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Activation of Bovine Factor VII by Hageman Factor Fragments

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During the early events of coagulation of human blood by the intrinsic pathway, factor XII is activated to a form which can activate factor XI, and is proteolytically fragmented to smaller species (30,000 daltons and 70,000 daltons) which have lost most of the ability to activate factor XI but which can activate prekallikrein rapidly. The effect of these fragments on factor VII was studied. It was found that these Hageman factor fragments promoted rapid proteolysis of one-chain factor VII to a more active two-chain form. The amino-terminal sequences of the chains of activated factor VII were found to be Ala-Asx-Gly- and Ile-Val-Gly-, the same as were earlier observed after activation of factor VII by activated factor X. This finding indicates that initiation of coagulation by the intrinsic pathway also primes the extrinsic pathway.

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THE INTRINSIC PATHWAY of coagulation is initiated by contact of Hageman factor (factor XII) with an appropriate surface such as collagen or kaolin and generation of "activated" Hageman factor (factor XII_a). It is unclear whether this "activation" involves proteolysis, but it is believed that the molecular weight of factor XII_a is similar to that of factor XII (80,000 daltons).[1,2] Factor XII_a is believed to activate factor XI, leading to eventual coagulation,[3] and plasminogen proactivator,[4,5] which leads to generation of plasmin. Some workers believe that plasminogen proactivator is identical to prekallikrein.[6] Plasmin is thought then to feed back on factor XII and generate proteolytic degradation fragments of factor XII_a, which have little activity toward factor XI but which are potent activators of prekallikrein.[7-10] Kallikrein, once formed, also possesses potent ability to fragment factor XII.[1,2,5,11] Two of these degradation fragments have been called prekallikrein activator (PKA; 30,000 daltons)[7,9] and large activator (70,000 daltons).[10,11]

In the present study we have examined the effect of these fragments of factor XII_a on factor VII, a single-chain molecule which, in the presence of tissue factor, initiates coagulation by the extrinsic pathway.[12,13] It has been shown that factor VII can be cleaved to a more active two-chain molecule by the action of factor X_a[13] and thrombin.[13,14] It has also been reported that kallikrein,[15-17] plasmin, factor IX_a, and Hageman factor fragments[17] increase the activity of factor VII, although detailed molecular changes have not been investigated.

We found that both PKA and large activator promoted rapid activation of factor VII with formation of a two-chain form suggesting that initiation of the intrinsic pathway not only leads to activation of factor XI, plasminogen proactivator, and prekallikrein, but also primes the extrinsic pathway.

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MATERIALS AND METHODS

Factors VII, X, and X_a were prepared from bovine plasma as described.[13] Factor XII was prepared from human plasma by the method of Cochrane and Wuepper[9] followed by disk gel electrophoresis.[11] PKA and large activator of prekallikrein were prepared by alcohol and salt fractionation, and ion-exchange and gel-exclusion chromatography from glass-contacted human plasma.[10] Human brain phospholipid was prepared by the method of Bell and Alton.[18] Bovine brain thromboplastin was prepared by the method of Quick.[19]

Factor VII was assayed by the method of Nemerson and Clyne.[20] Factor XII was assayed by a modified partial thromboplastin time in the presence and absence of kaolin (1 mg/ml).[21] One ml of normal plasma was defined to contain 100 units of factors VII and XII. Prekallikrein-activating ability was assayed by measuring the increase in hydrolysis of tosyl arginine methyl ester (TAMe) (due to released kallikrein) by citrated normal plasma after preincubation with the activator species.[10,22]

The sources of other reagents and the procedures for analytical and preparative sodium dodecyl sulfate (SDS) slab gel electrophoresis have been reported.[13] Radioautography of SDS gels of ^{32}P-diisopropylfluorophosphate (DF^{32}P)-labeled proteins was performed as previously described.[13] Amino-terminal sequences were determined by the dansyl-SDS procedure of Weiner et al.[23]

RESULTS

The factor XII used in these studies had 30 U/ml of coagulant activity in the presence of kaolin and 0.3 U/ml in its absence. It also possessed 40 U/ml of prekallikrein-activating ability, implying the presence of degradation fragments.[11] When this material was reacted with DF^{32}P and electrophoresed on SDS-acrylamide gels, radioautography revealed DF^{32}P-containing bands at 30,000 and 70,000 daltons. Approximately equal amounts of these species were present. No bands were seen by staining with Coomassie blue due to the low protein concentration (approximately 10 μg/ml). The PKA and large activator preparations were largely free of factor XII coagulant activity but contained minor amounts of each other as contaminants.[10] The factor VII preparation displayed a single band on SDS-acrylamide gel electrophoresis.[13]

When factor XII, PKA, or large activator was added to factor VII, factor VII coagulant activity increased rapidly at rates proportional to the amounts of added activator. Figure 1 shows the time course of activation by PKA and Fig. 2 shows the course of activation by varying amounts of factor XII. A similar family of curves was obtained by the action of a large activator preparation

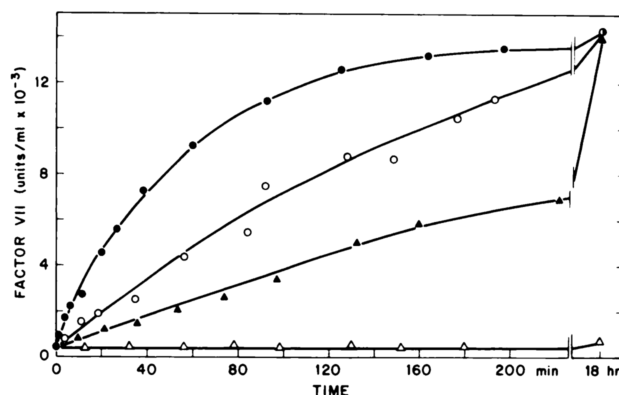

Fig. 1. Activation of factor VII by PKA preparation. Reactions contained 0.25 ml of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin, and factor VII (400 U/ml, 2 μg/ml) in plastic tubes at 37°C. PKA was added to produce final concentrations of 17 U/ml (▲), 30 U/ml (○), 75 U/ml (●), or was not added (△).

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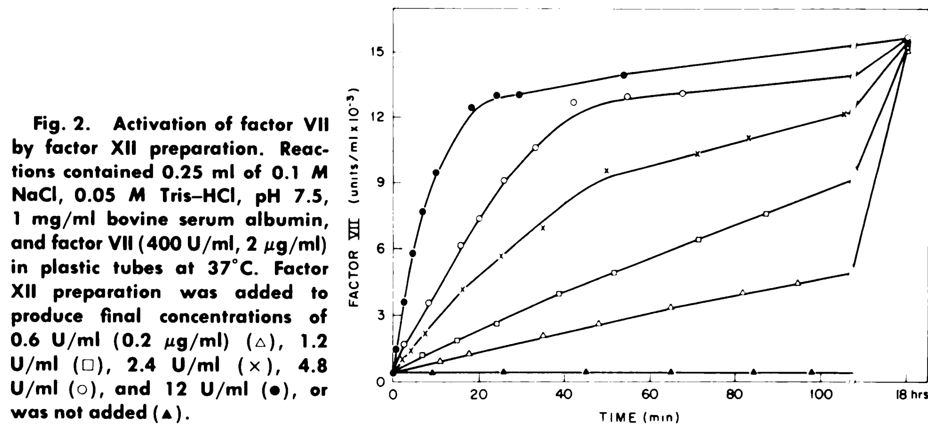

Fig. 2. Activation of factor VII by factor XII preparation. Reactions contained 0.25 ml of 0.1 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.5, 1 mg/ml bovine serum albumin, and factor VII (400 U/ml, 2 μg/ml) in plastic tubes at 37°C. Factor XII preparation was added to produce final concentrations of 0.6 U/ml (0.2 μg/ml) (△), 1.2 U/ml (□), 2.4 U/ml (×), 4.8 U/ml (○), and 12 U/ml (●), or was not added (▲).

containing equivalent prekallikrein-activating potency. In each case the extent of activation was dependent only on the starting level of factor VII, and the degree of activation (40-fold) was the same as was seen after activation with factor X_a or thrombin.[13] When the rates obtained were extrapolated to the situation of complete fragmentation of factor XII, approximately at plasma concentration, a rate of activation of 20-fold per minute was calculated.

When the time course of activation was followed by SDS gel electrophoresis (Fig. 3) it was observed that after 1 hr of incubation the single-chain factor VII was fully cleaved to a two-chain species whose peptide chains co-electrophoresed with the chains generated by the action of factor X_a on factor VII (center gel slot). The heavy chain (29,500 daltons) and light chain (23,500 daltons) were found to display amino-terminal sequences of Ile-Val-Gly and Ala-Asx-Gly-, respectively, the same as found for the chains generated by activation with factor X_a.[24] The light chains stained poorly with Coomassie blue and are not well visualized in the figure. In contrast to the reaction with factor X_a, the two-chain molecule was not readily destroyed by further cleavage of the heavy chain by factor XII fragments. This difference was not due to loss of activator activity, since additions were made at intervals to maintain the factor XII level.

The effects of possible cofactors and inhibitors on the activation by the factor XII preparation were studied. The rate of activation of factor VII was not affected by the presence of kaolin (0.25 mg/ml), calcium chloride (5 m*M*), or calcium chloride (5 m*M*) plus phospholipids (0.2 mg/ml). Benzamidine (10 m*M*) inhibited the reaction rate by 90%. Polybrene (0.5 mg/ml) caused complete inhibition after a several minute lag period. Trypsin inhibitors from soybean and bovine pancreas at 100 μg/ml caused 18% and 12% inhibition, respectively, of the reaction rate. Pretreatment of the factor XII preparation with DFP (1 m*M* or 5 m*M*) for 16 hr at 4°C had no effect on the coagulant activity but eliminated the ability to activate factor VII. This finding again implicated the degradation fragments as the activating species.

This conclusion was also supported by experiments performed using human Hageman factor kindly supplied by Dr J. Griffin and Dr. C. G. Cochrane of the Department of Experimental Pathology, Scripps Clinic and Research Founda-

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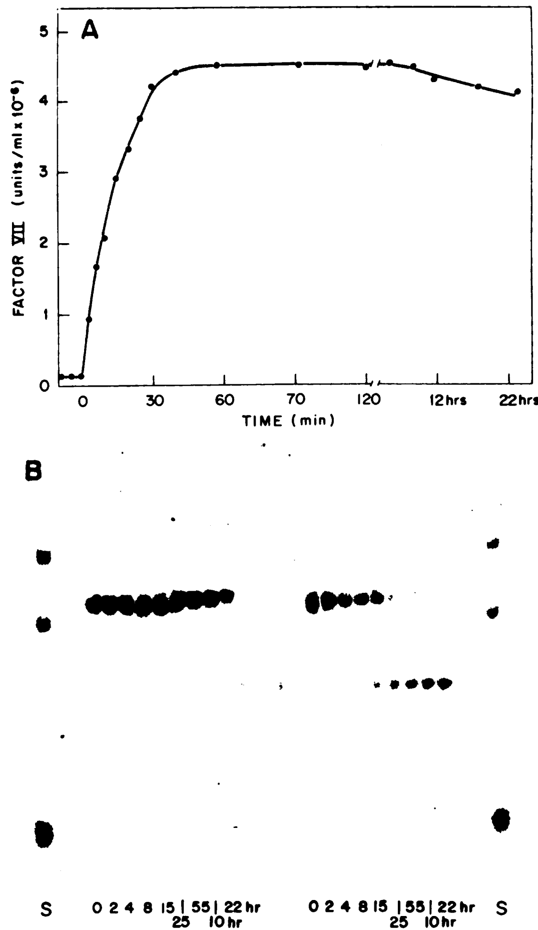

Fig. 3. Activation of factor VII by factor XII preparation (A) At time zero 20 μl of factor XII preparation (30 U/ml) was added to 1 ml of factor VII (0.5 mg/ml) in 0.1 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.5, in a plastic tube at 20°C. Additional factor XII was added at 3-hr intervals. (B) At indicated times aliquots were removed and heated at 100°C for 3 min with an equal volume of 4 *M* urea and 2% sodium dodecylsufate (SDS) and electrophoresed in a 7½% polyacrylamide slab gel containing tris-acetate buffer, pH 7.5, and 0.1% SDS. Samples on the right also contained 5% 2-mercaptoethanol. The center sample was a mixture of reduced two-chain and three-chain factor VII generated by the action of factor X_a, calcium ions, and phospholipids. Molecular weight standards were reduced bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C. Coomassie blue.

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tion, La Jolla, Calif. Their factor XII, purified in the presence of inhibitors to a single electrophoretic band (95%), did not activate factor VII. After brief preincubation with trypsin and inactivation of the trypsin by a 100-fold excess of orosomucoid trypsin inhibitor (w/w), activation of factor VII was observed at a rate of 20-fold per minute using the cleaved factor XII at plasma concentration. The control containing trypsin plus a 100-fold excess of orosomucoid trypsin inhibitor did not cause activation of factor VII.

When aliquots of the Factor XII preparation were added to citrated bovine plasma, rapid activation of factor VII was observed for several minutes followed by a plateau (Fig. 4), which probably reflected inhibition of the factor XII fragments by plasma inhibitors such as C1INH.[25] The assumed inhibition took longer at 0°C than at 37°C and the resultant degree of activation after each addition was therefore greater at 0°C. This difference may reflect the same phenomenon as Gjonnaess's observation that factor VII was activated in certain plasmas incubated overnight at 0°C, but not at 20°C.[15]

Figure 5 indicates that addition of factor XII to citrated human plasma was followed by activation of factor VII. Activation of human plasma factor VII

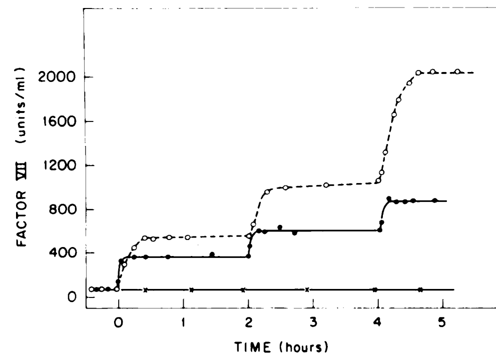

Fig. 4. Effect of temperature on activation of factor VII in citrated bovine plasma by factor XII preparation. At 0, 2, and 4 hr 15 μl of factor XII preparation (30 U/ml) were added to 0.25 ml of citrated bovine plasma at 0°C (○) and 37°C (●), or were not added (×). Plastic tubes were used.

35-fold and activation of bovine plasma factor VII 55-fold were obtained. Factor VII was assayed in the presence of tissue factor. No acceleration of coagulation was detected when tissue factor was omitted from the assay.

DISCUSSION

The data presented are consistent with the interpretation that fragments of factor XII cause up to a 55-fold enhancement of the coagulant activity of factor VII by proteolysis at the same Arg-Ile bond shown to be cleaved by factor X_a in the presence of calcium ions and phospholipids.[24] Calculation indicates that with reactants at approximate plasma concentrations an activation rate of 20-fold per minute at 37°C can be expected. This activation rate is similar to that observed for activation by factor X_a and is rapid enough to be of possible physiologic significance. It was observed that activation could be produced in both bovine and human citrated plasma. This finding indicates that the observations made with purified bovine factor VII can be extrapolated to human factor VII as well.

Specific assays did not reveal the presence of other coagulation factors in the factor XII preparation.[11] The fact that calcium ions and phospholipids did not enhance the rate of reaction argues against the possibility that contamination with factor X_a was involved. The action of thrombin would be far too slow[13] to explain the observed rate of activation. Kallikrein[15,16] and plasmin[17] have been suggested to be activators of factor VII and their presence as minor con-

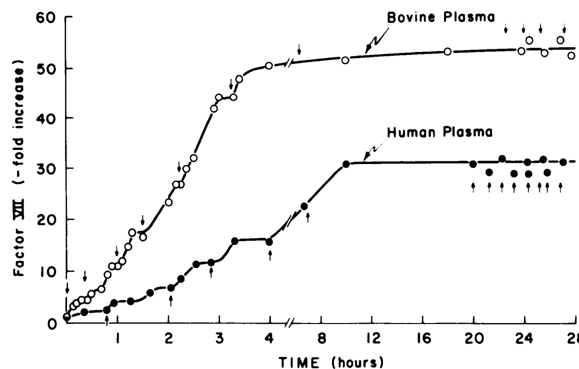

Fig. 5. Effect of factor XII preparation on factor VII in citrated bovine plasma (○) and citrated human plasma (●). Additions of 5 μl of factor XII (30 U/ml) were made to 0.3 ml of plasma at 0°C at times indicated by arrows up to 3 hr, after which time 20-μl aliquots were added. Corrections were made for dilution. Plastic tubes were used.

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taminants in the factor XII preparation must be considered. Kallikrein, however, is strongly inhibited by soybean trypsin inhibitor[26] and plasmin has been shown to rapidly form an inactive stoichiometric complex with both soybean and pancreatic trypsin inhibitors.[27] Since these two inhibitors caused only a weak inhibition of the activation of factor VII when present at high concentrations, it is unlikely that kallikrein or plasmin was responsible.

Several lines of evidence suggest that native factor XII is not an activator of factor VII: (1) The activation by the factor XII preparation was eliminated by pretreatment with $DF^{32}P$, whereas the factor XII coagulant activity remained intact. (2) When the factor XII preparation was allowed to react with $DF^{32}P$ and was electrophoresed in SDS-acrylamide gels, radioautography revealed bands at 70,000 and 30,000 daltons, whereas human factor XII migrates at a position corresponding to 80,000 daltons.[1,11] (3) When the activating species was added to citrated plasma, it was rapidly "inhibited" by a plasma constituent, in contrast to factor XII, which is stable in plasma. (4) Factor VII in plasma is in a single-chain form, whereas if plasma factor XII could cause rapid activation it would exist in its two-chain form. (5) The ability of the preparations of factor XII, PKA, and large activator to activate factor VII paralleled their ability to activate prekallikrein, not their coagulant activity. At the present time, we cannot say which fragmentation product(s) of factor XII are of physiologic significance. It is clear, however, that proteolysis of factor XII results in species which activate factor VII.

The present data indicate that the intrinsic pathway and extrinsic pathway are connected at their initiation points. While the physiologic significance of this interaction remains to be established, it is clear that the two pathways do not function in isolation. Fragmentation of Hageman factor appears to generate enzymes of broad specificity which can initiate the kinin system and the fibrinolytic system and prime the extrinsic coagulation pathway.

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