Interaction of Calcium with Bovine Plasma Protein C†

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ABSTRACT: The binding of $^{45}Ca^{2+}$ to bovine plasma protein C (PC) and to activated bovine plasma protein C (APC) has been examined by equilibrium ultrafiltration at pH 7.4 and 25 °C. Under these conditions, PC possesses 16.0 ± 2.0 equivalent Ca^{2+} binding sites, of average K_D (8.7 ± 1.5) × 10^{-4} M, and APC contains 9.0 ± 1.0 equivalent Ca^{2+} binding sites, with an average K_D of (4.3 ± 1.1) × 10^{-4} M. Both Mn^{2+} and Sr^{2+} were capable of ready displacement of Ca^{2+} from a Ca^{2+}–PC complex, while Mg^{2+} was less effective in this regard. The α-thrombin-catalyzed activation of PC was inhibited by the presence of Ca^{2+}. A kinetic analysis of this effect demonstrated that it was, in large part, due to an increase in the K_m of the reaction. Addition of other divalent cations, e.g., Mn^{2+}, Sr^{2+}, and Mg^{2+}, in place of Ca^{2+} also resulted in inhibition of the α-thrombin-catalyzed activation of PC in a manner which paralleled their ability to displace Ca^{2+} from a Ca^{2+}–PC complex. On the other hand, the activation of PC by the coagulant protein from Russell's Viper venom was augmented by the presence of Ca^{2+}. Other divalent metal ions, such as Sr^{2+} and Mn^{2+}, in the absence of Ca^{2+}, also weakly stimulated this reaction. Mg^{2+} was without notable effect.

Protein C is a γ-carboxyglutamic acid containing glycoprotein, which has been isolated from bovine (Stenflo, 1976) and human (Kisiel, 1979) plasma. In each case, the purified protein consisted of a heavy chain, of molecular weight 41 000, disulfide linked to a light chain, of molecular weight 21 000 (Kisiel et al., 1977; Kisiel, 1979).

It has been demonstrated that bovine protein C exists in plasma as a zymogen which can be converted, by limited proteolysis, into an enzyme, activated protein C, capable of incorporating diisopropyl fluorophosphate (iPr_2PF)[1] (Esmon et al., 1976). Activated protein C is formed as a result of liberation of a tetradecapeptide from the heavy chain of protein C (Kisiel et al., 1976). Both α-thrombin (Kisiel et al., 1977) and the factor X activating enzyme from Russell's Viper venom (RVV-X) (Kisiel et al., 1976) catalyze cleavage of the Arg–Ile bond, which is responsible for formation of activated protein C (Kisiel et al., 1976). The iPr_2PF-reactive serine residue has been located in the heavy chain of the enzyme (Kisiel et al., 1976). Activated protein C has been shown to possess amidolytic activity (Kisiel et al., 1976, 1977) and esterolytic activity (Steiner et al., 1980) toward synthetic substrates in reactions requiring the presence of monovalent cations (Steiner et al., 1980). In whole plasma, activated protein C functions as an anticoagulant (Kisiel et al., 1977). The basis of this effect appears to reside in the ability of activated protein C to inactivate factor V_a (Kisiel et al., 1977; Walker et al., 1979) and factor VIII (Vehar & Davie, 1980). These latter reactions are augmented by the presence of Ca^{2+} and phospholipid. In addition, activated protein C inhibits the prothrombinase-converting activity of platelets (Comp & Esmon, 1979), possibly through inactivation of platelet-associated factor V_a. It has recently been shown (Walker, 1980) that plasma protein S may be a cofactor for the inactivation of factor V_a by activated protein C.

The complete amino acid sequence of the light chain of protein C has been determined (Fernlund et al., 1978). The first 11 glutamic acid residues exist as γ-carboxyglutamic acid, a feature which is homologous to other vitamin K dependent coagulation proteins, such as prothrombin and factor X. This γ-carboxyglutamic acid containing light chain has been shown to be capable of interaction with Ca^{2+} (Stenflo, 1976).

The object of the present investigation was to quantitatively determine the nature of binding of Ca^{2+} to protein C and activated protein C and to evaluate the role of divalent metal cations in protein C activation. These studies are a requisite to our understanding the function of metal cations in blood coagualtion reactions.

Materials and Methods

Proteins. Protein C was prepared from fresh bovine plasma by the procedures of Stenflo (1976) and Kisiel et al. (1977). Activated protein C was prepared by activation of protein C with insolubilized RVV-X as described earlier (Steiner et al., 1980). Bovine α-thrombin was prepared as described by Lundblad et al. (1975), following activation of prothrombin by a factor X_a–factor V–Ca^{2+}–phospholipid mixture. The specific activity of the α-thrombin was 2500 NIH units mg^{-1} of protein. RVV-X was purified by the procedure of Furie et al. (1974). All proteins were homogeneous when examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Protein concentrations were determined employing an $\epsilon^{1\%}_{1cm}$, at 280 nm, of 13.7 for protein C and activated protein C (Kisiel et al., 1976), 19.5 for α-thrombin (Winzor & Scheraga, 1964), and 13.4 for RVV-X (Furie et al., 1974).

Ca^{2+} Binding Studies. All protein and buffer solutions were first passed over a column of Chelex 100 (Bio-Rad) in order to remove trace contamination by divalent metal ions. Binding of $^{45}Ca^{2+}$ was quantitated by the Paulus ultrafiltration method (Paulus, 1969). Our exact methodology has been described previously (Amphlett et al., 1978, 1979).

Atomic Absorption Analysis. The Ca^{2+} content of solutions of interest was evaluated with the aid of a Varian Techtron Model 1200 atomic absorption spectrometer by use of an air–acetylene flame. Our procedures have been described previously (Byrne et al., 1980).

Sedimentation Velocity. Sedimentation coefficients of bovine protein C and activated protein C were determined in the usual manner (Schachman, 1952), using a Beckman Model

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[1] Abbreviations used: iPr_2PF, diisopropyl fluorophosphate; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; RVV-X, factor X activating enzyme isolated from the venom of Russell's Viper; $NaDodSO_4$, sodium dodecyl sulfate.

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E analytical ultracentrifuge. For protein concentrations below 1.0 mg/mL, the photoelectric scanning optical system was used on the ultracentrifuge, and, for concentrations above 1.0 mg/mL, the Schlieren optical system was employed.

Circular Dichroism. Spectra were obtained by using a Cary Model 60 spectropolarimeter. The value of the molecular ellipticity [θ] at each wavelength was calculated as described previously (Amphlett et al., 1979).

Iodination of Protein C. Protein C was radioiodinated with $Na^{125}I$ by using the solid-state lactoperoxidase procedure of David (1972). The final specific activity of the iodinated protein was approximately 1000 dpm/μg. The labeled protein was activated at the same rate as unlabeled protein C, and each activated protein had a similar specific activity toward BAEE.

$NaDodSO_4$-Polyacrylamide Gel Electrophoresis. The procedure used was similar to that described by Weber & Osborn (1969).

Assays for Activated Protein C. The extent of activation of protein C in samples of interest was monitored by measuring the rate of hydrolysis of the synthetic ester substrate BAEE. A small aliquot of the protein solution (containing up to 1 μg of activated protein C) was added to 1 mL of 1.0 mM BAEE and 0.1 M NaCl in a Radiometer pH-stat titration vessel maintained at 30 °C under a N_2 atmosphere. The presence of Na^+ was essential for the esterase activity of activated protein C to be expressed (Steiner et al., 1980). Esterase activity was measured by automatic titration, to pH 8.0, with standardized 2.5 mM NaOH. At the conclusion of activation experiments, samples from the incubation mixtures were also subjected to reduced and nonreduced $NaDodSO_4$ gel electrophoretic analysis in order to compare the esterase activity developed with the extent of activation and to ensure that no products other than activated protein C had been formed. When the incubation mixtures contained divalent metal ions, it was necessary to add EDTA (final concentration 5 mM) to the gel samples before the start of electrophoresis (if EDTA was omitted, only a fraction of the light chain migrated into the gel). In some cases ^{125}I-labeled protein C was included in the activation mixtures (the extent of activation was then determined precisely by counting slices of reduced $NaDodSO_4$ gels of such samples in a Beckman Model 4000 γ counter).

Materials. $^{45}CaCl_2$ and $Na^{125}I$ were obtained from New England Nuclear. Crude Russell's Viper venom was purchased from Calbiochem, and lactoperoxidase was obtained from Sigma.

Results

Prior to studying the binding of Ca^{2+} to protein C, the protein was subjected to Chelex 100 treatment to remove endogenous metal ions. Atomic absorption analysis showed that after this treatment less than 0.05 mol of Ca^{2+}/mol of protein C remained.

A Scatchard plot representative of binding of $^{45}Ca^{2+}$ to bovine protein C is shown in Figure 1. These data were obtained by equilibrium ultrafiltration at two different initial protein concentrations, viz., 0.10 and 0.24 mg/mL, and also by equilibrium dialysis at a protein concentration of 2.2 mg/mL. Linear least-squares analysis of the data yields a model of 16.0 ± 2.0 mol of Ca^{2+} binding per mol of protein C, with an average K_D of (8.7 ± 1.5) × 10^{-4} M. A replot of the binding data in the form of a Hill plot gives a straight line with a slope of 1.0 (Figure 1, inset).

Since the Paulus ultrafiltration method necessarily results in a much larger protein concentration at the conclusion of the binding experiment, we have evaluated the possibility of aggregation of protein C, at high concentrations, in the absence

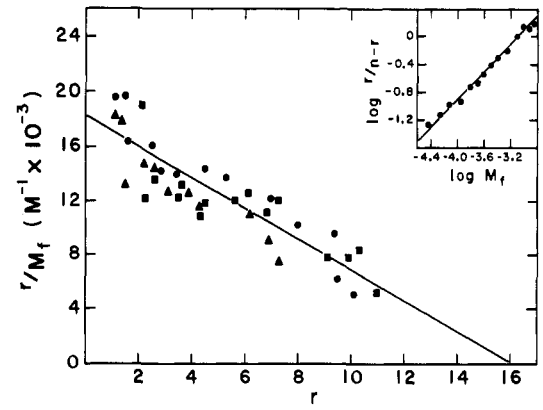

FIGURE 1: Scatchard plot representing the binding of $^{45}Ca^{2+}$ to bovine protein C at 25 °C. The buffer used was 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4. M_f refers to the free Ca^{2+} concentration, and r represents the amount of Ca^{2+} bound to protein C (mol/mol). The line shown drawn through the data points was generated by least-squares linear regression analysis of the data. (●) Binding determined by ultrafiltration at an initial protein concentration of 0.24 mg/mL; (▲) binding determined by ultrafiltration at an initial protein concentration of 0.10 mg/mL; (■) binding determined by equilibrium dialysis at a protein concentration of 2.2 mg/mL. (Inset) Hill plot of the binding data obtained by equilibrium ultrafiltration at an initial protein C concentration of 0.24 mg/mL. n refers to the total number of Ca^{2+} sites on the protein C molecule (in this case, assumed to be 16).

Table I: $s_{20,w}$ Values of Bovine Protein C at Various Concentrations

protein C concn (mg/mL)	Ca^{2+a} (2.5 mM)	$s_{20,w}$ (S)
0.38	−	4.11 ± 0.1
0.38	+	4.18 ± 0.1
1.98	−	4.25 ± 0.1
1.98	+	4.54 ± 0.1
4.28	−	4.00 ± 0.1
4.28	+	4.29 ± 0.1

[a] The buffer employed was either 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4 (−), or 0.05 M Tris-HCl, 0.1 M NaCl, and 0.0025 M $CaCl_2$, pH 7.4.

of Ca^{2+} and in the presence of a sufficient quantity of Ca^{2+} to saturate its sites on the protein. As can be observed from the data of Table I, no significant change in the value of the $s_{20,w}$ of protein C occurs, outside of normal regression, over a protein concentration range of 0.38–4.28 mg/mL, in the presence or absence of Ca^{2+}. This latter protein concentration would be approximately the final concentration in the equilibrium ultrafiltration experiments. It should be noted that a maximum in the $s_{20,w}$ of protein C occurs at approximately 2.0 mg/mL of protein C in the presence of 2.5 mM Ca^{2+}. This effect, also noted in the case of bovine prothrombin (Jackson et al., 1979), cannot reflect concentration-induced protein aggregation since the maximum $s_{20,w}$ value is not of sufficient magnitude to suggest dimer formation and also since the value decreases upon further increase in protein concentration. Also, from the data in Table I, it would appear that the values of the $s_{20,w}$ of protein C, at each protein concentration, in the presence of Ca^{2+} are slightly larger than those in the absence of Ca^{2+}. However, the $s^0_{20,w}$ in each case is 3.9 ± 0.1 S, indicating that the differences observed at each protein concentration reflect dissimilar regressions of the $s^0_{20,w}$ with protein concentration.

The binding isotherm of $^{45}Ca^{2+}$ to activated protein C is represented by the Scatchard plot of Figure 2, using equilibrium ultrafiltration to generate the data points. Two different

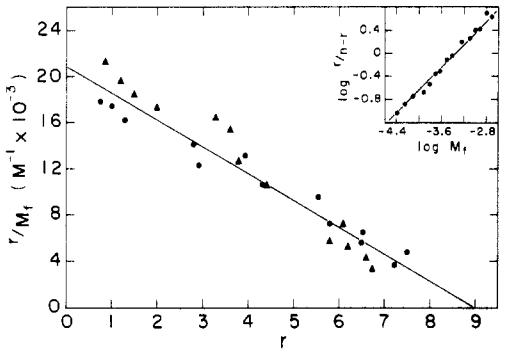

FIGURE 2: Scatchard plot representing the binding of $^{45}Ca^{2+}$ to activated bovine protein C at 25 °C. The buffer used was 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4. M_f refers to the free Ca^{2+} concentration, and r represents the amount of Ca^{2+} bound to activated protein C (mol/mol). The line shown drawn through the data points was generated by least-squares linear regression analysis of the data. (●) Binding determined by ultrafiltration at an initial protein concentration of 0.25 mg/mL; (▲) binding determined by equilibrium ultrafiltration at an initial protein concentration of 0.14 mg/mL. (Inset) Hill plot of the binding data obtained by ultrafiltration at an initial protein concentration of 0.25 mg/mL. n refers to the total number of Ca^{2+} sites on the activated protein C molecule (in this case, assumed to be 9).

Table II: $s_{20,w}$ Values of Activated Bovine Protein C at Various Concentrations

activated protein C concn (mg/mL)	Ca^{2+a} (2.5 mM)	$s_{20,w}$ (S)
0.41	−	4.10 ± 0.1
0.41	+	4.10 ± 0.1
1.2	−	4.32 ± 0.1
1.2	+	4.39 ± 0.1
7.3	−	4.18 ± 0.1
7.3	+	4.21 ± 0.1

[a] The buffer employed was either 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4 (−), or 0.05 M Tris-HCl, 0.1 M NaCl, and 0.0025 M $CaCl_2$, pH 7.4.

initial protein concentrations, 0.14 and 0.25 mg/mL, were employed. Here, a total of 9.0 ± 1.0 sites, with an average K_D of (4.3 ± 1.1) × 10^{-4} M, are found. A Hill plot of the binding data (Figure 2, inset) yields a straight line with a slope of 1.0. Again, as was the case with protein C, the $s^0_{20,w}$ values of activated protein C suggest that significant aggregation is not occurring in the absence or presence of 2.5 mM Ca^{2+}, at least up to a protein concentration of 7.3 mg/mL (Table II). This latter value of the protein concentration is larger than the final concentration expected in the ultrafiltration experiments. The response of the $s_{20,w}$ of activated protein C to protein concentration and to the presence of Ca^{2+} is similar to that of protein C, and similar interpretations are applicable. The $s^0_{20,w}$ value calculated for activated protein C is 3.9 ± 1.0 S in the presence or absence of 2.5 mM Ca^{2+}.

The circular dichroism (CD) spectra of protein C, in the absence of Ca^{2+} and in the presence of 2.5 mM Ca^{2+}, are illustrated in Figure 3. In the absence of Ca^{2+}, the data could be best fit (Chen et al., 1974) to a model in which the protein consists of approximately 9% α helix, 37% β structure, and 54% random structure. A small increase in ellipticity is noted for protein C in the presence of Ca^{2+} at wavelengths below 225 nm. In the case of activated protein C, the spectra do not significantly differ from those of protein C, in the presence or absence of Ca^{2+}, respectively.

The ability of other divalent cations to displace Ca^{2+} from protein C has been examined. A $^{45}Ca^{2+}$–protein C complex consisting of 7.8 mol of Ca^{2+}/mol of protein C has been

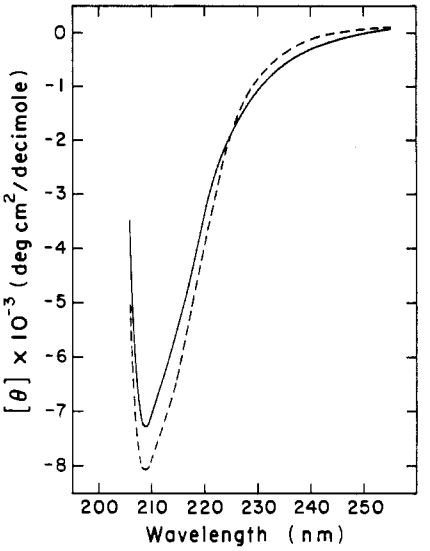

FIGURE 3: Circular dichroism spectra of bovine protein C in the absence (—) and presence (- - -) of 5 mM Ca^{2+}. The plot presents the variation in molecular ellipticity [θ] vs. the wavelength. The buffer used was 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4.

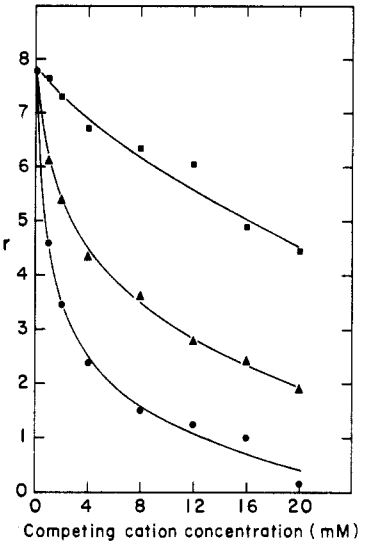

FIGURE 4: Displacement of Ca^{2+} from bovine protein C by other divalent cations. An initial complex of 7.8 mol of $^{45}Ca^{2+}$ bound per mol of protein C was filtrated with varying levels of competing cation, and the amount of $^{45}Ca^{2+}$ remaining bound per mole of protein C (r) was determined by the Paulus ultrafiltration procedure. The competing cations were (●), Mn^{2+}, (▲) Sr^{2+}, and (■) Mg^{2+}.

prepared. In separate experiments, various concentrations of Mn^{2+}, Sr^{2+}, and Mg^{2+} were added to the complex, and the amount of $^{45}Ca^{2+}$ remaining bound to protein C was evaluated by equilibrium ultrafiltration. The data obtained are shown in Figure 4. In the case of Mn^{2+}, full displacement of Ca^{2+} is effected at concentrations of 20 mM Mn^{2+}. Sr^{2+} was less effective than Mn^{2+} in displacement of Ca^{2+}. At levels of 20 mM Sr^{2+}, an average of approximately two sites on protein C remained saturated with Ca^{2+}. Mg^{2+} was least effective of the metal cations tested in that an average of four to five sites on protein C contained Ca^{2+}, at levels of 20 mM Mg^{2+}.

The effect of the above metal cations on the activation of protein C by thrombin is shown in Figure 5. Two important features of the assay system require mention. Activations of protein C by thrombin were carried out in the presence of 0.1 M NaCl. At lower NaCl concentrations, slower activation rates of protein C were observed (75% of the activation rate was obtained in the absence of NaCl). This effect was not specific for Na^+ in that the activation rate in 0.1 M choline

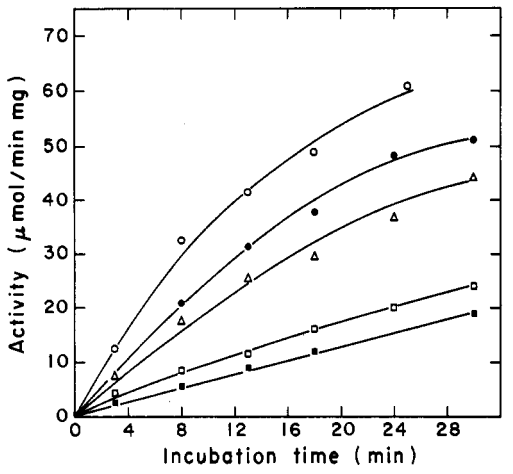

FIGURE 5: Inhibition of the thrombin-mediated activation of bovine protein C by Ca^{2+}. Chelex-treated protein C (final concentration 1.25 mg/mL) and thrombin (final concentration 0.033 mg/mL) were incubated at 37 °C in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4, in the presence of the desired concentration of metal ions. Production of activated protein C was assessed by monitoring the α-*N*-benzoyl-L-arginine ethyl ester (BAEE) esterase activity of small aliquots of the incubation mixture as described under Materials and Methods. An activity of approximately 80 μmol of BAEE cleaved min^{-1} (mg of protein C)$^{-1}$ corresponded to 100% activation of protein C, under the conditions used. The esterase activity due to the added thrombin was negligible. Ca^{2+} concentrations used were (○) no Ca^{2+} (similar results were obtained in the presence of 2 mM EDTA), (●) 0.4 mM, (△) 0.8 mM, (□) 1.6 mM, and (■) 4.5–8 mM.

Table III: K_m and V_{max} Values for the Activation of Protein C by Thrombin at Various [Ca^{2+}]

[Ca^{2+}] (mM)	K_m (μM)	V_{max}[a]
0	1.8 ± 0.1	0.53 ± 0.03
0.5	3.2 ± 0.2	0.45 ± 0.03
1.0	9.6 ± 0.3	0.42 ± 0.03

[a] Expressed as mol of protein C cleaved min^{-1} (mol of thrombin)$^{-1}$.

chloride was identical with that of 0.1 M NaCl. In addition, since it has been shown that the esterolytic and amidolytic activity of activated protein C is influenced by monovalent cations (Steiner et al., 1980), assays of activated protein C were performed in the presence of 0.1 M NaCl (see Materials and Methods for details). The data of Figure 5, obtained after elimination of the above potential difficulties, conclusively show that the activation of protein C by thrombin is inhibited by Ca^{2+} over a range of Ca^{2+} concentrations at which binding of Ca^{2+} to protein C, but not to thrombin, results. Lineweaver–Burk plots of the data at three different Ca^{2+} concentrations, viz., none, 0.5, and 1.0 mM, are presented in Figure 6. The data obtained are summarized in Table III. The major reason for the Ca^{2+}-induced inhibition of the reaction appears to reside in an increased K_m value in the presence of Ca^{2+}. The V_{max} for the reaction decreases only by a small amount in the presence of Ca^{2+}.

The data of Figure 7 illustrate the effect of other divalent metal cations, Mn^{2+}, Sr^{2+}, and Mg^{2+}, on the activation of protein C by thrombin. It appears as though their effectiveness in inhibition of this reaction parallels their ability to displace Ca^{2+} from a Ca^{2+}–protein C complex. None of the metal ions used, at these low concentrations, affected the esterase activity of thrombin.

Finally, the influence of Ca^{2+} on the RVV-X catalyzed activation of protein C has been examined. The activation rate is stimulated by the presence of Ca^{2+}, as can be seen from the data presented in Figure 8. Here, however, stimulation of

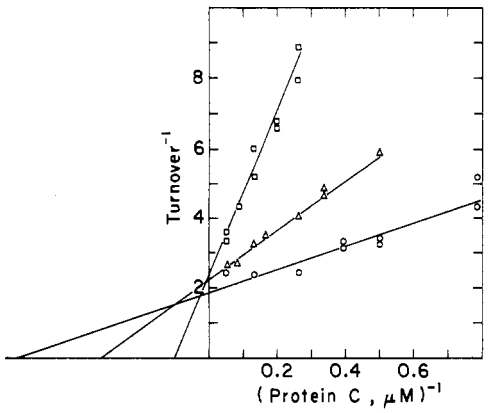

FIGURE 6: Lineweaver–Burk plot showing the effect of varying protein C concentration on the rate of activation by thrombin in the presence of three different Ca^{2+} concentrations. Incubations were conducted at 37 °C in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4, with a final thrombin concentration of 0.006 mg/mL. The rate of production of activated protein C, at each protein C and Ca^{2+} concentration used, was assessed by monitoring the BAEE esterase activity of small aliquots of the incubation mixture as described under Materials and Methods. The linear part of each activation curve was then used to calculate turnover of thrombin [expressed as mol of protein C cleaved min^{-1} (mol of thrombin)$^{-1}$] under each of the conditions used [the activity of fully activated protein C was taken to be 80 μmol of BAEE cleaved min^{-1} (mg protein C)$^{-1}$]. The amount of thrombin used in the activations did not interfere with the assays for activated protein C. The lines shown drawn through the data points were generated by least-squares linear regression analysis of the data. The Ca^{2+} concentrations used were (○) no Ca^{2+}, (△) 0.5 mM, and (□) 1.0 mM.

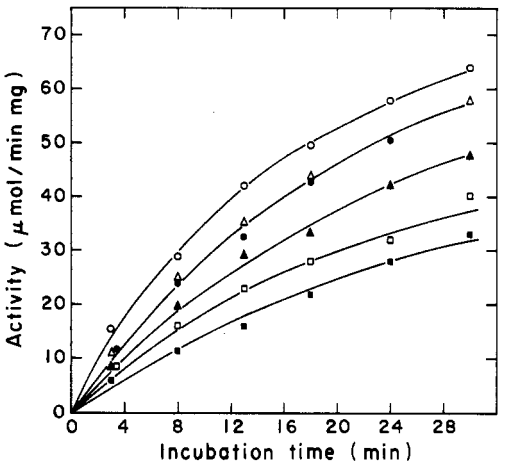

FIGURE 7: Inhibition of the thrombin-mediated activation of bovine protein C by Mn^{2+}, Sr^{2+}, and Mg^{2+}. Incubation and assay conditions were as in Figure 5. The cations added were (○) none, (□) 1.6 mM Mn^{2+}, (■) 9.1 mM Mn^{2+}, (△) 3.2 mM Sr^{2+}, (▲) 9.1 mM Sr^{2+}, and (●) 9.1 mM Mg^{2+}.

the rate occurs over a very narrow concentration range of Ca^{2+} (0.46–0.88 mM). This is reflected in the double-reciprocal plot of the activation data (Figure 8, inset). Regarding the effect of other metal cations on this activation, Mn^{2+}, at its maximal effective concentration, is approximately 15% as effective as an optimal level of Ca^{2+}. Sr^{2+} is similar to Mn^{2+} except that a higher level of Sr^{2+} is necessary to produce the maximum whereas Mg^{2+} does not stimulate the activation by RVV-X, up to levels of 10 mM. The effectiveness of these metal ions parallels, once again, their ability to displace Ca^{2} from its complex with protein C. As was the case with the thrombin-catalyzed activation of protein C, this rate was decreased by 75% in the absence of NaCl when compared to the rate obtained in 0.1 M NaCl. However, the activation rates obtained with 0.1 M NaCl and 0.1 M choline chloride were virtually identical, suggesting that the cation effect is not

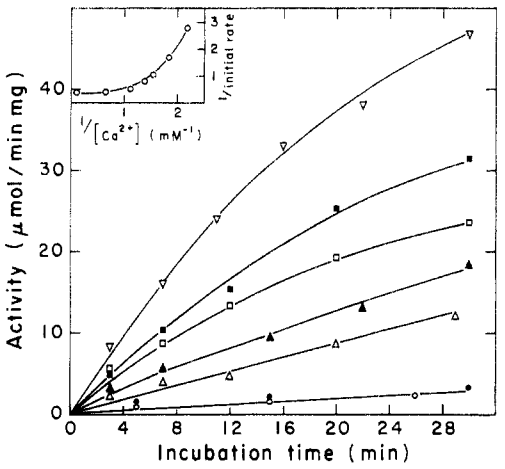

FIGURE 8: Effect of Ca^{2+} concentration on the activation of bovine protein C by RVV-X. Chelex-treated protein C (final concentration 1.3 mg/mL) and RVV-X (final concentration 0.006 mg/mL) were incubated at 37 °C in 0.05 M Tris-HCl and 0.1 M NaCl, pH 7.4, in the presence of the desired concentration of metal ions. The extent of activation of protein C was monitored as described in the legend to Figure 5. Ca^{2+} concentrations were (○) no Ca^{2+}, (●) 0.28 mM, (△) 0.46 mM, (▲) 0.54 mM, (□) 0.64 mM, (■) 0.71 mM, and (▽) 0.88–10 mM. (Inset) Double-reciprocal plot showing the relationship between the initial activation rate (in μmol of BAEE cleaved min^{-1} mg^{-1}) and Ca^{2+} concentration.

specific and likely reflects an enhancement of the activation rate by the ionic strength of the medium.

Discussion

As a consequence of the isolation of protein C, a novel series of blood-coagulation reactions has been formulated. In contrast to other vitamin K dependent coagulation proteins, protein C appears to function as an anticoagulant rather than a procoagulant. As with other γ-carboxyglutamic acid containing blood coagulation proteins, it has been suggested (Stenflo, 1976) that protein C is capable of interaction with Ca^{2+}. This being the case, it was of interest to us to evaluate the mode of binding of Ca^{2+} to protein C and activated protein C and to evaluate the role of Ca^{2+} in the activation of protein C.

Protein C appears to possess a single class of approximately 16 Ca^{2+} binding sites, as determined by the Paulus equilibrium ultrafiltration technique in combination with equilibrium dialysis studies. Since the ultrafiltration method results in a significant concentration of the protein at the conclusion of the experiment (however, the final protein concentration is approximately the same as would be needed for equilibrium dialysis, given the nature of the binding of Ca^{2+}), it is necessary to evaluate whether any concentration-dependent alterations occur in the protein, in the absence and presence of the ligand. In the case of protein C (and activated protein C), analysis of the $s_{20,w}$ of the protein under various conditions (Table I and II) leads us to conclude that no significant changes which would compromise the binding data occur. Additionally, since the Ca^{2+} binding parameters obtained by equilibrium ultrafiltration are virtually identical for two different initial protein concentrations and are in full agreement with the same parameters obtained from equilibrium dialysis, we feel that the model proposed has been established on a strong basis. The binding parameters obtained for Ca^{2+} with bovine protein C closely resemble these parameters, as obtained for human factor IX.[2] Other vitamin K dependent blood coagulation proteins, such as bovine factor IX and bovine factor VII, contain at least two classes of noninteracting Ca^{2+} sites (Amphlett et al., 1978; Strickland & Castellino, 1980) whereas both bovine prothrombin and bovine factor X appear to interact in a cooperative (positive) fashion with Ca^{2+} (Stenflo & Ganrot, 1973; Bajaj et al., 1975).

The differences in Ca^{2+} binding properties of the vitamin K dependent proteins are of interest in view of the considerable sequence homology that exists between the N-terminal regions of these proteins [see, for instance, Katayama et al. (1979)]. These homologous regions contain the full complement of γ-carboxyglutamic acid residues in each protein and are presumably involved in metal ion binding. Other regions of the various proteins must, therefore, be important in specifying the nature of the metal ion binding. It is also notable that the number of γ-carboxyglutamic acid residues per protein molecule is between 10 and 12 for all the vitamin K dependent proteins whose sequences have been determined. These γ-carboxyglutamic acid residues would be able to coordinate a maximum of five to six Ca^{2+} ions if the findings of Sperling et al. (1978), concerning the binding of lanthanide ions to γ-carboxyglutamic acid in solution, are directly applicable to the binding of Ca^{2+} to γ-carboxyglutamic acid in proteins. Since protein C has been shown in this study to have 16 Ca^{2+} binding sites, some of the Ca^{2+} cannot be coordinated through γ-carboxyglutamic acid. Because the sites are equivalent, however, the nature of the coordination is apparently not important.

In the case of activated protein C, fewer Ca^{2+} sites exist, of slightly higher affinity. Since there is only a very small molecular weight difference between protein C and activated protein C, it does not seem likely that the mass removed contains Ca^{2+} binding regions. It is likely, however, that a subtle conformational alteration occurs as a result of removal of the activation tetradecapeptide which leads to the loss of several Ca^{2+} binding sites.

A surprising finding resulting from this study was the inhibition of the rate of thrombin activation of protein C by Ca^{2+}. The cleavage by thrombin of prothrombin to yield prethrombin 1 and fragment 1 has been observed previously to be inhibited by Ca^{2+} (Silverberg, 1979). In that case, the inhibition was caused by an increase in the K_m of thrombin for prothrombin. Regarding protein C, kinetic evidence again suggests that Ca^{2+}-bound protein C possesses a higher K_m for thrombin than does protein C, in the absence of Ca^{2+}. Since thrombin does not interact with Ca^{2+} (Bajaj et al., 1975), it appears as though the decreased K_m is a result of modification of the substrate (protein C) and not the result of formation of a new enzyme. Other metal ions, such as Mn^{2+}, Sr^{2+}, and Mg^{2+}, also inhibit the thrombin-catalyzed activation rate of protein C. The effectiveness of this inhibition appears to correlate with the ability of the cations to displace Ca^{2+} from a Ca^{2+}–protein C complex. Although we cannot rule out the possibility that binding of these metal ions at sites other than Ca^{2+} sites in protein C are responsible for their inhibition properties, it is reasonable to suggest that the Ca^{2+} sites, when occupied by other metal ions, likely lead to the observed inhibition. Thus, it would appear as though the inhibition is a general effect of occupancy of the Ca^{2+} binding sites.

The effect of Ca^{2+} on the RVV-X catalyzed activation of protein C shows opposite behavior to that seen for the thrombin-catalyzed activation. Here, stimulation of the activation rate is observed. Some explanation is needed, however, for the observed nonlinearity of the double-reciprocal plot (Figure 8, inset) of initial activation rate vs. $[Ca^{2+}]$. It is possible that a few cooperative Ca^{2+} sites exist which are

[2] Manuscript submitted for publication.

important for the kinetic effects and that these are not observed by binding techniques due to the large number of noncooperative sites. Another possibility is that formation of the complex of RVV-X and protein C induces cooperative Ca^{2+} binding to protein C (or RVV-X), which is necessary for release of the products of the reaction. Studies in this laboratory[3] demonstrate that RVV-X also binds Ca^{2+}. However, the effect of Ca^{2+} on the enzymatic activity of RVV-X cannot be easily investigated since all of the substrates for the enzyme are proteins that themselves bind Ca^{2+}. Even if there are no cooperative Ca^{2+} sites essential for activation, linearity of the double-reciprocal plot depends on additional considerations. First, the binding of Ca^{2+} on the rate of product release from the complex should be linear, such that the rate of release is proportional to the fraction of the Ca^{2+} sites occupied, and, second, the effect of Ca^{2+} on the rate of product release from the complex should be linear, such that the rate of release is proportional to the fraction of the Ca^{2+} sites occupied. Extensive kinetic studies are necessary to distinguish between the above possibilities.

The effect of other metal ions, e.g., Mn^{2+}, Sr^{2+}, and Mg^{2+}, on the rate of activation of protein C by RVV-X suggests that some degree of specificity exists in this regard. Both Mn^{2+} and Sr^{2+} can substitute, maximally, to only 15% of the optimal stimulation capability of Ca^{2+}. Higher concentrations of Sr^{2+} are required to achieve this effect when compared to Mn^{2+}. On the other hand, Mg^{2+} is not particularly effective in this regard. Again, there is a parallel between the ability of these metal cations to displace Ca^{2+} from a Ca^{2+}–protein C complex and their efficacy in stimulation of the reaction.

In conclusion, we have forwarded a model for the binding of Ca^{2+} to protein C and activated protein C which has been of considerable utility in analysis of the inhibitory effect of metal ions in activation of protein C by thrombin. These same metal sites appear to function in a stimulatory sense in activation of protein C by RVV-X. The inhibition of thrombin activation by Ca^{2+} may be important in controlling the anticoagulant effect of protein C.

References

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 6774–6779.

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1979) *J. Biol. Chem. 254*, 6333–6336.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250*, 2150–2156.

[3] Manuscript in preparation.

Byrne, R., Amphlett, G. W., & Castellino, F. J. (1980) *J. Biol. Chem. 255*, 1430–1435.

Chen, Y.-H., Yang, J. T., & Chau, H. K. (1974) *Biochemistry 13*, 3350–3359.

Comp, P. C., & Esmon, C. T. (1979) *Blood 54*, 1272–1281.

David, G. S. (1972) *Biochem. Biophys. Res. Commun. 48*, 464–471.

Esmon, C. T., Stenflo, J., Suttie, J. W., & Jackson, C. M. (1976) *J. Biol. Chem. 251*, 3052–3056.

Fernlund, T., Stenflo, J., & Tufvesson, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5889–5992.

Furie, B. C., Furie, B., Gottlieb, A. J., & Williams, W. J. (1974) *Biochim. Biophys. Acta 365*, 121–132.

Jackson, C. M., Peng, C. W., Brenckle, G. M., Jonas, A., & Stenflo, J. (1979) *J. Biol. Chem. 254*, 5020–5026.

Katayama, K., Ericsson, L. H., Enfield, D. L., Walsh, K. A., Neurath, H., Davie, E. W., & Titani, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4990–4994.

Kisiel, W. (1979) *J. Clin. Invest. 64*, 761–769.

Kisiel, W., Ericsson, L. H., & Davie, E. W. (1976) *Biochemistry 15*, 4893–4900.

Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16*, 5824–5831.

Lundblad, R. L., Uhteg, L. C., Vogel, C. S., Kingdon, H. S., & Mann, K. G. (1975) *Biochem. Biophys. Res. Commun. 66*, 482–489.

Paulus, H. (1969) *Anal. Biochem. 32*, 91–100.

Schachman, H. K. (1952) in *Ultracentrifugation in Biochemistry*, p 82, Academic Press, New York.

Silverberg, S. A. (1979) *J. Biol. Chem. 254*, 88–94.

Sperling, R., Furie, B. C., Blumenstein, M., Keyt, B., & Furie, B. (1978) *J. Biol. Chem. 253*, 3898–3906.

Steiner, S. A., Amphlett, G. W., & Castellino, F. J. (1980) *Biochem. Biophys. Res. Commun. 94*, 340–347.

Stenflo, J. (1976) *J. Biol. Chem. 251*, 355–363.

Stenflo, J., & Ganrot, P. (1973) *Biochem. Biophys. Res. Commun. 50*, 98–104.

Strickland, D. K., & Castellino, F. J. (1980) *Arch. Biochem. Biophys. 199*, 61–66.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401–410.

Walker, F. J. (1980) *J. Biol. Chem. 255*, 5521–5524.

Walker, F. J., Sexton, P. W., & Esmon, C. J. (1979) *Biochim. Biophys. Acta 571*, 333–342.

Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.

Winzor, D. J., & Scheraga, H. A. (1964) *Arch. Biochem. Biophys. 104*, 202–207.