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# DEVELOPMENT OF A RADIOIMMUNOASSAY FOR [DES-ARG$^9$]-BRADYKININ

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**Abstract**—Antisera to [des-Arg$^9$]-bradykinin were elicited in rabbits immunized with the peptide conjugated to thyroglobulin and/or ovalbumin. Sera were screened for the presence of antibody with three radioactive antigens, mono-$^{125}$I-labeled derivatives of [Tyr$^1$,des-Arg$^{10}$]-kallidin, [Tyr$^5$,des-Arg$^9$]-bradykinin, and [Tyr$^8$, des-Arg$^9$]-bradykinin that were prepared by treating mono-$^{125}$I-labeled [Tyr$^1$]-kallidin, [Tyr$^5$]-bradykinin, and [Tyr$^8$]-bradykinin with carboxypeptidase B. Of the six animals immunized, five produced antibodies to [des-Arg$^9$]-bradykinin as evidenced by the ability of their sera to bind at least 33% of the added radioactivity at a final dilution of 1:500. Sensitivity and specificity studies were performed with each labeled antigen and a dilution of antiserum that bound 30–50% of the radioactivity. The best labeled antigen–antibody combination, with respect to titer, sensitivity, and specificity was obtained with [mono-$^{125}$I-Tyr$^8$,des Arg$^9$]-bradykinin and serum from a rabbit immunized with [des-Arg$^9$]-bradykinin conjugated to ovalbumin with toluene diisocyanate. The lowest concentration of [des-Arg$^9$]-bradykinin inhibiting 50% of this radioactive antigen binding was 0.23 ng/ml and the lowest concentration which could be distinguished from no [des-Arg$^9$]-bradykinin added was 67 pg/ml. This antiserum cross-reacts with bradykinin and lysyl-bradykinin about 9% but not with methionyl-lysyl-bradykinin.

[des-Arg$^9$]-Bradykinin‖, bradykinin that has had its C-terminal arginine removed, has been thought of as a biologically inactive fragment of bradykinin because of its relative lack of activity when assayed on the isolated rat uterus, a conventional biological assay system for kinins [1]. However, it has been found that BK(1–8) is at least six times more potent than bradykinin in contracting the isolated rabbit aorta, an unconventional preparation for assaying kinins, and that specific inhibitors could be synthesized that block the action of kinins on this tissue without blocking the action of kinins on isolated rat uterus. These data strongly suggest that there are at least two types of bradykinin receptors [2]. In contrast to bradykinin, which is readily inactivated in the lung, BK(1–8) is resistant to pulmonary inactivation [1, 3] and, therefore, could be a circulating active component of the kallikrein–kinin system. Blood contains the enzymes (kallikreins) and substrates (kininogens) necessary for bradykinin formation. It also contains carboxypeptidase N, which can cleave arginine from bradykinin to yield BK(1–8) [4].

In this paper we report the development of a radioimmunoassay for BK(1–8). The application of this assay could eventually lead to a better understanding of the role played by BK(1–8) in physiological and pathological conditions.

## MATERIALS AND METHODS

*Materials.* [Tyr$^1$]-kallidin, [Tyr$^5$]-bradykinin, and [Tyr$^8$]-bradykinin were purchased from Peninsula Laboratories, Inc., San Carlos, CA; bradykinin from Boehringer Mannheim Biochemicals, Indianapolis, IN; and kallidin and methionyl-lysyl-bradykinin from Vega Biochemicals, Tuscon, AZ. All other bradykinin analogues used in this investigation were prepared by the Merrifield solid phase method [5]. Crystalline bovine serum albumin, chicken egg ovalbumin, porcine thyroglobulin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl and diisopropyl fluorophosphate (DFP)-treated porcine pancreatic carboxypeptidase B were purchased from the Sigma Chemical Co., St. Louis, MO; tolyene-2,4-diisocyanate (also called toluene diisocyanate) from the Eastman Kodak Co., Rochester, NY; 1,10-phenanthroline monohydrochloride monohydrate from the Aldrich Chemical Co., Inc., Milwaukee, WI; complete Freund's adjuvant from Difco Laboratories, Detroit, MI; casein-Hammersten from the United States Biochemical Corp., Cleveland, OH; dextran T70 and CM-Sephadex C-25 from Pharmacia Fine Chemicals, Piscataway, NJ; Norit A charcoal from Pfanstiehl Laboratories, Inc., Waukegan, IL; sodium iodide-125 from the New England Nuclear Corp., Boston, MA; and cellulose acetate test tubes, 9/16 inch by 4 inches, from the Petro Packaging Co., Inc., Cranford, NJ. All other reagents were of the highest quality available and were obtained from commercial sources.

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‖ Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe, or BK(1–8).

*Methods.* BK(1–8) was coupled to ovalbumin and thyroglobulin with carbodiimide and glutaraldehyde as previously described [6] and to ovalbumin with toluene diisocyanate as reported by Talamo *et al.* [7]. Two rabbits, a male New Zealand black (-9CEO1) and a female New Zealand red (-9CEO2), were immunized with the conjugate prepared with toluene diisocyanate. Four rabbits, two female New Zealand white (-9CEO3 and -9CEO5), a male New Zealand black (-9CEO4), and a female New Zealand red (-9CE06), were immunized with the conjugates prepared with carbodiimide and glutaraldehyde. On immunization days each rabbit was injected with between 0.5 and 0.75 mg of the BK(1–8)-protein conjugate suspended in 1.25 ml of a mixture of equal amounts of saline and complete Freund's adjuvant. Primary immunizations were into the hind toe pads. Subsequent immunizations were alternated between multiple subcutaneous injections along the backs of the animals and intraperitoneal injections. Immunizations were at monthly intervals. Sera were heated at 60° for 30 min before analysis to minimize any kininase acitivity.

[Tyr$^1$]-Kallidin, [Tyr$^5$]-bradykinin, and [Tyr$^8$]-bradykinin were labeled with $^{125}$I and purified on CM-Sephadex C-25 columns as described previously [8], except that the columns were equilibrated and eluted with 0.05 M ammonium bicarbonate buffer, pH 8.75. Although this elution buffer does not separate di-iodinated peptide very well from unreacted iodide, it does separate the mono-iodinated peptide from unlabeled material, unreacted iodide, and di-iodinated analogue when 2.5-ml fractions are collected. Mono-iodinated analogues can be eluted within 3 hr of sample application with this buffer in contrast to the almost 24 hr required when 0.05 M ammonium acetate buffer, pH 8.0, is used. Aliquots of presumed mono-iodinated derivatives (0.15 ml, 5–10 $\mu$Ci) were prepared and to each was added 0.5 ml of a solution containing 0.8 M glycine, 0.2 M NaCl, 0.01 M sodium phosphate, pH 7.5, and crystalline bovine serum albumin, 0.25%. To remove the C-terminal arginines, each aliquot was incubated at room temperature for 15 min with DFP-treated carboxypeptidase B (10 units in 0.01 ml of 0.1 M NaCl). One unit will hydrolyze 1.0 $\mu$mole of hippuryl-L-arginine per min at pH 7.65 at 25°. That the reaction had gone to completion was assessed in a descending paper chromatography system capable of separating the radioactive starting material from its product (Whatman 3MM paper, 0.2 M acetic acid adjusted to pH 5.0 with pyridine). Aliquots were stored at −70°.

Radioimmunoassay mixtures contained 0.1 ml of a dilution of $^{125}$I-labeled peptide yielding approximately 10,000 cpm (10 pg), 0.1 ml of an antiserum dilution, and 0.1 ml of buffer or a dilution of unlabeled peptide in buffer. All dilutions were made in sodium phosphate buffer, 0.01 M, pH 7.0, that contained 1,10-phenanthroline, 0.01 M, and casein (Hammersten), 0.01%. After 16–18 hr of incubation at 4–10° in cellulose acetate test tubes, the bound radioactivity was separated from free radioactivity using dextran-coated charcoal. Dextran-coated charcoal was prepared by dissolving 2.5 g of dextran T70 in 1 liter of 0.01 M potassium phosphate buffer, pH 7.6, and then adding 12.5 g of Norit A charcoal. The charcoal suspension was stirred overnight in the cold room before use. This preparation was found to be stable for at least 2 months when stored in the cold room. Two milliliters of a 1:10 dilution of this charcoal suspension in the potassium phosphate buffer was added to incubation tubes. After centrifugation at 4° for 10 min at 1000 *g*, the supernatant fractions were aspirated and the charcoal pellets were counted in a well-type automatic gamma counter (Nuclear Chicago, model 1065).

Assays were run in triplicate and one set of tubes contained normal rabbit serum instead of immune serum to determine the correction for "co-precipitate" or "co-absorption".

## RESULTS

*Carboxypeptidase B treatment of iodinated kinins.* In Fig. 1 is shown the paper chromatographic

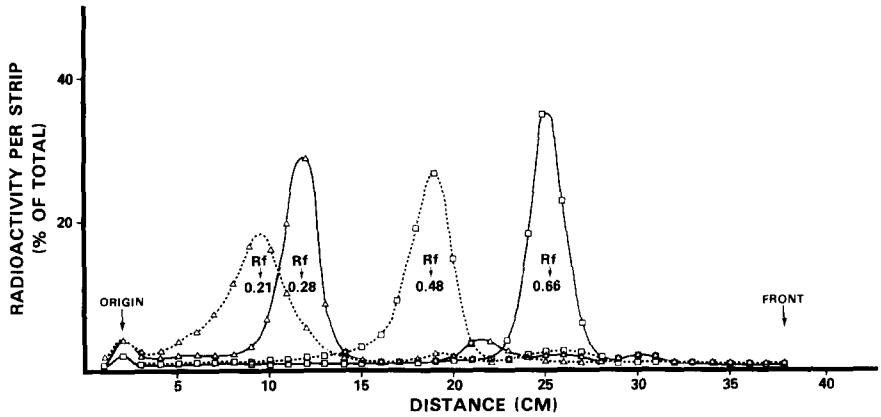


Fig. 1. Paper chromatography of carboxypeptidase B-treated and non-treated mono-$^{125}$I-labeled bradykinins. The mono-$^{125}$I-labeled derivatives of [Tyr$^8$]-bradykinin (□) and [Tyr$^1$]-kallidin (△) were incubated with carboxypeptidase B as described in Methods. The dashed lines correspond to the starting materials and the solid lines to the products of carboxypeptidase B treatment. Approximately 50,000 cpm of each sample was applied to the paper.

migration patterns of intact mono-$^{125}$I-labeled derivatives of [Tyr$^1$]-kallidin and [Tyr$^8$]-bradykinin and the products of their digestion with carboxypeptidase B, CBT1K and CBT8BK, respectively. The peptides migrate further after enzyme treatment. [Mono-$^{125}$I-Tyr$^5$]-bradykinin and its carboxypeptidase B product, CBT5BK, migrate exactly like the corresponding derivatives of [Tyr$^8$]-bradykinin. When authentic [Tyr$^5$,des-Arg$^9$]-bradykinin was iodinated and then subjected to paper chromatography, it migrated in the same position as CBT5BK, $R_f$ = 0.66. In this paper chromatographic system, iodide migrates with $R_f$ = 0.80, mono-iodo-tyrosine with $R_f$ = 0.71, and di-iodo-tyrosine with $R_f$ = 0.59. There is apparently only one radioactive product, which migrates in a position different from the starting materials, obtained after carboxypeptidase B treatment of the mono-$^{125}$I-labeled kinins. This indicates that the reaction with carboxypeptidase B has gone to completion. The co-chromatography of CBT5BK with [mono-$^{125}$I-Tyr$^5$,des-Arg$^9$]-bradykinin strongly suggests that the products obtained with carboxypeptidase B treatment are, in fact, the corresponding mono-$^{125}$I-labeled kinins lacking C-terminal arginines.

*Selection and characteristics of antibody–antigen pairs.* Shown in Fig. 2 are the results obtained when serial dilutions of one antiserum, -9CEO2, were incubated with each of the radioactive antigens. At any given dilution, more CBT8BK was bound by the antiserum than either of the other radioactive polypeptides. The dilutions of -9CEO2 binding 3.3 pg of the 10 pg of each radioactive antigen available were estimated from the curves shown in Fig. 2. These dilutions, along with those similarly obtained for four other sera, are recorded in Table 1. Three antisera bound CBT8BK and two antisera bound CBT1K at a higher dilution than they bound either of the other two radioactive antigens.

Table 1. Titer of [des-Arg$^9$]-bradykinin antisera as a function of antigen structure

| | Final dilutions binding 3.3 pg of $^{125}$I-labeled antigen* | | |
|---|---|---|---|
| Antiserum | CBT1K | CBT5BK | CBT8BK |
| -9CEO1 | 1:1,580 | 1:490 | 1:1,725 |
| -9CEO2 | 1:32,000 | 1:660 | 1:64,000 |
| -9CEO3 | 1:710 | 1:290 | None |
| -9CEO5 | 1:510 | 1:310 | 1:225 |
| -9CEO6 | 1:720 | 1:800 | 1:2,500 |

* Abbreviations: CBT1K, CBT5BK, and CBT8BK represent the products of carboxypeptidase B treatment of the mono-$^{125}$I-labeled derivatives of [Tyr$^1$]-kallidin, [Tyr$^5$]-bradykinin, and [Tyr$^8$]-bradykinin respectively.

To determine the sensitivity of each antigen–antibody combination to inhibition by unlabeled polypeptide, serial dilutions of BK(1–8) were incubated with 10,000 cpm (10 pg) of labeled antigen and antiserum dilutions that bind between 30 and 50% of the labeled peptide in the absence of unlabeled BK(1–8). The most sensitive labeled antigen–antibody combination was obtained with CBT8BK and -9CEO2 antiserum. A composite of nine BK(1–8) inhibition curves obtained with this labeled antigen–antibody combination is shown in Fig. 3. The lowest concentration of BK(1–8) that can be determined reliably is 67 pg/ml. The concentration of BK(1–8) inhibiting 50% of the labeled antigen

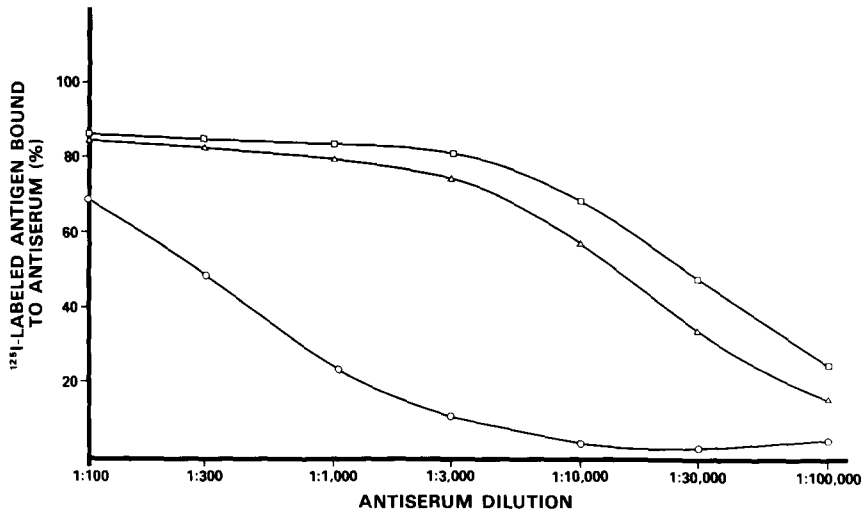


Fig. 2. Binding of $^{125}$I-labeled antigens to BK(1–8) antiserum, -9CEO2. $^{125}$I-Labeled bradykinins were purified and treated with carboxypeptidase B as described in Methods. Binding to antibody was determined by incubating the radioactive antigen with antiserum at the dilutions indicated for 18 hr and then separating bound from free radioactivity with dextran-coated charcoal. The ordinate is the amount of radioactivity not bound to charcoal, expressed as a percentage of the total radioactivity added, corrected for binding that occurs in the absence of antiserum. The results with carboxypeptidase B-treated [mono-$^{125}$I-Tyr$^8$]-bradykinin (□), [mono-$^{125}$I-Tyr$^1$]-kallidin (△), and [mono-$^{125}$I-Tyr$^5$]-bradykinin (○) are shown.

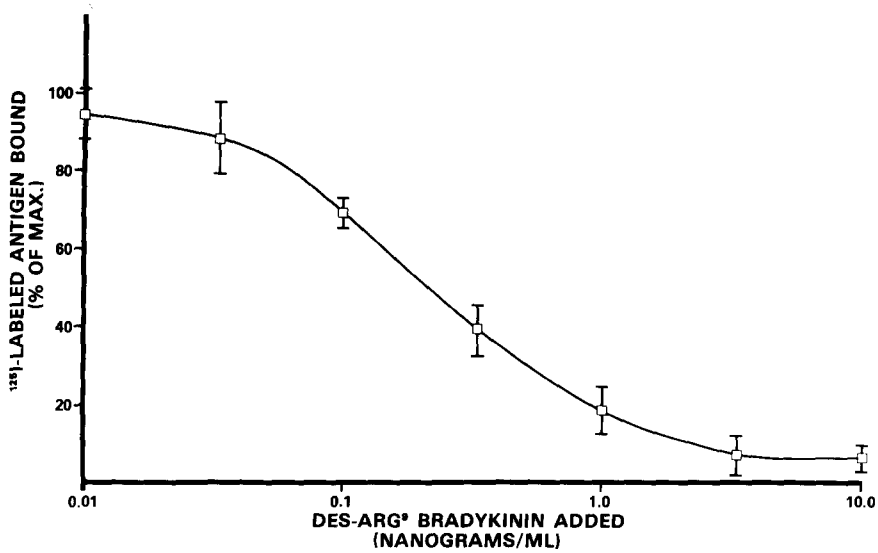


Fig. 3. Composite of nine BK(1–8) inhibiton curves obtained with -9CEO2 antiserum (final dilution 1:80,000) and carboxypeptidase B-treated [mono-$^{125}$I-Tyr$^8$]-bradykinin as labeled antigen. Brackets represent one standard deviation from the mean. Incubation and separation were performed as described in Methods.

binding was 0.23 ng/ml. Other labeled antigen–antibody combinations were evaluated and the results are shown in Table 2. Although for -9CEO2 the labeled antigen allowing for the greatest dilution of antiserum yielded the most sensitive labeled antigen–antibody combination, this was not necessarily the case, as demonstrated by -9CEO6, where the labeled antigen–antiserum combination allowing for the greatest dilution of antiserum required the largest concentration of BK(1–8) to inhibit 50% of the labeled antigen binding.

*Specificity.* Hapten inhibition curves were obtained using BK(1–9), lysyl-BK(1–9), methionyl-lysyl-BK(1–9), lysyl-BK(1–8), methionyl-lysyl-BK(1–8), BK(2–8), and BK(1–7) for each labeled antigen–antibody combination listed in Table 2. Concentrations of these peptides inhibiting 50% of the binding of labeled antigen to antiserum were compared to those concentrations of BK(1–8) yielding a comparable degree of inhibition. The results of these comparisons are recorded in Table 3. The specificity of an antiserum was found to vary with the labeled antigen employed. This was most apparent with -9CEO1. The greatest overall specificity

Table 2. Concentration of [des-Arg$^9$]-bradykinin inhibiting 50% of labeled antigen binding as a function of labeled antigen–antiserum combination

| $^{125}$I-labeled antigen* | Antiserum | Dilution of serum (to bind 30–50% of labeled antigen) | [des-Arg$^9$]-Bradykinin to inhibit 50% of binding (ng/ml) |
|---|---|---|---|
| CBT1K | | 1:1,200 | 17.2 |
| CBT5K | -9CEO1 | 1:300 | 18.0 |
| CBT8BK | | 1:1,400 | 4.5 |
| CBT1K | | 1:20,000 | 4.1 |
| CBT5BK | -9CEO2 | 1:600 | 5.3 |
| CBT8BK | | 1:80,000 | 0.23 |
| CBT1K | | 1:500 | 2.4 |
| CBT5BK | -9CEO3 | 1:200 | 4.4 |
| CBT1K | | 1:500 | 7.3 |
| CBT5BK | -9CEO5 | 1:250 | 7.6 |
| CBT8BK | | 1:200 | 9.1 |
| CBT1K | | 1:700 | 6.7 |
| CBT5BK | -9CEO6 | 1:800 | 4.2 |
| CBT8BK | | 1:2,500 | 13.0 |

* Abbreviations: CBT1K, CBT5BK, and CBT8BK represent the products of carboxypeptidase B treatment of the mono-$^{125}$I-labeled derivatives of [Tyr$^1$]-kallidin, [Tyr$^5$]-bradykinin, and [Tyr$^8$]-bradykinin respectively.

Table 3. Specificity of [des-Arg$^9$]-bradykinin antisera

| $^{125}$I-labeled antigen–antiserum combination* | Relative potencies of peptides as inhibitors of the binding of radioactive antigen ([des-Arg$^9$]-bradykinin, BK(1–8), = 100)† | | | | | | |
|---|---|---|---|---|---|---|---|
| | Lysyl-BK(1–8) | Methionyl-lysyl-BK(1–8) | BK(2–8) | BK(1–7) | BK(1–9) | Lysyl-BK(1–9) | Methionyl-lysyl-BK(1–9) |
| CBT1K–9CEO1 | 516.8 | 1091 | 19.2 | <1.5 | 6.6 | 47.5 | 28.0 |
| CBT5BK–9CEO1 | 204.0 | 534.9 | 63.3 | <1.7 | 1.4 | 6.6 | 2.6 |
| CBT8BK–9CEO1 | 3.4 | 10.9 | <0.3 | 24.4 | 30.6 | 2.1 | 1.2 |
| CBT1K–9CEO2 | 93.3 | 48.2 | 0.2 | 0.2 | 5.6 | 4.2 | 0.2 |
| CBT5BK–9CEO2 | 67.1 | 41.5 | 0.6 | 18.8 | 12.2 | 2.4 | 0.3 |
| CBT8BK–9CEO2 | 100 | 12.4 | 0.02 | 8.4 | 8.6 | 9.1 | 0.4 |
| CBT1K–9CEO3 | 162 | 248.4 | 127 | <0.3 | 2.4 | 4.5 | 2.2 |
| CBT5BK–9CEO3 | 165 | 296.4 | 95.9 | <0.6 | 1.3 | 2.8 | 1.6 |
| CBT1K–9CEO5 | 18.8 | 22.8 | <1.0 | 59.8 | 52.6 | 5.1 | 3.2 |
| CBT5BK–9CEO5 | 34.0 | 64.7 | <1.7 | 1.0 | 10.2 | 4.9 | 4.1 |
| CBT8BK–9CEO5 | 28.4 | 31.0 | <1.4 | 34.7 | 48.8 | 5.2 | 4.4 |
| CBT1K–9CEO6 | 95.6 | 149.8 | <0.7 | 12.9 | 16.6 | 29.5 | 21.1 |
| CBT5BK–9CEO6 | 194.4 | 400.0 | 40.0 | <0.5 | 5.6 | 9.3 | 4.2 |
| CBT8BK–9CEO6 | 97.1 | 146.2 | 19.2 | 13.8 | 92.8 | 63.5 | 61.9 |

* See Table 2 for antiserum dilutions and definition of abbreviations.
† Determined at the 50% inhibition point.

was obtained with -9CEO5 and CBT5BK and with -9CEO2 and CBT8BK. The least specificity was displayed by -9CEO6 with CBT8BK.

## DISCUSSION

Biological assays have until now, been the only means for measuring BK(1–8). The isolated rabbit aorta, in which the BK(1–8) receptor was first described [2], is two orders of magnitude less sensitive for BK(1–8) bradykinin that the radioimmunoassay we report in this paper. In general, the advantages that radioimmunoassays have over biological assays include: greater ease in processing large numbers of samples, increased specificity, i.e. chemically unrelated compounds with similar biological activities are not measured, less inter-assay variation in response, and greater sensitivity. The main disadvantage of radioimmunoassays is that they measure immunological rather than biological activity and, therefore, chemically related compounds lacking biological activity may be measured, i.e. biologically inactive fragments of a peptide might have immunological activity.

Since this is the first report on the development of a radioimmunoassay for BK(1–8), it is not possible to compare our findings directly with any others. However, comparisons can be made with reports of radioimmunoassays for BK(1–9). The finding that the five BK(1–8) antisera differed with respect to their binding capacities for the three carboxypeptidase B-treated $^{125}$I-labeled kinins is consistent with results obtained with BK(1–9) antisera and $^{125}$I-labeled kinins [8, 9]. Although the most sensitive antigen–antibody combination obtained was one where the labeled antigen employed allowed for the greatest dilution of antiserum, this was not necessarily the case (Table 2). That is to say, titer and affinity varied independently of each other. This is the opposite of results obtained with one set of BK(1–9) antisera [8].

We plan to use a combination of CBT8BK with -9CEO2 antiserum for our BK(1–8) radioimmunoassay. The lowest concentration of BK(1–8) that can be detected by the assay is 67 pg/ml (Fig. 3). If BK(1–8) levels are comparable to those reported for BK(1–9) in normal blood, 70 pg/ml [10], the assay should be able to measure BK(1–8) in normal blood. BK(1–9) antisera have been found to show varying degrees of cross-reactivity with biologically active kinins and biologically inactive bradykinin fragments [8]. The BK(1–8) antisera also display these properties. The labeled antigen–antibody combination used in our assay cross-reacts 100% with lysyl-BK(1–8) and 12.4% with methionyl-lysyl-BK(1–8), kinin analogues that are twenty and seven times, respectively, more potent that BK(1–8) in contracting the isolated rabbit aorta [3]‡. Thus, measurements made by radioimmunoassay may yield values that underestimate the biological activity of kinins, lacking a C-terminal arginine, in the samples. The cross-reactivities of BK(1–9) (8.6%) and lysyl-BK(1–9) (9.1%) in this assay may make it necessary to carry out independent determinations of these peptides in biological samples when BK(1–9) or lysyl-BK(1–9) levels exceed BK(1–8) by as much as 10-fold. The extent to which the 8.4% cross-reactivity with BK(1–7) will complicate measurements of BK(1–8) in biological fluids will depend to a large extent on the relative concentrations of these two bradykinin fragments in the samples. It might be possible to distinguish the two peptides by analyzing the same sample with other labeled antigen–antibody combinations that have different specificities, e.g. CBT1K and -9CEO2 antiserum.

The application of the BK(1–8) radioimmunoassay could lead to a better understanding of the role this

‡ It should be noted that, in previous reports on the biological activity of methionyl-lysyl-BK(1–8), the results for this peptide were mistakenly interchanged with those for methionyl-lysyl-BK(1–9).

peptide plays in physiological and pathological conditions. BK(1–8) might be involved in the inflammatory process since BK(1–8) receptors are formed in response to tissue damage [11].

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