Kinetics of Human Factor VII Activation[†]

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ABSTRACT: In this study the activation of human factor VII by a variety of potential activators in the presence and absence of mixed phospholipid vesicles [25% phosphatidylserine (PS), 75% phosphatidylcholine (PC)] is evaluated. At the plasma concentration of factor VII, 10 nM, the activation rate of the zymogen by 0.05 nM factor Xa is anionic phospholipid (PCPS) dependent and achieves a maximum value of 18 pM/s at 5−20 μM PCPS; further increases in the levels of PCPS decrease the activation rate of factor VII. The maximum activation rate of factor VII (10 nM) by the factor VIIa−tissue factor complex (0.1 nM), 0.76 pM/s, is achieved at 200 μM PCPS. No detectable activation of 10 nM factor VII is observed under similar conditions when either thrombin (0.1 nM) or factor IXa (0.1 nM) is used as an activator. Factor VIIa (10 nM) and factor XIa (1 nM) are not observed to activate factor VII at detectable rates. The observed Michaelis−Menten constants (K_M) for factor VII activation in the presence of PCPS at optimal concentrations vary from 1.2 μM for factor Xa to 3.2 μM for the factor VIIa−tissue factor complex. The highest catalytic constant (k_{cat}) value (15.2 s^{-1}) is observed for factor Xa−PCPS. The factor VIIa−tissue factor complex, factor IXa, and thrombin k_{cat} values are 1.4, 0.32, and 0.061 s^{-1}, respectively. Tissue factor does not increase the factor VII activation rate by factor Xa, factor IXa, or thrombin. Factor VIIIa in the presence of PCPS has no effect on factor VII activation by factor IXa. In contrast, factor Va decreases the factor VII activation rate by factor Xa, reaching saturation at concentrations consistent with complete prothrombinase complex formation. The formed prothrombinase complex activates factor VII at approximately 30% the rate of factor Xa bound to phospholipids. These data allow us to conclude that the predominant physiological factor VII activator is, most likely, membrane-bound factor Xa.

Factor VII is a single-chain vitamin K dependent protein that circulates in blood as a zymogen and may be converted to a two-chain serine protease, factor VIIa, by cleavage of a single peptide bond located at Arg_{152}-Ile_{153} (Hagen et al., 1986). Although Zur and Nemerson (1978) and Jesty and Morrison (1983) concluded that single-chain factor VII has significant enzymatic activity, experimental data published from various laboratories lead to the conclusion that factor VII is a true zymogen (Williams et al., 1989; Wildgoose et al., 1990; Lawson et al., 1992; Chaing et al., 1994) and has no amidolytic activity toward either natural or synthetic substrates. Other studies have proposed that approximately 1% of factor VII exists in plasma in the activated form as the enzyme factor VIIa (Morrissey et al., 1993). Plasma factor VIIa most likely escapes inhibition by the natural inhibitors present in blood due to the low activity of this enzyme in the absence of tissue factor. Lawson et al. (1993a) demonstrated that factor VIIa may be inhibited by antithrombin III−heparin only in the presence of tissue factor. Similarly, the inhibition of factor VIIa by the tissue factor pathway inhibitor occurs efficiently only in the presence of tissue factor and factor Xa (Broze et al., 1988). The poor factor VIIa amidolytic activity toward synthetic substrates and the low concentration of factor VII in blood have made analyses of the activation of this zymogen at physiologically relevant enzyme and substrate concentrations difficult. The development of sensitive fluorogenic substrates for factor VIIa which contain the aminonaphthalenesulfonamide detecting group (Butenas et al., 1992, 1993) permits evaluation of factor VIIa amidolytic activity at low nanomolar and subnanomolar concentrations of this enzyme (Lawson et al., 1992; Butenas et al., 1994; this study) and provides for appropriate quantitation of factor VIIa production under conventional "initial rate" and steady state conditions.

It has been hypothesized that factor VIIa in complex with tissue factor and a supporting surface in the presence of Ca^{2+} initiates the coagulation cascade by cleavage of factor X and factor IX (Jesty & Silverberg, 1979; Broze, 1982; Nemerson, 1988; Bom & Bertina, 1990; Komiyama et al., 1990; Lawson & Mann, 1991; Krishnaswamy, 1992). Many protease(s) have been suspected to play the critical role in factor VII activation *in vivo*. It has been reported that the serine proteases thrombin (Radcliffe & Nemerson, 1975), factor IXa (Seligsohn et al., 1979; Wildgoose & Kisiel, 1989), factor Xa (Radcliffe & Nemerson, 1976; Bajaj et al., 1981a; Rao & Rapaport, 1988a), factor XIIa (Kisiel et al., 1977; Seligsohn et al., 1979; Broze & Majerus, 1980), and factor XII fragments (Radcliffe et al., 1977) are biologically relevant. In addition, Nemerson and Esnouf (1973), Sakai et al. (1989), Nakagaki et al. (1991), Yamamoto et al. (1992), Ruf et al. (1992), and Fiore et al. (1994) have reported that factor VII may also be activated by the factor VIIa−tissue factor complex. Nonphysiological activators include hepsin (Kazama et al., 1995) as well as the prothrombin activator from the venom of Taipan snake (Nakagaki et al., 1992).

In the studies mentioned above, the conditions of factor VII activation such as the enzyme/substrate ratio and their concentrations, the nature and concentration of the supporting

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surface, and the factor VIIa assay methodology vary from publication to publication and, occasionally, within a publication. Additionally, there are controversial data about the influence of tissue factor on factor VII activation by factor Xa. In the present study, we evaluate the activation of factor VII under identical conditions by suspected potential *in vivo* activators of this zymogen, in the absence or presence of cofactors, and in various enzyme combinations. Our results permit conclusions concerning the preferred activator of factor VII.

EXPERIMENTAL PROCEDURES

Materials. The fluorogenic substrate 6-(D-Phe-Pro-Arg)-amino-1-naphthalenebutylsulfonamide (FPR-nbs) was synthesized, characterized, and employed for factor VIIa assay as described previously (Butenas et al., 1992, 1993). Final concentration of substrate in all experiments was 80 μM. Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Sigma. Phospholipid vesicles (PCPS) composed of 75% PC and 25% PS were prepared as described (Higgins & Mann, 1983). The buffer used for all experiments was composed of 20 mM HEPES, 0.15 M NaCl, 5 mM Ca^{2+}, 0.1% PEG 6000, pH 7.4 (HBS). All factor VII experiments were done at 25 °C.

Proteins. The thrombin inhibitor hirudin (Stone & Hofsteenge, 1986) was from Genentech, factor Xa inhibitor TAP (tick anticoagulant protein) (Krishnaswamy et al., 1994) was from CORVAS International Inc. Recombinant human tissue factor 1−242 and recombinant human factor VIII (free of albumin) were provided by Shu-Len Liu (Hyland Division, Baxter Healthcare Corp.). Human thrombin and factors Va, VIIa, Xa, and IXa were provided as gifts from Haematologic Technologies Inc. while factor VII was purchased from the same source. Human plasma factor IX was isolated from fresh frozen plasma by previously reported methods (Bajaj et al., 1981b; Lawson & Mann, 1991) or was purified from factor IX concentrate "Proplex" (provided as a gift from Roger Lundblad of Baxter Healthcare Corp.) on an anti-factor IX(a) heavy chain [αHFIX(a)$_{HC}$#40]-Sepharose antibody column (Jenny et al., 1986). Factor IX from both sources as well as activation product factor IXa were identical on SDS−PAGE gels and in clotting assays (Bajaj & Birktoft, 1993). Factor IX was activated to factor IXa by factor XIa using the general methods of Lindquist et al. (1978). The factor IXa produced was purified on a αHFIX(a)$_{HC}$#40-Sepharose antibody column. This antibody was provided by William Church (University of Vermont).

Factor VIIa−Tissue Factor Complex Formation. Tissue factor was relipidated on PCPS vesicles as reported previously (Lawson et al., 1993b). Factor VIIa at the desired concentration was added to relipidated tissue factor and incubated for 20 min. In experiments performed in the absence of PCPS, detergent-solubilized tissue factor and factor VIIa were added to HBS simultaneously and incubated for 20 min. Similar stoichiometry and affinity of factor VIIa for tissue factor on PCPS and PC vesicles or in the absence of the supporting surface (Lawson et al., 1992; Krishnaswamy, 1992) provide evidence that all tissue factor relipidated on PCPS vesicles is accessible for factor VIIa binding, i.e., the extracellular domain of tissue factor is located outside of PCPS vesicles.

Factor VIII Activation. Factor VIII activation was accomplished by the modified procedure of Fay et al. (1991) as follows: 10 nM factor VIII was incubated with 0.3 nM thrombin for 3 min in the presence of 20 μM PCPS. Hirudin at 100 nM concentration was added, and freshly formed factor VIIIa was immediately used.

Factor VII Activation by Factor VIIa. Factor VII at 10 nM or 1 μM concentration was added to 200 μM PCPS and factor VIIa (0.5 or 10 nM, respectively). Amidolytic activity of the reaction solution was evaluated at various time points within 4 h for 1 μM factor VII or within 24 min for 10 nM factor VII. No factor VII activation by factor VIIa was observed for up to 4 h.

Evaluation of Initial Factor VII Activation Rates. A. For studies of the activation of factor VII at physiological substrate concentration by factor IXa, factor Xa, factor VIIa−tissue factor complex, and thrombin, factor VII (10 nM final concentration) was added to the desired concentration of PCPS (0, 20, 60, or 200 μM) and either 0.5 nM/0.05 nM or 0.5 nM/0.1 nM of factor VIIa/tissue factor, respectively, 0.05 nM or 0.1 nM factor Xa, 0.1 nM factor IXa, or 0.1 nM thrombin were added. An excess of factor VIIa was used for the factor VIIa−tissue factor complex experiments to allow for complete incorporation of all tissue factor into the complex with factor VIIa due to the high affinity of these two proteins (Schullek et al., 1994). This approach was chosen since factor VIIa even at 10 nM was not able to activate factor VII (1 μM) at a detectable rate. Thus, in all cases we have only one active enzyme (the factor VIIa−tissue factor complex) present at a concentration equal to the concentration of tissue factor. The complex enzyme concentration was constant during the experiment and was not increased by generated additional factor VIIa. Aliquots, 1190 μL, of the activation mixture were removed at 1, 5, 10, 16, and 24 min, the corresponding inhibitor (hirudin for thrombin or TAP for factor Xa) and FPR-nbs were rapidly added, and the rate of substrate hydrolysis was evaluated. The amount of factor VIIa formed at each time point was established from a standard curve, and the initial rate of factor VIIa generation was evaluated using the nonlinear least-squares fitting program ENZFITTER (Elsevier-BIOSOFT, Cambridge, U.K.).

B. The activation of factor VII by factor Xa in the presence of tissue factor was studied in the following fashion: 10 nM factor VII was added to 0.05 nM tissue factor relipidated on 20 μM PCPS followed by the addition of 50 pM factor Xa. The factor VIIa generation rate was evaluated as described above.

Kinetic Constants for Factor VII Activation. Factor VII at varying concentrations (0.25−3.5 μM) was added to 20 or 200 μM PCPS followed by addition of experimentally established, practical concentrations of enzyme (0.1 nM for factor Xa, 2 nM for the factor VIIa−tissue factor complex, 10 nM for thrombin, and 5 nM for factor IXa). Aliquots of 12 μL were taken at 1 and 5 min and diluted 100-fold with HBS that contained FPR-nbs and the corresponding inhibitor, and the rate of substrate hydrolysis was evaluated. Quantitation and kinetic constants of factor VIIa formation were evaluated as described above.

Factor VII Activation in the Presence of Tissue Factor, Factor Va, or Factor VIIIa. The concentration of factor VII in these experiments was 1 μM. In control experiments, evaluations of factor VII activation rates by enzymes in the

absence of cofactors and by cofactors in the absence of enzymes were performed.

A. For studies of the factor VII activation by factor Xa in the presence of factor Va and/or tissue factor, factor VII was added to 20 μM PCPS, 0.1 nM factor Xa, and variable concentrations of factor Va (0−4 nM) or 0.5 nM relipidated tissue factor. The rate of factor VII generation was evaluated as described above. A similar activation experiment was also performed in the presence of 0.5 nM tissue factor, 3 nM factor Va, and 0.1 nM factor Xa.

B. Factor VII activation by factor IXa in the presence of tissue factor or factor VIIIa was studied as follows: factor VII was added to 200 μM PCPS, 2 nM factor IXa and 1 nM relipidated tissue factor or 10 nM freshly activated factor VIIIa. The rates of factor VIIa generation were evaluated.

C. Factor VII activation by thrombin in the presence of tissue factor was evaluated in the following fashion: factor VII was added to 200 μM PCPS, 2 nM thrombin, and 2 nM tissue factor. The rate of factor VIIa generation was evaluated.

D. For studies of the activation of factor VII by a mixture of factor VIIa, factor IXa, and factor Xa in the presence of tissue factor, factor Va and factor VIII(a), factor VII was added to 20 or 200 μM PCPS, 0.1 nM factor Xa, 0.1 nM factor VIIa−tissue factor complex, 0.1 nM factor IXa, 1 nM factor VIII, and 2 nM factor Va. Rates of factor VIIa generation were evaluated.

RESULTS

Initial Analyses of Factor VII Activation Rates. These experiments were designed to evaluate factor VII activation rates when the substrate is present at the plasma concentration of this protein and at relatively low concentrations of activating enzymes which, presumably, are generated during the initiation phase of blood coagulation (Lawson et al., 1994; Jones & Mann, 1994). Factor VIIa was not observed to activate 10 nM factor VII at enzyme concentrations as high as 0.5 nM. This observation led us to select conditions for the factor VIIa−tissue factor complex formation in which the tissue factor concentration was limiting and factor VIIa was in excess. Thus, the concentration of the factor VIIa−tissue factor complex is dictated by the tissue factor concentration. The factor VIIa−tissue factor complex at 0.05 nM concentration in the absence of PCPS did not activate factor VII at a detectable rate. Similar results were observed in the presence of 20 μM PCPS. In the presence of 200 μM PCPS, factor VII activation was just detectable. Thus, the next series of experiments were conducted at a higher factor VIIa−tissue factor complex concentration (0.1 nM). At this enzyme concentration and 200 μM PCPS the factor VII activation rate was 0.0076 pM/s per pM enzyme (pM/s/pM).

At potential physiologic concentrations, factor Xa was found to be a more effective activator of factor VII than the factor VIIa−tissue factor complex. The initial rates of 10 nM factor VII activation at 0.1 nM factor Xa and 20 μM PCPS were too rapid to permit evaluation. At 0.05 nM factor Xa and 20 μM PCPS, the factor VII activation rate was 0.36 pM/s/pM, i.e., almost 50-fold higher than the factor VIIa−tissue factor complex. The factor Xa activation of factor VII at plasma concentrations of the substrate was dependent upon phospholipid concentration (Figure 1). The maximum

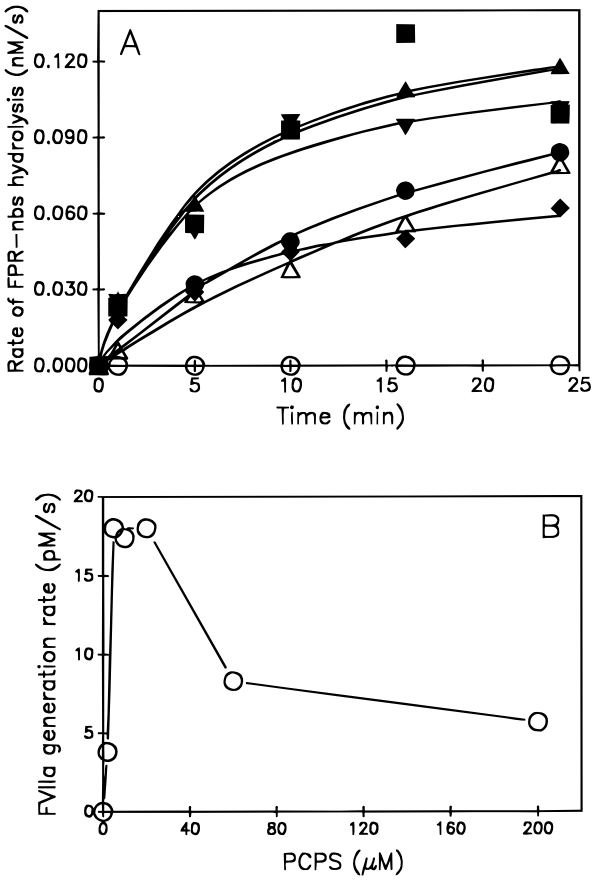

FIGURE 1: Factor VII activation by factor Xa; PCPS dependence. 50 pM factor Xa was added to HBS containing 10 nM factor VII and the following PCPS concentrations: 0 μM (open circles), 2 μM (filled diamonds), 5 μM (filled triangles), 10 μM (filled squares), 20 μM (filled inverted triangles), 60 μM (filled circles), 200 μM (open triangles). Aliquots of 1190 μL were removed at 1, 5, 10, 16, and 24 min, 10 nM TAP and 80 μM FPR-nbs were added, and the rate of substrate hydrolysis was evaluated (panel A). Initial rates of factor VIIa generation were calculated from a standard line (not shown), and the rate dependence of factor VIIa generation on PCPS concentration was plotted in panel B.

activation rate was observed at 5−20 μM PCPS and decreased with either decreased or increased PCPS levels. At 2 μM PCPS the factor VII activation rate by factor Xa was 0.076 pM/s/pM, at 60 μM and 200 μM PCPS this rate was 0.17 pM/s/pM and 0.11 pM/s/pM, respectively (Figure 1). However, even at relatively high phospholipid concentrations, i.e., the conditions optimal for factor VII activation by the factor VIIa−tissue factor complex, factor Xa activated factor VII at a 15-fold higher rate than the factor VIIa−tissue factor complex. Factor VII activation by factor Xa was not observed in the absence of PCPS/Ca^{2+}. The rate of factor VII activation by factor Xa was also not altered by the presence of tissue factor relipidated on 20 μM PCPS. At 1 μM concentration, the factor VIIa generation rates by 0.1 nM factor Xa were 0.61 and 0.63 nM/s in the presence of 20 and 200 μM PCPS, respectively.

Other reported activators of factor VII, factor IXa and thrombin, did not activate factor VII at detectable rates when concentrations of 0.1 nM enzyme and 10 nM factor VII were used. These observations were valid in the absence of phospholipids and for both PCPS concentrations chosen. Factor XIa at 1 nM did not activate 1 μM factor VII in the presence of 20 or 200 μM PCPS at a detectable rate.

Table 1: Kinetic Constants for Factor VII Activation

activator	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	ratio Xa/E
1. factor Xa[a]	1.2 ± 0.02	15.2 ± 0.1	1.3×10^7	
2. factor VIIa/TF[b]	3.2 ± 0.6	1.4 ± 0.16	4.4×10^5	30
3. factor IIa[b]	2.7 ± 0.1	0.061 ± 0.002	2.3×10^4	570
4. factor IXa[b]	1.7 ± 1.2	0.32 ± 0.16	1.9×10^5	68
5. factor XIa[a,b]		no activation		
6. factor VIIa[b]		no activation		

[a] 20 μM PCPS. [b] 200 μM PCPS.

Kinetic Constants for Factor VII Activation. The four enzymes reported to be involved in blood coagulation and the factor VII activation reaction were used at various concentrations (factor Xa, 0.1 nM; factor VIIa−tissue factor complex, 2 nM; thrombin, 10 nM; and factor IXa, 5 nM) for the evaluation of factor VII (0.25−3.5 μM) activation by the parameters of Michaelis−Menten kinetics (Table 1). The Michaelis−Menten constants (K_M) for factor Xa, factor VIIa−tissue factor complex, thrombin, and factor IXa varied in a relatively narrow range from 1.2 μM for factor Xa to 3.2 μM for the factor VIIa−tissue factor complex. Differences in the capability of the enzymes presented in Table 1 to activate factor VII are defined mostly by their ability to cleave this protein, i.e., by their catalytic constants (k_{cat}). Thus, the catalytic constant for factor Xa had a value of 15.2 s^{-1} and is approximately 1 order of magnitude higher than that observed for the factor VIIa−tissue factor complex. The catalytic constants of 0.32 s^{-1} observed for factor IXa and 0.061 s^{-1} observed for thrombin were only 2% and 0.4% of that for factor Xa, respectively. The limited proteolytic abilities of factor IXa and thrombin toward factor VIIa explains the lack of factor VII activation by these enzymes in the preliminary experiments discussed previously. The ratios of second-order rate constants for factor Xa *vs* the factor VIIa−tissue factor complex were similar to the ratios of initial rate studies for 10 nM factor VII, i.e., 30 and 50, respectively.

Influence of Tissue Factor and Factors Va and *VIIIa on Factor VII Activation.* Factor VIIa, in the absence of tissue factor, failed to activate factor VII at detectable rates despite the relatively high concentration of enzyme used (up to 10 nM) and long incubation times (up to 240 min). Tissue factor increased the ability of factor VIIa to activate factor VII to approximately 2%−3% of that observed for factor Xa. On the other hand, tissue factor neither enhanced nor depressed the rate of factor VII activation by the three other enzymes tested in this experiment, i.e., thrombin, factor IXa, and factor Xa.

Factor Va, an essential cofactor to factor Xa in forming *prothrombinase* (Nesheim et al., 1979), was tested for the influence on factor VII activation by factor Xa. We found that factor Va progressively decreased the efficiency of factor Xa to approximately 30% of that obtained in the absence of factor Va (Figure 2). Saturation of the factor VIIa generation rate inhibition process by factor Va was reached at 1 nM when factor Xa was present at 0.1 nM and PCPS at 20 μM. This saturation point corresponds to that for quantitative formation of the prothrombinase complex. Thus, we may conclude that prothrombinase can activate factor VII to factor VIIa but that the efficiency of the prothrombinase catalyst is depressed to approximately 30% of that for factor Xa−PCPS. The simultaneous involvement of both factor Va and tissue factor in factor VII activation by factor Xa did not alter the activation capability of the prothrombinase complex. Factor VIIIa had no detectable influence on the ability of factor IXa to activate factor VII.

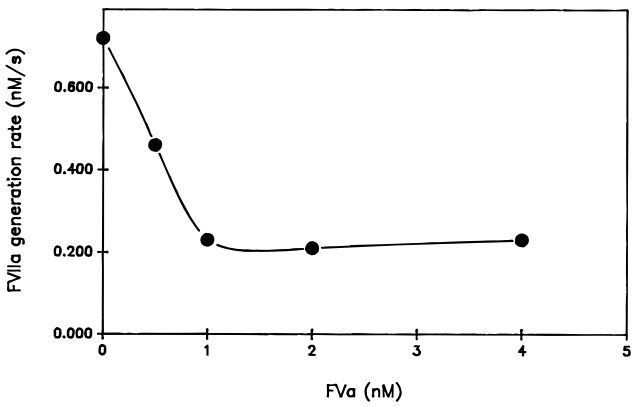

FIGURE 2: Factor Xa activity titration by factor Va. Factor VII, 1 μM, was added to HBS containing 20 μM PCPS, 0.1 nM factor Xa and various concentrations of factor Va (0−4 nM). Rates of factor VIIa generation were calculated as described in Experimental Procedures.

To investigate the possibility of yet undiscovered interactions between the potential factor VII activators and various cofactors which may influence the factor VII activation process, we designed experiments in which equimolar concentrations (0.1 nM) of three enzymes, factor IXa, factor Xa, factor VIIa−tissue factor complex, and excess concentrations of three cofactors, factor Va, factor VIIIa, and tissue factor (2, 1, and 0.5 nM, respectively), were mixed and provided as activators at 20 μM PCPS. No activation of factor VII in excess of that provided by the prothrombinase complex was observed at these experimental conditions. In control experiments factor Xa activated factor VII at a rate of 0.61 nM/s. In the presence of all the proteins mentioned above, the activation rate observed was 0.19 nM/s which was approximately 30% of factor Xa activity.

In the presence of 200 μM PCPS, the only factor VII activators were factor Xa and the factor VIIa−tissue factor complex which functioned as two independent enzymes. The factor VIIa generation rate by factor Xa alone was 0.63 nM/s. In the presence of the six proteins mentioned above, the factor VII activation rate increased to 0.89 nM/s. This rate can be accounted for by the sum of factor VIIa generation by the factor VIIa−tissue factor complex and by factor Xa. In a control experiment the factor VII activation rate by 0.5 nM factor VIIa−tissue factor complex was 0.18 nM/s. Thus, using a mixture of three enzymes and three cofactors, we have not observed any unexpected changes in the factor VIIa generation rates.

DISCUSSION

The fluorogenic substrate, FPR-nbs, permits the direct assay of free factor VIIa at nanomolar and subnanomolar concentrations. This approach allowed us to follow factor VII activation at plasma concentrations of this protein. The results of this experiment, presented in Table 1, permit the conclusion that the most productive activator, factor Xa-PCPS, is significantly more efficient than the factor VIIa−tissue factor complex. In contrast to prior reports, factor VIIa (in the absence of tissue factor) and factor XIa will not support factor VII activation.

In experiments designed to evaluate the Michaelis−Menten kinetics of factor VII activation the lowest substrate/enzyme ratio we employed varied from 25 to 350 for thrombin as an activator. For other enzymes used in this study the substrate/enzyme ratio was always over 100, reaching 35 000 for factor Xa. In terms of activation efficiency (Table 1), factor Xa is the major activator of factor VII at the enzyme concentration potentially achieved *in vivo*. Factor Xa has the highest catalytic constant and the highest second-order rate constant. The catalytic constant dominates the comparison because all the enzymes used in this study display similar Michaelis−Menten constants. Additionally, the precursor of factor Xa, factor X, circulates in blood plasma at approximately 170 nM concentration which is approximately 17-fold higher than the plasma concentration of factor VII (DiScipio et al., 1977). Thus, the potential concentration of factor Xa is significantly higher than the potential concentration of factor VIIa. Moreover, after activation, factor VIIa has to complex with tissue factor. Factor IX (80 nM) would compete with factor VII (10 nM) as a substrate for the active complex. Factor IXa and α-thrombin are unlikely factor VII activator candidates due to their low proteolytic activity toward this protein.

Factor Xa present as a component of the prothrombinase complex can also serve as an activator of factor VII; however, the activity of the factor VII activator is depressed 70% as compared to factor Xa−phospholipid. This "Prothrombinase switch" may be an important regulatory step in directing the specificity of factor Xa away from other procoagulant substrates such as factor VII, and factor IX (Lawson, 1992) and toward prothrombin as a substrate. During the last three decades several serine proteases involved in the blood coagulation process were identified as factor VII activators (Altman & Hemker, 1967; Nemerson & Esnouf, 1973; Radcliffe & Nemerson, 1975; Seligsohn et al., 1979; Nakagaki et al., 1991). Several studies describing activation of factor VII in the presence of various enzyme cofactors have been published as well. Most of those publications, however, lack an appropriate kinetic evaluation of factor VII activation. In a study published by Matsushita et al. (1994) the Michaelis−Menten equation was applied to evaluate substrate (factor VII) hydrolysis at an enzyme (factor Xa) concentration exceeding that of the substrate. Interpreting Michaelis−Menten kinetic experiments requires steady state conditions relative to substrates, i.e., constant enzyme concentration and infinitesimal consumption of substrate so that $[S] \simeq [S]_0$ during the experiment. This situation can only be approached in initial rate studies at relatively high substrate/enzyme ratios. In other studies, factor VII activation kinetics by the factor VIIa−tissue factor complex were evaluated employing increasing concentrations of enzyme (Yamamoto et al., 1992; Neuenschwander et al., 1993; Fiore et al., 1994). These approaches were used because of the relatively low rates of synthetic peptidyl substrate hydrolysis by factor VIIa.

The influence of tissue factor on factor VII activation by factor Xa has been investigated in a few laboratories. Wildgoose and Kisiel (1989) concluded that tissue factor does not influence factor VII activation by factor Xa either on carcinoma cells (J82) or on phospholipids. Our experimental data support this observation. We have not detected any increase in the factor VIIa generation rate by factor Xa when relipidated tissue factor was added to the activation mixture. This observation was valid for concentrations of factor VII extending from 10 nM (plasma) to 1 μM. The reported increase in the factor VII activation rate by factor Xa in the presence of tissue factor (Nemerson & Repke, 1985; Rao & Rapaport, 1988b; Neuenschwander & Morrisey, 1992; Matsushita et al., 1994) is most likely the result of factor VII activation by the increasing concentrations of the factor VIIa−tissue factor complex rather than by increased factor Xa activity. The presence of factor X in the factor Xa preparation may also be the cause for the observed increase in the factor VIIa generation rate due to activation of endogenous factor X by the factor VIIa−tissue factor complex.

Factor Va and factor VIIIa are known cofactors for factor Xa and factor IXa and, because of cooperative complex formation with phospholipids, increase the activity of those enzymes toward their natural substrates (prothrombin and factor X, respectively) by more than 5 orders of magnitude (Nesheim et al., 1979; van Dieijen et al., 1981). When factor VII is used as a substrate for factor IXa or factor Xa, no increase in the amidolytic activity was observed in the presence of factor VIIIa or factor Va. This observation is in agreement with data published previously (Masys et al., 1982; Rao & Rapaport, 1988a; Rao et al., 1988; Wildgoose & Kisiel, 1989). Moreover, the formation of the factor Va−factor Xa−phospholipid complex (prothrombinase) results in impaired ability to activate factor VII. Similar results are presented by Rao and co-workers (1988). During the titration of the factor Va, half-saturation effect was achieved at approximately 0.5 nM of this protein. This value is similar to the K_d reported for the factor Va−factor Xa−membrane complex (Nesheim et al., 1979; Krishnaswamy, 1990) at similar PCPS concentrations.

In previous publications which investigated the influence of phospholipids on prothrombin activation by factor Xa it was shown that in the presence of Ca^{2+}, phospholipids increased the rate of thrombin generation either by factor Xa alone or in the presence of factor Va (Nesheim et al., 1979; Rosing et al., 1980). Further increases in the concentration of phospholipids, however, decreased the ability of factor Xa to activate prothrombin (Rosing et al., 1980). This effect is most likely caused by the dilution of enzyme and substrate on the phospholipid vesicles (Nesheim et al., 1984). As the surface area increases, the local concentration of enzyme and substrate decreases and, as a consequence, an increase in the apparent K_M of the substrate hydrolysis is observed (Rosing et al., 1980).

Factor VII activation by factor Xa is also dependent upon phospholipid concentration. At 2 μM PCPS the rate of factor VII activation is only 21% of maximal. Further increase in the PCPS concentration may cause the dilution of factor Xa because of a relatively low K_d for factor Xa-PCPS binding, 0.25−0.47 μM (Nelsestuen et al., 1978; Krishnaswamy et al., 1992) and, thus, a decrease in the local concentration of this enzyme. This process is compensated by increased factor VII binding to the phospholipids because of the higher K_d for factor VII-PCPS binding (15 μM) (Nelsestuen et al., 1978). As a result of these two events, the rate of factor VIIa generation increases and reaches a plateau at approximately 5 μM PCPS. An increase in phospholipid concentration from 5 to 20 μM generates an equilibrium situation when the loss in factor VIIa generation rate due to the factor Xa−membrane dilution on a phospholipid surface

is compensated at the same extent by increased factor VII binding to PCPS. At the protein concentration used, when the phospholipid concentration is further increased (over 20 μM), the factor Xa membrane dilution effect exceeds the increase in the factor VII binding to PCPS which causes a decreased factor VIIa generation rate. Further increases in the phospholipid concentration result in dilution of both components, i.e., enzyme and substrate on the PCPS surface and, as a consequence, a further decrease in the factor VII activation rate. Thus, from data presented here we conclude that for efficient factor VII activation both proteins, factor Xa and factor VII, must be located on a phospholipid vesicle. This influence of PCPS on factor VIIa generation by factor Xa is not observed when the substrate (factor VII) is used at 100-fold higher, i.e., 1 μM, concentration.

Although the mechanism suggested above explains the influence of phospholipid concentration on the factor VII activation by factor Xa, the reported dissociation constant for factor VII and phospholipids is puzzling considering the plasma concentration of this protein. Factor VII circulates in plasma at 10 nM concentration which is the lowest among the vitamin K dependent proteins and is involved in surface dependent interactions. The K_d for phospholipids is, however, one of the highest and exceeds the plasma concentration of factor VII 1500-fold (Nelsestuen et al., 1978). The K_d value for the factor VIIa−tissue factor complex is dependent upon assay conditions, particularly upon protein concentrations and varies from 2.6 pM (Schullek et al., 1994) to 21 nM (Toomey et al., 1991). The nanomolar values were obtained at relatively high protein concentrations. Similar dependence may appear during evaluation of the dissociation constant for factor VII and phospholipids, giving an elevated K_d value due to the relatively high factor VII concentrations used for this assay. The ability of factor Xa to effectively activate factor VII in the phospholipid dependent manner when this enzyme is present at a concentration 5000−10000-fold lower than the K_d reported for the factor Xa−PCPS complex (evaluated at relatively high phospholipid concentration as well) also raises reasonable doubts about this dissociation constant.

Data presented in this study allow us to conclude that the predominant biological factor VII activator is most likely factor Xa−PCPS. While factor VIIa−tissue factor complex, factor IXa, and thrombin also activate factor VII *in vitro*, they are unlikely activators of factor VII *in vivo* due to their low proteolytic activity toward factor VII.

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