Direct Detection of Activated Protein C in Blood From Human Subjects

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The antithrombotic enzyme, activated protein C (APC) was measured in blood using an enzyme capture assay (ECA). The ECA involved (1) collection of blood into anticoagulant containing a reversible inhibitor of the enzyme, (2) specific affinity capture of the enzyme by an immobilized antibody that does not inhibit the enzyme, (3) removal of the reversible inhibitor by washing, and (4) direct assay of the captured enzyme's amidolytic activity. The ECA for APC used benzamidine for inhibition, anti-PC light-chain monoclonal antibody for capture, and the oligopeptide substrate S-2366 for enzyme assay. The sensitivity of this assay was 5 pmol/L

ACTIVATED protein C (APC) is a natural anticoagulant serine protease that is generated from its vitamin K-dependent plasma precursor, protein C (PC), on enzymatic activation by thrombin.1 The following clinical and experimental findings suggest that APC is an important regulator of thrombosis. The incidence of hereditary PC deficiency among thrombophilic patients2 is higher than in the normal population,3 and many patients have been described with heterozygous PC deficiency and familial thrombophilia. Complete deficiency of PC activity, whether inherited, experimental or acquired, represents a potentially fatal condition. Thrombotic complications of PC deficiency can be controlled with PC or APC replacement1,2,12 or liver transplantation.13 The presence of measurable quantities of APC-inhibitor complexes in plasma samples from normal subjects and from patients with intravascular coagulation, as well as measurements of a PC activation peptide, suggest that the enzyme is generated in vivo. Thus, direct assays for circulating APC and the laboratory diagnosis of APC deficiency in patients would be very useful and important.

Because, at pharmacological doses, APC is an antithrombotic agent that was successfully used in humans17 and nonhuman primates18,19 our goal was to develop a specific assay to determine the circulating concentrations of the enzyme from blood samples. There are several limitations of assaying serine proteases in biological samples. Among these limitations are enzyme-substrate or enzyme-inhibitor interactions subsequent to sample collection, as well as the problems of attaining high sensitivity and a high degree of specificity. For example, the PC zymogen can be activated by trace amounts of thrombin associated with thrombomodulin, and APC is inactivated in plasma by at least four protease inhibitors: protein C inhibitor (PCI), α-antitrypsin (α1AT), α2-antiplasmin (α2AP), and α2-macroglobulin (α2M). Inhibition of APC in vitro would lower the value measured. Also, there is no substrate for APC cleavage which is both specific and easily measured, and previously described assays are not sufficiently sensitive to detect active enzyme in vivo.

Here we describe an enzyme capture assay (ECA) for the specific determination of the concentration of APC in blood, plasma, or any solution. The ECA was developed and extended from previous methods. In the APC ECA described here, the enzyme in the sample is reversibly inhibited at the time of blood drawing using high concentrations of benzamidine to block irreversible inhibition of APC by plasma proteins. PC, APC, and APC-inhibitor complexes are captured from solution using immobilized antibodies, and then the amidolytic activity of the immobilized APC toward a peptide chromogenic substrate is measured. The sensitivity and specificity of the ECA was tested and the assay was used to detect endogenous APC activity in samples from healthy blood donors and to monitor the inhibition of exogenous APC activity in human blood.

MATERIALS AND METHODS

Mono Q-HR5/5 column and CNBr-activated Sepharose 4B (CNBr Sepharose, Pharmacia, Uppsala, Sweden), synthetic peptide chromogenic substrate, <Glu-Pro-Arg-pNA (S-2366), and S-2238, and S-2251 (Kabi Vitrum, Stockholm, Sweden), normal human plasma (NHP), and PC-depleted plasma (George King Biomedical, Overland Park, KS), and 96-well, flat-bottom Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA) were obtained as indicated. Pooled normal baboon plasma was prepared from 0.5 mL aliquots of quick-frozen baboon plasma samples from three animals. Normal pooled plasma (NPP) was prepared as described below. All reagents were the best grade available. Human PC and APC were prepared from factor IX complex concentrate and characterized as described.13,19 Immunoaffinity-purified anti-PC polyclonal antibodies (aPC-Pab) were purified from sheep IgG fraction as previously described. Anti-PC heavy chain monoclonal antibodies (C1-Mab)22 were purified using immunoaffinity chromatography as described for C3-Mab below. The specific immunoaffinity activity of PC18 was 250 U/mg and the APC amidolytic activity was indistinguishable in the ECA described below from that of the plasma-derived APC preparation.
kindly provided by the American Red Cross (Rockville, MD). Buffers and substrates were prepared using sterile water, filtered through 0.1 or 0.22 μm pore-size filters before use, and stored at 0 to 4°C unless otherwise stated. The principles of the ECA are summarized in Table 1.

Immobilization of Anti-PC Monoclonal Antibodies

An IgG fraction containing monospecific anti-human PC light chain murine monoclonal antibodies designated C3-Mab was purified from murine ascites fluid using union exchange chromatography (Mono Q, FPLC, Pharmacia) as previously described.23,25 The Mono Q-purified IgG fraction containing the C3-Mab was immunoadfinity-purified using purified human PC (30 mg) coupled to CNBr-activated Sepharose beads (10 mL). The Mab was adsorbed to the immobilized PC in 0.01 mol/L Tris, pH 7.4, 0.14 mol/L NaCl (TBS), and 0.02% Na-azide, and subsequently eluted using either 5 mol/L Na-thiocyanate in 0.05 mol/L Tris, pH 7.4, 1.0 mol/L NaCl, and 0.02% Na-azide or 0.1 mol/L glycine, pH 2.5, and 0.1 mol/L NaCl. The thiocyanate eluate was dialyzed against 0.05 mol/L Tris, pH 7.4, and 0.5 mol/L NaCl, and the glycine eluate was dialyzed against TBS and stored at −70°C until use. These two antibody preparation methods gave equivalent results. C1-Mab was purified from murine ascites without the Mono Q prepurification step and with glycine elution from the PC-Sepharose column. C1-Mab and C3-Mab recognize the heavy chain and the light chain of human PC, respectively, in Western blots.25 The PC binding properties of the C3-Mab under solid-phase assay conditions were described in a previous report,23 which showed that immobilized C3-Mab binds PC and APC without affecting its interaction with a snake venom activator enzyme (Porcine, American Diagnostica, Greenwich, CT), with PCI, or with the chromogenic substrate S-2366. Microtiter plate wells were coated with 200 to 250 μL of immunoadfinity-purified C3-Mab (50 to 100 μg/mL) in coating buffer, 0.01 mol/L sodium carbonate, pH 9.2, and 0.02% Na-azide, or coating buffer alone for 14 hours at 4°C. The plates were blocked with 1% casein in coating buffer (300 μL/well) for at least 1 hour at 37°C, or overnight at 4°C. The C3-Mab–coated plates were washed and stored at 4°C. Treatment of the C3-Mab–coated, casein-blocked microplates with irreversible serine protease inhibitors (diisopropylfluorophosphate [DFP] or p-amidinophenylmethyl sulfonyl fluoride [pAPMSF]) significantly decreased background amidolytic activity derived from either the C3-Mab or the casein (data not shown). Therefore, in experiments that were designed to detect APC activity in plasma samples from normal donors, the blocked C3-Mab microplates were treated with 250 μL/well of either DFP (10 μg/mL in 0.14 mol/L NaCl), or pAPMSF (10 μg/mL in 0.1 mol/L sodium acetate, pH 6.0) for 30 minutes. The wells then were washed with dilution buffer of 0.05 mol/L HEPES, pH 7.24, 0.2 mol/L NaCl, 0.05 mol/L benzamidine, 0.02 mol/L EDTA, 0.4% casein, 0.6% ovalbumin, 2% bovine serum albumin (BSA), 0.04% Na-azide, and 0.05% Tween-80 and stored at 4°C with wells containing 300 μL washing buffer of 0.02 mol/L Tris, pH 7.4, 0.15 mol/L NaCl, 0.02 mol/L EDTA, 0.02% Tween 20, and 0.02% Na-azide for no longer than 1 month.

Sample Preparation

Inhibition of APC. The effect of 0.03 mol/L benzamidine on the inhibition of purified human APC by protease inhibitors in plasma was examined using the ECA. APC (10 μL of 0.21 ng/mL) was added to 190 μL of dilution buffer or citrated plasma with or without benzamidine at 37°C and the inhibition of APC was assessed by measuring the residual APC activity in the reaction mixture at various time points, 10 μL aliquots were withdrawn and diluted into 200 μL of dilution buffer at 0°C to minimize further inhibition of the enzyme. The diluted samples were then assayed in the ECA. The inhibition of exogenously added APC (19 ng/mL) was also measured in studies where APC was added to plasma that was prepared from nine parts of blood drawn into one part of either 0.11 mol/L trisodium citrate or citrate containing 0.3 mol/L benzamidine. In controls, APC in dilution buffer (9.8 ng/mL) was treated similarly to the plasma mixtures and the APC activity was determined using the ECA.

Standards. Standards for quantitative measurement of APC in various samples were prepared using purified human plasma-derived APC or the endogenous APC in normal pooled plasma. Standard serial dilutions of purified human APC9,19 in the range of 38 ng/L to 10 mg/L were made in dilution buffer, and aliquots were frozen in liquid nitrogen and stored at −70°C. These standards were used in each separate ECA microtiter plate for determining the APC amidolytic activity in samples. Standards containing exogenously added purified APC (0.01 to 2.0 μg/mL) were also prepared in normal human and baboon plasma and in PC-depleted plasma in the presence of 0.03 mol/L benzamidine with the purpose of testing the PC antigen binding capacity of the C3-coated wells and the recovery of APC. Normal plasma standards were prepared from the venous blood of nine healthy volunteers. Blood samples of 1.8 mL were drawn directly into a 0.2 mL anticoagulant mixture of 0.3 mol/L benzamidine, 0.13 mol/L trisodium citrate, 0.1 mol/L HEPES, pH 6.8, and 0.02% sodium azide. Then the plasma samples were pooled. Aliquots of plasma dilutions in the range of 6.125% to 150% were made from the pool of plasma samples (NPP) and were used as standards in the ECA for determining circulating levels of APC in humans. The aliquots of NPP standards were stored at −70°C.

Effect of citrate. The ECA was used to study inhibition of APC activity in citrated blood in vitro. Venous blood samples of 4.5 mL from seven healthy volunteers (three females, four males) were drawn using a 19-gauge winged infusion set (Surflo, Terumo, Tokyo, Japan) into siliconized glass tubes (Sigmacoat; Sigma, St Louis, MO) containing 0.5 mL 0.05 mol/L Tris, pH 7.4, and 0.14 mol/L trisodium citrate at 37°C without the use of tourniquets or syringes in order to minimize prothrombin activation. The hematocrit was not determined. Immediately after blood drawing, 0.06 mL of APC (0.21 mg/mL) was added to the blood and gently mixed for a few seconds. Subsequently, 0.45-mL aliquots were taken at various times (0 to 50 minutes) into 0.05 mL anticoagulant containing 0.3 mol/L benzamidine and 0.14 mol/L citrate, pH 6.8, and stored in an ice bath to minimize further inhibition of the APC. The plasma was prepared by centrifugation (3,000g, 3 minutes, 4°C) and was stored frozen at −70°C before the ECA.

Detection of APC in blood. For assays of APC activity in plasma from normal blood donors, venous blood (4.5 mL) from 22 healthy, fasting volunteers (14 female, eight male) was collected into blood collection tubes (Vacutainer; Baxter, McGaw Park, IL) by standard venipuncture after informed consent at the General Clinical Research Center at Scripps Clinic. The blood was transferred into a polyethylene centrifuge tube holding 0.5 mL of 0.3 mol/L

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<th>Table 1. Main Steps in the ECA for APC</th>
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<td>1. Coating Anti-PC monoclonal antibodies were immobilized in microplates, and the surface was blocked.</td>
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<td>2. Binding Samples containing APC and benzamidine, a reversible inhibitor of APC, were incubated in the wells for capture of APC antigen.</td>
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<td>3. Washing Unbound sample constituents and the benzamidine were removed by extensive washing.</td>
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<td>4. Developing The amidolytic activity of the captured APC was measured using chromogenic substrate.</td>
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benzamidine, 0.14 mol/L Na-citrate, yielding a final concentration of benzamidine 0.03 mol/L. Then the plasma was prepared by centrifugation within 60 minutes of blood drawing, and frozen and stored at −70°C. Blood samples from healthy volunteers (13 females, six males) were also collected directly into citrate/benzamidine anticoagulant, and plasma was prepared as described above. Blood was similarly obtained from three related individuals, the proband, his son, and his brother, two of whom have type I heterozygous PC deficiency, and their plasmas were stored frozen for APC determinations.

Capture of APC and PC Antigen From Plasma

Standards and plasma samples were further diluted in a dilution microplate using one part sample and 20 to 30 parts dilution buffer. To capture APC and PC antigen, aliquots (50 to 200 μL) from the diluted APC standards, controls, and test samples were transferred to C3-Mab-coated microtiter plates containing an appropriate quantity of dilution buffer to reach or surpass the coating volume and then incubated at room temperature for 1 to 1.5 hours or overnight at 4°C with equivalent results. Each plate had at least one series of purified APC standards besides the unknown samples and NPP standards. Following the adsorption step, the unbound proteins and benzamidine were washed away using washing buffer. The plates with the buffer were vigorously shaken on a rocking table (2 to 5 minutes, 260 rpm), and then the washing buffer was removed by rapping the inverted plates in layers of paper towels for thorough removal of residual liquid. The washing cycle was repeated at least five times within 30 minutes for more complete removal of the benzamidine and especially for removal of contaminating plasma enzymes that could nonspecifically bind to the plate. In case of higher than average background in the C3-Mab–free wells (see below), the washing procedure was repeated.

Measurement of the Amidolytic Activity of the Immobilized Enzyme

Following the last washing cycle, a synthetic oligopeptide chromogenic substrate for APC, S-2366 (0.4 to 1.0 mmol/L in TBS, pH 7.4 or 8.0, 0.05% Na-azide) was added to the wells. The hydrolysis of the substrate was monitored at 405 nm or at a dual wavelength setting of 405/630 nm over time and recorded using Bio-Tek (Winooski, VT) Microplate Autoreaders EL 309 or EL 312. In some experiments, substrates other than S-2366 for APC were used with satisfactory results. When the standard APC concentration range covered more than three orders of magnitude, the plates were prepared with duplicate samples from various normal individuals, as well as NPP standards and purified APC standards. In the final step of the ECA, C1-Mab was present in the chromogenic substrate prepared with duplicate samples from various normal individuals, as well as APC dilutions of APC standards or of 22 normal plasma samples were preincubated with or without immunoaffinity-purified polyclonal anti-PC antibodies (aPC-Pab, 381 μg/mL final concentration in the dilution plate) for at least 20 minutes at ambient temperature. This antibody treatment was performed to prevent recognition and binding of the APC antigen by the C3-Mab–coated wells in the capture step, and/or to inhibit the activity of the APC in the sample. This polyclonal aPC-Pab inhibited the anticoagulant activity of human APC (data not shown).

Specificity of the assay for APC amidolytic activity was further investigated by inhibition of APC amidolytic activity using the monoclonal anti-PC heavy chain antibody, C1-Mab, in the ECA. C1-Mab has been previously shown to bind to the heavy chain of PC and to inhibit APC anticoagulant activity. The ability of C1-Mab to inhibit the amidolytic activity of APC, plasmin, or thrombin was examined by incubation of APC (at 10 nmol/L or 167 nmol/L), plasmin (56 nmol/L), or thrombin (6.6 nmol/L) with increasing concentrations of C1-Mab (0 to 450 nmol/L) in washing buffer for 30 minutes at ambient temperature. Chromogenic substrates (S-2366 for APC, S-2238 for thrombin, S-2251 for plasmin) were subsequently added to the reaction mixtures, and the residual amidolytic activities of the enzymes at various C1-Mab concentrations were determined and calculated as percentage of the activity without C1-Mab.

C1-Mab was also tested in the APC-ECA for its ability to block the C3-Mab–captured APC amidolytic activity. Microplates were prepared with duplicate samples from various normal individuals, as well as NPP standards and purified APC standards. In the final step of the ECA, C1-Mab was present in the chromogenic substrate at 40 nmol/L in one well with each sample, while no C1-Mab was present in the duplicate wells. The inhibition by C1-Mab of the amidolytic activity captured by C3-Mab was then measured in the ECA.

Data Analysis

The changes in absorbance (ΔA405/time) were plotted versus the known APC concentrations of the standards to give standard curves. The concentration of APC in the unknown samples was estimated from the average of changes in absorbance of two or more measurements of duplicate samples using the standard curves for the corresponding time points. The values for multiple measurements were expressed as the mean ± SD unless otherwise stated. Comparative or noncomparative analysis of the results was performed by analysis of variance, Student’s t test, Pearson’s correlation coefficient, and linear regression using EPISSTAT, a statistical program package by TL Gustafson (Round Rock, TX). N represents the number of variables, and P the probability. P < .01 was considered statistically significant. The interassay coefficient of variation is given as a percentage. The correlation coefficient and the significance of the regression were calculated for each standard. Only data from plates with APC standards exhibiting Δ A values of greater than .95 and P < .01 were used for further analysis.
ACTIVATED PROTEIN C IN CIRCULATING BLOOD

Fig 1. Benzamidine protection of APC activity from inhibition by protease inhibitors in citrated plasma. The amidolytic activity of APC (0.3 μmol/L) was assayed at various times using the ECA, as described in Materials and Methods, in citrated normal human plasma (NHP) alone (●), in NHP containing 30 mmol/L benzamidine (△), or in dilution buffer containing 30 mmol/L benzamidine (■). No amidolytic activity was detected when plasma samples containing benzamidine and APC were incubated in wells that were not coated with C3-Mab (□).

RESULTS

To assess the ability of the reversible inhibitor, benzamidine (30 mmol/L), to protect APC in NHP from irreversible plasma protease inhibitors, APC was added to NHP in the presence or absence of benzamidine, and residual APC activity was measured over time in aliquots from the reaction mixtures using the ECA. As seen in Fig 1, APC (0.3 μmol/L) activity was progressively neutralized in NHP with 50% inhibition at 37 minutes (r = .996, P < .01). APC was not irreversibly inhibited in NHP containing benzamidine and in dilution buffer. In similar studies, APC at 0.3 nmol/L in citrated plasma was completely protected from irreversible inhibition by 30 mmol/L benzamidine (data not shown). Thus, benzamidine in NHP prevented irreversible inhibition of added exogenous APC by plasma protease inhibitors at 37°C. Therefore, the benzamidine/citrate mixture was used as anticoagulant for sample collection based on the assumption that it would protect endogenous APC in fresh blood samples from irreversible inhibition in plasma.

The sensitivity and the linearity of the ECA for APC is shown in Fig 2 with standard curves for various APC concentration ranges. Hydrolysis of the chromogenic substrate was linear with the concentration of APC at different assay times (r > .95, P < .01 for each displayed standard curve). Figures 2A and B present APC standard curves obtained using immunoaffinity-purified C3-Mab for coating plates. The development of statistically significant changes in absorbance at very low APC concentrations, as compared with control wells with no APC added (Fig 2A) or with no capture antibody coating (data not shown), employed prolonged incubations at 4°C; however, shorter incubation times at 37°C also generated similar results. Thus, the ECA for APC was sensitive to at least 38 pg/mL of purified APC, and linear standard curves covering 38 pg/mL to 10 μg/mL APC were generated.

The specificity of this ECA for APC was assessed in a variety of ways. First, when wells were blocked but not coated with C3-Mab and were incubated with plasma containing either 0.3 μmol/L APC or 0.3 nmol/L APC, no amidolytic activity over background was detected in the ECA, suggesting that the C3-Mab was required to capture the amidolytic activity of purified APC and of the enzyme(s) in plasma. Second, before the ECA, APC was exposed to polyclonal anti-PC antibodies (aPC-Pab) that neutralize APC anticoagulant activity. The hydrolysis of S-2366 was reduced by greater than 95% when APC in a concentration range of 0.038 ng/mL to 5 pg/mL was preincubated with aPC-Pab (P < .01 for all measurements) (Fig 2B). Assuming that the aPC-Pab either prevented APC binding to the C3-Mab capture antibody or neutralized the amidolytic activity of APC, this indicated that the measured amidolytic activity in the ECA was due to APC. Third, a monoclonal antibody previously reported to inhibit APC25 was tested for its ability to quench purified APC amidolytic activity in fluid phase by measuring APC activity following preincubation with varying C1-Mab concentrations as described in Materials and Methods. For the C1-Mab concentrations tested, the inhibition of APC exhibited a negative linear correlation with antibody concentra-

Fig 2. APC standard curves in the ECA. (A) Amidolytic activity toward S-2366 substrate observed in the ECA at 4°C for APC in a wide concentration range. The change in absorbance at 405 nm was determined for the indicated times. (B) APC standards (●) and effect of pretreatment of APC standards with polyclonal anti-PC antibodies (aPC-Pab) before the ECA (△).
was present in the final step with the S-2366 substrate, APC infra). Plasma was assessed in a variety of ways. First, microplate low as 1:20,000 of plasma PC zymogen levels.

ECA for purified APC is indeed due to APC and not to any contaminants, and, moreover, that the C1-Mab is a useful specific inhibitor of immobilized APC in the ECA (vide infra).

To determine if endogenous APC was detectable in normal plasma, dilutions of NPP without addition of exogenous APC were assayed by the ECA (Fig 3A). The observed amidolytic activity was linear with plasma dilution down to 6% of NPP, with a correlation coefficient of .998 (P < 10⁻⁵). To quantitate APC in NPP, a careful comparison of NPP dilutions to standard curves was made. Figure 3B shows that the amidolytic activity of 12.5%, 25%, 50%, 100%, and 150% NPP dilutions corresponded to 0.30 ± 0.052, 0.60 ± 0.096, 1.19 ± 0.13, 2.27 ± 0.080, and 3.16 ± 0.22 ng/mL of a purified plasma-derived human APC standard, respectively (N = 6 for each, r = .977, P < 10⁻⁶). Thus, APC amidolytic activity of 100% normal pooled plasma corresponded to 2.27 ng/mL APC, equivalent to 38 pmol/L purified APC. The interassay coefficient of variation of the ECA was 6.4%. Since NPP contains 4.3 μg/mL PC zymogen (70 nmol/L), the APC ECA was sensitive for enzyme concentrations as low as 1:20,000 of plasma PC zymogen levels.

The specificity of the ECA for endogenous APC in plasma was assessed in a variety of ways. First, microplate wells that were not coated with the capturing C3 antibody did not exhibit significant amidolytic activity from 100% NPP. Second, the anti-PC monoclonal antibody, C1-Mab, that inhibits purified APC in the ECA (vide supra) was used in the ECA for various dilutions of normal plasma (Fig 4). When C1-Mab was present in the substrate solution, the amidolytic activity observed in the ECA for APC was reduced by greater than 97% (Fig 4), showing that the observed activity of pooled normal plasma is due to APC. To verify further that the capture antibody, C3-Mab, removes APC from plasma, treatment of plasma previously depleted of PC containing 5.0 or 0.05 mg/L of exogenously added APC by C3-Sepharose was performed for 30 minutes as described in the Methods. This treatment reduced the amidolytic activity of the plasma samples by greater than 95% as measured using the ECA. Negative controls that showed no decrease in amidolytic activity of exogenously added APC included treatment with Sepharose that was coupled to purified high molecular weight kininogen or blocked with monoethanolamine. Control samples to which APC was not added had no significant amidolytic activity over background in the ECA (P > .1, N = 3 for both controls and C3-Sepharose treatment samples). Thus, immobilized C3-Mab removed exogenously added APC from plasma samples and removed the amidolytic activity observed in the ECA. These specificity studies lend further support to the argument that the ECA is specific for APC.

Experiments were performed using the ECA to measure the range and variability of APC activity in plasma samples prepared from the venous blood of 41 healthy blood donors and to demonstrate that the ECA is specific for APC. The plasma levels of APC are shown in Fig 5A and B. The amidolytic activity in the experiment shown in Fig 5A averaged 27.3 ± 3.2 mAU/10 days in 22 samples compared with a background of 6.9 ± 1.3 for no plasma or 7.3 ± 0.8 for wells that were not precoated with the capturing C3-Mab before incubation with plasma samples. The APC levels ranged from 70% to 143%, averaging 104.9% ± 19.6% (SD). Similar results, i.e., APC activity in the range of
Fig 4. Inhibition of APC by C1-Mab in the ECA. Captured enzymatic activity of a normal pooled plasma standard in the presence (A) or in the absence (B) of C1-Mab (6.4 μg/mL) in the chromogenic substrate. Background absorbance changes observed for samples lacking APC on C3-lab were subtracted from the observed values.

64% to 131% (Fig 5B), were obtained in a separate series of experiments with 19 different normal adult subjects whose blood was collected directly into the citrate/benzamidine mixture. The specificity of the ECA for APC in the plasma of each of these 41 subjects was demonstrated using two different antibody preparations. First, for the subjects studied in Fig 5A, pretreatment of the plasma samples with the sheep polyclonal αPC-Pab resulted in a significant decrease in the observed APC amidolytic activity in the ECA, similar to the inhibition observed for incubation of purified APC standards with αPC-Pab (vide supra). In the experiment shown in Fig 5A, the observed amidolytic activity for 20 of the 22 samples following this treatment was within the mean background ± 2 SD, suggesting that APC activity was almost completely blocked or removed by αPC antibodies. For samples I and K, in the first experiment performed, the antibody treatment did not reduce the observed enzyme activity to background level. Repeated antibody treatment of samples I and K with αPC-Pab in a subsequent experiment, as shown in Fig 5A, reduced APC activity to low or background activity levels. Second, for the subjects studied in Fig 5B, the effect of the presence of the inhibitory monoclonal antibody, C1-Mab, in the chromogenic substrate step of the APC ECA is shown. The antibody reduced the observed amidolytic activity in all 19 samples to background level. Since this monoclonal antibody specifically inhibits purified APC and APC in pooled plasma (vide supra and Fig 4), these results suggest that the ECA is detecting APC in normal subjects and that at least 95% of the observed amidolytic activity (Fig 5B) is due to APC. These results demonstrate the presence of APC in the plasma of 41 normal subjects with an observed range of 64% to 143% of a pool of normal plasmas.

The APC levels of members of a family with heterozygous PC deficiency were measured. The proband with a history of venous thrombosis whose antigenic PC was 50% and functional PC level was 44% had 42% APC activity in the ECA. The asymptomatic brother of the proband with 61% PC antigen and 50% functional PC had 60% circulating APC levels in the ECA. A son of the proband with 110% PC antigen and 102% functional PC had 126% circulating APC. Both the proband and his brother were identified as being heterozygous for a point mutation in the PC gene (Tsay, Greengard, and Griffin, unpublished data). These results show that two related individuals with half-normal levels of the PC zymogen have half-normal levels of circulating APC and suggest that the level of APC under basal conditions in the absence of hemostatic stress may be proportional to the circulating PC level.

To study the inhibition of APC in vitro in whole blood from different individuals, APC was added to fresh venous blood from seven individuals and the inhibition of APC was assessed by measuring residual activity in the blood throughout 20 minutes using the ECA. The results demonstrated that exogenous APC activity was inhibited as a function of time in citrated whole blood in vitro (Fig 6). The $t_{1/2}$ of APC

Fig 5. APC amidolytic activity in plasma samples from 41 healthy blood donors as measured by the ECA. The APC amidolytic activity in the ECA is displayed as cross-hatched bars for each letter coded (A) or numbered (B) plasma sample in percentage of the pooled plasma standard. Solid bars represent the APC amidolytic activity observed for specificity controls: (A) the activity of the corresponding individual plasma samples following polyclonal anti-PC antibody (αPC-Pab) pretreatment; (B) the activity measured in the presence of monoclonal anti-PC heavy-chain antibodies (C1-Mab) that are known to inhibit APC activity. For details see text.
activity calculated from results of measurements using samples taken from whole citrated blood averaged 27.1 ± 10.3 minutes, with a range of 14 to 44 minutes. Assuming there is a large excess of plasma APC inhibitors, the average pseudo-first-order rate constant for the inhibition can be calculated as $3.72 \times 10^{-4} \text{s}^{-1}$. The results demonstrate significant individual differences in the inhibition of pharmacologic concentrations of exogenous APC in citrated whole blood.

**DISCUSSION**

The zymogen form of the antithrombotic plasma factor, PC, has been assayed using various antigenic and functional assays.\(^4,23,33-36\) Inactive byproducts of PC activation such as the PC activation peptide\(^{10}\) and APC-inhibitor complexes have been measured in order to infer PC activation.\(^14,15,21,22,37,38\) Because APC is a powerful anticoagulant, the concentration of exogenously added APC at pharmacologic levels was successfully determined using clotting assays of blood drawn from animals receiving APC infusions.\(^18,24\) However, clotting assays of plasma containing APC are not specific for APC and are not linearly sensitive to very low or very high APC concentrations, ie, APC less than 0.05 mg/L or greater than 2.0 mg/L, and clotting assays have to be performed immediately following blood sampling to avoid progressive inactivation of the APC by plasma protease inhibitors. We recently showed that amido lytic assays of APC captured from frozen plasma samples using the ECA approach were in excellent agreement with APC assays based on clotting assays that were performed within 5 minutes of blood drawing.\(^15,24\) Clinical and experimental data on PC deficiency support the hypothesis that a deficiency of APC, whether due to impaired PC activation, PC zymogen deficiency, or enhanced APC inhibition, may result in a prethrombotic state. Thus, the direct detection of basal levels of circulating APC and of its deficiency, as well as the measurement of the inhibition of APC, could be useful for diagnosis of a prethrombotic state. This rationale led us to methods to quantitate low levels of APC in plasma and demonstrate that APC is a normal component in the circulating blood of 41 normal subjects.

Since APC activity is progressively inhibited in blood or plasma, special procedures were introduced in blood drawing and sample processing to avoid underestimation of the circulating levels of APC. In the ECA presented here, protection of APC from plasma protease inhibitors and prevention of in vitro generation of APC were achieved using a combination of citrate with high levels of benzamidine, a potent reversible inhibitor of trypsin-like proteases including APC and thrombin. Benzamidine at 30 mmol/L prevented any detectable irreversible inhibition of exogenously added APC by serine protease inhibitors in citrated plasma and it probably also inhibited any activation of PC ex vivo in blood by thrombin or trypsin-like enzymes. Mixing of blood with the inhibitors was optimally achieved by drawing blood directly into tubes containing the citrate/benzamidine anticoagulant, and it was assumed that no significant amount of APC was inhibited or generated within the few seconds needed for blood to mix with the inhibitors.

The sensitivity and specificity of the ECA for APC was established. The use of immobilized, immunoaffinity-purified, high-affinity C3-Mab against PC allowed specific capture and separation of the various forms of PC antigen from other plasma components. Vigorous washing of the C3-Mab-coated wells removed interfering enzymes or constituents of the plasma specimen, including benzamidine. The immobilized C3-Mab does not inhibit the amido lytic activity of APC\(^{23}\) and the antibody-bound enzyme catalyzes the cleavage of the chromogenic substrate after benzamidine is removed by washing. Because the maximum PC antigen (20 ng) in a well was less than the binding capacity of the C3-Mab-coated well (at least 40 ng),\(^{23}\) the APC standard curve prepared in dilution buffer or NHP or PC-depleted plasma were parallel or indistinguishable in the ECA (data not shown). Sample and standard APC concentrations in the 0.05 to 10 μg/mL range yielded detectable color development of substrate within minutes, while lower APC concentrations in the 0.05 to 50 ng/mL range generated change in absorbance of greater than 5 mOD over background over hours to days. Naturally, any method or modification that would capture more enzyme, eg, by increasing the surface and the sample volume, could increase the sensitivity. The use of fluorogenic substrates and suitable equipment could also enhance the sensitivity of the ECA. The current ECA is suitable for quantitatively determining APC levels in plasma greater than 0.3 ng/mL (5 pmol/L), or approximately 0.008% of the normal average PC zymogen level (4.3 μg/mL, 70 nmol/L).\(^4\)

A variety of tests of capture and assay specificity were performed. The following results indicate that the APC ECA measured APC activity in the samples. When the capturing C3-Mab was immobilized on Sepharose, it adsorbed exogenously added APC from plasma. Microtiter plate wells without C3-Mab coating did not bind measurable amido lytic activity from APC standards or plasma samples with or without exogenously added APC. Pretreat-
ment of plasma samples or of APC standards in dilution buffer or in plasma using immunoaffinity-purified polyclonal sheep anti-PC antibodies inhibited greater than 95% of the amidolytic activity in the ECA. The presence of a monoclonal anti-PC antibody that blocks the APC amidolytic activity inhibited greater than 98% of the amidolytic activity of samples and standards. APC levels in the plasma of heterozygous PC-deficient individuals were proportional to the PC levels in these samples. C3-Mab does not recognize antigens in commercial PC-depleted plasma. These observations provide evidence that the activity observed in the APC ECA is indeed due to APC.

Based on ECA results, the APC amidolytic activity of NPP in the ECA corresponded to the amidolytic activity of 38 pmol/L purified APC. The observed range for APC activity in the 41 samples from normal individuals was 64% to 143% of the average. Because inhibition of APC by plasma is slow in the presence of citrate and benzamidine, the measured APC levels are very likely to represent the in vivo APC levels.

Demonstration of APC enzymatic activity in plasma provides direct evidence for the continuous in vivo activation of the anticoagulant PC pathway and for circulating APC as a normal plasma component. The previous findings in normal subjects of APC:inhibitor complexes and of PC activation peptide suggested that PC activation is part of normal physiology, but gave no indication of whether APC circulates in the fluid phase of blood under normal physiology. The measured 38 pmol/L average APC activity in NPP is in good agreement with differences from the APC-α1-AT complexes and from the activation peptide data. For example, APC-α1-AT complexes average 60 pmol/L in normal plasma, with a reported half-life of 140 minutes in baboons. The PC activation peptide is 1 to 5 pmol/L in human plasma, with a half-life in nonhuman primates of 5 minutes. The reported half-life of APC infused into humans is 22 minutes.

The level of APC might have been expected to show wide variation depending on many factors including the PC zymogen level, factors responsible for APC generation including thrombin generation and thrombomodulin availability, and the inhibition of APC by plasma protease inhibitors. However, the range for APC levels in 41 normal individuals was 64% to 143% of pooled plasma (1 SD = 19%) and was typical of many plasma components, strongly suggesting that the level of circulating APC is biologically regulated. Moreover, the preliminary finding of half-normal APC levels in plasma from two heterozygous PC-deficient individuals suggests that APC levels may be proportional to PC levels.

The ECA for APC also provides a useful approach to study the inhibition of APC, because dilution of the sample into dilution buffer containing benzamidine quenches the inhibition of the enzyme and allows convenient assays at later times. The results for inhibition of APC in citrated blood from seven individuals with an average half-life of 27 minutes were in good agreement with published data on inhibition of APC in citrated plasma. Significant differences in the rate of inhibition of APC by blood from different individuals were observed (Fig 6), and this may have important implications in both the pharmacologic administration of APC and the regulation of endogenous APC activity. Whether unfavorable regulation of endogenous APC levels by plasma inhibitors might be related to increased risks of thrombotic disease remains to be clarified.

It is not known how circulating APC might change in various disease states. We speculate that the release or generation of circulating thrombin would increase endogenous APC. Data from non-human primate experiments indicate that circulating APC levels significantly increase during experimental nonocclusive thrombus formation, and that low-dose thrombin infusions increase circulating APC levels over 100-fold. We also speculate that inherited or acquired deficiency of thrombomodulin would result in a decrease in circulating APC levels. Clinical studies are required to determine whether measuring APC levels in normals or in various disease states will have prognostic or diagnostic value for thrombotic disorders.

In summary, development of the sensitive and specific ECA for APC provides direct evidence for the presence of an active endogenous vitamin K-dependent serine protease, APC, in plasma samples indicating that APC is a normal component of human blood. The principles of the ECA for APC could serve for development of highly sensitive and specific assays for other enzymes that may be present in the circulation. An active site-specific immunoassay for APC was recently described that might be adaptable for APC measurements. The physiologic significance of circulating APC remains to be established, although it is tempting to speculate that it represents an important endogenous circulating anticoagulant that directly reflects the functional balance between the thrombomodulin mechanism for PC activation and the ability of plasma protease inhibitors to neutralize APC. Since a prethrombotic state may represent an unfavorable shift between prothrombotic and antithrombotic mechanisms, it will be interesting to assess whether measurement of either endogenous APC levels or of APC inhibition will be useful in the evaluation of prethrombotic states.

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