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SPECIFIC, HIGH-AFFINITY BRADYKININ BINDING BY PURIFIED PORCINE KIDNEY POST-PROLINE CLEAVING ENZYME

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Abstract—Post-proline cleaving enzyme (PPCE) was purified from porcine kidney cytosol. The purified enzyme bound [^{125}I-Tyr^5]-bradykinin but neither [^{125}I-Tyr^1]-kallidin nor [^{125}I-Tyr^8]-bradykinin. Scatchard analysis of the data was consistent with a single class of binding sites with a $K_{assoc} = 1.3 \pm 0.1 \times 10^8\ M^{-1}$. The optimal pH for [^{125}I-Tyr^5]-bradykinin binding was 6.8. The specificity of binding was evaluated with sixty-seven bradykinin analogs. The catalytic activity of the enzyme was measured with *N*-benzyloxycarbonyl-Gly-Pro-methylcoumarinyl-7-amide (Z-Gly-Pro-MCA). The optimal pH for hydrolysis of this substrate was broad and centered at 8.3. The apparent K_m and V_{max} were obtained from Lineweaver and Burk plots and were $4.8 \pm 0.4 \times 10^{-5}$ M and $42 \pm 5\ \mu moles \cdot mg^{-1} \cdot min^{-1}$ respectively. The IC_{50} values for bradykinin, diisopropylfluorophosphate (DFP), and *N*-benzyloxycarbonyl-Pro-Prolinal (Z-Pro-Prolinal) to inhibit Z-Gly-Pro-MCA hydrolysis by PPCE were $5.9 \pm 1.4 \times 10^{-7}$ M, $8.8 \pm 3.1 \times 10^{-7}$ and $7.9 \pm 0.3 \times 10^{-9}$ M respectively. Corresponding values for inhibition of [^{125}I-Tyr^5]-bradykinin binding by PPCE were $5.1 \pm 2.3 \times 10^{-9}$ M, $1.2 \pm 0.3 \times 10^{-6}$ M and $1.4 \pm 0.6 \times 10^{-8}$ M.

During the course of our work to solubilize and purify a bradykinin (BK†) receptor-like binding activity from bovine uterine myometrium [1], we discovered a kinin binding protein that displayed high affinity for BK, $K_d \simeq 1 \times 10^{-9}$ M. Results of preliminary specificity studies indicated that the binding activity was probably post-proline cleaving enzyme (PPCE). PPCE is a prolyl endopeptidase (EC 3.4.21.26) that degrades a number of proline-containing peptides by cleavage of the peptide bond at the carboxyl side of the proline residues in the peptide chain [2]. With BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) as substrate, it cleaves initially between Pro^7-Phe^8 and subsequently between Pro^3-Gly^4 [3]. The specific activity of a rabbit brain PPCE preparation with BK as substrate is $0.78\ \mu mol \cdot mg^{-1} \cdot min^{-1}$, and the K_m value for BK is 3.5×10^{-5} M [4]. The importance of PPCE in the metabolism of BK *in vivo* is not known. We have purified PPCE from a porcine kidney and characterized it with respect to its kinin binding. The results of these studies are the subject of this report.

MATERIALS AND METHODS

Porcine kidneys were obtained from the Bloomfield Locker Plant, Inc., Bloomfield, IN. Chromatography media were obtained as follows: DE 23 cellulose from Whatman, Inc., Clifton, NJ; AcA 44 and AcA 34 Ultrogel and HA-Ultrogel from LKB Instruments, Inc., Rockville, MD; Polybuffer 74 and Polybuffer exchanger PBE 94 from Pharmacia Fine Chemicals, Inc., Piscataway, NJ; and Affi-Gel 501 from Bio-Rad Laboratories, Richmond, CA. Protein assay reagents were also from Bio-Rad Laboratories. Crystalline bovine serum albumin (A 7638), 7-amino-4-methylcoumarin, angiotensin I, luteinizing hormone-releasing hormone, substance P, p Glu-Lys-Trp-Ala-Pro (BPP_{5a}), [Arg^8]-vasopressin, DFP, bacitracin (B 0125), and *N*-tosyl-phenylalanine chloromethyl ketone (TPCK) were from the Sigma Chemical Co., St. Louis, MO. Piperazine-*N*,*N*-bis-2-ethanesulfonic acid (PIPES) and dithiothreitol (DTT) were from Research Organics Inc., Cleveland, OH. Lys-BK and *N*-benzyloxycarbonyl-Gly-Pro-methylcoumarinyl-7-amide (Z-Gly-Pro-MCA) were purchased from Vega Biochemicals, Tuscon, AZ. Z-Gly-Pro-MCA was also purchased from Cambridge Research Biochemicals, Ltd., Atlantic Beach, NY. Met-Lys-BK, neurotensin, [Arg^8]-vasopressin, and Arg-Pro-Pro were from the Chemical Dynamics Corp., South Plainfield, NJ. BK was from Serva Fine Biochemicals, Inc., Long Island, NY. Angiotensins I and II, [Tyr^1]-kallidin, [Tyr^5]-BK, and [Tyr^8]-BK

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† Abbreviations: BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; OMT, *O*-methyltyrosine; ThiAla, β-(2-thienyl)-alanine; Aib, α-aminoisobutyric acid; Abu, γ-aminobutyric acid; Tos, *p*-toluenesulfonyl; MePhe, α-methylphenyl-alanine; MeArg, α-methylarginine; p Glu, pyroglutamic acid; ACE, angiotensin I converting enzyme; BPP_{5a}, p Glu-Lys-Trp-Ala-Pro; SQ20881, p Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; Z-Pro-Prolinal, *N*-benzyloxycarbonyl-Pro-Prolinal; Z-Gly-Pro-MCA, *N*-benzyloxycarbonyl-Gly-Pro-methylcoumarinyl-7-amide; MCA, 7-amino-4-methylcoumarin; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; PIPES, piperazine-*N*,*N*-bis-2-ethanesulfonic acid; PPCE, post-proline cleaving enzyme; and TPCK, *N*-tosyl-phenylalanine chloromethyl ketone.

were from Peninsula Laboratories, Inc., San Carlos, CA. Cellulose acetate tubes (9/16 × 4 in.) were from the Petro Packaging Co., Inc., Cranford, NJ; Norit A activated charcoal was from Pfanstiehl Laboratories, Inc., Waukegan, IL; and dextran T-70 from Pharmacia Fine Chemicals, Piscataway, NJ. [Orn^1]-BK, [Lys^1]-BK and [*p*-fluoro-Phe^8]-BK, gifts of Dr. E. D. Nicolaides, were from Parke, Davis & Co., Ann Arbor, MI. [Sar^4]-BK, [MePhe^5]-BK, [MePhe^8]-BK and [MeArg^9]-BK were supplied by G. D. Searle & Co., Skokie, IL. [Abu^6]-BK, [D-Phe^8]-BK, [des-Arg^{11}]-Met-Lys-BK, [des-Arg^{11}]-Lys-Lys-BK, [des-Arg^{10}]-Lys-BK, [des-Arg^{11}, Leu^{10}]-Lys-Lys-BK and [des-Arg^{11}-Leu^{10}]-Met-Lys-BK were provided by Drs. Jean Barabe and Domenico C. Regoli, Sherbrooke, Q.B., Canada; all the other BK analogs used in these studies were provided by Drs. John M. Stewart and Raymond J. Vavrek, Denver, CO. p Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (SQ20881) was a gift of the Squibb Institute for Medical Research, Princeton, NJ. *N*-Benzyloxycarbonyl-Pro-Prolinal (Z-Pro-Prolinal) was a gift from Dr. Sherwin Wilk, New York, NY. All other reagents were of the highest quality available and were obtained from commercial sources.

Purification of PPCE. Fourteen porcine kidneys were obtained from a local abattoir on the day of slaughter and transported on ice to our laboratory. Renal capsules were removed and the kidneys were cut in half longitudinally and placed on ice. Intrarenal fat and pelvises were removed and discarded. The remaining tissue was diced with a knife and then rinsed with 20 liters of cold saline; 2.03 kg of tissue was obtained. This was separated into four equal fractions (500 g) and stored frozen overnight at −35°. Aliquots were thawed and then homogenized with 0.8 liter of 20 mM PIPES, pH 6.8, containing 1 mM EDTA and 1 mM sodium azide (Buffer A) in an ice water-jacketed commercial Waring blendor at the top speed for 4 min. The homogenate was centrifuged at 4° for 10 min at 1000 *g* in RC2-B Sorvall refrigerated centrifuges. The resulting supernatant fraction was centrifuged for 40 min at 10,000 *g*. The supernatant fraction from this spin was centrifuged for 5 hr at 10,000 *g*. PPCE was purified from the supernatant fraction (2.7 liters) from this last spin. Solid ammonium sulfate (524 g) was added, and the solution was stirred overnight in a cold-room, 10°. The suspension was centrifuged for 20 min at 10,000 *g*. To the supernatant fraction (2.62 liters) from this spin was added 862 g of solid ammonium sulfate, and the mixture was stirred overnight in the cold-room. The suspension obtained was centrifuged as before. Pelleted proteins were solubilized in a final volume of 1.36 liters of Buffer A. All the following steps were performed in the cold-room. The reconstituted sample was equilibrated to Buffer A by concentrating the sample to 0.15 liter in a Millipore Minitan ultrafiltration apparatus equipped with filter packets having a nominal molecular weight cut-off of 100,000. The volume was brought up to 1.5 liters with Buffer A, and the sample was again concentrated to 0.15 liters. This step was repeated and the final sample was brought up to a volume of 3.05 liters. The sample was incubated over 3 days in the cold-room with 750 g wet weight of DE 23 cellulose resin that had been equilibrated to Buffer A. The resin was separated from the solution by filtration under vacuum on a sintered-glass funnel. The resin was washed with 1 liter of Buffer A and then resuspended in 2 liters of Buffer A containing 0.4 M KCl. After sitting in this buffer for 2 hr in the cold-room, the supernatant fraction was removed by filtration on the sintered-glass funnel. The resin was washed with 1 liter of Buffer A containing 0.4 M KCl and combined with the 0.4 KCl eluate. The supernatant fraction eluted from the DE 23 resin prior to treatment with KCl-containing buffer was incubated a second time with recharged DE 23 resin, and the 0.4 M KCl eluted material from this treatment was combined with that from the first treatment. The 6 liters of 0.4 KCl eluted material from the DE 23 cellulose was concentrated to 2 liters with the Minitan and applied to an Affi-Gel 501 column (5 × 2.5 cm) at a flow rate of 60 ml/hr. After washing the column with Buffer A, the PPCE was eluted with 0.5 liter of Buffer A containing 3 mM DTT. This step was repeated for a total of three times, and the DTT eluates were combined (1.5 liters) and concentrated to 50 ml with the Minitan. The sample was equilibrated to 0.2 mM sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide (Buffer B) in the Minitan as had been done before when equilibrating the sample to Buffer A. The equilibrated sample (0.995 liter) was applied to an HA-Ultrogel column (5 × 40 cm) at 100 ml/hr. After washing the column with the starting buffer (Buffer B), PPCE was eluted with a linear gradient consisting of 1.5 liters of Buffer B and 1.5 liters of 0.15 M sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide. Fractions containing PPCE activity (fractions eluting between 0.2 and 17.6 mM) were pooled (0.37 liter) and concentrated to 24 ml in an Amicon stirred-cell equipped with a PM 30 membrane. The sample was applied to an AcA 34 Ultrogel column (5 × 93 cm) and eluted at a flow rate of 50 ml/hr with Buffer A. PPCE-containing fractions were pooled (0.15 liter) and concentrated to 10 ml in the Amicon. The sample was applied to a chromatofocusing column, PBE 94, 1.2 × 16.5 cm, equilibrated with 25 mM histidine–HCl, pH 6.2. The column was eluted with 62.5 ml of Polybuffer 74 that had been adjusted to pH 4.0 with HCl after being diluted 1:8 with deionized water. The flow rate was 15 ml/hr, and 3.3-ml fractions were collected. The peak of PPCE activity eluted at pH 4.2. PPCE-containing fractions were pooled (19 ml) and concentrated to 3.9 ml in the Amicon. The sample was applied to an AcA 44 Ultrogel column (2.5 × 100 cm) and eluted with Buffer A at a flow rate of 50 ml/hr. Fractions containing the peak of PPCE activity were pooled and applied to an Affi-Gel 501 column (1.2 × 9 cm). The column was eluted at a flow rate of 30 ml/hr with Buffer A. PPCE activity was eluted by adding 3 mM DTT to Buffer A. The fractions containing PPCE were pooled (23 ml) and concentrated to 4 ml in the Amicon. The sample was applied to the AcA 44 Ultrogel column and run as before. The peak of PPCE activity was pooled (16.8 ml) and was the source of enzyme used in these studies. The absorbance measured at 280 nm was used to estimate protein concentrations during the

course of purification. A 1 ng/ml solution was assumed to have an extinction coefficient of 1.0. However, the protein concentrations for specific activities were measured by the procedure of Bradford [5] as adapted by Bio-Rad Laboratories. The molecular weight of PPCE was estimated to be 66,200 by gel filtration and 69,500 by sodium dodecyl sulfate (SDS)–polyacrylamide slab gel electrophoresis. A minor band that corresponds to a molecular weight of 37,000 was also seen on gel electrophoresis. This represented less than 10% of the coomassie blue staining material detected.

Enzymatic assay for PPCE. The buffer used for the PPCE enzymatic assay was 20 mM Tris–HCl, containing 1 mM EDTA and 1 mM sodium azide, pH 8.3. To 12 × 75 mm borosilicate glass test tubes run in triplicate were added: 890 μl of buffer, 50 μl of peptide or other substance to be tested, dissolved in buffer, 10 μl of a 14.8 mM stock solution of Z-Gly-Pro-MCA in methanol, and 26.5 ng of PPCE in 50 μl of buffer containing 10 μg crystalline bovine serum albumin and 200 mM DTT. After incubation at 37° for 20 min, the reactions were terminated with the addition of 2 ml of 1 M sodium acetate buffer, pH 4.2, containing 1 mM sodium azide. The fluorescence intensity was measured at 445 nm with excitation at 383 nm on an Aminco SPF-500 spectrofluorometer. The fluorescence was converted into nanomoles of 7-amino-4-methylcoumarin by using a standard curve prepared with the latter compound under corresponding control conditions. Under these assay conditions, the reaction rate was linear, and less than 20% of the substrate added was hydrolyzed. This statement is also true for those assays run to monitor PPCE activity during the course of purification of the enzyme and in kinetic studies.

Kinin binding assay for PPCE. Monoiodinated derivatives of [Tyr^1]-kallidin, [Tyr^5]-BK and [Tyr^8]-BK were prepared [6] and purified [7] as previously described. Specific activities of the iodinated peptides were determined by the self-displacement method [8] using purified PPCE. The binding assay buffer was 15 mM PIPES, containing 1 mM EDTA and 1 mM sodium azide, pH 6.8. To 9/16 × 4 inch cellulose acetate tubes run in triplicate were added: 900 μl of buffer, 100 μl of peptide or other substance to be tested, dissolved in buffer, 100 μl of buffer containing 10 μg of crystalline bovine serum albumin, 50,000 cpm of ^{125}I-labeled kinin (0.02 pmole), and 1.2 mM DTT, and 100 μl of buffer containing 21.2 ng of purified PPCE. After incubation at 0° for 1 hr the bound radioactivity was separated from free radioactivity with the addition of 1 ml of dextran-coated charcoal solution. Dextran-coated charcoal was prepared by dissolving 5.0 g of dextran T70 in 1 liter of 0.01 M potassium phosphate buffer, pH 7.6, and then adding 25 g of Norit A activated charcoal. Tubes were centrifuged at 4° for 15 min at 1000 *g*, and 1.6 ml of each supernatant fraction was transferred into a new tube and counted in a well-type automatic gamma counter (Nuclear Chicago, model 1065, ^{125}I-counting efficiency = 83.5%). Saturable binding was defined as the difference in the amount of radioactivity bound in the absence of unlabeled BK and the amount bound in the presence of an excess of unlabeled BK (5.0 μg). Under these assay conditions, 5–6% of the [^{125}I-Tyr^5]-BK incubated in the absence of nonradioactive competitors was bound to PPCE.

Calculations. In all hydrolytic assays, controls were run in which PPCE was omitted from the incubation mixtures. Results obtained for these controls were subtracted from those obtained in the presence of PPCE. In binding assays non-saturably bound counts were subtracted from those counts obtained when ^{125}I-labelled kinin and PPCE were incubated together, with or without unlabeled competitors. When various concentrations of nonradioactive compounds were tested for their abilities to inhibit Z-Gly-Pro-MCA hydrolysis or [^{125}I-Tyr^5]-BK binding, the results were normalized to controls which contained only PPCE and Z-Gly-Pro-MCA or the radioactive kinin. The concentrations of compounds that inhibit 50% of the hydrolysis or binding by PPCE were calculated from plots of the normalized values versus the $\log_{10}$ of the concentrations of inhibitors. Results are expressed as the mean ± SD and were determined with a Texas Instruments model 58 calculator. Linear regression analyses on the data from the kinetic experiments were performed using the statistics package for this calculator and the equation for the best straight line corresponding to the data points determined. Data from the Scatchard analyses were analyzed using the LIGAND program by Munson and Rodbard [9] run on an IBM PC computer.

RESULTS

PPCE was purified 5010-fold from porcine kidney cytosol following a 10-step purification scheme (Table 1). The purified enzyme bound [^{125}I-Tyr^5]-BK but neither [^{125}I-Tyr^1]-kallidin nor [^{125}I-Tyr^8]-BK. The binding of [^{125}I-Tyr^5]-BK as a function of time is shown in Fig. 1. Maximum binding was obtained after 60 min, and all subsequent binding experiments were incubated for this length of time. The optimal pH for [^{125}I-Tyr^5]-BK binding was 6.8 (Fig. 2). In contrast, the optimal pH for Z-Gly-Pro-MCA hydrolysis by the enzyme was broad and centered at 8.3 (Fig. 2). The apparent K_m and V_{max} for Z-Gly-Pro-MCA with PPCE were obtained from Lineweaver and Burk plots and were $4.8 \pm 0.4 \times 10^{-5}$ M and 42 ± 5 μmoles·mg^{-1}·min^{-1} respectively.

Scatchard analyses [5], like that shown in Fig. 3, were performed on [^{125}I-Tyr^5]-BK binding data. These analyses were consistent with a single type of binding site having a $K_{assoc} = 1.3 \pm 0.1 \times 10^8$ M^{-1} and a B_{max} of $5.6 \pm 1.0 \times 10^{-10}$ M.

A comparison of the abilities of various concentrations of BK to inhibit either [^{125}I-Tyr^5]-BK binding or Z-Gly-Pro-MCA hydrolysis by PPCE is shown in Fig. 4. The IC_{50} for BK inhibition of [^{125}I-Tyr^5]-BK binding was $5.1 \pm 2.3 \times 10^{-9}$ M and is in good agreement with the K_{assoc} determined by Scatchard analysis. The specificity of [^{125}I-Tyr^5]-BK binding was evaluated with 67 BK analogs or compounds structurally related to BK by comparing the IC_{50} values for these compounds to that for BK (Table 2). ThiAla^5 (compound 8) and ThiAla^{5,8} (compounds 2, 6 and 7) substitutions for Phe in position(s) 8 and/or 5 are compatible with binding to PPCE. Although

Table 1. Purification of post-proline cleaving enzyme from porcine kidney

	Step	Total protein (mg)	Total activity (μmoles MCA*/min)	Yield (%)	Specific activity (μmoles MCA/mg per min)	Purification (fold)
1.	10,000 $g \times 5$ hr Supernatant	81,810	775	100	0.00947	1
2.	$(NH_4)_2SO_4$ fractionation (35–85%)	60,297	619	80	0.0103	1.1
3.	DE-23 chromatography	17,028	380	49	0.0223	2.4
4.	Affi-Gel 501 chromatography	2,388	363	47	0.152	16
5.	HA-Ultrogel chromatography	296	315	41	1.06	112
6.	AcA 34 Ultrogel chromatography	165	220	28	1.33	140
7.	Chromatofocusing chromatography	8.5	82.4	11	9.69	1,023
8.	AcA 44 Ultrogel chromatography	2.9	66.2	8	22.83	2,411
9.	Affi-Gel 501 chromatography	1.3	47.6	6	36.62	3,867
10.	AcA 44 Ultrogel chromatography	0.9	42.7	6	47.44	5,010

* MCA = 7-amino-4-methylcoumarin.

extensions at the N-terminus with basic amino acids result in compounds that are as potent or more potent than BK, compounds 1, 7 and 10, this modification does not necessarily result in compounds as potent or more potent than BK, compounds 22–25 and 38. Fragments of BK, compounds 57, 64, 67 and 68, and BK analogs containing D amino acids in position 7, compounds 48, 52, 54, 58 and 61, or position 6, compounds 51, 56, 59, 60, 62, 63 and 66, were approximately 2 orders of magnitude less potent than BK at inhibiting [^{125}I-Tyr^5]-BK binding by PPCE.

BK was less potent as an inhibitor of Z-Gly-Pro-MCA hydrolysis than as an inhibitor of [^{125}I-Tyr^5]-BK binding (Fig. 4). This was also true for a number of compounds structurally unrelated to BK (Table 3), although Z-Pro-Prolinal, neurotensin, and DFP were equipotent in the two assays. Although an exact comparison of our data with the literature is not possible because of differences in enzyme sources and purities, assay buffers, and substrates, in most cases similar similar results were obtained [4, 10–17].

DISCUSSION

PPCE was purified over 5000-fold from porcine kidney cytosol. This compares favorably with the 1000-fold purification reported by Soeda *et al.* [18], who purified the enzyme from the same source. Part of the difference in fold purification may be due to the fact that these investigators used Suc-$(Ala)_3$-pNA to monitor purification of PPCE, whereas in the present study Z-Gly-Pro-MCA was employed. Suc-$(Ala)_3$-*p*-nitroanilide is probably not as selective a substrate for PPCE as Z-Gly-Pro-MCA is. Z-Gly-Pro-MCA was originally introduced by Yoshimoto *et al.* [19], as a fluorogenic substrate for PPCE. The K_m for this substrate with purified lamb kidney PPCE

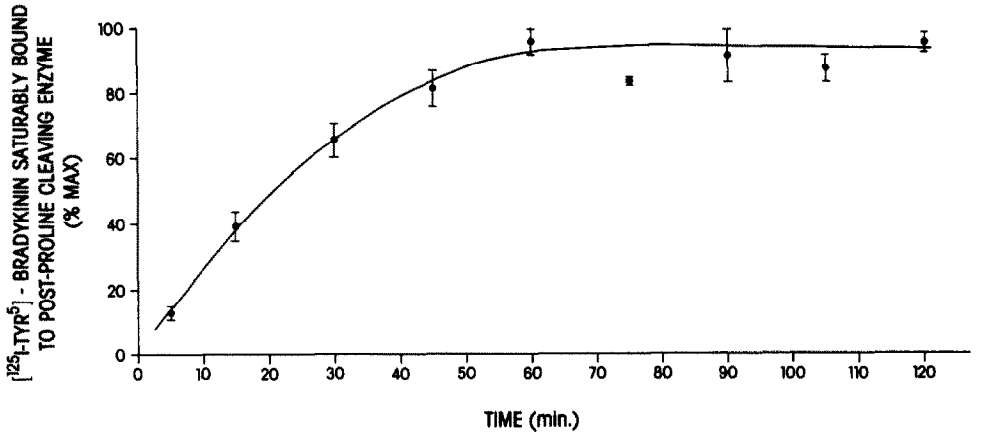

Fig. 1. Binding of [^{125}I-Tyr^5]-bradykinin to purified porcine kidney post-proline cleaving enzyme (PPCE) as a function of time. Thirty picograms (50,000 cpm) of [^{125}I-Tyr^5]-bradykinin was incubated for 5, 15, 30, 45, 60, 75, 90, 105 and 120 min with purified PPCE as described in Materials and Methods. The maximum saturable binding of [^{125}I-Tyr^5]-bradykinin in a given experiment was assigned a value of 100%, and all other time points in that experiment were expressed as a percent of this maximum. Results plotted in this figure are the means of three experiments. The brackets enclose a standard deviation on each side of these means.

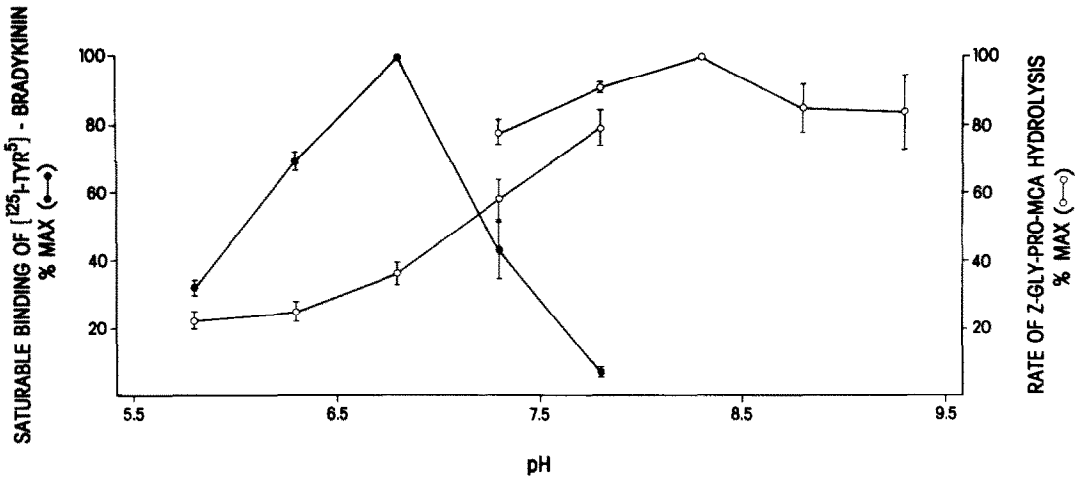

Fig. 2. pH optimum for [^{125}I-Tyr^5]-bradykinin binding and Z-Gly-Pro-MCA hydrolysis by purified porcine kidney post-proline cleaving enzyme (PPCE). [^{125}I-Tyr^5]-bradykinin binding to PPCE (●) was tested at pH values of 5.8, 6.3, 6.8, 7.3 and 7.8 using 15 or 20 mM PIPES buffer adjusted to the appropriate pH with KOH. The hydrolysis of Z-Gly-Pro-MCA by PPCE (○) was tested at pH values of 5.8, 6.3, 6.8, 7.3 and 7.8 using 20 mM PIPES buffer adjusted to the appropriate pH with KOH or at pH values of 7.3, 7.8, 8.3, 8.8 and 9.3 using 20 mM Tris buffer adjusted to the appropriate pH with HCl. The binding and enzymatic assays were performed as described in Materials and Methods. The curve for binding activity is a composite of results from four experiments, whereas curves for hydrolytic activity are a composite of data from three experiments. The brackets enclose a standard deviation on each side of the means of these data. The maximal activity was taken as 100%.

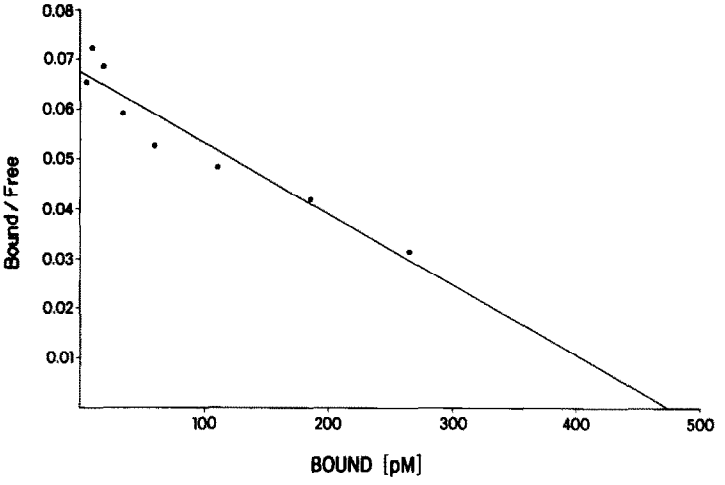

Fig. 3. Scatchard plot of [^{125}I-Tyr^5]-bradykinin binding to purified porcine kidney post-proline cleaving enzyme (PPCE). Serial dilutions of [^{125}I-Tyr^5]-bradykinin containing approximately, 1,000,000, 500,000, 250,000, 125,000, 65,000, 50,000, 25,000 and 12,000 cpm in 0.1 ml were incubated with 21.2 ng purified PPCE for 1 hr at 0° in a final volume of 1.2 ml as described in Materials and Methods. Dextran-coated charcoal was used to separate bound from free radioactivity, and each of these fractions was counted in a Nuclear Chicago gamma counter (83.5% counting efficiency for ^{125}I). The specific activity of [^{125}I-Tyr^5]-bradykinin (0.06 μCi/pmole) was determined by comparing the effects of labeled and unlabeled peptide on the bound/free ratio [8]. Calculations assume that [^{125}I-Tyr^5]-bradykinin and [Tyr^5]-bradykinin had identical effects on the bound/free ratio. The data presented in this figure are from a single experiment that is representative of five experiments performed. The data were analyzed using the LIGAND program by Munson and Rodbard [9] run on an IBM PC computer.

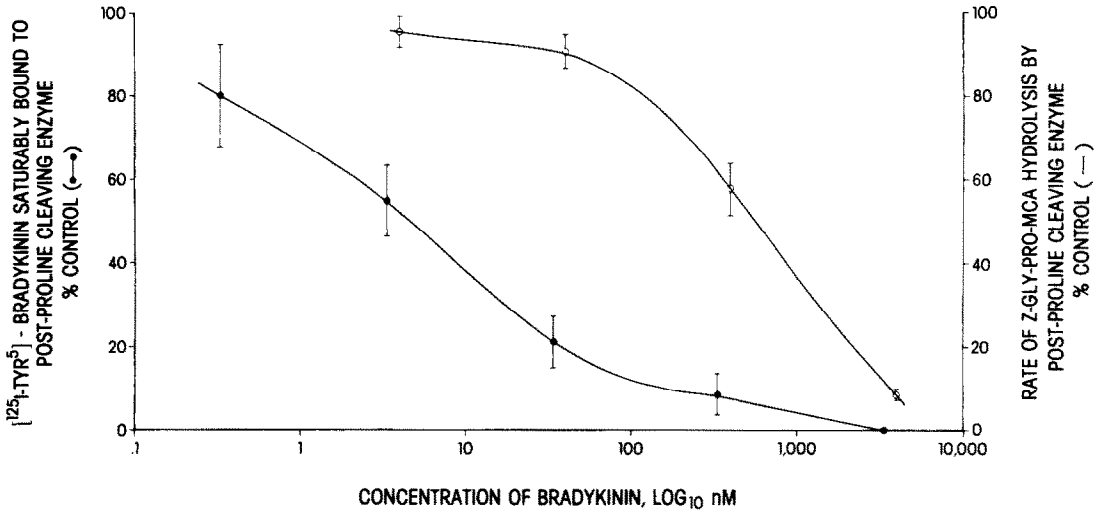

Fig. 4. Bradykinin (BK) inhibition of [^{125}I-Tyr^5]-bradykinin binding and Z-Gly-Pro-MCA hydrolysis by purified porcine kidney post-proline cleaving enzyme (PPCE). Serial dilutions of BK were incubated with [^{125}I-Tyr^5]-bradykinin (●) or Z-Gly-Pro-MCA (○) and PPCE as described in Materials and Methods. The curves are composites of results from 71 and 20 experiments with [^{125}I-Tyr^5]-bradykinin and Z-Gly-Pro-MCA respectively. The brackets enclose a standard deviation on each side of the means of these data.

Table 2. Specificity of kinin binding by post-proline cleaving enzyme (PPCE)

	Compounds*	IC_{50} (nM) for the binding of [^{125}I-Tyr^5]-bradykinin by porcine kidney PPCE
1	Lys-OMT-Lys-BK	0.7 ± 0.3
2	ThiAla^{5,8}-BK	1.2 ± 0.7
3	Aib^3-BK	1.6 ± 1.2
4	Lys(Tos)^6-BK	1.7 ± 0.3
5	Tyr^5-BK	2.1 ± 0.9
6	Hypro^2-ThiAla^{5,8}-BK	2.5 ± 1.1
7	Lys-Lys-ThiAla^{5,8}-BK	2.7 ± 1.2
8	ThiAla^5-BK	3.2 ± 1.8
9	Thr^6-BK	3.4 ± 2.3
10	des-Arg^{11}-Met-Lys-BK	4.2 ± 1.3
11	BK	5.1 ± 2.3
12	Tyr^8-BK	5.9 ± 0.6
13	Hypro^3-BK	6.2 ± 2.8
14	MeArg^9-BK	6.8 ± 0.6
15	des-Arg^{11}-Lys-Lys-BK	7.0 ± 5.0
16	des-Arg^{10}-Lys-BK	7.2 ± 4.3
17	DPhe^8-BK	7.3 ± 2.3
18	des-Phe^8-Arg^9-BK	8.3 ± 2.1
19	Phe^9-BK	8.9 ± 7.0
20	*p*-Fluoro-Phe^8-BK	9.3 ± 2.2
21	Phe^6-BK	9.7 ± 2.2
22	Lys-Lys-BK	9.8 ± 1.1
23	Lys-BK	10.3 ± 2.3
24	Met-Lys-BK	10.6 ± 3.9
25	des-Arg^{11}-Leu^{10}-Lys-Lys-BK	10.8 ± 1.8
26	Sar^4-BK	11.1 ± 3.9
27	des-Arg^9-BK	12.8 ± 4.7
28	Orn^1-BK	13.0 ± 4.5
29	D-Ala^4-BK	13.1 ± 1.6
30	Leu^8-BK	14.7 ± 3.2
31	MePhe^5-BK	15.7 ± 9.7
32	Gly^6-Tyr^8-BK	15.9 ± 2.7
33	Lys^1-BK	17.2 ± 9.5
34	Aib^7-BK	20.3 ± 11.5
35	D-Pro^{2,3}-BK	20.9 ± 4.2
36	Aib^2-BK	22.1 ± 5.8

(*continued*)

Table 2 (*continued*)

	Compounds*	IC_{50} (nM) for the binding of [^{125}I-Tyr^5]-bradykinin by porcine kidney PPCE
37	Tyr-BK	22.9 ± 11.5
38	des-Arg^{11}-Leu^{10}-Met-Lys-BK	24.1 ± 11.8
39	Cl-Acetyl-Arg^1-BK	25.5 ± 15.4
40	Gly^{6}Aib^7-BK	27.0 ± 12.6
41	Leu^5-BK	32.6 ± 12.7
24	Phe^{4,6}-BK	32.9 ± 10.8
43	D-Pro^3-BK	33.6 ± 18.2
44	Ile-Ser-BK	40.8 ± 5.3
45	Acetyl-Arg^1-D-Pro^3-BK	40.9 ± 3.5
46	Acetyl-Arg^1-D-Pro^3-OMT^8-BK	51.1 ± 37.8
47	D-Pro^2-BK	105 ± 30
48	D-Pro^{3,7}-BK	205 ± 65
49	Abu^6-BK	279 ± 84
50	des-Arg^1-BK	299 ± 176
51	D-Trp^6-Aib^7-BK	309 ± 117
52	ThiAla^{5,8}-D-Phe^7-BK	317 ± 98
53	D-Pro^{2,7}-BK	360 ± 85
54	D-Pro^{2,3,7}-BK	470 ± 93
55	MePhe^8-BK	480 ± 117
56	*p*-Chloro-D-Phe^6-Aib^7-BK	507 ± 259
57	Gly-Phe-Ser-DehydroPro	631 ± 322
58	D-Pro^7-BK	715 ± 316
59	D-Phe^6-Aib^7-BK	719 ± 254
60	D-Trp^6-BK	773 ± 176
61	Acetyl-Arg^1-D-Pro^7-BK	911 ± 345
62	D-Phe^6-BK	925 ± 233
63	*p*-Chloro-D-Phe^6-BK	934 ± 327
64	Gly-*p*-ChloroPhe-Ser-Pro	1040 ± 447
65	Gly^{5,6,8}-BK	1582 ± 688
66	D-Ser^6-BK	1647 ± 311
67	Gly-Phe-Ser-Pro	1708 ± 685
68	Arg-Pro-Pro	>8531

Values are means ± SD, N = a minimum of three determinations.

* Abbreviations: BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; OMT, *O*-methyltyrosine; ThiAla, β-(2-thienyl)-alanine; Aib, α-aminoisobutyric acid; Abu, γ-aminobutyric acid; Tos, *p*-toluenesulfonyl; MePhe, α-methylphenylalanine; and MeArg, α-methylarginine.

is 20 μM and the pH optimum is 7.0 [19]. In contrast, the K_m for this substrate with purified porcine kidney PPCE was $4.8 \pm 0.4 \times 10^{-5}$ M and the pH optimum was broad and centered at 8.3. While the differences between the results could be attributed to different sources of enzyme and incubation conditions, it should be noted that pH optima of 8.3 have been reported for other fluorogenic substrates using purified rabbit [20] and rat [21] brain PPCE, and it may be that the original report [18] was in error.

We have shown previously [22] that angiotensin I converting enzyme (ACE), a metallopeptidase, binds [^{125}I-Tyr^1]-kallidin but only when ACE is catalytically inactive, i.e. in the presence of EDTA. Results of the present study indicate that radioactive kinin binding is not limited to ACE but also occurs with PPCE, a serine peptidase. However, in contrast to ACE, PPCE was only capable of binding kinins when the enzyme was in a catalytically active conformation, i.e. DFP-treated PPCE did not bind kinins. In general, higher concentrations of compounds were required to decrease Z-Gly-Pro-MCA hydrolysis than were needed to inhibit [^{125}I-Tyr^5]-BK binding by purified porcine kidney PPCE (Table 3). This result is similar to what was reported for ACE using [^{3}H]-hippuryl-glycylglycine ([^{3}H]-HGG) to measure hydrolysis and [^{125}I-Tyr^1]-kallidin (TIK) to measuring binding by the enzyme [22]. In the ACE report, because the concentrations of radioactive ligands used were several orders of magnitude below their reported or expected K_m values with ACE; the enzyme concentrations in the hydrolytic and binding assays were the same; and, with the exception that the binding assay buffer contained 3.3 mM EDTA, the assay buffers were identical, it was speculated that the primary reason why higher concentrations of peptides were required to decrease [^{3}H]-HGG hydrolysis than TIK binding was because the peptides were being hydrolyzed in the catalytic assays to products that had lower affinities for ACE than the intact peptides, whereas in the binding assays the peptides remained intact and consequently more effective at inhibiting ACE. In the present report, Z-Gly-Pro-MCA was used at a concentration three times larger

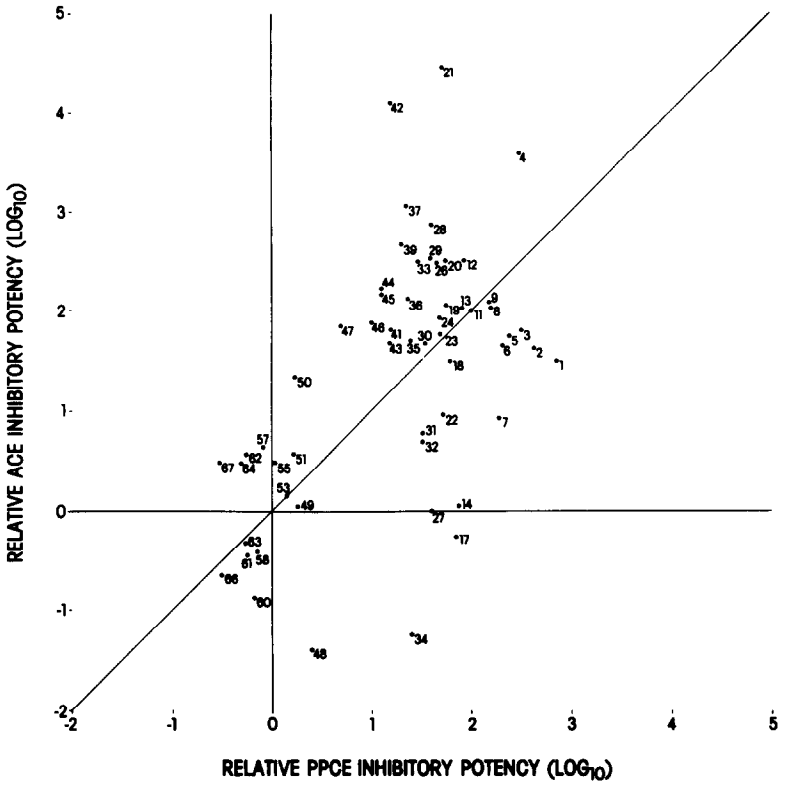

Fig. 5. Comparison of the relative potencies of bradykinin (BK) analogs to inhibit [^{125}I-Tyr^1]-kallidin binding by purified porcine kidney angiotensin I converting enzyme (ACE) with their relative potencies to inhibit [^{125}I-Tyr^5]-bradykinin binding by purified porcine kidney post-proline cleaving enzyme (PPCE). The numbers in the figure correspond to the BK analogs listed in Table 2. Data for [^{125}I-Tyr^1]-kallidin binding to ACE were taken from the literature [22, 27] and for [^{125}I-Tyr^5]-bradykinin binding to PPCE from Table 2. The potencies of the analogs relative to BK (100%) were calculated, and the $\log_{10}$ transformations of these data plotted in this figure. The line drawn through the origin represents the "line of identity". An analog having the same relative potency for inhibition of ^{125}I-labeled kinin binding by ACE and PPCE would lie on this line.

than its K_m for PPCE, whereas [^{125}I-Tyr^5]-BK was undoubtedly used at a concentration orders of magnitude lower than its K_m for the enzyme. For this reason alone, higher concentrations of compounds might be required to decrease Z-Gly-Pro-MCA hydrolysis than are required to decrease [^{125}I-Tyr^5]-BK binding. Other factors, which might explain the differences, are the fact that the concentration of PPCE in the binding assay was two-thirds that used in the catalytic assay and the binding assay buffer was pH 6.8, while the pH of the catalytic assay was 8.3. To try and evaluate to what extent hydrolysis of compounds in the catalytic assay might contribute to their lesser potencies in this assay versus the binding assay, a single experiment was done in which IC_{50} concentrations of the compounds for Z-Gly-Pro-MCA hydrolysis were preincubated under catalytic assay conditions for 20 min with PPCE and then Z-Gly-Pro-MCA was added and the incubation continued for another 20 min. The inhibitory effects of the compounds under these conditions were compared in the same experiment to the inhibitory effects of the compounds not preincubated with the enzyme. Z-Pro-Prolinal, BPP_{5a}, and DFP were more effective inhibitors of Z-Gly-Pro-MCA hydrolysis when preincubated with PPCE than when they were not preincubated with the enzyme. Preincubation had no effect on the inhibitory actions of bacitracin, whereas all the other compounds listed in Table 3 were less effective after preincubation with PPCE than when they were added at the same time Z-Gly-Pro-MCA was added to the reaction mixtures. So, for these compounds, hydrolysis in the catalytic assay to products which are less effective inhibitors than the intact compounds or instability under catalytic versus binding assay conditions could explain in part why they are less potent at inhibiting Z-Gly-Pro-MCA hydrolysis than [^{125}I-Tyr^5]-BK binding by PPCE. The effectiveness of BK decreased more than the other compounds after preincubation with PPCE. This indicates that, under the assay conditions employed, BK is probably hydrolyzed at a faster rate than the other compounds tested. It should be noted that the reason BK appears to be hydrolyzed under these catalytic assay conditions but not under binding assay conditions is because the BK concentration used (5.9×10^{-7} M) is approximately 5 orders of magnitude greater than the concentration of [^{125}I-Tyr^5]-BK used in the binding assay.

In the first paper on the effects of BPP_{5a} and BPP_{9a}

Table 3. Inhibition of post-proline cleaving enzyme (PPCE) by bradykinin (BK) and compounds structurally unrelated to BK

Compounds*	IC_{50} (nM) for: Binding of [^{125}I-Tyr^5]-BK by porcine kidney PPCE	IC_{50} (nM) for: Rate of Z-Gly-Pro-MCA hydrolysis by porcine kidney PPCE
BK	5.1 ± 2.3	590 ± 140
Z-Pro-Prolinal	14 ± 6	7.9 ± 0.3
Angiotensin I	2.4 ± 3	6,200 ± 1,200
Angiotensin II	66 ± 29	11,000 ± 1,000
Neurotensin	120 ± 30	120 ± 10
Luteinizing hormone-releasing hormone	130 ± 20	18,000 ± 3,000
SQ20881	220 ± 80	8,700 ± 1,300
Substance P	270 ± 20	2,700 ± 400
BPP_{5a}	330 ± 60	39,000 ± 5,000
Arg^8-Vasopressin	770 ± 310	35,000 ± 7,000
DFP	1,200 ± 300	880 ± 310
Bacitracin	2,200 ± 100	9,900 ± 1,200
Z-Gly-Pro-MCA	2,800 ± 800	
TPCK	2,900 ± 700	94,000 ± 20,000

Values are means ± SD, N = a minimum of three determinations.

* Abbreviations: BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; Z-Pro-Prolinal, *N*-benzyloxycarbonyl-Pro-Prolinal; SQ20881, p Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; BPP_{5a}, p Glu-Lys-Trp-Ala-Pro; DFP, diisopropylfluorophosphate; Z-Gly-Pro-MCA, *N*-benzyloxycarbonyl-Gly-Pro-methylcoumarinyl-7-amide; TPCK, *N*-tosyl-phenylalanine chloromethyl ketone; and p Glu, pyroglutamic acid.

(SQ20881) on PPCE activity, it was reported that BPP_{5a} at 10^{-3} M does not inhibit the hydrolysis of BK 8×10^{-5} M, whereas BPP_{9a} has a K_i of 3×10^{-6} M [4]. So the fact that BPP_{5a} had one-fourth the potency of SQ20881 (BPP_{9a}) in our catalytic assay is surprising. However, in a paper on the effects of BPP_{5a} and BPP_{9a} (SQ20881) on angiotensin II metabolism by PPCE [14], it was reported that BPP_{5a} is about 1/100 as potent as BPP_{9a} at inhibiting angiotensin II cleavage by PPCE. It may be that the potency of BPP_{5a} relative to BPP_{9a} (SQ20881) for inhibition of PPCE is a function of the substrate, BK [4], angiotensin II [14], or Z-Gly-Pro-MCA, as well as the enzyme source, rabbit brain [4, 14] or porcine kidney. The greatest discrepancy between out data and one previous report [15] was obtained with TPCK, $9.4 \pm 2.0 \times 10^{-5}$ M versus 2×10^{-9} M. The only other paper that bears on this point is by Yoshimoto *et al.* [23] who reported that ZPCK (0.5 mM) does not inhibit purified lamb kidney PPCE peptidase activity. There appears to be considerable variability in the susceptibility of various PPCE preparations to inhibition by these chymotrypsin inhibitors. The finding that DFP inhibited the binding of [^{125}I-Tyr^5]-BK to PPCE was not entirely expected, even though PPCE is a serine peptidase, because DFP-inhibited PPCE has been reported to still bind to an affinity resin prepared by coupling Z-Pro-D-Ala, a PPCE inhibitor, to poly (Lys) Sepharose [23]. Apparently, this small peptide can still bind to the DFP-inhibited enzyme, whereas [^{125}I-Tyr^5]-BK cannot.

The results of kinetic experiments done with PPCE using synthetic peptide esters and amides extended N-terminally from the scissile bond and peptides extended C-terminally from the scissile bond have indicated that PPCE has five binding sites, S_3, S_2, S_1, S_1' and S_2'*, and results from experiments using substrates containing D amino acids have demonstrated that only subsites S_2, S_1, and S_1' possess stereospecificity [25]. From the data in Table 2 it can be proposed that, for BK binding to PPCE, the S_2 subsite displays greater stereospecificity than the S_1 subsite. Compare [D-Ser^6]-BK, compound 66, and [D-Pro^7]-BK, compound 58, with each other and with BK, compound 11. While these findings confirm the stereospecificity of S_1 and S_2 as determined with synthetic peptides, the stereospecificity of the PPCE S_1' subsite was not confirmed. Compare [D-Phe^8]-BK, compound 17, with BK compound 11. This could be due to the fact that [D-Phe^8]-BK has arginine in the P_2' position and this P_2'–S_2' interaction may influence the stereospecificity of the S_1' subsite. The decreased potencies of [D-Pro^3]-BK, compound 43, [D-Pro^2]-BK, compound 47, and [des-Arg^1]-BK, compound 50, relative to BK, compound 11, suggest that, for BK, PPCE may also contain binding subsites, S_7, S_6, S_5, and S_4. So, for BK, PPCE may contain as many as nine binding subsites.

The initial cleavage products of the hydrolysis of ^{125}I-labeled kinins by PPCE and ACE migrate in the

* The nomenclature of Schechter and Berger [24] is used to describe the interaction of substrate with enzyme. Amino acid residues are designated P_1, P_2 in the N-terminal direction and P_1', P_2' in the C-terminal direction of the scissile bond. The corresponding subsites of the enzyme are designated S_1, S_2, etc.

same positions in a descending paper chromatography system that separates the intact peptides from their fragments [7, 26] (unpublished observations). Since ACE and PPCE both cleave the Pro[7]-Phe[8] bond in BK to release Phe-Arg and a heptapeptide, it is quite likely that the kinin binding specificity of these enzymes may display some similarities. To facilitate a comparison of the kinin binding specificites of these kininases, the relative potencies of the BK analogs to inhibit ^{125}I-labeled kinin binding by each enzyme were calculated and $\log_{10}$ transformations of the data plotted (Fig. 5). Linear regression analysis on the data for the 56 analogs plotted in Fig. 5 resulted in a correlation coefficient of 0.54. Although this indicates a significant correlation between the relative kinin binding specificities of ACE and PPCE, it is not as impressive as if attention is only focused on compounds 8, 9, 11, 13, 18, 19, 23, 24, 30, 35, 49, 53, 58, 61, 63, and 66. The correlation coefficient for these sixteen compounds was 0.99. The greatest difference in specificity between the enzymes was seen with [Phe[4,6]]-BK, compound 42, and [Aib[7]]-BK, compound 34, [Phe[4,6]]-BK being more selective for ACE and [Aib[7]]-BK being more selective for PPCE.

The IC_{50} concentration for BK to inhibit TIK binding to ACE is $1.6 + 0.3 \times 10^{-8}$ M [22], whereas the IC_{50} concentration of BK to inhibit [^{125}I-Tyr[5]]-BK binding to PPCE was found to be $5.1 + 2.3 \times 10^{-9}$ M (Table 2). The high affinity of BK binding by these kininases was not expected, given the reported K_m values of these enzymes for BK, PPCE = 3.5×10^{-5} M [4] and ACE = 0.85×10^{-6} M [28]. ACE and PPCE are the only kininases that have been characterized with respect to their kinin binding affinities. The extent to which PPCE and/or ACE and other less characterized kininases complicate direct binding studies for BK receptors in crude tissue preparations is not known. However, our experiences with kinin binding by ACE and PPCE lead us to caution those investigators performing or contemplating direct binding studies for BK receptors to be very attentive to the possibility that binding to high-affinity, non-receptor binding sites, e.g. kininases, could interfere with their studies. This warning extends to all those doing direct binding studies for receptors, not just those working in the kinin area. It is likely that degradative enzymes will display much higher affinities for the ligands under investigation than would be expected based on the K_m values of the enzymes for the ligands being studied. Both metallo and serine classes of peptidases, as evidenced by ACE and PPCE, respectively, are capable of displaying these ligand binding properties.

The relative importance of PPCE in modulating the activity of BK *in vivo* is not known. The predominant cytosolic localization of PPCE makes it a likely candidate to be involved in the inactivation of BK should BK–receptor complexes be internalized, as has been described for a number of hormone–receptor complexes [29].

In addition to its cytosolic localization, PPCE has been found in renal brush border membranes [30] and thus may also be involved extracellularly in the renal inactivation of BK. The use of Z-Pro-Prolinal [31] in *in vivo* experiments should help to delineate the importance of PPCE in the metabolism of BK.

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