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INTERACTIONS OF KININS WITH ANGIOTENSIN I CONVERTING ENZYME (KININASE II)*

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Abstract—Angiotensin I converting enzyme (ACE) was purified to homogeneity from porcine kidney in order to determine whether iodobradykinins bind to the enzyme and, if so, whether pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, SQ20881, a competitive ACE inhibitor, changes the conformation of the enzyme in such a way that it binds kinins with an affinity and specificity expected of a bradykinin (BK) receptor, i.e. where the BK potentiating action of SQ20881 involves an increase in the number of BK receptors due to a conformational change in ACE. ^{125}I-Labeled derivatives of [Tyr^1]-kallidin and [Tyr^8]-bradykinin bound to the EDTA-inhibited enzyme, and binding was inhibited by nonradioactive BK. [^{125}I-Tyr^5]-BK was not bound by the enzyme. Specificity of [^{125}I-Tyr^1]-kallidin (TIK) binding was tested with forty-eight BK analogs, and the concentrations of analogs that inhibited 50% of TIK binding were determined. BK at $1.6 \pm 0.3 \times 10^{-8}$ M inhibited 50% of TIK binding. In addition, the concentrations of analogs that decreased by 50% the rate of [^{3}H]-Hip-Gly-Gly ([^{3}H]-HGG) hydrolysis by ACE were assessed. BK at $1.2 \pm 0.2 \times 10^{-6}$ M decreased the rate of [^{3}H]-HGG hydrolysis by 50%. A comparison between these concentrations of analogs for inhibition of TIK binding and [^{3}H]-HGG hydrolysis yielded a high correlation coefficient ($r = 0.85$). The specificity of ACE binding was clearly different from that expected of a BK receptor. Compounds structurally unrelated to BK, such as SQ20881, pGlu-Lys-Trp-Ala-Pro-OH (BPP_{5a}) and angiotensin I, inhibited TIK binding and [^{3}H]-HGG hydrolysis by ACE.

Angiotensin I converting enzyme (ACE, EC 3.4.15.1) is a peptidyldipeptide hydrolase that releases angiotensin II, a potent vasopressor, by cleavage of His-Leu from the C-terminus of angiotensin I and that inactivates bradykinin (BK), a potent vasodepressor, by cleavage of Phe-Arg from its C-terminus [1]. Inhibitors of ACE have antihypertensive actions that may be attributed to their abilities to inhibit the formation of angiotensin II and to their potentiation of the actions of BK. pGlu-Trp-Pro-Arg-Gln-Ile-Pro-Pro, SQ20881, a competitive inhibitor of ACE, potentiates the actions of BK in a number of biological systems. While protection of BK from destruction by ACE is probably involved in the mechanism of potentiation by SQ20881, this is probably not the sole mechanism [2, 3].

In our initial attempts to study directly the interactions of BK with its receptors, commercially available [^{125}I-Tyr^8]-BK was used as the radioactive probe for BK receptors in a particulate subcellular fraction from porcine kidney medulla [4]. Binding of the radioactive peptide to this subcellular fraction was saturable, i.e. unlabeled BK was able to inhibit binding of radioactivity to the particulate matter. However, most, but not all, of the saturable binding was inhibited by SQ20881 [5]. This was interpreted as indicating that most of the binding was to ACE and/or to some other kinin binding site that could be blocked by SQ20881. Another interpretation is that ACE, when it is inhibited by SQ20881, binds kinins in a receptor-like manner.

The present studies were undertaken to see whether ^{125}I-labeled kinins bind to ACE purified from porcine kidney and, if they do, to determine the specificity of that binding. In addition, we wanted to test the hypothesis that ACE, inhibited by SQ20881, undergoes a conformational change such that the enzyme now displays a kinin binding affinity and specificity expected of a bradykinin receptor, i.e. part of the BK potentiating action of SQ20881 involves an increase in the number of BK receptors that are formed as a result of a conformational change in ACE.

MATERIALS AND METHODS

Materials

Porcine kidneys were obtained from the Stadler Packing Co., Inc., Columbus, IN. Chromatography media were obtained as follows: DE 23 cellulose from Whatman, Inc., Clifton, NJ; AcA 54 and AcA 34 Ultrogel and HA-Ultrogel from LKB Instruments, Inc. Rockville, MD; and Polybuffer 74 and Polybuffer exchanger PBE 94 from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. Neuraminidase Type X, crystalline bovine serum albumin, substance P, and phosphoramidone were from the Sigma Chemical Co., St. Louis, MO. 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) was from Research Organics, Inc., Cleveland, OH. Hippu-

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rylglycylglycine (HGG) was from Pfaltz & Bauer, Inc., Stamford, CT, or Vega Biochemicals, Tuscon, AZ. The latter company was also the source of Methionyl-Lysyl-BK, Lysyl-BK, and neurotensin. Methionyl-Lysyl-BK and neurotensin were also purchased from the Chemical Dynamics Corp., South Plainfield, NJ, as were vasopressin and Arg-Pro-Pro. [^{3}H]-HGG (sp. act. 25 Ci/mmole) and Scintillation Cocktail No. 1 were from Ventrex Laboratories, Inc., Portland, ME. [Tyr^1]-Kallidin, [Tyr^5]-BK, [Tyr^8]-BK, and human angiotensins I and II were purchased from Peninsula Laboratories, Inc., San Carlos, CA. BK was from Boehringer Mannheim Biochemicals, Indianapolis, IN, or Serva Fine Biochemicals, Inc., Long Island, NY. [Orn^1]-BK, [Lys^1]-BK, and [Phe(F)^8]-BK, gifts of Dr. E. D. Nicolaides, were from Parke, Davis & Co., Ann Arbor, MI. All other BK analogs used in this investigation were prepared by the Merrifield solid phase method [6]. pGlu-Lys-Trp-Ala-Pro-OH, BPP$_{5a}$, was purchased from Bachem Fine Chemicals, Inc., Torrance, CA. pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-OH, SQ20881, and captopril, D-3-mercapto-2-methylpropanoyl-L-proline, SQ14225, were gifts of the Squibb Institute for Medical Research, Princeton, NJ. *N*-(1-*S*-1-Carboxy-3-phenylpropyl)-L-Ala-L-Pro, MK-422, and its ethyl ester, MK-421, were gifts of the Merck Institute for Therapeutic Research, West Point, PA. Trasylol (registered trade name for Bayer AG) was a gift from Bayer Ag, Wuppertal-1, West Germany. Econo-Column polypropylene columns were purchased from Bio-Rad Laboratories, Richmond, CA. All other reagents were of the highest quality available and were obtained from commercial sources.

Methods

Purification of ACE. ACE was purified from porcine kidney using a modification of the procedure of Oshima *et al.* [7]. Briefly, ACE was extracted with sodium desoxycholate from a "microsomal" fraction of a homogenate of the combined cortical and medullary regions of fresh porcine kidneys. The detergent extract was equilibrated with 5 mM Tris–HCl, pH 7.4, containing 0.1 mM $CoCl_2$, in a DC-2 Amicon hollow-fiber ultrafiltration apparatus equipped with 50,000 mol. wt (M_l) cut-off cartridges. The solution retained by the Amicon was applied to a DE-23 cellulose column equilibrated with the same buffer. ACE was eluted with a linear NaCl gradient. Fractions that contained ACE were concentrated and then equilibrated with 0.2 mM sodium phosphate buffer, pH 6.8, in the Amicon. The sample was applied to an HA-Ultrogel column equilibrated with the same buffer. ACE was not retained under these conditions. Fractions that contained ACE were concentrated by ultrafiltration with the DC-2 Amicon and then further concentrated by ultrafiltration in a 202 Amicon stirred cell equipped with a YM-30 membrane before application to an AcA 34 Ultrogel column. The column was eluted with 50 mM Tris–HCl, pH 7.4, that contained 0.2 M NaCl and 0.1 mM $CoCl_2$. Fractions that contained ACE were concentrated by ultrafiltration in the 202 Amicon and then equilibrated with 25 mM histidine–HCl, pH 6.2. ACE was further purified by chromatofocusing. A column of PBE 94, 1.2 × 11.5 cm, was equilibrated with the histidine buffer. After the ACE sample was applied, the column was eluted with 125 ml of Polybuffer 74 that had been adjusted to pH 4.0 with HCl and then diluted 1:8 with deionized water. The column was eluted at a flow rate of 15 ml/hr and 3.3-ml fractions were collected. The peak of ACE activity eluted at pH 4.8 and had a specific activity of 29 μmoles of HGG cleaved in 1 min at 37° per mg of protein. The molecular weight of this ACE preparation was estimated to be 280,000 by gel filtration on a calibrated AcA 34 Ultrogel column and 165,000 by sodium dodecyl sulfate (SDS)-polyacrylamide disc gel electrophoresis with 7.5% gels. Standard gel electrophoresis of the purified enzyme yielded two protein bands, but a single band was obtained after treatment of the enzyme preparation with neuraminidase.

Enzymatic assay for ACE. The buffer used for the ACE enzymatic assay was that found by Dorer *et al.* [8] to be optimal for the hydrolysis of HGG: 0.05 M HEPES, pH 8.0, that contained 0.6 M Na_2SO_4, 0.1 M NaCl, 0.1 mM $CoCl_2$, and 2 mM NaN_3. To 2 ml glass scintillation vials run in triplicate were added: 0.0143 μg ACE in 0.05 ml buffer; 100,000 cpm (4 pmoles) of [^{3}H]-HGG in 0.02 ml buffer; 5 μg crystalline bovine serum albumin in 0.02 ml buffer; and 0.01 ml water, peptide dissolved in water, or other substances to be tested. After incubation at 37° for 1 hr, reactions were terminated by the addition of 0.05 ml of 0.5 N HCl. [^{3}H]-Hippuric acid was separated from unreacted substrate with 1.5 ml of Ventrex scintillation Cocktail No. 1 and counted for 1 min in a Beckman model LS 8100 beta counter. The reaction rate was linear over 1 hr and 25% of the substrate was consumed in that time. The initial concentrations of ACE and [^{3}H]-HGG in the assay were 8.7×10^{-10} M and 40×10^{-9} M respectively. When the assay was used to monitor ACE activity during the course of enzyme purification, 400 nmoles of nonradioactive HGG was included in each reaction mixture.

Kinin binding assay for ACE. Monoiodinated derivatives of [Tyr^1]-kallidin, [Tyr^5]-BK and [Tyr^8]-BK were prepared [9] and purified [10] as previously described. Conditions for binding of kinins to ACE were the same as those listed above for ACE enzymatic activity except: the incubation buffer included 3.3 mM EDTA; borosilicate glass 12 × 75 mm test tubes replaced the 2 ml scintillation vials; 30,000 cpm (0.015 pmole) of ^{125}I-labeled kinin replaced the [^{3}H]-HGG; and the length of incubation was 2 hr instead of 1 hr. After the incubation at 37°, the contents of the test tubes were transferred with 0.1 ml disposable glass micropipettes to polypropylene columns, 0.8 × 4 cm, of AcA 54 Ultrogel that were equilibrated and subsequently eluted at room temperature with 50 mM Tris–HCl, pH 7.4, that contained 0.1 M NaCl and 1 mM NaN_3. Before using them for the first time, columns were pretreated with 1 ml of a 1 mg/ml solution of crystalline bovine serum albumin in the elution buffer. Radioactivity bound to ACE was eluted in the void volume of the columns, 1.5 ml. The unbound radioactivity was eluted with an additional 3.0 ml. The bound and free radioactivity were counted for 1 min in an automatic

well-type gamma counter (Nuclear Chicago, model 1065). The total radioactivity from each column was calculated, and the amount present in the void volume was expressed as a percentage of the total. Under these assay conditions, 16% of the [^{125}I-Tyr^1]-kallidin incubated in the absence of nonradioactive competitors was bound to ACE.

Calculations. In all assays, controls were run in which ACE was omitted from the incubation mixtures. Results obtained for these controls were subtracted from those obtained in the presence of ACE. When various concentrations of nonradioactive compounds were tested for their abilities to inhibit [^{3}H]-HGG hydrolysis or ^{125}I-labeled kinin binding, the results were normalized to controls which contained only ACE and the radioactive material. The concentrations of compounds that inhibit 50% of the hydrolysis or binding by ACE were calculated from plots of the normalized values versus the $\log_{10}$ of the concentrations of inhibitors. Results are expressed as the mean ± S.D. and were determined with a Texas Instruments model 58 calculator. Linear regression analyses were performed using the statistics package for this calculator.

RESULTS

Figure 1 shows the binding of the mono-^{125}I-labeled derivatives of [Tyr^1]-kallidin, [Tyr^5]-BK, and [Tyr^8]-BK to purified porcine kidney ACE. [^{125}I-Tyr^1]-Kallidin (T1K) bound to a greater extent than [^{125}I-Tyr^8]-BK and there was essentially no [^{125}I-Tyr^5]-BK binding. BK and SQ20881 were able to inhibit completely the binding of iodokinins to ACE, i.e. there was no nonsaturable binding of radioactivity to ACE. "Binding" seen in the presence of excess BK or SQ20881 was equal to the amount of radioactivity that elutes from the gel filtration columns in the absence of enzyme. The binding of T1K to ACE as a function of time is shown in Fig. 2. Maximum binding was obtained after 15 min. An arbitrary length of incubation of 2 hr was selected for subsequent experiments in which nonradioactive compounds were tested for their abilities to inhibit T1K binding to ACE.

A comparison of the abilities of various concentrations of BK to inhibit either T1K binding or [^{3}H]-HGG hydrolysis by ACE is shown in Fig. 3. BK was approximately 100 times more potent as an inhibitor of T1K binding than as an inhibitor of [^{3}H]-HGG hydrolysis. The concentrations of BK that inhibit 50% of these ACE activities (IC_{50}) were read from the graph and are recorded in Table 1 along with data obtained in a similar manner for forty-seven BK analogs or compounds structurally related to BK. Also recorded in Table 1, for the sake of comparison, are the relative biological potencies of these compounds on isolated rat uterus. A plot of the $\log_{10}$ of the IC_{50} values for [^{3}H]-HGG hydrolysis versus the $\log_{10}$ of their respective IC_{50} values for T1K binding is shown in Fig. 4. Linear regression analysis of these transformed data revealed a highly significant correlation, $r = 0.85$. A similar analysis of the $\log_{10}$ transformations of the IC_{50} values of the BK analogs for T1K binding versus their respective biological activities yielded an r value of -0.04, indicating the lack of a relationship.

Compounds structurally unrelated to BK were also evaluated for their abilities to inhibit T1K binding or [^{3}H]-HGG hydrolysis by ACE. These data are recorded in Table 2 along with selected data on some of these compounds taken from the literature.

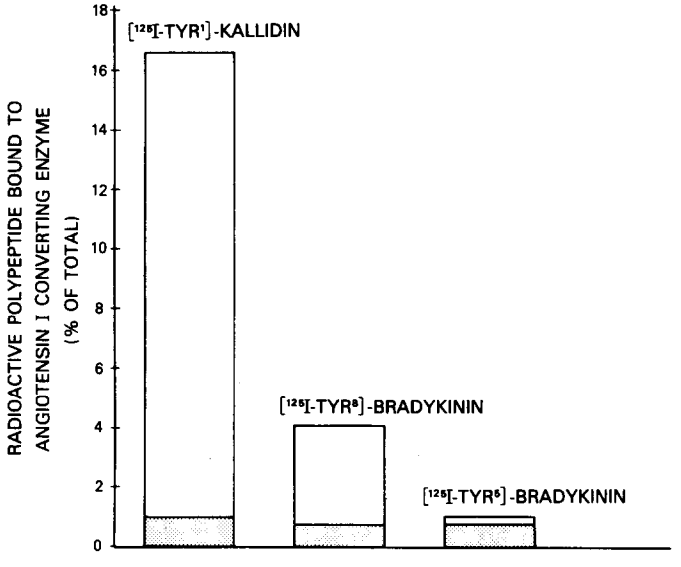

Fig. 1. Binding of ^{125}I-labeled kinins to purified porcine kidney angiotensin I converting enzyme (ACE). Thirty picograms (30,000 cpm) of [^{125}I-Tyr^1]-kallidin, [^{125}I-Tyr^5]-bradykinin or [^{125}I-Tyr^8]-bradykinin was incubated for 2 hr with purified ACE as described in Methods. The amounts of radioactive polypeptides bound to ACE in the absence of bradykinin (BK) or SQ20881 (total bar) and with 10 μg BK or SQ20881 in the incubation media (shaded area) were expressed as percentages of the total amounts of radioactivity eluted from the gel filtration columns used to separate bound from free radioactivity.

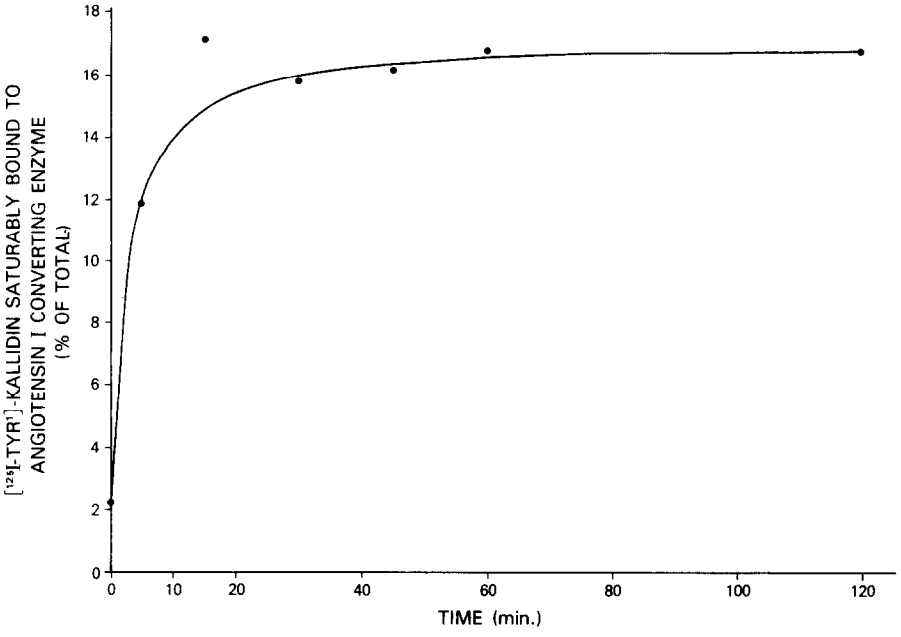

Fig. 2. Binding of [^{125}I-Tyr^1]-kallidin (T1K) to purified porcine kidney angiotensin I converting enzyme (ACE) as a function of time. Thirty picograms (30,000 cpm) was incubated for 5, 15, 30, 45, 60 and 120 min with purified ACE as described in Methods. The amounts of T1K bound to ACE after each of these incubations were expressed as percentages of the total amounts of radioactivity eluted from the gel filtration columns used to separate bound from free radioactivity.

Vasopressin and Trasylol were also tested and found not to inhibit binding or hydrolysis by ACE at 9.2×10^{-5} M and 3.1×10^{-5} M respectively. 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, and their concentrations, it can be seen that there are no striking differences between our results and some of those previously reported.

DISCUSSION

Purified porcine kidney ACE was found to bind ^{125}I-labeled kinins saturably (Fig. 1). The spectrum of saturable binding of the radioactive peptides is similar to that obtained with a bradykinin receptor-like binder from bovine uterine myometrium [5]. ACE can easily be distinguished from the receptor-like binder of myometrium, since SQ20881

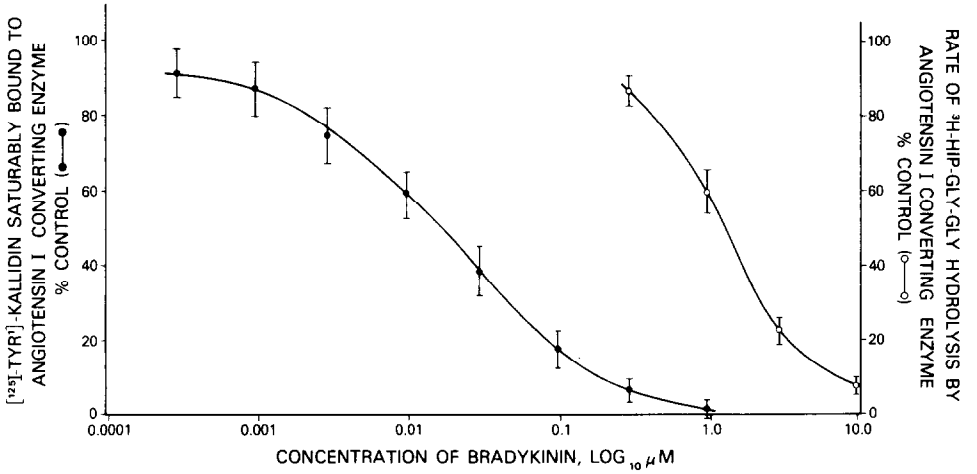

Fig. 3. Bradykinin (BK) inhibition of [^{125}I-Tyr^1]-kallidin (T1K) binding and [^{3}H]-Hip-Gly-Gly ([^{3}H]-HGG) hydrolysis by purified porcine kidney angiotensin I converting enzyme (ACE). Serial dilutions of BK were incubated with T1K (●) or [^{3}H]-HGG (○) and ACE as described in Methods. The curves are composites of results from thirty-two and thirty-one experiments with T1K and [^{3}H]-HGG respectively. The brackets enclose a standard deviation on each side of the means of these data.

Table 1. Inhibition of angiotensin I converting enzyme (ACE) by bradykinin (BK) and structurally related compounds

Compound	Relative biological potency on isolated rat uterus [Ref.]	IC_{50} (M) for: The binding of [^{125}I-Tyr^1]-kallidin by porcine kidney ACE inhibited by EDTA	IC_{50} (M) for: The rate of [^{3}H]-Hip-Gly-Gly hydrolysis by porcine kidney ACE
1 Lys (Tos)^6-BK	0.05 [11]	$4.2 \pm 4.0 \times 10^{-10}$	$9.2 \pm 1.3 \times 10^{-8}$
2 Phe^6-BK	0.1 [11]	$5.6 \pm 5.7 \times 10^{-11}$	$1.7 \pm 0.3 \times 10^{-7}$
3 Phe^{4,6}-BK	0.01 [12]	$1.3 \pm 1.0 \times 10^{-10}$	$2.3 \pm 0.9 \times 10^{-7}$
4 D-Ala^4-BK	0.01, 0 [12]	$4.7 \pm 0.3 \times 10^{-9}$	$3.4 \pm 0.7 \times 10^{-7}$
5 Aib^2-BK	0.5 [13]	$1.2 \pm 0.6 \times 10^{-8}$	$4.7 \pm 1.0 \times 10^{-7}$
6 Cl-Acetyl-Arg^1-BK	1000 [12]	$3.4 \pm 0.8 \times 10^{-9}$	$4.8 \pm 0.3 \times 10^{-7}$
7 Thr^6-BK	100, 33 [11]	$1.3 \pm 0.4 \times 10^{-8}$	$5.8 \pm 1.3 \times 10^{-7}$
8 ThiAla^5-BK	200 [12]	$1.5 \pm 0.2 \times 10^{-8}$	$5.8 \pm 0.2 \times 10^{-7}$
9 Hypro^3-BK	100, 114 [12]	$1.5 \pm 0.3 \times 10^{-8}$	$6.5 \pm 0.3 \times 10^{-7}$
10 Lys^1-BK	0.2 [11]	$5.2 \pm 0.6 \times 10^{-9}$	$7.6 \pm 0.2 \times 10^{-7}$
11 Acetyl-Arg^1-D-Pro^3-OMT^8-BK	0.2 [13]	$2.1 \pm 0.7 \times 10^{-8}$	$7.7 \pm 0.6 \times 10^{-7}$
12 D-Pro^2-BK	0.2 [11]	$2.3 \pm 0.3 \times 10^{-8}$	$7.8 \pm 0.4 \times 10^{-7}$
13 Thr^6-OMT^{5,8}-BK	3.3 [5]	$3.7 \pm 0.3 \times 10^{-8}$	$7.8 \pm 0.9 \times 10^{-7}$
14 Aib^3-BK	7.7 [14]	$2.5 \pm 1.2 \times 10^{-8}$	$9.6 \pm 0.2 \times 10^{-7}$
15 Acetyl-Arg^1-BK	50 [11]	$3.4 \pm 1.3 \times 10^{-9}$	$1.0 \pm 0.2 \times 10^{-6}$
16 des-Arg^1-BK	0.00001 [12]	$7.3 \pm 0.4 \times 10^{-8}$	$1.0 \pm 0.1 \times 10^{-6}$
17 Acetyl-Arg^1-D-Pro^3-BK	0.05 [13]	$1.1 \pm 0.0 \times 10^{-8}$	$1.1 \pm 0.1 \times 10^{-6}$
18 Phe (F)^8-BK	150 [12]	$5.0 \pm 0.4 \times 10^{-9}$	$1.2 \pm 0.0 \times 10^{-6}$
19 BK	100	$1.6 \pm 0.3 \times 10^{-8}$	$1.2 \pm 0.2 \times 10^{-6}$
20 Leu^5-BK	5 [12]	$2.6 \pm 0.1 \times 10^{-8}$	$1.2 \pm 0.3 \times 10^{-6}$
21 D-Pro^{2,3}-BK	0.01 [11]	$3.2 \pm 1.2 \times 10^{-8}$	$1.2 \pm 0.2 \times 10^{-6}$
22 Leu^8-BK	0.3 [12]	$3.4 \pm 1.0 \times 10^{-8}$	$1.2 \pm 0.4 \times 10^{-6}$
23 D-Pro^3-BK	0.01 [14]	$3.4 \pm 1.1 \times 10^{-8}$	$1.2 \pm 0.1 \times 10^{-6}$
24 Tyr^1-Kallidin	40, 95 [12]	$1.4 \pm 0.5 \times 10^{-9}$	$1.3 \pm 0.3 \times 10^{-6}$
25 Tyr^8-BK	24 [5]	$5.0 \pm 1.5 \times 10^{-9}$	$1.3 \pm 0.4 \times 10^{-6}$
26 Lysyl-BK	50–66, 10 [11]	$2.8 \pm 0.8 \times 10^{-8}$	$1.4 \pm 0.1 \times 10^{-6}$
27 Tyr^5-BK	0.2, 0.3 [11]	$2.9 \pm 1.0 \times 10^{-8}$	$1.4 \pm 0.3 \times 10^{-6}$
28 D-Pro^3-OMT^8-BK	0.1 [5]	$3.4 \pm 0.5 \times 10^{-8}$	$1.7 \pm 0.0 \times 10^{-6}$
29 Orn^1-BK	0.1 [11]*	$2.2 \pm 0.2 \times 10^{-9}$	$1.8 \pm 0.1 \times 10^{-6}$
30 Hypro^{2}ThiAla^{5,8}-BK	100 [12]	$3.6 \pm 0.9 \times 10^{-8}$	$2.5 \pm 0.1 \times 10^{-6}$
31 ThiAla^{5,8}-BK	1000 [12]	$3.8 \pm 0.1 \times 10^{-8}$	$2.8 \pm 0.8 \times 10^{-6}$
32 Methionyl-Lysyl-BK	25, 33 [11]	$1.9 \pm 0.1 \times 10^{-8}$	$2.9 \pm 1.0 \times 10^{-6}$
33 Abu^6-BK	51 [15]†	$1.4 \pm 0.4 \times 10^{-6}$	$4.1 \pm 1.2 \times 10^{-6}$
34 Phe^9-BK	<0.01 [12]	$1.4 \pm 0.4 \times 10^{-8}$	$4.4 \pm 0.2 \times 10^{-6}$
35 des-Arg^9-BK	1 [12]	$1.6 \pm 0.1 \times 10^{-6}$	$8.8 \pm 0.5 \times 10^{-6}$
36 D-Pro^7-OMT^8-BK	0.033 [5]	$2.8 \pm 0.4 \times 10^{-7}$	$1.2 \pm 0.1 \times 10^{-5}$
37 Arg-Pro-Pro	0 [12]	$>2.0 \pm 0.0 \times 10^{-4}$	$1.4 \pm 0.0 \times 10^{-5}$
38 des-Phe^8-Arg^9-BK	0 [16]	$5.0 \pm 1.0 \times 10^{-8}$	$1.9 \pm 0.1 \times 10^{-5}$
39 D-Ala^6-BK	0.7 [15]†	$3.5 \pm 0.3 \times 10^{-6}$	$2.9 \pm 0.2 \times 10^{-5}$
40 Aib^7-BK	67 [14]	$2.8 \pm 0.2 \times 10^{-5}$	$5.2 \pm 0.3 \times 10^{-5}$
41 D-Pro^7-BK	1.3, 1.5 [11]	$4.1 \pm 1.0 \times 10^{-6}$	$5.3 \pm 0.1 \times 10^{-5}$
42 D-Phe^8-BK	15 [12]	$2.9 \pm 1.6 \times 10^{-6}$	$>9.4 \pm 0.0 \times 10^{-5}$
43 Acetyl-Arg^1-D-Pro^7-BK		$4.5 \pm 0.3 \times 10^{-6}$	$>0.7 \pm 0.0 \times 10^{-4}$
44 Gly^6-Aib^7-BK	2.5 [13]	$>0.7 \pm 0.0 \times 10^{-4}$	$>0.7 \pm 0.0 \times 10^{-4}$
45 D-Pro^{2,3,7}-BK	0.0005 [11]	$>0.8 \pm 0.0 \times 10^{-4}$	$>0.8 \pm 0.0 \times 10^{-4}$
46 D-Pro^{2,7}-BK	0.01 [11]	$1.1 \pm 0.0 \times 10^{-6}$	$>0.8 \pm 0.0 \times 10^{-4}$
47 D-Pro^{3,7}-BK	0.001 [11]	$4.0 \pm 1.0 \times 10^{-5}$	$>0.8 \pm 0.0 \times 10^{-4}$
48 Gly^{5,6,8}-BK	0.01 [12]	$>1.0 \pm 0.0 \times 10^{-4}$	$>1.0 \pm 0.0 \times 10^{-4}$

* Guinea pig broncho-construction.
† Cat ileum strip.

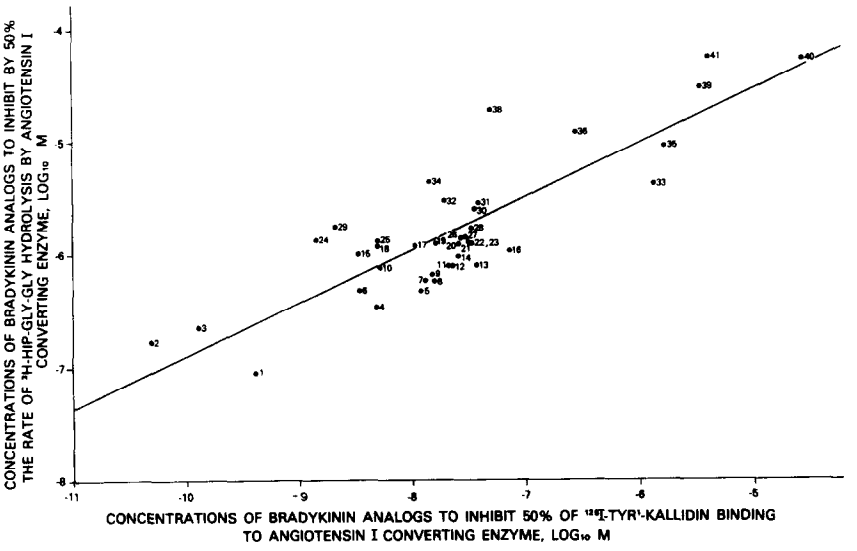

Fig. 4. Comparison of the concentrations of bradykinin analogs that decrease by 50% the rate of [^{3}H]-Hip-Gly-Gly hydrolysis by purified porcine kidney angiotensin I converting enzyme (ACE) with those that inhibit 50% of [^{125}I-Tyr^1]-kallidin binding to ACE. Logarithmic transformations of the data from Table 1 were used to construct this figure. The numbers correspond to the analogs listed in Table 1. The line was obtained from linear regression analysis of these transformed data and has a correlation coefficient, r, of 0.85.

Table 2. Inhibition of angiotensin I converting enzyme (ACE) by bradykinin (BK) and compounds structurally unrelated to BK

Compound	IC_{50} (M) for: The binding of [^{125}I-Tyr^1]-kallidin by porcine kidney ACE inhibited by EDTA	IC_{50} (M) for: The rate of [^{3}H]-Hip-Gly-Gly hydrolysis by porcine kidney ACE	The rate of substrate hydrolysis by ACE* [Ref.]
			0.85×10^{-6} [17] 2.6×10^{-6} [18]
BK	$1.6 \pm 0.3 \times 10^{-8}$	$1.2 \pm 0.2 \times 10^{-6}$	9.0×10^{-6} [19]
Angiotensin I	$3.2 \pm 2.3 \times 10^{-6}$	$1.5 \pm 0.1 \times 10^{-5}$	1×10^{-5} [17] 5×10^{-5} [19]
Angiotensin II	$4.8 \pm 2.2 \times 10^{-5}$	$2.4 \pm 0.5 \times 10^{-5}$	1.9×10^{-5} [18] 4.0×10^{-5} [19]
BPP_{5a}	$5.2 \pm 2.3 \times 10^{-11}$	$4.9 \pm 0.7 \times 10^{-7}$	6.0×10^{-8} [17] 7.9×10^{-8} [18]
SQ20881	$5.3 \pm 1.0 \times 10^{-9}$	$1.8 \pm 0.4 \times 10^{-9}$	1.5×10^{-9} [8] 3.6×10^{-8} [20]
SQ14225	$>4.6 \pm 0.0 \times 10^{-4}$	$7.2 \pm 1.8 \times 10^{-10}$	3.0×10^{-9} [21] 2.0×10^{-8} [20]
MK 421	$>2.5 \pm 0.0 \times 10^{-4}$	$4.0 \pm 1.3 \times 10^{-7}$	1.2×10^{-6} [20]
MK 422	$5.5 \pm 4.8 \times 10^{-6}$	$1.4 \pm 0.3 \times 10^{-10}$	1.2×10^{-9} [20]
Val-Trp	$1.5 \pm 0.7 \times 10^{-7}$	$6.3 \pm 1.1 \times 10^{-7}$	3×10^{-7} [17]
Phosphoramidone	$4.6 \pm 0.5 \times 10^{-5}$	$4.5 \pm 1.0 \times 10^{-6}$	
Neurotensin	$6.8 \pm 1.2 \times 10^{-7}$	$2.5 \pm 0.6 \times 10^{-5}$	
Substance P	$4.2 \pm 0.0 \times 10^{-6}$	$7.4 \pm 0.0 \times 10^{-5}$	9.1×10^{-6} [22] 5.0×10^{-5} [23]

* Data are taken from the literature and were obtained with other ACE preparations: porcine lung [8] and plasma [20], rabbit lung [17, 19], guinea pig lung [18], human kidney [21], canine lung [22], and rat striatum [23], and other substrates: Hip-Gly-Gly [8], Hip-His-Leu [17–19, 23], Boc-Phe-His-Leu [20], randomly tritiated Hip-Gly-Gly [21], and [^{14}C]-Hip-His-Leu [22].

causes a small increase in saturable binding of T1K to myometrium but completely inhibits T1K binding to ACE. SQ20881 did not cause ACE to bind kinins with a receptor-like specificity, i.e. the bradykinin potentiating action of SQ20881 does not involve conversion of ACE to a BK receptor. The binding of T1K by membranes from human umbilical vessels reported by Trapeznikova *et al.* [24] could be to ACE or some other enzyme(s) involved in the metabolism of both BK and angiotensin, since [Tyr^1]-kallidin, [des-Arg^1]-BK and angiotensin II were able to inhibit binding of radioactive kinin by the membranes. The inability of these authors to demonstrate binding of T1K to endothelial cells, a known source of ACE, could be due to incomplete inhibition of the enzyme. We were not able to demonstrate binding of ^{125}I-labeled kinins to ACE when it was enzymatically active, presumably because they were metabolized to products with lower affinities for ACE. It should be noted in this regard that the spectrum of iodokinin binding (Fig. 1) is not a function of difference in the susceptibility of these compounds to hydrolysis by ACE, since analyses of the radioactivity that was incubated with the EDTA-inhibited enzyme demonstrated that the ^{125}I-labeled peptides were still intact.

The importance of the various amino acid residues in BK for binding to ACE can be assessed by examination of the effects that changes in the BK sequence have on its K_i for ACE. The IC_{50} values for [^{3}H]-HGG hydrolysis recorded in Table 1 for BK analogs equal the K_i values for these compounds, if inhibition is competitive, since ACE enzymatic activity was measured under conditions where the substrate concentration was much lower than its K_m, i.e. 40×10^{-9} M vs 1×10^{-3} M. For linear competitive inhibition, $IC_{50} = K_i\ (1 + S/K_m)$ [25]; since $S \ll K_m$, $IC_{50} = K_i$. Little is known about the binding specificity of ACE for BK. Sander *et al.* [26] found that BK, [Tyr^8]-BK, and Methionyl-Lysyl-BK inhibited angiotensin I hydrolysis by crude rabbit lung ACE with the same rank order of potency that we find for [^{3}H]-HGG hydrolysis by pure porcine kidney ACE, i.e. [Tyr^8]-BK $\geq$ BK > Methionyl-Lysyl-BK. Arg-Pro-Pro competitively inhibited HGG hydrolysis by purified porcine lung ACE with an $IC_{50} = 1 \times 10^{-5}$ M in one report [27], while others obtained an $IC_{50} = 2.3 \times 10^{-6}$ M [28]. Kariya *et al.* [29] reported a $K_i = 5.9 \times 10^{-6}$ M for this tripeptide with a microsomal ACE fraction from rat brain and Hip-His-Leu as substrate. Their value for BK was 7.6×10^{-7} M. Cheung *et al* [17] obtained a K_i for BK of 8.5×10^{-7} M with purified rabbit lung ACE and Hip-His-Leu substrate. These values for Arg-Pro-Pro and for BK are comparable to ours, 1.4×10^{-5} and 1.2×10^{-6} M respectively.

Substitution of D-Pro for L-Pro^7, D-Phe for L-Phe^8, or L-Phe for L-Arg^9 in BK, compounds 41, 42 and 34, respectively, leads to an increase in the K_i of the compounds relative to BK. Although these changes in K_i are in the same direction as those reported by Rohrbach *et al.* [30] for systematically modified tri- and tetrapeptides, the magnitude of the changes is different. These differences could be due to our use of longer peptides that contain additional groups for interaction with ACE. For example, replacement of Ser in position 6 of BK with aromatic residues resulted in compounds, 1, 2, and 3, that bind to ACE with approximately ten times greater affinity than BK. This result is consistent with others that suggest that there is a hydrophobic region in the vicinity of the catalytic site of ACE [21, 31–34] that might be involved in the binding of substrates and inhibitors to the enzyme.

The reason why higher concentrations of BK and its analogs are required to inhibit [^{3}H]-HGG hydrolysis by ACE than are required to inhibit T1K binding to the EDTA-inhibited enzyme (Table 1) could be due to the hydrolysis of these compounds to products which are less effective as inhibitors of [^{3}H]-HGG hydrolysis than are the intact peptides. To test this possibility, [Phe^6]-BK, BK, [Abu^6]-BK, and [D-Pro^7]-BK, at concentrations that inhibited 50% of the rate of [^{3}H]-HGG after 1 hr of incubation, were also examined after 30 and 90 min of incubation. Compounds which are being hydrolyzed to less active products should be more effective as inhibitors after shorter incubations than after longer incubations. Conversely, compounds resistant to hydrolysis should be equally effective at all lengths of incubation. The effectiveness of [D-Pro^7]-BK as an inhibitor did not vary with length of incubation, while the other analogs were all less effective with increasing length of incubation. Results for BK and [D-Pro^7]-BK are consistent with a previous report in which BK was found susceptible and [D-Pro^7]-BK resistant to hydrolysis by purified porcine lung ACE [35]. However, the fact that the concentration of [D-Pro^7]-BK to inhibit T1K binding is about ten times less than that required to inhibit [^{3}H]-HGG hydrolysis indicates that an additional factor(s) must account for the differences obtained. Perhaps the presence of the metal decreases the binding affinity of the enzyme for some BK analogs. Further experiments are required in order to make a more definitive explanation.

Of the compounds listed in Table 2, probably SQ14225 (captopril) and MK 421 (enalapril) are of the greatest clinical interest because they are ACE inhibitors that are effective when administered orally and are able to decrease blood pressure in hypertensives [36]. MK421, its free acid MK422, and SQ14225 appear to require the presence of a free metal in order to bind to ACE, since these compounds were orders of magnitude less potent as inhibitors of T1K binding to EDTA-inhibited ACE than they were as inhibitors of [^{3}H]-HGG hydrolysis. In contrast, BPP_{5a} and SQ20881, peptides that were originally isolated from *Bothrops jararaca* venom, do not. They were about as potent or more potent as inhibitors of T1K binding than as inhibitors of [^{3}H]-HGG hydrolysis. Phosphoramidone, *N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp, was initially evaluated because its C-terminus resembles Val-Trp, the most potent dipeptide inhibitor of ACE known [36]. Phosphoramidone is a potent inhibitor of thermolysin [37], whose active site has several features in common with that of carboxypeptidase A [38]. Since SQ14225 was developed as a result of a comparison of ACE to carboxypeptidase A [36], it is not surprising that phosphoramidone inhibits ACE. Phosphoramidone appears to have

less of a metal requirement for binding to ACE than MK421, MK422, and SQ14225. While the importance of ACE in the metabolism of BK and angiotensin I is recognized [1], its potential involvement in the metabolism of other biologically active peptides needs further study. The IC_{50} values of neurotensin and substance P for [^{3}H]-HGG hydrolysis are comparable to those for the angiotensins, which suggests that their interactions with ACE may be of physiological importance. The reader is cautioned not to interpret our data as indicating that substance P and the other peptides tested are substrates for ACE, because the susceptibility of these peptides to hydrolysis by ACE was not assessed. It is unlikely that substance P is a substrate for ACE, since it does not have a free C-terminal carboxyl group. If any of the peptides are substrates for ACE, the IC_{50} values for [^{3}H]-HGG hydrolysis by ACE should be close approximations of the K_m values of these compounds with ACE [17, 30, 39].

IC_{50} Values were obtained for some compounds that were less than the presumed concentration of ACE in the reaction mixtures. These data indicate either that the enzyme is not pure or that some of the enzyme is no longer active. It is unlikely that the enzyme is not pure, since the specific activity of the ACE used in these studies, 29 μmoles HGG cleaved in 1 min at 37° per mg protein, is comparable to the 27 [40] and 30 [41] for pure porcine kidney ACE from two other laboratories. Our ACE preparation was stable for more than 15 months of storage at 2°, and no data were obtained that would suggest that ACE was being inactivated under our assay conditions. Since ACE is an amphiphilic protein it may form aggregates under our assay conditions that do not have their active sites exposed and this could account for the lower number of active sites than the molar enzyme concentration. Alternately, the enzyme may be inactivated during the course of purification. Sodium desoxycholate was used to solubilize the enzyme in this and previous reports [40, 41] on purification of ACE from porcine kidney and may be responsible for inactivation of the enzyme [42]. If this explanation is correct, solubilization of the porcine renal enzyme with Nonidet P-40 or some other nonionic detergent should result in an ACE preparation that has a higher specific activity than those previously reported. Consistent with this proposal is the report by Takada *et al.* [43] who solubilized ACE from porcine kidney by treatment with trypsin and subsequently purified it to yield a preparation that had a specific activity with hippurylhistidylleucine that was 3.6 times higher than that previously reported by Nagamatsu *et al.* [41] for pure porcine ACE.

Radioactive kinins have been used in experiments to study directly the interactions of BK with its receptors [5, 13]. One of the problems with this approach to the study of receptors is the possibility that the presence of non-receptor kinin binding sites could interfere with the characterization of the receptor-like binding sites. This was apparently the case in our initial attempts to study kinin receptors in porcine kidney [4, 5]. The results of this report unequivocally identify ACE as being capable of binding kinins and with a specificity that clearly distinguishes it from BK receptors. Our findings may have far reaching implications. For example, isolated rat uterus is used frequently to assess the biological activities of BK analogs [11, 12]. It is also reported to demonstrate very little kininase activity [44]. However, ACE is probably present in rat uterus. SQ20475 (pGlu-Lys-Trp-Ala-Pro-OH, BPP_{5a}), an ACE inhibitor, displaces the dose–response curve of angiotensin I on isolated rat uterus to the right, which suggests that the response to angiotensin I is due, at least in part, to its conversion to angiotensin II by ACE in the tissue [45]. Since BPP_{5a} does not potentiate the action of BK on rat uterus [44, 46], ACE might not be considered to limit the actions of kinins on this tissue. However, fourteen of the forty-eight BK analogs we tested were more potent than BK with respect to their ACE interactions. So, their actions on isolated rat uterus might be potentiated in the presence of BPP_{5a}, SQ20881 or some other ACE inhibitor. Conversely, the reported biological activities of these analogs on rat uterus may be less than their actual activities because of their interaction with ACE. Another factor that should be considered in this discussion is that, even if a biological assay preparation does not display kinin hydrolyzing activity, it may still contain non-receptor, kininase, binding sites that can complicate BK receptor studies. All the T1K binding by ACE described in this report occurred in the absence of kinin hydrolyzing activity.

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