IDENTIFICATION AND CHARACTERIZATION OF PROLYLCARBOXYPEPTIDASE AS AN ENDOTHELIAL CELL PREKALLIKREIN ACTIVATOR

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Running Title: HUVEC PRCP activates prekallikrein

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ABBREVIATIONS

PK: prekallikrein
HK: high molecular weight kininogen
uPAR: urokinase plasminogen activator receptor
CK1: cytokeratin 1
FXI: factor XI
FXII: factor XII
PKA: endothelial cell prekallikrein activator
PRCP: prolylcarboxypeptidase
HUVEC: human umbilical vein endothelial cells
CTI: corn trypsin inhibitor
HCB: HEPES-carbonate buffer
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
LAMP1: lysosomal-associated membrane protein 1
SUMMARY

Recent investigations postulate an endothelial cell (HUVEC)-associated prekallikrein activator (PKA). When prekallikrein (PK) assembles on high molecular weight kininogen (HK) on HUVEC, PK is activated to kallikrein. PKA is found in the 15,800 X g pellet of HUVEC lysates using an assay that measures PK activation only when bound to HK linked to microtiter plates. Sequential DEAE, wheat germ lectin affinity, and hydroxyapatite chromatography result in 4 protein bands on SDS-PAGE. One protein in the 73 kDa band is identified by amino acid sequencing as prolylcarboxypeptidase (PRCP). On gel filtration, PKA activity is a single homogenous peak identical in migration to the 73 kDa immunoblot of PRCP. Anti-PRCP inhibits PKA activity and PK activation on HUVEC. Purified PKA is blocked by DFP (1 mM), PMSF (3 mM), leupeptin (100 µM) antipain (IC₅₀=2 µM), HgCl₂ (IC₅₀=500 µM), Z-Pro-Pro-aldehyde-dimethyl acetate (IC₅₀=1 µM), and corn trypsin inhibitor (IC₅₀=40 nM). PKA does not correct the coagulant defect in factor XII deficient plasma, is purified from HUVEC cultured in factor XII deficient serum, is not detected by antibody to factor XII, does not activate FXI, and is not inhibited by a neutralizing antibody to FXII. Angiotensin II (IC₅₀=2 µM) or bradykinin (IC₅₀=100 µM), but not angiotensin II₁₋₇ or bradykinin₁₋₅, and the prolyl oligopeptidase inhibitor, Fmoc-Ala-Pyr-CN (IC₅₀=50 nM), also block purified PKA activation of PK. The $K_m$ of PK activation by PRCP is 6.7 nM. PRCP antigen is present on the membrane of fixed but not permeabilized HUVEC. PRCP appears to be a HUVEC-associated PK activator.
INTRODUCTION

The physiologic initiator of activation of the plasma kallikrein/kinin system has not been identified. Recent evidence indicates that the assembly of prekallikrein (PK) and high molecular weight kininogen (HK) on endothelial cells results in kallikrein formation that is blocked by antipain (1-3). On endothelial cells, HK assembles on a multiprotein receptor that consists of at least gC1qR, urokinase plasminogen activator (uPAR), and cytokeratin 1 (CK1) (4-8). Moreover, CK1 colocalizes on endothelial cells with uPAR but not gC1qR (9).

HK, the parent protein for bradykinin, is found primarily in plasma and some tissues (10). The majority of plasma PK and factor XI (FXI) circulates in a complex with HK allowing for HK bound to its multiprotein receptor to serve as the PK and FXI receptor on endothelial cells (3,11,12). Activation of FXI on endothelial cells requires factor XIIa (FXIIa), either exogenously added or endogenously derived from the bovine enzyme present in bovine serum in the culture media (12). Alternatively, PK activation on endothelial cells occurs in the absence of endogenous or exogenous FXII or its activated forms (3). FXII does not autoactivate on endothelial cells as fast as PK activation when PK is assembled on HK (13). However, activated FXII associated with endothelial cells amplifies PK activation (14). These combined data suggest that there is a prekallikrein activator (PKA) associated with endothelial cells in culture independent of factor XIIa. These investigations show that the serine protease, prolylcarboxypeptidase (lysosomal carboxypeptidase, angiotensinase C, PRCP), an enzyme of the renin angiotensin system, is an endothelial cell PK activator.
EXPERIMENTAL PROCEDURES

Materials: Frozen human umbilical vein endothelial cells (HUVEC), endothelial cell growth medium (EGM), trypsin-EDTA and trypsin neutralizing buffer were purchased from Clonetics (San Diego, CA). Wheat germ immobilized on 6% agarose macrobeads, CM Cellulose, SP-Sephadex, and hydroxyapatite (calcium phosphate hydroxide, Type 1) were obtained from Sigma Chemical Co (St Louis, MO). DEAE (diethylaminoethyl) cellulose was purchased from Whatman (Fairfield, NJ). Triton X-100 was purchased from Boehringer-Mannheim. Prestained and low molecular weight standards, nitrocellulose, polyacrylamide and Biobeads SM2 were purchased from Bio-Rad (Richmond, CA). HK, PK, corn trypsin inhibitor (CTI), plasma kallikrein, and antibody to human factor XII were purchased from Enzyme Research Laboratory (South Bend, IN). HD-Pro-Phe-Arg-paranitroanilide (S2302) was from DiaPharma (Franklin, OH). Peptides angiotensin II, angiotensin II₁₋₇, bradykinin, bradykinin₁₋₅, mercuric chloride, iodoacetic acid, iodoacetamide, N-ethylmalimide, o-phenanthroline, leupeptin, and benzamidine were purchased from Sigma. Antipain and phenylmethylsulfonylfluoride (PMSF) were obtained from Calbiochem (San Diego, CA). Antibody to LAMP1 was obtained from Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA. Monoclonal antibody (HKL16) to the PK binding site on HK was generous provided by Dr. Werner Muller-Esterl, Frankfurt, Germany. Peptide SDD31 (S⁵⁶⁵DDDWPMDIQTDPMGNLSFNP-ISDFPDTPSPK⁵⁹⁵) from HK’s domain 6 and which is the PK binding site on HK was synthesized at Multiple Peptide Systems, San Diego, CA (12). Peptides K⁶⁶⁻TFNQRYLVDK⁸¹ and R⁴⁷⁹⁻HMKNWIRDFYDSAGKH⁴⁹⁶ from mature PRCP were synthesized and used to prepare antisera in goats by QCB Biochemicals, Hopkinston, MA.
**PK Activator Assay:** The activity of the endothelial cell prekallikrein activator (PKA) was determined in a solid phase assay. PKA activity was measured in 100 µl reaction mixtures consisting of enzyme in cell lysate or purified protein in HEPES-carbonate buffer (HCB) (137 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 14.7 mM Hepes, 5.5 mM dextrose and 0.1% gelatin, pH 7.1 containing 10 µM CaCl₂, and 1 mM MgCl₂). In preparation for the assay, HK in 0.1 M Na₂CO₃, pH 9.6 was linked to a 96 well microtiter plates by overnight incubation at 4°C. Preliminary experiments revealed that linking HK in such a manner did not result in its proteolysis and the amount of HK linked to microtiter plate was constant from experiment to experiment. After incubation, the wells were washed with HCB and then blocked with 1% gelatin. Samples containing endothelial cell lysate or partially purified enzyme were added in 100 µl aliquots to the cuvette wells in HCB containing 20 nM PK and incubated for 1 h at 37°C. Triton X-100 in cell lysates was removed using Bio-Beads SM2 adsorbent (Bio-Rad, Hercules, CA). After incubation, the wells were washed to remove unbound activator and PK and the enzyme reaction was initiated by addition of 100 µl of S2302 (0.8 mM). Activation of PK was observed at 405 nm for 1 h at 37°C. The amount of kallikrein formed in the presence of the activator was determined by comparing it with the hydrolysis of S2302 by known amounts of plasma kallikrein (0.01-2.0 nM) in HCB for 1 h. Only 20% of the added substrate were hydrolyzed under the conditions of the assay and the substrate hydrolysis was linear for an incubation time up to 120 min.

**Endothelial Cell Culture.** Human umbilical vein endothelial cells (HUVEC) were obtained and cultured in growth medium (EGM) containing bovine brain extract, according to the recommendations of Clonetics. Cells between the 1st-3rd passage were subcultured onto fibronectin-treated T-175 flasks, 2 days prior to the start of the experiment as previously reported (12). Cell viability was determined using trypan blue exclusion.
Extraction and purification of endothelial cell PKA from human umbilical vein endothelial cells: The entire purification process was performed at 4°C. Fifty-100 flasks (T-175) of confluent monolayers of HUVEC (20-50 mg total protein) in passage 2 or 3 were removed and homogenized for 3 rounds of 20 strokes each with a hand homogenizer in 5 volumes of homogenizing buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.5 mM EGTA). The crude homogenate then was centrifuged for 10 min at 1,200 X g and the supernatant was collected. The pellet was re-homogenized in 3 volumes of homogenizing buffer and its supernatant was combined with the first supernatant. The combined supernatants were then centrifuged for 20 min at 15,800 X g. The resulting pellet was dissolved in 10 ml of 0.3% Triton X-100 in homogenizing buffer and the detergent/membrane mixture was gently rocked for 40 min at 4°C. The mixture was then centrifuged for 60 min at 105,000 X g. The supernatant was the Triton X-100 solubilized granule-vesicular fraction of the 15,800 X g pellet. Its pellet was Triton X-100 insoluble granule-vesicular membrane proteins. The Triton X-100 solubilized granule-vesicular fraction and not its pellet or the supernatant of the 15,800 X g centrifugation, contained most of the PKA activity and was subjected to further purification.

A DEAE cellulose column was packed and equilibrated in 10 mM Tris-HCl, pH 7.4, 0.05 M NaCl containing 0.01% Triton X-100, 0.5 mM EGTA. The Triton X-100-solublized 15,800 X g pellet was loaded onto the DEAE column (1 by 10 cm). After sample loading, the column was washed with 30 ml of starting buffer followed by a 15-ml linear gradient of the same buffer containing 1 M NaCl and then a 10 ml wash with the 1 M NaCl buffer. The PKA was not adsorbed onto the DEAE column. The fractions containing PKA were pooled and concentrated to 5 ml using the centriplus concentrators (YM 10), Amicon (Bedford, MA).
Fractions from the DEAE cellulose flow-through were loaded onto a 5 ml wheat germ agglutinin agarose column equilibrated in 10 mM Tris-HCl, 50 mM NaCl, pH 7.4 containing 0.5 mM EGTA. After adsorption of the sample, the column was washed with 20 ml starting buffer followed by a nonlinear gradient consisting of 15 ml 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 containing 0.5% NP-40, 0.1% SDS and 5 mM EDTA. PKA was then eluted with 15 ml of 50 mM Tris-HCl, 500 mM NaCl, pH 7.4 containing 0.5% Triton-X100, 0.5 mM EDTA. LAMPl, a lysosomal protein that co-purified with PKA through this point, was eluted from the wheat germ agglutinin column with 15 ml 10 mM Tris-HCl, pH 8.0 containing 100 mM N-acetylglucosamine.

Fractions from the wheat germ agglutinin column with PKA activity were pooled, concentrated with centriplus concentrators (YM 10, Amicon) and dialyzed against 10 mM Tris-HCl, 50 mM KCl, pH 7.4, containing 0.5 mM EGTA. After which they were loaded onto a 2 ml hydroxyapatite (calcium phosphate hydroxide) column equilibrated in the same buffer. Flow-through fractions containing PKA activity were pooled and concentrated to 3 ml using the centriplus concentrators (YM 10, Amicon).

PKA from the hydroxyapatite chromatography or purified by the technique of Odya et al. or Tan et al. was applied to a 24-ml prepacked Superdex 200 HR 24/30 column (1.0 X 24 cm) (Pharmacia) equilibrated with 6 bed volumes of 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 at 0.3 ml/min on an AKTA FPLC system (Amersham Pharmacia Biotech, Piscataway, NJ) (15,16). This column had been calibrated using molecular weight standards: bovine thyroid thyroglobulin (669,000), horse spleen ferritin (440,000), bovine liver catalase (232,000), rabbit muscle aldolase
(158,000), bovine serum albumin (67,000), hen egg ovalbumin (43,000), bovine pancreas chymotrypsinogen A (25,000) and bovine pancreas ribonuclease A (13,700). Fractions of the gel filtration that contained PKA were pooled and concentrated in a Centricon-3 ultrafiltration device and frozen at -20°C until assay.

**Protein Sequencing:** Protein sequence analysis was performed at the Harvard Microchemistry Facility (Harvard University, Cambridge, MA). After tryptic digestions of protein eluted from bands of the SDS-PAGE, the protein sequencing was performed by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer (17-19).

**Gel electrophoresis and immunoblot studies:** Proteins in purification fractions were applied directly or were precipitated with 5% deoxycholate:trichloroacetic acid followed by solubilization in 15 µl of 2X SDS sample buffer containing 2% β-mercaptoethanol and boiled for 5 min. The proteins were separated on an 8 or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was stained with Colloidal-Blue (Novex Technical Service, San Diego, CA) or silver (Bio-Rad). In certain experiments, proteins were separated on a 10% SDS-PAGE and then transferred to nitrocellulose membranes at 8 mA overnight. The membranes then were incubated in blocking buffer [5% (wt/v) dry milk with 0.1% (w/v) BSA, 0.05% Tween 20, 0.15 M NaCl, and 20 mM Tris-HCl, pH 7.4] for 1 h (20). The nitrocellulose membranes were incubated with a rabbit antibody against prolylcarboxypeptidase (PRCP) (1:1,500) that was generously provided by Dr. Randal Skidgel, University of Illinois, Chicago IL, or a goat anti-PRCP peptide antisera (1:100) for 1 h at room temperature. After washing, the nitrocellulose was then incubated with an anti-rabbit or anti-
goat antibody horseradish peroxidase conjugate, respectively. The specific reactivity of antibody with electroblotted sample was detected with the ECL system from Amersham (Arlington Height, IL). In certain experiments, the immunoblot was scanned by densitometer (Model GS 300 Hoefer Scientific Instruments, San Francisco, CA) in the transmittance mode to determine the band intensity and thus relative amounts of the protein present.

**Laser scanning confocal microscopy.** Monolayers of non-permeabilized HUVEC grown on glass slides were washed and fixed in 2% paraformaldehyde for 15 min at 37°C. After washing the slides with 50 mM glycine in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) for 5 min at room temperature, the cells were incubated with rabbit antiserum to PRCP (1:100) or normal rabbit serum (1:100) in PBS containing 1 mg/ml human γ-globulin and 1 mg/ml glucose for 1 h at 37°C. Rabbit antibody attached to the HUVEC was identified by incubating a goat anti-rabbit IgG conjugated with FITC (1:50) (Calbiochem) for 1 h at room temperature. After incubation, the cells were washed, covered with antifading mounting medium (Molecular probe, Eugene, OR), and analyzed on the laser scanning confocal microscopy as previously described (9).

**Characterization of the PKA:** Initial experiments determined the inhibitory spectrum of isolated PKA. The ability of rabbit antisera or antibody to block PK activation by purified PRCP or endothelial cell-associated PKA was determined. The ability of purified PRCP to change the structure of PK bound to HK on plastic was determined in the absence or presence of 1 mM DFP, 3 mM PMSF, 300 µM angiotensin II, or 0.2 mg/ml neutralizing antibody to factor XIIa by immunoblot using an antibody to PK (Enzyme Research Labs) of the reaction mixture after reduced 10% or 12% SDS-PAGE (3,8). Further studies determined the ability of 100 µM leupeptin or antipain, 1 mM o-phenanthroline and
EDTA, 3 mM N-ethylmalimide, or 5 mM iodoacetic acid or iodoacetamide to inhibit PKA activation of PK. Antipain, HgCl$_2$, benzamidine, corn trypsin inhibitor (CTI), or Z-Pro-Pro-aldehyde-dimethyl acetate also were mixed in increasing concentrations with PKA in HCB for 1 min prior to the addition of PK to microtiter plates wells coated with HK. After 1 h incubation at 37°C, the wells were washed and the formed kallikrein bound to HK was detected by hydrolysis of the 0.8 mM chromogenic substrate HD-Pro-Phe-Arg-pNA (S2302). Further investigations determined if increasing concentrations of angiotensin II, angiotensin II$_{1-7}$, bradykinin, bradykinin$_{1-5}$ (peptide RPPGF), or Fmoc-Ala-Pyr-CN inhibited PK activation by the PKA. Fmoc-Ala-Pyr-CN is a prolyl oligopeptidase inhibitor that was generously provided by Dr. Sherwin Wilks, Mount Sinai Medical School, New York, NY (21). The ability of the partially purified PKA to activate FXI and to clot factor XII-deficient plasma (George King, Overland Park, KS) was performed by an amidolytic assay using 0.8 mM L-pyroglutamyl-L-prolyl-L-arginine-pNA (S2366, Dia-Pharm, Franklin, OH) and by factor XII coagulant assay, respectively (20). PKA was also examined on immunoblot using antibody to factor XII. Last, the ability of a neutralizing anti-FXII antibody (0.1-0.5 mg/ml) to block PK activation by purified PKA was determined. The ability of this antibody to inhibit the factor XII coagulant activity of 50% normal human plasma (181 nM factor XII) was determined in an activated partial thromboplastin time coagulant assay using reagents from Organon Teknika, Durham, NC.

*Properties of the PKA:* The $K_m$ of PK activation by the PKA was determined by incubating increasing concentrations of PK with PKA at 37°C. Kallikrein formed was detected by hydrolysis of 0.8 mM S2302. The optimal pH for PKA activity also was determined from a pH range of 5.5 to 10.5. MES was used to buffer the pH range from 5.5 through 6.8; HEPES was used from pH 7 to 7.4; Tris was used from pH 7.8 to 8.5 and Na$_2$CO$_3$ was used from pH 9-10.5. Activities did not vary more than
10% between buffers at the crossover pH values 6.8 and 7.4. Additional studies determined the influence of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) ions on PKA activity.

**HK and PK binding to HUVEC and PK activation on HUVEC.** Biotinylated-HK or -PK binding to HUVEC in culture was performed as previously reported (3,8). PK activation on endothelial cells also was performed as previously reported (3,9). Additional studies determined if angiotensin II, angiotension II\(_{1-7}\), Fmoc-Ala-Pyr-CN, or Z-Pro-Pro-aldehyde-dimethyl acetate blocked PK activation when bound to HK on HUVEC (3).

**Protein Assay:** Protein concentration was determined by the method of Bradford utilizing dye reagent from Bio-Rad (Hercules, CA) and bovine serum albumin (BSA) as a standard. Concentration of the purified PK activator also was determined by measuring UV absorption at 205 nm with the same BSA as the standard and the eluting buffer as the blank.
RESULTS

Nature of HUVEC PK activator (PKA). Previous investigations indicated that the endothelial cell PK activator was inhibited by antipain, cysteine, HgCl₂, dithiothreitol, or glutathione, but not by 1 mM PMSF or benzamidine (3). Since PK activation on HK bound to endothelial cells required the binding of HK to HUVEC and the binding of PK to HK, any agent that interfered with these activities also would interfere with PK activation on endothelial cells. Initial investigations determined if any of the above agents interfered with HK binding to HUVEC. In the presence of glutathione, cysteine, or dithiothreitol, HK binding to HUVEC was inhibited 50, 75, and 87%, respectively (data not shown). Alternatively, antipain and HgCl₂ did not inhibit HK binding to HUVEC. HgCl₂, however, was found to inhibit PK binding to HK on HUVEC by 25% (data not shown). Last, experiments determined that 3 mM PMSF and 1 mM DFP, but not 1 mM PMSF as previously reported (3), prevented the conversion of PK to kallikrein on HUVEC as seen on reduced SDS-PAGE of solubilized cells with bound PK/kallikrein (data not shown). These combined data suggested that the hypothesized PK activator (PKA) was a serine protease.

Development of the PKA assay: In order to isolate the PKA from endothelial cells, a specific assay was developed. The assembly of HK followed by PK and PKA in microtiter plate cuvette wells resulted in PK activation to kallikrein with hydrolysis of the chromogenic substrate, S2302 (Figure 1). If PK was excluded from the mixture or HK was not linked to cuvette wells, no PK activation occurred. Similarly, exclusion of the PKA or the chromogenic substrate for kallikrein resulted in no detectable hydrolysis. If the complex between HK and PK is blocked by the addition of peptide SDD31, the PK binding site on HK, or a monoclonal antibody (HKL16) directed to the PK binding
site on HK, PK activation also was prevented. These data indicated that PK must be bound to HK for activation by the PKA.

*Isolation of the PKA from endothelial cells:* HUVEC were homogenized and subjected to differential centrifugation to locate the fraction containing PKA using the above assay. The PKA activity primarily was associated with the 15,800 X g pellet, the fractions of HUVEC lysates enriched for granule or lysosomal material. Confirmation that PKA was from the granule-lysosomal compartment of HUVEC was indicated by the presence of lysosomal-associated membrane protein 1 (LAMP1) by immunoblot in the same fractions (data not shown).

A series of sequential column chromatography purification steps were performed to isolate the PKA. PKA and LAMP1 did not bind to DEAE Sephadex. After the DEAE affinity chromatography, there was a large increase in the specific activity of the PKA with an apparent increase in yield suggesting that this isolation step eliminated an endogenous inhibitor of the enzyme (Table I). Wheat germ agglutinin bound both PKA and LAMP1; however, PKA eluted before LAMP1. Using size exclusion chromatography on hydroxyapatite, eluted PKA was purified 27,019-fold with 25% recovery of activity (Table I). The hydroxyapatite preparation of PKA on SDS-PAGE consisted of 4 bands at 96, 73, 48, and 24 kDa (data not shown). PKA activity was mostly associated with the 73 and 48 kDa (data not shown).

Amino acid sequencing of the four bands seen on SDS-PAGE using the microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry technique resulted in the identification of 48 individual proteins. In the 73 kDa fraction, 12 distinct protein fragments were
sequenced. One protein identified by the amino acid sequencing was prolylcarboxypeptidase (lysosomal carboxypeptidase, angiotensinase C, PRCP), a serine protease of the renin angiotensin system. PRCP is known to be present in endothelial cell lysosomes, but also present on endothelial cell membranes because it is a physiologic converting enzyme of angiotensin II to angiotensin II_1-7 (22). In order to examine whether PRCP was a candidate PK activator, PRCP was purified from HUVEC by the technique used by Odya et al. (15). Using the assay to detect PKA in the Odya purification schema (DEAE, CM-cellulose, SP-Sephadex, CM-cellulose and hydroxyapatite chromatography), PKA was found in the identical fractions where PRCP was to be found. The data suggested that PKA and PRCP are the same protein.

Characterization of PKA as PRCP. Investigations were performed to determine if PKA and PRCP co-eluted on FPLC (Figure 2A). Final fractions of PKA from either the present purification schema or that of Odya et al. were applied to a Superdex 200 FPLC. A single peak of PKA was detected at fraction 32 (fractions 30-34) (Figure 2A). This location corresponded to a molecular weight on gel filtration of 62 ± 12 kDa (n = 23 separate fractionations). The fractions from the Superdex 200 FPLC were also immunoblotted with a rabbit antibody to PRCP (Figure 2B) and a goat anti-PRCP peptide antisera (Figure 2C). PRCP antigen at 73 kDa was found in the identical fractions with fraction 32 containing the highest concentration of PRCP antigen (Figures 2A-2C). Further, increasing concentrations of rabbit antisera to PRCP blocked PKA activation of purified PK on HK in microtiter plates whereas rabbit serum at the same dilution had little inhibitory effect (Figure 3A). Likewise, increasing concentrations of purified rabbit IgG against PRCP blocked PK activation on HUVEC when the PK was bound to HK (Figure 3B). These combined data suggested that PKA and PRCP antigen were the same proteins.
Studies determined the inhibitory profile of isolated PKA. Since serine protease inhibitors block both PKA activation of PK and formed kallikrein activity, investigations determined if DFP or PMSF blocked the formation of kallikrein by PKA, as detected by reduced SDS-PAGE followed by immunoblot (Figure 4A). Under the conditions of the assay, PKA-treated PK resulted in 15% kallikrein formation as indicated by the presence of a 50 kDa heavy chain and two light chains at 42 and 38 kDa on this gel electrophoresis system (Figure 4A). In the presence of 1 mM DFP or 3 mM PMSF, kallikrein did not form. These data suggested that the PKA was a serine protease. Further studies were performed against other inhibitors. Leupeptin and antipain both at 100 µM neutralized the kallikrein forming activity of PKA (Figure 4B). One mM EDTA or o-phenanthroline inhibited about 2% or 12% of PKA activity, respectively (Figure 4B). Three mM N-ethylmalimide reduced PKA activity by 30%. However, 5 mM iodoacetic acid or iodoacetimide had no inhibitory activity on PKA. These combined studies indicated that PKA was a serine protease.

Investigations next were performed to determine if the inhibitory profile of PKA was consistent with that of PRCP. As previously reported, antipain inhibited PKA with an IC₅₀ of 2 µM, but benzamidine, had no effect at 10 mM (3) (Figure 5). HgCl₂ inhibited PRCP with an IC₅₀ of 500 µM. The prolyl oligopeptidase inhibitor, Z-Pro-Pro-aldehyde-dimethyl acetate, inhibited PKA with an IC₅₀ of 1 µM (Figure 5). The serine protease inhibitor, corn trypsin inhibitor (CTI) also was an inhibitor of PKA with an IC₅₀ of 40 nM (Figure 5). This latter result suggested that PKA might be contaminated with trace amounts of factor XIIa. However, purified PKA did not correct the coagulant defect of factor XII-deficient plasma and did not hydrolyze the factor XIIa substrate H-D-Pro-Phe-Arg-pNA. Further, PKA was isolated from endothelial cells grown in 2% human factor XII
deficient serum. Antibody to human factor XII did not recognize purified PKA from HUVEC on immunoblot. A neutralizing antibody to factor XII, which at 0.1 to 0.5 mg/ml concentrations completely inhibited the coagulant activity of 50% normal human plasma, had no specific inhibitory activity against PKA. Last, isolated PKA was unable to activate factor XI bound to HK on a microtiter plate. These data and the fact that PKA is not inhibited by benzamidine indicated that PKA is not factor XIIa or PRCP is not contaminated with catalytic amounts of factor XIIa.

Further evidence that the isolated PKA was PRCP was determined by substrate inhibition studies (Figure 6). Two μM angiotensin II, the preferred known substrate of PRCP, inhibited isolated PKA by 50% (Figure 6). Alternatively angiotensin II1-7, which does not have the carboxyterminal phenylalanine of angiotensin II, did not inhibit PKA at 150 μM. Similarly, bradykinin another substrate of PRCP, but not bradykinin1-5, inhibited PKA with an IC50 of 100 μM (Figure 6) (23). Last the prolyl oligopeptidase inhibitor, Fmoc-Ala-Pyr-CN, inhibited the PKA with an IC50 of 50 nM (Figure 6). These combined data also indicated that PKA was PRCP.

Investigations were performed to determine of known substrates and inhibitors of PRCP would inhibit PK activation when assembled on HK on cultured endothelial cells (Figure 7). Angiotensin II at 100 μM, but not angiotensin II1-7, blocked PK activation on HUVEC with 100% inhibition at 100 μM (Figure 7). Fmoc-Ala-Pyr-CN and Z-Pro-Pro-aldehyde-dimethyl acetate also completed inhibited PK activation on HUVEC 100% at 3 and 10 mM concentration, respectively.

Properties of PKA (PRCP): The Km of PRCP for PK is 6.7 nM (Figure 8). The PKA was stable at 37°C with a half-life of 240 min. At 95°C, all activity was lost after 3 min of incubation. The PKA-
induced activation of PK exhibited a high activity (>75% of maximum) between pH 6.8 and 7.4, with an optimum at pH 7.1. PKA activity was optimal with 10 μM CaCl₂ and 1 mM MgCl₂ whereas various concentrations of BaCl₂, NiCl₂, CdCl₂, CuCl₂ and MnCl₂ had little influence on PKA activity. Last, investigations were performed to determine the structure of PRCP- versus factor XIIa-formed plasma kallikrein (Figure 9). Both PRCP and factor XIIa produced a 50 kDa heavy chain and 39 and 36 kDa light chains of reduced plasma kallikrein (Figure 9A). However, angiotensin II at 300 μM blocked PRCP the cleavage and activation of PK, but not the cleavage and activation produced by factor XIIa (Figure 9A and 9B). Alternatively, neutralizing antibody to factor XIIa blocked factor XIIa’s cleavage and activation of PK, but did not inhibit PRCP cleavage and activation (Figures 9A and 9B). Under the conditions of the assay in Figure 9B, 40 pM of factor XIIa alone was insufficient to hydrolyze the chromogenic substrate itself (data not shown). These data suggested that PRCP and factor XIIa were distinctly different kallikrein-forming enzymes.

PRCP is expressed on HUVEC: Investigations were performed to determine the expression of PRCP on fixed, but non-permeabilized HUVEC in culture (Figure 10). On laser scanning confocal microscopy, PRCP antigen is expressed on non-permeabilized HUVEC membranes (Figure 10). These data indicated that a portion of the lysosomal carboxypeptidases is constitutively expressed on the membrane of cultured endothelial cells.
DISCUSSION

Several novel observations emerge from these studies. PK activation on HUVEC is initiated by a membrane-associated enzyme called prekallikrein activator (PKA). This enzyme is the first identified cell-associated activator of the plasma kallikrein/kinin system that is better known as the "contact system". These studies also provide evidence that the HUVEC PKA is prolylcarboxypeptidase (PRCP). The evidence that PKA is PRCP is that they overlap on gel filtration, antibody to PRCP inhibits PKA and PK activation on HUVEC, and substrates of PRCP block PK activation by partially purified endothelial cell PKA and on endothelial cells themselves. PRCP is a ubiquitous enzyme which cleaves C-terminal to proline whose known substrates are angiotensin I and angiotensin II converting both to the angiotensin II1-7 (15,16,23). As a converting enzyme for angiotensins, PRCP changes the blood pressure elevating and prothrombotic effects of angiotensin II to a vasodilatory peptide, angiotensin II1-7 that directly stimulates NO and prostacyclin formation (24-26). Recognition that PRCP also activates PK is the first evidence to our knowledge that this enzyme is an endoproteinase. It also indicates that this enzyme indirectly produces bradykinin that potentiates the vasodilatory effects of angiotensin II1-7 (27). These data suggest that the regulation of expression of PRCP is important in maintaining vasodilatory activity in the intravascular compartment. Recognition that PRCP of the renin angiotensin system is a plasma prekallikrein activator indicates a new and potentially important interaction between these two systems.

It was an initial concern that the PKA was found to be inhibited by corn trypsin inhibitor (CTI). Corn trypsin inhibitor is only known as an inhibitor to factor XIIa. Although unpublished studies from our laboratory show that CTI also inhibits urokinase and tissue plasminogen activator, no
other enzymes have been described as CTI sensitive. However, the partially isolated PRCP did not hydrolyze a factor XIIa substrate or correct the coagulant defect of factor XII deficient plasma, was present when purified from endothelial cells that were cultured in human factor XII deficient serum, was not recognized by antibody to human factor XII, was not inhibited by a neutralizing antibody to factor XII, and did not activate factor XI. Last, factor XII would have bound to the DEAE column under the conditions used in the purification of PKA whereas PRCP does not (15,28). These combined data indicated that PKA is not factor XIIa and factor XIIa in catalytic amounts is not present in the PRCP preparation.

PK activation when bound to HK occurs by the presence of a membrane-associated enzyme. It seems a paradox to identify the PKA as PRCP, lysosomal carboxypeptidase, i.e., an enzyme purified from the lysosomal fraction of endothelial cells. However, PRCP itself is known to be present on membranes of cells since it is the physiologic converter of plasma angiotensin II to angiotensin II₁₋₇ (22,29). On laser scanning confocal microscopy PRCP is constitutively expressed on the external membrane of cultured endothelial cells. These data indicate a portion of the lysosomal pool of PRCP is expressed upon the cell membrane (30). Recent cell biology studies suggest that the lysosomal compartment in all eukaryotic cells is an endomembrane system that is intimately involved in the export of internal constituents (31).

The properties of PRCP are not fully known. On gel filtration, the PK activation activity and antigen appear as a 62 ± 12 kDa protein consistent with its predicted molecular mass of 55.8 kDa of the full-length, unprocessed precursor. This molecular size is similar to its migratory features on reduced SDS-PAGE where it is a 73 kDa protein. This latter molecular size is similar to the SDS-
PAGE migration of PRCP isolated from human kidney (Dr. R. Skidgel, personal communication). The inhibition of PRCP by Fmoc-Ala-Pyr-CN or Z-Pro-Pro-aldehyde-dimethyl acetate, two prolyl oligopeptidase inhibitors, provides evidence for its varied features. However, the relatively flat slope of inhibition produced by Fmoc-Ala-Pyr-CN probably reflects the fact that this inhibitor was developed as one against prolyl oligopeptidases not prolylcarboxypeptidase (21). How PRCP cleaves PK to activate it to kallikrein is not completely known. The factor XIIa catalytic site on PK is \( R^{371}-I^{372} \). The structure of kallikrein generated by PRCP on reduced SDS-PAGE is similar to that produced by factor XIIa-activated PK (3). These data suggest that the PRCP may be cleaving PK at the same \( R^{371}-I^{372} \) site as factor XIIa. It is of interest that prorenin is activated by cleavage to an aspartyl protease at more than 1 site (32). Further investigations are needed to determine the exact site of PRCP activation of PK. Last, previous studies have shown that the physiologic substrates of PRCP are angiotensin II (\( K_m = 0.2 \) mM) and bradykinin (\( K_m = 1 \) mM) (15,16). The finding that the \( K_m \) of PRCP for PK is 6.7 nM indicates that PK also is an important substrate for this enzyme.

The recognition that PRCP is both a PK activator and an angiotensin II inactivator indicates another previously unappreciated interaction between the plasma kallikrein/kinin and the renin angiotensin systems. Recent data suggest the renin angiotensin system along with its ability to elevate blood pressure is independently prothrombotic by the ability of angiotensin II to stimulate plasminogen activator inhibitor 1 release (24,33). Alternatively, the plasma kallikrein/kinin system through its ability to generate bradykinin that stimulates tissue plasminogen activator liberation, prostacyclin formation, and NO synthesis and kininogen that interferes with thrombin activity has been proposed to be profibrinolytic and anticoagulant (34-37). This interpretation suggests that the physiologic role of the plasma kallikrein/kinin system is as a counterbalance to the renin angiotensin
system. Recognition that PRCP is both an activator of PK as well as an inactivator of angiotensin II supports this notion.
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FIGURE LEGENDS

**Figure 1.** *Prekallikrein activator assay.* HK (1 µg/100 µl) was linked to microtiter plate in 0.1 M Na₂CO₃ pH 9.6 overnight at 4°C. After removing unbound HK, the cuvette wells were blocked with 1% gelatin for 1 h at 37°C. In each reaction 20 nM PK in the presence of prekallikrein activator (PKA) in HEPES-carbonate buffer (HCB) was added to the wells in the absence (HK+PK+PKA) or presence of 100 µM peptide SDD31 or 100 nM anti-HKL16 antibody. After 1 h of incubation at 37°C, the wells were washed followed by the addition of 0.8 mM S2302. Hydrolysis of S2302 was monitored at 405 nm for 1 h at 37°C. In certain experiments, wells were not linked with HK (PK+PKA) or PK (HK+PKA), PKA (HK+PK), or the chromogenic substrate (HK+PK+PKA-S2302) were left out. The results shown is a representative experiment of two showing generated kallikrein activity by PKA in the absence and presence of all the controls at the same time.

**Figure 2.** *Characterization of PKA on FPLC.* Panel A: the formed kallikrein activity as result of the presence of PKA (●) was plotted versus the FPLC fraction number. Also, the intensity of PRCP antigen as detected by immunoblot (■) was plotted against the FPLC fraction number. The peak fraction for both PKA activity and immunoblot identification of PRCP was Fraction 32. PKA activity was measured by its ability to activate PK bound to HK in microtiter plates (See EXPERIMENTAL PROCEDURES). The results shown are one representative experiment of five. The immunochemical reactivity of these fractions to anti-PRCP was determined by immunoblot, quantified by densitometer, and expressed as an arbitrary unit of density. Panel B, the Western blot of Fraction 32 using rabbit anti-PRCP is shown. The immunoblot detects a 73 kDa protein. Panel C, the
Western blot of Fraction 32 using goat anti-PRCP peptide is shown. The immunoblot also detects a 73 kDa protein.

**Figure 3.** *Anti-PRCP antibody inhibits PK activation.* Panel A: Microtiter plate cuvette wells coupled with 20 nM HK were incubated for 1 h at 37°C with 20 nM PK and PKA in HCB in the absence or presence of increasing concentrations of rabbit anti-PRCP antiserum (●) or preimmune rabbit serum (■). Panel B: Monolayers of HUVEC were incubated with 20 nM HK followed by 20 nM PK in the absence or presence of increasing concentrations of purified rabbit anti-PRCP (●) or preimmune IgG (■). At the end of the incubation, the wells were washed and the percent inhibition of PK activation was determined by the amount of residual kallikrein hydrolysis of 0.8 mM S2302 compared with an uninhibited sample. The results shown are a representative experiment of two.

**Figure 4.** *Characterization of isolated PKA.* Panel A: *The ability of serine protease inhibitors to block PK activation by PKA.* Purified PK (20 nM) was incubated in microtiter plates cuvette wells pre-coated with 20 nM HK in the absence or presence of 1 mM DFP or 3 mM PMSF. After incubation, the wells were solubilized with sample buffer for SDS-PAGE, reduced with 2% β-mercaptoethanol and boiled, and electrophoresed on a 10% SDS-PAGE. The electrophoresed proteins were then transferred onto nitrocellulose followed by immunoblot with a polyclonal antibody to human PK. The numbers to the left of the gel represent molecular mass standards in kilodaltons. Panel B: *The influence of various inhibitors on PKA activation of PK.* The ability of PKA to activate PK bound to HK in the absence or presence of 100 μM leupeptin or antipain, 1 mM o-phenanthroline or EDTA, 3 mM N-ethylmalimide, or 5 mM iodoacetamide or iodoacetic acid. At the end of the incubation, the wells were washed and the amount of kallikrein activity determined by hydrolysis of
0.8 mM S2302 was compared with an uninhibited sample. The results shown are the mean ± SEM of three experiments.

**Figure 5.** *Specific inhibitors of PKA.* Microtiter plate cuvette wells were coupled with HK. After blocking with 1% gelatin, 20 nM PK and PKA were incubated in the wells in the absence or presence of increasing concentration of antipain (●), benzamidine (■), Z-Pro-Pro-aldehyde-dimethyl acetate (Z-Pro-Pro) (▲), corn trypsin inhibitor (CTI) (▼) or HgCl₂ (♦). The amount of kallikrein activity formed in the presence of each of these inhibitors was determined by hydrolysis of 0.8 mM S2302 and compared with an uninhibited control. The results are the mean data using PK activator from 3 separate purifications and are expressed as % prekallikrein activation.

**Figure 6.** *Substrate inhibition of PKA.* Microtiter plate cuvette wells were coupled with HK and incubated with 20 nM PK and PKA in the absence or presence of increasing concentrations of angiotensin II (●), angiotensin II₁₋₇ (■), bradykinin (BK) (▲), bradykinin₁₋₅ (BK₁₋₅) (▼), or Fmoc-Ala-Pyr-CN (♦). The degree of PK activation in the presence of PKA and the various substrates of PRCP was compared to the amount of kallikrein-formed in the absence of these substrates. The results, which are the mean data using PKA from 3 separate purifications, are expressed as % prekallikrein activation.

**Figure 7:** *The ability of PRCP inhibitors to block PK activation on HUVEC.* Purified PK (20 nM) and HK (20 nM) in HCB were incubated with HUVEC monolayers in microtiter plate wells in the absence or presence of 100 µM angiotensin II, 100 µM angiotensin II₁₋₇, 3 mM Fmoc-Ala-Pyr-CN, or 10 mM Z-Pro-Pro-aldehyde-dimethyl acetate (Z-Pro-Pro) for 1 h at 37°C. At the end of the
incubation, the cells were washed with HCB and 0.8 mM S2302 was added and the hydrolysis of the substrate was monitored for 1 h. The results presented are the mean ± SEM of three separate experiments.

**Figure 8:** *The Km of PKA (PRCP) for prekallikrein.* The *Km* of PRCP activation of PK (■) was determined by incubating PRCP for 1 h at 37°C in the presence of increasing concentrations of PK (2.5 to 100 nM) in the microtiter plates coupled with HK. In other experiments increasing concentrations of PK alone was incubated in microtiter plates coupled with HK in the absence of PRCP (●). The amount of formed kallikrein activity was determined by incubating each well with 0.8 mM S2302. In the Inset, a double-reciprocal plot of the kinetic data is fitted to a straight line. The estimated apparent *Km* with respect to prekallikrein was 6.7 nM, and the apparent *Vmax* was 4.5 fmol/min.

**Figure 9:** *The structure and activity of PRCP-formed kallikrein.* Two µg HK was linked overnight to microtiter plate wells. After washing, all wells were incubated 1 h at 37°C with 20 nM prekallikrein (PK) in the absence or presence of PRCP (PKA) or factor XIIa (FXIIa) (40 pM) in the absence or presence of 300 µM angiotensin II (AgII) or 0.2 mg/ml neutralizing antibody to factor XIIa (Ab). In Panel A at completion of the incubation, the reactions were stopped by the addition of sample buffer for SDS-PAGE and the solubilized reactions were reduced with 2% β-mercaptoethanol and boiled, and applied to a 12% SDS-PAGE for electrophoresis. The samples were then transferred by electroblot onto nitrocellulose followed by immunoblot with a polyclonal antibody to human prekallikrein. The formed kallikrein was detected by a second antibody conjugated with horseradish peroxidase followed by chemiluminescence and autoradiography. The numbers to the right of the gel
represents molecular mass standards in kilodaltons. In Panel B, additional wells with the same conditions as in Panel A were prepared at the same time and after washing 0.8 mM S2302 was added and hydrolysis proceeded for 1 h at 37°C. The data presented are means of triplicate wells of a representative experiment.

**Figure 10.** Laser scanning confocal microscopy of HUVEC PRCP. Paraformaldehyde-fixed but not permeabilized resting HUVEC grown on glass slides were incubated with rabbit anti-human PRCP antibody (lower panel) or with preimmune rabbit serum (upper panel) at 1:100 dilution. The binding of the rabbit anti-PRCP or normal rabbit antibody was detected with a secondary goat anti-rabbit IgG labeled with fluorescein isothiocyanate. The figure, which is a laser scanning confocal micrograph, is a representative experiment of two.
| Step                  | PKA Specific Activity (units/mg) | Total Activity (units) | Protein vol. (ml) | Total Protein Activity (units) | Protein vol. (mg) | Yield (%) | Purification Factor |
|-----------------------|----------------------------------|------------------------|-------------------|-------------------------------|-------------------|-----------|---------------------|
| Cell homogenate       | 1.56                             | 550                    | 25                | 22                            | 39                | 100       | 1.00                |
| 15,800 x g pellet     | 0.508                            | 472.8                  | 12                | 39.4                          | 6.096             | 86        | 11.5                |
| DEAE cellulose        | 0.262                            | 754.2                  | 4.5               | 167.6                         | 1.179             | 137       | 252                 |
| Wheat germ agglutinin | 0.054                            | 391.8                  | 3                 | 130.6                         | 0.162             | 71        | 1429                |
| Hydroxyapatite        | 0.0091                           | 138.7                  | 1                 | 138.7                         | 0.0091            | 25        | 27019               |
Identification and characterization of prolylcarboxypeptidase as an endothelial cell prekallikrein activator
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