THE SIGNIFICANCE OF CIRCULATING FACTOR IXa IN BLOOD

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SUMMARY

The presence of activation peptides (AP) of the vitamin K-dependent proteins in the phlebotomy blood of human subjects suggests that active serine proteases may circulate in blood as well. The goal of the current study was to evaluate the influence of trace amounts of key coagulation proteases on tissue factor independent thrombin generation using three models of coagulation. With procoagulants and select coagulation inhibitors at mean physiological concentrations, concentrations of factor IXa, factor Xa and thrombin were set either equal to those of their AP or to values which would result based upon the rates of AP/enzyme generation and steady state enzyme inhibition. In the latter case, numerical simulation predicts that sufficient thrombin to produce a solid clot would be generated in ~2 min. Empirical data from the synthetic plasma suggest clotting times of 3-5 min, which are similar to that observed in contact pathway inhibited whole blood (4.3 min) initiated with same concentrations of factors IXa and Xa and thrombin. Numerical simulations performed with the concentrations of two of the enzymes held constant and one varied suggest that the presence of any pair of enzymes is sufficient to yield rapid clot formation. Modeling of states (numerical simulation and whole blood) where only one circulating protease is present at steady state concentration shows significant thrombin generation only for factor IXa. The addition of factor Xa and thrombin has little effect (if any) on thrombin generation induced by factor IXa alone. These data indicate that: 1) concentrations of active coagulation enzymes circulating in vivo are significantly lower than can be predicted from the concentrations of their AP; 2) expected trace amounts of factor IXa can trigger thrombin generation in the absence of tissue factor.
INTRODUCTION

Five vitamin K-dependent proteins serve as precursors for serine proteases, which are essential for normal hemostasis (factors VII, IX, and X, prothrombin and protein C) (1). The zymogens are converted to fully functional enzymes by the proteolytic removal of corresponding activation peptides. The only exception is factor VII, which is converted to an active enzyme by a single cleavage not followed by the release of an activation peptide (2). For the release of activation peptides from factor X, protein C and prothrombin, single cleavages are required, whereas the activation peptide of factor IX is generated by two proteolytic cleavages (3). The length of activation peptides varies from 12 amino acids for protein C to 271 amino acids for prothrombin (Fragment 1.2). There are no structural domains observed for activation peptides of factor IX, factor X and protein C. However, fragment 1.2 of prothrombin contains a Gla-domain and two kringle domains. The former one is rich in γ-carboxyglutamic acid residues and facilitates a calcium-dependent binding of prothrombin to the membranes containing acidic phospholipids (1). The kringle domain 1 contains two of three prothrombin glycosylation sites (4).

The detection of activation peptides of the vitamin K-dependent proteins in plasma of human subjects suggests that active serine proteases may circulate in blood either as a result of continuous in situ production (“idling motor”) or as a consequence of production during localized coagulation (“historical record”). The relationship between the concentrations of enzymes and their corresponding activation peptides is not clear in part because the source(s) of these peptides is not identified. A possible trigger for the generation of these peptides and corresponding enzymes can be active tissue factor hypothetically circulating in blood (5). Alternatively, they can be generated due to the initiation of the coagulation cascade during
phlebotomy and processing of blood. The key problem for the evaluation of active enzymes is that direct assays of these serine proteases are difficult (if possible at all) due to their rapid neutralization by the natural inhibitors present in blood.

To circumvent this obstacle, assays for the activation peptides released during the generation of serine proteases involved in blood coagulation as well as for the reaction and inhibition products of these enzymes have been developed (6-11). Products, including activation peptides of prothrombin, factor X and factor IX, related to the processes leading to thrombin generation and clot formation have been used as markers for the assessment of the coagulation state of human beings. Elevated levels of activation peptides are observed in patients suffering from disseminated intravascular coagulation (11, 12), deep vein thrombosis (13, 14), coronary heart disease (15, 16), unstable angina, myocardial infarction (17, 18), and other coagulation-related disorders (19). The concentrations of activation peptides are also affected by deficiencies or mutations in procoagulants and coagulation inhibitors (10, 20-24), by smoking (25) and by age (26).

Although there is a strong correlation between the prothrombotic state of individuals and estimated levels of activation peptides, the question whether these levels reflect concentrations of active enzymes circulating in vivo remains open. The validity of this question can be illustrated by the observation that estimated activation peptide levels are dependent upon the conditions of sample collection (27, 28).

In the current study we evaluated the capability of thrombin, factor Xa and factor IXa to trigger thrombin generation and clot formation, when these enzymes are present at concentrations
relevant to those reported for their activation peptides in healthy individuals. Three *in vitro* models of coagulation developed in our laboratory were used to achieve this goal (29-34).
EXPERIMENTAL PROCEDURES

Materials— Human coagulation factors VII, X, IX, and prothrombin, were isolated from fresh frozen plasma using the general methods of Bajaj et al. (35), and were purged of trace contaminants and traces of active enzymes as described (36). Human factor V and AT-III were isolated from freshly frozen plasma (37, 38). Recombinant factor VIII and recombinant tissue factor (TF) (residues 1-242) were provided as gifts from Drs. Shu Len Liu and Roger Lundblad (Hyland division, Baxter Healthcare Corp, Duarte, CA). Recombinant human factor VIIa was provided as a gift from Dr. Ula Hedner (Novo Nordisk, Denmark). Recombinant full-length TFPI produced in Escherichia coli was provided as a gift by Dr. K. Johnson (Chiron Corp, Emeryville, CA). Factor IXa, factor Xa and α-thrombin were provided as a gift by Dr. R. Jenny (Haematologic Technologies, Essex Junction, VT). Corn trypsin inhibitor (CTI) was isolated from popcorn as described elsewhere (34). Washed platelets were prepared by the procedure of Mustard et al. (39). Preparation of the TF/lipid reagent was done as described elsewhere (34). 1,2-Dioleoyl-sn-Glycero-3-Phospho-L-Serine (PS) and 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL), and EDTA (Ca++ quencher) was purchased from Sigma (St Louis, MO). Phospholipid vesicles (PCPS) composed of 25% PS and 75% PC were prepared as described (40). Spectrozyme TH was purchased from American Diagnostica, Inc (Greenwich, CT). d-Phe-Pro-ArgCH2Cl (FPRck) and the fluorogenic substrate 6-(d-Val-Pro-Arg)amino-1-naphthalene(n-butyl)sulfonamide (VPRnbs) (41) were synthesized in house. Recombinant hirudin was a gift from Genentech. ELISA thrombin-AT-III (TAT) kit (Enzygnost TAT) was purchased from Behring (Marburg, Germany).
Numerical simulation—The present effort is based upon prior publications by Jones et al. (29) and Hockin et al. (30). An additional step of factor X activation by factor IXa was incorporated using kinetic constants published by Walsh and coworkers (42) (K_M=1.4x10^{-7} M; k_cat=8.0x10^{-4} s^{-1}).

Synthetic coagulation model—The procedure used is a modification of Lawson et al. (31) and van 't Veer et al. (36).

I. Procofactor solution. 4x10^8/ml Platelets or 4 μM PCPS are incubated in HBS (20 mM HEPES and 150 mM NaCl, pH 7.4), 2 mM CaCl_2 for 10 min at 37°C. When desired, 10 pM relipidated TF is added (molar ratio PCPS:TF=5000). Factor V (40 nM) and factor VIII (1.4 nM) are added prior to the initiation of the reaction.

II. Zymogen-inhibitor solution. Prothrombin (2.8 μM), factors VII (20 nM), VIIa (0.2 nM), X (340 nM), IX (180 nM) and XI (60 nM), TFPI (5 nM), and AT-III (6.8 μM) are preheated in HBS, 2 mM CaCl_2 at 37°C for 3 min. Thrombin, factor IXa and factor Xa are added at 2x desired concentrations prior to the initiation of the reaction.

The reaction is started by mixing equal volumes of both solutions resulting in physiological concentrations of the zymogens, pro-cofactors, inhibitors and platelets (or 2 μM PCPS), and desired concentrations of enzymes. Following initiation of the reaction, at selected time points, 10 μl aliquots are withdrawn from the reaction mixture and quenched in 20 mM EDTA in HBS (pH 7.4) containing 0.2 mM Spectrozyme TH and assayed immediately for thrombin activity. The hydrolysis of the substrate is monitored by the change in absorbance at 405 nm using a V_{max} spectrophotometer (Molecular Devices Corp., Menlo Park, CA). Thrombin generation is calculated from a standard curve prepared by serial dilutions of α-thrombin.
Whole blood model—The protocol used is a modification of Rand et al. (33). Two healthy donors were recruited, advised according to a protocol approved by the University of Vermont Human Studies Committee (33, 34) and their consent was obtained. Individual selected exhibited normal values for the parameters of blood coagulation, protein levels and platelet counts. Experiments were performed in tubes placed on a rocking table enclosed in a 37°C temperature-controlled glove box using fresh CTI-inhibited (100 μg/ml CTI) blood. Blood was drawn by venipuncture and immediately delivered into the reagent-loaded tubes.

I. Time-course. All tubes (four series per experiment) are loaded with CTI. No additional reagents are added to the phlebotomy control series (4 tubes). Six tubes are loaded with relipidated TF (TF/PCPS 5 pM/25 nM) in HBS, 2 mM CaCl₂, eight tubes with 97 pM factor IXa, and another eight tubes with 97 pM factor IXa, 5.5 pM factor Xa and 5.3 pM thrombin. The zero-time tube of each series is pretreated with 1 ml of 50 mM EDTA and 10 μl of 10 mM FPRck (diluted in 10 mM HCl). After blood is delivered, the tubes are periodically (2-20 min) quenched with EDTA and FPRck.

II. Final levels of the TAT. In these experiments all tubes are loaded in duplicates with CTI and desired concentrations of thrombin, factor IXa and factor Xa (individually or all three together). The TF control tubes are loaded with 5 pM relipidated TF. Tubes are quenched with EDTA and FPRck 20 min after fresh blood was added.

In all experiments, no more than 35 μl of reagents are loaded in each tube. The clotting time is observed visually by two observers and is called when "clumps" are observed on the side of the tube. After the experiment, tubes are centrifuged and the supernatants are aliquoted and analyzed for the TAT concentration.
Plasma preparation for the fluorogenic assay. Blood (10 ml) from a healthy volunteer was drawn into a syringe loaded with 10 ml HBS containing 50 mM EDTA, pH 7.4. The platelet poor plasma (PPP) was made by centrifugation at 3,000 rpm for 20 min followed by the centrifugation at 11,000 rpm (20,000 x g) for 10 min. When kept on ice, plasma did not clot for at least 10 h. One fraction of the PPP was used for the fluorogenic assay immediately and another one was spiked with 1 pM α-thrombin and kept for 1 h before the assay.

Fluorogenic thrombin activity assay in the PPP. Fluorogenic substrate VPRnbs at a final 50 μM concentration was added to a cuvette containing PPP. This substrate allows the quantitation of α-thrombin at concentrations as low as 20 fM (41). Change in fluorescence over time representing substrate hydrolysis was monitored in a spectrofluorometer FluoroMax-2 (Jobin Yvon-Spex Instruments S. A., Inc., NJ) at 25°C for 15 min using λ_ex 350 nm and λ_em 470 nm. Light-scattering artifacts were minimized with a 450 nm cutoff filter in the emission light beam. Hirudin at 1 μM was added to the reaction mixture and a change in fluorescence was monitored again. Rates of substrate hydrolysis were established from a calibration line constructed using the detecting group [6-amino-1-naphthalene(n-butyl)sulfonamide].

Estimation of the in vivo concentrations of free enzymes—The concentration of a given free enzyme in a dynamic system containing stoichiometric inhibitors is determined by the balance between the rate of enzyme generation and the rate(s) of inhibition. Assuming that the concentrations of inhibitors are relatively constant in blood under normal flow, the concentration of free enzyme can be described by the following equation: v = k_1[I_1] · [E_f] + k_2[I_2] · [E_f] + ... +
\[ k_n[I_n] \cdot [E_f], \text{ where } v \text{ is rate of enzyme generation, } k_1, k_2, \ldots, k_n \text{ are the second order rate constants defining the rates of reactions of inhibitors } I_1, I_2, \ldots, I_n \text{ with a particular enzyme} (30), \text{ and } E_f \text{ is free enzyme.} \]

The rate of generation of each enzyme was assumed to be equal to the rate of removal of its activation peptide from blood (19). The latter was assumed to be a first order process and the rate constant \( k_{AP} \) could be calculated using the reported half-life for each activation peptide (AP) (see Table 1). Ongoing rates of activation peptide removal and equal rates of enzyme formation are then defined as the product \( k_{AP}[AP] \) using reported steady state concentrations of activation peptides (13-17, 20-26). These rate estimates are used to calculate \([E_f]\) for each enzyme using the above equation and reported concentrations of inhibitors and their second order rate constants of inhibition for the particular enzyme.

**Operational definitions.** The initiation phase of thrombin generation was defined as a time interval from the start of the reaction (represented by 0 on the x-axis in figures) to the point of intersection of the x-axis and a tangent to the maximum slope of thrombin generation. It is characterized by relatively slow prothrombin activation and the proteolytic processing steps required for the assembly of the vitamin K-dependent protein activating complexes. The propagation phase of thrombin generation is defined as a time interval from the end of the initiation phase to the maximum thrombin concentration. It is characterized by robust prothrombin activation principally by factor Xa-factorVa-membrane, i.e. the prothrombinase complex with factor Xa provided predominantly by the factor IXa-factor VIIIa complex, the intrinsic factor Xase.
RESULTS

Numerical simulations predicting the influence of simultaneous variations of thrombin, factor Xa and factor IXa on thrombin generation in the absence of TF—Figure 1 represents thrombin generation over time initiated with the mixture of all three enzymes. Reported concentrations of activation peptides of serine protease zymogens involved in blood coagulation circulating in vivo for healthy individuals vary from 94 pM for factor X to 210 pM for factor IX and to 1.0 nM for prothrombin (F1.2). In the presence of serine proteases at these concentrations, thrombin generation enters the propagation phase after a very short initiation phase, i.e. blood containing these concentrations of enzymes would clot in a few seconds (Figure 1, —). The maximum concentration of active thrombin produced reaches almost 1.0 μM with the maximum rate of generation as high as 28 nM/s. A decrease in the concentration of all three enzymes by one order of magnitude (9.4 pM for factor Xa, 21 pM for factor IXa and 100 pM for thrombin) prolongs the initiation phase to 100 s and decreases both maximum concentration and maximum rate of thrombin generation to 630 nM and 8.5 nM/s, respectively (— —). A further decrease of the enzyme concentrations to 1 % of those reported for activation peptides (0.94 pM factor Xa, 2.1 pM factor IXa and 10 pM thrombin) prolongs the initiation phase to 600 s, decreases maximum concentration of active thrombin to 260 nM and provides a maximum rate of thrombin generation of 1.1 nM/s (*). No active thrombin is observed in 20 min of the reaction when concentrations of all three enzymes are decreased to 0.1% (1 pM for thrombin, 94 fM for factor Xa, 0.21 pM for factor IXa and 1 pM for thrombin) of those detected for their activation peptides (— — —).
The enzyme concentrations selected for the initial studies of thrombin generation were either equal to those of activation peptides or decreased in sequential studies by orders of magnitude. However, the free enzymes continuously generated \textit{in vivo}, would be inhibited by the inhibitors present in blood, primarily by AT-III and TFPI. Our calculations based upon activation peptide/enzyme generation and enzyme inhibition rates suggest that the steady state concentrations of coagulation serine proteases for the “idling motor” assumption should be 5.3 pM for thrombin, 5.5 pM for factor Xa and 97 pM for factor IXa (see Experimental Procedures and Table 1). At these enzyme concentrations, the initiation phase duration would be 160 s (Figure 1, □). Thus blood containing these enzyme concentrations would clot in less than 3 min. The maximum concentration of active thrombin achieved is 780 nM and the maximum rate of thrombin generation is 17 nM/s.

\textit{Variations of a single enzyme concentration in numerical simulations in the presence of two other enzymes}—In an attempt to determine which one enzyme of the mixture of three potential resident enzymes would have the most significant impact on thrombin generation, the concentration of the selected enzyme was varied, while concentrations of the other two were kept at the levels of their activation peptides.

Figure 2 represents thrombin generation initiated with varying concentrations of thrombin, 94 pM factor Xa and 210 pM factor IXa (panel A), with varying concentrations of factor Xa, 210 pM factor IXa and 1.0 nM thrombin (panel B), and varying concentrations of factor IXa, 94 pM factor Xa and 1.0 nM thrombin (panel C).
In the simulation of thrombin titration (panel A), thrombin generation during the propagation phase is almost not affected by the amount of this enzyme used as an initiator. The maximum concentration of active thrombin produced (~900 nM) is similar at 1.0 nM (100%) thrombin (panel A, —) and without any exogenous thrombin (○). The maximum rate of thrombin generation is also little affected by the presence of exogenous thrombin (28 nM/s at 1.0 nM exogenous thrombin and 24.5 nM/s in the absence of it). The initiation phase of thrombin generation is more dependent upon exogenous thrombin, especially at the high end of the range. At 1.0 nM thrombin, the initiation phase is 10 s (—). A decrease of the thrombin concentration by one order of magnitude (to 100 pM) triples (30 s) the initiation phase duration (— —). A decrease of exogenous thrombin to 10 pM adds 20 more seconds to the initiation phase, i.e. extends it to 50 s (◇). Further decreases in thrombin concentration have almost no effect on the duration of the initiation phase (see — — — at 1 pM thrombin and ○ without exogenous thrombin).

When thrombin generation is initiated with varying concentrations of factor Xa and steady concentrations of exogenous thrombin (1.0 nM) and factor IXa (210 pM) (panel B), thrombin generation is almost independent of the presence of factor Xa at concentrations tested. Only a limited decrease in thrombin generation rate is observed during the initiation phase and the inception of the propagation phase when factor Xa is omitted [compare — for 94 pM (100%) exogenous factor Xa and ○ without it].

The most pronounced effect on thrombin generation was observed in a hypothetical factor IXa titration at constant concentrations of exogenous thrombin and factor Xa (1.0 nM and 94 pM,
respectively) (panel C). A decrease in exogenous factor IXa concentration from 210 pM (100%) (—) to 21 pM (10%) (— —) causes decreases in both the maximum concentration of active thrombin (from 940 nM to 670 nM) and the maximum rate of thrombin generation (from 28 nM/s to 10 nM/s). A decrease in factor IXa concentration to 2.1 pM (1%) (∗) leads to a further decrease in the maximum thrombin concentration and generation rate (to 380 nM and 2.4 nM/s, respectively). In the presence of 0.21 pM exogenous factor IXa (0.1% of maximum), the highest concentration of active thrombin is decreased to 180 nM and the maximum rate of thrombin generation to 1.8 nM/s (— — —). No further decrease in the maximum rate of thrombin generation is observed when the factor IXa concentration is decreased below 0.21 pM. Thrombin generation profiles in the presence of 21 fM factor IXa (+) and in the absence of factor IXa (O) are almost identical. The variations in factor IXa concentration within the tested range (from 0 to 210 pM) have no effect on the initiation phase of thrombin generation.

The data presented in Figure 2 (panels A-C) show that any two enzymes present at concentrations identical to those reported for their activation peptides are able to initiate thrombin generation. Decreasing the concentration of exogenous thrombin or factor Xa has little effect (if any) on thrombin generation when the other two enzymes are present as initiators of thrombin generation. In contrast, a decrease in exogenous factor IXa leads to pronounced impairments in both maximum thrombin concentration and the generation rate. These data suggest that factor IXa, if present in blood, would play the major role in the initiation of thrombin generation.
Thrombin generation initiated with a single enzyme in the absence of other enzymes—After it was established in the numerical simulations, that the presence of any combination of two enzymes could function as an initiator of thrombin generation, producing amounts of thrombin substantial enough for clot formation, the ability of a single exogenous enzyme in the absence of TF to trigger thrombin generation was tested in three models of coagulation.

A. The influence of thrombin and factor Xa in numerical simulations. Data presented in Figure 3 show thrombin generation over time at varying concentrations of exogenous thrombin (panel A), factor Xa (panel B) and factor IXa (panel C). No other enzymes were present at the initiation of the reaction.

When exogenous thrombin is used as a trigger of thrombin generation in the absence of tissue factor (panel A), no additional thrombin is produced. Thrombin introduced into the reaction mixture is inhibited by AT-III at a thrombin concentration-dependent rate. The maximum inhibition rate is 18 pM/s when 1.0 nM (100%) thrombin is added (—) and 2 pM/s for 0.1 nM thrombin (— —). These data indicate that thrombin alone would not be able to trigger its own generation.

Data of panel B predict that exogenous factor Xa alone at 94 pM (100%), in the absence of exogenous thrombin and factor IXa, would be able to trigger thrombin generation at concentrations, which would yield clot formation, i.e. >10 nM (—). The propagation phase of thrombin generation starts after a relatively short (20 s) initiation phase with the maximum rate of thrombin generation of 0.9 nM/s. The concentration of active thrombin reaches only 84 nM
(compare with almost 1.0 \( \mu M \) in Figure 1, when all three enzymes are present). Any further thrombin generation is prevented due to the inhibition of factor Xa. When the concentration of exogenous factor Xa is decreased by one order of magnitude (to 9.4 pM), thrombin generation is almost completely abolished (— —). These data suggest that the presence of factor Xa in blood at the concentration reported for the factor X activation peptide would cause clot formation in less than 1 min. On the other hand, at concentrations \( \leq 9.4 \) pM, factor Xa alone would not be able to trigger thrombin generation above the coagulation threshold. It will be inhibited by TFPI and AT-III before producing enough thrombin required for its own amplification.

B. The influence of factor IXa on thrombin generation. In contrast to thrombin and factor Xa, factor IXa alone, in the absence of other enzymes, can trigger an efficient thrombin generation comparable with that observed in the presence all three enzymes. This observation is valid for both computer simulations and empirical models.

I. Numerical simulations (Figure 3, panel C). In the presence of 210 pM (100\%) exogenous factor IXa, thrombin generation occurs at a maximum 24 nM/s rate and the maximum concentration of thrombin reaches 800 nM (—). These parameters of thrombin generation are comparable with those observed when the mixture of all three enzymes at their maximum concentrations is used to trigger thrombin generation (28 nM/s and 940 nM, respectively; see — in Figure 1). The duration of the initiation phase, however, is significantly prolonged (160 s for factor IXa alone vs 20 s for all three enzymes). A decrease in exogenous factor IXa to 10\% (21 pM) of maximum more than triples the initiation phase (to 500 s) and significantly decreases maximum rate of thrombin generation (to 6.5 nM/s) (— —). The maximum concentration of
active thrombin produced (540 nM) is affected by this decrease in factor IXa concentration to a lesser extent than the initiation phase duration and thrombin generation rate. No active thrombin is observed in 20 min when the reaction is initiated with 2.1 pM (1%) factor IXa (*). When thrombin generation is initiated with the steady state concentration of factor IXa (97 pM), the profile of the process is similar to that observed at 210 pM factor IXa, although the initiation phase is prolonged from 160 s to 240 s (□).

II. Empirical models. The data generated in computer simulations and presented in Figures 1-3 suggest that, in the absence of tissue factor, the major trigger of thrombin generation in vivo should be factor IXa if present at concentrations related to the reported amount of factor IX activation peptide. Thrombin and factor Xa play an accessory role to the factor IXa induced thrombin generation. To test the validity of predictions obtained in computer simulations, two empirical models of blood coagulation were used: synthetic plasma and whole blood.

In the synthetic plasma reconstituted with washed platelets at mean physiologic concentration (2x10^8/ml) and in the absence of TF, factor IXa, factor Xa and thrombin at the initiation of the reaction (Figure 4), no thrombin generation is observed over a period of 840 s (○). When calculated steady state concentrations of thrombin, factor Xa and factor IXa (5.3 pM, 5.5 pM and 97 pM, respectively) are used to initiate the reaction, thrombin generation enters the propagation phase after an initiation phase of 190 s (□). Thrombin generation occurs at a maximum 0.9 nM/s rate with the maximum concentration of active thrombin reaching 130 nM. The omission of thrombin and factor Xa has almost no effect on thrombin generation, i.e. thrombin generation initiated with 97 pM factor IXa alone (◆) is comparable to that observed in the presence of all
three enzymes (□). The initiation phase is slightly prolonged (from 190 s to 210 s), the maximum rate of thrombin generation and maximum concentration of thrombin are slightly increased (to 1.3 nM/s and 160 nM, respectively).

In an attempt to evaluate the influence of exogenous thrombin, factor Xa and factor IXa on thrombin generation \textit{in vitro} evaluated as the thrombin-antithrombin III complex (TAT) formation under the conditions approaching those present \textit{in vivo}, the three enzymes were tested in fresh CTI-inhibited whole blood (Figure 5). The propagation phase of thrombin generation initiated in whole blood in the absence of tissue factor with 5.3 pM thrombin, 5.5 pM factor Xa and 97 pM factor IXa starts after the initiation phase of ~4 min (panel A; □). Thrombin is generated (TAT formation) at a maximum rate of 1.7 nM/s and the final TAT concentration reaches 800 nM. The omission of thrombin and factor Xa has little effect on thrombin generation. When 97 pM factor IXa alone is added to the blood (●), the initiation phase of thrombin generation is ~3 min, the maximum rate of thrombin generation and final concentration of the TAT complex are 1.3 nM/s and 720 nM, respectively. For comparison, thrombin generation in CTI-inhibited whole blood induced with 5 pM relipidated TF, with no exogenous enzymes added, enters the propagation phase following the initiation phase of ~4 min (○). No thrombin generation is observed during 20 min of the reaction when neither enzyme(s) nor TF are added to the blood (●).

Panel B of Figure 5 shows whole blood clotting times induced with relipidated TF, thrombin, factor Xa and factor IXa as well as with the mixture of all three enzymes.
When no activator is added to the CTI-inhibited fresh blood, it does not clot in 1200 s (the duration of the experiment). The initiation of the reaction in whole blood with 5 pM relipidated TF causes clot formation in 240 s. Comparable clotting times (119-188 s) are observed when individual enzymes are added at their activation peptide concentrations. However, clotting times are significantly prolonged when either 5.3 pM thrombin or 5.5 pM factor Xa (calculated steady-state concentrations) are added to the CTI-inhibited whole blood (1080 and 860 s, respectively). In contrast, the initiation of whole blood with 97 pM factor IXa leads to clot formation in 188 s, i.e. identical to that observed with 210 pM factor IXa and slightly faster than with 5 pM TF. The addition of all three enzymes to whole blood at their activation peptide concentrations simultaneously shortens clotting time to 86 s, whereas the addition of these enzymes at their calculated steady-state concentrations does not decrease the clotting time (220 s) below that observed for factor IXa alone.

The above empirical results together with computer simulations indicate that factor IXa at the estimated steady state in vivo concentration (97 pM) should cause at physiologic conditions a robust thrombin generation leading to a relatively rapid (3-4 min) clot formation. Thrombin and factor Xa at the estimated in vivo concentrations have quite limited (if any) effect on factor IXa induced thrombin generation.

*The concentration of active thrombin in non-stimulated whole blood*—All three models of blood coagulation were used so far to evaluate thrombin generation initiated with factor IXa, factor Xa and thrombin at concentrations estimated from activation peptide measurements as circulating in vivo. The availability of efficient fluorogenic substrates (41) allows direct detection of picomolar
amounts of certain coagulation enzymes. One of these substrates, VPRnbs, was used for the
detection of enzymatic activity in fresh, EDTA-containing plasma. No detectable substrate
hydrolysis is observed over 15 min of the assay, when VPRnbs is added to the PPP (platelet poor
plasma). The addition of purified human α-thrombin to this plasma at a final 1 pM concentration
leads to the substrate hydrolysis at a 0.23 nM/s rate. The subsequent addition of 1 μM hirudin
quenches enzymatic activity completely. A possible explanation for the lack of enzymatic
activity in fresh plasma is that active enzymes are inhibited by their endogenous inhibitors during
the processing of blood. To address this issue, plasma was spiked with 1 pM α-thrombin and
incubated for 1 hr (time required to make the PPP). The rate of substrate hydrolysis observed
after this incubation was 0.16 nM/s, indicating that ~70% of added thrombin remained active.
The results of this experiment suggest that the concentration of active thrombin in whole blood is
significantly lower than that estimated for steady state conditions (5.3 pM) on the basis of
reported Fragment 1.2 concentration.
DISCUSSION

Activation peptides of the vitamin K-dependent proteins are reported to be present in the blood of normal individuals (6, 10-26); altered levels of these peptides correlate with various coagulation disorders (11-19) and risk factors related to these disorders (20-26).

One hypothesis ("idling motor") proposes that systemic, continuous activation of serine protease zymogens is a normal feature of the vasculature. Implied in this idea is the presence of active enzymes at levels insufficient to trigger clot formation. Studies in whole blood indicate that enzymatic activity, if present in blood, is below the activation threshold required for coagulation, because unstimulated, contact pathway inhibited blood remains fluid in vitro for >20 min (33, 34, 43, current study) with no measurable thrombin observed. The data of the current study show, that the addition of three enzymes of coagulation (thrombin, factor Xa and factor IXa) at estimated steady state concentrations causes solid clot formation in ~200 s. Similar estimated “ clotting times” (the duration of the initiation phase) (32, 33) were observed in the other two models of blood coagulation used in this study – empirical (synthetic) and mathematical (computational) (190 and 160 s, respectively). Moreover, the addition of any single enzyme to whole blood at the steady state concentration leads to the clot formation in less than 1200 s with factor IXa showing the highest efficiency (clotting time 190 s). The two other models predict a similar clotting time for this factor IXa concentration (210-240 s). For comparison, whole blood initiated to react with 5 pM relipidated tissue factor clots in 240 s and does not clot for >1200 s in its absence. These data suggest that concentrations of active enzymes (thrombin, factor Xa and factor IXa) circulating in blood are significantly lower (if present at all) than those estimated on the basis of reported in vivo concentrations of corresponding activation peptides and rates of their
generation/clearance as well as the known kinetics of enzyme inhibition by their in vivo inhibitors. Thus, an additional pathway for the clearance of thrombin, factor IXa, and factor Xa has to exist in vivo. It can be related to the binding of these serine proteases to cell-surface receptors with subsequent internalization via endocytosis (44-47). Additionally, the binding of AT-III and TFPI to their target serine proteases could be altered in vivo by proteoglycans present on endothelial cells (48, 49).

Several observations related to the mechanism of thrombin generation can be made based upon the data of the current study. Only factor IXa at the picomolar concentrations relevant to the reported concentration of factor IX activation peptide is able to trigger thrombin generation similar to that observed with the mixture of all three enzymes used as an initiator. Factor IXa at those concentrations generates enough factor Xa for the production of initial amounts of thrombin. The latter amplifies its own generation through the activation of factor V and factor VIII. Although factor Xa is efficiently inhibited by TFPI (50), a relatively poor inhibition of factor IXa by antithrombin-III (51) allows generation of factor Xa for a prolonged period of time keeping prothrombin activation alive. A decrease in factor IXa concentration prolongs the initiation phase of thrombin generation due to the slower factor X activation.

In the reaction system lacking tissue factor, i.e. an essential component of the physiologic activator of factor IX and factor X, factor Xa can trigger thrombin generation above the coagulation threshold (>10 nM) (33, 43) only when present at the highest concentration tested (94 pM). The maximum concentration of thrombin produced, however, is much lower than that observed in the presence of factor IXa alone. At a lower factor Xa concentration (9.4 pM), factor
Xa is inhibited by TFPI and AT-III (51) prior to the generation of detectable amounts of thrombin.

In the numerical simulations, thrombin even at the highest concentration used (1 nM) does not trigger its own generation. However, whole blood initiated with 1 nM thrombin in the absence of tissue factor clotted in 119 s suggesting relatively rapid thrombin generation. This discrepancy between the two models occurs due to the absence of factor XI in the numerical simulations, and, as a consequence, the absence of an efficient pathway of factor IX and factor X activation because the only enzymes present in the system are thrombin and 0.1 nM factor VIIa. The numerical model is an evolving entity, and new proteins, reactions and their kinetic constants have been and will be added to this model.

The data obtained when two enzymes are used simultaneously as a trigger of thrombin generation show that only the concentration of factor IXa has a pronounced effect on the process. Although factor Xa and thrombin initiate prothrombin activation in the absence of factor IXa, the maximum concentration of thrombin produced is relatively low, resembling that observed in hemophilia (29, 34). The absence of factor Xa when thrombin and factor IXa are present, has almost no effect on prothrombin activation, presumably due to the rapid activation of factor V and factor VIII by thrombin (30, 31-33) and, as a result, robust factor Xa generation by the factor IXa/factor VIIIa complex. The absence of initiating thrombin when factor IXa and factor Xa are present as initiators of thrombin generation has almost no effect on rates of prothrombin activation and maximum levels of thrombin achieved. The initiation phase, however, is slightly prolonged in the absence of thrombin. This delay in the onset of the propagation phase is related
to the time required to generate initial amounts of thrombin by factor Xa. When produced, thrombin amplifies its own generation by activating factor V and factor VIII.

In conclusion, the data of this study suggest that concentrations of coagulation enzymes circulating \textit{in vivo} are significantly lower than can be predicted from the reported concentrations of their activation peptides and rates of their inhibition by AT-III and TFPI. These peptides thus probably represent a “historical record” rather than an “idling motor”. Our results suggest that in prothrombotic disease states, which are characterized by increased levels of activation peptides, exceptional attention has to be paid to the concentration of the factor IX activation peptide due to the high efficiency of factor IXa in initiation of thrombin generation.

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FIGURE LEGENDS

Figure 1. Numerical simulations. Thrombin generation initiated by factors IXa and Xa and thrombin in the absence of TF. Coagulation enzymes are present at the concentrations identical to the physiologic concentrations of their activation peptides (1.0 nM for thrombin, 94 pM for factor Xa and 210 pM for factor IXa) (—), at 10% (— —), 1% (*) and 0.1% (— — —) of those or at concentrations which would occur based upon the rates of enzyme generation and inhibition in a steady state situation (5.3 pM for thrombin, 5.5 pM for factor Xa and 97 pM for factor IXa) (□). Prothrombin, factors V, VIII, VII, VIIa, IX, and X, AT-III and TFPI are present at their mean physiological concentrations.

Figure 2. Numerical simulations. Titration of a single enzyme in the presence of two others at the concentrations identical to those of their activation peptides (100%) in the absence of TF. A. Thrombin titration, B. Factor Xa titration, C. Factor IXa titration. Titrants are present at 100% (1.0 nM for thrombin, 94 pM for factor Xa and 210 pM for factor IXa) (—), 10% (— —), 1% (*), 0.1% (— — —), and 0% (○). Prothrombin, factors V, VIII, VII, VIIa, IX, and X, AT-III and TFPI are present at their mean physiological concentrations.

Figure 3. Numerical simulations. Titration of a single enzyme in the absence of other enzymes and TF. A. Thrombin titration, B. Factor Xa titration, C. Factor IXa titration. Titrants are present either at 100% concentrations of their activation peptides (1.0 nM for thrombin, 94 pM for factor Xa and 210 pM for factor IXa) (—), 10% (— —), 1% (*), 0.1% (— — —), and 0% (○) or at steady state concentrations (5.3 pM for thrombin, 5.5 pM for factor Xa and 97 pM for factor IXa)
Prothrombin, factors V, VIII, VII, VIIa, IX, and X, AT-III and TFPI are present at their mean physiological concentrations.

**Figure 4.** Synthetic plasma in the presence of $2 \times 10^8$/ml platelets and in the absence of TF. Thrombin generation in the absence of an initiator (●), initiated by 5.3 pM thrombin; 5.5 pM factor Xa and 97 pM factor IXa (□) and by 97 pM factor IXa alone (◆). Prothrombin, factors V, VIII, VII, VIIa, IX, and X, AT-III and TFPI are present at their mean physiological concentrations.

**Figure 5.** Whole blood. Thrombin generation (TAT formation) over time (A) and clotting times (B) in the contact pathway inhibited whole blood (0.1 mg/ml CTI) in the absence of an initiator (●), initiated by 97 pM factor IXa (◆) or by 97 pM factor IXa, 5.5 pM factor Xa, and 5.3 pM thrombin (□) in the absence of TF or by 5 pM relipidated TF (⊙). Clotting times are indicated on the top of bars (B).
Table 1. Estimated in vivo concentrations of active enzymes.

<table>
<thead>
<tr>
<th>Zymogen</th>
<th>Activation peptides</th>
<th>Conc. (M)</th>
<th>Half-life (s)</th>
<th>Removal rate (M/s)</th>
<th>[I] (M)</th>
<th>k (M⁻¹ s⁻¹)</th>
<th>E₀ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin</td>
<td></td>
<td>1.0x10⁻⁹</td>
<td>5400</td>
<td>1.3x10⁻¹³</td>
<td>3.4x10⁻⁶</td>
<td>7.1x10³</td>
<td>5.3</td>
</tr>
<tr>
<td>Factor IX</td>
<td></td>
<td>2.1x10⁻¹⁰</td>
<td>900</td>
<td>1.6x10⁻¹³</td>
<td>3.4x10⁻⁶</td>
<td>4.9x10²</td>
<td>97</td>
</tr>
<tr>
<td>Factor X</td>
<td></td>
<td>9.4x10⁻¹¹</td>
<td>1800</td>
<td>3.6x10⁻¹²</td>
<td>3.4x10⁻⁶</td>
<td>1.5x10³</td>
<td></td>
</tr>
</tbody>
</table>

  |        |                    |           |                |                    |        |            |        |
  |        |                    |           |                |                    |        | 2.5x10⁻⁹  | 9.0x10⁵ | 5.5     |

a from (19); b equal to the enzyme generation rate; c from (50-52); d free enzyme; e AT-III; f TFPI
Figure 2B

Thrombin (nM) vs. Time (sec)

B
Figure 2C

Thrombin (min)

Time (sec)
Figure S4

A

Thrombin (nM)

Time (sec)
The significance of circulating factor IXa in blood
Saulius Butenas, Thomas Orfeo, Matthew T. Gissel, Kathleen E. Brummel and Kenneth G. Mann

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