Activation of Factor VIII by Factor IXa

By Margaret E. Rick

Thrombin causes an increase in factor VIII coagulant (VIII:C) activity, which is followed by a decay of VIII:C activity to below baseline levels. It has been suggested that a similar interaction of trace amounts of thrombin and factor VIII is a necessary prerequisite before factor VIII can participate in the coagulation cascade. In the current study, factor IXa, a serine protease with structural similarities to thrombin, is shown to cause an increase and subsequent fall in VIII:C in a manner qualitatively similar to the reaction with thrombin. The reaction is inhibited by a human inhibitor to factor IX and the interaction appears to involve only VIII:C, since factor-VIII-related protein (VIII:RP) is not changed on polyacrylamide gel electrophoresis (PAGE) or radioimmunooassay during the reaction. Phospholipid increases the activation of factor VIII by factor IXa, and high concentrations of diisopropylfluorophosphate and hirudin inhibit the reaction. The physiologic significance of the interaction of factor IXa with factor VIII is not entirely clear since the concentration of factor IXa needed for activation is much greater than the concentration of thrombin required for similar activation of factor VIII. Factor IXa is most likely to play a role in the intrinsic cascade acting as an initial activator of factor VIII, since factor IXa precedes thrombin in this clotting sequence. In addition, factor IXa may be important wherever relatively high local concentrations of factor IXa and factor VIII occur, particularly in the presence of phospholipid, which may serve to localize the coagulation factors.

Factor VIII is thought to be a complex of two proteins that circulate together in plasma: factor VIII procoagulant (VIII:C) and factor-VIII-related protein (VIII:RP). Factor VIII:C functions as a regulatory protein in the intrinsic coagulation cascade, apparently acting as a cofactor with factor IXa, calcium ions, and phospholipid to activate factor X.23 The VIII:RP has a separate role, functioning in the formation of the primary hemostatic plug by promoting the adherence of platelets to exposed subendothelium (von Willebrand factor activity).45

The precise mechanism by which VIII:C performs its cofactor function is not understood; however, several investigations have suggested that the molecule must be altered ("activated") by thrombin or other proteolytic enzymes before it can participate in the reaction. Factor VIII:C is exquisitely sensitive to very low concentrations of thrombin. This reaction has been studied in both plasma and purified systems,6,8-10 and the investigations have shown that VIII:C activity is initially enhanced and then inactivated following its interaction with thrombin, thus allowing VIII:C to exert both a positive and negative regulation. However, thrombin is not generated until after the activation of factor X in the intrinsic pathway. In addition, thrombin and factor IXa are both serine proteases that have structural similarities,1 suggesting that possibly factor IXa might share the ability to activate VIII:C. Factor IXa would also be in a reasonable position to react with VIII:C, since the formation of a complex between VIII:C, factor IXa, calcium, and phospholipid might bring together relatively high local concentrations of VIII:C and factor IXa. We have therefore examined the effects of the reaction of factor IXa with factor VIII using a system comprised of purified coagulation factors.

These studies show that relatively high concentrations of factor IXa do indeed enhance VIII:C activity in a reaction pattern similar to that seen with thrombin, demonstrating an initial increase in VIII:C activity followed by a decrease in the activity to below baseline levels. The reaction does not affect VIII:RP as judged by PAGE and radioimmunooassay. The effects of phospholipid, calcium ions, and several inhibitors of serine proteases have also been evaluated with regard to the interaction of factor IXa and factor VIII.

MATERIALS AND METHODS

Sephadex G-150, QAE Sephadex, and Sepharose CL-4B were purchased from Pharmacia (Piscataway, N.J.) and Boehringer ASm (6% agarose) from Bio Rad (Richmond, Calif.). Human a-thrombin was a gift of Dr. John W. Fenton, II, Division of Laboratories and Research, N.Y. State Department of Health, Albany, N.Y. Hirudin was obtained from Pentapharm Ltd., Basle, Switzerland. Soybean trypsin inhibitor (SBTI), diisopropylfluorophosphate (DFP), benzamidine, and phosphatidyl serine ( Folch fraction III, bovine brain) were purchased from Sigma Chemical Co. (St. Louis, Mo.); Russell's viper venom from Calbiochem-Behring (La Jolla, Calif.); aprotinin from Boehringer Mannheim (Indianapolis, Ind.); and bovine albumin, fraction V, RIA grade, from U.S. Biochemical Corp. (Cleveland, Ohio). Phosphatidyl ethanolamine (egg) was obtained from P-L Biochemicals (Milwaukee, Wisc.). Factor-VIII and factor-IX-deficient substrate plasmas were obtained from George King Biomedical, Inc. (Overland Park, Kans.).

Factor VIII Purification

Factor VIII was purified from commercially available concentrates (Hyland Laboratories, Costa Mesa, Calif.) by modifications of the method of Shapiro et al.11 The basic procedure included dissolution of the factor VIII concentrate in deionized water, acidifi-
cation to pH 6.5 with citric acid, and addition of 0.5 M sodium citrate, to a final concentration of 0.02 M sodium citrate, precipitation by 5% (w/v) polyethylene glycol (PEG) 4000 with removal of the precipitate, and precipitation of the remaining supernatant by addition of PEG 4000 (w/v) to a final concentration of 12%. The final precipitate was dissolved in veronal-buffered saline (VBS, 0.015 M barbital, 0.010 M Na barbital, 0.125 M NaCl, pH 7.5) and chromatographed in the same buffer on 6% agarose. Void volume fractions containing factor VIII activity were pooled and concentrated by dialysis against 35% (NH₄)₂SO₄ for 16 hr at 4°C. The precipitate was collected and brought up in 1/10 the original volume in VBS. A typical preparation contained 32 U/ml VIII:C activity, 68 U/ml VIII:RP by RIA, and 53 U/ml von Willebrand factor activity; the protein concentration was 0.9 mg/ml. Assessment by SDS-PAGE showed that no protein entered the gel under nonreducing conditions and that a major single band of approximately 200,000 daltons appeared after reduction of disulfide bands; bands of lower molecular weight, constituting less than 8% of the protein, were seen under reducing conditions.

**VIII:C Preparation**

VIII:C was isolated by gel filtration chromatography in the same buffer on 6% agarose. Void volume fractions containing VIII:C activity were collected and brought up in 1/10 the original volume in VBS. A typical preparation contained 32 U/ml VIII:C activity, 68 U/ml VIII:RP by RIA, and 53 U/ml von Willebrand factor activity; the protein concentration was 0.9 mg/ml. Assessment by SDS-PAGE showed that no protein entered the gel under nonreducing conditions and that a major single band of approximately 200,000 daltons appeared after reduction of disulfide bands; bands of lower molecular weight, constituting less than 8% of the protein, were seen under reducing conditions.

**Factor IXa Preparation**

Purified bovine factor IXa was the gift of Dr. Walter Kisiel, Seattle, Wash., and was purified according to the method of Fujikawa et al., except that the final gel filtration step was omitted. The IgG fraction formed a precipitin line with purified factor VIII (0.8 mg/ml) at a dilution of 1/16 (immunodiffusion) and had an inhibitory titer of 8 Bethesda units. This IgG fraction was coupled to crosslinked agarose.

VIII:C was prepared from 200 ml of fresh frozen citrated plasma using 50 ml of the antibody beads according to the method of Hoyer et al., except that the final gel filtration step was omitted. Briefly, 2 mM DFP was added to the thawed plasma, which was then applied to the column. After extensive washing to reduce the OD 280 readings to less than 0.02 nm, the VIII:C was eluted with 0.24 M CaCl₂/0.05 Mimidazole buffer, pH 7.4, containing 0.1 mg/ml bovine serum albumin and 2 mM DFP. Fractions containing VIII:C activity were concentrated with 25% (w/v) PEG 6000 with stirring for 30 min at 4°C; after centrifugation, the precipitate was brought up in 1/10 the original volume in VBS and dialyzed against 1000 vol VBS/2 mM DFP at 4°C for 10 hr to remove residual calcium ions and then against VBS for 5 hr at 4°C. The resulting VIII:C activity was 1.8 U/ml. VIII:RP measured by radioimmunoassay was 0.01 U/ml.

**Activation of Factor VIII by Factor IXa**

Purified factor VIII or the isolated VIII:C was mixed with 1/10 volume of factor IXa. The sample was quickly mixed, and a subsample was removed for dilution in VBS and factor VIII assay. The remaining sample was placed at 37°C (or other designated temperature), and further subsamples were removed at various time points for factor VIII assay. The factor IXa was used at the concentrations noted in the results section, which were such that, after dilution for the factor VIII assay, they did not shorten the clotting time of the assay more than a 1/500 dilution of PNP. Varying factor VIII concentrations were also used. In experiments where phospholipid was added, 1/20 volume of phospholipid was mixed with the factor VIII prior to factor IXa addition; alternatively, an appropriate quantity was added to factor IXa so that the same final concentration of phospholipid would be present in the incubation mixtures. VBS was substituted for phospholipid in control incubations with each experiment. The final concentrations of phospholipid were approximately 0.3-0.4 mg/ml and 0.03-0.04 mg/ml. The phospholipid was prepared by mixing 50 mg phosphatidyl ethanolamine and 22 mg of phosphatidyl serine in 0.05 M Tris, 0.15 M NaCl, pH 7.4, and sonicating at 4°C in 30-sec bursts until uniformly dispersed; the mixture was diluted in the same buffer. In experiments where Ca²⁺ was added, an appropriate volume of 0.3 M or 1.0 M CaCl₂ was added to the factor VIII prior to factor IXa addition; the volumes of Ca²⁺ ranged from 1/10 to 1/100 of the factor VIII volume. In experiments where both Ca²⁺ and phospholipid were added, the Ca²⁺ was added to the factor VIII and the phospholipid was added either to the factor IXa or the factor VIII in the concentrations and volumes noted above.

In experiments where radioimmunoassays of PAGE were to be performed, aliquots of the factor IXa-factor VIII incubation mixture were removed at each time point, snap frozen, and stored at -70°C until assay. Agglutination assays were performed at the usual time points during the experiment.

**Anti-Factor IX: Effect on Factor IXa-Factor VIII Interaction**

A human inhibitor of factor IX (George King Biomedical) with 110 Bethesda units (against human factor IX) was added to an equal volume of bovine factor IXa (0.013 mg/ml) and incubated for 8 min at 37°C prior to incubation with factor VIII (0.5 U/ml). In this experiment, equal volumes (rather than 1/10 volume) of the factor IXa-antibody or factor IXa-VBS control mixtures were added to factor VIII; serial time points were assayed for both the antibody and control (VBS) tubes. In order to verify the specificity of the factor IX antibody, a control experiment was performed in which...
thrombin (0.4 U/ml) was substituted for factor IXa in the above experiment.

Inhibitors of Serine Proteases: Effect on Factor IXa–Factor VIII Interaction

The effects of DFP, soybean trypsin inhibitor, benzamidine, and hirudin were assessed by preincubating 1/10 volume of each inhibitor with factor IXa for 10 min at room temperature prior to addition of the factor IXa to factor VIII. Final concentrations of reactants are shown in Table 2. In the case of DFP, the DFP–factor-IXa mixture was subsequently dialyzed for 2 hr at room temperature prior to addition to factor VIII. For control incubations, VBS was added to factor IXa. The concentrations of inhibitors used did not interfere with the factor VIII assay after a final dilution of the factor IXa–inhibitor–factor VIII mixture was made for the assay.

Sequential Activation of Factor VIII by Factor IXa and Thrombin

Factor VIII (1 U/ml) was activated with factor IXa (0.026 mg/ml) at 37°C and factor VIII was assayed at subsequent time points until peak activation had occurred. Thrombin (0.1 U/ml final concentration) was then added in 1/10 volume to the reaction mixture, which was vortexed, and at 30 sec and each subsequent 3 min, aliquots were taken for dilution and factor VIII assay. In a separate experiment, factor VIII (1 U/ml) was initially activated with thrombin (0.05 U/ml) and after peak activation had occurred, factor IXa (0.026 mg/ml) was added and factor VIII assays were performed at the same time points noted above. In a third experiment, factor VIII was initially activated by factor IXa under the same conditions as above, then a second aliquot of factor IXa was added and factor VIII was assayed at subsequent time points.

Assays

Factor VIII coagulant assays were performed as previously reported, except that platelet plus activator (General Diagnostics, Morris Plains, N.J.) was substituted for kaolin-gliddex. Factor VIII:RP and VIII:C are measured by radioimmunoassay according to the methods of Hoyer, Lazarchick and Hoyer, respectively. von Willebrand factor was measured using a formaldehyde-fixed platelet assay. Factor IX was assayed using a one-stage method similar to the factor VIII assay, except that factor IX substrate was substituted for factor VIII substrate. Factor IXa was assayed in the same way, except that platelet without activator was used, the incubation of substrate and platelet was shortened to 2 min, and the test was performed in 12 x 75 mm polystyrene tubes. SDS-PAGE was performed by the method of Weber and Osborn. Proteins were measured using the Lowry method with ovalbumin as standard.

RESULTS

Activation of Factor VIII by Factor IXa

The general pattern of activation followed by decay of factor VIII activity to below baseline levels is seen in Fig. 1. When the factor VIII concentration is held constant, and factor IXa concentrations are varied, progressively less activation is seen with lower levels of factor IXa. In addition, at the lowest concentration of factor IXa shown, the peak activity achieved is delayed. The decay of factor VIII activity also varies with the factor IXa concentration; the T½ for decay decreases with lower factor IXa concentrations (Table 1). At even lower concentrations of factor IXa (not shown), the decay is slower and the activation phase is not seen.

When the factor IXa concentration is held constant and the factor VIII concentration is varied, relatively less absolute activation occurs as the factor VIII concentration is raised (Fig. 2). With factor VIII at 10 U/ml, an activation of only 2.8-fold over baseline is achieved, while at factor VIII concentrations of 5 and 2.5 U/ml, increases of 4-fold and 5.7-fold over baseline are seen. Also, the peak of activation is delayed at the highest factor VIII concentration.

The effect of temperature is seen in Fig. 3. At 37°C and room temperature, the peak of activation occurs at 3 min and 6 min, respectively, while at 4°C, the
FACTOR IXa ACTIVATION OF FACTOR VIII

Table 1. Inactivation of Factor VIII: Varying Concentrations of Factor IXa

<table>
<thead>
<tr>
<th>Factor IXa Concentration</th>
<th>T% Decay of Factor VIII</th>
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<tbody>
<tr>
<td>0.133 mg/ml</td>
<td>4 min</td>
</tr>
<tr>
<td>0.065 mg/ml</td>
<td>8 min</td>
</tr>
<tr>
<td>0.026 mg/ml</td>
<td>11 min</td>
</tr>
<tr>
<td>0.016 mg/ml</td>
<td>20 min</td>
</tr>
</tbody>
</table>

The decay of factor VIII (5 U/ml) after incubation with varying concentrations of factor IXa is noted.

activation is prolonged to 24–36 min. The extent of activation is also decreased at room temperature and 4°C, and the decay is markedly prolonged at 4°C.

SDS-PAGE of samples obtained during the time course of activation and inactivation of factor VIII (5 U/ml) by factor IXa (0.13 mg/ml) showed no detectable change in the migration of either protein under nonreducing or reducing conditions. Likewise, radioimmunoassay of VIII: RP showed no detectable change during the time course. Radioimmunoassay for VIII:C Ag showed a very slight decrease to 76% of the original baseline value over a 24-min time course.

When isolated VIII:C is incubated with factor IXa a similar pattern of activation and inactivation of factor VIII activity is seen (Fig. 4).

No activation of factor VIII was seen when the control protein (BSA), which had been incubated with the RVV-X beads, was mixed with factor VIII.

Effect of Phospholipid and Ca++

Addition of 5 or 10 mM Ca++ to the reaction mixture does not change the time course or extent of activation of factor VIII by factor IXa. However, 25 or 50 mM Ca++ decreases the magnitude of activation and delays both the peak time point (36 min) and decay of factor VIII activity. One-hundred millimolar Ca++ completely abolishes the reaction.

When phospholipid (0.3–0.4 mg/ml) is added to factor VIII, the extent of activation by factor IXa is enhanced twofold over the peak value for control activations without phospholipid (average of 4 experiments); at a concentration of 0.03–0.04 mg/ml of phospholipid, the extent of activation is 1.6 times baseline. When phospholipid is added to factor IXa prior to addition to factor VIII, the peak factor VIII activity is also double that of the control incubations, but the peak time point is delayed to 12 min (versus 3 min for control or phospholipid-factor-VIII experiments).

Addition of both Ca++ (5 or 10 mM) and phospholipid to the reaction components gives results similar to that seen with phospholipid additions alone, whether added to factor VIII or to factor IXa.

Sequential Activation of Factor VIII by Factor IXa and Thrombin

When factor VIII is activated by factor IXa, the subsequent addition of thrombin produces no further increase in factor VIII activity (Fig. 5A). Similarly, when factor VIII is initially activated by thrombin, the later addition of factor IXa does not increase factor VIII activity (Fig. 5B). Also, a second addition of factor IXa (after an initial activation by factor IXa) has no effect on factor VIII activity (Fig. 5C).
Anti-Factor IX: Effect on Factor IXa–Factor VIII Interaction

When antibody to human factor IX is preincubated with bovine factor IXa, the activation of factor VIII by factor IXa is abolished (Fig. 6). This experiment gave similar results when repeated on two occasions. Lower concentrations of both factor IXa and factor VIII were utilized because of the limited cross-reactivity of the anti-human factor IX. The control experiment in which the factor IX antibody was preincubated with thrombin and then the thrombin–antibody mixture was added to factor VIII showed no inhibition of the thrombin activation of factor VIII. The factor IX antibody thus is specific for factor IX and does not cross-react with thrombin.

Inhibitors of Serine Proteases: Effect on Factor IXa–Factor VIII Interaction

Preincubation of factor IXa with benzamidine (20 mM) and SBTI (20 mg/ml) does not inhibit the activation of factor VIII by factor IXa (Table 2). However, 10 mM DFP does abolish the activation. Hirudin does not interfere with the activation of factor VIII when 4.5 U/ml hirudin is preincubated with 1.33 mg/ml factor IXa, but partial inhibition is seen when 0.5 U/ml hirudin is added to 0.06 mg/ml factor IXa. In this experiment, peak activation occurred at 18 min (versus 6 min in the control) and the inactivation of factor VIII was delayed. Further dose response was not investigated.

DISCUSSION

Previous studies have indicated that factor VIII acts as a cofactor with Ca$^{2+}$ and phospholipid during the activation of factor X by factor IXa,$^{39,23}$ and recent studies using highly purified coagulation factors have confirmed this concept, showing that the $V_{max}$ for the reaction is markedly enhanced by factor VIII.$^{24}$ Most investigations have indicated that factor VIII itself does not have intrinsic enzyme activity, though Vehar and Davie have shown inhibition of thrombin-activated factor VIII by DFP implying that the activity of the
FACTOR IXa ACTIVATION OF FACTOR VIII

Fig. 5. Sequential activation of factor VIII by factor IXa and thrombin. (A) Factor VIII (1 U/ml) was activated with factor IXa (0.026 mg/ml); at 11.5 min, thrombin (0.1 U/ml final concentration) was added as 1/10 volume of the incubation mixture and factor VIII coagulant assays were continued. (B) Factor VIII (1 U/ml) was activated with thrombin (0.05 U/ml final concentration), and at 8.5 min, factor IXa (0.026 mg/ml final concentration) was added. (C) Factor VIII (1 U/ml) was activated by factor IXa (0.026 mg/ml); at 8.5 min, a second aliquot of factor IXa (0.026 mg/ml) was added.

Fig. 6. Effect of anti-factor IX on the factor IXa–factor VIII interaction. Anti-factor IX (---0-----) or VBS (- - - -) was preincubated with factor IXa prior to addition to factor VIII (0.5 U/ml), as noted in Materials and Methods.

thrombin–factor VIII mixture is a serine protease.²⁵
With regard to the activation of factor X by the factor IXa–factor VIII–Ca²⁺–phospholipid complex, the majority of studies have shown that to be active in factor X activation, factor VIII itself must be activated by thrombin.⁶,⁹,²⁴ Factor VIII can also be activated by factor Xa,³ though whether the factor VIII activated by factor Xa can carry out the same cofactor role in the factor X activator complex is not known. The structural similarities of factor IXa to factor Xa and thrombin²⁶ suggest that factor IXa might also be capable of activating factor VIII, and the current study was undertaken to test this hypothesis.

The present study shows that factor IXa activates and degrades factor VIII in a reaction that is qualitatively similar to that seen with thrombin. The extent of activation and rate of degradation are dependent on the relative concentrations of factor IXa and factor VIII as seen with thrombin: the higher the concentration of factor IXa relative to factor VIII, the greater the activation and the more rapid the decay of VIII:C. However, the concentration of factor IXa required for VIII:C activation is much greater than the concentration of thrombin necessary for activation of factor VIII. Whereas the concentration of thrombin utilized for factor VIII activation in purified systems usually

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Table 2. Effect of Inhibitors on Factor IXa Activation of Factor VIII

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor Final Concentration</th>
<th>Factor IXa Concentration Incubated With Inhibitor</th>
<th>Effect on Factor VIII Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzamidine</td>
<td>20 mM</td>
<td>1.33 mg/ml</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>1.33 mg/ml</td>
<td>None</td>
</tr>
<tr>
<td>SBTI</td>
<td>20 mg/ml</td>
<td>1.33 mg/ml</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>0.2 mg/ml</td>
<td>1.33 mg/ml</td>
<td>None</td>
</tr>
<tr>
<td>DFP</td>
<td>10 mM</td>
<td>1.33 mg/ml</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Hirudin</td>
<td>4.5 U/ml</td>
<td>1.33 mg/ml</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>0.5 U/ml</td>
<td>0.06 mg/ml</td>
<td>Partial inhibition</td>
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</table>

Preincubation of inhibitor with factor IXa was carried out as noted in Materials and Methods prior to addition to factor VIII. One-tenth volume of the inhibitor–factor IXa mixture was added to factor VIII (5 U/ml, except 0.5 U/ml in last line). Control preincubations were done with VBS and factor IXa.
approximates 1–10 nM, the concentration of factor IXa utilized in these experiments was 0.25–2 μM. Therefore, the physiologic significance of the factor IXa activation of factor VIII may be small when compared to that by thrombin. On the other hand, factor IXa may be more important for factor VIII activation in the intrinsic coagulation cascade, since factor IXa precedes thrombin in this pathway. Furthermore, relatively high local concentrations of factor IXa and factor VIII may occur at areas of active coagulation, particularly in the presence of phospholipid, which may serve to localize the coagulation factors. In this study, a small increase (twofold) in the activation of factor VIII was seen when phospholipid was added to the activation mixture, although a systematic study of different phospholipids and dose responses has not been examined.

That the current experiments are specific for factor IXa and do not reflect contaminating thrombin is shown by the inhibition of the factor VIII activation by a human inhibitor to factor IX. The inhibitor did not inactivate thrombin in the reaction, verifying its specificity for factor IX. Also, at low concentrations of factor IXa, only loss of factor VIII activity is observed, in contrast to the reported biphasic effect of thrombin on factor VIII evaluated by one-stage factor VIII assays; additionally, 100 mM Ca⁺⁺ abolishes the activation of factor VIII by factor IXa, a phenomenon that does not occur with the bovine thrombin–factor VIII reaction.  

Factor IXa appears to affect only VIII:C and not VIII:RP as judged by the failure of factor IXa to produce a change in VIII:RP on PAGE and radioimmunoassay. Also, the pattern of activation and inactivation of isolated VIII:C is similar to that of factor VIII. Whether there is a significant alteration in the VIII:C Ag is not certain; in the present study, there was a modest decrease in the VIII:C Ag to 76% of initial values. Studies seeking proteolytic degradation products that still retain VIII:C Ag have not yet been performed.

Sequential activation of factor VIII by factor IXa and thrombin shows that after peak activation, no further enhancement of factor VIII activity occurs, whether factor IXa is the initial activator followed by thrombin or vice versa. This suggests that either the initial site involved in the activation of factor VIII is the same for both factor IXa and thrombin, or the intrinsic lability of activated factor VIII ²⁻²⁷ obviates further activation by the second protease.

The inhibitor studies show that high concentrations of DFP and hirudin (relative to factor IXa) inhibit the activation of factor VIII when they are preincubated with factor IXa. A high concentration of DFP was utilized in the current study, since it was known that factor IXa is relatively resistant to DFP in terms of its coagulation activity. ²⁸ Likewise, hirudin in a high concentration relative to factor IXa showed inhibition of the activation of factor VIII. This is consistent with the previously described inhibition of hirudin on the coagulant activity of factor IX. ²⁹ High concentrations of SBTI do not inhibit the activation, showing that contaminating factor Xa is not responsible for the reaction. Benzamidine at moderate concentrations (20 mM) also does not inhibit the factor IXa activation of factor VIII. Benzamidine at that concentration also fails to inhibit thrombin-mediated activation of factor VIII, ³⁰ even though it does inhibit thrombin action on fibrinogen.

The mechanism by which factor VIII performs its regulatory function in the intrinsic system is not known, but its activation and subsequent decrease in activity by serine proteases appear to play an important role. The relative contributions of factor IXa, factor Xa, and thrombin to the process are not established and will need to be studied in a reconstituted plasma system where platelet membrane phospholipids and normal plasma inhibitors are also present.

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REFERENCES


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