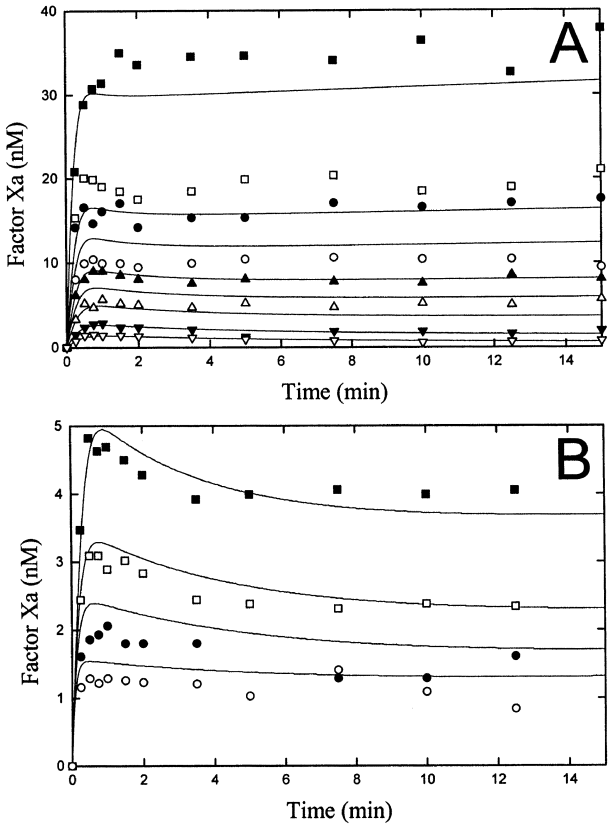


Fig. 3. Computational simulation of the experimental curves for the factor X activation carried out with the help of the hypothesis of X–VIIa–TF inhibition by Xa–TFPI (model 3). (A) (see [8]) Activation of 170 nM of factor X by VIIa–TF (1024, 512, 384, 256, 192, 128, 64 and 32 pM from top to bottom) in the presence of TFPI (2.4 nM). (B) (see [8]) Activation of 170 nM of factor X by VIIa–TF (128 pM) in the presence of 2.4 nM of TFPI preincubated with factor Xa (0, 0.25, 0.5, 1 nM from top to bottom). Theoretical curves were obtained by digital integration of Eqns 1b–9b (Scheme 3A). The values of the constants for the hypothetical reactions were: $k_a^{Xa-VIIa-TF,TFPI} = 6$ nM^{-1}·min^{-1}, $k_d^{TFPI-Xa-VIIa-TF} = 0.02$ min^{-1}, $k_{+1}^{X-VIIa-TF,TFPI} = 20$ nM^{-1}·min^{-1}, $k_{-1}^{TFPI-Xa-VIIa-TF,X} = 0$ min^{-1}. All other constants are listed in Table 1. Experimental data are reproduced by kind permission of the American Society of Biochemistry and Molecular Biology, Copyright 1998, from [8].

studies [19,20] at the same values of kinetic constants which give the best description of the experiments from the study [8] (not shown).

The second version suggesting that Xa–TFPI binds Xa–VIIa–TF, also can describe the experiments of series 1, 2 from the previous study [8] (data not shown). However, one has to assume the binding constant $k_{+1}^{Xa-VIIa-TF,Xa-TFPI}$ to be equal to 60 nM^{-1}·min^{-1}. This value is near diffusion-limited. As $k_a^{Xa-VIIa-TF,TFPI}$, it depends on assumed $k_d^{Xa-VIIa-TF}$ according to the equation: $k_{+1}^{Xa-VIIa-TF,Xa-TFPI} \propto k_d^{Xa-VIIa-TF}$. But, even being several-fold lower, it still would be much larger than the values of other association constants involved in the TFPI pathway. So this version looks less plausible.

The important question is how significant the role of each hypothetical reaction considered above is in the overall inhibition process. The calculations allow us to suggest (data not shown), that series 1 and 2 could be approximately described with the help of the single hypothesis of the interaction between X–VIIa–TF (or Xa–VIIa–TF) and Xa–TFPI, with slight variation of the constants of the two-step pathway. However, the description of the systems of studies [19,20] requires direct one-step inhibition by TFPI. No feedback, requiring factor Xa and subsequent Xa–TFPI formation, can slow down thrombin formation to the same extent as TFPI does in the experiments [19,20] (inhibition of X–VIIa–TF by Xa–TFPI suggested in the present study is also this kind of feedback).

The main conclusion is the explanation of all experiments requires both direct inhibition of Xa–VIIa–TF by TFPI and inhibition of X–VIIa–TF (or Xa–VIIa–TF) by Xa–TFPI.

Possible contradictions with the observations of other studies

The activation of factor X (50 pM) by the VIIa–TF complex (10 pM) in the presence of the increasing concentrations

of Xa–TFPI was investigated in a previous study [18]. The results obtained were used for the determination of the rate constants of Xa–TFPI binding to VIIa–TF. Model 3 of the present study, in which Xa–TFPI can inhibit not only VIIa–TF, but also X–VIIa–TF, predicts much more efficient inhibition than that observed in [18], if Xa–TFPI interaction is considered to be one-step. If we consider it to be two-step, the following explanation becomes possible. In a previous study [8] Xa and TFPI were preincubated for 2 h, while in another previous study [18] their preincubation lasted only 15 min. A plausible explanation is that binding observed in [8] was really complete while in [18] most Xa–TFPI was in its intermediate state, which maybe is not as efficient an inhibitor of VIIa–TF. For the purposes of simplicity we suggested that the intermediate of Xa–TFPI formation cannot inhibit VIIa–TF or Xa–VIIa–TF at all. If model 3 is changed so that factor Xa inhibition occurs in a two-step fashion with the constant for the second step about 0.1 min^{-1}, and we approximate that at the start of the experiment in [18] Xa–TFPI is totally in the intermediate state, we shall be able to obtain good description of inhibition (Fig. 4). For the purpose of better perception we presented theoretical and experimental data on different panels. The description of the results of other studies [8,19,20] with the help of this modified model did not change significantly (not shown).

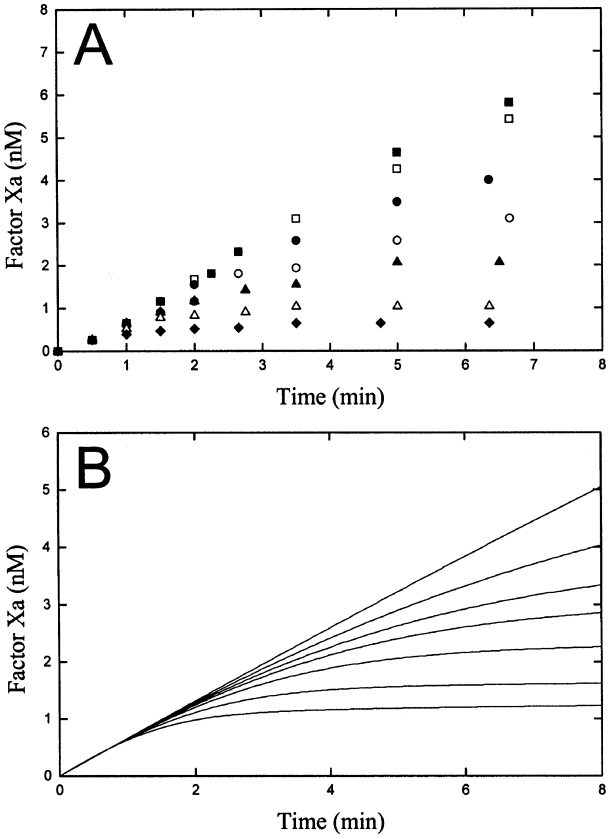


Fig. 4. Inhibition of the factor X activation by Xa–TFPI. Activation of factor X (50 nM) was conducted by 10 pM of VIIa–TF in the presence of (from top to bottom) 0, 0.1, 0.2, 0.3, 0.1, 1 and 2 nM of Xa–TFPI. Xa and TFPI were preincubated for 15 min. (A) Experimental data from [18] are reproduced by kind permission of the American Chemical Society, Copyright 1994. (B) Corresponding theoretical calculations carried out by digital integration of Eqns 1b–9b modified by addition of the second step of Xa/TFPI binding to explain slow inhibition of (A). All the constants are listed in Table 1, with the exception of the constants for the second step of Xa:TFPI binding, which were: $k_{+}^{Xa,TFPI} = 0.1$ min^{-1}, $k_{-}^{Xa-TFPI} = 0.01$ min^{-1}.

Verification of the hypotheses considered in the present study

The main conclusion of the present study is that good quantitative description of all experimental data can be achieved with the help of two hypothetical reactions: (a) the enzyme–product Xa–VIIa–TF complex inhibition by TFPI, and (b) the enzyme–substrate X–VIIa–TF and/or the enzyme–product Xa–VIIa–TF complex inhibition by Xa–TFPI.

If Xa–VIIa–TF concentration is large enough, the existence of these reactions can be verified experimentally. One possible way to do this is to create an excess concentration of one of the components of the Xa–VIIa–TF complex (Xa or VIIa–TF) so that a significant part of another component will be in the complex. Hypothetical inhibition pathways, which involve this complex and are normally efficient only during ongoing factor X activation, will then be visible. Specific organization of the experiments is presented below.

The Xa–VIIa–TF:TFPI binding verification

Suppose that 1 nM of Xa, 1 nM of TFPI, 0 or 5 nM of the VIIa–TF complex are mixed together and activity of factor Xa is monitored. In the absence of VIIa–TF, slow inhibition of Xa by TFPI will be observed (Fig. 5A, the first curve from the top). On the other hand, there are two possibilities in case of addition of 5 nM of VIIa–TF.

If the hypothetical reaction of Xa–VIIa–TF/TFPI binding does not exist, then during the first few seconds factor Xa concentration will slightly decrease because of its binding into Xa–VIIa–TF. Then the slow inhibition of Xa will start, as in the absence of VIIa–TF (Fig. 5A, the second curve from the top).

If the binding between Xa–VIIa–TF and TFPI does exist and is significant, then adding of VIIa–TF will cause potent inhibition of factor Xa (Fig. 5A, the third curve from the top).

Feasibility of the experiment depends on possibility of creation of a Xa–VIIa–TF concentration high enough to make this hypothetical pathway visible. Evidently, it depends on the Xa:VIIa–TF equilibrium constant, whose value is unknown. We varied it to evaluate the effect. If it is smaller than the value used in the model (Table 1) then one has to use higher concentration of VIIa–TF to maintain Xa–VIIa–TF concentration. This concentration of VIIa–TF is defined by Eqn A22 (see Appendix).

The criterion for the possibility to detect the reaction was twofold change of factor Xa concentration by the end of the experiment in the presence of VIIa–TF. Evidently only the VIIa–TF concentration, which can be modified, limits this possibility.

The Xa–VIIa–TF/Xa–TFPI interaction verification

Suppose that 1 nM of VIIa–TF, 1 nM of preformed Xa–TFPI, 0 or 100 nM of Xa are mixed together and the factor VIIa–TF activity is monitored. In the absence of Xa, slow inhibition of Xa by VIIa–TF will be observed (Fig. 5B, the first curve from the top). There are two possibilities in case of addition of 100 nM of Xa: (a) If there is no interaction between Xa–VIIa–TF and Xa–TFPI, rapid decrease of VIIa–TF activity will be observed because of its binding into the Xa–VIIa–TF complex. It will be followed by slow inhibition (Fig. 5B, second curve from the top). (b) If the interaction exists, the inhibition will be very rapid in the presence of high Xa concentrations (Fig. 5B, third curve from the top).

By analogy with the previous case, the possibility of detecting this reaction is defined by the maximal possible Xa concentration. Xa–TFPI is saturated and its concentration does not change after the addition of Xa, so it inhibits VIIa–TF at a constant rate. If even weak interaction between Xa–VIIa–TF and Xa–TFPI exists, it can be visualized by increasing factor Xa (and thus Xa–VIIa–TFPI) concentration. Theoretical analyses given in the Appendix shows that visibility of the reaction is defined by the correlation similar to Eqn A22 (see Eqn A23).

X–VIIa–TF/Xa–TFPI interaction test

As stated above, this reaction appears to be more possible than Xa–VIIa–TF inhibition by Xa–TFPI because of the more plausible values of its kinetic constants. It allows similar good explanation of the experiments of factor X activation in the presence of Xa–TFPI [8]. Unlike the previous two reactions, its direct verification with the help of natural proteins is difficult because of factor X cleavage and, thus, instability of the X–VIIa–TF complex. However, this difficulty can be overcome by using some artificial species of factor X that cannot be cleaved by VIIa–TF. It can provide convincing evidence of the existing of this reaction in the experiment similar to the experiment of the previous section (where factor Xa is substituted with modified factor X).

Indeed, let us take 1 nM of VIIa–TF, 1 nM of preformed Xa–TFPI, 0 or 100 nM of modified factor X and monitor VIIa–TF activity. By analogy with the previous case, in the absence of factor X, slow inhibition of VIIa–TF will occur. In the presence of high concentration of modified factor X there will the same two possibilities, which will let us decide whether the hypothetical reaction exists.

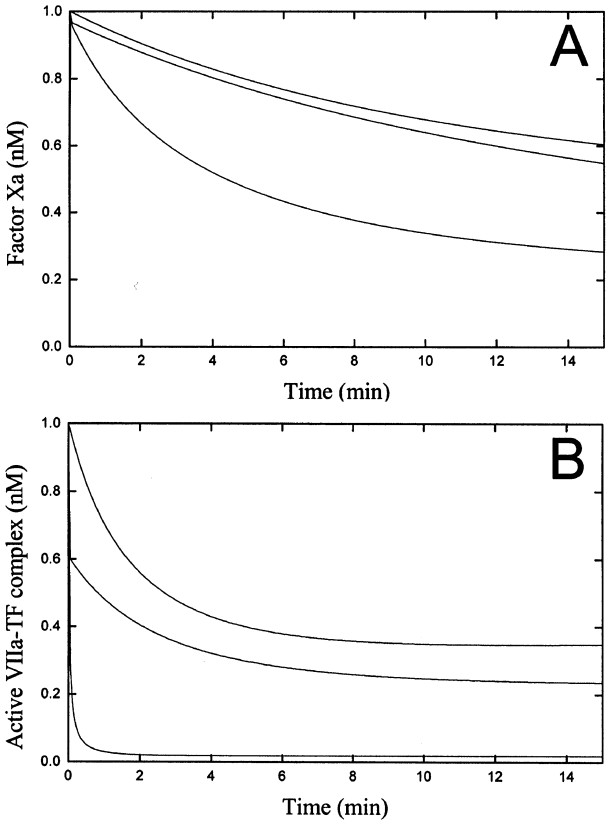


Fig. 5. Verification of the hypotheses for TFPI inhibitory mechanism. (A) Verification of the hypothesis of the enzyme–product complex inhibition by TFPI. Factor Xa is inhibited by TFPI in the presence or in the absence of VIIa–TF. The concentrations of the reagents are: [Xa] = 1 nM, [TFPI] = 1 nM, [VIIa–TF] = 0 or 5 nM. From top to bottom: VIIa–TF is absent; VIIa–TF is present, the hypothetical reaction is absent; VIIa–TF is present, the hypothetical reaction is present. The curves are drawn according to the digital integration of Eqns 1b–9b. $k_a^{Xa-VIIa-TF,TFPI} = 6\ \text{nM}^{-1}\cdot\text{min}^{-1}$, $k_d^{TFPI-Xa-VIIa-TF} = 0.02\ \text{min}^{-1}$, $k_{+1}^{X-VIIa-TF,Xa-TFPI} = 20\ \text{nM}^{-1}\cdot\text{min}^{-1}$, $k_{-1}^{TFPI-Xa-VIIa-TF,x} = 0\ \text{min}^{-1}$. All other constants are listed in Table 1. (B) Verification of the hypothesis of the enzyme–product complex inhibition by Xa–TFPI. VIIa–TF is inhibited by Xa–TFPI in the presence or in the absence of factor Xa. The concentrations of the reagents are: [TF] = 1 nM, [Xa–TFPI] = 1 nM, [Xa] = 0 or 100 nM. From top to bottom: factor Xa is absent; Xa is present, the hypothetical reaction is absent; Xa is present, the hypothetical reaction is present. The curves are drawn by digital integration of Equations 1b–9b with a single modification: Xa–TFPI inhibits Xa–VIIa–TFPI and not X–VIIa–TFPI. $k_a^{Xa-VIIa-TF,TFPI} = 6\ \text{nM}^{-1}\cdot\text{min}^{-1}$, $k_d^{TFPI-Xa-VIIa-TF} = 0.02\ \text{min}^{-1}$, $k_{+1}^{Xa-VIIa-TF,Xa-TFPI} = 60\ \text{nM}^{-1}\cdot\text{min}^{-1}$, $k_{-1}^{TFPI-Xa-VIIa-TF,Xa} = 0\ \text{min}^{-1}$. All other constants are listed in Table 1.

DISCUSSION

The objective of the study was to simulate various mechanisms of TFPI action, which had been discussed previously, and test their ability to describe existing experimental data. TFPI is unique in its ability to interact with both the enzyme VIIa–TF and its product factor Xa. TFPI has three Kunitz-type domains. The first domain binds to and inhibits factor VIIa, the second inhibits factor Xa. Inhibition of VIIa can proceed only after preliminary Xa binding. It was shown [6] to result in the quaternary Xa–TFPI–VIIa–TF complex formation. The hypothesis of Baugh *et al.* [8] suggests that this inhibitory complex can be the result of binding of TFPI to the enzyme–product Xa–VIIa–TF complex and this is the main pathway of inhibition of VIIa–TF during ongoing factor X activation. Analysis of the present study supports this conclusion. Results of a recent study [16] show that factor Xa binds to VIIa–TF with the affinity, which is similar to that of factor X. According to Eqn A19, it means that concentration of Xa–VIIa–TF during factor X activation is similar to that of free VIIa–TF, which is also an evidence of the hypothesis.

An attempt to visualize possible intermolecular interactions between the proteins involved in these reactions is shown in Scheme 4. In the final inhibitory complex the enzyme, E, is bound to the first Kunitz-domain of the inhibitor, while the product, P, is bound to the second one. Two-step Scheme 1 suggests consecutive binding of P to I and then of the PI complex to E. In the final complex the inhibitor mediates interaction between the product and the enzyme, being bound to both. If the reaction of Xa–VIIa–TF inhibition by TFPI proceeds through inhibition of factor Xa bound to enzyme and results in the final complex formation, then some intramolecular conversion should occur. Binding of inhibitor to the enzyme–substrate complex will lead to the formation of the intermediate complex (EPI), where the product is bound both to the enzyme and to the second Kunitz-domain of the inhibitor (Scheme 4B). After intramolecular conversion the final inhibitory complex appears, where the enzyme is bound to the first domain of the inhibitor. One can speculate that the third Kunitz-type domain plays a role of some joint or anchor, which allows the conversion without dissociation. It is possible that it interacts not only with the enzyme but also with surface structures of the membrane in which the enzyme is integrated.

Mathematical simulation of the study has shown that the hypothesis of Baugh *et al.* [8] can describe a series of experimental data (Fig. 2A), which could not be explained by the two-step model suggesting consecutive inhibition of factor Xa and VIIa–TF by TFPI (Fig. 1A,C). However, the factor Xa generation in the presence of the preformed Xa–TFPI complex cannot be explained by this hypothesis.

The contradiction can be overcome if we suppose that there is an interaction between Xa–TFPI and some VIIa–TF-containing species, e.g. the enzyme–substrate complex X–VIIa–TF or the enzyme–product complex Xa–VIIa–TF. The similarity between these complexes [16] suggests that Xa–TFPI can interact with both complexes. It is obvious that if the product-inhibitor complex displaces the substrate from the enzyme, this can result either in the intermediate inhibitory complex formation (Scheme 4C, lower pathway) or direct generation of the final complex (Scheme 4C, upper pathway). Our model shows that such interaction between Xa–TFPI and the enzyme–substrate complex can explain the experiment (Fig. 3A,B). Note that slow reaction of Xa:TFPI binding will possibly decrease significance of this pathway in comparison with direct inhibition of Xa–VIIa–TF by TFPI under physiological conditions. Simulation of the model systems of proteins mimicking blood coagulation system [19,20] supports this conclusion.

In the study of Jesty *et al.* [18], the constants for the VIIa–TF inhibition by Xa–TFPI were measured by analysis of factor Xa generation curves in the presence of Xa–TFPI. If the hypothetical reaction of Xa–VIIa–TF inhibition by Xa–TFPI is efficient as we suppose, it must have been detected in such an experiment. A possible explanation of the contradiction is the low duration (15 min) of Xa:TFPI preincubation in the study [18]. As for other Kunitz-type inhibitors TFPI inhibits Xa in a two-step fashion. It is possible that if the reaction is not completed then VIIa–TF inhibition by Xa–TFPI will be weak. The calculations show that this explanation can describe the experiments accurately (Fig. 4).

In general, various hypotheses of TFPI action investigated in the present study provide good explanations of the existing experiments. However, there are not enough data to make a final choice between them to evaluate the kinetic parameters of these reactions. However, the calculations show that the parameters can be determined directly by experiment. Using excess concentrations of Xa or VIIa–TF, it is possible to create a high Xa–VIIa–TF concentration (in comparison with the concentration of the limiting component). If the reaction exists then after addition of the inhibitor (Xa–TFPI or TFPI, respectively) of the limiting component the rate of inhibition of the limiting component will substantially increase in comparison with the rate observed in the absence of excess component.

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This study was motivated in part by the pioneering work of Baugh, R. J., Broze, G. J. Jr. & Krishnaswamy, S. [8], and therefore we would like to acknowledge their intellectual contribution. We thank O. N. Izakova for help in the translation of the manuscript and M. V. Ovanesov for helpful comments.

APPENDIX

Kinetic constants for the enzyme–substrate X–VIIa–TF and the enzyme–product Xa–VIIa–TF complexes formation/dissociation are unknown. If the values of these constants are in the range of: $k_a^{VIIa-TF,Xa} = 0$–10 нМ$^{-1}$·min^{-1}; $k_a^{VIIa-TF,X} = 2$–10 нМ$^{-1}$·min^{-1}; $k_d^{X-VIIa-TF}, k_d^{Xa-VIIa-TF} = 200$–$2000$ min^{-1}, it can be shown that steady-state assumption can be used. The kinetics of model 1 under these condition does not depend on their values and is defined only by Michaelis and catalytic constants. In addition, reduction of the model system allows us to obtain several correlations for models 1, 2 described in Results (Equations A20, A22, A23).

Reduction of the differential equations system of model 1

To perform reduction we shall use the following dimensionless variables: $\tau = \frac{t}{k_a^{VIIa-TF,X}[VIIa-TF]_0}$, $e = \frac{[VIIa-TF]}{[VIIa-TF]_0}$, $es = \frac{[X-VIIa-TF]}{[VIIa-TF]_0}$, $ep = \frac{[Xa-VIIa-TF]}{[VIIa-TF]_0}$, $eip = \frac{[Xa-TFPI-VIIa-TF]}{[VIIa-TF]_0}$, $s = \frac{[X]}{[X]_0}$, $p = \frac{[Xa]}{[X]_0}$, $i = \frac{[TFPI]}{[TFPI]_0}$, $pi = \frac{[Xa-TFPI]}{[TFPI]_0}$. In these equations $[VIIa-TF]_0$ is a typical concentration of the enzyme, $[X]_0$and $[TFPI]_0$ are initial concentrations of the substrate and the product, respectively, in the experiments considered. Then the system of equations of model 1 (Eqns 1–8) will have the form:

$$\frac{[VIIa-TF]_0}{[X]_0}\frac{de}{d\tau} = -e\cdot s + \frac{k_d^{X-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}es - \frac{k_a^{VIIa-TF,Xa}}{k_a^{VIIa-TF,X}}e\cdot p + \frac{k_d^{Xa-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}ep - \frac{k_a^{Xa-TFPI,VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}pi\cdot e + \frac{k_d^{Xa-TFPI-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}pie; \quad (A1)$$

$$\frac{ds}{d\tau} = -e\cdot s + \frac{k_d^{X-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}es; \quad (A2)$$

$$\frac{[VIIa-TF]_0}{[X]_0}\frac{des}{d\tau} = e\cdot s - \frac{k_d^{X-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}es - \frac{k_{cat}^{X,VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}es; \quad (A3)$$

$$\frac{[VIIa-TF]_0}{[X]_0}\frac{dep}{d\tau} = \frac{k_{cat}^{X,VIIa-TF}}{k_a^{VIIa-TF,X}[S]_0}es + \frac{k_a^{VIIa-TF,Xa}}{k_a^{VIIa-TF,X}}e\cdot p - \frac{k_d^{Xa-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}ep; \quad (A4)$$

$$\frac{dp}{d\tau} = -\frac{k_a^{VIIa-TF,Xa}}{k_a^{VIIa-TF,X}[X]_0}e\cdot p + \frac{k_d^{Xa-VIIa-TF}}{k_a^{VIIa-TF,X}[X]_0}ep - \frac{k_a^{Xa,TFPI}[TFPI]_0}{k_a^{VIIa-TF,X}[VIIa-TF]_0}p\cdot i + \frac{k_d^{Xa-TFPI}[TFPI]_0}{k_a^{VIIa-TF,X}[X]_0[VIIa-TF]_0}pi; \quad (A5)$$

$$\frac{di}{d\tau} = -\frac{k_a^{Xa,TFPI}[X]_0}{k_a^{VIIa-TF,X}[VIIa-TF]_0}p\cdot i + \frac{k_d^{Xa-TFPI}}{k_a^{VIIa-TF,X}[VIIa-TF]_0}pi; \quad (A6)$$

$$\frac{dpi}{d\tau} = \frac{k_a^{Xa,TFPI}[X]_0}{k_a^{VIIa-TF,X}[VIIa-TF]_0}p\cdot i - \frac{k_d^{Xa-TFPI}}{k_a^{VIIa-TF,X}[VIIa-TF]_0}pi - \frac{k_a^{Xa-TFPI,VIIa-TF}}{k_a^{VIIa-TF,X}}pi\cdot e + \frac{k_d^{Xa-TFPI-VIIa-TF}}{k_a^{VIIa-TF,X}[TFPI]_0}pie; \quad (A7)$$

$$\frac{dpie}{d\tau} = \frac{k_a^{Xa-TFPI,VIIa-TF}[TFPI]_0}{k_a^{VIIa-TF,X}[VIIa-TF]_0}pi\cdot e - \frac{k_d^{Xa-TFPI-VIIa-TF}}{k_a^{VIIa-TF,X}[VIIa-TF]_0}pie; \quad (A8)$$

If we use the constants listed in Tables 1 and a typical concentration of enzyme, 0.1 nM, the variables *e*, *es* and *ep* will have a small parameter $[VIIa-TF]_0/[X]_0 \approx 0.1\ \text{nM}/170\ \text{nM} < 0.001$ in the left part of their equations. It means that they are rapid in comparison with the others. So we can use Tikhonov's theorem [22] to consider more rough time scale. Variables *e*, *es*, *ep* reach their steady-state values exponentially. Characteristic time for this process can be obtained from Equations A1–A8. As the variables *s*, *p*, *i*, *ip*, and *eip* are practically constant during this characteristic time, Eqns A3, A4 become:

$$\frac{des}{dt} = A + \alpha es + \beta ep; \quad (A9)$$

$$\frac{dep}{dt} = B + \gamma es + \delta ep; \quad (A10)$$

where A, B, α, β, γ, δ are constant.

Using standard techniques for the solution of systems of linear differential equations (e.g [22]). we obtain the characteristic time $\tau = -\frac{2}{\alpha+\delta\pm\sqrt{(\alpha+\delta)^2-4(\alpha\delta-\beta\gamma)}} \approx 0.001$ min.

In the new time scale, on times larger than *t*, steady-state assumption can be used for the variables *e*, *es*, and *ep*. It means that on times larger than 0.001 min, we can substitute the small parameter $[VIIa-TF]_0/[X]_0$ with 0 and thus obtain the concentrations of the complexes from Eqns A1–A8. Going back to dimensional variables we obtain:

$$[X-VIIa-TF] = \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right)\cdot[X]}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}; \quad (A11)$$

$$[Xa-VIIa-TF] = \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right)}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}\cdot\left(\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}[X]+\frac{K_M^{X,VIIa-TF}}{K_D^{Xa-VIIa-TF}}[Xa]\right); \quad (A12)$$

$$[VIIa-TF] = \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right)}{\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}; \quad (A13)$$

Substituting these values to Eqns 2, 5, 6, 7, 8 we obtain the reduced system:

$$\frac{d[X]}{dt} = -\frac{k_{cat}^{X,VIIa-TF}\cdot\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)\cdot[X]}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}; \tag{A14}$$

$$\frac{d[Xa]}{dt} = \frac{k_{cat}^{X,VIIa-TF}\cdot\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)\cdot[X]}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)} - k_a^{Xa,TFPI}[Xa]\cdot[TFPI] + k_d^{Xa-TFPI}[Xa-TFPI]; \tag{A15}$$

$$\frac{d[TFPI]}{dt} = -k_a^{Xa,TFPI}[Xa]\cdot[TFPI]+k_d^{Xa-TFPI}[Xa-TFPI]; \tag{A16}$$

$$\frac{d[Xa-TFPI]}{dt} = k_a^{Xa,TFPI}[Xa]\cdot[TFPI]-k_d^{Xa-TFPI}[Xa-TFPI]-k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI]\cdot\frac{\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)}{\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}+k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \tag{A17}$$

$$\frac{d[Xa-TFPI-VIIa-TF]}{dt} = \frac{\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)}{\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}\cdot k_a^{Xa-TFPI,VIIa-TF}[Xa-TFPI] - k_d^{Xa-TFPI-VIIa-TF}[Xa-TFPI-VIIa-TF]; \tag{A18}$$

where $K_D^{Xa-VIIa-TF}=\frac{k_d^{Xa-VIIa-TF}}{k_a^{Xa,VIIa-TF}}$.

From this system we see that influence of $k_a^{VIIa-TF,X}$ is small because of its absence in the reduced system. $k_a^{VIIa-TF,Xa}$ and $k_d^{Xa-VIIa-TF}$ within the ranges specified above only modestly affect the kinetics for they are present only in the denominator $\left(1+[Xa]/K_D^{Xa-VIIa-TF}+[X]/K_M^{X,VIIa-TF}\left[1+k_{cat}^{X,VIIa-TF}/k_d^{Xa-VIIa-TF}\right]\right)$ and concentration of the product $[Xa]$ is low in comparison with the expected values for the equilibrium dissociation constant $K_D^{Xa-VIIa-TF}$ of the enzyme–product complex.

Notice that the behavior of the system with the enzyme–product complex stage included is still equivalent to that of Michaelis scheme although the expression for the effective K_M has multiplicator of $\left[1+k_{cat}^{X,VIIa-TF}/k_d^{Xa-VIIa-TF}\right]$ in comparison with classical Michaelis pathway.

The results of the reduction

From Eqn A12, which defines steady-state concentration of the enzyme–product complex during ongoing factor X activation it is readily seen that:

$$[Xa-VIIa-TF] = \frac{\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)} \cdot\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\left([X]+\frac{K_M^{X,VIIa-TF}\cdot k_a^{VIIa-TF,Xa}}{k_{cat}^{X,VIIa-TF}}[Xa]\right)\approx\frac{\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\left([VIIa-TF]_0-[Xa-TFPI-VIIa-TF]\right)\cdot[X]}{K_M^{X,VIIa-TF}\cdot\left(1+\frac{[Xa]}{K_D^{Xa-VIIa-TF}}+\frac{[X]}{K_M^{X,VIIa-TF}}\left[1+\frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)}; \tag{A19}$$

Approximate Eqn A19 is valid for the description of initial kinetics of inhibition when [X] ≫ [Xa] and we can consider the second term in the numerator to be small. It can be shown that this equation is also valid for model 2 when Xa–VIIa–TF is directly inhibited by TFPI. It follows that $[Xa-VIIa-TF]\propto 1/k_d^{Xa-VIIa-TF}$, and inhibition efficiency is inversely proportional to $k_d^{Xa-VIIa-TF}$. The rate of inhibition is equal to the concentration of the enzyme–product complex $[Xa-VIIa-TF]$ multiplied to the constant of inhibition. Under fixed concentrations and Michaelis constants there exists a proportionality: $k_a^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF]\propto k_a^{Xa-VIIa-TF,TFPI}/k_d^{Xa-VIIa-TF}$.

The more $k_d^{Xa-VIIa-TF}$ is the more inhibition constant $k_a^{Xa-VIIa-TF,TFPI}$ should be to obtain the same inhibition rate:

$$k_a^{Xa-VIIa-TF,TFPI}\propto k_d^{Xa-VIIa-TF} \tag{A20}$$

In addition, from Eqn A19 under condition [X] ≪ [Xa] two more proportionalities, Eqns A22,A23, follow. For example let us consider the analysis leading to Eqn A22. Factor Xa can lose its activity either because of its binding to TFPI or because

of inhibition of Xa–VIIa–TFPI by TFPI. In the first case the rate of inhibition is $v_1 = k_a^{Xa,TFPI}[Xa] \cdot [TFPI]$, while the rate of the enzyme–product complex inhibition by TFPI is:

$$\begin{aligned} v_2 &= k_{+1}^{Xa-VIIa-TF,TFPI}[Xa-VIIa-TF] \cdot [TFPI] = k_{+1}^{Xa-VIIa-TF,TFPI} \frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}} \\ &\cdot \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right)}{K_M^{X,VIIa-TF} \cdot \left(1 + \frac{[Xa]}{K_D^{Xa-VIIa-TF}} + \frac{[X]}{K_M^{X,VIIa-TF}}\left[1 + \frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)} \cdot \left([X] + \frac{K_M^{X,VIIa-TF} \cdot k_a^{VIIa-TF,Xa}}{k_{cat}^{X,VIIa-TF}}[Xa]\right) \cdot [TFPI] \\ &\approx \frac{k_{+1}^{Xa-VIIa-TF,TFPI}\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right) \cdot \frac{K_M^{X,VIIa-TF}}{K_D^{Xa-VIIa-TF}}[Xa]}{K_M^{X,VIIa-TF} \cdot \left(1 + \frac{[Xa]}{K_D^{Xa-VIIa-TF}} + \frac{[X]}{K_M^{X,VIIa-TF}}\left[1 + \frac{k_{cat}^{X,VIIa-TF}}{k_d^{Xa-VIIa-TF}}\right]\right)} \cdot [TFPI] \\ &\propto \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right) \cdot [Xa]}{K_D^{Xa-VIIa-TF}}; \end{aligned} \tag{A21}$$

To make the latter reaction visible one has to make increase of inhibition after VIIa–TF addition significant. It means that v_2 must have the same order as v_1. Their ratio must therefore be constant (the value of this constant is defined by the minimal difference of factor Xa inhibition rate which is possible to measure). So, $\frac{v_2}{v_1} = \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right) \cdot [Xa]}{k_a^{Xa,TFPI}[Xa] \cdot [TFPI] \cdot K_D^{Xa-VIIa-TF}} \propto \frac{\left([VIIa-TF]_0 - [Xa-TFPI-VIIa-TF]\right)}{K_D^{Xa-VIIa-TF}} = const$. From this equation it follows that the concentration of VIIa–TF to be added for the detection of the hypothetical reaction is defined by the correlation:

$$[VIIa-TF]_0 \propto K_D^{Xa-VIIa-TF} = \frac{k_d^{Xa-VIIa-TF}}{k_a^{VIIa-TF,Xa}} \tag{A22}$$

In a similar fashion, the analysis of VIIa–TF inhibition by Xa–TFPI or with the help of hypothetical reaction of the enzyme–product complex inhibition by Xa–TFPI gives us dependence of factor Xa concentration, which is necessary to detect this hypothetical reaction on the equilibrium dissociation constant for the enzyme–product complex:

$$[Xa]_0 \propto K_D^{Xa-VIIa-TF} = \frac{k_d^{Xa-VIIa-TF}}{k_a^{VIIa-TF,Xa}} \tag{A23}$$

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