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Heparin binding of protein-C inhibitor

Analysis of the effect of heparin on the interaction of protein-C inhibitor with tissue kallikrein

Sonja ECKE, Margarethe GEIGER and Bernd R. BINDER

Department of Vascular Biology and Thrombosis Research, University of Vienna, Vienna, Austria

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The non-specific serine-protease inhibitor protein-C inhibitor (PCI) inactivates its target enzymes by forming stable 1:1 complexes. Heparin stimulates most PCI/protease reactions, but interferes with the inhibition of tissue kallikrein by PCI by a hitherto unknown mechanism. In this study we analyzed the inhibitory effect of heparin on the tissue-kallikrein–PCI interaction. Free PCI and tissue-kallikrein · PCI complexes but not free tissue kallikrein bound to heparin-Sepharose, implying that the inhibitory effect of heparin cannot be caused by a tissue-kallikrein–heparin interaction. Heparin did not dissociate tissue-kallikrein · PCI complexes, making it unlikely that in the presence of heparin PCI becomes a substrate for, rather than an inhibitor of, tissue kallikrein. However, heparin-bound PCI, which was able to form complexes with ^{125}I-urokinase, did not form complexes with ^{125}I-tissue-kallikrein. This suggests that the inhibitory effect of heparin is either based on the neutralization of positive charges in the PCI molecule, which might be required for the interaction of PCI with the acidic protease tissue kallikrein, or on a change in reactivity of PCI upon heparin binding, making heparin-bound PCI no longer a tissue-kallikrein inhibitor. Neutralization of basic amino acids in the PCI molecule by glutamic acid, which prevented in a dose-dependent way the inhibitory effect of heparin, did not have any effect on the tissue-kallikrein–PCI interaction. Therefore, direct involvement of basic amino acid residues present in the heparin-binding site of PCI in the tissue-kallikrein–PCI interaction can be excluded. Heparin binding might rather cause a change in reactivity of PCI (e.g. by inducing a conformational change or by steric interference), thereby preventing its interaction with tissue kallikrein.

Keywords: tissue kallikrein; protein-C inhibitor; heparin; serpin; glycosaminoglycan.

Protein-C inhibitor (PCI) is a heparin-binding serine-protease inhibitor (serpin) [1, 2] present in plasma (5 μg/ml), urine (200 ng/ml), and several other body fluids and secretions [3–7]. PCI was described originally in plasma as an inhibitor of the anticoagulatory serine protease activated protein-C [6, 7]. Since then, it has been shown that PCI inhibits a variety of other serine proteases including thrombin [8, 9], factor Xa [8, 9], factor XIa [9, 10], plasma kallikrein [9, 10], trypsin [8], chymotrypsin [8] and urokinase [9, 11, 12]. PCI is therefore a non-specific serpin and its physiological role has not been defined. PCI interacts with its target proteases by forming stable 1:1 complexes. Upon complex formation the reactive-site peptide bond of PCI is cleaved by the protease, and the C-terminal peptide is released [1]. Depending on the protease involved, complexes dissociate at differing rates, resulting in the release of active enzyme and inactive cleaved PCI [1]. Many of the PCI/protease reactions are stimulated by heparin and other glycosaminoglycans [7–13]. This stimulatory effect of heparin has been explained by a ternary-complex model, in which protease and inhibitor bind to the same glycosaminoglycan molecule and are thereby brought into close vicinity, resulting in a more rapid interaction [13]. However, formation of a ternary-complex does not seem to be the only mechanism involved in the stimulation of PCI activity by glycosaminoglycans, and direct alteration of PCI reactivity by saccharides has been discussed [14]. The inhibition-rate enhancement of PCI by heparin is relatively poor [8–10, 13] compared with those of other heparin-binding serpins, e.g. antithrombin III [15, 16] or heparin cofactor II [16, 17]. It has therefore been speculated that heparin binding to the putative heparin-binding site of PCI (H-helix, [13]), which is located in a different part of the serpin molecule than the heparin-binding site of antithrombin III and heparin cofactor II (D-helix), might result in a less favorable orientation of serpin and protease [14].

We have shown previously that PCI is an inhibitor of the trypsin-like serine-protease tissue kallikrein [18]. The primary physiological function of this enzyme seems to be kinin release from high-M_r and low-M_r kininogens by the cleavage of two peptide bonds within the kininogen molecules [19]. Since kinins have a variety of biological activities, including vasodilation and contraction of visceral smooth muscles, regulation of tissue kallikrein activity by serpin-type inhibitors might be physiologically important. The tissue-kallikrein–PCI interaction differs from all other PCI–target-enzyme interactions studied in that heparin does not enhance but interferes with the inhibition of tissue kallikrein by PCI [18], and high concentrations of heparin completely abolish the tissue-kallikrein–PCI interaction. This inhibitory effect is not restricted to heparin, but is also seen with

Correspondence to M. Geiger, Department of Vascular Biology and Thrombosis Research, Schwarzspanierstrasse 17, A-1090 Vienna, Austria

Fax: +43 1 4087500.

E-mail: margarethe.geiger@univie.ac.at

URL: http://www.univie.ac.at

Abbreviations. ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); KIU, kallikrein inhibitory unit; PCI, protein-C inhibitor; S-2266, D-valyl-L-leucyl-L-arginyl-*p*-nitroaniline.

Enzymes. Tissue kallikrein (EC 3.4.21.35); urokinase (EC 3.4.21.73).

other glycosaminoglycans [20]. In a purified system containing urokinase and tissue kallikrein, heparin shifts the target-enzyme specificity of PCI from a tissue-kallikrein inhibitor in the absence of heparin to a urokinase inhibitor in the presence of heparin [20, 21]. Other than PCI, no serpin-type inhibitor has been described whose protease-neutralizing activity could be stimulated or abolished by heparin, depending on the target protease. We therefore analyzed the inhibitory effect of heparin on the tissue kallikrein−PCI interaction in more detail employing urokinase as a control target protease.

EXPERIMENTAL PROCEDURES

Materials. Human urinary tissue kallikrein (Protogen AG); 5-oxoprolyl-glycyl-L-arginyl-*p*-nitroaniline and D-valyl-leucyl-arginyl-*p*-nitroaniline (S-2266) (Chromogenix), heparin, sodium salt (M_r 4000−6000, ≈140 U/mg; Fluka AG), SDS, acrylamide, bisacrylamide and stained M_r standards for SDS/PAGE (Bio-Rad), rainbow-protein M_r markers for SDS/PAGE (Amersham), heparin-Sepharose CL-6B (Pharmacia), aprotinin (Bayer), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt (ABTS, Boehringer Mannheim), and ^{125}I (New England Nuclear) were obtained as indicated. Urokinase was from Serono. It migrated on SDS/PAGE [22] as a doublet (M_r 52000 and 43000) and contained one additional faint band (M_r 32000). All bands were detected in immunoblots by monoclonal anti-urokinase IgG (MPW5UK) [23] and were functionally active on fibrin autography [24]. PCI was purified from human urine [18]. Routine chemicals were of the purest grade available and were purchased from Merck.

Analytical methods. Molar concentrations of tissue kallikrein and urokinase were evaluated from the cleavage of S-2266 and 5-oxoprolyl-glycyl-L-arginyl-*p*-nitroaniline, respectively, using ΔA_{405} values of 2.75 min^{-1} · cm^{-1} · μM^{-1} tissue kallikrein and 10 min^{-1} · cm^{-1} · μM^{-1} urokinase, respectively, as given by the manufacturer. The molar concentration of purified urinary PCI was determined from its absorbance at 280 nm using an absorption coefficient (1 cm, 1%) of 14.1 [1] and an M_r of 57000. SDS/PAGE (10% acrylamide) was performed according to Laemmli [22] using 1.5-mm slab. After electrophoresis, gels were fixed with acetic acid/methanol/water (10:50:40), dried, and exposed to Kodak X-Omat AR films.

Radiolabelling of urokinase and tissue kallikrein. ^{125}I-labelling of urokinase and tissue kallikrein was performed using Iodo-beads iodination reagent (Pierce) following the manufacturer's instructions. Excess low-M_r iodine was separated from the labelled proteins by means of an acrylamide buffer-exchange column (Beckman) equilibrated in 0.01 M Tris/HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4, and dialysis against the same buffer. ^{125}I-tissue-kallikrein was further purified by aprotinin-Sepharose affinity chromatography [25]. The specific activities of the labelled proteins were 13−39 μCi/nmol for ^{125}I-urokinase and 6−48 μCi/nmol for ^{125}I-tissue-kallikrein.

Affinity chromatography of ^{125}I-urokinase, ^{125}I-tissue-kallikrein and their complexes with PCI on heparin-Sepharose CL-6B. ^{125}I-urokinase (230 μl, 100 nM) and ^{125}I-tissue-kallikrein (160 μl, 98 nM) were incubated separately with PCI (800 μl, 1.25 μM each) in 0.01 M Tris/HCl, 0.1 M NaCl, pH 7.4. After 60 min at 37°C both incubation mixtures were pooled and applied to a heparin-Sepharose CL-6B column (1 cm×5 cm) equilibrated in 0.01 M Tris/HCl, 0.01% Tween 80, pH 7.4. After loading, the column was washed with equilibration buffer until the radioactivity in each fraction was below 100 cpm, and developed with a gradient from 0 to 2 M NaCl in equilibration buffer (50 ml each) at a flow rate of 40 ml/h. Fractions (1 ml) were collected from the loading of the column on and the ^{125}I radioactivity was measured in a Beckman 8000 γ-counter. Aliquots of the following species were subjected to SDS/PAGE and autoradiography: ^{125}I-tissue-kallikrein incubated with PCI (20 μl, ≈4000 cpm); ^{125}I-urokinase incubated with PCI (20 μl, ≈4000 cpm); the mixture of both, corresponding to the material applied to the heparin-Sepharose column (20 μl, ≈ 4000 cpm); and aliquots of the peak ^{125}I fractions (fraction 9, 100 μl, 1300 cpm; fraction 33, 150 μl, 1370 cpm; and fraction 38, 70 μl, 1800 cpm; Fig. 1A).

Complex formation of ^{125}I-urokinase and ^{125}I-tissue-kallikrein with PCI bound to heparin-Sepharose CL-6B. Heparin-Sepharose CL-6B (200 μl) was incubated with purified PCI (170 nM, 600 μl) for 1 h at 22°C in 0.01 M Tris/HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4, in 1.5-ml Eppendorf tubes. The supernatants were separated from the Sepharose beads by centrifugation and removed. PCI-treated heparin-Sepharose was washed twice with equilibration buffer and incubated with ^{125}I-urokinase (100 μl, 9.8 nM) or with ^{125}I-tissue-kallikrein (100 μl, 5 nM) in the same buffer at 22°C. After 1 h, supernatants were removed as above and the Sepharose was washed twice with equilibration buffer. The radioactivity in each supernatant, each washing fluid, and the washed Sepharose beads was measured in a Beckman 8000 γ-counter. 50 μl 0.26 M Tris/HCl, pH 6.8, 2.5% SDS, 25% glycerol was added to each tube containing Sepharose beads. Aliquots of the unbound ^{125}I-radioactivity, i.e. aliquots of the supernatants of the incubation mixtures of PCI/heparin-Sepharose with ^{125}I-tissue-kallikrein and ^{125}I-urokinase (^{125}I-urokinase 580 cpm; ^{125}I-tissue-kallikrein 2050 cpm), and the material eluted from the beads by SDS treatment (^{125}I-urokinase 1500 cpm; ^{125}I-tissue-kallikrein <100 cpm) were analyzed by SDS/PAGE and autoradiography performed as described above.

Effect of heparin on ^{125}I-tissue-kallikrein · PCI complexes. Five aliquots of PCI (0.37 μM, 80 μl) were incubated with ^{125}I-tissue-kallikrein (98 nM, 5 μl) in 0.01 M Tris/HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4, in 1.5-ml Eppendorf tubes at 37°C. After 2 h, aprotinin [50 μl, 10000 kallikrein-inhibitory units, (KIU)/ml] was added to one tube to stop the reaction. Into the remaining tubes heparin (10 μl, 3 mg/ml) was added, and the reactions were terminated in these tubes by addition of aprotinin (50 μl, 10000 KIU/ml) after incubation for 10, 30, 60 or 120 min, respectively. 50 μl 0.26 M Tris/HCl, pH 6.8, 2.5% SDS, 25% glycerol was added to each sample, and samples were heated in a boiling-water bath for 10 min and subjected to SDS/PAGE and autoradiography as described above.

Amidolytic assay for tissue kallikrein activity. Inhibition of the amidolytic activity of purified tissue kallikrein by purified PCI was tested on 96-well microtiter plates. Tissue kallikrein (2.3 nM) was incubated without or with PCI (86.5 nM) in the absence or presence of heparin (6 nM to 6 μM) and in the absence and presence of lysine, glutamic acid or glycine (2.5−80 mM each) at 37°C in 50 μl 0.01 M Tris/HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4. After 30 min 50 μl S-2266 (0.4 mM final), dissolved in 0.05 M Tris/HCl, pH 8.3, was added to each well and after incubation for 3−5 h at 37°C, the A_{405nm} was determined in an ELISA reader (Anthos Reader 2001).

Generation of tissue kallikrein amidolytic activity from tissue kallikrein · PCI complexes was studied in the following way. Tissue kallikrein (2.7 nM) was incubated without or with PCI (72 nM) in 50 μl 0.01 M Tris/HCl, 0.1 M NaCl, 0.01% Tween 80, pH 7.4, in wells of a microtiter plate. After 1 h at 37°C, 10 μl heparin was added (to 60 μM) to each well immediately or after incubation for up to 25 min. 5 min after the addition of heparin to the last wells, i.e. after a total incubation period of 1 h in the absence of heparin and up to 30 min in the presence

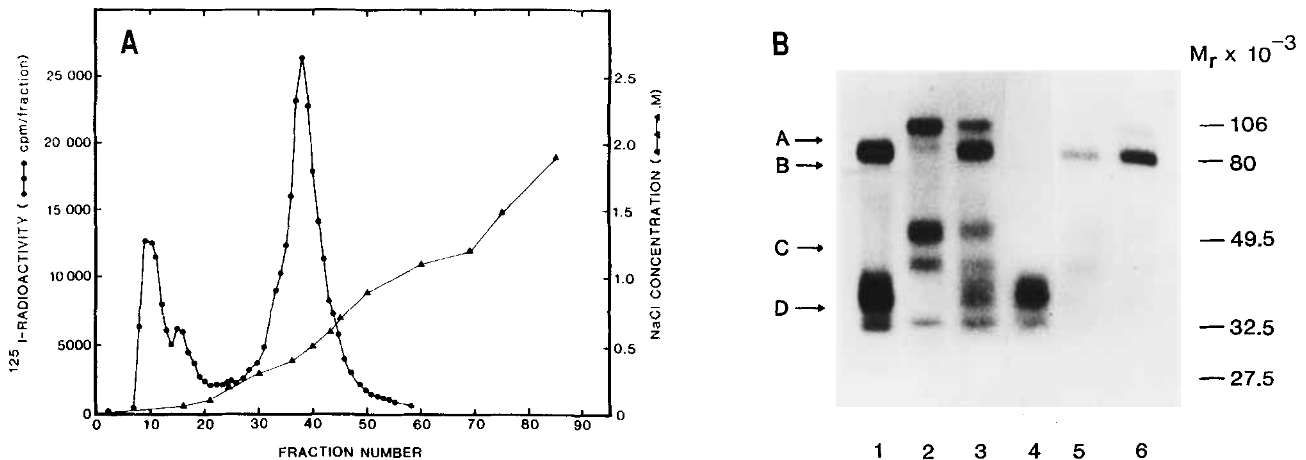


Fig. 1. Affinity chromatography of ^{125}I-tissue-kallikrein, ^{125}I-urokinase and their complexes with PCI on heparin-Sepharose CL-6B. A mixture containing ^{125}I-urokinase, ^{125}I-tissue-kallikrein, ^{125}I-urokinase · PCI, ^{125}I-tissue-kallikrein · PCI and PCI was subjected to affinity chromatography on a heparin-Sepharose CL-6B column (1 cm×5 cm) as described in Experimental Procedures. Elution was performed with a linear gradient from 0 to 2 M NaCl in 0.01 M Tris/HCl, 0.01% Tween 80, pH 7.4. (A) Elution profile of the heparin-Sepharose CL-6B column. (B) Autoradiograph of a SDS/PAGE gel (10% acrylamide) showing ^{125}I-tissue-kallikrein incubated with PCI (20 µl, ≈4000 cpm; lane 1), ^{125}I-urokinase incubated with PCI (20 µl, ≈4000 cpm; lane 2); a mixture of the material shown in lanes 1 and 2, corresponding to 20 µl of the material applied to the heparin-Sepharose column (lane 3), and the following fractions from the heparin-Sepharose column in (A) fraction 9 (100 µl, lane 4); fraction 33 (150 µl, lane 5); and fraction 38 (70 µl, lane 6). Migration distances of M_r markers are shown on the right. The arrows on the left indicate the migration distances of urokinase · PCI (A), tissue-kallikrein · PCI (B), urokinase (C) and tissue kallikrein (D).

of heparin, 50 µl S-2266 (0.4 mM final) dissolved in 0.05 M Tris/HCl, pH 8.3, was added to each well and substrate cleavage was determined as described above.

Binding of tissue kallikrein to immobilized PCI. Wells of a microtiter plate were coated overnight at 4°C with 100 µl of monoclonal anti-PCI IgG (4PCI) [18], which had been treated with 0.02 M glycine/HCl, pH 2.5, for 20 min at 37°C [26], and diluted to 10 µg/ml in 5.6 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.01% thimerosal, pH 9.6. Remaining binding sites were blocked with 1% BSA in 0.01 M Tris/HCl 0.14 M NaCl, pH 7.4 (Tris/NaCl), for 1 h at 37°C. The wells were rinsed three times with 0.5% Tween 20 in Tris/NaCl, and incubated with 50 µl PCI (288 nM) in Tris/NaCl containing 1% BSA, for 2 h at 37°C. The wells were rinsed as above and incubated for 1 h at 37°C either with 50 µl tissue kallikrein (0–35 nM) in Tris/NaCl, 1% BSA, or with 50 µl tissue kallikrein (0–27.5 nM) in Tris/NaCl, 1% BSA, containing 50 KIU aprotinin. After rinsing the plate as above, wells were incubated with rabbit anti-(tissue kallikrein) IgG (10 µg/ml) for 1 h at 37°C and, after additional washing as above, with peroxidase linked goat anti-rabbit-Ig (1:1000; Amersham). The plate was washed as above and bound peroxidase was quantified by incubating each well with 100 µl 0.1% ABTS in 0.11 M Na_2HPO_4, 0.5 M citric acid, pH 4.0, containing 0.03% H_2O_2. After 15 min the reactions were stopped by addition of 100 µl 0.32% NaF, and the absorbances at 405 nm were determined in an ELISA reader (Anthos Reader 2001).

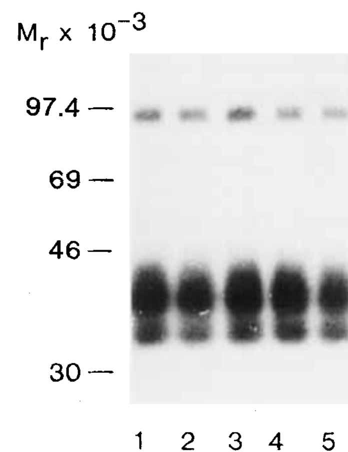


Fig. 2. Effect of heparin on ^{125}I-tissue-kallikrein · PCI complexes. ^{125}I-tissue-kallikrein (5 µl, 98 nM) was incubated with PCI (80 µl, 0.37 µM) at 37°C as described in Experimental Procedures. After 2 h, 50 µl aprotinin (10000 KIU/ml) was added to the sample shown in lane 1, and 10 µl of heparin (600 µM) each to samples shown in lanes 2–5. To these samples, 50 µl of aprotinin (10000 KIU/ml) was added after incubation at 37°C for 10 min (lane 2), 30 min (lane 3), 60 min (lane 4) or 120 min (lane 5), respectively. SDS/PAGE (10% acrylamide) and autoradiography were performed as described in Experimental Procedures. Migration distances of M_r markers are shown on the left.

RESULTS

To analyze affinities of tissue kallikrein and tissue kallikrein · PCI complexes for heparin, we studied binding of ^{125}I-tissue-kallikrein and ^{125}I-tissue-kallikrein · PCI complexes to a heparin-Sepharose CL-6B column equilibrated in 0.01 M Tris/HCl, 0.01% Tween 80, pH 7.4. ^{125}I-urokinase and ^{125}I-urokinase · PCI complexes were used as controls in the same experiment. Two peaks of ^{125}I-radioactivity were obtained; one represented the unbound material, the other material eluted between 0.3 M and 0.6 M NaCl (Fig. 1A). Aliquots of the material applied to the column (Fig. 1B) and aliquots of fractions of each of the two ^{125}I-radioactivity peaks were analyzed by SDS/PAGE and autoradiography. PCI complexes with both proteases, ^{125}I-urokinase and ^{125}I-tissue-kallikrein, and uncomplexed ^{125}I-urokinase were found in the peak eluted between 0.3 M and 0.6 M NaCl, whereas free ^{125}I-tissue-kallikrein was only present in the unbound peak (Fig. 1B). When uncomplexed ^{125}I-tissue kallikrein alone at a higher concentration (2 µM, 200 µl) was subjected to affinity chromatography on heparin-Sepharose CL-6B under the same experimental conditions, coinciding peaks of amidolytic activity (cleavage of S-2266) and radioactivity were obtained in the non-bound fraction, and neither radioactivity nor amidolytic activity was present in the fractions between 0.3 M and 0.6 M

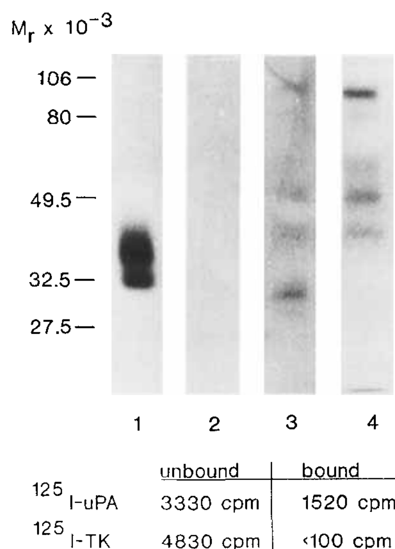


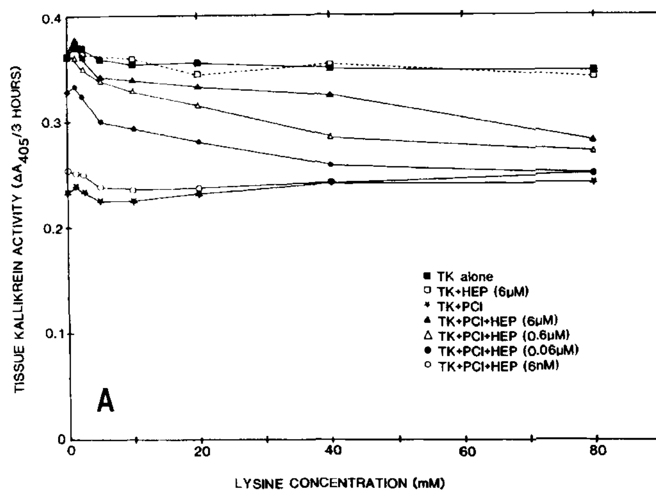
Fig. 3. Binding of ^{125}I-tissue-kallikrein and ^{125}I-urokinase to PCI immobilized on heparin-Sepharose CL-6B. 200 µl heparin-Sepharose CL-6B were incubated with PCI (170 nM, 600 µl) as described in Experimental Procedures, then with either ^{125}I-tissue-kallikrein (^{125}I-TK; 5 nM, 100 µl) or ^{125}I-urokinase (^{125}I-uPA; 9.8 nM, 100 µl) for 1 h. Unbound and bound radioactivities were determined and are shown for both enzymes at the bottom of the figure. Aliquots of unbound and bound material were analyzed by SDS/PAGE. Lanes 1 and 2, ^{125}I-tissue-kallikrein incubated with PCI-treated heparin-Sepharose, unbound material (2050 cpm, lane 1), bound material (< 100 cpm, lane 2); lanes 3 and 4, ^{125}I-urokinase incubated with PCI-treated heparin-Sepharose, unbound material (580 cpm, lane 3), bound material (1500 cpm, lane 4). Migration distances of M_r markers are indicated on the left.

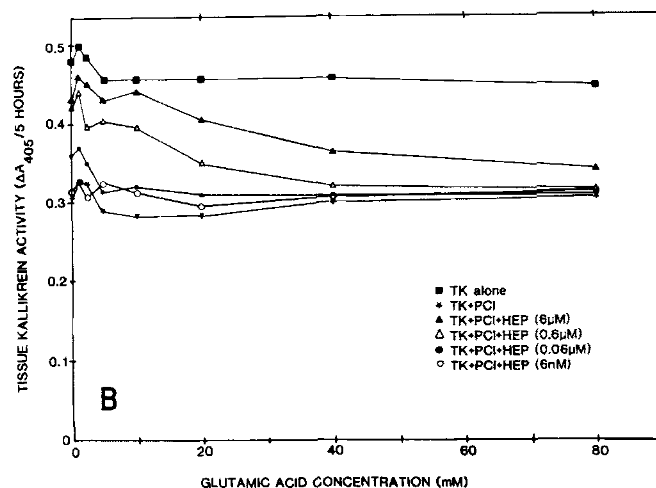
NaCl (data not shown). These data indicate that tissue kallikrein does not bind to heparin and that the inhibitory effect of heparin on the tissue-kallikrein–PCI interaction can only be caused by an interaction of heparin with PCI and/or with the tissue-kallikrein · PCI complex.

The effect of heparin on the dissociation of tissue-kallikrein · PCI complexes was analyzed in a functional assay, and by SDS/PAGE and autoradiography. In the functional assay PCI (72 nM) was incubated with tissue kallikrein (2.7 nM) in wells of a microtiter plate to allow complex formation. After 1 h at 37°C heparin (60 µM) was added for different times as described in Experimental Procedures. These experiments revealed that the amidolytic activity of wells incubated with heparin for 30 min was only slightly higher (ΔA_{405} = 0.092 h^{-1}) than the amidolytic activity of wells incubated for 30 min without heparin (ΔA_{405} = 0.081 h^{-1}). In controls containing only tissue kallikrein and no PCI ΔA_{405} increased from 0.103 h^{-1} in the absence of heparin to 0.118 h^{-1} after incubation with heparin for 30 min.

The amount of SDS-stable ^{125}I-tissue-kallikrein · PCI complexes present after 2 h ^{125}I-tissue-kallikrein–PCI interaction was unaltered by the addition of heparin up to 2 h (Fig. 2). Therefore, functional and SDS/PAGE data indicate that heparin does not dissociate tissue-kallikrein · PCI complexes.

The interaction of tissue kallikrein with heparin-bound PCI was studied by incubating ^{125}I-tissue-kallikrein with PCI bound to heparin-Sepharose CL-6B beads. In control experiments, binding of ^{125}I-urokinase to heparin-bound PCI was studied in the same way. More than 98% ^{125}I-tissue-kallikrein was recovered in the unbound fraction as free enzyme and no ^{125}I-tissue-kallikrein band was visible on SDS/PAGE in the lane corresponding to the material bound to PCI · heparin-Sepharose and eluted by SDS, whereas 30% of the applied ^{125}I-urokinase radioactivity bound to PCI · heparin-Sepharose (Fig. 3). Unbound ^{125}I-urokinase consisted mainly of several molecular forms of uncomplexed ^{125}I-urokinase, whereas the bound material that was eluted with SDS contained radioactive bands corresponding





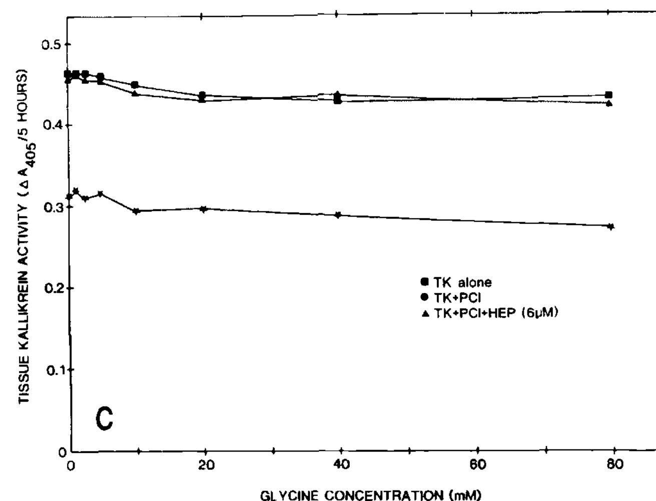


Fig. 4. Effect of amino acids on the inhibition of tissue kallikrein by PCI. Tissue kallikrein (TK; 2.3 nM) was incubated without or with PCI (86.5 nM) in the absence or presence of different concentrations of heparin (HEP) and in the absence and presence of different concentrations of lysine (A), glutamic acid (B) or glycine (C) in wells of a microtiter plate. After 30 min at 37°C, S-2266 was added, and the amidolytic activity was determined. Details are given in Experimental Procedures.

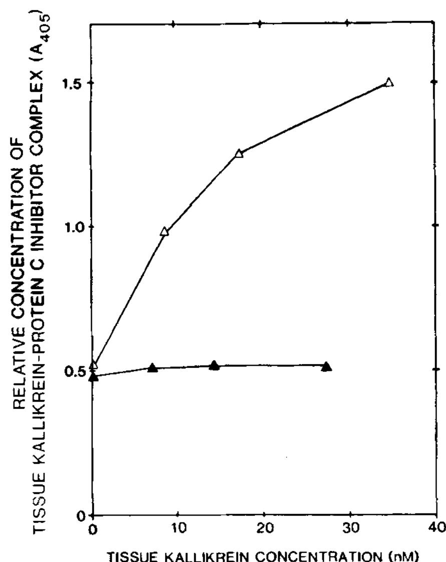


Fig. 5. Binding of tissue kallikrein to immobilized PCI. PCI was immobilized on a microtiter plate coated with monoclonal anti-PCI IgG and then incubated with different concentrations of tissue kallikrein (as indicated) in the absence (△) or presence (▲) of aprotinin (1000 KIU/ml). Bound tissue kallikrein was quantified using rabbit anti-(tissue kallikrein) IgG and peroxidase-linked anti-rabbit Ig. Bound peroxidase was quantified with the peroxidase substrate ABTS as described in Experimental Procedures. The obtained absorbance at 405 nm was used as the relative amount of tissue-kallikrein · PCI complex. Reagent blanks were not subtracted.

to free ^{125}I-urokinase (mainly M_r 52000) and several molecular entities of complexed ^{125}I-urokinase (mainly M_r 95000). Complex formation of urokinase and PCI during or after elution can be excluded, since the elution conditions employed (SDS) do not allow serine-protease–serpin interactions. These binding data therefore indicate that heparin-bound PCI is functionally active but is not able to form an SDS-stable complex with ^{125}I-tissue-kallikrein.

We studied the effect of basic and acidic amino acids on tissue kallikrein inhibition by PCI using glycine, which had no effect on the tissue-kallikrein–PCI or tissue-kallikrein–PCI–heparin interactions (Fig. 4C), as a control amino acid. In the absence of heparin, inhibition of tissue kallikrein by PCI was not influenced by lysine (Fig. 4A) or glutamic acid (Fig. 4B). However, both amino acids interfered in a dose-dependent manner with the inhibitory effect of heparin on the tissue-kallikrein–PCI interaction (Fig. 4A, B). Depending on the heparin/amino acid concentration ratio, both amino acids were able to completely reverse the inhibitory effect of heparin. These results indicate that acidic and basic amino acids are able to inhibit heparin-binding to PCI, either by blocking heparin-binding sites in the PCI molecule or by blocking binding sites for PCI in the heparin molecule. Such a neutralization of heparin-binding sites in the PCI molecule, however, does not seem to affect binding of tissue kallikrein to PCI. Therefore direct involvement of the heparin-binding site of PCI in PCI binding to tissue kallikrein does not seem to be very likely. Additional experiments were performed to study binding of tissue kallikrein to PCI in the absence and in the presence of aprotinin, which blocks the active site of tissue kallikrein [25]. No tissue-kallikrein–PCI complexes were measurable when tissue kallikrein was incubated with PCI in the presence of aprotinin (Fig. 5), while a tissue-kallikrein-dose-dependent increase in A_{405} was seen when tissue kallikrein was incubated with PCI in the absence of aprotinin. Therefore tissue kallikrein does not seem to bind to PCI when its active site is blocked, further supporting the idea that there is no major second binding site involved in the tissue-kallikrein–PCI interaction.

DISCUSSION

In this study we analyzed the inhibitory effect of heparin on the tissue-kallikrein–PCI interaction. This inhibitory effect of heparin on the tissue-kallikrein–PCI interaction is unusual since all other PCI–target-protease interactions studied are either unaffected [9, 10] or stimulated by heparin [7–13]. Furthermore, no other serpin has been described whose protease-neutralizing activity could either be stimulated or inhibited by heparin depending on the target protease. In our experiments we used urokinase as a control target protease of PCI, since the urokinase–PCI interaction and stimulation of this interaction by heparin has been studied in detail before [9, 11–13, 27]. Heparin-Sepharose binding of urokinase, PCI and urokinase · PCI complexes has also been shown previously [13]. Here we showed that tissue-kallikrein–PCI complexes bound to heparin-Sepharose and were eluted between 0.3 M and 0.6 M NaCl, while uncomplexed tissue kallikrein did not bind (Fig. 1). These data suggest that the inhibitory effect of heparin on the tissue kallikrein inhibition by PCI cannot be caused by an interaction of free tissue kallikrein with heparin, but rather by heparin-binding to free PCI and/or heparin-binding to tissue-kallikrein–PCI complexes. We therefore analyzed the following hypotheses: a) positively charged amino acids in the heparin-binding domain of PCI could be involved in binding of PCI to the acidic protease tissue kallikrein, and by neutralizing these positive charges, heparin could interfere with the enzyme-inhibitor interaction; b) heparin binding could cause a change in reactivity of the PCI molecule, which is not compatible with a tissue-kallikrein–PCI interaction, or could sterically hinder the tissue-kallikrein–PCI interaction; and c) tissue-kallikrein · PCI complexes could dissociate more rapidly in the presence than in the absence of heparin and thereby release active tissue kallikrein and cleaved PCI [8, 18].

The latter explanation for the inhibitory effect of heparin on PCI–tissue-kallikrein interactions would suggest that in the presence of heparin PCI becomes a substrate for rather than an inhibitor of tissue kallikrein. Experiments (Fig. 2) do not support the idea that in the presence of heparin PCI becomes a substrate for tissue kallikrein. They are confirmed by data shown in Fig. 1, indicating that intact ^{125}I-tissue-kallikrein · PCI complexes but no free ^{125}I-tissue-kallikrein were eluted from heparin-Sepharose by increasing the NaCl concentration, and by previous findings showing that heparin not only interfered with the inhibition of tissue kallikrein by PCI and with complex formation of tissue kallikrein with PCI, but also with the cleavage of PCI from its 57-kDa form to its 54-kDa form by tissue kallikrein [18]. Increased cleavage of PCI by tissue kallikrein would occur if heparin would cause rapid dissociation of the tissue-kallikrein · PCI complex. It is therefore unlikely that in the presence of heparin PCI becomes a substrate for rather than an inhibitor of tissue kallikrein. It is also unlikely that the heparin effect results from a heparin interaction with the tissue-kallikrein · PCI complex, but rather from an interaction of heparin with free PCI.

To analyze the hypothesis that heparin-binding sites of PCI, which contain basic amino acids [13, 28], might be directly involved in binding of PCI to the acidic protease tissue kallikrein (e.g. by representing an additional binding site), we studied the effect of positively and negatively charged amino acids on the inhibition of tissue kallikrein by PCI.

That positively charged and negatively charged amino acids interfered with the inhibitory effect of heparin suggests that

these amino acids either neutralize the heparin-binding site in the PCI molecule or the PCI-binding sites in heparin. None of the amino acids analyzed, however, had an effect on the inhibition of tissue kallikrein by PCI in the absence of heparin (Fig. 4). Glutamic acid, which presumably binds to positively charged amino acids in the PCI molecule and thereby interferes with heparin-binding to PCI, had no effect on the tissue-kallikrein–PCI interaction in the absence of heparin. These data suggest that the heparin-binding domain of PCI is not directly involved in PCI binding to tissue kallikrein. This is supported by the finding that enzymatically active tissue kallikrein, but not active-site-blocked tissue kallikrein, is able to bind to PCI (Fig. 5). Therefore, the possibility of a second major binding site for tissue kallikrein that might be blocked by heparin can be excluded.

We conclude that heparin-binding of free PCI is responsible for the inhibitory effect of heparin on the tissue-kallikrein–PCI interaction. The underlaying mechanism may be a change in reactivity of heparin-bound PCI, which could involve a conformational change of the PCI molecule. Alternatively, heparin-binding to PCI may sterically interfere with the tissue-kallikrein–PCI interaction. It is of interest that an inhibitory effect of heparin has been described for the interaction of tissue kallikrein with another serpin-type inhibitor, i.e. kallistatin (or kallikrein-binding protein), which is a tissue-kallikrein-inhibiting serpin closely related to PCI [29, 30]. In a purified system kallistatin inhibits tissue kallikrein with a similar rate constant as PCI does [29], and it is present in plasma in a concentration similar to that of PCI [29]. However, for the kallistatin–tissue-kallikrein interaction the mechanism of the inhibitory effect of heparin has not been elucidated so far. The modulation of PCI activity by heparin might represent an example for a mechanism by which the target-enzyme specificity of non-specific serpins, such as PCI, could be regulated.

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