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Articles

Inactivation of Human Plasma Kallikrein and Factor XIa by Protein C Inhibitor†

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ABSTRACT: The inhibition of kallikrein and factor XIa by protein C inhibitor (PCI) was studied. The method of Suzuki et al. [Suzuki, K., Nishioka, J., & Hashimoto, S. (1983) *J. Biol. Chem. 258*, 163–168] for the purification of PCI was modified in order to avoid the generation of proteolytic activity and subsequent inactivation of PCI. With the use of soybean trypsin inhibitor, an efficient inhibitor of kallikrein and factor XIa, the generation of proteolytic activity was avoided. The kinetics for the inactivation of activated protein C (APC), kallikrein, and factor XIa by PCI were determined. In the absence of heparin, no inactivation of APC was observed, in contrast to kallikrein and factor XIa, which are inhibited with second-order rate constants of $(11 \pm 4) \times 10^4$ and $(0.94 \pm 0.07) \times 10^4$ M^{-1} s^{-1}, respectively. Addition of heparin potentiated the inhibition of APC [$(1.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1}] and factor XIa [$(9.1 \pm 0.7) \times 10^4$ M^{-1} s^{-1}] by PCI, whereas the inhibition of kallikrein by PCI was unchanged [$(10 \pm 1) \times 10^4$ M^{-1} s^{-1}]. The second-order rate constants for the inhibition of kallikrein or factor XIa by PCI were similar to the second-order rate constants for the inhibition of their isolated light chains by PCI, indicating a minor role for the heavy chains of both molecules in the inactivation reactions. With sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis and immunoblotting, complex formation of APC, kallikrein, and factor XIa with PCI could be demonstrated. APC and kallikrein formed 1:1 molar complexes with PCI. Factor XIa formed 1:1 and 1:2 molar complexes with PCI, indicating that both active sites on the factor XIa molecule can be inactivated by PCI. On the basis of second-order rate constant calculations, PCI would account for 7% of the inactivation of kallikrein and 5% of the inactivation of factor XIa in plasma, indicating that PCI can be physiologically important in the inhibition of both proteinases. Moreover, because of the high second-order rate constants for the inactivation of kallikrein and factor XIa by PCI and the low concentration of PCI in plasma, the PCI activity in plasma could be regulated by these proteinases of the contact system.

Activated protein C (APC)[1] is a vitamin K dependent serine protease that plays an important role in the regulation of blood coagulation (Stenflo, 1976; Kisiel et al., 1977; Kisiel, 1979; Dahlback & Stenflo, 1980; Vehar & Davie, 1980; Marlar et al., 1982, Suzuki et al., 1983b). In 1980, Marlar and Griffin described an inhibitor of activated protein C in plasma, the protein C inhibitor (PCI). PCI has been purified from human plasma by Suzuki et al. (1983a). It is a single-chain glycoprotein with a molecular weight of 57 000. PCI forms a 1:1 molar complex with APC. At the same time a small peptide at the carboxyl terminus is cleaved from PCI, resulting in a modified, inactive PCI (Suzuki et al., 1984). PCI inactivates not only APC but also thrombin and factor Xa. The action of protein C inhibitor is enhanced by heparin (Suzuki et al., 1984) and dextran sulfate (Suzuki, 1985). Recently, the cDNA for human protein C inhibitor has been characterized, and the amino acid sequence of PCI shows a high degree of homology with members of the superfamily of serine protease inhibitors, the serpins (Suzuki et al., 1987).

In this study we describe a purification procedure for PCI based on the method of Suzuki et al. (1983a). Plasma kallikrein and activated factor XI formed complexes with PCI. By use of specific inhibitors for these enzymes, inactivation of PCI during the purification was avoided. The kinetic

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[1] Abbreviations: εACA, ε-aminocaproic acid; APC, activated protein C; BSA, bovine serum albumin; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; M_r, molecular weight; PAA, polyacrylamide; PCI, protein C inhibitor; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminoethane.

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constants and the molecular mechanism for the inactivation of APC, kallikrein, and factor XIa by PCI were determined.

Materials and Methods

Materials. DEAE-Sephadex A-50, heparin–Sepharose, DEAE-Sephacel, CNBr-activated Sepharose, and a mono S column were obtained from Pharmacia (Uppsala, Sweden). Dextran sulfate agarose was prepared as described by Kisiel (1979) and was only used if the binding capacity exceeded 0.15 mg/mL cytochrome *c*. All chemicals obtained were of the best grade available.

Purification of Proteins. All proteins were of human origin. Protein C was purified and activated as described by Koedam et al. (unpublished results). Factor XI and prekallikrein were purified and activated as described before (Bouma et al., 1983; van der Graaf et al., 1982a, 1983a). Kallikrein light chain and factor XIa light chain were purified as described by van der Graaf et al. (1982b, 1983b). Protein concentrations, except for PCI, were determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference. The concentration of PCI was determined with $E^{1\%}_{280nm}$ = 14.1 (Suzuki et al., 1983a).

Assay for Protein C Inhibitor. During the purification the presence of protein C inhibitor was determined with the following assay: Samples were dialyzed against 50 mM Tris, pH 7.4, and 150 mM NaCl. To a 30-μL sample was added 6 μL of 2% BSA (w/v) in Michaelis buffer (28.5 mM sodium acetate, 28.5 mM sodium barbital, pH 7.35, and 116 mM NaCl), 3 μL of heparin (167 units/mL; Organon, Oss, The Netherlands), and 3 μL of APC (40 μg/mL). After 60 min at 37 °C, this mixture was added to 0.5 mL of 0.4 mM S2366 (KabiVitrum) in 50 mM Tris, pH 7.8, and 150 mM NaCl. The change in absorbance at 405 nm was followed continuously by using a Beckman Model 3600 double-beam spectrophotometer. The amount of PCI was related to the amount of PCI in a plasma pool of 40 healthy donors, 1 unit being the amount present in 1 mL of plasma. The calibration curve of this assay was linear between 0.25 and 1 unit/mL.

Purification of Protein C Inhibitor. Fresh frozen plasma (1.5 L) was obtained from the local blood bank and thawed at 37 °C. To the plasma were added 100 mg/L SBTI, 15 mM benzamidine, 8 mM εACA, 1 mM PMSF, and 1.5 mM DFP. After incubation for 60 min at room temperature, the plasma was dialyzed at 4 °C against buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM benzamidine, 5 mM εACA, and 1 mM PMSF). All following steps, except FPLC, were performed at 4 °C. The dialyzed plasma was applied to two columns of DEAE-Sephadex (5 × 30 cm, 750 mL of plasma each, 175 mL/h) previously equilibrated in buffer A. After application of the plasma, the columns were washed with 250 mL of buffer/column. The flow-throughs of the two columns were combined, and 60 mg/L SBTI was added before application to a column of heparin–Sepharose (5 × 15 cm, 80 mL/h) previously equilibrated in buffer A containing 100 mg/L SBTI. After application of the sample, the column was washed with 100 mL of buffer A, followed by 250 mL of buffer A with 170 mM NaCl, both buffers also containing 100 mg/L SBTI. PCI was eluted with a linear gradient (170–800 mM NaCl in buffer A containing 100 mg/L SBTI, 300 mL each chamber). Fractions of 10 mL were collected. PCI eluted in the gradient at 0.25 M NaCl (Figure 1), just after the protein peak containing prekallikrein and factor XI (data not shown). Fractions containing PCI were pooled, and after addition of SBTI (400 mg/L), the mixture was dialyzed against buffer B (50 mM Tris, pH 7.0, 150 mM NaCl, 10 mM benzamidine, 5 mM εACA, and 1 mM PMSF). After dialysis, the sample was applied to a column of dextran sulfate agarose (2.5 × 20 cm, 30 mL/h) previously equilibrated in buffer B. After application, the column was washed with buffer B containing 400 mg/L SBTI. PCI was eluted with a linear gradient of 150 mM–1.2 M NaCl in buffer B containing 400 mg/L SBTI (150 mL each chamber) (Figure 2). Fractions (6 mL) containing PCI were pooled and dialyzed against buffer C (50 mM Tris–phosphate, pH 9.0, 1 mM NaCl, 1 mM benzamidine, and 0.5 mM εACA). After dialysis, the sample was applied to a column of DEAE-Sephacel (2.5 × 20 cm, 30 mL/h) previously equilibrated in buffer C. After application, the column was washed with buffer C containing 50 mg/L SBTI. PCI was eluted in a pH gradient in buffer C containing 50 mg/mL SBTI (pH 9.0–6.0, 200 mL each chamber). PCI eluted in two peaks (Figure 3) as described by Suzuki et al. (1983a). The peaks were pooled separately and dialyzed against buffer D (50 mM sodium phosphate, pH 7.0, 2 mM EDTA, and 0.02% sodium azide). After dialysis, each sample was applied to a mono S column by using FPLC equipment (Pharmacia). PCI was eluted in a linear gradient of sodium chloride (0–0.5 M, 35 mL) with a flow rate of 60 mL/h and a fraction size of 1 mL (Figure 4).

Preparation of Monoclonal Antibodies. A Balb/c mouse was immunized by repeated intraperitoneal injections with PCI. Three days after the final injection, spleen cells were fused with Ag 8.653 myeloma cells. Fusion and hybridoma selection were performed according to standard procedures. Culture supernatants were screened for the presence of specific antibodies by an enzyme-linked immunosorbent assay (ELISA), in which PCI was used as antigen. Bound antibodies were detected with peroxidase-conjugated rabbit antibodies against mouse immunoglobulins (DAKO, Denmark). Hybridoma cells producing anti-PCI antibodies were cloned by limiting dilution. The characterization of the monoclonal antibodies will be described elsewhere.

Monoclonal antibodies against kallikrein were similarly prepared.

Kinetic Studies of the Inactivation of APC, Kallikrein, and Factor XIa by PCI. APC, kallikrein or its light chain, and factor XIa or its light chain were incubated in the presence or absence of heparin (Organon, Oss, The Netherlands) in 0.05 M Tris, pH 7.4, 0.15 M NaCl, and 0.1% (w/v) bovine serum albumin at 37 °C. After addition of PCI, the inactivation of kallikrein or its light chain was followed by adding a 25-μL sample from the incubation mixture to a plastic cuvette containing 475 μL of 0.2 mM H-D-Pro-Phe-Arg-*p*-nitroanilide (S2302, KabiVitrum, Stockholm, Sweden) in 0.05 M Tris and 0.15 M NaCl, pH 7.8, at various times. The inactivation of APC and factor XIa or its light chain was followed similarly by adding a 25-μL sample from the incubation mixtures to 475 μL of 0.4 mM pyroGlu-Pro-Arg-*p*-nitroanilide (S2366). The change in absorbance at 405 nm was followed continuously by using a Beckman Model 3600 double-beam spectrophotometer. The observed ΔA per minute was converted to the percent of maximum activity by comparison with the ΔA per minute of the sample that did not contain PCI. Second-order rate constants (k_1) were obtained in two ways. (1) Pseudo-first-order conditions: For each inhibitor concentration used, the half-life ($t_{1/2}$) is determined from a linear semilogarithmic plot of residual enzyme activity vs time. k_1 is then obtained by dividing the apparent rate constant ($k_{app} = \ln 2/t_{1/2}$) by the inhibitor concentration. (2) Second-order conditions with equimolar active concentrations of enzyme and inhibitor: k_1 is determined as the slope of a linear plot of 1/[E] vs time, according to the equation (Beatty et al., 1980) 1/[E]

Table I: Purification of PCI

	vol (mL)	protein[a] (mg)	act. (units)	sp act. (units/mg)	purification (x-fold)
plasma	1500	85 500	1500	0.018	1
DEAE-Sephadex	2100	56 290	1500	0.027	1.5
heparin–Sepharose	100	450	400	0.89	49
dextran sulfate agarose	84	130	315	2.4	134
DEAE-Sephacel	177	37	115	3.1	172
FPLC	6	0.28[b]	53	190	10 600

[a] Determined by using $E^{1\%}_{280nm} = 10$. [b] Determined by using $E^{1\%}_{280nm} = 14.1$ (Suzuki et al., 1983a).

$= k_1t + 1/[E]^0$, where $[E]^0$ is the initial enzyme concentration and [E] is the concentration of free enzyme measured at different time points by using the chromogenic substrate.

Immunoblotting Studies on the Interaction of PCI with APC, Kallikrein, and Factor XIa. APC (80 nM) was incubated with 160 nM PCI in the presence or absence of 5 units/mL heparin in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.02% bovine serum albumin, and 0.02% sodium azide in a volume of 300 μL at 37 °C. At different time points 95-μL samples were added to 25 μL of a SDS solution (13 mM Tris, pH 6.8, 3% w/v SDS, 0.01% w/v bromophenol blue, and 10% v/v glycerol) and incubated for 5 min at 100 °C.

In the same way, 125 nM kallikrein was incubated with 125 nM PCI, and 25 nM factor XIa was incubated with 25 nM PCI.

SDS–PAA slab gel electrophoresis was performed on 8% slab gels according to the method of Laemmli (1970). After electrophoresis, the gels were immunoblotted essentially as described by Towbin et al. (1979). The nitrocellulose blots were incubated with monoclonal antibodies against PCI (mixture of API-25, -29, and -88, 1/250 dilution of ascites fluid), kallikrein (1/50 dilution of ascites fluid), APC (C3, generously provided by Dr. J. H. Griffin, Scripps Clinic, La Jolla, CA, 30 μg/mL), or goat anti-human factor XI antibodies [1/100 dilution of a γ-globulin fraction prepared as described before (Bouma et al., 1983)]. Peroxidase-labeled rabbit anti-mouse immunoglobulins (DAKO) or a peroxidase-labeled rabbit anti-goat antiserum (Nordic, Tilburg, The Netherlands) were used as second antibody.

Results

Purification of PCI. In this study we describe a modification of the method described by Suzuki et al. (1983a) for the purification of PCI. Using the original method of Suzuki, we observed that proteolytic activity was generated on the dextran sulfate column and that this resulted in an inactivation of PCI. In our present method plasma is first incubated with several inhibitors before it is applied to a DEAE-Sephadex column. This column is used to remove the bulk of plasma proteins, including the vitamin K dependent proteins, from the flow-through fractions, which contain PCI and the contact system proteins prekallikrein and factor XI. Two affinity chromatography steps are then used. First, a heparin–Sepharose column is used to obtain partial separation of prekallikrein and factor XI from PCI. PCI elutes in the gradient (Figure 1) just after the protein peak containing prekallikrein and factor XI (data not shown). Originally, proteolytic activity was generated on this column, which resulted in a low recovery of PCI. After the addition of SBTI during this step, the recovery of PCI was greatly improved. Second, dextran sulfate agarose chromatography was performed (Figure 2). Generation of proteolytic activity on this column was also avoided by addition of SBTI. PCI elutes in the salt gradient and was further purified by DEAE-Sephacel chromatography (Figure 3). As described by Suzuki et al. (1983a), PCI eluted in two peaks. The peaks were pooled separately and applied to a

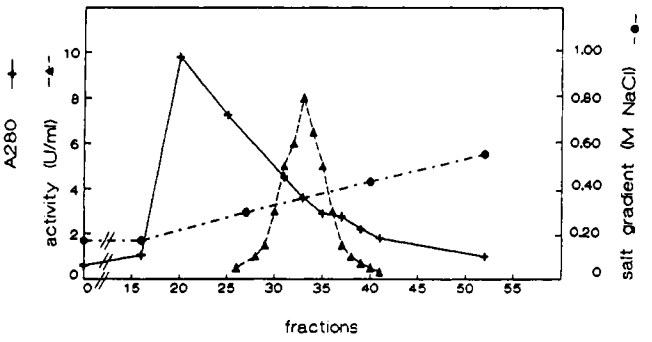

FIGURE 1: Purification of PCI using heparin–Sepharose chromatography at pH 7.4. Elution was effected by using an increasing NaCl gradient as described under Materials and Methods. Protein and PCI determinations and salt gradient are indicated.

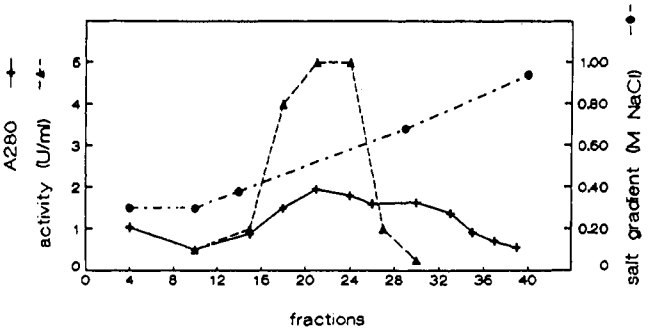

FIGURE 2: Purification of PCI using dextran sulfate agarose chromatography at pH 7.0. Elution was effected by using an increasing NaCl gradient as described under Materials and Methods. Protein and PCI determinations and salt gradient are indicated.

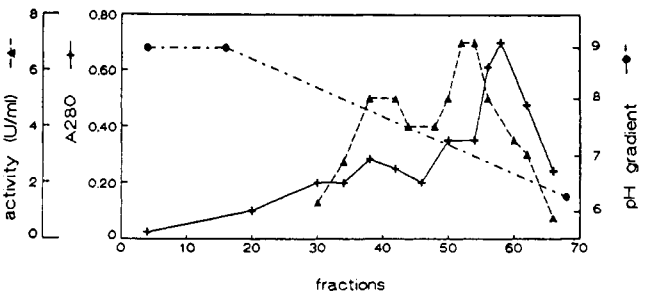

FIGURE 3: Purification of PCI using DEAE-Sephacel chromatography. Elution was effected by using a decreasing pH gradient as described under Materials and Methods. Protein and PCI determinations and pH gradient are indicated.

mono S column by using FPLC (Figure 4). No differences were found between PCI from the first or second peak. Probably, they represent two forms of PCI with different isoelectric points. The purification of PCI is summarized in Table I.

PCI appeared to be homogeneous on SDS–PAA slab gel electrophoresis (Figure 5) and had a M_r of 54 000, under both reduced and nonreduced conditions. The specific activity was 190 units/mg.

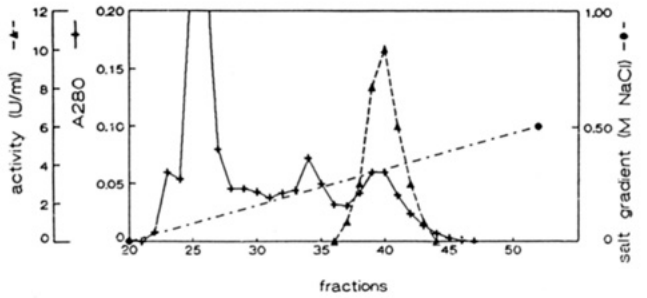

FIGURE 4: Purification of PCI using fast protein liquid chromatography. Elution was effected by using an increasing NaCl gradient as described under Materials and Methods. Protein and PCI determinations and salt gradient are indicated.

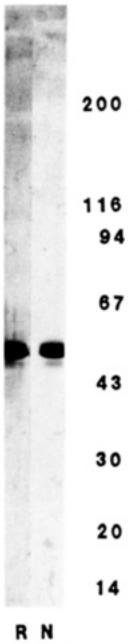

FIGURE 5: SDS–PAA 3–25% slab gel electrophoretic analysis of purified PCI (1.1 μg) under nonreduced (N) and reduced (R) conditions. Protein was visualized by silver staining. Molecular weights are expressed in kilodalton.

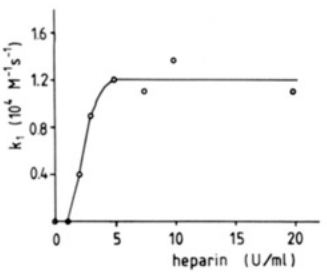

FIGURE 6: Effect of heparin on the second-order rate constant for the inactivation of APC (25 nM) by PCI (75 nM). Second-order rate constants were calculated as described under Materials and Methods.

Kinetic Studies of the Inactivation of APC, Kallikrein, and Factor XIa by PCI. The second-order rate constant for the inactivation of APC by PCI was determined. Figure 6 shows the second-order rate constants of the inactivation of APC by a 3-fold molar excess of PCI as a function of the heparin concentration. In the absence of heparin, no second-order rate constant could be determined, not even in a 12-fold molar excess of PCI (data not shown). Heparin increased the second-order rate constant for the inactivation of APC by PCI, with an optimum of 5 units/mL of heparin. Therefore, all the experiments in the presence of heparin were performed with a concentration of 5 units/mL.

Table II: Second-Order Rate Constants for the Inactivation of Enzymes by PCI[a]

	−heparin	+heparin[b]
APC	ND[c]	1.2 ± 0.2
kallikrein	11 ± 4	10 ± 1
kallikrein light chain	19 ± 5	15 ± 4
factor XIa	0.94 ± 0.07	9.1 ± 0.7
factor XIa light chain	1.5 ± 0.1	8.0 ± 0.4

[a] The data represent k_1 ($\times 10^4$ M^{-1} s^{-1}) and are means ± SD of at least four independent experiments. [b] In the presence of 5 units/mL heparin. [c] ND, not detectable with a 12-fold molar excess of inhibitor.

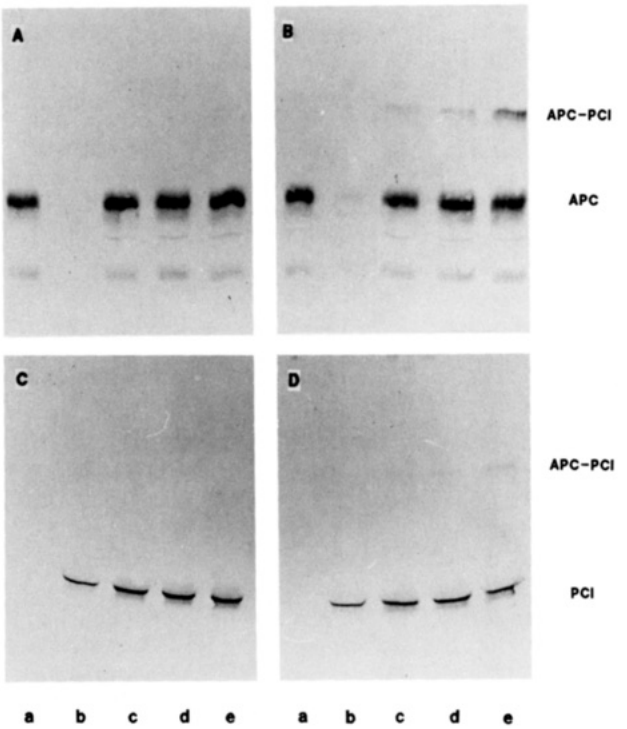

FIGURE 7: Immunoblotting analysis of the reaction of APC with PCI. APC (80 nM) was incubated with 160 nM PCI in the absence (A, C) or presence (B, D) of 5 units/mL heparin. At different times samples were taken and subjected to SDS–PAA slab gel electrophoresis and immunoblotting under nonreduced conditions. The immunoblot was incubated with anti-protein C antibodies (A, B) or anti-PCI antibodies (C, D). The time points were 0 min, APC alone (a), 0 min, PCI alone (b), 5 min (c), 15 min (d), and 30 min (e). APC, PCI, and the formed complex (APC–PCI) are indicated.

The second-order rate constants for the inactivation of APC, kallikrein and its light chain, and factor XIa and its light chain by PCI in the presence and absence of heparin are shown in Table II.

Heparin has an enhancing effect on the inactivation of APC, factor XIa, and factor XIa light chain by PCI but not on the inactivation of kallikrein and kallikrein light chain by PCI. The second-order rate constants for the inactivation of kallikrein and factor XIa are similar to their respective light chains, indicating a minor role for the heavy chains of both molecules in the inactivation reactions.

Immunoblotting Studies on the Inactivation of APC, Kallikrein, and Factor XIa by PCI. To investigate the interaction of APC, kallikrein, and factor XIa with PCI, PCI was incubated with APC, kallikrein, or factor XIa in the absence or presence of 5 units/mL heparin at 37 °C. At different time points samples were taken and analyzed by using SDS–PAA slab gel electrophoresis and immunoblotting. The proteins were visualized with specific antibodies against both the enzyme and the inhibitor.

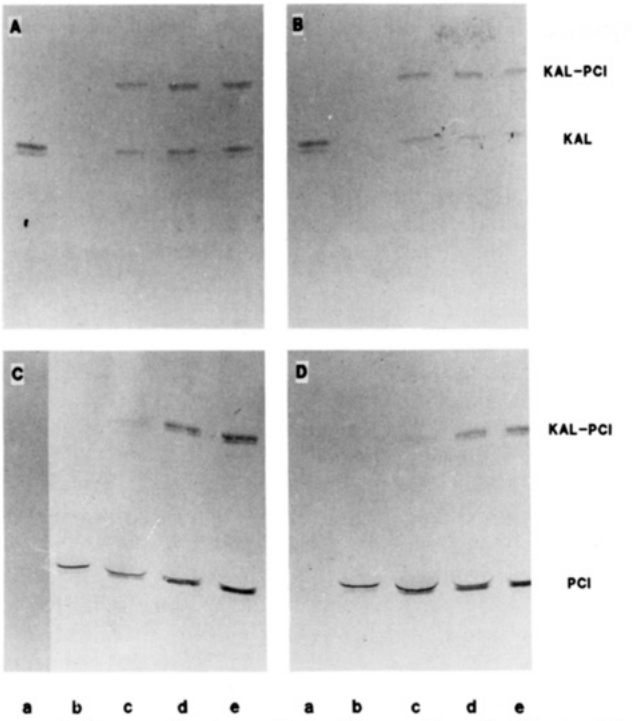

FIGURE 8: Immunoblotting analysis of the reaction of kallikrein with PCI. Kallikrein (25 nM) was incubated with 125 nM PCI in the absence (A, C) or presence (B, D) of 5 units/mL heparin. At different times samples were taken and subjected to SDS–PAA slab gel electrophoresis and immunoblotting under nonreduced conditions. The immunoblot was incubated with anti-kallikrein antibodies (A, B) or anti-PCI antibodies (C, D). The time points were 0 min, kallikrein alone (a), 0 min, PCI alone (b), 1 min (c), 5 min (d), and 10 min (e). Kallikrein (KAL), PCI, and the formed complex (KAL–PCI) are indicated.

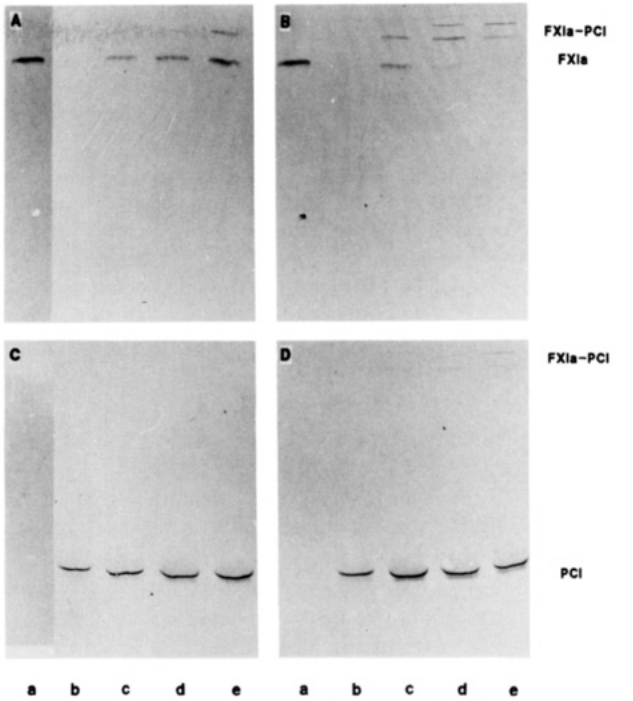

FIGURE 9: Immunoblotting analysis of the reaction of factor XIa with PCI. Factor XIa (25 nM) was incubated with 25 nM PCI in the absence (A, C) or presence (B, D) of 5 units/mL heparin. At different times samples were taken and subjected to SDS–PAA slab gel electrophoresis and immunoblotting under nonreduced conditions. The immunoblot was incubated with anti-factor XI antibodies (A, B) or anti-PCI antibodies (C, D). The time points were 0 min, factor XIa alone (a), 0 min, PCI alone (b), 1 min (c), 5 min (d), and 10 min (e). Factor XIa (FXIa), PCI, and the formed complexes (FXIa–PCI) are indicated.

Figure 7 shows the interaction of APC with PCI. In the absence of heparin, no complexes are formed. In the presence of heparin, a complex with a M_r of 112000 is visualized both with anti-protein C antibodies and with anti-PCI antibodies, indicating that a 1:1 molar complex between APC (M_r 64000) and PCI (M_r 54000) is formed.

In the inactivation of kallikrein by PCI a complex is formed in both the absence and presence of heparin at a similar rate (Figure 8). Both the anti-kallikrein and anti-PCI antibodies recognize the formed complex (M_r 132000), indicating the formation of a 1:1 molar complex between kallikrein (M_r 82000) and PCI (M_r 54000).

Figure 9 shows the interaction of factor XIa with PCI. In the absence of heparin, a single complex with a M_r of 205000 is formed, reacting with both anti-factor XI and anti-PCI antibodies. In the presence of heparin, this complex is also formed but decreases in time to form a higher molecular weight complex with a M_r of 255000 (Figure 9B). This indicates that in the inactivation of factor XIa (M_r 155000) by PCI (M_r 54000) first a 1:1 molar complex is formed between factor XIa and PCI, after which the second active site of factor XIa (Kurachi & Davie, 1977) also binds a PCI molecule, resulting in a 1:2 molar complex between factor XIa and PCI. Heparin has an enhancing effect on the inactivation of factor XIa by PCI.

Discussion

In this study we describe a modification of the method described by Suzuki et al. (1983a) for the purification of PCI. Using the original method of Suzuki, we observed that proteolytic activity was generated on the dextran sulfate column and that this resulted in an inactivation of PCI. In the first step of the purification procedure, PCI elutes from the DEAE-Sepharose column together with the contact factors prekallikrein and factor XI, and since dextran sulfate is known to activate the contact system (Kluft, 1978), we included inhibitors of plasma kallikrein and factor XIa during the purification procedure to avoid contact activation. In addition, precipitation steps were omitted. With the ample use of specific proteinase inhibitors, the recovery of PCI could be greatly enhanced. Especially SBTI, which is known to be a good inhibitor for kallikrein and factor XIa, prevented the inactivation of PCI on the heparin–Sepharose and dextran sulfate columns. Probably, a high local concentration of prekallikrein on the column leads to autoactivation.

To investigate if kallikrein and factor XIa indeed can react with PCI, the second-order rate constants for the inactivation of both enzymes by PCI were investigated. PCI was found to be a good inhibitor for both kallikrein and factor XIa, in contrast to APC, which is not inactivated by PCI in the absence of heparin (Table II). Heparin increased the inactivation rate of factor XIa, factor XIa light chain, and APC with PCI but not the inactivation rate of kallikrein and its light chain.

Suzuki et al. (1984) described the inactivation of several coagulation enzymes (APC, thrombin, and factor Xa) by PCI and determined the second-order rate constants for these reactions. Our second-order rate constant for the inactivation of APC by PCI in the presence of 5 units/mL of heparin was 6-fold lower than the value obtained by Suzuki et al. (1984). In the absence of heparin we were unable to determine a second-order rate constant, not even in a 12-fold molar excess of PCI. This difference may be explained by the presence of a heparin-independent protein C inhibitor (van der Meer et al., 1987). Since Suzuki et al. (1983a) used a functional assay without heparin, they may have copurified this heparin-in-

dependent inhibitor, whereas we strictly selected for the heparin-dependent inhibitor, because heparin was included in the assay.

The potentiating effect on the interaction between APC and PCI by heparin was also demonstrated by using SDS–PAA slab gel electrophoresis and immunoblotting. With both anti-protein C and anti-PCI antibodies a complex with a M_r of 112000 could only be visualized in the presence of heparin (Figure 7). The formed complex is a 1:1 molar complex between APC and PCI as has been shown before (Suzuki et al., 1984). In this reaction a band with a slightly lower M_r than that of PCI can also be observed on the anti-PCI immunoblot (Figure 7D). This inactive PCI is characteristic for proteolytically modified PCI (Suzuki et al., 1984).

PCI is a good inhibitor for kallikrein. The second-order rate constant for the inactivation of kallikrein by PCI is approximately twice the second-order rate constant for the inactivation of C1 inhibitor, the major inhibitor for kallikrein (van der Graaf et al., 1983a).

Heparin has no influence on this inactivation (Table II, Figure 8). It is not understood why heparin has no effect on the inactivation of kallikrein by PCI while on the other hand the inhibition of kallikrein by antithrombin III is greatly enhanced by the addition of heparin (Burrowes et al., 1975; Venneröd & Laake, 1975; Venneröd et al., 1976). In the reaction between kallikrein and PCI, a complex with a M_r of 132000 is formed that is recognized by both anti-kallikrein and anti-PCI antibodies (Figure 8). This indicates that a 1:1 molar complex has been formed. The kinetic inactivation of kallikrein and its light chain by PCI are similar, indicating that a complex has been formed between the light chain of kallikrein with PCI and that the heavy chain of kallikrein is not involved in complex formation. This has been shown before for the inactivation of kallikrein by C1 inhibitor (van der Graaf et al., 1983a) and α_2-macroglobulin (van der Graaf et al., 1984). In the reaction between kallikrein and PCI, PCI is modified by kallikrein to a molecule with a slightly lower M_r (Figure 8C,D).

The second-order rate constant for the inactivation of factor XIa by PCI is $(0.94 \pm 0.07) \times 10^4$ M^{-1} s^{-1} (Table I), which is much higher than previously described second-order rate constants for the inactivation of factor XIa by α_1-antitrypsin, antithrombin III, and C1 inhibitor (Scott et al., 1982; Soons et al., 1987; Meijers et al., 1988). It can be calculated from the second-order rate constant for the inactivation of factor XIa by PCI, the plasma concentration of PCI (Suzuki et al., 1983a), the second-order rate constant for the inactivation of factor XIa by C1 inhibitor (Meijers et al., 1988), the plasma concentration of C1 inhibitor (Harpel, 1982), and the observation that since C1 inhibitor accounts for 24% of the inactivation in plasma (J. C. M. Meijers, R. A. A. Vlooswijk, and B. N. Bouma, unpublished observation) that PCI would account for 5% of the inactivation of factor XIa in plasma.

Heparin increased the second-order rate constant for the inactivation of factor XIa by PCI about 10-fold. The inactivation of factor XIa by antithrombin III was increased 26-fold in the presence of heparin (Soons et al., 1987).

The inactivation of factor XIa light chain is similar to the whole molecule, indicating a minor role for the heavy chain of factor XIa in the inactivation process. This has been observed before for the inactivation of factor XIa by C1 inhibitor (Meijers et al., 1988). Factor XIa is a dimeric molecule with two identical protein chains with M_r's of 80000 held together by disulfide bonds. It consists of two heavy chains (M_r 50000) and two light chains (M_r 33000) and contains an active site on each light chain (Bouma & Griffin, 1977; Fujikawa et al., 1986). In the reaction between factor XIa and PCI, first a 1:1 molar complex is formed, after which an additional PCI molecule can bind to the second active site of the factor XIa molecule (Figure 9). This has been shown before for the inactivation of factor XIa by antithrombin III (Kurachi & Davie, 1977; Soons et al., 1987) and C1 inhibitor (Meijers et al., 1988). In the reaction between factor XIa and PCI, PCI is modified by factor XIa to a molecule with a slightly lower M_r (Figure 9C,D).

The interference of kallikrein during the purification of PCI occurs most likely by inactivation of PCI by kallikrein, resulting in a 1:1 molar complex between PCI and kallikrein or resulting in a modified, inactive inhibitor. The influence of factor XIa will be much smaller, due to the low amount of factor XI present in plasma.

Whether PCI plays an important role in the inactivation of kallikrein and factor XIa in physiologic conditions remains to be investigated. Van der Graaf et al. (1983c) described the presence of an unknown kallikrein inhibitor in antithrombin III deficient plasma with a molecular weight similar to that of antithrombin III, accounting for 7% of the inactivation of kallikrein. It can be calculated from the second-order rate constant for the inactivation of kallikrein by PCI, the plasma concentration of PCI (Suzuki et al., 1983a), the second-order rate constant for the inactivation of kallikrein by C1 inhibitor (van der Graaf et al., 1983a), the plasma concentration of C1 inhibitor (Harpel, 1982), and the observation that since C1 inhibitor accounts for 52% of the inactivation in plasma (van der Graaf et al., 1983c) that PCI would account for 7% of the inactivation of kallikrein in plasma. This suggests that PCI is the unknown inhibitor of kallikrein, originally described by van der Graaf et al. (1983c), and can play a physiologically important role in the inactivation of kallikrein and factor XIa. Furthermore, because of the high second-order rate constants of the inactivation of kallikrein and factor XIa by PCI and the low concentration of PCI in plasma, these proteinases of the contact system could regulate the PCI activity, thereby forming a link between the contact system and the protein C pathway.

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Isolation and Characterization of an Insulin-Degrading Enzyme from *Drosophila melanogaster*†

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ABSTRACT: An insulin-degrading enzyme (IDE) from the cytoplasm of *Drosophila* Kc cells has been purified and characterized. The purified enzyme is a monomer with an *s* value of 7.2 S, an apparent K_m for porcine insulin of 3 μM, and a specific activity of 3.3 nmol of porcine insulin degraded/(min·mg). N-Terminal sequence analysis of the gel-purified enzyme gave a single, serine-rich sequence. The *Drosophila* IDE shares a number of properties in common with its mammalian counterpart. The enzyme could be specifically affinity-labeled with [^{125}I]insulin, has a molecular weight of 110K, and has a p*I* of 5.3. Although *Drosophila* Kc cells grow at room temperature, the optimal enzyme activity assay conditions parallel those of the mammalian IDE: 37 °C and a pH range of 7–8. The *Drosophila* IDE activity, like the mammalian enzymes, is inhibited by bacitracin and sulfhydryl-specific reagents. Similarly, the *Drosophila* IDE activity is insensitive to glutathione as well as protease inhibitors such as aprotinin and leupeptin. Insulin-like growth factor II, equine insulin, and porcine insulin compete for degradation of [^{125}I]insulin at comparable concentrations (approximately 10^{-6} M), whereas insulin-like growth factor I and the individual A and B chains of insulin are less effective. The high degree of evolutionary conservation between the *Drosophila* and mammalian IDE suggests an important role for this enzyme in the metabolism of insulin and also provides further evidence for the existence of a complete insulin-like system in invertebrate organisms such as *Drosophila*.

Identification and characterization in lower organisms of homologues of mammalian proteins is an approach that can yield information on the role and relative importance of proteins conserved during evolution. One system that appears to be highly conserved between mammals and *Drosophila* is that of insulin and its related proteins. An insulin-like activity in protein extracts from *Drosophila* has been described (Meneses & Ortiz, 1975), and *Drosophila* homologues of the human insulin receptor have recently been identified (Petruzzelli et al., 1985a,b, 1986).

In vertebrates, insulin is an important hormone that has pleiotropic effects on cellular metabolism and growth, including regulation of glucose homeostasis and stimulation of cell proliferation [reviewed in Czech (1985)]. Insulin-induced

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