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BRADYKININ RECEPTOR-LIKE BINDING STUDIED WITH IODINATED ANALOGUES*

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Abstract— The biological potencies of iodinated derivatives of Tyr[1] kallidin, Tyr[5] bradykinin, and Tyr[8] bradykinin were determined on isolated rat uteri and bovine uterine strips. Monoiodotyr[1] kallidin was the most potent of the derivatives (0.9 times as active as bradykinin on rat uterus). Monoiodotyr[8] bradykinin was 0.2 and iodotyr[5] bradykinin was less than 0.001 times as active as bradykinin. Using mono-[^{125}I]-Tyr[1] kallidin as a receptor probe, we demonstrated receptor-like binding in a subcellular fraction from bovine myometrium. Binding of bradykinin showed an apparent $K_{assoc.}$ of 10^{10} M^{-1}, and was highly specific. The optimum pH for saturable binding of [^{125}I]-Tyr[1] kallidin to the homogenized myometrium was 6.0 to 6.5. Several cations inhibited binding, with calcium the most effective (ID_{50} = 20 mM), and potassium the least effective (ID_{50} = 220 mM). The problems encountered in using radioactive bradykinins to search for and study receptors in bradykinin target tissues are discussed.

Potent hormones like bradykinin§ that are site-specific and act at very low concentrations are assumed to initiate or regulate events in target cells by interacting with specialized molecules called receptors. The interaction with receptors presumably includes a binding reaction or association. From what is known of its kinetics, bradykinin probably exerts its characteristic effects by interacting with a small number of receptors at each target cell.

If the above assumptions are true, then an experiment designed to find and study kinin receptors must meet several requirements. First, the chemical probe used to detect receptors, such as a radioactive analogue, must be "recognized" by the receptor. This implies that it must have biological activity as an agonist or specific antagonist. The probe must also be experimentally detectable in small quantities, such as the low concentrations of bradykinin that are biologically effective and the small quantities of responsive sites that are probably involved in its actions. Finally, the experiments must be designed to permit the probe to interact with the receptor, yet enable differentiation between that interaction and others such as transport or degradation.

Peptide hormones (e.g. angiotensin, oxytocin, glucagon, ACTH and insulin) labeled with ^{125}I have been used successfully to study their interactions with receptors [1]. The high specific activity of ^{125}I (2200 Ci/milliatom) and the efficiency with which its gamma radiation can be detected make it possible to measure picogram quantities of peptides labeled with this atom.

Bradykinin does not contain a readily iodinated amino acid. To prepare potential receptor probes that incorporate ^{125}I, we used the analogues Tyr[1] kallidin, Tyr[5] bradykinin, and Tyr[8] bradykinin. In this paper, we report the biological potencies of these analogues and their iodinated derivatives, and their use in direct binding studies of the kinin receptor.

MATERIALS AND METHODS

Materials

Bradykinin and methionyl–lysyl–bradykinin were purchased from Schwarz/Mann, Orangeburg, NY. Kallidin, i.e. lysyl-bradykinin, and (des–Arg[1], des–Arg[9])-bradykinin were the gift of Dr. E. D. Nicolaides, Parke, Davis & Co., Ann Arbor, MI. All other bradykinin analogues used in this investigation were prepared by the Merrifield solid phase method [2]. Protease inhibitors were purchased from the following companies: a nonapeptide from *Bothrops jararaca* venom, BPP_{9a}, that inhibits angiotensin I-converting enzyme (kininase II) was supplied as SQ 20881 by Squibb Pharmaceuticals, Inc., Princeton, NJ; the chymotrypsin inhibitor, L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK), from Sigma Chemical Co., St. Louis, MO; ε-aminocaproic acid (EACA), an inhibitor of kininase I, from CalBiochem, La Jolla, CA; sodium iodide-125 from New England Nuclear Corp., Boston, MA; and CM-Sephadex C-25 from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. All other reagents were of the highest quality available and were obtained from commercial sources.

Methods

Bovine uteri from pregnant cows were removed and placed on ice at the Oscar Mayer Co., Madison, WI, within 1 hr of the death of the animal. The endometrium was separated from the myometrium

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§ Bradykinin is H–Arg–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH. Kalidin is lysyl-bradykinin. Tyr[1] kallidin is tyrosyl-bradykinin.

and discarded. The myometrium was washed five times with an ice-cold, calcium-free buffered salt solution (115 mM NaCl, 4.6 mM KCl, 22 mM $NaHCO_3$, 10 mM NaH_2PO_4, 1.0 mM $MgSO_4 \cdot 7\ H_2O$, 5.5 mM glucose, and 13.4 mM Na_2 EDTA, pH 6.7), minced with scissors, and then weighed. Four hundred g (wet weight) of tissue were homogenized at top speed in a Waring blendor (model 5011) for 0.5 min. Four hundred ml of buffer were added to the homogenate, and the homogenization was continued for an additional 2 min. The crude homogenate was centrifuged for 10 min at 1000 *g* at 4°, and the supernatant fraction was stored overnight at −20°. The supernatant fraction was thawed and rehomogenized for 2 min. The homogenate was centrifuged for 20 min at 20,000 *g* at 4°, and the pellet was discarded. The supernatant fraction was centrifuged at 50,000 *g* for 1 hr at 4°. The resulting pellet was stored at −70° and was the subcellular fraction used in most of the binding experiments described in this paper. A freeze–thaw step at any point before the final homogenization clearly increased the yield of receptor-like binding sites from the myometrium.

Iodinations. Tyr^1 kallidin, Tyr^5 bradykinin, and Tyr^8 bradykinin were iodinated with ^{127}I and a trace of ^{125}I according to the method of Simpson and Vallee [3]. A freshly prepared solution (0.2 ml) of iodine (0.1 μg) in KI (0.5 M) was added to a glass-scintillation vial containing 3.0 ml of sodium veronal (0.02 M)–NaCl (2 M) buffer, pH 8.5, 5.0 mg of peptide, and 5×10^6 cpm of ^{125}I. The reaction mixture was magnetically stirred for 4 hr at 4°. The reaction was terminated by adding sufficient sodium thiosulfate (0.1 M) to decolorize the brown solution.

Peptides were isolated from reaction mixtures by saturating the mixture with NaCl, adjusting it to pH 1.5 with 2 N HCl, and then extracting it four times with 3-ml aliquots of *n*-butanol [4]. The butanol extracts were frozen and lyophilized. Then, they were taken up in 4.5 ml of water, and the pH was adjusted to 8.0 with concentrated ammonium hydroxide. The samples were applied to columns of CM-Sephadex C-25 and eluted with ammonium acetate buffer, 0.05 M, pH 8.0. The column dimensions were 1 × 40 cm with a bed volume of 25 ml. They were eluted at 10° at a flow rate of 20 ml/hr. Three-ml fractions were collected. Separate columns were prepared for each iodinated bradykinin analogue. Fractions that contained the iodinated derivatives were pooled, lyophilized, taken up in water, and stored at −20°. Amino acid composition was determined after hydrolysis for 72 hr at 105° in 6 N HCl containing 1 mg/ml of phenol and 1 mg/ml of 2-mercaptoethanol. Analyses were performed on a Spackman–Stein–Moore apparatus [5].

Carrier-free ^{125}I-labeled derivatives of Tyr^1 kallidin, Tyr^5 bradykinin, and Tyr^8 bradykinin were prepared, purified, and stored as described previously [6]. The monoiodo derivatives of the kinin analogues were used in the direct binding assays described in this paper.

An alternate method of iodination was to use solid-phase lactoperoxidase-glucose oxidase as supplied by Bio-Rad Laboratories, Richmond, CA [7]. A more rapid method of purification after iodination was passage over anion exchange resin [8]. These two modifications were used to iodinate Tyr^1 kallidin for the experiments measuring affinity by Scatchard analysis. Specific activities of the iodinated peptides were determined by the self-displacement method using antibody or the subcellular fraction from bovine myometrium [9].

Biological assays. Biological potencies of the ^{127}I-labeled derivatives were determined on the isolated estrus rat uterus [10] and on a bovine uterine strip preparation. Bovine myometrium from uteri that contained fetuses ranging in crown–rump length from 30 to 50 cm was dissected free from endometrium and washed three times with ice-cold de Jalon's solution (154 mM NaCl, 5.6 mM KCl, 5.9 mM $NaHCO_3$, 0.5 mM $CaCl_2$, and 2.8 mM glucose). It was refrigerated overnight in de Jalon's solution. The next day, strips of myometrium (about 0.5 × 2.0 cm) were cut.

Muscle was suspended in 10 ml of de Jalon's solution which was aerated with 95 per cent O_2, 5 per cent CO_2 at 25°. The bovine uterus strips or rat uterus horns were stretched under 1 g of tension, with frequent changes of solution, until spontaneous contraction ceased and no further stretching occurred (1–2 hr). Isometric contractions were measured for 2 min each, with a Grass strain gauge (model FT03), and the maximum response was recorded on a Beckman Dynograph (Type R). Five min elapsed between sample applications. Standard bradykinin doses were interspersed between unknowns.

Binding assays. Binding reactions were performed at 4° in polypropylene test tubes (Nalgene 3110, 3.5 ml capacity). To prevent adsorption of peptide, the tubes were coated for 1 hr at 25° with a freshly prepared, boiled solution of 0.1 per cent casein (Hammersten) in 0.01 M sodium phosphate buffer, pH 7.6, and then rinsed with water. The assay buffer in initial experiments was the buffered saline solution described above for preparation of uteri. It was supplemented with 14 μM TPCK and 6.3 μM SQ 20881. After the experiments with cations described below, the buffer was modified to contain only K_2HPO_4, 0.025 M, pH 6.3, and the two enzyme inhibitors.

An aliquot of buffer with or without unlabeled peptide, followed by a radioactive kinin (approximately 20 pg), and an aliquot of subcellular particles (0.5 to 1.0 mg protein) were added to each tube. The final volume was 0.7 ml. After 15 min of incubation at 4°, the tubes were centrifuged for 1 hr at 27,000 *g* at 4°. The supernatant fractions were aspirated and the tubes were rinsed gently with 3.5 ml of ice-cold incubation buffer to remove any radioactive polypeptide adhering to the test tube walls, taking care not to dislodge the pelleted subcellular particles. The radioactivity bound to the particles was counted in a well-type gamma counter.

When frozen particles were used in our binding assays, they were first thawed at room temperature, suspended with a Teflon-glass homogenizer, and dispensed into reaction tubes within 10 min. An alternate method of resuspending frozen particles was to homogenize them in a chilled Waring blendor, and then allow the suspension to stand at 4° for 30 min. This method resulted in greater binding than simple homogenization and immediate incubation.

Tubes were prepared in triplicate. In all binding experiments, one set of tubes was included to control for non-saturable binding. This set contained the radioactive polypeptide, subcellular particles, and an excess (5 μg) of native bradykinin. The amount of radioactivity bound under these conditions was subtracted from the amounts of radioactivity bound when lesser amounts of unlabeled peptide were present. The difference represented "saturable binding".

Protein was measured by the method of Lowry *et al.* [11]. The method of Scatchard [12] was used for kinetic analysis of binding data.

RESULTS

Purification and characterization of iodobradykinins.

Figure 1 shows the elution profile obtained when the products of an iodination reaction mixture that contained Tyr^8 bradykinin, ^{127}I and a trace of ^{125}I were passed over a CM-Sephadex C-25 column. A similar pattern was observed with iodination reactions involving the other analogues. Four peaks of coincident radioactivity and optical density were observed, along with a fifth peak of optical density that did not have an associated peak of radioactivity.

Because cation-exchange columns retain basic compounds, and the dissociation of the phenolic proton of tyrosine is favored by the addition of iodine in the ortho positions, we believe that the first peak of radioactivity was free iodide, the second small peak was an unknown compound, the third peak was diodinated peptide, and the final radioactive peak was the monoiodinated derivative. Since conditions were chosen to favor complete conversion of analogues to iodinated derivatives, only a small peak of optical density corresponding to unreacted peptide was obtained. The identity of the peaks was studied in experiments described below, and the results supported the proposed assignments.

Peaks I and II were not active when tested on the isolated rat uterus preparation. Material in these peaks did not inhibit the binding of ^{125}I-labeled bradykinin to bradykinin antibody, and no amino acids could be detected. Finally, when a mock iodination was performed, omitting the bradykinin analogue, peaks I and II were obtained but none of the other peaks were detected.

Peaks III and IV were biologically active when tested on rat uterus and bovine uterine strip preparations. Peak III was less potent than IV. Material in these peaks inhibited the binding of ^{125}I-labeled bradykinin to bradykinin antibody, with peak III less potent than peak IV. Amino acid analyses of peaks III and IV revealed that each peak contained the same amino acid composition as the initial peptides.

Iodotyrosine is converted to tyrosine by the acid hydrolysis we employed, so amino acid analyses could not characterize the iodinated peptides unambiguously. To test the extent of iodination, ^{125}I-labeled derivatives of Tyr^1 kallidin and Tyr^8 bradykinin that eluted in the region corresponding to peak IV in Fig. 1 were hydrolyzed by purified aminopeptidase or carboxypeptidase respectively. The radioactivity that was isolated co-chromatographed with monoiodotyrosine in a descending paper chromatography system capable of separating monoiodotyrosine, diodotyrosine and free iodide (Whatman 3 MM paper, 0.2 M acetic acid adjusted to pH 5.0

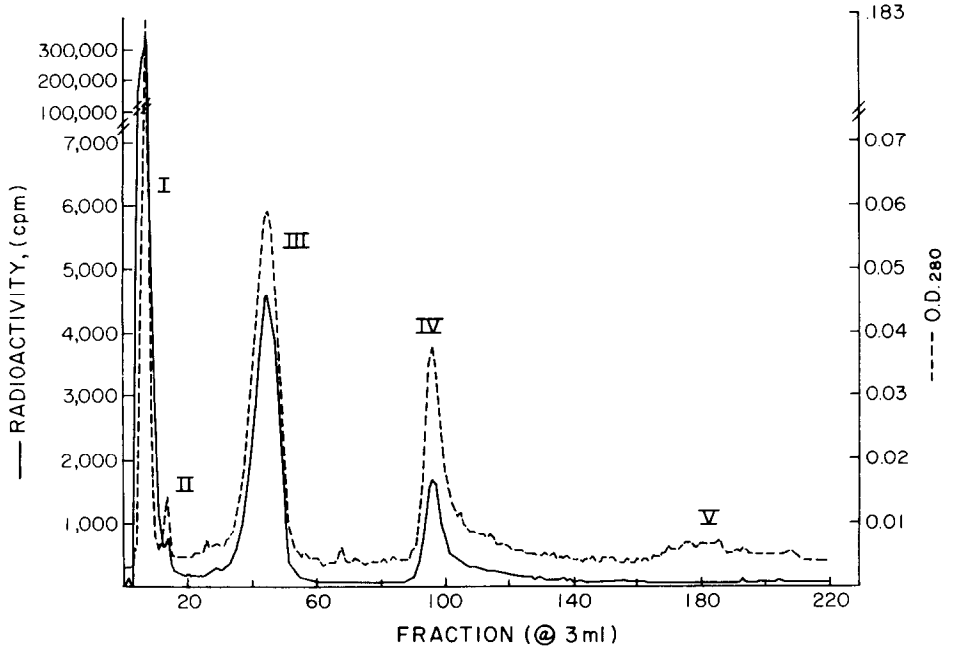

Fig. 1. Column chromatographic purification of ^{127}I-labeled Tyr^8 bradykinin. The column contained CM-Sephadex C-25 cation exchange resin and was 1 cm in diameter and 40 cm long. It was eluted at 20 ml/hr at 10° with ammonium acetate buffer, 0.05 M, pH 8.0. Three-ml fractions were collected. The solid line represents the radioactivity from a trace amount of ^{125}I added to the original iodination mixture.

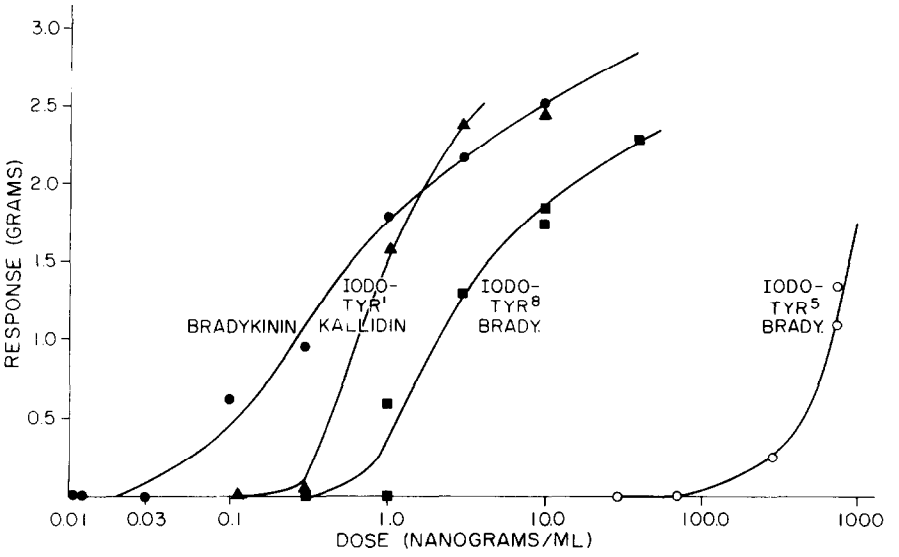

Fig. 2. Dose–response curves of bovine uterine strips contracting in the presence of bradykinin and non-radioactive monoiodo-bradykinins. Two strips of bovine uterine myometrium, 0.5 × 2.0 cm, from pregnant cows were suspended in de Jalon's solution aerated with 95 per cent O_2–5 per cent CO_2. The contractile responses to bradykinin (●), iodotyr[1] kallidin (▲) iodotyr[8] bradykinin (■) and iodotyr[5] bradykinin (○) were measured. The data plotted in this figure were obtained in one experiment with two uterine strips. The symbols represent the individual data points.

with pyridine). The specific activities of the proposed mono-^{125}I-labeled derivatives of Tyr[1] kallidin, Tyr[5] bradykinin, and Tyr[8] bradykinin ranged between 1.2 and 2.2 Ci/μmole. The theoretical maximum specific activity obtainable by incorporating one atom of carrier-free iodine per molecule of peptide is 2.2 Ci/μmole.

Biological potencies of iodobradykinins

The relative biological potencies of the synthetic polypeptides were determined by comparing the steep portions of each dose–response curve to that for bradykinin. Figure 2 depicts the results of an experiment with three iodinated bradykinin analogues and bradykinin itself in two bovine uterine strips. The mean relative potencies found in two experiments on a total of four strips appear in Table 1. Table 1 also lists the results of experiments on isolated rat uteri in which a total of nine bradykinin analogues were tested. Tyr[1] kallidin, Tyr[8] bradykinin, and the monoiodinated derivatives of these two analogues were far more potent than Tyr[5] bradykinin and its iodinated derivatives. Diiodinated derivatives of all analogues were less potent than the corresponding monoiodinated forms. This difference was most apparent with the Tyr[8] bradykinin analogue, and least apparent with Tyr[1] kallidin. The results also show that rat and bovine uteri reacted similarly to bradykinin and the other analogues we tested.

Binding reactions with bovine uterus

Subcellular particles prepared from bovine uterine myometrium from pregnant cows were exposed to each of the three bradykinin analogues that con-

Table 1. Relative biological potencies of iodinated bradykinins*

Polypeptide	Mean relative biological potency ± S.E.M. Rat uterus	N	Bovine uterine strip	N
Bradykinin	1.00		1.00	
Tyrosine[1] kallidin	1.02 ± 0.10	6		
Monoiodotyrosine[1] kallidin	0.88 ± 0.13	6	0.69 ± 0.08	4
Diiodotyrosine[1] kallidin	0.62 ± 0.07	6		
Tyrosine[5] bradykinin	0.006 ± 0.002	7		
Monoiodotyrosine[5] bradykinin	0.0008 ± 0.00006	4	0.0009 ± 0.0003	4
Diiodotyrosine[5] bradykinin	0.00003 ± 0.0000002	2		
Tyrosine[8] bradykinin	0.24 ± 0.04	5		
Monoiodotyrosine[8] bradykinin	0.20 ± 0.02	6	0.15 ± 0.03	4
Diiodotyrosine[8] bradykinin	0.0008 ± 0.0001	6		

* The responses of two different uterine preparations to the administration of bradykinin and its iodinated derivatives were measured. N refers to the number of uterine horns or uterine strips upon which the polypeptide was assayed.

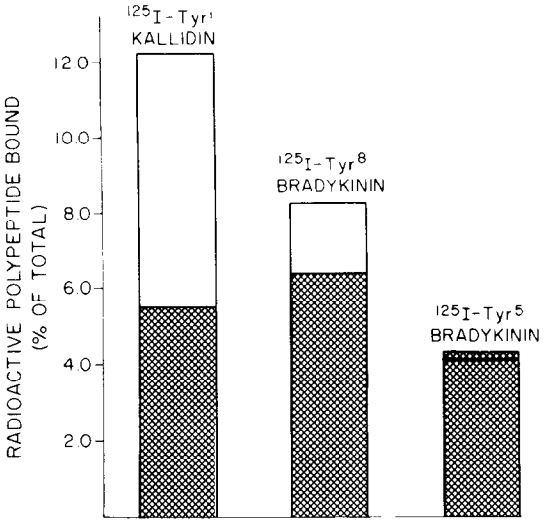

Fig. 3. Binding of three radioactive iodobradykinins to subcellular particles from bovine uterine myometrium. Twenty pg (20,000 cpm) of radioactive monoiodotyr^{1} kallidin, monoiodotyr^{5} bradykinin, and monoiodotyr^{8} bradykinin were incubated with subcellular particles from uterine myometrium from pregnant cows. See Methods for details of the binding assay. The amounts of radioactive polypeptides bound to the particles in the absence of native bradykinin (total bar) and with 5.0 μg of native bradykinin in the incubation media (hatched area) were expressed as percentages of the total radioactivity added to each of the incubation mixtures.

tained radioactive monoiodotyrosine. In all experiments, non-saturable binding was determined by binding reactions to which unlabeled bradykinin itself was added in a relatively large amount (5 μg). The results are shown in Fig. 3. In the absence of native bradykinin, particles bound more of the [^{125}I]-Tyr^{1} kallidin than either of the other iodinated peptides followed by [^{125}I]-Tyr^{8} bradykinin and then [^{125}I]-Tyr^{5} bradykinin. Non-saturable binding, measured in the presence of excess native bradykinin, was roughly the same for the three radioactive analogues. Saturable binding of [^{125}I]-Tyr^{1} kallidin was greater than that of [^{125}I]-Tyr^{8} bradykinin, while [^{125}I]-Tyr^{5} bradykinin showed no saturable binding at all.

Monoiodo derivatives of bradykinin analogues that contained non-radioactive iodine were tested for their ability to inhibit bindiing of [^{125}I]-Tyr^{1} kallidin to bovine uterus particles. The results are shown in Fig. 4. Similar experiments were done with many other bradykinin analogues. The analogues and their relative binding inhibitory activities, expressed as ID_{50} concentrations, are summarized in Table 2. This table also shows the relative biological potencies of the analogues. These data were obtained either in our laboratory using rat uterus and bovine uterine strip preparations or by other investigators using rat uterus or guinea pig bronchus preparations.

The comparison between biological activity and binding inhibition potency in our study is depicted graphically in Fig. 5. The line in Fig. 5 is the line on which analogues would lie if they were equally active

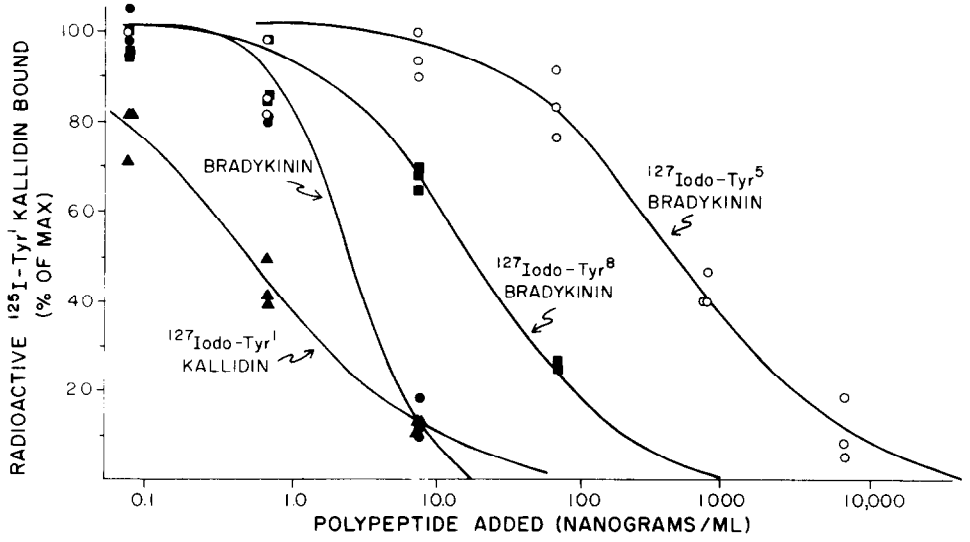

Fig. 4. Inhibition of the binding of radioactive monoiodotyr^{1} kallidin to bovine uterine subcellular particles. Serial dilutions of bradykinin (●), mono[^{127}I]Tyr^{1} kallidin (▲), mono[^{127}I]Tyr^{8} bradykinin (■), and mono[^{127}I]Tyr^{5} bradykinin (○) were incubated with radioactive monoiodotyr^{1} kallidin and subcellular particles from bovine uterine myometrium, as described in the text. The amounts of radioactivity bound to the particles in the presence of competing non-radioactive peptides are expressed as percentages of the maximum radioactivity bound to the particles. All data were corrected for the amount of radioactivity bound in the presence of 5 μg of unlabeled bradykinin. The symbols represent the individual data points. The curves were computer-fitted and represent the results of weighted analyses of the data.

Table 2. Relative biological and binding activities of bradykinin analogues

Polypeptide	Biological activity (smooth muscle)	Binding activity (bovine uterine particles)	Code*	Reference for biological activity data
5,8-Dithienylalanine bradykinin	8	0.37	TH5,8	13
8,Thienylalanine bradykinin	4	0.70	TH8	13
5-Thienylalanine bradykinin	2	1.07	TH5	13
2-Hydroxyproline bradykinin	2	1.07	H2	14
8-Parafluorophenylalanine bradykinin	1.4†	1.53	F8	15
Bradykinin	1	1	B	
1-Monoiodotyrosine kallidin	0.69	5.10	TIM	‡
Kallidin	0.5	1.61	K	15
Acetyl-bradykinin	0.5	0.06	A1	15
Methionyl-lysyl-bradykinin	0.25	0.26	MLB	15
8-Monoiodotyrosine bradykinin	0.15	0.14	T8M	‡
6-Threonine-5,8-di(*O*-methyl-tyrosine)bradykinin	0.0333	0.0066	T5,6,8	§
2-D-Proline-8-(*O*-methyl-tyrosine)bradykinin	0.02	0.029	D2,8	§
7-D-Proline bradykinin	0.0133	0.0089	D7	15
9-Desarginine bradykinin	0.01	0.00047	−9	13
1-Lysine bradykinin	0.002	0.001	L1	15
2-D-Proline bradykinin	0.002	0.0026	D2	15
1-Ornithine bradykinin	0.001†	0.00085	O1	15
6-Phenylalanine bradykinin	0.001	0.0028	P6	15
3-D-Proline-8-(*O*-methyl-tyrosine)bradykinin	0.001	0.008	D3,8	§
5-Monoiodotyrosine bradykinin	0.0009	0.005	T5M	‡
7-D-Proline-8-(*O*-methyl-tyrosine)bradykinin	0.00033	0.0008	D7,8	§
3-D-Proline bradykinin	0.0001	0.003	D3	15

* The code is the abbreviation for each analogue used in Fig. 5.
† Biological activity was determined in guinea pig bronchus.
‡ Biological activity was measured in our laboratory in the bovine uterine strip.
§ Biological activity was measured in our laboratory in isolated rat uterus.

in the biological and binding assays. With few exceptions, rank orders in the two assays matched.

Several compounds with biological activity in smooth muscle but structurally unrelated to bradykinin were tested in the uterine particle binding assay. Angiotensin I, angiotensin II, oxytocin, and serotonin at concentrations of 7 μg/ml did not inhibit binding of [^{125}I]-Tyr^1 kallidin to the particles. Biologically inactive elision analogues of bradykinin were also inactive in the binding assay. These included peptides with the bradykinin sequence lacking the following amino acids: 1 and 2; 1, 2, and 3; 1, 2, 3, and 4; 1 and 9; 1, 2, and 9; and 8 and 9.

Experiments were performed to determine optimum conditions for receptor-like binding in bovine uterus particles. We tested effects of pH and ionic composition on the saturable and non-saturable binding of the biologically active analogue, [^{125}I]-Tyr^1 kallidin, and compared these results to those obtained with the biologically inactive analogue [^{125}I]-Tyr^5 bradykinin. We sought reaction conditions that would maximize saturable binding of [^{125}I]-Tyr^1 kallidin while minimizing its non-saturable binding and the total binding of [^{125}I]-Tyr^5 bradykinin.

The optimum pH for the saturable binding of [^{125}I]-Tyr^1 kallidin was 6.0 to 6.5 (Fig. 6). The optimum pH for non-saturable binding was lower. The pH did not affect the binding of [^{125}I]-Tyr^5 bradykinin until pH 7.0 and then binding decreased with increasing pH.

Cations inhibited binding with varying effectiveness. These results are summarized in Fig. 7. Calcium, with an ID_{50} of 20 mM, was most effective in inhibiting [^{125}I]-Tyr^1 kallidin binding. Potassium was least effective, with an ID_{50} of 220 mM. Sodium inhibited [^{125}I]-Tyr^5 bradykinin binding more than it inhibited [^{125}I]-Tyr^1 kallidin binding. The inhibitory effects of ions could not be duplicated by adding sucrose or mannitol at concentrations as high as 250 mM.

Varying the length of incubation between 5 and 30 min at 4° caused no significant change in the amount of [^{125}I]-Tyr^1 kallidin saturably bound by bovine uterus particles. For our convenience, we chose an incubation time of 15 min.

In forty-seven separate experiments, bovine uterine myometrial particles, sedimenting at 27,000 *g* for 1 hr, bound 11.5 ± 1.7 per cent of the radioactive iodotyr^1 kallidin added to assay tubes. Of the radioactivity bound, 62.3 ± 1.8 per cent was displaced by unlabeled bradykinin.

Binding reactions with other tissues

Employing optimal buffer and incubation conditions for receptor-like binding in bovine uterine myometrium particles, we surveyed other bovine tissues. We sought binding sites with two characteristics: a preference for [^{125}I]-Tyr^1 kallidin

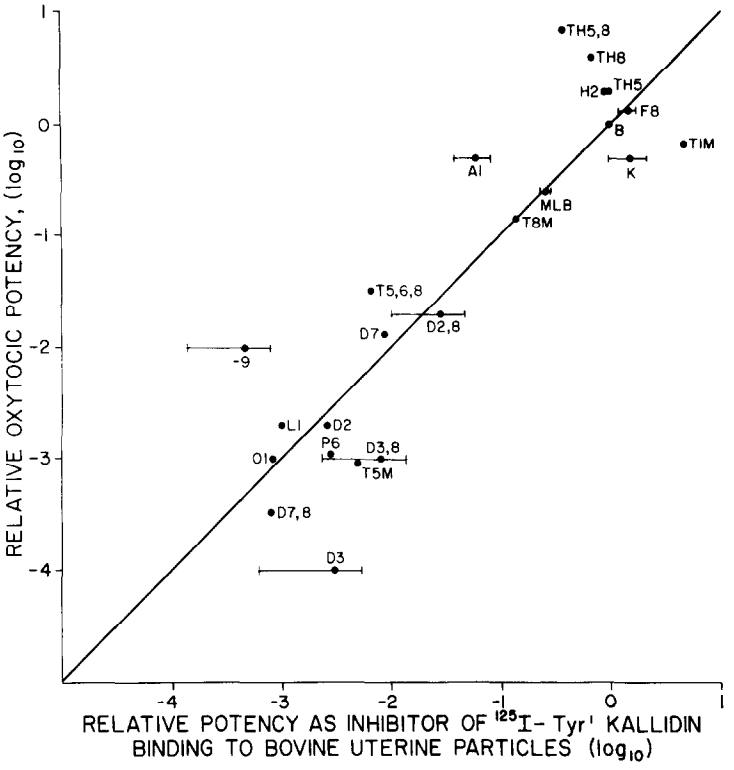

Fig.5. Comparison of the oxytocic potencies of bradykinin analogues with their potencies as inhibitors of [^{125}I]Tyr^1 kallidin binding to subcellular particles from bovine uterine myometrium. Logarithmic transformations of the data from Table 2 for twenty-three bradykinin analogues were used to construct this figure. The individual analogues are designated according to the code in Table 2. The relative oxytocic potency of each analogue (ordinate) is plotted against the relative potency of the analogue as an inhibitor of [^{125}I]Tyr^1 kallidin binding to bovine uterine myometrium particles (abscissa). The line drawn through the origin represents the "line of identity". An analogue having the same oxytocic and binding inhibitory potency would be on this line. The bracketed symbols represent the means of two values and the brackets indicate the range of the values.

Table 3. Saturable binding of [^{125}I]Tyr^1 kallidin by homogenates of bovine tissues*

Tissue	No. of preparations tested	Saturable binding of [^{125}I]Tyr^1 kallidin (% of total radioactivity added)	Saturable binding as a proportion of total binding (%)
Myometrium	47	11.55 ± 1.7	62.3 ± 1.8
Esophagus (mucosa)	6	4.28 ± 1.5	45.5 ± 5.7
Ductus arteriosus (fetal)	3	3.20 ± 1.5	52.7 ± 13.1
Heart (left ventricle)	3	2.23 ± 1.0	11.7 ± 5.8
Lung (parenchyma)	4	1.27 ± 0.4	11.5 ± 3.5
Bronchi	3	1.02 ± 0.2	10.0 ± 2.5

* Organs were homogenized in a Waring blendor, filtered through cheesecloth, and divided into two particulate fractions by centrifugation at 1,000 *g* and 40,000 *g*. Aliquots containing approximately 1 mg protein were exposed to approximately 100 pg of labeled peptide in the absence or presence of unlabeled bradykinin (5 μg). The radioactivity bound in the absence of bradykinin was called "total binding", and the difference caused by unlabeled bradykinin was called "saturable". Details of the incubation procedure are described in the text. Data are expressed as mean ± S.E.M. Organs without saturable binding included adrenal cortex, spleen, thymus and testis.

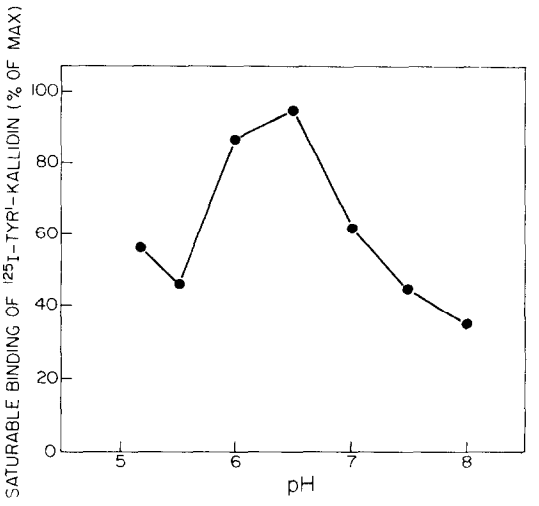

Fig. 6. Effect of pH on binding of [^{125}I]Tyr^1 kallidin to subcellular particles from bovine uterine myometrium. All points have been corrected for non-saturable binding.

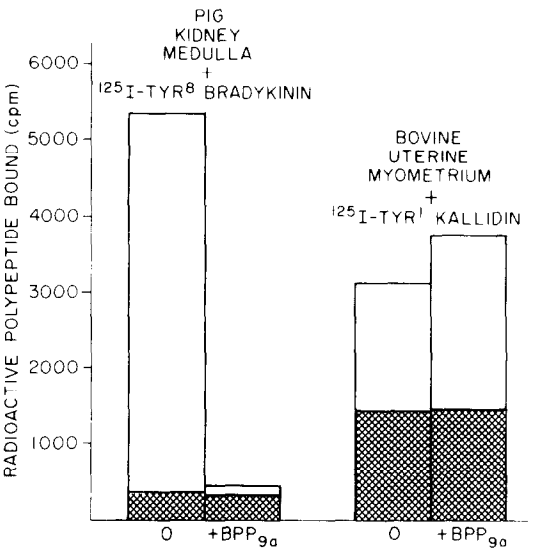

Fig. 8. Effect of BPP$_{9a}$ (SQ 20881), a potent kininase II inhibitor, on the binding of [^{125}I]Tyr^8 bradykinin to subcellular particles from pig kidney medulla and the binding of [^{125}I]Tyr^1 kallidin to subcellular particles from bovine uterine myometrium. The radioactive polypeptides were incubated with subcellular particles either with (hatched area) or without (total bar) 5 μg/ml of native bradykinin, and with or without 5 μg/ml of BPP$_{9a}$.

over [^{125}I]-Tyr^5 bradykinin, and saturability by native bradykinin.

Tissues were homogenized in 4 vol. of 0.02 M potassium phosphate buffered saline, pH 6.3. Crude homogenates were centrifuged, and particles sedimenting between 1,000 and 40,000 $g \times 20$ min were incubated with radioactive and native bradykinin in the same way as with bovine uterine particles. The incubation buffer contained 20 mM potassium phosphate buffer (pH 6.3), 1 mM EDTA, 14 μM TPCK, and 6.3 μM SQ 20881. Incubations were for 15 min at 4°. The results are shown in Table 3. The most consistent sources of saturable, specific binding sites for the biologically active kinin analogue, [^{125}I]-Tyr^1 kallidin, were the pregnant uterus and the mucosal layer of the esophagus.

Effects of kininase II inhibitor

Figure 8 shows that SQ 20881 increased the saturable binding of [^{125}I]-Tyr^1 kallidin to a subcellular fraction from bovine myometrium. By contrast, this enzyme inhibitor decreased the saturable binding of [^{125}I]-Tyr^8 bradykinin to a subcellular fraction of porcine renal medulla [16].

Kinetic analysis

Experiments were performed to measure the

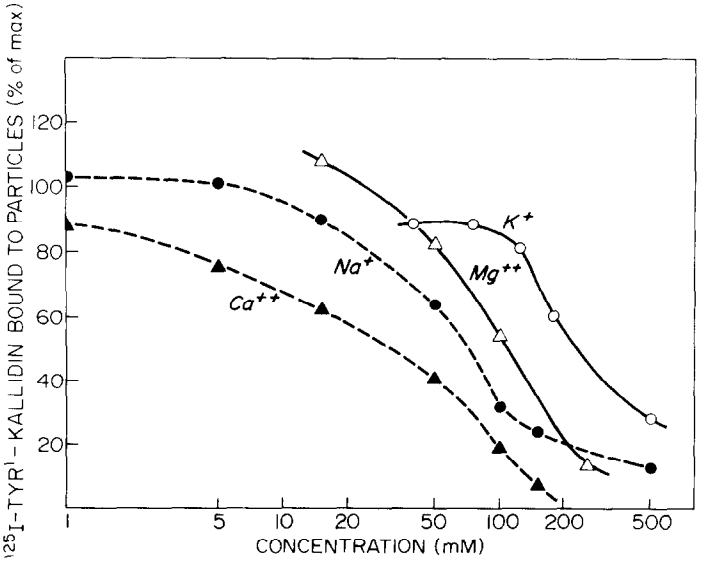

Fig. 7. Effects of cations on binding of [^{125}I]Tyr^1 kallidin to subcellular particles from bovine uterine myometrium. All points have been corrected for non-saturable binding.

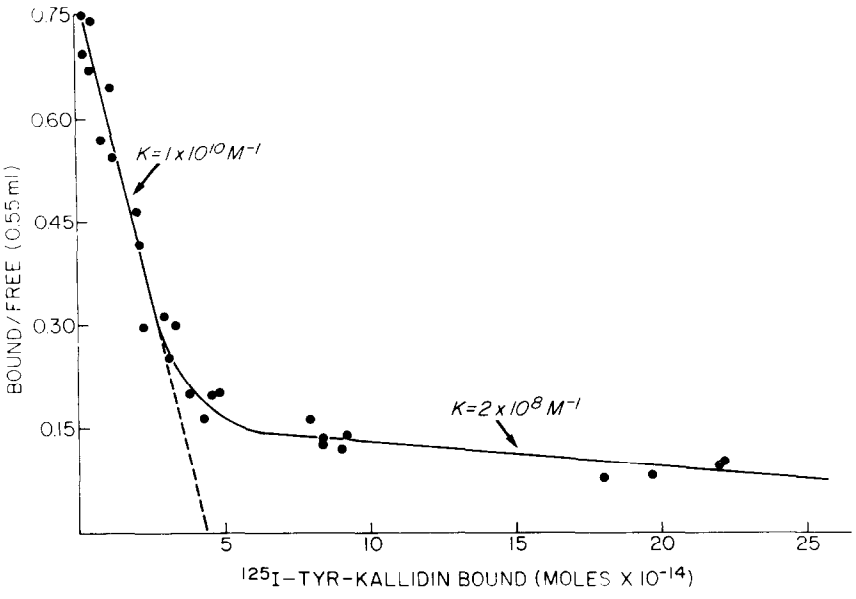

Fig. 9. Scatchard plot of binding of [^{125}I]Tyr^1 kallidin to subcellular particles from bovine myometrium. Serial dilutions of labeled peptide were added to aliquots of particles, and incubated for 15 min in a final volume of 0.55 ml. Each aliquot of particles contained 0.27 mg protein. The incubation was terminated by centrifugation, and the bound and the free radioactivity were counted. The specific activity of the labeled peptide was determined by comparing the effects of labeled and unlabeled peptide on the bound/free ratio [9]. Calculations assume that the iodinated tyrosine analogue and the native peptide had identical effects on the bound/free ratio. The data presented are the composite of four separate experiments and each point is the mean of six assay tubes. Graphic analysis is according to the method suggested by Scatchard [12].

affinity of binding sites for kinins in bovine myometrium. Optimum conditions were employed as defined above, with low ionic strength and enzyme inhibitors in the buffer. The final volume was 0.55 ml. Pooled results of four experiments are displayed in Fig. 9. Two types of binding are suggested, one with an apparent association constant of $10^{10}M^{-1}$, and one with an affinity 200 times less avid. The number of the more avid sites in our preparation of bovine myometrium was 1.6×10^{-13} moles/mg of protein.

DISCUSSION

These experiments show that a molecule containing the amino acid sequence of bradykinin with tyrosine added to its amino terminus was as potent as bradykinin on the isolated rat uterus. Furthermore, addition of a single iodine atom to this tyrosylated peptide did not impair its activity significantly. When it contained ^{125}I, this analogue was found to bind avidly, saturably and specifically to targets of bradykinin, notably myometrium. We believe that [^{125}I]-Tyr^1 kallidin is a useful probe of kinin receptors. By contrast, iodinated derivatives of Tyr^5 bradykinin were essentially inert in bioassays, and did not bind saturably to bovine myometrium. [^{125}I]-Tyr^8 bradykinin was intermediate in biological activity and saturable binding.

The presence of potent kininases is likely to complicate studies of bradykinin receptors, and undoubtedly confused the interpretation of our own early work in which we employed the ^{125}I-labeled derivative of Tyr^8 bradykinin [16,17]. We found high affinity binding sites in porcine renal medulla, presumably the site of kinin-stimulated prostaglandin production [18]. However, the specificity of binding differed from that expected of receptors. For example, most of the saturable binding of [^{125}I]-Tyr^8 bradykinin to pig kidney medulla particles was inhibited by SQ 20881, a competitive inhibitor of kininase II (Fig. 8). This result suggested that most of the binding we observed was to a kininase. Chiu *et al.* [19] then reported that [^{125}I]-Tyr^8 bradykinin was a good substrate of kininase II in cultures of pulmonary endothelial cells. The evidence supports the conclusion that binding of [^{125}I]-Tyr^8 bradykinin to crude homogenates of porcine medulla in large part reflected binding to degradative enzymes (presumably, binding of [^{125}I]-Tyr^5 bradykinin would exclusively reflect sites different from receptors).

The results of our initial experiments with enzyme inhibitors suggested that some reagents, such as chelating agents, can allow binding of bradykinin to enzymes even though they may inhibit hydrolysis. We then tested more specific inhibitors of three bradykininases: SQ 20881, a competitive inhibitor of kininase II; epsilon-aminocaproic acid, an inhibitor of kininase I; and TPCK, an irreversible inhibitor of chymotrypsin-like enzymes.

In contrast to its effects on binding of Tyr^8 bradykinin to pig kidney, SQ 20881 increased binding of Tyr^1 kallidin to bovine myometrium (Fig. 8). This suggested that the inhibitor protected the peptide

without inhibiting its binding to receptors in that tissue. a competitive inhibitor of kininase II was added in all subsequent experiments. Epsilon-aminocaproic acid increased non-saturable binding without affecting saturable binding. It was not used thereafter. TPCK had no effect on binding of Tyr^1 kallidin to one preparation of bovine myometrium, but it was included in subsequent experiments to inhibit chymotrypsin-like enzymes that might occur in other specimens.

Two other enzymes that might confuse studies of bradykinin receptors are kinin-converting aminopeptidase, the enzyme that cleaves kallidin to bradykinin, and collagen proline hydroxylase, an enzyme that can hydroxylate position 3 of bradykinin. Since purified kinin-converting aminopeptidase does not possess bradykinin-hydrolyzing activity [20], and since bradykinin is not known to inhibit or enhance enzyme activity, bradykinin should not be bound by this aminopeptidase. Although collagen proline hydroxylase has greater affinity for Tyr^1 kallidin than for bradykinin (2×10^3 M^{-1} vs 1×10^3 M^{-1}) [21], these affinities are seven orders of magnitude less than those displayed by the uterine particles. Thus, the binder we studied probably is not collagen proline hydroxylase.

Specificity of the putative receptors was tested by measuring inhibition of binding of [^{125}I]-Tyr^1 kallidin to bovine myometrium. Table 2 and Fig. 5 show that there was a direct correlation between the potencies of most analogues as inhibitors of binding and as stimulators of smooth muscle contraction.

A comparison of the activities of substances in binding assays and bioassays can be a useful screen for potential receptor antagonists [22]. Antagonists would be expected to have activity in a binding assay, but little or no stimulatory activity in bioassays. We found that 3-D-proline bradykinin had this combination of properties. However, Stewart [23] did not observe bradykinin antagonism by this peptide. 3-D-Proline bradykinin may be a partial agonist whose antagonistic activity is obscured by its agonistic effects.

9-Desarginine bradykinin was less active as a binding inhibitor in bovine myometrium than as an agonist in rat uterus. There are several possible explanations for this finding: (1) the desarginine analogue may have easier access to the receptor as it exists in the intact organ than would be predicted from its binding to broken cells; (2) the desarginine analogue may be less readily degraded by intact tissue than the parent peptide, giving it increased relative potency; and (3) the receptors in rat uterus may be different from the receptors in bovine myometrium. In reference to the third possibility, Regoli *et al.* [24] reported that 9-desarginine bradykinin was six times more potent than bradykinin in the isolated rabbit aorta, in contrast to its weak activity in rat uterus.

Our finding of a bradykinin binding site in bovine uterus homogenates with an apparent $K_{assoc.}$ of 10^{10} M^{-1} is consistent with data obtained by Reissmann *et al.* [25]. They used tritium-labeled acetyl bradykinin analogues to study binding to plasma membranes purified from rat uterus and reported that [^{3}H] acetyl-(8-erythro-amino-β-phenylbutyric acid)-bradykinin was bound to these membranes at a site with an apparent $K_{assoc.}$ of 3.6×10^9 M^{-1}. The comparable value obtained for [3H]acetyl-(1,9-di-norarginine)-bradykinin was 2.2×10^{10} M^{-1}. Lower affinity sites with apparent $K_{assoc.}$ of 10^8 M^{-1} were also found. Specificity of the binding was not described.

The fact that saturable [^{125}I]-Tyr^1 kallidin binding was optimal at a slightly acid pH is interesting in view of the acid pH optimum of enzymes that form kinins and of inflamed areas where they are purported to act.

Our experiments demonstrate the existence of specific, saturable binding sites for bradykinin and related polypeptides. The location of the binding sites in known target cells, their specificity for active polypeptides, and their high affinity were consonant with properties expected of receptors. However, we did not prove that these binding sites include receptors that transduce binding of certain concentrations of kinins into visible responses. Such proof awaits discovery of specific binding inhibitors that also inhibit kinin responses, elucidation of the intimate mechanism of hormone action and association of the mechanism with the binding site, or isolation of binding sites that can confer responsiveness on unresponsive systems.

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