Activated Protein C Inhibits Platelet Prothrombin-Converting Activity

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Bovine platelets that have been activated by thrombin facilitate the conversion of prothrombin to thrombin in the presence of calcium ions and factor Xa. Activated protein C, a vitamin-K-dependent plasma protein, inhibits this platelet prothrombin-converting activity. The inhibition is time dependent and is not reversed by increasing concentrations of factor Xa. However, factor Xa is able to protect the platelet prothrombin-converting activity from inactivation by activated protein C. The activated protein C causes a parallel loss of factor Xa receptor sites and platelet prothrombin-converting activity. Activated protein C may contribute to the regulation of clotting through inactivation of the platelet prothrombin-converting activity.

PROTEIN C is a vitamin-K-dependent plasma protein. The activated form of the protein (APC) is antigenically and structurally identical to the autoprotrombin II-A described by Seegers. In contradistinction to the vitamin-K-dependent clotting factors, activated protein C is a potent anticoagulant. Seeger's group demonstrated that APC serves as a competitive inhibitor of factor Xa and that inhibition of clotting could be overcome by increasing concentrations of factor V. Kisiel and coworkers found that APC could inhibit factor V in the presence of phospholipid and calcium ions. This finding has been extended by the observation that factor Va, not factor V, is inhibited by APC.

Previous studies on the inhibition of factor Va by activated protein C have examined the effect of APC on purified plasma factor V or Va and the effect of phospholipids on the reaction. Recently, a number of investigators have demonstrated that factor Va is bound either in or on platelets. The platelet factor V may accelerate prothrombin conversion to thrombin more efficiently than factor-V-phospholipid mixtures. This platelet-associated factor V activity can be inhibited by anti-factor-V antibodies. The platelet-factor-V activity can be expressed by freeze-thawing platelet suspensions or by thrombin treatment of the platelets. Expression of platelet factor V activity during platelet activation or aggregation appears to correlate with the formation of a high affinity receptor for factor Xa on the platelet surface.

Since APC is a selective inhibitor of factor Va, we felt that the effects of APC on platelet-mediated prothrombin activation could be used to further characterize the relationship between factor Va activity and the factor Xa receptor. We find that the ability of platelets to catalyze prothrombin activation is blocked by low levels of APC, with a corresponding loss in factor Xa receptor activity.
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MATERIALS AND METHODS

Reagents

Bovine blood was a generous gift of the Wilson Food Corporation, Oklahoma City, Okla. Agarose (Bio-Gel A-15 m, 50–100 mesh) was purchased from Bio-Rad Laboratories, Richmond, Calif. Bovine fibrinogen (type IV), bovine albumin (fraction V), and factor-X-deficient plasma were purchased from Sigma Chemical Corporation, St. Louis, Mo. Other reagents were the best grade commercially available. Phospholipid was prepared by the method of Bligh and Dyer.

Preparation of Proteins

Activated factor X (Xa) was prepared from purified bovine factor XI by activation with the factor X activator from Russell’s viper venom. The factor X activator from Russell’s viper venom was removed from the factor Xa following the activation by ion-exchange chromatography on QAE Sephadex.

Prothrombin was prepared as previously described. Thrombin was isolated following activation of prothrombin with factor Xa, factor Va, phospholipid, and Ca2+ as described previously. The preparations had specific activities of 2400 NIH U/mg.

Protein C was isolated by a modification of the method of Stenflo. Bovine plasma preparation and BaSO4 absorption and elution were performed as described previously. The eluate from 45 liters of plasma was diluted 1:1 with distilled water and passed through a 2 × 5 cm column of agarose-immobilized soybean trypsin inhibitor. The filtrate was absorbed onto QAE Sephadex Q50 (400 ml) equilibrated in 0.2 M NaCl, 20 mM Tris-HCl, 1 mM benzamidine HCl, pH 7.5. The QAE Sephadex was washed extensively and poured into a 5 × 120 cm column that had been packed to a height of 90 cm with QAE Sephadex Q50 in the same buffer. The column was eluted with a linear gradient from 0.28 M to 0.6 M NaCl (20 mM Tris-HCl, 1 mM 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 eluted fractions was followed by sodium dodecyl sulfate gel electrophoresis following disulfide bond reduction. Biologic activity of protein C was determined following activation of the protein C with thrombin. A sample (100 μl) from each column fraction was incubated with thrombin (20 μl, 0.15 mg/ml) for 30 min at 37°C. The thrombin was inhibited by the addition of purified antithrombin III (20 μl, 1.2 mg/ml) and incubation was continued for 30 min at 37°C. The anticoagulant activity of protein C was detected by monitoring the increase in the clotting time in the following assay mixture: 100 μl of citrated bovine plasma, 100 μl of a mixture of purified factor X activator from Russell’s viper venom (30 ng/ml) and cephalin (0.5 mg/ml), 100 μl of a 1:10 dilution of the protein C activation mixture diluted into 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, containing 1 mg/ml bovine serum albumin, and 100 μl of 25 mM CaCl2. Clotting times were determined by measuring the time required for visual fibrin formation following addition of CaCl2. The clotting time of the control was adjusted to 30 sec by diluting the factor X activator with cephalin as required. Only those samples that gave clotting times of 50 sec or longer and contained 25% or more protein C were pooled for subsequent chromatography. The pooled fractions were dialyzed against 50 mM imidazole, 10 mM CaCl2, 1 mM benzamidine HCl, pH 6.0, and chromatographed on a 1.5 × 30 cm column of heparin-agarose. The column was developed at 4°C with a linear gradient of 0–0.6 M NaCl (50 mM imidazole, 10 mM CaCl2, 1 mM benzamidine HCl, pH 6.0, 200 ml/reservoir). The purity of the protein C was assessed by disc gel electrophoresis in the presence and absence of sodium dodecyl sulfate.

Activation of Protein C

Protein C (0.5 mg/ml, 5 mg) in 0.1 M NaCl, 20 mM Tris-HCl, 1 mM benzamidine HCl, 10 mM CaCl2, pH 7.4, was activated at 25°C with the factor X activator from Russell’s viper venom (25 μg/ml final concentration). The time course of protein C activation was followed by measuring the increase of anticoagulant activity in a factor Xa one-stage clotting assay. The assay contained 100 μl factor-X-deficient bovine plasma to which was added sequentially 100 μl cephalin (0.5 mg/ml), 100 μl of 25 mM CaCl2, and 100 μl of factor Xa diluted to give a clotting time of 18–20 sec. The anticoagulant activity in the protein C activation mixture was measured by adding 5 μl of that mixture to the factor-X-deficient plasma prior to the addition of cephalin, CaCl2, and factor Xa. Protein C activation was judged to be complete when maximal prolongation of the clotting time was obtained. This required 15–18 min. Activated protein C was separated from the venom protein by ion-exchange chromatography on a 0.9 × 3 cm column of QAE Sephadex Q50 at 25°C. The column was equilibrated at 25°C in 0.1 M
NaCl, 20 mM Tris-HCl, 1 mM benzamidine HCl, pH 7.4, and following sample application, was washed with 10 ml of this buffer, followed by 15 ml of 0.2 M NaCl, 20 mM Tris-HCl, 1 mM benzamidine HCl, pH 7.4. These steps removed all detectable factor X activator. The activated protein C was eluted from the column with 0.6 M NaCl, 20 mM Tris-HCl, 1 mM benzamidine HCl, pH 7.4.

Protein was monitored by absorbance at 280 nm. The molecular weights and extinction coefficients, respectively, used were as follows: APC, 56,000, $E_{280}^{1%}$ 13.7; prothrombin, 72,000, $E_{280}^{1%}$ 15.5; factor Xa, 45,000, $E_{280}^{1%}$ 12.4.

Platelet Collection and Gel Filtration

Nine parts bovine blood was collected by jugular venipuncture into 1 part 3.8% sodium citrate, pH 5.5. Platelet-rich plasma was obtained by centrifuging the blood for 5 min at 895 g at 25°C. The platelet-rich plasma was drawn off and 8 ml layered on a 3 x 25 cm column of Bio-Gel A-15 m equilibrated with a modified Tangen-HEPES buffer containing 5 mg/ml bovine albumin and gel filtered in the same buffer. The collection and isolation of platelets was in plastic.

Iodination of Factor X

The method of Hunter was used to iodinate factor X. The iodination was performed at room temperature with 1 mCi Na$^{131}$I added to 300 µg factor X in 1.2 ml of buffer. Final chloramine T concentration was 0.71 mM and sodium meta-bisulfite 1.26 mM. Chloramine T was added 3.5 sec prior to the sodium meta-bisulfite. Immediately following iodination, the factor X was chromatographed on a 0.9 x 20 cm Sephadex G 25 column equilibrated with 0.1 M NaCl, 20 mM Tris-HCl, and 1 mM benzamidine, pH 7.4, to remove the unbound $^{131}$I. The $^{131}$I-factor-X was then activated with the factor X activator from Russell’s viper venom. The factor Xa activity resulting was identical to that generated from noniodinated control factor X. Activation of the $^{131}$I-factor-X was also complete as judged by disc gel electrophoretic analysis with reduced samples on sodium dodecyl sulfate containing gels. The resulting specific radioactivity was 600–700 cpm/ng.

Binding Assays

The binding of factor Xa to platelets was determined by centrifugation of the platelets through oil to separate the platelets from unbound factor Xa. The method described by Miletich et al. was employed, except a mixture of 11 parts Dow Corning 702 diffusion pump oil and 2 parts isooctane was used as the oil phase. Platelets were activated directly with thrombin. Prothrombin was not present during the binding studies.

Characterization of the Assay for Platelet Prothrombin-Converting Activity

The ability of thrombin-treated platelets to enhance the conversion of prothrombin to thrombin was measured as follows: gel filtered platelets were diluted to 10$^9$/ml in 0.15 M NaCl, 20 mM Tris HCl buffer, pH 7.4, containing 1 mg/ml bovine albumin and to 100 µl of the diluted platelets, 0.1 U thrombin (10 µl) was added. Then following incubation at 37°C for 45 sec, 100 µl 25 mM CaCl$_2$ was added, and 15 sec later, 100 µl factor Xa at 2 µg/ml was added. The mixture was incubated for 1 min and then 100 µl of prothrombin at 200 µg/ml was added. After 60-sec incubation, 100 µl fibrinogen at 6 mg/ml was added and the clotting time determined. Thrombin units generated were determined by reference to a standard curve. Under these conditions, 5.4–5.8 U of thrombin were generated per minute per 10$^9$ platelets. This platelet cofactor activity will be referred to as platelet prothrombin-converting activity (PPCA).

In this assay, thrombin formation was directly proportional to the length of incubation with prothrombin (Fig. 1). This permitted incubation of the activated platelets with calcium, factor Xa, and prothrombin for periods of time up to 3 min in order to obtain clotting times that could be easily interpreted using the standard curve in cases where PPCA was markedly diminished. Platelet prothrombin-converting activity expressed was presumably dependent on the extent to which the platelet

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*Platelet prothrombin-converting activity (PPCA) is defined as the activity associated with platelets that accelerates the activation of prothrombin by factor Xa. No measurable prothrombin activation occurs in the absence of added factor Xa.
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Fig. 1. Time course of prothrombin activation in PPCA assay. Platelets diluted to $10^8$/ml were treated with thrombin (0.2 U/ml). Following activation, the platelets were incubated with calcium ions (5 mM) and factor Xa (400 ng/ml). Prothrombin (40 ng/ml) was then added and incubated for progressively longer times prior to the addition of fibrinogen. Thrombin formation was assayed at 37°C, as described in Materials and Methods.

factor Xa receptor was filled (Fig. 2). With 30-min incubation of the thrombin-treated platelets with factor Xa in the presence of calcium ions, maximal PPCA was reached at 1–2 ng/ml factor Xa. With 1-min incubation, 10–20 ng/ml factor Xa were needed to reach maximal activity. Therefore, factor Xa at 400 ng/ml was routinely used for PPCA determination with 1-min preincubation of the factor Xa with the platelets and calcium prior to prothrombin addition. The supernatant from thrombin-activated platelet suspensions centrifuged at 10,000 g for 5 min contained no measurable PPCA. Freeze-thawing the gel-filtered platelets, as described by Osterud et al., resulted in the generation of 5.4–5.8 thrombin U/min/10^8 platelets with 400 ng/ml factor Xa.

RESULTS

Effect of APC on Platelet Prothrombin-Converting Activity

Activated protein C was incubated with thrombin-activated platelets in the presence of calcium prior to the addition of factor Xa and subsequent measurement of thrombin-generating activity (Fig. 3). Loss of PPCA activity was dependent both on APC concentration and on the length of incubation. The rate of inactivation decreased after a rapid initial loss of PPCA activity over the first 10–15 min. Nonthrombin-treated platelets were also treated with the same concentrations of

Fig. 2. Effect of length of incubation of factor Xa with activated platelets on thrombin generation in the PPCA assay. Factor Xa at the concentrations indicated was incubated at 37°C with activated platelets for either 1 min (○) or 30 min (■) in the presence of calcium prior to prothrombin addition. Factor Xa concentrations ranged from 0.1 ng/ml to 200 ng/ml, prothrombin 40 μg/ml, thrombin 0.2 U/ml, calcium 5 mM.
Fig. 3. The time-dependent inactivation of platelet prothrombin-converting activity by activated protein C. Activated protein C was added to activated platelets at 0 ng/ml (*), 0.1 ng/ml (●), 1 ng/ml (□), 10 ng/ml (●), 100 ng/ml (+), 1 µg/ml (○), and 10 µg/ml (△) at 37°C for the times indicated prior to the addition of factor Xa (400 ng/ml) for 1 min and then the sequential addition of prothrombin and fibrinogen. Dipropylfluorophosphate-inactivated APC was also incubated with activated platelets at 100 ng/ml (■). Protein concentrations are as indicated in Fig. 1.

APC for the same periods of time prior to thrombin activation. PPCA activity did not show a time-dependent decrease during incubation of nonthrombin-treated platelets with APC (data not shown). Determination of factor V activity associated with the activated platelets using tissue thromboplastin and factor-V-deficient plasma showed that platelet factor V activity and PPCA were inhibited at similar rates in the presence of APC (data not shown).

Effect of Factor Xa Concentration of APC Inhibition of PPCA

The addition of increasing concentrations of factor Xa to activated platelets in the absence of APC showed maximal thrombin generation of 5.8 thrombin

Fig. 4. Effect of increasing factor Xa concentration on the platelet prothrombin-converting activity generated by platelets treated with activated protein C. Activated platelets were treated with 0 ng/ml APC (●) or 10 ng/ml APC (■) for 1 min in the presence of calcium prior to incubation with factor Xa for 1 min and then sequential addition of prothrombin and fibrinogen. Factor Xa concentrations ranged from 0 to 400 ng/ml. Thrombin, calcium, and prothrombin concentrations are as in Fig. 2.
Protection of Platelet Prothrombin-Converting Activity by Factor Xa

Factor Xa is thought to bind tightly to the platelet factor V. To determine if factor Xa could protect the platelet prothrombin-converting activity from inactivation by the activated protein C, the order of addition of APC and factor Xa was reversed so that factor Xa was added first (Fig. 5). The presence of factor Xa slowed the rate of inhibition of platelet prothrombin-converting activity by APC. When 200 ng/ml factor Xa were added to the activated platelets prior to addition of APC, only 11% of original PPCA activity was lost after 4-min incubation with the APC, whereas 59% of the initial PPCA was lost with the platelets that had not been incubated with factor Xa prior to the APC. The protection of PPCA by factor Xa appeared to saturate at about 20 ng/ml factor Xa; factor Xa concentrations of 20 ng/ml and 200 ng/ml afforded the same degree of protection. Factor Xa at 2 ng/ml resulted in PPCA only slightly higher than the platelets not receiving factor Xa. Factor X did not protect the platelet prothrombin-converting activity from APC.

Effect of Activated Protein C on Factor Xa Binding

The binding of $^{125}$I-factor-Xa to gel-filtered bovine platelets is shown in Fig. 6. The nonspecific binding was measured by including a 100-fold excess of unlabeled factor Xa in a parallel platelet incubation. The corrected factor Xa binding was obtained by subtracting the second curve from the first and was saturated between
2.0 and 4.0 ng $^{125}$I-factor-Xa/ml at $10^8$ platelets/ml. Analysis of a Scatchard plot of the data from three sets of experiments (Fig. 6, inset) indicated an association constant of $1.2 \times 10^{10} M^{-1}$ and 300 molecules of $^{125}$I-factor-Xa bound per platelet were calculated. These values are in agreement with those of Dahlback and Stenflo, which were $2.8 \times 10^9 - 1.0 \times 10^{10} M^{-1}$ and 290–420 molecules/platelet, respectively.\(^{11}\)

To determine if inhibition of PPCA correlated with loss of factor Xa receptor sites on the platelet, the ability of platelets treated with APC to bind factor Xa was measured (Fig. 7). Platelets were diluted to a final concentration of $10^8$/ml and were incubated with 1 U/ml thrombin for 3 min at 25°C. Following this, activated protein C was added at concentrations from 0 to 20 ng/ml. The activated protein C and platelets were incubated for 30 min at 25°C. Then $^{125}$I-factor-Xa was added
and incubated for an additional 30 min at 25°C. The specific factor Xa binding was then measured. There was a progressive loss in factor Xa binding, with increasing concentrations of activated protein C. Activated protein C, which had been inactivated with diisopropylfluorophosphate, had no effect on factor Xa binding. Platelet prothrombin-converting activity also decreased progressively with the loss in factor Xa binding (Fig. 8) and the loss in PPCA paralleled the loss in Factor Xa binding. Incubation of the platelets with the diisopropylfluorophosphate-treated activated protein C did not result in a loss of platelet prothrombin-converting activity.

DISCUSSION

Activated protein C has been shown previously to inhibit plasma factor Va activity.6,7 This study demonstrates that the platelet prothrombin-converting activity can also be inhibited by activated protein C. The ability to inhibit PPCA may be important, if, as suggested by Miletich and Majerus, platelet-associated factor V activity is sufficient to facilitate normal or near normal prothrombin activation.10 Our findings indicate that APC can play a role in regulating both plasma and platelet-mediated prothrombin activation.

The ability of APC to inactivate platelet prothrombin-converting activity is consistent with the idea that factor Va, or a protein similar to factor Va, is responsible for much of this platelet activity. If factor Va constitutes the factor Xa receptor, then it would seem likely that inactivation of the cofactor activity would correlate with loss of factor Xa receptor activity. Our results indicate that the high affinity receptor for factor Xa is destroyed by activated protein C during the inactivation of platelet prothrombin-converting activity. Increasing concentration of factor Xa does not restore lost PPCA activity, suggesting the action of APC is
not competitive with factor Xa. APC may inhibit PPCA and the binding activity by destroying the binding site in situ on the platelet or by cleaving it from the platelet surface. The factor Xa concentration required for maximal PPCA (Fig. 4) is lower than that required for maximal factor Xa binding (Fig. 7). This may reflect the different conditions in the PPCA and binding assays and is being investigated.

Factor Xa has been found to slow the rate of inactivation of PPCA by APC. The simplest explanation for this observation is that factor Xa binds to a site at, or near, the site at which APC binds. The amount of factor Xa required to saturate this protective function appears to be greater than the amount required to saturate either the receptor or the functional site on the platelet. This could be due to a competitive displacement of factor Xa by APC or a requirement for factor Xa binding to lower affinity sites distinct from the receptor. The idea of competition was investigated directly by studying the ability of inactivated APC (DIP-APC) to displace factor Xa. In these experiments, DIP-APC was found to be ineffective in displacing factor Xa. This indicates either that direct competitive binding is not involved or that the DIP-APC has lost its capacity to bind to this site. Also, a purely competitive protection of the platelet prothrombin-converting activity due to displacement of APC is not consistent with the observation that the protection of the platelet activity did not become complete with increasing concentrations of factor Xa. In direct binding experiments of APC to activated platelets we have yet to demonstrate a high affinity receptor for APC (unpublished data). The possibility that factor Xa protection of PPCA involves binding to secondary sites remains open, but no direct data support this conclusion.

The question of the role of protein C in the inhibition of blood coagulation remains open. It is clear that under conditions where significant quantities of protein C are activated, the protein will function as an inhibitor. At the level of prothrombin activation, activated protein C can clearly inhibit both plasma and platelet-mediated clotting. The effectiveness of APC as an anticoagulant will be dependent on many parameters, including the activation of factor X and the extent of protein C activation.

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