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Simulation Model for Thrombin Generation in Plasma

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Key Words. Prothrombin activation · Computer simulation model · Regulation model · Factor V activation · Hirudin · Activation threshold · Down-regulation by inhibitors

Abstract. A simulation model for the production of thrombin in plasma is presented. Values of the reaction rate constants as determined in purified systems are used and the model is tested by comparison of simulations of factor Xa, factor Va and thrombin generation curves with experimental data obtained in thromboplastin-activated plasma. Simulations of the effect of hirudin indicate that factor V is predominantly activated by thrombin and not by factor Xa. The model predicts a threshold value for the factor Xa production which, if exceeded, results in explosive and complete activation of prothrombinase. The dependence of this threshold value on different negative feedback reactions, e.g. the inactivation of thrombin and factor Xa by antithrombin III (+ heparin), is investigated. The threshold value, for control plasma in the range of 1–10 pM total factor Xa production, can be raised two orders of magnitude by accelerated inactivation of factor Xa and prothrombinase but is hardly affected by a tenfold increase in the rate of thrombin inactivation or by increased production of activated protein C. This latter effect, however, results in a more gradual input-response relation between factor Xa input and the extent of prothrombinase activation.

Introduction

The dual requirements of rapid clot formation in response to vascular lesions and prevention of spontaneous formation or expansion of clots suggest an intricate, highly nonlinear, regulation of thrombin production. Extensive research during past decades has produced detailed knowledge at the molecular level of the reaction mechanisms involved in the successive activation of the clotting (co)factors [1]. Thus emerged the concept that the explosive amplification of procoagulant activity is due to the cascade of successive activations of the serine proteases with positive feedback through activation of

cofactors. Assembly with its cofactor, in enzyme-cofactor complexes adsorbed to the procoagulant lipid surface, greatly enhances the conversion capacity of the enzyme. The response is limited by localization of the amplified response to the lipid surface at the site of injury. This localized response is regulated downward by rather effective scavenging of the proteases by antithrombin III (ATIII), and α_2-macroglobulin inactivation of the cofactors Va and VIIIa by activated protein C, and inhibition of tissue factor by extrinsic pathway inhibitor (EPI). Studies of clotting factor activation in plasma support the above-mentioned picture and especially stress the pivotal role of the feedback activation of, e.g., factor V for the amplification of the response [2–6]. However, apparent discrepancies between data from purified systems and observations in plasma have also arisen. In purified systems, for instance, it was shown that factor Xa activates factor V as effectively as thrombin [7], while it was concluded from experiments in plasma that thrombin is the only effective activator of factor V [5].

Due to the multiple and interacting protein conversions, the interpretation of data on thrombin generation in plasma requires a dynamic model. Therefore a model for the generation of prothrombinase activity in plasma was constructed, using reaction rates derived from experiments in purified systems. The response on a transient input of factor Xa into plasma is considered as a function of thrombin generation by factor Xa and prothrombinase, feedback activation of factor V by thrombin, assembly of prothrombinase, elimination of factor Xa and thrombin by ATIII, and inactivation of factor Va by thrombin-activated protein C (APC). In these respects, it deviates from

earlier models that either did not account for feedback activation of factor V by prothrombin [8–10] or assumed constant inactivation of factor Va [11].

Methods

The model

The various mechanisms included in the simulation model are summarized in the following scheme (see also table 1):

Activation		Inactivation	
(1a)	$F(t) \rightarrow Xa$	(1b)	$Xa + ATIII \rightarrow$ Xa-ATIII
(2a)	$V \xrightarrow{IIa} Va$	(2c)	$Va \xrightarrow{APC}$ Va-inactive
(2b)	$V \xrightarrow{Xa} Va$		
(3a)	$Va + Xa \longleftrightarrow$ Va-Xa	(3b)	$Va\text{-}Xa + ATIII \rightarrow$ Va + Xa-ATIII
		(3c)	$Va\text{-}Xa \xrightarrow{APC}$ Va-inactive + Xa
(4a)	$II \xrightarrow{Xa} IIa$	(4c)	$IIa + ATIII \rightarrow$ IIa-ATIII
(4b)	$II \xrightarrow{Va\text{-}Xa} IIa$		
(5a)	$C \xrightarrow{IIa} APC$	(5b)	$APC \rightarrow$ APC-inactive
(6)	$Fibrinogen \xrightarrow{IIa} Fibrin$		

Generation and Inactivation of Factor Xa. The factor Xa generation, induced by thromboplastin, is considered to be a given input $F(t)$ simulated by:

$$F(t) = Q_{Xa}\, \alpha \cdot exp(-\alpha t),$$

where Q_{Xa} is the total amount factor Xa produced and α the mono-exponential decay rate associated with the disappearance of the tissue factor VIIa activity. The inactivation of factor Xa by ATIII is modelled by a first-order reaction with rate constant k_i. The rate enhancement of this reaction by heparin is reflected in higher values of this parameter [13]. The

Table 1. Reactions rates of the simulation model

Reaction		Kinetic parameters	References
(1a)	M^a	$Q_{Xa} = 20$ nM; $\alpha = 1$ min^{-1}	estimated, see text
(1b)		$k_i = 0.25$ min^{-1}	estimated, see text
(2a)	M	$k_{cat} = 14$ min^{-1}; $K_m = 72$ nM	7
(2b)	M	$k_{cat} = 2.6$ min^{-1}; $K_m = 10$ nM	7
(2c)	M	$k_{cat} = 24$ min^{-1}; $K_m = 20$ nM	16
(3a)	S	$k_p = 10$ nM^{-1} min^{-1};	15
(3b)	F	$k_i = 0.05$ min^{-1};	17
(3c)	M	$k_{cat} = 24$ min^{-1}; $K_m = 20$ nM	16
(4a)	M	$k_{cat} = 2.3$ min^{-1}; $K_m = 58$ nM	18
		1 nM lipid binding sites for factor Xa, $K_d = 50$ nM	15
(4b)	M	$k_{cat} = 2,000$ min^{-1}; $K_m = 210$ nM	18
		1 nM lipid binding sites for prothrombinase, $K_d = 0.01$ nM	15
(4c)	F	$k_i = 1.3$ min^{-1}	12
(5a)	F	$k = 0.0014$ min^{-1}	estimated, see text
(5b)	F	$k_i = 0.35$ min^{-1}	estimated, see text
(6)	M	$K_m = 2$ μM (corrected for multiple cleavage sites)	12

a Kinetics:

M = Michaelis-Menten mechanism: $V = k_{cat} \cdot E \cdot S/(S + K_m)$

F = First-order reaction: $V = k \cdot E$

S = Second-order reaction: $V = k \cdot Va \cdot Xa$

It was assumed in the simulations that no activated clotting factors were present before addition of the thromboplastin trigger, i.e. the initial values ($t = 0$) for the activated factors were set to zero. Initial values for the unactivated proteins were: prothrombin: 1 μM; factor V: 30 nM; protein C: 100 nM; fibrinogen: 10 μM.

values of Q_{Xa} and α are obtained by fitting F(t) to the observed time-activity curve of factor Xa.

Generation and Inactivation of Factor Va. Activation of factor V by factor Xa and thrombin are both included in the model [7, 14]. In these reactions, we must account for competition between prothrombin and factor V for factor Xa. For two substrates with concentrations S_1 and S_2, competing for the same enzyme with concentration E, the conversion rate of S_1 is given by:

$$k_{cat1} \cdot E \cdot S_1/[S_1 + K_{m1}(1 + S_2/K_{m2})],$$

where k_{cat1} is the maximal turnover rate of S_1 by the enzyme and K_{m1}, K_{m2} are the Michaelis constants of the conversion of S_1 resp. S_2 by E. Activated factor V is degraded by activated protein C (APC).

Generation and Inactivation of Prothrombinase. Because initially the factor Xa and factor Va concentrations can both be very low (< 0.01 nM) and the second-order association rate constant on small unilamellar vesicles is estimated to be $k_p = 10$ nM^{-1} min^{-1} [15] the assembly rate easily becomes rate limiting. Therefore, instantaneous equilibrium (quasi steady state) cannot be assumed and the transient kinetics must be included in the model. The inactivation rate of prothrombinase as a result of factor Va inactivation by APC is assumed to be identical to the inactivation rate of uncomplexed factor Va. This may represent an overestimation because some protection of factor Va by factor Xa has been reported [16]. The factor Xa complexed in the prothrombinase can be inactivated by ATIII (+ heparin) although at a about

80% lower rate than lipid-bound or free factor Xa [17].

Generation and Inactivation of Thrombin. The catalytic efficiency of both factor Xa and the factor Xa-Va complex increases enormously when bound to phospholipid [18]. Therefore it is assumed that the conversion rate is determined by the lipid-bound factor Xa and Va-Xa and the contribution of free factor Xa and Va-Xa is neglected. The association to the lipid generally is rapid [19] and an instantaneous equilibrium is assumed. The number of bindings sites then becomes an important determinant of the maximal prothrombinase activity.

Generation and Inactivation of Activated Protein C. Both activation of protein C by thrombin and inactivation of APC are described by apparent first-order reactions and the rate constants are adjusted by fitting the factor Va disappearance curve.

Conversion of Fibrinogen by Thrombin. This reaction is included to account for the reduction of the activation rate of factor V by thrombin, caused by the competition of fibrinogen with factor V for thrombin.

The enzymatic conversions and the inactivation of thrombin and factor Xa by ATIII require at least two steps, a bimolecular reaction resulting in complex formation followed by a rate-limiting final step, such as proteolytic cleavage of the substrate. In order to avoid unwarranted complexity, these reactions, with the exception of the assembly of prothrombinase, are approximated by using the quasi-steady-state approximation. This is justified at a time scale of minutes, as the concentration of the abundant reactant (the substrate or ATIII) in these reactions exceeds 30 nM so that, even for values as low as 5×10^{-3} nM^{-1} s^{-1} for the second-order rate constant of the association reaction, the half-time for equilibration is less than 0.06 min. Thus the enzymatic reactions are described by the classical Michaelis-Menten model, and the inactivation reactions are considered as apparent first-order reactions.

Numerical Integration. As the set of differential equations is moderately to very stiff (with hirudin), a stiffly stable integration routine, the variable-order variable-step implementation of the backward integration formula supplied as routine D02EAF in the NAG library [20] was employed for numerical calculation of the solution. The accuracy, target 10^{-4} nM, of the simulations was checked for several representative cases by comparison of the results for several values of the error control parameter.

Results and Discussion

Generation of Thrombin and Factor Va in Thromboplastin-Activated Plasma

Figure 1 shows the response of the complete simulation model on a trigger of transient factor Xa production. Using the model parameters presented in table 1, the total amount of factor Xa production (Q_{Xa}) and the decay rate (α) of the factor Xa production were estimated from the factor Xa time-activity curve. Then the activation and inactivation rate constants of protein C were adjusted in order to obtain agreement between model prediction and observed factor Va activities. The activation pathway of factor V by factor Xa proved totally irrelevant in this simulation, values of k_{cat} between 0 and 3 min^{-1} did not change the result. Fitting of the thrombin generation curve revealed that the quantity of available lipid binding sites for prothrombinase had to be limited to 1 nM (about 0.5 μM lipid), otherwise thrombin peaked too early. There are no data in the literature on this parameter but lipid limitation has been demonstrated in thromboplastin-activated plasma [21]. The observed trombin curve was measured by an amidolytic assay (S2238) in which thrombin inactivated by α_2-macroglobulin has about 50% of the activity of thrombin and this causes the plateau level of thrombin activity in the experimental curve. The lower peak value of the measured thrombin activity compared to the simulation is probably related to clot formation after about 1 min and binding of part of the thrombin to this clot. Beyond these differences, that affect only the measurement of thrombin activity and not the parameter fit based on the factor Va and factor Xa plasma activities, a good agreement between model and experimental data is apparent.
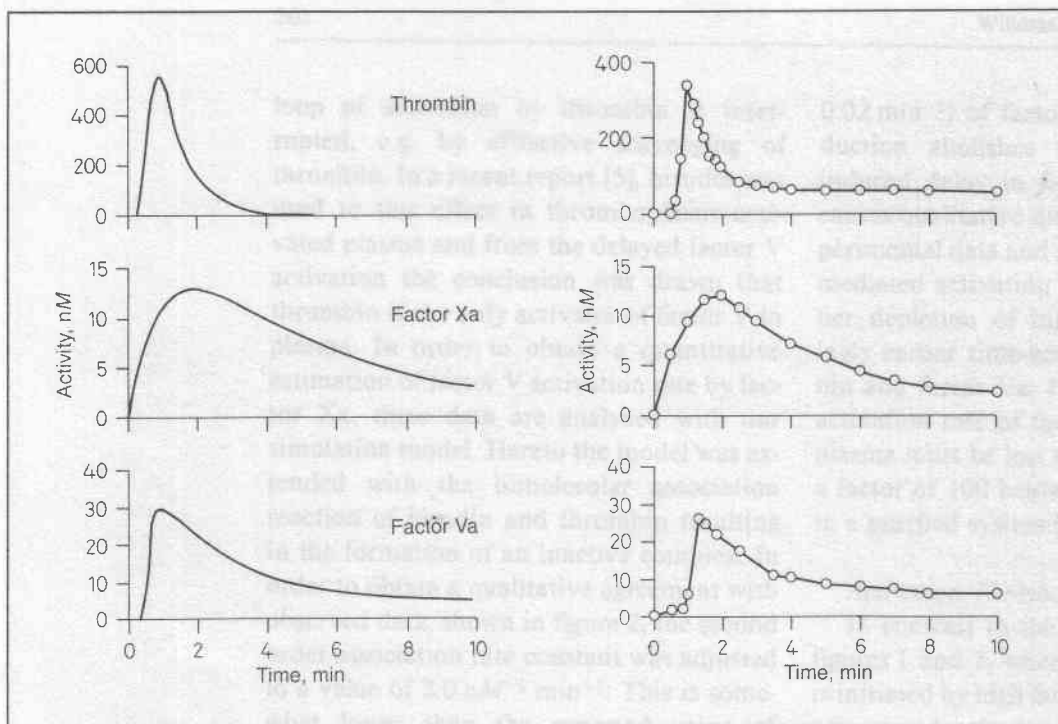
Fig. 1. Model simulations (left panel) and measured plasma activities (right panel) of thrombin, factor Xa and factor Va. Plasma, activated with 1/30 (vol/vol) thromboplastin. Experimental data from Pieters [4].

Time Delay of Prothrombin Activation

The time lag of thrombin generation was found qualitatively to be largely determined by the product of three reaction rates involved in the feedback activation loop of factor V by thrombin, that is the product $k_p \times k_{cat}$ (2a)$\times k_{cat}$ (4b) (table 1). Reduction of each of these reaction rates results in similar extensions of the time lag. Reduction of prothrombinase activity appears to cause the largest delay for a given proportional reduction of the reaction rates. The model behavior is relatively insensitive to small changes in these parameters. The time lag of 1 min, as observed in plasma, (fig. 1), required a reduction of one of these reaction rates by

nearly one order of magnitude, compared to the values in table 1. However, limitation of the number of lipid binding sites for prothrombinase to the plausible value of 1 nM offers a much simpler explanation. Although this issue requires further experiments, at the moment the lipid limitation appears the best explanation, and is incorporated in the model on the sequel of this paper.

Inhibition of Thrombin by Hirudin:
Factor V in Plasma Is Not Activated by
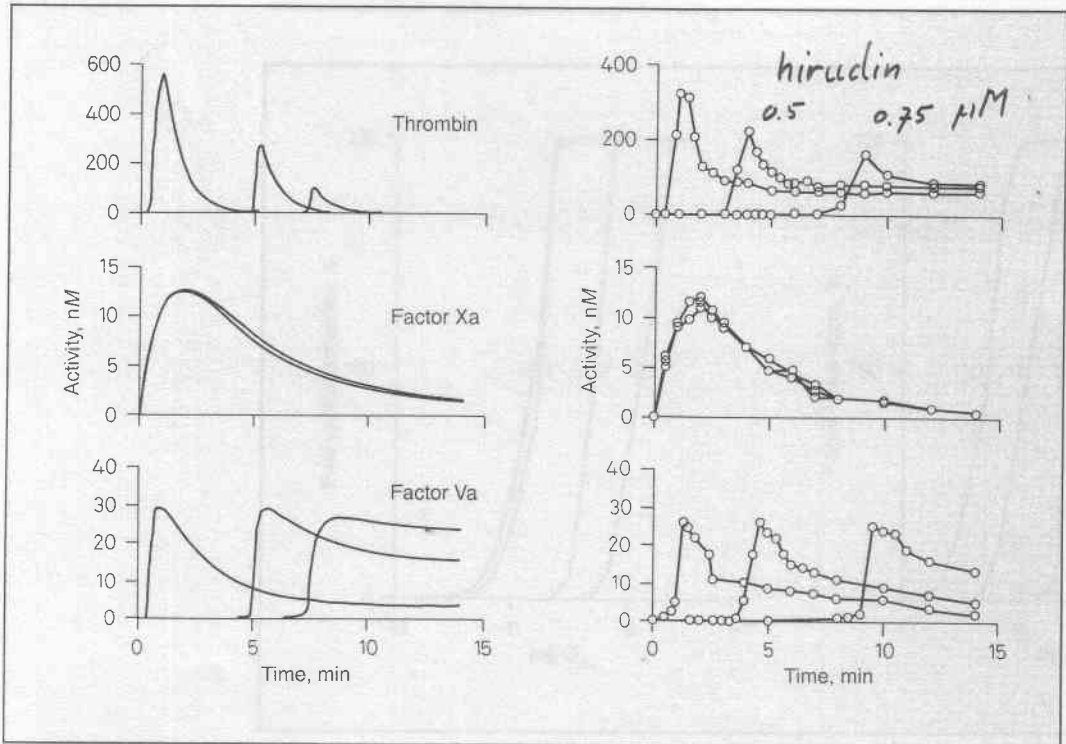*Factor Xa*

The simulations in figure 1 showed that the contribution of factor V activation by factor Xa is unimportant unless the feedback

loop of activation by thrombin is inter-
rupted, e.g. by efffective scavenging of
thrombin. In a recent report [5], hirudin was
used to this effect in thromboplastin-acti-
vated plasma and from the delayed factor V
activation the conclusion was drawn that
thrombin is the only activator of factor V in
plasma. In order to obtain a quantitative
estimation of factor V activation rate by fac-
tor Xa, these data are analyzed with our
simulation model. Hereto the model was ex-
tended with the bimolecular association
reaction of hirudin and thrombin resulting
in the formation of an inactive complex. In
order to obtain a qualitative agreement with
observed data, shown in figure 2, the second
order association rate constant was adjusted
to a value of 2.0 nM^{-1} min^{-1}. This is some-
what lower than the reported value of
5.3 nM^{-1} min^{-1} [22], but this is consistent
with the sensitivity of this reaction to the
ionic strength also shown in that report. Fig-
ure 2 shows an excellent qualitative agree-
ment between model simulations when it is
assumed that only thrombin contributes to
factor V activation (left panel) and observed
(right panel) plasma activity curves. It ap-
pears that despite the virtually immediate
elimination of thrombin by hirudin, the re-
maining small amounts of thrombin activate
sufficient factor V for the generation of
considerable prothrombinase activity. After
5 min, nearly 500 nM thrombin is formed in
the presence of 500 nM hirudin, whereas the
factor Xa uncomplexed with factor Va could
only produce 100 nM of thrombin in this
time. This thrombin production results ulti-
mately in nearly complete depletion of hiru-
tin, followed by a rapid rise of plasma
thrombin and complete activation of factor
V. The simulations in figure 3 show that
even a minute catalytic activity (k_{cat} =

0.02 min^{-1}) of factor Xa in factor Va pro-
duction abolishes most of the hirudin-
induced delay in factor V activation and
causes qualitative discrepancies between ex-
perimental data and simulations. Factor-Xa-
mediated activation of factor V causes ear-
lier depletion of hirudin and correspond-
ingly earlier time-activity curves of throm-
bin and factor Va. It is concluded that the
activation rate of factor V by factor Xa in
plasma must be less than 0.02 min^{-1}, that is
a factor of 100 below the value determined
in a purified system [7].

Activation Threshold

In contrast to the situation presented in
figures 1 and 2, where thrombin generation
is initiated by high factor Xa generations, we
now consider the response to very low factor
Xa generations. For small total factor Xa
generations of finite duration, the activation
delay may become so long that the factor Xa
concentration has dropped to negligible lev-
els before the appearance of factor Va. Then
the assembly rate of prothrombinase, pro-
portional to the product of the factor Va and
Xa concentrations becomes rate limiting, the
feedback loop is interrupted and only very
limited activation occurs. This effect was
studied quantitatively by using the simula-
tion model to predict the system response
upon a factor Xa input, F(t) = $Q_{Xa} \cdot \alpha \cdot$
$\exp(-\alpha t)$ with α = 1.0 min^{-1}, as a function of
the total factor Xa production (Q_{Xa}). Lipid
binding sites for factor Xa and prothrom-
binase were kept constant at 1 nM. The
simulations thus purely reflect the effect of
variable production capacity of factor Xa,
for instance due to different amounts of tis-
sue factor. Figure 4 indeed shows that for
total factor Xa inputs below threshold values
of about 0.5 and 2 pM, the activation of

Fig. 2. Effect of addition of hirudin on the activation of thromboplastin-triggered plasma. Left panel shows model simulations, assuming no activation of factor V by factor Xa, and the right panel the observed plasma activities of thrombin, factor Xa and factor Va. Hirudin concentrations were 0, 500 and 750 n*M*. Experimental data are from Pieters [5].

Fig. 3. The effect of the rate of factor V activation by factor Xa. Indicated are simulations of plasma activity curves for a hirudin concentration of 750 n*M* and for values of k_{cat} of factor V activation by factor Xa equal to 0.0, 0.02, 0.1, 0.5, and 2.6 min^{-1}.
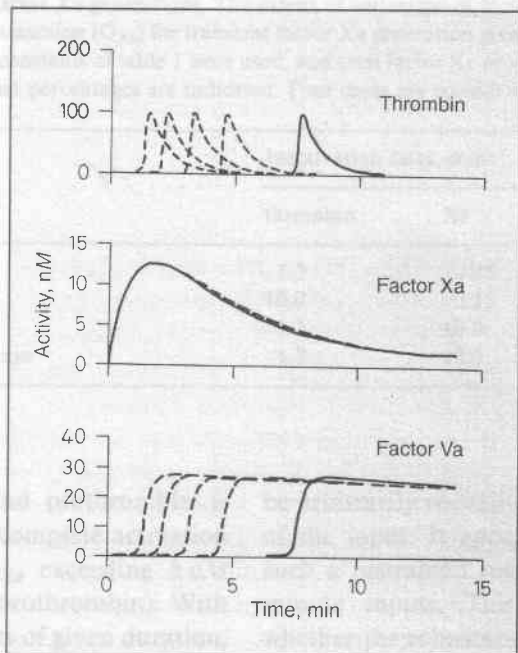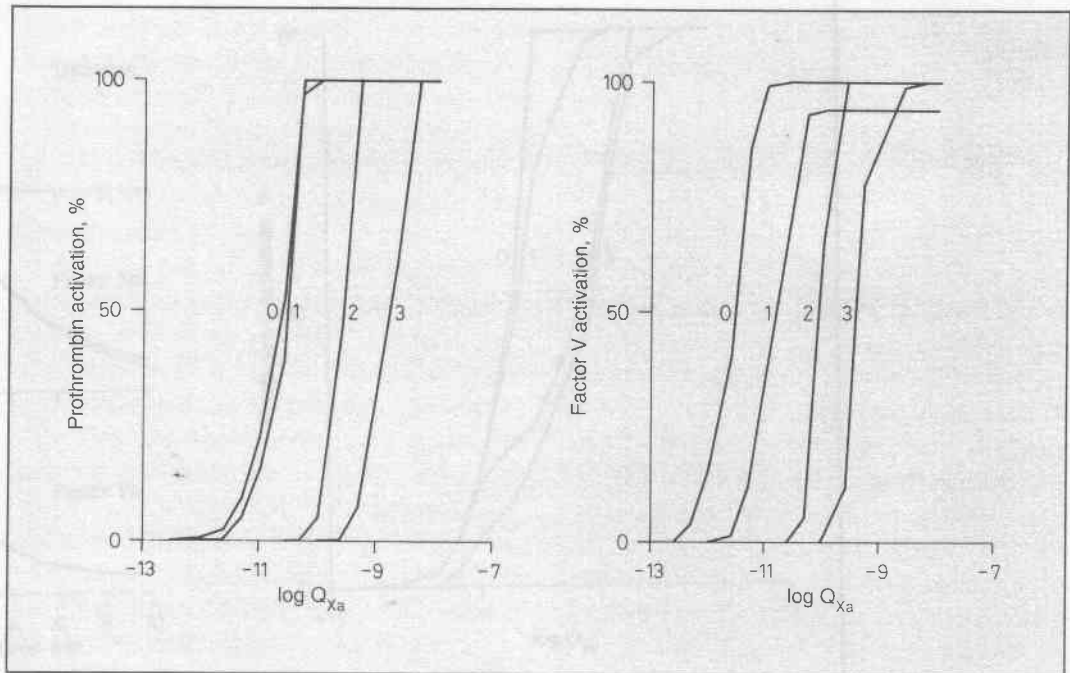
Fig. 4. Response on low factor Xa generations. The extent of activation of factor V and prothrombin as a function of total factor Xa production (Q_{Xa}) for transient factor Xa generation given by $F(t) = Q_{Xa} \, \alpha \cdot \exp(-\alpha t)$ with $\alpha = 1$. The reaction rate constants of table 1 were used, and total factor Xa production, Q_{Xa}, was varied as indicated. Ultimately activated percentages are indicated. Four cases are considered:

		Inactivation rates, min^{-1}		
		thrombin	Xa	prothrombinase
0	Control	1.3	0.25	0.05
1	Thrombin inhibition	10.0	0.25	0.05
2	Xa inhibition	1.3	10.0	0.05
3	+ Prothrombinase inhibition	1.3	10.0	2.00

respectively factor V and prothrombin is negligible, while nearly complete activation results for values of Q_{Xa} exceeding 5 pM (factor Va) and 20 pM (prothrombin). With respect to transient inputs of given duration, the system thus appears to be stable, in contrast to systems in which the response cannot be arbitrarily restrained by limiting the size of the input. It appears from figure 4 that such a restrained response results only for minute inputs. This raises the question whether the robustness of the system can be influenced by down-regulation, for instance by enhancing the inactivation rates of factor
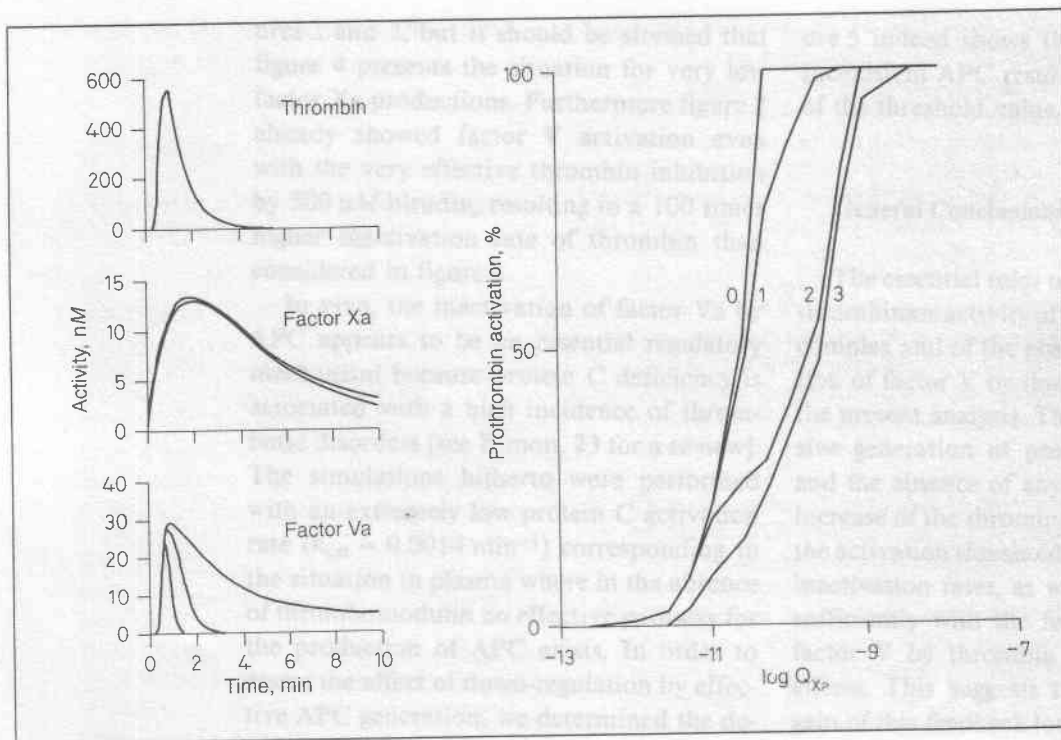
Fig. 5. The effect of the activation rate of protein C. Left panel shows the simulated plasma activities of thrombin, factor Xa and factor Va after a trigger of Xa production ($Q_{Xa} = 20.0$ nM) for three activation rates (0) 0.0014, (1) 0.0070 and (2) 0.035 min^{-1}. Right panel shows the effect of the increased activation rate of protein C on the extent of prothrombin activation as a function of the factor Xa generation. Also shown is the input–response relation for $k_{cat} = 0.035$ min^{-1} for a steady-state base level of 1 nM APC (3).

Xa and/or thrombin. Therefore the input–response relation was also determined for the following conditions: (1) enhanced inactivation of thrombin with a rate constant of 10 min^{-1}; (2) enhanced factor Xa inactivation with a rate constant of 10 min^{-1}; (3) both enhanced factor Xa inactivation with a rate constant of 10 min^{-1} and enhanced prothrombinase inactivation with a rate constant of 2.0 min^{-1}. These values of rate constants are chosen in the range of the observed inactivation rates in the presence of therapeutic concentrations of standard hepa-

rin [3]. Figure 4 shows that increased thrombin inhibition does not affect the input–response relation for prothrombin activation and causes only a small shift of this relation for factor V activation. In contrast, anti-factor Xa activity increases the robustness of the system enormously with an upward shift of the activation thereshold of 2 orders of magnitude. Additional inhibition of prothrombinase reinforces this effect. This is seemingly in contrast with the predominant role of thrombin in the generation of prothrombinase activity as discussed at fig-

ures 2 and 3, but it should be stressed that figure 4 presents the situation for very low factor Xa productions. Furthermore figure 2 already showed factor V activation even with the very effective thrombin inhibition by 500 nM hirudin, resulting in a 100 times higher inactivation rate of thrombin than considered in figure 4.

In vivo, the inactivation of factor Va by APC appears to be an essential regulatory mechanism because protein C deficiency is associated with a high incidence of thrombotic disorders [see Esmon, 23 for a review]. The simulations hitherto were performed with an extremely low protein C activation rate (k_{cat} = 0.0014 min^{-1}) corresponding to the situation in plasma where in the absence of thrombomodulin no effective pathway for the production of APC exists. In order to assess the effect of down-regulation by effective APC generation, we determined the dependence of the input-response relation on the protein C activation rate by thrombin. The simulations in the left panel of figure 5 show that a 5- or 25-fold increase of the protein C activation rate has an enormous effect on the factor Va activity curves but leaves the thrombin generation curve unaffected. The right panel of this figure shows, however, that the input-response relation becomes more gradual for high protein C activation rates. Up to factor Xa productions of 10 pM, the responses practically coincide (<20% prothrombin activation), but for higher values of Q_{Xa} the prothrombin activation lags behind for high protein C activation rates (0.035 min^{-1}). This raises the question whether the system response for low values of Q_{Xa} can be further limited by assuming that, in addition to this high activation rate, a small percentage of protein C is always present in the activated state. Fig-

ure 5 indeed shows that an amount of 1% preexistent APC results in a moderate shift of the threshold value.

General Conclusions

The essential roles of the tremendous prothrombinase activity of the factor Va-Xa-lipid complex and of the positive feedback activation of factor V by thrombin are stressed by the present analysis. This explains the explosive generation of prothrombinase activity and the absence of any effect of a moderate increase of the thrombin inactivation rate on the activation threshold. Only extremely high inactivation rates, as with hirudin, interfere sufficiently with the feedback activation of factor V by thrombin to cause significant effects. This suggests that reduction of the gain of this feedback loop, for instance by an immediate reduction of prothrombinase activity, as reported for ATIII + heparin in a purified system [17], could represent an effective mechanism to reduce the explosivity of the system. Furthermore this analysis indicates that the possible contribution of factor Xa to the activation of factor V is negligible unless the factor Xa concentration is very low or thrombin inactivation proceeds at unrealistic high rate constants, exceeding 200 min^{-1}. The existence of an activation threshold for transient factor Xa generation is demonstrated and this stresses the relevance of effective degradation of the factor-Xa-generating capacity of tissue factor VIIa [24]. The good agreement between model predictions and measured activities in plasma suggests that the proposed model is essentially valid. However, the model parameters used were obtained from studies in purified systems and further identification of these parameters di-

rectly from the plasma activation curves is a next step to consolidate the model.

Acknowledgments

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