We studied the inhibition of tissue kallikrein by protein C inhibitor (PCI), a relatively unspecific heparin-dependent serine protease inhibitor present in plasma and urine. PCI inhibited the amidolytic activity (cleavage of H-D-valyl-L-leucyl-arginine-p-nitroaniline) of urinary kallikrein with an apparent second order rate constant of \(2.3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) and formed stable complexes (85 kDa) with urinary kallikrein as judged from silver-stained sodium dodecyl sulfate-polyacrylamide gels. Complex formation was time-dependent and was paralleled by a decrease in the intensity of the main PCI protein band \((M_\text{r} = 57,000)\) and an increase in the intensity of the lower \(M_\text{r}(54,000)\) PCI form (cleaved inhibitor). Heparin interfered with the inhibition of tissue kallikrein by PCI and with the formation of tissue kallikrein-PCI complexes in a dose-dependent fashion and completely abolished PCI-tissue kallikrein interaction at 300 \(\mu \text{g/ml}\). This is in contrast to findings on the interaction of PCI with all other target proteases studied so far (i.e. stimulation of inhibition by heparin) but is similar to the reaction pattern of \(^{125}\text{I}-\text{Iabeled tissue kallikrein with so called kallikrein binding protein described in serum and other systems. To study a possible relationship between PCI and this kallikrein binding protein we incubated \(^{125}\text{I}-\text{Iabeled urinary kallikrein in serum and in PCI-immunodepleted serum in the absence and presence of heparin and analyzed complex formation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In normal serum, formed complexes co-migrated with complexes of purified PCI and \(^{125}\text{I}-\text{kallikrein and were less intense in the presence of heparin. No complex formation at all was seen in PCI-depleted serum. Our data indicate that PCI may be a physiologically important endogenous inhibitor of tissue kallikrein and provide evidence that PCI may be identical to the previously described kallikrein binding protein.}

Tissue/glandular kallikreins are trypsin-like serine proteases that have been isolated from several tissues, including kidney, salivary glands, and pancreas (1–6). They are also present in the exocrine secretions of the respective organs (2, 6, 7). The primary physiological function of tissue kallikreins seems to be kinin release from high and low \(M_\text{r}\), kininogens by the cleavage of two peptide bonds within the kininogen molecules (2). Kinins have a variety of species and tissue-specific biological activities, including vasodilation and contraction of visceral smooth muscles (2). In addition to kinin generation, kallikrein may also be involved in renin release (8) and angiotensin II generation (9–11). Therefore, the regulation of kallikrein activity seems to be of major physiological importance. So far, no efficient endogenous inhibitor of tissue kallikrein has been identified. The only proteinase inhibitor exhibiting some inhibitory activity toward tissue kallikrein is \(\alpha\)-protease inhibitor (12). However, the interaction of tissue kallikrein with \(\alpha\)-protease inhibitor is slow, with a half-life of several hours (12, 13). Recently, a tissue kallikrein binding protein has been described in several biological systems including serum (13–15). This binding protein interacts with tissue kallikrein by forming SDS-stable complexes with a \(M_\text{r}\) of 92,000. Complex formation is only observed with enzymatically active kallikrein (14) and is, at least in human systems, abolished in the presence of heparin (13–15). The identity of this kallikrein binding protein has not yet been defined. However, its reaction pattern suggests that it may be a serine protease inhibitor with a \(M_\text{r}\) of approximately 50,000 and with affinity for heparin.

Protein C inhibitor (PCI; \(M_\text{r} = 57,000\)) is a relatively nonspecific heparin-dependent serine protease inhibitor (serpin) that inhibits a variety of proteases (16–18). The presence of PCI has been described in plasma (5 \(\mu \text{g/ml}\) (16) and urine (19–21), and the urinary concentration is higher than expected from plasma filtration (200 ng/ml, 21). So far, PCI has mainly been studied with respect to its interaction with enzymes involved in coagulation and fibrinolysis (16–23). It has been shown that PCI inhibits activated protein C, thrombin, factor Xa, factor Xla, plasma kallikrein, and urokinase by forming SDS-stable complexes (16–18). The reaction of PCI with its target proteases is stimulated by heparin (16–18) and other glycosaminoglycans (24). Although activated protein C-PCI complexes have been shown in plasma samples from patients with disseminated intravascular coagulation (25) and urokinase-PCI complexes in plasma samples from patients receiving exogenous urokinase therapy (26), the physiological role of PCI is still unclear. The presence of PCI in urine combined with the fact that urine is a major source...
of tissue kallikrein prompted us to investigate the possible inhibition of tissue kallikrein by PCI.

**Experimental Procedures**

Materials—Sepharose 4B, activated CH-Sepharose 4B, and heparin-Sepharose CL-6B (Pharmacia, Sweden); C8Nbr (Merck, Germany); heparin sodium salt (≥140 units/mg; Fluka, Switzerland); acrylamide, bisacrylamide, SDS, and calibration proteins for SDS-PAGE (Bio-Rad); H-D-Val-Leu-Arg-pNA (S-2266) and <Glu-Pro-Arg-pNA (S-2366) (Kabi, Sweden); and activated protein C (Technoclone GmbH, Austria) were obtained as indicated. A monoclonal antibody against protein C inhibitor (4PCI) was obtained by immunizing Balb/c mice with purified urinary PCI (24). Immunization of mice, fusion of mouse spleen cells, and isolation of single anti-PCI IgG secreting clones were performed as described previously (27), except that X63-Ag8.653 mouse myeloma cells were used as fusion partners. One clone producing anti PCI-IgG (4PCI) was selected and injected intraperitoneally into Pristan-primed Balb/c mice. Ascitic fluid was collected and 4PCI-IgG (4PCI) was purified and stored in aliquots at -70°C until further use. PCI preparation.

Eluted with a protein peak at 0.35 M NaCl, 0.01% Tween 80, pH 7.4, and subjected to heparin-Sepharose CL-GB chromatography extensively against 0.05 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, pH 7.4. The first flow-through fraction (protein content 2 mg/ml) was used as PCI immunodepleted serum.

Control serum (protein content 2.5 mg/ml) was prepared in the same way but a Sepharose 4B column was used instead of 4PCI-IgG-Sepharose. PCI-immunodepleted serum contained less than 1% of PCI activity present in control serum as determined by enzyme-linked immunosorbant assay using 4PCI IgG as catching antibody and peroxidase-labelled immunopurified rabbit anti-PCI IgG as detecting antibody (lower detection limit = 3 ng/ml).

**Purification of Urinary Protein C Inhibitor**—The PCI purification protocol described previously (24) was modified in such a way that affinity chromatography on 4PCI-IgG Sepharose 4B was used as a first step and that affinity chromatography on anti-urokinase-IgG Sepharose and ion exchange chromatography on Q-Sepharose fast flow were omitted. Briefly, freshly voided human urine (21) was collected (10 ml final concentration) and protein (100 KIU/ml final concentration), adjusted to a pH between 7.0 and 7.4 by addition of 1 M NaOH and incubated, and end-over-end rotated overnight with 4PCI-IgG-Sepharose 4B (40 ml settled volume) equilibrated in 0.01 M Tris-Cl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. Unbound material was separated from the Sepharose gel by means of a sintered glass filter. The 4PCI-IgG-Sepharose was washed extensively with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, 10 mM benzamidine, 100 KIU/ml aprotinin, pH 7.4. Unbound material was separated from the Sepharose gel by means of a sintered glass filter. The 4PCI-IgG-Sepharose was washed extensively with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, 10 mM benzamidine, 100 KIU/ml aprotinin, pH 9.0, on the same filter. Thereafter the gel suspension was filled into a column and washed with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, pH 9.0, until the A280 was below 0.01. Elution was performed with 0.05 M triethylamine buffer, pH 11.5, containing 2.5% SDS and 100 KIU/ml aprotinin (100 KIU/ml final concentration), adjusted to a pH between 7.0 and 7.4 by addition of 1 M NaOH and incubated, and end-over-end rotated overnight with 4PCI-IgG-Sepharose 4B (40 ml settled volume) equilibrated in 0.01 M Tris-Cl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. Unbound material was separated from the Sepharose gel by means of a sintered glass filter. The 4PCI-IgG-Sepharose was washed extensively with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, 10 mM benzamidine, 100 KIU/ml aprotinin, pH 7.4. Unbound material was separated from the Sepharose gel by means of a sintered glass filter. The 4PCI-IgG-Sepharose was washed extensively with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, 10 mM benzamidine, 100 KIU/ml aprotinin, pH 9.0, on the same filter. Thereafter the gel suspension was filled into a column and washed with 0.01 M Tris-Cl, 1.0 M NaCl, 0.01% Tween 80, pH 9.0, until the A280 was below 0.01. Elution was performed with 0.05 M triethylamine buffer, pH 11.5, containing 0.5 M NaCl. Fractions (3-4 ml each) were collected into tubes containing 1 ml of 0.01 M Tris-Cl, pH 7.5, to neutralize pH immediately. Protein containing fractions (A280 ≥ 0.03) eluted at pH 11.5 were pooled and stored at -70°C until further use. Eluates obtained in this way from three to five 4PCI-IgG-Sepharose steps were pooled, dialyzed extensively against 0.05 M Tris-Cl, 0.2 M NaCl, 0.01% Tween 80, pH 7.4, and subjected to heparin-Sepharose CL-6B chromatography as described previously (24). Elution of the heparin-Sepharose column was performed with a linear gradient between 0.2 and 0.8 M NaCl at 0.03 M Tris-Cl, 0.01% Tween 80. Fractions (2.5 ml) were collected. Fractions were screened for protein (A280) and inhibition of activated protein C (24). PCI activity co-eluted with a protein peak at 0.35 M NaCl. PCI-containing fractions were pooled and stored in aliquots at -70°C until further use. PCI purified by this procedure was homogeneous and migrated on silver-stained SDS-PAGE gels as a doublet with a major band corresponding to M, of 57,000 and a minor band (M, = 54,000). The yield of this purification procedure was ≈100 μg PCI/liter urine as compared with ≈55 μg PCI/liter urine obtained with the previously described purification protocol (24).

**Analytical Methods**—Molar concentrations of purified active kallikrein were determined from the cleavage of S-2266 using the change in absorbance at 405 nm/min and cm (ΔA405/min/cm) of 2.75 μM enzyme as given by the manufacturer. The concentration of purified PCI was determined from its absorbance at 280 nm using an A ε280 of 14.1 (16). M, of 57,000 was used to calculate molar concentrations; SDS-PAGE (10% acrylamide) was performed according to Laemmli (29) using 1.5-mm slab gels. After electrophoresis gels were stained by the silver stain method (30). Each lane of the silver-stained gels was scanned densitometrically at 523 nm using a Hirschmann Elscript 400 densitometer and peaks corresponding to the stained protein bands were obtained. The relative intensity of each stained band, represented by the size of the area of the corresponding peak, was expressed as percent of the total intensity of all stained bands present in a lane. Gels containing 125I-kallikrein were fixed with acetic acid/methanol/water (10:50:40), dried, and exposed to Kodak X-omat AR films.

**Amidolytic Assay for Inhibition of Tissue Kallikrein Activity by PCI**—Inhibition of the amidolytic activity of purified urinary kallikrein by purified urinary PCI was tested on 96-well microtiter plates. Urinary kallikrein (3 nM final concentration) was incubated without or with different concentrations of PCI (30, 15, and 7.5 nM final concentration) in 50 μl of a first flow-through fraction at 37°C in 50 μl of 0.01 M Tris-Cl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. After 30 min, 50 μl of S-2266 (0.4 mM final concentration) dissolved in 0.05 M Tris-Cl, pH 8.3, was added to each well and after additional incubation for 2 h at 37°C, the Aε280 was determined in an enzyme-linked immunosorbent assay reader (Antos reader 2001).

**Kinetic Analysis of Tissue Kallikrein Inhibition by PCI**—Urinary kallikrein (4 nM final concentration) was incubated without or with different concentrations of PCI (30, 15, and 7.5 nM final concentration) in 100 μl of 0.01 M NaCl, 0.01% Tween 80, pH 7.4, in wells of a 96-well microtiter plate. After different time periods (0, 5, 10, 20, 40, and 80 min) heparin (10 μl, 3 mg/ml final concentration) was added to each well in order to stop the enzyme-inhibitor interaction. After stopping the last wells (80 min) 50 μl of S-2266 (final concentration 0.4 mM) dissolved in 0.05 M Tris-Cl, pH 8.3, was added to each well and the amidolytic activity was measured as described above. Kallikrein activity [E] was determined for each PCI concentration and each incubation time period and plotted either as [E]/[E0] versus incubation time or as ln[E]/[E0] versus incubation time, [E] representing the initial amidolytic activity of kallikrein and [E0] for each PCI concentration by linear regression analysis of the data using the initial linear parts of the ln[E]/[E0] versus time plots. The apparent second order rate constant (k) was obtained by dividing the pseudo first order rate constants by the molar concentrations of PCI used.

**Bioassay for Kallikrein Activity**—For control purposes, kinin generation by urinary kallikrein in the absence or presence of PCI and in the absence or presence of heparin was tested using the rat uterus bioassay system (31). Urinary kallikrein (2.5 nM final concentration) was incubated at 37°C in 100 μl of 0.01 M NaCl, 0.01% Tween 80, pH 7.4. Incubations were stopped at various times (0-4 h) by the addition of 35 μl of 0.25 M Tris-Cl buffer, pH 6.8, containing 2.5% SDS and 0.05 M Tris-HCl, pH 7.4. The mixture was applied to the rat uterus bioassay system and kallikrein generated was quantified using known bradykinin standards.
Complex Formation of 125I-Urinary Kallikrein in Serum and PCI-immunodepleted Serum—125I-Labeled urinary kallikrein (4.5 nM, 6000 cpm) was incubated with control serum (3 μl), with PCI-immunodepleted serum (3 μl), or with purified PCI (270 nM final concentration) in the absence or presence of heparin (3 and 300 μg/ml final concentrations) in a total volume of 63 μl 0.01 M Tris-HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. After 1 h the reactions were stopped by the addition of 40 μl 0.26 M Tris-HCl buffer, pH 6.8, containing 2.5% SDS and 25% glycerol and heating the samples in a boiling water bath for 10 min. SDS-PAGE and autoradiography were done as described above.

RESULTS

As shown in Fig. 1, purified urinary PCI inhibited the amidolytic activity of urinary kallikrein and heparin decreased this inhibition in a dose-dependent manner. Furthermore, no concentration of heparin studied (0.03-300 μg/ml) had an effect on the amidolytic activity of kallikrein itself. In control experiments the effect of PCI on the kinin-generating activity of urinary kallikrein was also tested using the rat uterus bioassay. In an assay system using 0.11 nM heat-inactivated plasma as a source of kininogen, PCI (10.9 nM final concentration) caused a decrease in kinin formed over 5 min from 10 to 5 ng/sample. When heparin was present in the preincubation mixture of PCI and urinary kallikrein at concentrations 5 μg/ml, PCI had no effect on kinin generation by urinary kallikrein.

Since high concentrations of heparin completely abolished the interaction between PCI and urinary kallikrein (Fig. 1), the time course of kallikrein inhibition by PCI was analyzed using heparin (3 mg/ml final concentration) to stop the enzyme-inhibitor interactions at defined intervals. Fig. 2 shows the time course of kallikrein inhibition by different concentrations of PCI. When these data were replotted as ln([E0]/[E]) versus time, linear plots were obtained for the first 10-20 min of each reaction. The apparent second order rate constant (k2) calculated from these plots as described under experimental procedures was $2.3 \times 10^4$ M⁻¹ s⁻¹.

The interaction of purified urinary PCI and purified urinary kallikrein was analyzed using silver-stained SDS-PAGE gels. As can be seen from Fig. 3 (panel A) the time-dependent appearance of a new band (M₅ = 85,000) was observed when purified urinary kallikrein (0.77 nM) and purified urinary PCI (200 nM) were incubated together. As determined densitometrically, the maximal relative intensity of this new band, observed after incubation periods of ≥2 h, was about 6-7% of the total intensity of all stained bands. Complex formation was paralleled by a decrease in the relative intensity of the main (M₅ = 57,000) PCI band and an increase in the relative intensity of the M₅ = 54,000 PCI band. The effect of heparin on kallikrein-PCI complex formation was also studied. Fig. 3 (panel B) shows the effect of increasing heparin concentrations on the interaction of PCI with urinary kallikrein using an incubation period of 4 h. It can be seen from this figure that heparin inhibited in a dose-dependent way the formation of the 85-kDa band and diminished the generation of the 54-kDa PCI band. 300 μg/ml heparin completely abolished the PCI-kallikrein interaction as judged from the fact that there was no 85-kDa band present and that the relative intensities of the 57- (81.5%) and the 54-kDa bands (18.5%) were comparable with those seen in the original PCI preparation (Fig. 3A, inset, lane 1).

We analyzed a possible relationship between PCI and the kallikrein binding protein present in serum (13-15) by incubating 125I-labeled tissue kallikrein with serum and with PCI-immunodepleted serum in the absence and presence of heparin. As can be seen from Fig. 4, 125I-kallikrein formed a complex with a component present in serum; this complex co-migrated with the complex formed between 125I-kallikrein and purified PCI, and its intensity was decreased by the presence of heparin. No 125I-kallikrein complex formation was observed in PCI-immunodepleted serum.

DISCUSSION

PCI is a relatively nonspecific heparin-binding serine protease inhibitor (16-18). It inhibits not only activated protein C, but also several other enzymes involved in coagulation (thrombin, factor Xa, factor Xla, plasma kallikrein) and fibrinolysis (urokinase) (16-18, 23). Its inhibitory activity is
FIG. 3. Complex formation of purified PCI with purified urinary kallikrein and cleavage of PCI by urinary kallikrein. Panel A, purified urinary PCI (200 nM final concentration) was incubated with purified urinary kallikrein (7.7 nM final concentration) in 70 μl of 0.01 M Tris-HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. The reactions were stopped after the intervals indicated on the abscissa by addition of 35 μl 0.26 M Tris-HCl, 2.5% SDS, 25% glycerol and putting the samples in a boiling water bath for 10 min. SDS-PAGE and silver staining of the gel were performed as described under "Experimental Procedures." Each lane of the stained gel was scanned densitometrically at 523 nm, and the relative intensity of each stained protein band was evaluated as described under "Experimental Procedures." Relative intensities of the stained bands (○, 57-kDa band; ▲, 54-kDa band; △, 85-kDa band) are shown on the ordinate. Inset, silver-stained SDS-PAGE gel. Lane 1, PCI alone (200 nM); lanes 2–7, PCI (200 nM) incubated with purified urinary kallikrein (7.7 nM final concentration) as described in panel A in the absence or presence of different concentrations of heparin (as indicated on the abscissa). Reactions were stopped after 4 h, and samples were subjected to SDS-PAGE. Silver-stained gels were scanned densitometrically as described under "Experimental Procedures." Relative intensities of the silver-stained bands (○, 57-kDa band; ▲, 54-kDa band; △, 85-kDa band) are shown on the ordinate. Inset, silver-stained SDS-PAGE gel of PCI (200 nM final concentration) incubated for 4 h with purified urinary kallikrein (7.7 nM final concentration) in the absence (lane 1) or presence (lanes 2–6) of different concentrations of heparin (0.05 μg/ml in lane 2; 0.3 μg/ml in lane 3; 3 μg/ml in lane 4; 39 μg/ml in lane 5; 300 μg/ml in lane 6).

FIG. 4. Complex formation of 125I-labeled tissue kallikrein in serum and in PCI-immunodepleted serum. 125I-Labeled urinary kallikrein (4.5 nM, 6000 cpm) was incubated without (lane 1) or with 270 nM purified PCI (lanes 2, 5, and 6) or with control serum (3 μl; lanes 4, 9, and 10) or with PCI-depleted serum (3 μl; lanes 3, 7, and 8) in 65 μl 0.01 M Tris-HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4, for 1 h at 37 °C, in the absence (lanes 1–4) or presence of heparin (lanes 5–10). Lanes 5, 7, and 9 contained 300 μg/ml heparin (final concentration) lanes 6, 8, and 10 contained 3 μg/ml heparin (final concentrations). SDS-PAGE and autoradiography were performed as described under "Experimental Procedures."

stimulated by heparin (16–18) and other glycosaminoglycans (24). The presence of naturally occurring stimulatory glycosaminoglycans might therefore play a crucial role for the in vivo regulation of PCI activity and possibly also enzyme specificity. However, so far the physiological role of PCI is still unclear.

PCI is present in urine in a surprisingly high concentration and could therefore be an important protease inhibitor in the urinary tract. We have shown that PCI is also present in saliva and intestinal fluid (32). Laurell et al. (33) have demonstrated recently that very high concentrations of PCI immunoreactivity can be found in seminal plasma, Graaf follicle fluid, and synovial fluid. These findings may indicate a more general role for this inhibitor in secretions. These secretions are also major sources of tissue kallikrein, an enzyme for which no efficient endogenous serpin-type inhibitor has been described. We therefore investigated a possible inhibition of tissue kallikrein by PCI.

In fact, using purified urinary kallikrein and purified urinary PCI, we were able to show that PCI inhibited kallikrein activity in a dose- and time-dependent fashion; furthermore, this interaction was accompanied by the formation of SDS-stable complexes (Mc = 85,000). As judged from densitometrically scanned silver-stained gels, the maximal amount of complex formed was somewhat higher (6–7% of total PCI) than expected using a %-fold excess of PCI over kallikrein judged from densitometric scans. The presence of naturally occurring stimulatory glycosaminoglycans might therefore play a crucial role for the in vivo regulation of PCI activity and possibly also enzyme specificity. However, so far the physiological role of PCI is still unclear.

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PC1 preparations used as shown in Fig. 3A, indicating that PCI might have been cleaved either in vivo or during the purification procedure. Kinetics for the inhibition of urinary kallikrein by PCI were determined using a high heparin concentration to stop the enzyme-inhibitor interaction after defined intervals, assuming that heparin has no effect on the stability of the kallikrein-PCI complex. Although the apparent second order rate constant (kₐ) calculated under these conditions for the inhibition of urinary kallikrein by PCI (2.3 × 10⁷ M⁻¹ s⁻¹) does not seem to be very high, it is in the same order of magnitude (18) or higher (24) than the kₐ calculated for the reaction of PCI with its hitherto known target proteases. This indicates that PCI may, in fact, be a physiologically important inhibitor of tissue kallikrein.

Analysis of the effect of heparin on the PCI-tissue kallikrein interaction revealed an inhibitory effect of heparin. This is in contrast to findings on the interaction of PCI with all other target proteases studied so far, where heparin enhanced the enzyme-inhibitor interaction. Such a reaction pattern, however, has been described by the group of Chao for the interaction of tissue kallikrein with its hitherto known target proteases. This indicates that PCI may, in fact, be a physiologically important inhibitor of tissue kallikrein.

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