

Factor XII-independent cleavage of high-molecular-weight kininogen by prekallikrein and inhibition by C1 inhibitor

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Background: Bradykinin formation typically requires interaction of Factor XII, prekallikrein (PK), and high-molecular-weight kininogen (HK) with negatively charged exogenous initiators or cell-surface proteins. Approximately 85% of plasma PK circulates as a complex with HK.

Nonenzymatic cell-derived initiators, such as heat shock protein 90, can activate the HK-PK complex to generate kallikrein, bradykinin, and cleaved HK, even in the absence of Factor XII.

Objective: We sought to determine whether PK, without activation to kallikrein, can digest HK to release bradykinin.

Methods: Kallikrein was measured by using a chromogenic assay, and bradykinin levels were determined by ELISA.

Cleavage of PK and HK were assessed by SDS-PAGE and Western blot analysis.

Results: Cleavage of HK by PK is demonstrated without any conversion of PK to kallikrein. HK cleavage by PK is distinguished from that of kallikrein by the following: (1) stoichiometric activation of HK by PK with release of bradykinin proportional to the PK input; (2) inhibition of PK cleavage of HK by corn trypsin inhibitor, which has no effect on kallikrein; and (3) inhibition of PK cleavage of HK by a peptide derived from HK, which inhibits binding of PK to HK. The same peptide has no effect on kallikrein activation of HK. C1 inhibitor (C1INH), the major control protein of the plasma bradykinin-forming cascade, inhibits PK cleavage of HK.

Conclusion: PK is an enzyme that can cleave HK to release bradykinin, and this reaction is inhibited by C1INH. This might account, in part, for circulating bradykinin levels and initiation of kinin formation in C1INH deficiency. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Factor XII, prekallikrein, kininogen, C1 inhibitor, angioedema

Contact activation of human plasma traditionally requires activation of Factor XII, conversion of prekallikrein (PK) to kallikrein by Factor XIIa, and digestion of high-molecular-weight kininogen (HK) by kallikrein.¹ Activation can be initiated

Abbreviations used

APMSF: (4-Amidinophenyl)-methanesulfonyl fluoride

C1INH: C1 inhibitor

CTI: Corn trypsin inhibitor

DFP: Diisofluorophosphate

HAE: Hereditary angioedema

HBS: HEPES-buffered saline

HK: High-molecular-weight kininogen

Hsp90: Heat shock protein 90

PK: Prekallikrein

S2302: H-D-prolyl-D-phenylalanyl-D-arginine p-nitrophenylester

by means of incubation of plasma with negatively charged inorganic surfaces (glass, kaolin, and dextran sulfate) or macromolecules with biologic properties (LPS and amyloid β protein). It can also occur on binding of each of the above plasma proteins to cells, such as platelets,²⁻⁵ neutrophils,⁶ or endothelial cells.⁷⁻⁹ However, activation of the plasma bradykinin-forming pathway on the surface of endothelial cells can be demonstrated in the absence of Factor XII,^{10,11} although a prominent augmentation is seen when Factor XII is present.¹²⁻¹⁴ In this instance the conversion of PK to kallikrein is dependent on interaction with heat shock protein 90 (Hsp90)¹⁵ or prolylcarboxypeptidase.¹⁶ Although the cleavage of PK observed is identical to that seen with Factor XIIa, Hsp90 is not known to be a proteolytic enzyme, and prolylcarboxypeptidase is an exopeptidase rather than an endopeptidase. Thus the active site that cleaves PK is not defined. Because the cleavage of PK by Hsp90 is stoichiometric (ie, the amount of kallikrein formed is proportional to the Hsp90 input),¹⁵ we theorized that PK might autoactivate when bound to Hsp90, suggesting the presence of a potential active site in PK.

In this article we demonstrate that PK is capable of digesting HK to release bradykinin. However, the reaction is stoichiometric, in contrast to kallikrein, which is catalytic. Because PK circulates bound to HK,¹⁷ this reaction could theoretically cleave much of the kininogen present. However, PK cleavage of HK is inhibited by C1 inhibitor (C1INH).

METHODS

Materials

Human plasma proteins, HK, PK, and kallikrein, were purchased from Enzyme Research Laboratories (South Bend, Ind). HK and PK were treated with 0.1 mmol/L (4-amidinophenyl)-methanesulfonyl fluoride (APMSF; Boehringer Mannheim, Indianapolis, Ind) before storage in 4 mmol/L sodium acetate buffer containing 0.15 mol/L NaCl (pH 5.5) at -70°C . Recombinant HK was purchased from R&D Systems (Minneapolis, Minn). The synthetic substrate H-D-prolyl-D-phenylalanyl-D-arginine p-nitrophenylester (S2302)

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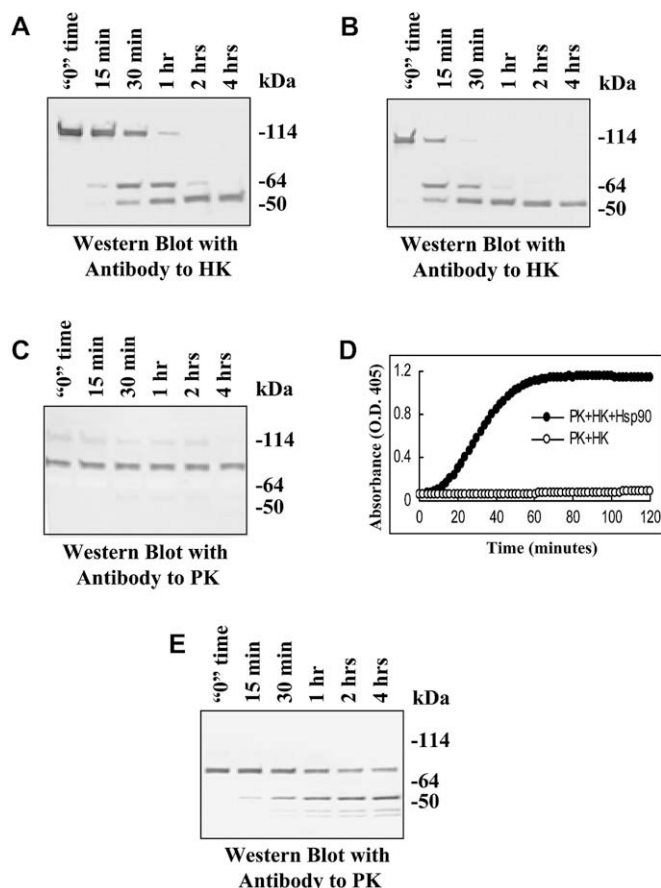


FIG 1. Comparison of proteolysis of HK by PK with that of kallikrein. **A**, Western blot analysis of HK (0.6 nmol/L) cleavage by PK (0.6 nmol/L) with antibodies to the light chain of HK. **B**, Western blot analysis of HK (0.6 nmol/L) cleavage by kallikrein (0.06 nmol/L). **C**, Western blot analysis of duplicate samples from Fig 1, A, with antibody to PK. **D**, HK and PK were incubated in the presence or absence of Hsp90, and the conversion of PK to kallikrein was monitored with a synthetic chromogenic substrate, S2302. **E**, Western blot analysis of samples from Fig 1, D, by using antibody to PK.

was purchased from Kabi Pharmacia, Inc (Franklin, Ohio). Purified Hsp90 was purchased from Calbiochem (La Jolla, Calif). Antibody to HK was prepared as described previously,¹⁸ and antibody to PK was obtained from Accurate Chemical and Scientific Corp (Westbury, NY). Protease inhibitors and other reagents were obtained from Sigma (St Louis, Mo).

PK activation assay

PK activation assays were performed in assay buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L D-glucose, and 1 mg/mL RIA-grade BSA), with a kallikrein-specific substrate (0.6 mmol/L S2302) in 96-well disposable polystyrene microtiter plates (Dynatech Laboratories, Chantilly, Va). These microtiter plates were pretreated with 1% polyethylene glycol (Aquacide III; Calbiochem, La Jolla, Calif) in HEPES-buffered saline (HBS; 10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, and 11 mmol/L D-glucose) for 2 hours to prevent adsorption of the proteins used for the assay. Just before the assay, all the proteins were treated with 2.0 mmol/L APMSF for 90 minutes at pH 5.5, after which they were diluted 1:100 with assay buffer and incubated for 30 minutes to allow for the decomposition of any unreacted APMSF at the neutral pH. Assay buffer was also pretreated with 0.4 μ mol/L APMSF to inactivate any serine protease activity present in the RIA-grade BSA. HK and PK were incubated in the assay buffer in the presence of S2302, and the kallikrein activity was determined by using

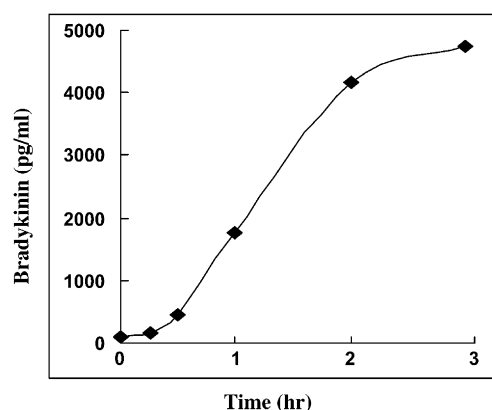


FIG 2. Bradykinin release from HK by PK. HK (0.6 nmol/L) was incubated with PK (0.6 nmol/L) at room temperature for 4 hours and assayed for bradykinin, as described in the Methods section.

color development. The absorbance (OD at 405 nm) was monitored at room temperature on a Molecular Devices (Sunnyvale, Calif) THERMOMax microplate reader.

Bradykinin assay

For bradykinin assay, samples were prepared by incubating HK and PK at room temperature and withdrawing aliquots at indicated time intervals. The protein in the aliquots was precipitated with ice-cold ethanol and centrifuged for 1 hour at 10,000 rpm in a microcentrifuge at 4°C, and the supernatants containing free bradykinin were collected. The supernatant was then evaporated with a centrifugal concentrator to dryness and resuspended in the EIA buffer. Bradykinin enzyme immunoassay was performed with an assay kit obtained from Peninsula laboratories (San Carlos, Calif). Briefly, bradykinin antibody was first bound to a specially treated 96-well plate. Then biotinylated peptide and samples or standards were added, mixed, and incubated for 2 hours at room temperature. After incubation, unbound peptides were removed by means of washing, and horseradish peroxidase-conjugated streptavidin was added and allowed to bind to the immobilized biotinylated peptide. The wells were again washed, and the peroxidase substrate 3,3',5,5'-tetramethyl benzidine dihydrochloride was added to react with the bound horseradish peroxidase. The color intensity is inversely proportional to the amount of peptide present. Concentration of the peptide present in samples was calculated using a standard curve.

SDS-PAGE and Western blotting

Samples were prepared by incubating desired protein mixtures at room temperature and withdrawing aliquots at indicated time intervals. The reaction was stopped by the addition of SDS sample buffer containing 5% 2-mercaptoethanol. SDS-PAGE was performed by using the buffer system of Laemmli.¹⁹ Gradient gels (4% to 15%) were used for separation of proteins. After electrophoresis, the gels were transferred to nitrocellulose membranes overnight. The membranes were then incubated with blocking buffer (1% BSA in PBS) for 1 hour. After blocking, the membranes were probed with mAbs for an additional 1 hour. Bound probes were visualized by incubating the membranes with alkaline phosphatase-conjugated secondary antibodies followed by color development in 5-bromo, 4-chloro, 3-indolylphosphate nitroblue tetrazolium.

Binding of PK to HK and its inhibition by WIP27

PK binding to HK was studied by using a direct binding assay. Microtiter plates were first coated with 1 μ g/mL HK overnight at 4°C. The plates were then washed 3 times with HBS and blocked with 3% milk in PBS for 1 hour at room temperature. The plates were again washed and incubated with various

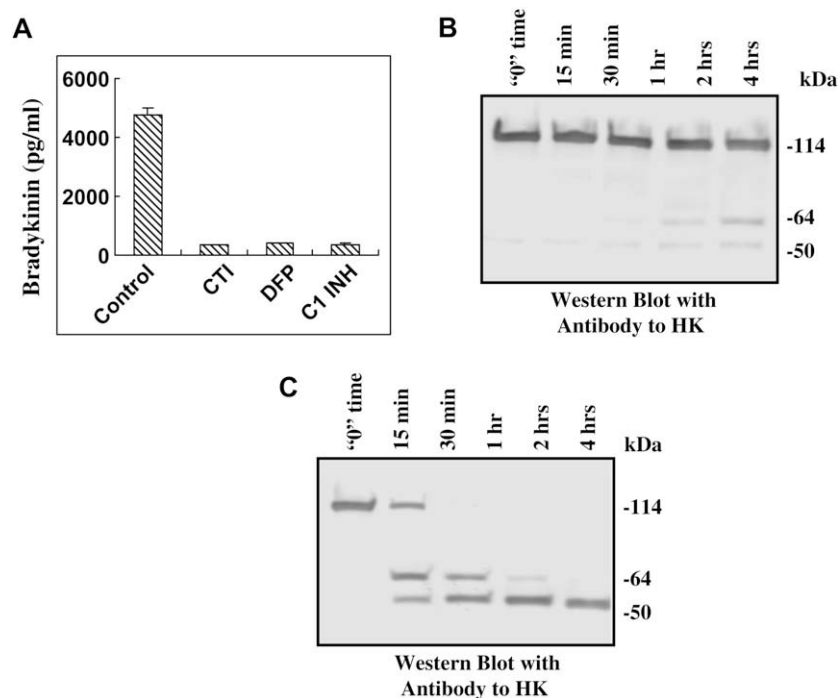


FIG 3. CTI inhibited the cleavage of HK by PK and not the kallikrein-mediated HK cleavage. **A**, Inhibition of bradykinin formation from HK by PK with various inhibitors. **B**, Western blot analysis showing inhibition of HK cleavage by PK with CTI (1.5 nmol/L). **C**, Western blot analysis showing absence of inhibition of HK cleavage by kallikrein with CTI (1.5 nmol/L).

concentrations of PK in the binding buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L D-glucose, and 1 mg/mL RIA-grade BSA) for 1 hour. The bound PK was detected with antibody to PK followed by peroxidase-conjugated secondary antibody and color development with Turbo-TMB ELISA (Pierce, Cheshire, United Kingdom). For inhibition studies, HK-coated plates were reacted with 1 μ g/mL PK in the presence of increasing concentrations of the peptide WIP27. The bound PK was then detected similarly.

Stoichiometry of HK and PK interaction

Three different concentrations of PK were incubated with increasing concentrations of HK (0.5–35 nmol/L) for 3 hours at room temperature. Similarly, 3 different concentrations of HK were incubated with increasing concentrations of PK (0.5–25 nmol/L). After 3 hours, samples were precipitated with ice-cold ethanol to separate free bradykinin from HK, and bradykinin was measured as described above.

RESULTS

Proteolytic cleavage of HK by PK

These 2 proteins were mixed in HEPES-buffered saline (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, and 11 mmol/L glucose, pH 7.4) containing 1 mg/mL BSA and incubated at room temperature to demonstrate the proteolytic cleavage of HK by means of the direct action of PK. Samples were collected at different time points, separated by means of SDS-PAGE, and analyzed by means of Western blotting with antibodies to the light chain of HK. Fig 1, A, shows the cleavage of HK and the formation of 2 forms of the light chain at 56 and 62 kd.²⁰ A comparison with proteolysis of HK by kallikrein (Fig 1, B) also shows the same pattern of cleavage; the fragments generated by PK and kallikrein were indistinguishable. However, the rate of HK cleavage by kallikrein was much faster, even though kallikrein was used at a

10-fold lower concentration compared with PK. We also confirmed that PK was not converted to kallikrein under these conditions when assessed by means of Western blot analysis (Fig 1, C) and by using a more sensitive chromogenic assay with a synthetic substrate specific for kallikrein (Fig 1, D). Because Hsp90 is reported to activate PK to kallikrein,¹⁵ we used Hsp90-mediated activation of PK to kallikrein as a control in the chromogenic assay (Fig 1, D). Proteolytic cleavage of PK and the formation of kallikrein by Hsp90 are also shown by Western blot with antibody to PK (Fig 1, E). The heavy chain of kallikrein is seen at about 50 kd, and the 2 forms of light chain are seen at 36 and 33 kd.²¹

We next measured the amount of bradykinin generated from HK by PK with an enzyme immunoassay. As shown in Fig 2, bradykinin was released on incubation of HK with PK, and the amount of kinin released was calculated to be proportional to the extent of proteolysis of HK, thus confirming that the cleavage of HK by PK was complete.

We considered the possibility that small amounts of kallikrein contaminating the PK preparation could be responsible for the activity, even though the PK preparation was treated with APMSF and had no discernable activity on pro-phe-arg-chloromethyl ketone.

The mechanism of HK cleavage by PK is different from that by kallikrein

In an effort to find a specific inhibitor for this reaction, we have tested various serine protease inhibitors. We found that C1INH, corn trypsin inhibitor (CTI), and diisofluorophosphate (DFP) inhibited the release of bradykinin from HK by PK (Fig 3, A). Inhibition of proteolysis of HK by PK in the presence of CTI is shown in Fig 3, B, whereas CTI had no effect on HK cleavage by kallikrein (Fig 3, C). These results suggested that the active

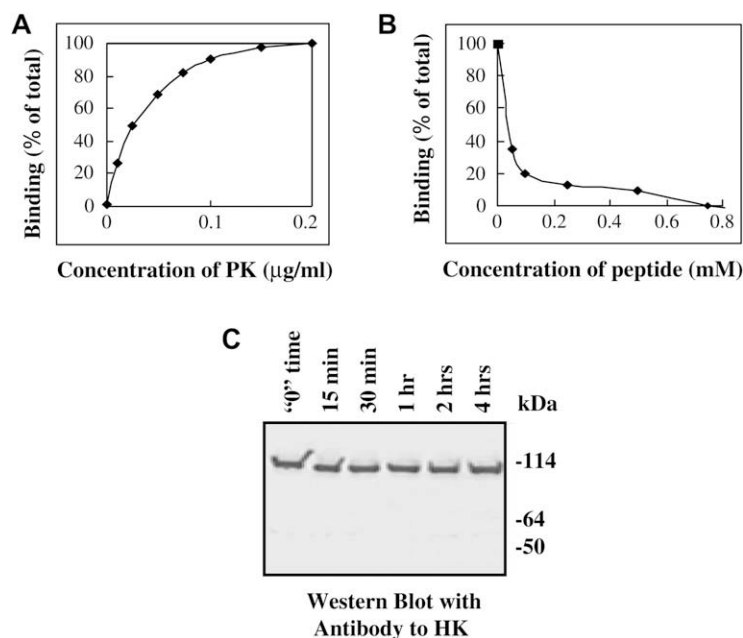


FIG 4. PK binding to HK and its inhibition by an inhibitory peptide, WIP27. **A**, PK binding to HK was performed as described in the Methods section. **B**, PK binding to HK in the presence of increasing concentrations of the inhibitory peptide WIP27. **C**, HK (0.6 nmol/L) was incubated with PK (0.6 nmol/L) at room temperature and analyzed by means of Western blotting with antibody to the light chain of HK.

sites required for HK cleavage by PK and kallikrein are different. Because CTI is also a specific inhibitor of Factor XIIa²²⁻²⁴ and Factor XIIa has been shown to slowly cleave HK and release bradykinin,²⁵ it was necessary to rule out any contamination by Factor XIIa in our experimental system. APMSF (p-amidinophenyl-methanesulfonyl fluoride) is an inhibitor of Factor XIIa, and addition of this inhibitor did not affect the PK-mediated HK cleavage. These experiments also confirmed that there is no contamination of kallikrein in our system because APMSF is known to inhibit kallikrein activity completely.^{26,27} C1INH, a plasma inhibitor of kallikrein²⁸ and Factor XIIa,^{29,30} inhibited the PK-mediated bradykinin formation (Fig 3, A).

HK and PK form a complex before HK is cleaved

To demonstrate that an interaction between HK and PK is necessary for HK cleavage, we incubated HK and PK in the presence of a peptide that is derived from the PK binding region of HK (WIP27; WIPDIQTDPNGLSFNPISDFPDTTSPK) and followed the pattern of cleavage. WIP27 has been shown to inhibit PK binding to HK.³¹ The binding of PK to HK is shown in Fig 4, A, and the inhibition of binding in the presence of WIP27 is shown in Fig 4, B. In the presence of excess WIP27 peptide, PK-mediated HK cleavage was completely blocked (Fig 4, C), suggesting that PK binding to HK is necessary for the cleavage of HK and the release of bradykinin. This peptide has no effect on the cleavage of HK by kallikrein.

Inhibition of bradykinin formation

To further understand the inhibition of PK-mediated cleavage of HK by C1INH, as well as DFP, we measured bradykinin formation in the presence of increasing concentrations of these inhibitors. Both inhibitors blocked bradykinin generation,

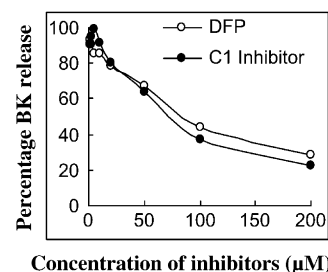


FIG 5. Inhibition of binding and bradykinin (BK) formation in PK-mediated HK cleavage by different serine protease inhibitors. Binding was performed as described for Fig 4. After incubation for 3 hours, the supernatants were collected and assayed for bradykinin as described for Fig 2.

achieving 80% inhibition at 200 μmol/L concentration of the inhibitors (Fig 5).

Stoichiometry of HK and PK interaction

To further demonstrate that PK-mediated cleavage of HK differs from kallikrein-mediated HK cleavage, we compared the stoichiometry of these 2 reactions. Different concentrations of PK (6.25, 12.5, and 25 nmol/L) were incubated with increasing concentrations of HK (0.5–35 nmol/L) at room temperature for 3 hours, and similarly, different concentrations of HK (8.7, 17.4, and 26.1 nmol/L) were incubated with increasing concentrations of PK (0.5–25 nmol/L). Bradykinin formed in each reaction was measured. When PK was kept constant at 6.25 nmol/L, the amount of bradykinin released never exceeded that concentration (Fig 6, A), even when the HK concentration was increased to 35 nmol/L. Similarly, the concentration of bradykinin did not exceed the level of PK with the PK concentration at 12.5 and 25 nmol/L. Fig 6, B, demonstrates the amount of bradykinin released when

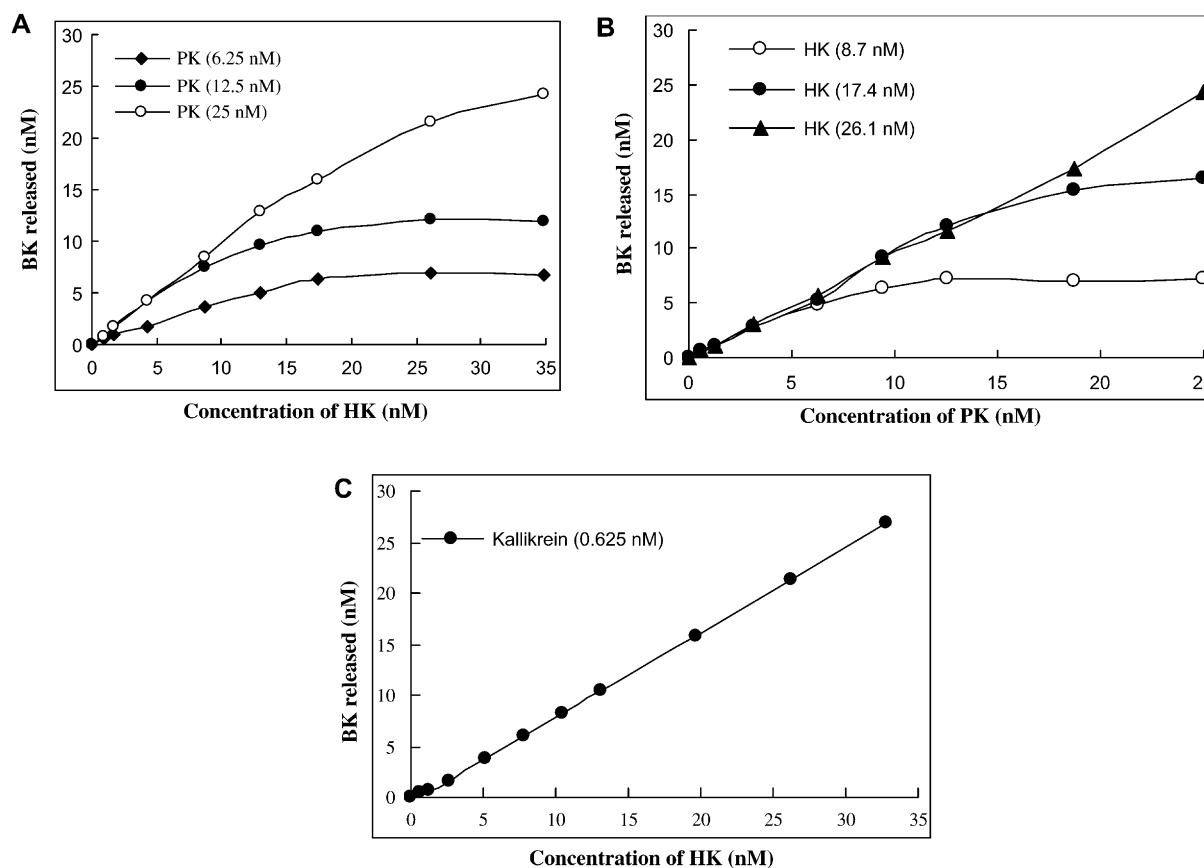


FIG 6. Stoichiometry of HK-PK interaction. **A**, PK (6.25, 12.5, and 25 nmol/L) was incubated with increasing concentrations of HK (0.5–35 nmol/L) for 3 hours at room temperature, and the bradykinin (BK) released was prepared and measured as described for Fig 2. **B**, HK (8.7, 17.4, and 26.1 nmol/L) was incubated with increasing amounts of PK. Samples were prepared and assayed for bradykinin as in Fig 2. **C**, Kallikrein (0.625 nmol/L) was incubated with increasing concentrations of HK at room temperature for 3 hours, and released bradykinin was measured.

HK was incubated with increasing concentrations of PK. As the PK concentration was increased, the amount of bradykinin formed increased up to, but not beyond, the molar input of HK. By contrast, the kinetics of kallikrein-mediated HK cleavage and bradykinin release (Fig 6, C) are very different. A 10-fold lower concentration of kallikrein (0.625 nmol/L) compared with the lowest concentration of PK (6.25 nmol/L) was sufficient for complete cleavage of HK and release of bradykinin. To further confirm the stoichiometry of the reaction, we prepared a 1:1 molar complex of PK with the light chain of HK and incubated this complex with additional native HK. There was no cleavage of HK or release of bradykinin (data not shown), which is consistent with cleavage of HK by PK, which is stoichiometric, and demonstrating that binding to the HK light chain precludes further proteolysis.

DISCUSSION

Although activation of the plasma bradykinin-forming pathway is initiated by Factor XII, recent studies of activation on endothelial cells have revealed activation of the PK-HK complex by cell-derived Hsp90¹⁵ or prolylcarboxypeptidase.¹⁶ The product of these interactions is kallikrein, cleaved HK, and bradykinin. When Factor XII is present, the initiating active site could be formed by Factor XII autoactivation on interaction with cell-

surface binding proteins³² or, alternatively, by kallikrein cleavage of Factor XII.¹² Thus the reciprocal activation involving Factor XII and PK, described decades ago,³³ is operative at the cell surface as well.

We have now found additional complexity regarding these interactions because PK has an active site that becomes evident when it is bound to HK. The resultant cleavage of HK by PK is stoichiometric and differs from that seen with kallikrein, which is catalytic. In fact, this difference is the reason why such an interaction might have been missed in the past. At the ratio (1:50) of kallikrein to HK commonly used in enzymatic reactions, PK would only activate 2% of the HK present, whereas kallikrein would activate 100%. Any PK contribution to the reaction would have been considered “background.” We have demonstrated that the active site within PK differs from that of kallikrein by the requirement for stable complex formation with HK (Fig 4) and by the inhibition of PK by CTI, which has no effect on kallikrein. DFP inhibits both, but PK inhibition requires higher molarity and much longer incubation (data not shown). It is critical that although PK and kallikrein are both inhibited by C1INH, digestion of HK by kallikrein is so much faster than that of PK that the presence of C1INH prevents bradykinin formation by PK. If PK is converted to kallikrein, C1INH then limits bradykinin formation by binding irreversibly to the kallikrein active site.

These data have relevance for hereditary angioedema (HAE; C1INH deficiency) because the extreme lability of this system might be due not only to the absence of the major inhibitor of Factor XIIa^{29,30} but also a missing control protein for stabilization of the PK-HK complex. With absent or dysfunctional C1INH, seemingly spontaneous generation of bradykinin is seen, even when incubated in nonactivating plastic containers.³⁵ Such lability might be due to the cleavage of HK by PK, which might contribute to attacks of swelling apart from the lack of inhibition of Factor XII autoactivation.^{36,37}

One might question why most of the HK is not cleaved at all times in plasma of patients with C1INH deficiency because 85% of PK circulates bound to HK and only 15% circulates free.³⁴ The answer is not known, but possibilities include the presence of alternative inhibitors, nonspecific effects of a high protein milieu, or both, which might prevent HK cleavage unless Factor XII is recruited and an episode of swelling ensues. On the other hand, plasma from patients with HAE, even under optimal circumstances, have increased bradykinin levels.^{35,38} Our most recent data indicate that half the HK in HAE plasma is cleaved in 15 minutes simply by means of incubation of the plasma at room temperature without addition of any activator.³⁸ Low levels of bradykinin present in normal plasma (10-25 pg/mL)^{20,39,40} had been assumed to be due to cleavage of low-molecular-weight kininogen by tissue kallikrein, but PK cleavage of HK may be responsible.^{41,42}

Factor XII, once converted to Factor XIIa by means of autodigestion,^{12,36} can serve as the initiator of the bradykinin-forming cascade, and any kallikrein produced markedly increases the rate of Factor XII activation.³³ However, if the PK-HK complex were able to activate Factor XII in addition to cleaving HK, the initiating active site would reside within PK. Absence of C1INH would eliminate control of these reactions in addition to effects on Factor XIIa, Factor XIIIf, or kallikrein. It will be of interest to assay the kallikrein inhibitor ecallantide⁴³ to determine whether it also inhibits the PK activity described herein. Finally, the addition of Hsp90 (or prolylcarboxypeptidase) to the PK-HK complex leads to cleavage of PK and conversion to kallikrein. Hsp90 is not known to have a proteolytic active site, and the amount of PK converted to kallikrein within the complex is directly proportional to the Hsp90 input; that is, there is 1:1 stoichiometry between Hsp90 and PK. We speculate that the active site within PK that is evident when it is bound to HK might lead to PK autoactivation when the PK-HK complex is bound to Hsp90. Hsp90 is released from endothelial cells by "stressful" conditions, such as hypoxia, or endothelial cell activation by cytokines. Perhaps episodes of swelling in cases of HAE initiated by trauma, infection, estrogen, or even emotional stress might lead to release of Hsp90 and activation of the bradykinin-forming cascade.

Clinical implications: The digestion of HK by PK and its inhibition by C1INH might contribute to low levels of bradykinin in normal plasma and to initiation of attacks of swelling in patients with HAE.

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