Tissue Factor-Dependent Activation of Tritium-Labeled Factor IX and Factor X in Human Plasma

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Recent investigations have suggested that the activation of factor IX by factor VIIa/tissue factor may be an important alternative route to the generation of factor Xa. Accordingly, we have compared the tissue factor-dependent activation of tritium-labeled factor IX and factor X in a human plasma system and have studied the role of proteases known to stimulate factor VII activity. Plasma was defibrinated by heating and depleted of its factors IX and X by passing it through antibody columns. Addition of human brain thromboplastin, Ca²⁺, and purified ³H-labeled factor X to the plasma resulted, after a short lag, in burst-like activation of the factor X, measured as the release of radioactively labeled activation peptide. The progress of activation was slowed by both heparin and a specific inhibitor of factor Xa. In this case, factor Xa could not be completely abolished by such inhibitors. In the case of ³H-factor IX activation, the rate also increased for ~3 min after addition of thromboplastin, but was not subsequently curtailed. A survey of proteases implicated as activators of factor VII in other settings showed that both factor Xa and (to a much smaller extent) factor IXa could accelerate the activation of factor IX. However, factor Xa was unique in obliterating activation when present at concentrations greater than ~1 nM. Heparin inhibited the tissue factor-dependent activation of factor IX almost completely, apparently through the effect of antithrombin on the feedback reactions of factors Xa and IXa on factor VII. These results suggest that a very tight, biphasic control of factor VII activity exists in human plasma, which is modulated mainly by factor Xa. Variation of the factor IX or factor X concentrations permitted kinetic parameters for each activation to be derived. At saturation of factor VIIa/tissue factor, factor IX activation was significantly more rapid than was previously found in bovine plasma under similar conditions. The activation of factor X at saturation was slightly more rapid than in bovine plasma, despite the presence of heparin.

FACTOR VIIa IN THE PRESENCE OF tissue factor can activate two zymogens of the coagulation pathways: factor X and factor IX. The latter reaction, a relative newcomer to formulations of the coagulation scheme, was first demonstrated by Ostertud and Rapaport¹ with proteins purified from human plasma. Its kinetic characterization was then undertaken by Zur and Nemerson,² using the purified bovine proteins, and by ourselves³ in bovine plasma. In both cases, radiometric assays that measure the release of tritiated activation peptides from factor IX² and factor X³ proved very useful, particularly because they are independent of the subsequent inhibition of the proteolytic products, factor Xa and factor IXa, in plasma.

It was important to establish the significance of factor IX activation by this alternative to the activation catalyzed by factor XIa, because it forms a link between the intrinsic and extrinsic pathways. Such a link could help to explain (formally at least) why certain defects of the intrinsic pathway (factor VIII and factor IX deficiencies) cannot be compensated by the extrinsic pathway, whereas defects in other preceding steps are either variable in severity or asymptomatic in vivo. Taking into account the kinetic constants for the activation of bovine factors IX and X by factor VIIa/tissue factor (TF) and the inhibition constants that defined the mutual competition of these substrates, the significance to the generation of bovine factor Xa of this means of factor IX activation seemed to us to be marginal. We proposed that the amount of factor Xa expected from the subsequent activation of factor X by factor IXa/VIII was slight compared to the amount expected in the same time from the direct activation of factor X by factor VIIa/TF.⁴

However, a number of complications and uncertainties cloud the picture. One is that bovine factor VII, if isolated with due regard for its susceptibility to proteolysis, has relatively poor coagulant activity until it has been exposed to traces of one of its products, factor Xa.⁵ In plasma, the kinetic state of affairs before such exposure could be different and crucial. We have recently attempted to eliminate the feedback activation of factor VII by factor Xa and other proteases in a bovine plasma system by the use of a spectrum of inhibitors.⁶ This study suggested that the poor activity of the unactivated enzyme toward one substrate, factor IX, lay in its low turnover number: the apparent $K_m$ values were actually disposed to favor factor IX activation slightly more when factor VII was not activated.
than when it was. However, the comparison with factor X activation could not be made because of the technical difficulty of blocking factor Xa activity completely when it is the reaction product (this article).

A second complication is some uncertainty about the extent to which factor IX activation is amplified in the subsequent factor VIII-dependent reaction of its product, factor IXa, with factor X. Although the reaction, under optimal conditions, appears no less efficient than others,7,8 these conditions (similarly) pertain only after the factor VIII has been exposed to products generated further along in the pathway—to wit, thrombin9 or factor Xa.7 Unactivated factor VIII supports little, if any, factor X activation by factor IXa.8

A third problem, which we approach in the present study, is how far it is warranted to extrapolate from bovine model systems to human hemostasis. Quite a small species difference in the partition of factor VIII(a)/tissue factor between the two substrates in question could change their relative activation rates substantially. In addition, such a shift has been reported to occur even in a bovine system in the presence of a constituent of heparin.9 We have therefore purified human factor IX and factor X and begun to study the activation of their tritium-labeled derivatives in a human plasma model system. The results give a Vmax for factor IX activation that is significantly greater than we and others obtained with the bovine protein. There are also important differences in how the apparent activity of factor VII is influenced by small species difference in the partition of factor IXa.9 Unactivated factor VIII supports little, if any, factor X activation by factor IXa.8

**Materials and Methods**

Materials

Materials for this study were obtained as follows: benzamidine. HCl and cyanogen bromide from Aldrich Chemical Co., Milwaukee, WI; dextran sulfate agaroze from Bethesda Research Laboratories, Bethesda, MD; Biogel A15M and hydroxylapatite from Biorad, Richmond, CA; Sepharose, Sephadex, and Sephacyrl products from Pharmacia Fine Chemicals, Piscataway, NJ; tritiated sodium borohydrate (40-60 Ci/mmmole) from New England Nuclear, Boston, MA; sodium metaperiodate from Mallinkrodt, St. Louis, MO; ammonium sulfate (ultrapure enzyme grade) from Schwarz-Mann, Spring Valley, NY; Freund’s adjuvants, complete and incomplete, from Calbiochem-Behring, La Jolla, CA; Inosithin (soybean phospholipid concentrate) from Associated Concentrates, Woodside, NY; ethylenediaminetetraacetic acid disodium salt (EDTA), Russell’s viper venom (RVV), bovine serum albumin (Type 6, fatty acid-free), II-VII-X-deficient and II-VII-deficient bovine plasmas, imidazole, and disopropylfluorophosphate (DFP) from Sigma Chemical Co., St. Louis, MO; Plateitin Plus and Simplastin from General Diagnostics, Morris Plains, NJ; British Drug Houses standards 1 M CaCl2 solution and sodium dodecyl sulfate from Gallard-Schlesinger, Carle Place, NY; factor XII, factor XI, factor VIII, factor IX, factor VII, and fibrinogen-deficient human plasmas from George King Biomedical, Overland Park, KA. The citrated fibrinogen-deficient plasma (lot no. GK103-7-15-82) had a prothrombin time of greater than 45 min, with other factors (as listed in Table 1) falling within normal limits. In particular, the factor VII assay results were identical to pooled normal plasma, also obtained from George King Biomedical. Other chemicals were the best available grade. Goat antisera to purified factor IX, factor X, antithrombin III, and prothrombin were raised, by Serasource Inc., Berlin, MA, in single animals against antigens supplied by us. The inhibitor 1,2-bis-(5-amidinobenzimidazole)-ethane (BABE) was a generous gift from Dr. J. D. Geratz, University of North Carolina, Chapel Hill, NC.

**Purification of Factors IX and X**

The starting material for most of the factors IX and X used in this study was a sample of prothrombin complex fractionated by anion exchange and generously supplied by Dr. Charles Heldebrant of Alpha Therapeutics, Los Angeles, CA. The method developed for this material consists of barium citrate adsorption/elution and chromatography on (1) DEAE-Sephadex, (2) dextran sulfate-agaroze, (3) antibody columns, and (4) hydroxylapatite. It is given in detail below. In early trials, we found greater Cohn fractions from other sources to be unsatisfactory because of substantial proteolysis having occurred, particularly of factor IX. In contrast, we have subsequently found the method to be suitable for purifications from fresh plasma obtained from patients undergoing plasmapheresis. All procedures were done at 0-4°C.

**Step 1: Barium citrate eluate** Six hundred milliliters of prothrombin complex, containing 104 U each of prothrombin, factor IX, and factor X, was diluted with 10 mM BZA to give a refractive increment against water of 1.7 x 10-3. Sodium citrate was then added to a concentration of 25 mM and the pH adjusted to 7.5. A 50 volume of 1.5 M BaCl2 was added slowly and the suspension stirred for 30 min. The barium citrate was removed by centrifugation at 5,000 g for 20 min and washed twice by blending briefly in 1 liter 5 M BaCl2/10 mM BZA and centrifuging. The protein was eluted by blending the precipitate briefly in 300 ml 35% saturated (NH4)2SO4/10 mM BZA, then stirring for 30 min. After centrifugation at 12,000 g for 20 min, the supernatant was made 65% saturated in (NH4)2SO4 and stirred for 30 min, then centrifuged again. The resulting precipitate was taken up in 100 ml 50 mM imidazole/HCl, pH 7.5, and treated with 10 mM DFP at room temperature for 1 hr. Benzamidine was then added to a concentration of 10 mM, and the solution was titrated to pH 6.0 with HCl and chilled. It was finally desalted on Sephadex G-25 (900-ml column, 300 ml/hr) equilibrated in 5 mM BZA/50 mM imidazole/HCl, pH 6.0.

**Step 2: DEAE-Sephadex.** The material from step 1 was applied directly at 30 ml/hr to a 200-ml column of DEAE-Sephadex A50 equilibrated in the same BZA/imidazole buffer. After washing with 100 ml 0.2 M NaCl/50 mM imidazole/HCl/5 mM BZA, pH 6.0, the column was eluted with a 1 liter gradient, 0.24-0.50 M NaCl in 50 mM imidazole/HCl/5 mM BZA, pH 6.0. Factors IX and X were eluted by clotting assay. Their elution position corresponded closely to that of prothrombin.

**Step 3: Dextran sulfate-agaroze.** The IX/X/prothrombin pool from step 2 was dialeyzed twice against 4 liters 20 mM citrate/NaOH/10 mM BZA, pH 7.5, and applied to an 80-ml column of dextran sulfate-agaroze equilibrated in the same buffer. Under these conditions prothrombin does not bind and can be pooled. At this point, it is >95% pure by gel electrophoresis. Factors IX and X were eluted from the column with a 600-ml gradient, 0-1.0 M NaCl in citrate/BZA, pH 7.5. They were located by assay and absorbance at 280 nm, pooled separately, and each dialyzed against 0.1 M phosphate/NaOH/10 mM BZA, pH 6.8.

Although, by assay, the factor IX pool was free of factor X and vice versa, each was now freed of any remaining traces of the other
by passage through columns of rabbit monospecific antibodies raised against the contaminating factor and coupled to agarose beads (see below). The columns were previously equilibrated in the same phosphate/BZA buffer.

The resulting pools, which still contained traces of prothrombin, were applied to separate 15-ml columns of hydroxylapatite equilibrated in the same buffer. These were eluted as follows: (A) factor IX with a 200-ml gradient, 0.15-0.40 M phosphate/KOH, pH 6.8, in 10 mM BZA; (B) factor X from its column with a 200-ml gradient, 0.10-0.35 M phosphate/KOH, pH 6.8, in 10 mM BZA. Active fractions from each column were analyzed by SDS gel electrophoresis for prothrombin contamination and pooled accordingly. If contamination of the pool by prothrombin was still detectable by assay, an antiprothrombin antibody column was used to remove it.

The factor IX and factor X pools were dialyzed against 2 x 1 liter Tris/saline/l mM EDTA, pH 7.5, assayed, and frozen at -70°C. Factor IX was obtained in a yield of 48 mg with a specific activity of 360 U/mg. The factor X yield was 32 mg, specific activity 200 U/mg. Both proteins were >95% pure by SDS gel electrophoresis, and, by assay, were free of each other, of factor VII, and of prothrombin. Although the specific activities quoted are valid for a particular preparation, we have found some variation, particularly of factor IX, which on occasion has exceeded 400 U/mg. This is, however, definitely not caused by contamination with factor IXa or factor Xa, which are absent by assay.

Factor IXa was made by accident as a failed preparation of factor IX from a poor sample of crude Cohn Fraction III. By SDS-gel electrophoresis it was identical to the two-chain protein produced by activating factor IX with factor Xa. Its activity was determined in a nonactivated partial thromboplastin time (PTT) against "standard" purified factor IXa that had been assayed by titration with bovine antithrombin III of known concentration. Human α-thrombin was prepared by the method of Fenton et al. and had a specific activity of 3,600 U/mg with National Institutes of Health thrombin, Lot J, as the standard. Human Factor Xa was prepared and assayed by methods described elsewhere. A mixture of activated and fragmented human factor XII (1:1) made by the method of Silverberg et al. was provided by Dr. Michael Silverberg. A saline extract of human brain acetone powder was made by the method of Quick. An enriched anticoagulant fraction of porcine heparin was isolated by gel filtration on Sephadex G-100 in 0.2 M ammonium bicarbonate. It had a specific activity of 230 U/mg.

Tritium Labeling of Factors IX and X

We have found the labeling protocol that was used for bovine coagulation factors to be unsuitable for human factor IX and factor X because of unacceptable losses of coagulant activity. Although the reasons have not been fully investigated, the loss appears to be due to a greater sensitivity of the human proteins to both periodate oxidation and, more importantly, reduction by sodium borohydride. The following procedure takes account of these problems, giving products with >90% of their original coagulant activity and with specific activities of 2 x 10^6 cpm/μg for factor X and 2 x 10^5 cpm/μg for factor IX when the freshest tritiated borohydride is used. Factor IX or factor X, 3 mg at a concentration of ~1.5 mg/ml, was dialyzed against 0.1 M NaCl/0.1 M acetate/NaOH, pH 5.8, then oxidized with sodium periodate, 1/100 volume of a freshly prepared 0.2 M solution, for 15 min in ice with stirring. The reaction was terminated by adding 5 μl ethylene glycol and stirring for 5 min. The oxidized protein was dialyzed at 4°C for 6 hr against 2 x 1 liter 0.1 M borate/NaOH, pH 8.5. It was then added to 20 μl 10 mM NaOH containing 100 mM tritiated sodium borohydride (nominally 40-60 Ci/m mole, but supplied as high as 90 Ci/m mole). The highest incorporation of label was obtained when this solution had been stored at ~70°C for less than 1 mo. Labeling was allowed to proceed for 20 min with stirring in ice, then the protein was applied immediately to a Sephadex G25 column (1.5 x 10 cm) leading into a Sephacryl S-200 column (1 x 20 cm), both equilibrated in 0.1 M NaCl/0.05 M Tris, pH 7.5, containing 1 mM EDTA and 0.01% serum albumin. The protein was eluted at 20 ml/hr and located from the peak of radioactivity corresponding to the peak absorbance at 280 nm. After pooling, the specific activity of the product was determined by liquid scintillation counting of replicate, 20-fold diluted samples and from the absorbance of the pool. The protein was then dialyzed against 50% glycerol/NaCl/Tris/1 mM EDTA and the final concentration of factor IX or factor X determined from the isotopic specific activity before dialysis. We find that, by the use of the adjunct S-200 column, traces of low molecular weight contaminants (perhaps labeled activation peptides) are removed, lowering the background (i.e., zero time) radioactivity in plasma rate determinations from ~2% to <0.5% of the total radioactivity.

The use of serum albumin in the column buffer increases the recoveries of starting material to at least 90%; it has the disadvantage that the (unlabeled) carrier remains in the labeled protein.

Preparation of Depleted Plasma

Human plasma was dialyzed, defibrinated, and depleted of factors IX and X, much as already described for bovine plasma. A unit of platelet-poor human plasma, fresh-frozen by the blood bank, was dialyzed overnight against 40 vol of NaCl/Tris/1 mM EDTA, pH 7.4. Fibrinogen was removed by precipitating it by heating at 36°C for 3 min. The plasma was passed through several layers of cheesecloth and then centrifuged for 20 min at 25,000 g to remove remaining insoluble debris and contaminating platelet material. It was then pumped at 5 ml/hr through two 20-ml columns, in series, of antibodies raised against purified human factor IX and factor X, respectively, coupled to agarose beads.

We have used, without apparent differences, antibodies raised in both rabbits and goats. The antibodies were heat-inactivated, then adsorbed with 5% (w/v) BaSO4 3 times. The immunoglobulin fraction was precipitated with ammonium sulfate (35% saturated for rabbit, 45% saturated for goat), then taken up in 1/60 the original volume of 0.2 M bicarbonate/NaOH, pH 8.5, before exhaustive dialysis versus this buffer. Coupling to CNBr-activated agarose beads (Bio gel A15M or Sepharose 4B) was done by the method of Parikh et al. at approximately 20 mg immunoglobulin fraction/ml packed beads. Columns were stripped between each use with a 3 M solution of potassium thiocyanate.

After passage through the antibody columns, the plasma was eluted with NaCl/Tris/1 mM EDTA. Only those fractions of maximum refractive index were included in the final pool to minimize dilution of the plasma. The pool was assayed for all coagulation factors and for antithrombin III (see Results). The plasma was stored in 2-ml portions at ~70°C. A small amount of plasma depleted of 95% of its heparin cofactor activity was prepared using a column of anti-antithrombin III antibodies, in addition to the columns described above. However, the method does not work well for removing this protein, requiring more than one cycle through the column because of its high plasma concentration, with the risk of changes in the coagulation factors (particularly factor VII).

Determination of Activation Rates

All activation mixtures consisted of 80% (v/v) depleted plasma plus additions. Phenomenologic studies of the progress of factor IX or factor X activation were done at 37°C in final volumes of 2 ml, with either labeled protein at 5 μg/ml. Rate determinations for kinetics were done in quadruplicate with 1.0-ml mixtures. Inosithin,
at 20 μg/ml was the phospholipid source. All experiments contained 6 mM CaCl₂, which, allowing for the presence of 1 mM EDTA and other plasma binding sites, gives approximately 3.5 mM free Ca²⁺. Except where noted, reactions were started by addition of the tritium-labeled substrate after a 1-min preincubation of the remaining constituents. Then, samples were withdrawn at the stated intervals into 1 vol 50 mM EDTA/50 mM BZA/NaCl/Tris, pH 7.4, on ice. At the end, each received 1 vol 15% (factor X) or 12% (factor IX) trichloroacetic acid. After centrifugation (8 min, Eppendorf Centrifuge 5413), 4 50-μl samples of supernatant were counted in 4 ml Liquiscint scintillation cocktail. The assays were calibrated as previously described. Complete activation released 48% and 33% of the total counts from factor IX and factor X, respectively.

RESULTS

Characterization of the Depleted Plasma

As in our previous study of bovine plasma, we were at pains to establish that the removal of fibrinogen and factors IX and X, by heating and antibody treatment, respectively, did not cause unacceptable changes in the behavior of the plasma, in particular in the levels of other coagulation factors. The results of a battery of coagulation assays (Table 1) showed that factor V became activated during the overnight dialysis against NaCl/Tris/1 mM EDTA and subsequently lost its activity. Some contact activation of factors XII and XI may also have occurred, although we will show that this does not bear on the behavior of factor IX in the plasma. The activity levels of prothrombin, factor VII, and factor X are somewhat less after dialysis than in the starting plasma. In general, it must be admitted that human plasma proved less robust in this series of treatments than bovine plasma had been, and we occasionally had to discard batches in which factor VII clotting activity had been reduced to less than 20% normal. However, lower factor VII activity does not reflect a loss of the ability to be activated. Thus, the activity ratio measured by coupled amidolytic and clotting assays as previously described. Complete activation released 48% and 33% of the total counts from factor IX and factor X, respectively.

Table 1. Levels of Clotting Factors at Stages of Plasma Preparation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Dialyzed†</th>
<th>Depleted†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VII</td>
<td>0.85</td>
<td>0.55</td>
</tr>
<tr>
<td>Factor X</td>
<td>0.73</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Factor IX</td>
<td>1.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>0.80</td>
<td>0.62</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>1.10</td>
<td>1.20</td>
</tr>
<tr>
<td>Factor XII</td>
<td>1.10</td>
<td>2.10</td>
</tr>
<tr>
<td>Factor XI</td>
<td>1.25</td>
<td>2.70</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>1.65</td>
<td>1.40</td>
</tr>
<tr>
<td>Factor V</td>
<td>&gt;3.00</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

†See Materials and Methods.

Tissue Factor-Dependent Activation of Factor X in Plasma

In the next part of the study we obtained progress curves for the TF-dependent activation of factor X in depleted plasma and compared them with the activation of factor X in plasma congenitally deficient in fibrinogen (so as to avoid clot formation) but otherwise normal. ³H-labeled factor X of high isotopic specific activity (2 x 10⁶ cpm/μg) was added as a tracer at 2 μg/ml to mixtures consisting of 80% plasma, phospholipids, and three levels of crude human brain thromboplastin. The phospholipids were present to provide a background level of this component in the face of varying levels of thromboplastin and to standardize the conditions with later experiments in which preincubations with phospholipids were required. The depleted plasma also received 5 μg/ml each of unlabeled factor IX and factor X so as to restore these factors at approximately their plasma concentrations. When 6 mM Ca²⁺ was added to initiate activation, the generation of labeled activation peptide followed a distinctive, burst-like pattern comprised of (1) a lag of a few seconds, (2) a rapid release of peptide over the next 2 min or so, and (3) a plateau (Fig. 1, A and B). The maximum rate and the extent of activation were both dependent on the TF concentration. It can also be seen that, at high TF, activation in depleted plasma was about as rapid and extensive as in plasma that had not been through heat treatment, dialysis, and affinity chromatography. At lower concentrations of TF, the depleted plasma gave similar lags, but subsequent

![Fig. 1. Progress of tissue factor-dependent factor X activation in human plasma. (A) ³H-factor X (2 μg/ml), 6 mM CaCl₂, 20 μg Inosithin/ml, and 5 μg/ml each of unlabeled factor IX and factor X were added to plasma depleted of factors IX and X. The plasma comprised 80% (v/v) of the final activation mixture, after addition of human brain thromboplastin to initiate activation at 1/3, 1/2, or 1/4 of the total volume. Tritium-labeled activation peptide was extracted into 5% trichloroacetic acid from samples withdrawn at the intervals shown and added to an equal volume of 50 mM BZA/50 mM EDTA/Tris/saline, pH 7.5, to arrest activation. (B) The same experiment done in fibrinogen-deficient plasma containing the same additions, but for factor IX and unlabeled factor X. The curves were drawn by inspection.](image-url)
Plasma hereafter refers to plasma that has been dialyzed, defibrinated, and depleted of its factor IX and factor X, as described in Materials and Methods.

Fig. 2. Effects on subsequent factor X activation of preincubating plasma, pretreated in various ways, with factor Xa. Activation of $^3$H-factor X (5 $\mu$g/ml) was measured as in Fig. 1, after prior treatment of the plasma as follows: (a) 1 $nM$ factor Xa for 1 min in the presence of 6 mM CaCl$_2$ and 20 $\mu$g Inosithin/ml; (b) no pretreatment; (c) plasma incubated overnight with 5 mM diisopropylfluorophosphate, then as in a; (d) (O—O) as in a, except that thromboplastin was omitted; (e) (I—I) as in b, except that thromboplastin was omitted; (f) (A—A) plasma adsorbed with barium sulfate (5% w/v), then as in a. The curves were drawn by inspection.

Activation was rather more extensive. We take these results to indicate that the plasma suffered no loss of factors likely to bear on the present studies. Although we cannot account for a greater responsiveness to tissue factor, one possible explanation is that both forms of plasma were from single donors and subject to some variation. These results are shown as percentage activation, partly as the assay could not be calibrated in the usual manner because of the presence of unlabeled factor X and also to illustrate that activation did not go to completion, even at the highest concentration of thromboplastin, despite the rapid rate at maximum.

A series of controls was done to find out whether peptide release depended exclusively on TF-dependent factor VII activity and also whether the factor Xa-dependent feedback component predicted from studies of purified human Factor VII$^{18}$ was significant in plasma. When plasma* containing 5 $\mu$g $^3$H-factor X/ml (2.7 $\times$ 10$^5$ cpm/$\mu$g) was preincubated with 1 $nM$ factor Xa (45 ng/ml) for 1 min in the presence of Ca$^{2+}$ and phospholipids, and TF was then added, the lag was abolished, but the plateau persisted (Fig. 2A). In contrast, when no factor Xa was added before initiation with tissue factor (Fig. 2B), activation resembled the corresponding (middle) curve of Fig. 1A. Only preincubation with factor Xa in the presence of a phospholipid source and Ca$^{2+}$ abolished the lag. After partial inhibition of the plasma factor VII by incubation overnight with 5 mM DFP, then pretreatment with factor Xa as described above, peptide release was much reduced and linear (Fig. 2C). The omission of TF either with (O—O) or without (●—●) added factor Xa, or the removal of all the vitamin K-dependent proteins with BaSO$_4$ before the addition of $^3$H-factor X and 1 $nM$ factor Xa (A—A), resulted in essentially no peptide release (Fig. 2, D, E, F). From these results we can conclude that (1) the proteolysis of factor X requires both TF and factor VII (poorly inhibited by DFP in the human case$^{18}$); (2) no release of radioactive peptide resulted from proteolysis of factor X by factor Xa; (3) the lag before activation may reflect a period during which factor VII is being activated by the factor Xa being formed. More data bearing on the last point appear below.

We also investigated a possible role for factor Xa in the premature cessation of activation. When another 0.05 vol of brain thromboplastin was added at the plateau (2.25 min in Fig. 2A) no further activation occurred (data not shown). Therefore, the loss of factor VII activity implied by the plateau is irreversible. Likewise, the addition of 1 $nM$ factor Xa at the same point had no effect, discounting the possibility that there was a pool of potentially reactive factor VII remaining in the plasma at this point. In plasma to which factors IX and X had been restored, as in Fig. 1A, the activation of factor X by Russell's viper venom over a wide range of concentrations gave linear progress curves with neither lags nor plateaux (Fig. 3). This was the case even at a concentration exceeding that of factors IX and X, which was intended to drive factor X activation as fast and as far as possible (O—O). Activation begins at the same rapid rate as was seen with high tissue factor (Fig. 1A, top curve)—$\sim$20%/min—but progressed 3 times further in 5 min, with little downward curvature.

These data provide evidence that both the delayed onset and the cessation of activation are peculiar to the

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*"Plasma" hereafter refers to plasma that has been dialyzed, defibrinated, and depleted of its factor IX and factor X, as described in Materials and Methods.
reaction catalyzed by factor VII(a)/tissue factor. Hence, the shape of the TF-dependent curves cannot be ascribed to an effect on, for example, the factor X. (This was a potential artifact because of the finding of Mertens and Bertina that the activation of human factor X is accompanied by an autocatalytic bond cleavage in which the active site serine is removed on a peptide of mol wt 13,000.)

Experiments were also done in which inhibitors of factor Xa were included in TF activations, on the grounds that these should interfere with any modulation of factor VII activity by factor Xa. The inhibitors used were heparin at 5 U/ml and/or 1.2-bis-(5-amidinobenzimidazole)-ethane (BABE) at 100 μM. The latter inhibits factor Xa reversibly (K_i ~ 3 μM) without a significant effect on factor VII(a) (K_i >200 μM). Figure 4 shows the progress curves obtained with heparin alone (BABE alone had identical effects, not shown) and with heparin and BABE together. The effect in either case was a slowing down of peptide release and abolition of the plateau; each phenomenon was more noticeable when both inhibitors were used. However, as in bovine plasma, it proved impossible to suppress factor X activation altogether by interfering with the putative feedback reactions of factor Xa upon factor VII.

Activation of Factor IX

Because of the difficulty of establishing the exact role of factor Xa in a system in which it is being rapidly and extensively formed, we turned to a similar examination of factor IX activation. Here, there is no major source of factor Xa, permitting the level of this enzyme to be controlled with more certainty. Upon addition of a 1/20 vol of thromboplastin to plasma containing 3H-factor IX (8 × 10^5 cpm/μg) at 5 μg/ml, the progress curve comprised a lag of ~2 min, followed by an upwardly curving segment, reaching ~5% activation in 5 min (Fig. 5A). This lag was longer if the plasma had been passed twice through the anti-factor X-Sepharose column, implying some role for traces of factor X(a) in the subsequent acceleration of factor IX activation. This was therefore the method used to prepare the plasma for initial experiments. Preincubation of the plasma with the amount of factor Xa that had abolished the lag before factor X activation (1 nM) showed a similar effect here; however, the ensuing activation was rapid and linear beyond 5 min, reaching ~10% in that time with no plateau (Fig. 5B). No peptide was released either when 1 nM factor Xa (Fig. 5C) or 50 nM factor Xa (data not shown) was present but thromboplastin was omitted, or after DFP treatment. Nor was peptide released after barium sulfate adsorption of the vitamin K-dependent proteins (Fig. 5, D and E). Therefore, peptide release from factor IX in the presence of factor Xa is not due to a direct effect of this protease on factor IX, nor can it result from the activation of factor IX by factor Xa. Rather, it is completely TF-dependent, thus reflecting the expression of factor VII activity augmented by a trace of factor Xa (1 nM or less).

In the linear activation of factor IX achieved with factor Xa, and the accelerating activation seen without added proteases, an anticoagulant fraction of heparin at 5 U/ml was now almost completely inhibitory (Fig. 6A), reducing the rate from ~2.5 to less than 0.1 nM/min. In the light of a report that a fraction of heparin changes the specificity of factor VIIa/tissue factor for its two substrates, we also tested the effects of the same fraction of heparin on factor IX activation in plasma that had been depleted of its antithrombin III (see Materials and Methods). Although heparin cofactor activity was reduced to 5% of normal levels

![Fig. 4. Effects of heparin and a specific inhibitor of factor Xa on the tissue factor-dependent activation of factor X in plasma. Activation was measured after incubation of the plasma with no inhibitor ( ), with purified heparin (5 U/ml, 230 U/mg) ( ), or with heparin plus BABE (100 μM) ( ). The plasma contained 3H-factor X (1 μg/ml), 6 mM CaCl_2, 20 μg Inosithin/ml, and 1/20 volume thromboplastin. The curves were drawn by inspection.](image-url)
These results suggest that most of the inhibitory effect of heparin is exerted through antithrombin III, and (2) the reactivity of factor VII toward thrombin, factor IXa, or factor XIIa was too low to detect by clotting assay in either case. This is consistent with some increase in reactivity of factor VII toward factor IX. We were not able to pursue this, however, for lack of sufficient plasma depleted of antithrombin III (see Materials and Methods).

We also investigated the origin of the accelerating peptide release seen in the absence of added factor X(a). From what is known of other proteases that can activate factor VII, we supposed that upward curvature might result from the parallel generation in the plasma of thrombin, factor IXa, or factor XIIa, as well as from very small amounts of factor X(a). We therefore tested the potency of these enzymes in promoting factor IX activation by preincubating the plasma with 0.2 nM factor Xa alone or with 0.2 nM factor Xa and heparin at 5 U/ml or 50 U/ml (Δ-Δ). The curves were drawn by inspection.

A 1:1 mixture of factors XIIa and XIIla was without effect at either 1 nM or 50 nM (Fig. 7C). A comparison of Fig. 7A with 7D shows that preincubation with 50 nM factor IXa was required to obtain the same enhancement of factor IX activation as was obtained with 0.2 nM factor Xa. However, for factor Xa, there is a superimposed phenomenon—that at concentrations at which factor IXa was too low to detect by clotting assay in either case. This is combined with a unique effect of factor Xa among the proteases tested: at higher concentrations it also, directly or indirectly, causes the loss of factor VII activity.
Kinetic Constants for Factor IX Activation

The foregoing results and those from our previous studies of the bovine system$^{1,6}$ led us to make the determination of kinetic parameters for factor IX activation after a 1-min preincubation of the plasma with 0.2 nM factor Xa in the presence of phospholipids and Ca$^2+$. The progress curves were thereby made linear at all the factor IX concentrations used, and we assume this to represent the activity of factor VIIa exclusively. The dependence of activation rate on factor IX at 8 concentrations between 1.5 and 52 μg/ml (0.03 and 0.97 μM) is shown in Fig. 8A, and the constants derived are in Table 2.

Kinetic Constants for Factor X Activation

The self-suppression of tissue factor-dependent factor X activation in the simple plasma system meant that a reaction rate was not readily obtainable and would have been of questionable validity. To obtain rates at all, we were forced to use heparin to prevent the rapid damping of activation that was apparent at all concentrations of thromboplastin (Fig. 1A), notwithstanding that heparin clearly also slows activation. With heparin at 5 U/ml, rates were measured 4 times each at 8 concentrations between 1.3 and 39 μg/ml (0.024 and 0.71 μM). Slopes were obtained by nonweighted linear regression. The relation of rate to factor X, fitted to a rectangular hyperbola (Fig. 8B), gave values for apparent $K_m$ and $V_{max}$ that appear in Table 2.

Table 2. Kinetic Constants for the Activation of Factor IX and Factor X in Plasma

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM) ± SD</th>
<th>$V_{max}$ (nM/min unit thromboplastin) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Factor X†</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Factor IX</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>Bovine</td>
<td>Factor X†</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Factor IX</td>
<td>0.073</td>
</tr>
</tbody>
</table>

†Maximum velocities are normalized to undiluted brain thromboplastin.
‡Apparent constants obtained in the presence of heparin (5 U/ml).
§Bovine constants taken from reference 3.

DISCUSSION

This study investigates some kinetic aspects of the activation of factor IX and factor X by TF-factor VII(a) in human plasma. The results of a survey of proteases expected to enhance the activity of factor VII toward these substrates are consistent with others in the literature. For example, the work of Seligsohn et al. suggested that factor IXa would be capable of potentiating its own formation when catalyzed by tissue factor-factor VII,$^{2}$ and Radcliffe and Nemerson compared the effects of thrombin and factor Xa in stimulating the activity of purified bovine factor VII.$^{4}$ A comparable effect of factor Xa on human factor VII, also concomitant with proteolytic cleavage, has subsequently been demonstrated by Broze and Majerus$^{18}$ and Bajaj et al.,$^{23}$ also in experiments using purified proteins. The results reported here permit a quantitative comparison of the net effects of these enzymes in plasma, i.e., in the presence of their plasma inhibitors (chiefly antithrombin III). We find the potency of factor IXa during a 1-min preincubation with plasma to be about 250-fold less than factor Xa on a molar basis. On the other hand, the same rate is eventually achieved when factor IXa is being generated from the plasma concentration of factor IX, although the accel-
eration may result partly from the effects of traces of factor Xa. This rather good agreement with the 800-fold difference in effectiveness between factor IXa and factor Xa recently reported for the activation of purified human Factor VII \(^{24}\) implies that, in fact, plasma inhibition of these two enzymes plays rather little role in modulating their feedback activity in the time covered. In contrast to its effect on purified bovine factor VII, \(^{4}\) thrombin did not enhance factor IX activation in human plasma; rather, thrombin showed a slight negative effect when present at a high concentration equivalent to \(\text{~15\%~activation~of~the~plasma~level~of~prothrombin.~(It~should~be~noted~that~little~thrombin~}
\)

can form endogenously in our system because of the lack of factor V, but the results also show that its effects in this area of coagulation are negligible anyway.) Also surprising was the lack of effect of \(4 \mu g\) factor XIIa/f/ml (in the absence of kaolin or a similar surface) over a short time. Although factor XIIa/f can lead to the activation of both bovine \(^{21}\) and human \(^{22}\) factor VII, Radcliffe et al. \(^{21}\) found that it required repeated additions of this enzyme over a period of hours to activate factor VII maximally in human plasma. Hence, the net effectiveness of these proteases in stimulating TF-dependent activation of factor IX in plasma can be ranked: Xa \(\gg\) IXa \(\gg\) thrombin \(\sim\) factor XIIa/f. Interestingly, both of the enzymes that were effective activators of factor VII bind to phospholipid, where they may be protected from inhibition by antithrombin III. \(^{25,26}\) In contrast, the two enzymes that were ineffective, despite their reactivity in a buffer system, do not have particular affinity for membranes. Also, if we take the size of the heparin effect on the maximum rate of activation of factor IX as indicating only stimulation by Xa, and allow for \(\sim\) 50\% inhibition of tissue factor-factor VIIa activity by heparin, \(^{18}\) the maximum enhancement of factor VII activity was at least 15-fold in plasma. This relatively close agreement with the \(\sim\) 20-fold enhancement obtained by others with purified proteins argues against factor VII having been activated substantially during processing of the plasma, as do the normal results for coupled amidolytic and clotting assays of factor VII. \(^{16}\)

The tight negative control that factor Xa exerts over its own generation and that of factor IXa was first suspected from the burst-like nature of the tissue factor-dependent activation of factor IX and was confirmed in experiments that showed almost complete abolition of factor IX activation by \(5 nM\) factor Xa, equivalent to \(\sim\) 5\% activation of plasma factor X. The failure of factor IX and factor X activation to proceed beyond \(\sim\) 30\% was also shown by Osterud and Rappaport \(^{27}\) at TF levels comparable to or higher than those used here. We have given circumstantial evidence that this represents an effect of factor Xa on human factor VII(a). The level of factor Xa added or produced, although low, is still at least 1 mole for every 2 of factor VII—a considerably higher ratio than was used in studies of the activation of purified factor VII. \(^{18,23}\) Thus, inactivation could have escaped detection in these investigations. In fact, we cannot tell whether factor Xa acts directly on factor VII(a) or requires other enzymes and zymogens not addressed in the survey of proteases and inhibitors, although the data mean that thrombin, factor IXa, factor X(a), and factor XII can be discounted from playing a major role. Although we cannot establish the nature of the destructive cleavage of factor VII, we note that an early preparation from serum showed a lower specific activity of factor VII and considerable fragmentation beyond its two-chain, most reactive form. \(^{28}\)

The self-limiting phenomenon was much more pronounced than in bovine plasma, \(^{3,6,28}\) and it has precluded kinetic studies in such detail. Thus, we did not study the inhibition of factor X activation by factor IX or vice versa, because the generation of Xa beyond trace levels prevented further expression of TF/factor VII(a) activity. A detailed comparison of the kinetic behavior of factors IX and X relative to one another is therefore not warranted, if only because of the need for an inhibitor of factor Xa in factor X activation, but its contraindication in factor IX activation. However, a worthwhile comparison with the bovine system can be made. The maximum rate of factor IX activation at saturation of TF/VIIa (under conditions where TF is limiting) is sixfold higher than in bovine plasma under similar conditions (Table 2). This finding confirms our suspicion that the bovine system was not a good model in this instance and complements a recent report \(^{10}\) that a significant part of the total activation of radiolabeled factor X in normal human plasma is absent in either factor IX- or factor VIII-deficient plasma when dilute thromboplastin is the initiator. The constants for factor IX activation are in tolerable, but not absolute, agreement with those that Bajaj et al. \(^{21}\) measured in a purified system, in that the value of \(K_m\) they obtained (0.25 \(\mu M\)) is within a factor of 2 of the present one. (It should be noted that the peculiar effects of phospholipid concentration upon the apparent \(K_m\) reported by Zur and Nemerson \(^{5}\) restrict the usefulness of this kinetic constant in defining a reaction that is occurring in the presence of an undefined level of phospholipid.) However, there is a greater discrepancy in \(V_{max}\), as the present value (obtained at nonsaturating thromboplastin) gives a \(k_{cat}\) of at least 68 min \(^{-1}\) rather than 13 min, if we assume for factor VII(a) a plasma concentration of 0.5 \(\mu g/ml\) or 1 \(nM.\) \(^{23}\) The origin of this difference between a purified system and plasma is unknown,
although variations in phospholipid or thromboplastin concentration may again provide an explanation.

We may also suppose that factor X activation at saturation is more rapid than bovine plasma because, even in the presence of heparin, the $V_{\text{max}}$ for factor X is not significantly different, but the activation of factor IX at its plasma concentration is rate-limiting to a greater degree through its $K_{\text{m}}$ being somewhat higher. Ignoring the presence of heparin in one activation, the similar $K_{\text{m}}$ values for factor IX and factor X suggest almost equal partitioning of these substrates in human plasma, whereas the similar values of $V_{\text{max}}$ imply equally rapid turnover at the active site of factor VIIa. This agrees with the previous observation of Osterud and Rapaport that the activations of IX and X were about equally favored in plasma at several tissue factor concentrations.  

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REFERENCES


Tissue factor-dependent activation of tritium-labeled factor IX and factor X in human plasma

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