Biochimica et Biophysica Acta, 571 (1979) 333—342

BBA 68865

THE INHIBITION OF BLOOD COAGULATION BY ACTIVATED PROTEIN C THROUGH THE SELECTIVE INACTIVATION OF ACTIVATED FACTOR V

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(Received May 2nd, 1979)

Key words: Blood coagulation; Prothrombin activation; Factor V; Protein C

Summary

Activated Protein C was found to inhibit Factor Xa initiated clotting of plasma. Activated Protein C did not inhibit prothrombin activation by Factor Xa and Ca^{2+} or Factor Xa, Ca^{2+} and lipid. However, activated Protein C did inhibit prothrombin activation by Factor Xa, Ca^{2+} and Factor Va or Factor Xa, Ca^{2+}, lipid, and Factor Va. Excess Factor Va could reverse the inhibition of prothrombin activation. Incubation of Factor V with activated Protein C had little effect on Factor V activity either in the presence or absence of phospholipid.

Preincubation of Factor V with activated Protein C had no effect upon the degree to which Factor V could be activated. When Factor V was activated with thrombin in the presence of activated Protein C, a rapid decline in Factor Va activity was observed. When activated Protein C was incubated with purified Factor Va in the absence of thrombin, a similar rapid decay in Factor Va activity was observed. Activated Protein C catalyzed decay of Factor Va activity was not obligately dependent on the presence of lipid. However, lipid enhanced the rate of inactivation.

Analysis of sodium dodecyl sulfate gels of either Factor V or Va treated with activated Protein C indicated that activated Protein C degraded a slow migrating band of the Factor V doublet and that it also was able to degrade both the heavy and light chains of Factor Va. The results indicated that activated Protein C can inhibit Factor Xa initiated clotting by degrading Factor Va.

Factor Va could be protected from activated Protein C inactivation by the presence of Factor Xa, suggesting that activated Protein C binds to Factor Va

at or near the Factor Xa binding site. The specificity of activated Protein C for Factor Va and the ability of Factor Xa to stabilize Factor Va may both play important functions in the regulation of blood coagulation.

Introduction

Protein C is one of the plasma proteins synthesized in response to vitamin K [1,2]. When activated, it is an inhibitor of blood coagulation. Seeger's group has reported that activated Protein C was a competitive inhibitor of Factor Xa [3]. They further reported that the inhibitory effects of activated Protein C could be overcome by increasing concentrations of Factor V [4]. The concept of activated Protein C functioning as a competitive inhibitor is supported by the sequence homology between Factor Xa [5] and activated Protein C [1]. However, this concept is difficult to reconcile with several observations which indicate that activated Protein C functions as a protease. The first of these observations is that diisopropylfluorophosphate could inactivate the anticoagulant activity of activated Protein C indicating that proteolytic activity is necessary for anticoagulation [5]. This view is also supported by Kisiel and coworkers' findings [6] that activated Protein C inhibited the activity of partially purified Factor V. The inhibitory capacity was blocked when activated Protein C was inhibited with diisopropylfluorophosphate. They also reported that the destruction of Factor V (Va activity) was dependent on the presence of phospholipid. From these studies they concluded that activated Protein C inhibited blood clotting by proteolytic inactivation of Factor V. However, they did not distinguish between the possibilities of proteolytic inactivation of Factor V or Factor Va.

In order to clarify and to explore further the mechanism of activated Protein C mediated inhibition of blood clotting, we have studied the effects of activated Protein C on Factor V and Factor Va structure and activity. In this paper we report differences in susceptibility of Factor V and Factor Va to activated Protein C and that Factor Xa can block the inactivation of Factor Va by activated Protein C.

Materials and Methods

Materials. Bovine blood was the generous gift of the Wilson Food Corporation (Oklahoma City, OK). Soybean trypsin inhibitor and QAE-Sephadex Q50 were purchased from Sigma Chemical Company (St. Louis, MO). DEAE-Sephacel was purchased from Pharmacia Fine Chemicals (Pascataway, NJ). Acrylamide was electrophoresis grade and purchased from Eastman Chemical Company (Rochester, NY). Agarose-immobilized Soybean trypsin inhibitor and agarose-immobilized heparin were prepared by the cyanogen bromide method [7]. All other reagents were of the highest grade commercially available.

Phospholipid preparation. Phospholipid was prepared from acetone dried bovine brain by the method of Bligh and Dyer [8]. Phospholipid vesicles were prepared by mixing the phospholipid in $CHCl_3$ and then drying under nitrogen onto the walls of a glass tube. The lipid was dispersed into buffer (0.1 M NaCl,

0.02 M Tris-HCl, pH 7.5) by sonicating with a Branson bath sonicator for 4 h at room temperature.

Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli [9]. Acrylamide gel electrophoresis in the absence of detergent was performed by the method of Davis [10]. Gels were stained with Coomasie blue.

Preparation of proteins. Activated Factor X (Xa) was prepared from purified Factor X [11] by activation of the Factor X with the Factor X activator from Russell's viper venom as described earlier [12]. 1 ng of the activated Factor X would form a clot in 0.1 ml bovine plasma, 0.1 ml 0.5 mg/ml phospholipid, 0.1 ml 0.025 M $CaCl_2$ in a final volume 0.4 ml in 30 s. Prothrombin and thrombin were prepared as described earlier [11]. Factor V was purified and converted to Factor Va by incubation with thrombin [13]. Factor Va was separated from activation products and thrombin by chromatography on QAE-Sephadex Q50 [13]. The Factor Va heavy chain and light chain were isolated by chromatography of Factor Va on QAE-Sephadex Q50 in the presence of EDTA [13]. The Factor X activator from Russell's viper venom was isolated by gel-filtration chromatography on Sephadex G-100 followed by ion exchange on QAE-Sephadex Q50 [12]. Bovine fibrinogen was prepared by the method of Straughn and Wagner [14] and was 96% clottable.

Protein C was isolated by a modification of the method of Stenflo [1]. Bovine plasma preparation and $BaSO_4$ absorption (25 mg/ml) and elution were performed exactly as described prevously [11]. The eluate from 45 l of plasma was diluted 1 : 1 with distilled water and passed over a 5 × 2 cm column of agarose immobilized soybean trypsin inhibitor. The filtrate was absorbed onto QAE-Sephadex Q50 (400 ml) equilibrated in 0.2 M NaCl, 0.02 M Tris-HCl, 0.001 M benzamidine HCl, pH 7.5. The QAE-Sephadex was washed extensively and layered over a bed of QAE-Sephadex (5 × 120 cm). The column was developed with a linear gradient from 0.28—0.6 M NaCl (0.02 M Tris-HCl, 0.001 M benzamidine HCl, pH 7.5, 4000 ml/reservoir). Protein C was eluted on the trailing edge of the prothrombin peak. The Protein C content of the fractions was evaluated qualitatively by sodium dodecyl sulfate gel electrophoresis following disulfide bond reduction of the sample. Fractions containing approx. 25% Protein C were pooled for further purification. These fractions were dialyzed against 0.05 M imidazole, 0.01 M $CaCl_2$, 0.001 M benzamidine HCl at pH 6.0, and chromatographed on a column (15 × 30 cm) of heparin-agarose. The column was developed at 4°C with a linear gradient from 0 to 0.6 M NaCl (0.05 M imidazole, 0.01 M $CaCl_2$, 0.001 M benzamidine-HCl, pH 6.0, 200 ml/reservoir). Purity of the fractions was monitored by gel electrophoresis in the absence and presence of sodium dodecyl sulfate.

Factor V assay. Factor V deficient plasma was prepared from aged, oxalated human plama. Factor V activity was assayed by the method of Kappeler [15]. Standard curves were prepared with bovine plasma as the Factor V source. Factor V and Factor Va activity were determined by reference to this standard curve. In our assay, bovine plasma contains 5—10 times the activity of human plasma. Therefore, the specific activities reported here are 5—10 times lower than if compared to a human standard. Bovine plasma was defined as containing 1 unit of Factor V/ml. Purified Factor V had a specific activity of 25—

50 units/mg when compared to bovine plasma. Bovine Factor Va had a specific activity of 300—500 units/mg based upon the same assay.

Factor Xa assay. Factor Xa was assayed by a modification of the method of Bachman [16]. In this modification, bovine plasma was used in place of Factor X deficient plasma and Russell' viper venom was deleted from the cephalin. Factor Xa was assayed by reference to a standard curve prepared by dilution of Factor Xa from a stock solution. The effect of activated Protein C on the apparent activity of Factor Xa was calculated by comparing the clotting time observed in the presence of activated Protein C to the clotting time observed for the same concentration of Factor Xa in the absence of activated Protein C. This effect was quantitated by reference to the standard curve prepared for Factor Xa.

Thrombin assay. Thrombin was assayed by its ability to clot fibrinogen. Clotting times were converted to thrombin activity by comparison with a reference curve prepared from prothrombin-*Echis carinatus* incubation.

Protein was monitored by absorbance at 280 nm. The molecular weights and extinction coefficients used for all protein components were as follows: prothrombin, 72 000, $E_{1\%}^{1cm}$ 15.5, Xa, 45 000, $E_{1\%}^{1cm}$ 12.4 [11], activated Protein C, 56 000, $E_{1\%}^{1cm}$ 13.7 [6], Factor V, 300 000, $E_{1\%}^{1cm}$ 10.0, Factor Va, 180 000, $E_{1\%}^{1cm}$ 10.0 [13].

Activation of Protein C. Protein C (6 mg, 0.5 mg/ml) in 0.1 M NaCl, 0.02 M Tris-HCl, 0.01 M $CaCl_2$, pH 7.5, was activated at 37°C with the Factor X activator from Russell's viper venom (12.5 μg/ml final concentration). The extent of activation was monitored by the hydrolysis of N^{α}-*p*-tosyl-L-arginine methyl ester, and activation was allowed to proceed until no further increase in hydrolytic activity occurred (usually 180 min).

Isolation of activated Protein C. Following complete activation of Protein C, the Factor X activator was quantitatively separated from activated Protein C by chromatography on a column (0.6 × 10 cm) of QAE-Sephadex. Prior to chromatography the reaction mixture was made $2.0 \cdot 10^{-3}$ M in benzamidine-HCl. The column was washed with 20 ml of 0.2 M NaCl, 0.02 M Tris-HCl, $2 \cdot 10^{-3}$ M benzamidine, pH 7.5 to remove the venom protein. Activated Protein C was eluted from the column with 0.6 M NaCl, 0.02 M Tris-HCl, $2 \cdot 10^{-3}$ M benzamidine, pH 7.5.

The effect of activated Protein C on the rate of prothrombin activation. The effect of activated Protein C on the activation of prothrombin was studied by examining its effect on the time course of prothrombin activation. All proteins were dialyzed into 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 before performing the activation studies. All prothrombin activation studies were performed in the presence of 10 mM $CaCl_2$ and the reactions were carried out at 37°C. Reactants were mixed together within 45 s in the following order: Prothrombin, $CaCl_2$, activated Protein C (where added), phospholipid (where added), Factor Va (where added) and Factor Xa, Factor Xa was added within 10 s of the Factor Va in order to minimize activity loss due to preincubation.

Results

Activated Protein C inhibited Factor Xa initiated clotting of plasma. The amount of inhibition was dependent on the concentration of activated Protein C. Since there are several mechanisms by which Factor Xa initiated clotting could be inhibited, including, the inhibition of lipid binding and the stimulation of the activity of a plasma protease inhibitor, we decided to study the effects of activated Protein C on the activation of prothrombin by purified clotting factors. In this system there are no plasma protease inhibitors present and the lipid dependence upon inhibition can be evaluated.

Activated Protein C had no effect on the activation of prothrombin by either [Xa,Ca^{2+}] or [Xa,phospholipid,Ca^{2+}] (Figs. 1A and 1B). This indicates that the inhibition of plasma clotting was not due to alterations in Xa-lipid binding. Activated Protein C inhibited prothrombin activation by both [Xa,Va,Ca^{2+}] and [Xa,Va,phospholipid,Ca^{2+}] (Figs. 1C and 1D). These results confirm that activated Protein C effects Factor Va function [6]. The inhibitory effects of activated Protein C on prothrombin activation could be minimized by increasing the Factor Va concentration (Fig. 2). The reversal of activated Protein C inhibition by Factor Va could also be demonstrated in plasma. Factor Xa initiated clotting of plasma became insensitive to activated Protein C when the plasma was supplemented with 1 unit/ml purified Factor Va.

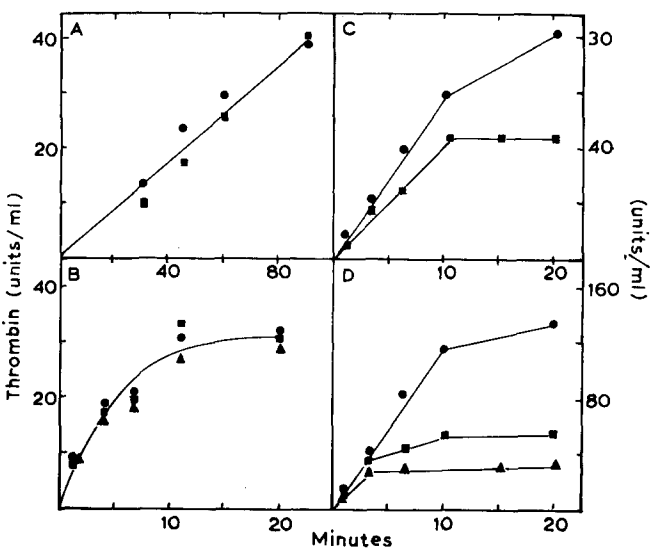

Fig. 1. The effect of activated Protein C on prothrombin activation by [Xa,Ca^{2+}], [Xa,phospholipid, Ca^{2+}], [Xa,Va,Ca^{2+}], and [Xa,Va,phospholipid,Ca^{2+}]. The reaction mixtures contained: (A) 330 μg/ml prothrombin, 3 μg/ml Xa, and 10 mM $CaCl_2$ with 10 μg/ml activated Protein C (■) and without activated Protein C (●). (B) 330 μg/ml prothrombin, 50 μg/ml phospholipid, 3 μg/ml Xa, 10 mM $CaCl_2$ and 19 μg/ml activated Protein C (●), 6 μg/ml activated Protein C (■) and without activated Protein C (▲). (C) 350 μg/ml prothrombin, 3 μg/ml Xa, 0.3 units/ml Va, 10 mM $CaCl_2$, with 8 μg/ml activated Protein C (■) and without activated Protein C (●). (D) 164 μg/ml prothrombin, 0.007 μg/ml Xa, 0.5 units/ml Va, 17 μg/ml phospholipid, 10 mM $CaCl_2$ and 9.7 μg/ml activated Protein C (■), 4.8 μg/ml activated Protein C (■) and without activated Protein C (●). The reaction was initiated with the addition of Factor Xa. At the times indicated samples were removed and thrombin was determined.

Although the anticoagulant effect of activated Protein C appears to be at the level of Factor V in the clotting cascade it is not clear whether Factor V or Factor Va were preferred substrates for activated Protein C. In order to see if activated Protein C could distinguish between Factor V or Factor Va we measured the effect of activated Protein C on the activity of both proteins. When Factor V was treated with activated Protein C either in the presence or absence of phospholipid, very little effect upon activity was observed (Fig. 3A). When the same experiment was repeated using Factor Va in the place of Factor V we observed a decline in the Factor Va activity. The rate of the decline in the Factor Va activity was enhanced by the addition of phospholipid (Fig. 3B).

In order to see if there may have been an effect on Factor V structure that did not affect the Factor V activity, a sodium dodecyl sulfate gel time course was run on the Factor V treated with activated Protein C. Although Factor V was not inactivated by activated Protein C, proteolysis of Factor V did occur.

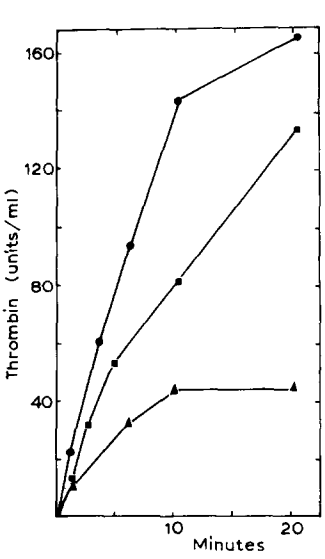

Fig. 2. Effect of Factor Va on the activated Protein C mediate inhibition of prothrombin activation by [Xa,Va,phospholipid,Ca^{2+}]. The reaction mixtures contained 164 μg/ml prothrombin, 0.007 μg/ml Xa, 17 μg/ml phospholipid, 10 mM $CaCl_2$, 9.7 μg/ml activated Protein C and 1 unit/ml Factor Va (▲), 20 units/ml Factor Va (■) and 1 unit/ml Factor Va with no activated Protein C (●). The reaction was initiated by the addition of Factor Xa. At the times indicated samples were removed and assayed for thrombin as described. The reaction was carried out at 37° C.

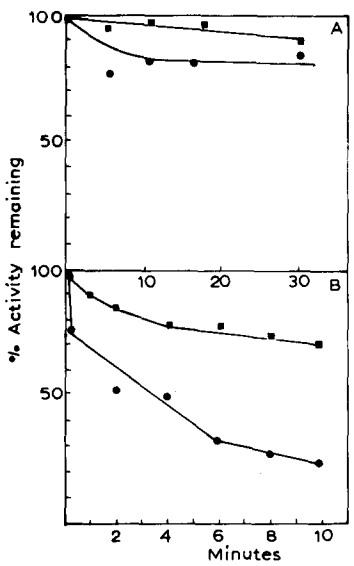

Fig. 3. (A) The effect of activated Protein C on the activity of Factor V. 13.5 units/ml (0.27 mg/ml) Factor V was incubated with 10 mM $CaCl_2$, without phospholipid (■), and with 50 μg/ml phospholipid (●) in 0.1 M NH_4Cl, 0.02 M Tris-HCl, pH 7.5. The reaction was initiated by the addition of 2.3 μg/ml activated Protein C and the Factor V activity was monitored by removing samples at the indicated times and assaying Factor V activity. The reaction was carried out at 37° C. (B) The effect of activated Protein C on the activity of Factor Va. 7.3 units/ml (16.2 μg/ml) Factor Va was incubated with 10 mM $CaCl_2$, without phospholipid (■) and with 50 μg/ml phospholipid (●) in 0.1 M NH_4Cl, 0.02 M Tris-HCl, pH 7.5. The reaction was initiated by the addition of 0.33 μg/ml activated Protein C and the Factor Va activity was monitored by removing samples and assaying the Factor Va activity. The reaction was performed at 37° C.

Activated Protein C appeared to cleave the slower migrating form of Factor V into the faster migrating band of the Factor V doublet. The apparent doublet in Factor V may arise as an artifact of purification or may exist in bovine plasma. Both forms are rapidly and completely cleaved by thrombin [13].

Though there was no effect of activated Protein C on the Factor V one-stage activity, the possibility exists that activated Protein C may alter the final extent by which Factor V could be activated by thrombin. After incubating Factor V with activated Protein C for one hour, thrombin was added to convert the

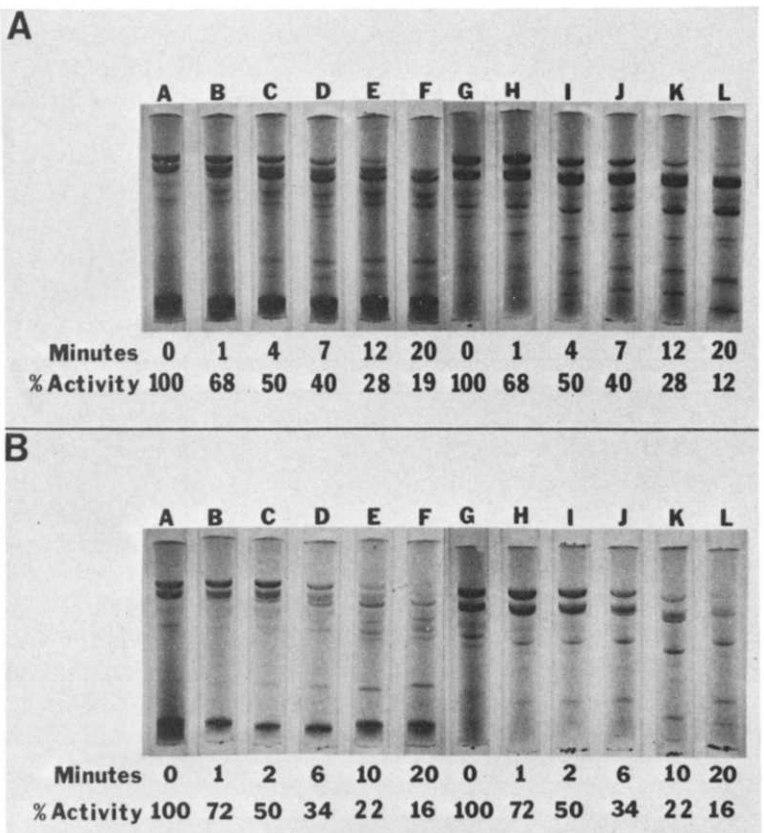

Fig. 4. (A) 64 units/ml (0.16 mg/ml) Factor Va was incubated with 14.5 μg/ml activated Protein C at 37°C in 0.1 M NH_4Cl, 0.02 M Tris-HCl, 0.01 M $CaCl_2$, pH 7.5. At the time indicated two samples were removed: one sample, 20 μg, was made 1% in sodium dodecyl sulfate and placed in a boiling water bath for 1 min; the other sample was diluted and assayed immediately for Factor Va activity. Samples were electrophoreses on gels containing 10% acrylamide. Samples for gels A-F were removed and electrophoresed on sodium dodecyl sulfated acrylamide gels without disulfide bond reduction. Samples for gels G-L were removed and electrophoresed as described above but the disulfide bonds were reduced prior to electrophoresis. The time of sample removal and the percent activity remaining at that time are indicated below each gel. (B) 64 units/ml (0.16 mg/ml) Factor Va was incubated with 7 μg/ml activated Protein C at 37°C in 0.1 M NH_4Cl, 0.02 M Tris-HCl, 0.01 M $CaCl_2$, pH 7.5, in the presence of 0.2 mg/ml phospholipid. Samples were removed and assayed or electrophoresed as described in Fig. 4A. Samples for gels A-F were prepared without disulfide bond reduction. Samples for gels G-L were prepared by reducing disulfide bonds prior to electrophoresis. The time the sample was removed and the activity remaining at that time are indicated below the gel.

Factor V to Factor Va. The treatment of Factor V with activated Protein C did not effect the extent of activation. However, immediately following activation, there was a rapid decay of the Factor Va activity. This decay could be attributed to the presence in the reaction mixture of activated Protein C since, in the absence of activated Protein C, Factor Va activity was essentially stable over the time period of the experiment.

The effect of activated Protein C on Factor Va structure was more extensive than seen on Factor V. The inactivation of Factor Va appeared to correlate with the cleavage of the heavy chain of Factor Va (Fig. 4A). From a qualitative analysis of the gel electrophoresis time course, it would appear that a small, but significant Factor V activity was retained even after the complete proteolysis of the Factor Va heavy chain. The rate of activated Protein C inhibition and proteolysis of Factor Va was enhanced by the presence of phospholipid. The presence of phospholipid both increased the rate of inhibition and the extent of Factor Va degradation (Fig. 4B).

Analysis of the Factor Va inactivation is complicated by the possibility that one of the cleavage products of the heavy chain might co-migrate with the light chain. This possibility was evaluated by cleaving the Factor Va heavy chain in the absence of the light chain. Activated Protein C degraded the Factor Va heavy chain to a fragment with a molecular weight of 85 000, that migrated at or near the position of the Factor V light chain. Two additional peptides were also observed at position corresponding to 53 000 and 22 000. This observation raised the question of whether the light chain also contained an activated Protein C sensitive bond. When the Factor Va light chain was incubated with activated Protein C the light chain was degraded into 53 000 molecular weight chain and a 22 000 molecular weight chain.

Activated Factor X and activated Protein C share many structural similarities [6] suggesting the possibility that these two enzymes might bind to the same site on Factor Va. If the two enzymes bind to the same site, then Factor Xa should protect Factor Va from inactivation by activated Protein C. Factor Xa was found to decrease the rate of activated Protein C inhibition of Factor Va. The extent of Factor Va protection was dependent on the concentration of Factor Xa. The protection by Factor Xa was specific since the precursor form of Factor Xa, Factor X had no effect on activated Protein C inhibition of Factor Va.

Discussion

We have confirmed that activated Protein C inhibits the clotting of plasma initiated by Factor Xa. Inspection of the time courses of prothrombin activation in the presence of activated Protein C (Figs. 1C and 1D) indicates that inhibition is time dependent. At early time points activated Protein C is relatively ineffective. It is not until later times that activated Protein C inhibition is fully expressed. This observation is consistent with the hypothesis that activated Protein C functions proteolytically and that inhibition of prothrombin activation is due to the specific degradation of activated Factor V. In contrast to the results of Kisiel et al. [6] the degradation of Factor Va is not obligately dependent on the presence of phospholipid, but the presence of

phospholipid causes a large enhancement in the rate of inactivation.

Activated Protein C appears to have a novel function in coagulation. Contrasting other vitamin K dependent proteins which are procoagulants, activated Protein C appears to be an anticoagulant. As a zymogen it can circulate having no effect on other circulating proteins, but upon activation it can rapidly inactivate Factor Va by proteolysis thereby rapidly shutting off prothrombin activation and clot formation. This is an interesting mechanism because by limiting the substrate specificity to only the activated form of Factor V the extent of prothrombin activation can be rapidly controlled without affecting Factor V levels and, therefore, the potential for the reinitiation of clotting is retained. By conserving the zymogen and inactivating only the activated protein, activated Protein C very selectively controls the rate and extent of prothrombin activation.

Activated Protein C appears to have a very narrow substrate specificity. Apparently the primary sensitive bonds in Factor V only become accessible after activation by thrombin. This indicates that the sensitive bonds must be buried in Factor V. It is surprising that given the inability of activated Protein C to cleave prothrombin, Factor X or fibrinogen [6], it is capable of cleaving both chains of Factor Va. This could reflect the presence of some common structural feature in each of the subunits.

The mechanism of activated Protein C inhibition of clotting differs from the mechanism by which plasma protease inhibitors inhibit clotting. These inhibitors form complexes with the proteases involved in coagulation, but do not degrade proteases [17]. Once formed, these complexes are rapidly cleared from the circulation. Although activated Protein C and plasma protease inhibitors work through different mechanisms they both share the property of only inhibiting the activated forms of the clotting proteins.

Our results help to explain the apparent discrepencies in the literature on how activated Protein C functions. Seegers' group [3] concluded that activated Protein C was a competitive inhibitor of prothrombin activation and that this inhibition could be overcome by increasing levels of Factor V. The competitive nature of the inhibition would appear to be inconsistent with the observation that the anticoagulant activity of activated Protein C was destroyed by the active site inhibitor diisopropylfluorophosphate [5] and that the inactivation of Factor V was time dependent [6]. However, the ability of Factor Xa to protect Factor Va from inactivation as demonstrated here, could create the appearance of a competitive inhibitor. This possibility is supported by the demonstration that the rate of Factor Va inactivation by activated Protein C is inversely related to the Factor Xa concentration present at the time of inactivation.

The anticoagulant properties of activated Protein C are subject to several levels of control. The protection of Factor Va by Factor Xa indicates that the Factor Xa concentration affects the rate of inactivation. The dependence of the rate of inactivate on the presence of phospholipid indicates that the availability of a lipid surface will regulate the activity. Finally, the rate of activation of activated Protein C from the zymogen, Protein C, will effect the appearance of the anticoagulant activity.

It is as yet still unclear as to the physiological activator and to what rate and

extent Protein C becomes activated during coagulation. Each of these parameters contribute to a complex control mechanism which is involved in the localization of the clot.

Acknowledgements

This work was supported by a grant-in-aid from the National Heart, Lung and Blood Institute, grant No. HL-17812. F.J.W. was supported by a post-doctoral fellowship from the Oklahoma Affiliate of the American Heart Association.

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