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FURTHER STUDIES OF MYOMETRIAL BRADYKININ RECEPTOR-LIKE BINDING

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Abstract—[^{125}I-Tyr^1]Kallidin (T1K), a bradykinin (BK) analog with biological potency comparable to BK, was used as a probe for BK receptor-like binding from bovine uterine myometrium. BK binding exhibited a high affinity, $K_{dissoc} = 1.65 \times 10^{-10}$ M. The specificity of T1K binding was examined with forty-four BK analogs. Comparison of the binding inhibitory potencies with the relative biological potencies of these analogs on isolated rat uterus resulted in a good correlation, $r = 0.87$. BK binding activity was solubilized with CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent. The solubilized binding activity exhibited a BK binding affinity, $K_{dissoc} = 2.25 \times 10^{-10}$ M, and a specificity for three ^{125}I-labeled kinins similar to those of the particulate BK receptor-like binding activity.

Kinin receptors have been classified as B1 (rabbit aorta) and B2 (rat uterus, cat ileum) based on biological assay data, and specific antagonists for the B1 receptor have been synthesized [1]. Specific antagonists for the B2 receptor have not been prepared [2]. Direct binding studies have utilized [^{125}I-Tyr^1]kallidin (T1K)§ [3] and [^{3}H]bradykinin [4] to examine B2 receptors from bovine uterine myometrium and guinea pig ileum respectively. In both cases, high affinity bradykinin‖ (BK) binding was observed, with binding specificity for BK analogs correlating well with biological assay data. These are necessary but not sufficient conditions for characterization of receptor-mediated binding. However, it is not clear that biological assay data on kinins accurately reflect the interactions of kinins with the B2 receptor. Potencies of BK analogs relative to BK measured on the same biological preparation but in different laboratories do not always agree [5]. When different biological preparations in the same laboratory were used to determine the relative potency of a single BK analog, different potencies were obtained [6, 7]. Most importantly, data from biological assays have not resulted in the synthesis of a competitive antagonist for the more prevalent B2 receptor [2]. The good correlation between binding data, obtained with crude particulate fractions, and biological assay data could result from the presence in both preparations of comparable amounts of receptor and non-receptor kinin binding sites. Two approaches can be taken to address this possibility. One approach is to use competitive inhibitors of non-receptor kinin binding proteins, e.g. kininases, to see whether they alter binding and biological potencies of BK analogs. The other approach is to solubilize, purify and characterize the putative kinin receptor. If the specificity of kinin binding by the purified "receptor" correlates with the biological assay data, then the assumption that biological assay data accurately reflect the BK receptor interaction would be supported. Alternatively, kinin binding specificity of the purified "receptor" might not correlate with biological assay data. However, if this binding specificity results in predictions about the B2 receptor that lead to the syntheses of competitive antagonists, the argument that biological assay data do not accurately reflect the BK receptor interaction would be supported.

This paper extends previous studies on the characterization of a particulate BK receptor-like binding activity from bovine uterine myometrium using T1K as the receptor probe [3]. In addition, solubilization of BK binding activity from bovine uterine myometrium has been accomplished with CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent. The soluble BK binding activity exhibited a specificity for three ^{125}I-labeled kinins and a BK affinity similar to those of the particulate BK receptor-like binding activity.

MATERIALS AND METHODS

Materials

[Tyr^1]kallidin, [Tyr^5]BK, and [Tyr^8]BK 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; and methionyllysyl-BK and kallidin were from Vega Biochemicals, Tucson, AZ. All other BK analogs used in this investigation were prepared by the Merrifield solid phase method [8]. CHAPS was purchased from Serva

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§ Abbreviations used: T1K, [^{125}I-Tyr^1]kallidin; BK, bradykinin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; and PIPES, piperazine-*N*,*N*′bis[2-ethanesulfonic acid].

‖ Bradykinin is H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH.

Biochemicals, and PIPES (piperazine-*N*,*N*′-bis [2-ethanesulfonic acid]) from Research Organics, Inc., Cleveland, OH. Polypropylene tubes were purchased from Sarstedt, Inc., Princeton, NJ; 9/16 × 4 inch cellulose acetate tubes from the Petro Packaging Co., Inc., Cranford, NJ; Norit A activated charcoal from Pfanstiehl Laboratories, Inc., Waukegan, IL; dextran T 70 from Pharmacia Fine Chemicals, Piscataway, NJ; tetramethylammonium hydroxide from the Sigma Chemical Co., St. Louis, MO; and protein assay reagents from the Bio-Rad Co., Richmond, CA. SQ20881 (teprotide), pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, was a gift from the Squibb Institute for Medical Research, Princeton, NJ. All other reagents were of the highest quality available and were obtained from commercial sources.

Methods

Preparation of the particulate myometrial fraction. Bovine uteri from pregnant cows were removed and placed on ice within 1 hr of the death of the animal at the Stadler Packing Co., Inc., Columbus, IN. Myometrium, dissected free of endometrial tissue, was thoroughly washed with cold homogenization buffer (115 mM NaCl, 4.6 mM KCl, 22 mM $NaHCO_3$, 10 mM NaH_2PO_4, 1.0 mM $MgSO_4 \cdot 7H_2O$, 5.5 mM glucose and 13.4 mM Na_2EDTA, pH 6.7) and minced with scissors. Wet myometrial tissue was homogenized with the above buffer (1:2 w/v) in an ice-water jacketed Waring Commercial Blender at top speed for 4 min. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fraction was discarded. The pellet was frozen at −10°. The pellet was thawed and 500 g was rehomogenized with 2.0 litres of ice-cold buffer in the Waring Blendor at top speed for 4 min. After centrifugation at 1000 *g* for 10 min, the pellet was discarded. The supernatant fraction was centrifuged at 10,000 *g* for 5 hr, and the resulting pellet was stored overnight at −10°. This pellet was resuspended in 70.0 ml of 20 mM potassium phosphate buffer, containing 1 mM EDTA, pH 6.3. Seventy-two aliquots were prepared in polypropylene test tubes and, after centrifugation at 25,000 *g* for 1 hr, the supernatant fractions were aspirated and the pellets were stored at −10° until used.

Conditions for solubilization of BK binding activity. Aliquots of uterine myometrial particulate fraction were suspended in 20 mM PIPES buffer, containing 1 mM 1,10-phenanthroline, pH 6.8. Various concentrations of CHAPS were added to yield final protein concentrations of 0.5 mg/ml. These mixtures were stirred slowly in an ice bath for 30 min and then centrifuged at 50,000 *g* for 1 hr at 0°. Supernatant fractions were carefully removed and assayed for BK binding activity.

Preparation of radioactive peptides. The monoiodinated derivatives of [Tyr^1]kallidin, [Tyr^5]BK and [Tyr^8]BK were prepared [9] and purified [10] as previously described. Iodinated peptides were prepared every 2 months and were stored at −70°.

Particulate binding assay conditions. Assays were performed in casein-coated 12 × 75 mm polypropylene test tubes, as previously described [3]. The assay buffer was either 20 mM potassium phosphate at pH 6.3, or 20 mM PIPES at pH 6.8, both containing 1 mM EDTA and 6.3 μM SQ20881. For the experiments with cations, the assay buffer was 20 mM PIPES, containing 6.3 μM SQ20881, and adjusted to pH 6.3 with tetramethylammonium hydroxide. To each tube was added 0.1 ml of assay buffer, with or without unlabeled peptide, 0.05 ml of cation, followed by 0.1 ml of the radiolabeled kinin (20,0000 cpm), and the reaction was initiated by addition of 0.45 ml of the myometrial particulate fraction suspended in assay buffer. After 15 min of incubation in an ice-bath, the tubes were centrifuged at 25,000 *g* for 1 hr at 0°. The supernatant fraction was aspirated, and pellet-associated radioactivity was counted in a Beckman Gamma 4000 counter.

Soluble binding assay conditions. The assays for soluble BK binding activity were performed in 9/16 × 4 inch cellulose acetate tubes. Radiolabeled kinin (0.1 ml, 50,000 cpm), with or without unlabeled BK (0.1 ml), was incubated with 0.5 ml of CHAPS myometrial extract at 4° for 20 hr. Bound radioactivity was separated from free radioactivity by addition of 1.0 ml of dextran-coated charcoal solution. Dextran-coated charcoal solution was prepared by dissolving 5.0 g of dextran T 70 in 1 liter of 0.01 M potassium phosphate buffer, pH 7.6, and then adding 25 g of Norit A activated charcoal [10]. Tubes were then centrifuged at 4° for 15 min at 1000 *g*, and 1.2 ml of each supernatant fraction was transferred into a new tube and counted in a well-type automatic gamma counter (Nuclear Chicago, model 1065).

Both particulate and soluble binding assays were run in triplicate. Saturable binding was defined as the difference in the amount of radioactivity bound in the absence of unlabeled BK and the amount bound in the presence of excess unlabeled BK (5.0 μg).

Protein was measured using the procedure of Bradford [11] as adapted by the Bio-Rad Co.

RESULTS

Bradykinin (BK) and forty-four BK analogs were assessed for their abilities to inhibit binding of T1K to a particulate fraction from bovine uterine myometrium. The binding inhibitory potencies of these analogs, expressed as the concentrations required to inhibit 50% of saturable T1K binding, are recorded in Table 1. The relative biological potencies of these analogs to contract rat uterus are also shown in this table. Linear regression analysis comparing the $\log_{10}$ of the 50% binding inhibitory concentrations with the $\log_{10}$ of the relative potencies of these analogs to contract rat uterus resulted in a good correlation, $r = 0.87$.

Both monovalent and divalent cations inhibit T1K binding to the myometrial particulate fraction. The concentrations of six cations that decreased by 50% the saturable binding of T1K are recorded in Table 2. The rank order of potency for these cations is as follows: $K^+ < Li^+ < Na^+ < Mg^{2+} < Ca^{2+} < Zn^{2+}$.

Solubilization of bradykinin binding activity from bovine uterine myometrium using CHAPS is shown in Fig. 1. T1K binding activity was optimally solubilized with 3 mM CHAPS. At higher detergent concentrations, greater amounts of protein were solubilized but the binding activity of the extracts was decreased.

Table 1. Binding and biological potencies of bradykinin analogs*

Analog	Concentration to inhibit 50% of [^{125}I-Tyr^1]kallidin binding to particulate bovine uterine myometrium (nM)	Relative biological potency on isolated rat uterus [Ref.]†
Lys-Lys-[ThiAla^{5,8}]BK	0.026	34‡
Lys-Lys-BK	0.032	33 [5]
[p-chloro-D-Phe^6,Aib^7]BK	0.044	97‡
[p-chloro-D-Phe^6]BK	0.058	52 [12]
BK	0.165	100
[Hypro^3]BK	0.17	100
Lys-OMT-Lys-BK	0.29	21‡
[D-Trp^6,Aib^7]BK	0.31	100‡
[D-Phe^6,Aib^7]BK	0.32	124‡
[Tyr^1]Kallidin	0.34	95
Ile-Ser-BK	0.40	66‡
[Thr^6]BK	0.41	33
[Hypro^2,ThiAla^{5,8}]BK	0.50	100
[Aib^3]BK	0.86	7.0
[D-Trp^6]BK	1.7	29
Suc-bis(EACA-EACA-BK)	2.0	15 [13]
Suc-bis(EACA-BK)	2.5	45 [13]
[Aib^7]BK	3.2	55
Suc-bis(BK)	3.6	9 [13]
[Tyr^8]BK	4.5	24
[D-Phe^6]BK	4.5	6 [12]
[Abu^6]BK	5.2	51§
[D-Phe^8]BK	5.8	15
Cl-acetyl-BK	6.3	1000
[Leu^5]BK	6.3	5
[D-Ala^6]BK	9.6	0.7§
[Gly^6,Tyr^8]BK	12	0.4 [2]
[Tyr^5]BK	22	0.2
[Gly^6,Aib^7[BK	46	2.5
[D-Ser^6]BK	58	5 [12]
[Lys(Tos)^6]BK	71	0.05
[Aib^2]BK	91	0.5 [14]
[Leu^8]BK	110	0.3
[D-Pro^{2,3}]BK	390	0.01
[Phe^9]BK	510	0.01
[Acetyl-Arg^1,D-Pro^7]BK	690	0.1‡
[D-Ala^4]BK	1,600	0.01
[D-Pro^{2,7}]BK	1,600	0.01
[Phe^{4,6}]BK	2,900	0.01
[D-Pro^{3,7}]BK	3,500	0.001
des-Arg^1-BK	4,600	0.00001
Acetyl-[D-Pro^3,OMT^8]BK	4,900	0.2
Acetyl-[D-Pro^3]BK	5,100	0.2
[D-Pro^{2,3,7}]BK	5,500	0.0005
[Gly^{5,6,8}]BK	15,000	0.01

* Abbreviations: OMT, *O*-methyltyrosine; Aib, α-aminoisobutyric acid; ThiAla, β-(2-thienyl)-alanine; Suc, succinyl; Abu, γ-aminobutyric acid; EACA, ϵ-aminocaproic acid; and Tos, *p*-toluenesulfonyl.

† Except where indicated, biological potencies of these analogs were taken from Ref. 15, wherein the original literature references can be found.

‡ Unpublished data.

§ Cat ileum.

The specificity of the soluble binding activity for three radioactive BK analogs was examined (Fig. 2). Saturable binding of T1K was greater than that of [^{125}I-Tyr^8]BK, while [^{125}I-Tyr^5]BK showed no saturable binding. Non-saturable binding was roughly the same for the three iodokinins. The ability of increasing concentrations of unlabeled BK to inhibit T1K binding to the soluble binding activity is shown in Fig. 3. T1K binding was inhibited 50% by a BK concentration of 2.25×10^{-10} M.

DISCUSSION

The present results are consistent with the previous report of BK receptor-like binding activity in a particulate fraction from bovine uterine myometrium

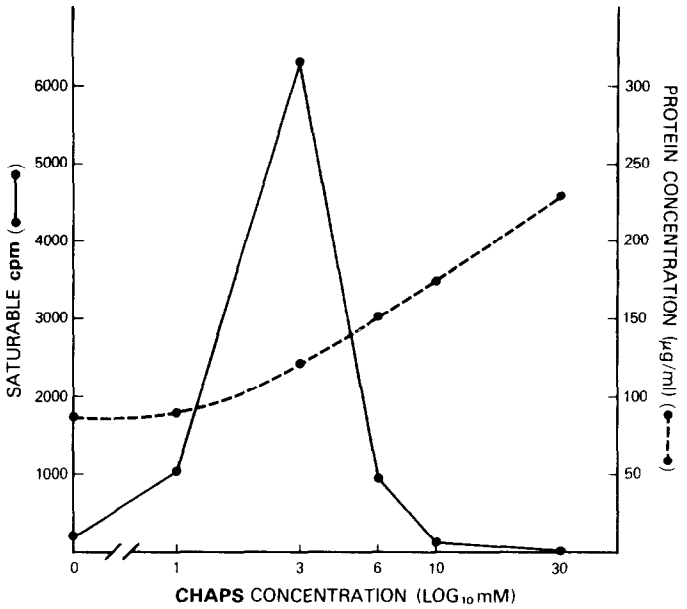

Fig. 1. Effect of CHAPS concentration on the solubilization of bradykinin binding activity and protein from bovine uterine myometrium. A particulate fraction of bovine uterine myometrium was extracted with various concentrations of CHAPS for 30 min at 0°. After centrifugation at 50,000 *g* for 60 min, the supernatant fraction was collected and assayed for saturable [^{125}I-Tyr^1]kallidin binding as described in Methods. Protein concentrations were measured using the Bio-Rad assay.

[3]. T1K binding inhibitory potencies of forty-four previously untested BK analogs correlated well with the biological potencies of these compounds. Because of differences in the literature concerning the inhibitory effects of various cations on the binding of radioactive probes to putative BK receptors [3, 4], the effects of cations on binding of T1K to particulate bovine uterine myometrium were re-evaluated. Previous reports did not discuss the mechanism of binding inhibition by cations but implied a direct effect on the peptide–receptor interaction. Yet many kininases are metal requiring enzymes [15]. The inhibition of T1K binding by Ca^{2+} and Zn^{2+} (Table 2) was due, at least in part, to degradation of the receptor probe,

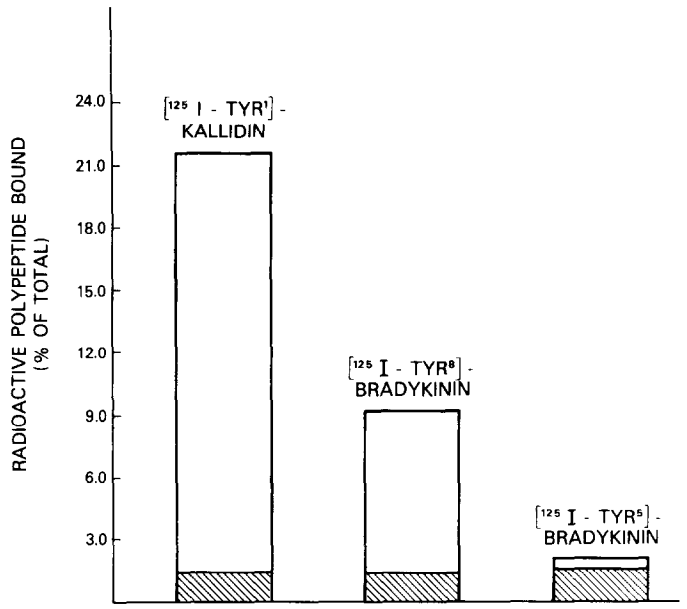

Fig. 2. Binding of three ^{125}I-labeled kinins by the 3 mM CHAPS extract of particulate bovine uterine myometrium. Fifty picograms (50,000 cpm) of [^{125}I-Tyr^1]kallidin, [^{125}I-Tyr^5]bradykinin and [^{125}I-Tyr^8]bradykinin were each incubated with 0.5 ml of the extract. The relative amounts of radioactive polypeptides bound in the absence of bradykinin (total bar) and with 5.0 μg of unlabeled bradykinin present (shaded area) were expressed as percentages of the total radioactivity added to each of the incubation mixtures.

Table 2. Inhibition of [^{125}I-Tyr^1]kallidin binding to particulate bovine uterine myometrium by monovalent and divalent cations*

Cation	IC_{50} for [^{125}I-Tyr^1] kallidin binding to bovine uterine myometrium (mM)
K^+	69
Li^+	51
Na^+	35
Mg^{2+}	7
Ca^{2+}	2
Zn^{2+}	0.03

* Serial dilutions of each cation were assayed for their inhibition of saturable [^{125}I-Tyr^1]kallidin binding, as described in Methods. For these ion experiments, the assay buffer consisted of 20 mM PIPES, containing 6.3 μM SQ20881, adjusted to pH 6.3 with tetramethylammonium hydroxide. Chloride salts of the cations were used. IC_{50} Values represent the means of at least three assays performed in triplicate.

while binding inhibition by K^+, Li^+, Na^+, and Mg^{2+} apparently was not due to degradation of T1K (unpublished observations). The nature of the radioactive peptide incubated in the presence of the cations and the particulate myometrial fraction was evaluated in a descending paper chromatography system capable of separating the intact peptide from its degradation products [9]. Inhibition of T1K binding was obtained with lower concentrations of cations than those previously required [3], and the rank order of potency of the cations was now the same as that reported by Innis *et al.* [4]. An EDTA-containing phosphate buffer was used before [3], and this may account for the differences. The lower cation concentrations required for the myometrial studies than those required for ileum [4] may be due to differences in the kininase activities and receptor-like binding activities in the two preparations. Data obtained from direct binding studies utilizing crude particulate preparations and from bioassay may be complicated by the presence of non-receptor kinin binding sites. Innis *et al.* [4] reported a good correlation of binding inhibition and biological potencies for BK analogs, yet later reported that their binding conditions allowed degradation of the radioactive receptor probe and presumably of the BK analogs tested [16]. Roscher *et al.* [17] reported a good correlation between inhibition of [^{3}H]BK binding by BK analogs and their abilities to stimulate PGI_2 release by intact human fibroblasts. At least an order of magnitude higher concentrations of peptides were required to stimulate PGI_2 release than were needed for [^{3}H]BK binding inhibition. The reasons for these differences are not known, but the assay conditions for PGI_2 release probably did not prevent degradation of the peptides. Even in binding studies where degradation of the receptor probe does not occur, saturable binding of the radiolabeled probe should not necessarily be interpreted as receptor binding. Saturable binding of [^{125}I-Tyr^8]BK by porcine kidney medulla occurs, but most of this binding can be inhibited by SQ20881, a competitive inhibitor of kininase II [3]. The kinin-binding specificity of purified porcine kidney kininase II was characterized recently using forty-seven BK analogs and shown to be clearly different from that expected of a BK receptor [18]. These binding studies were performed on the EDTA-inhibited, catalytically inactive kininase II, i.e. the peptides were not being hydrolyzed by the enzyme. One implication of that report is that even in a biological preparation devoid of kinin hydrolyzing activities, kinin binding by these non-receptor sites may occur, i.e. catalytically inactive enzymes that are capable of

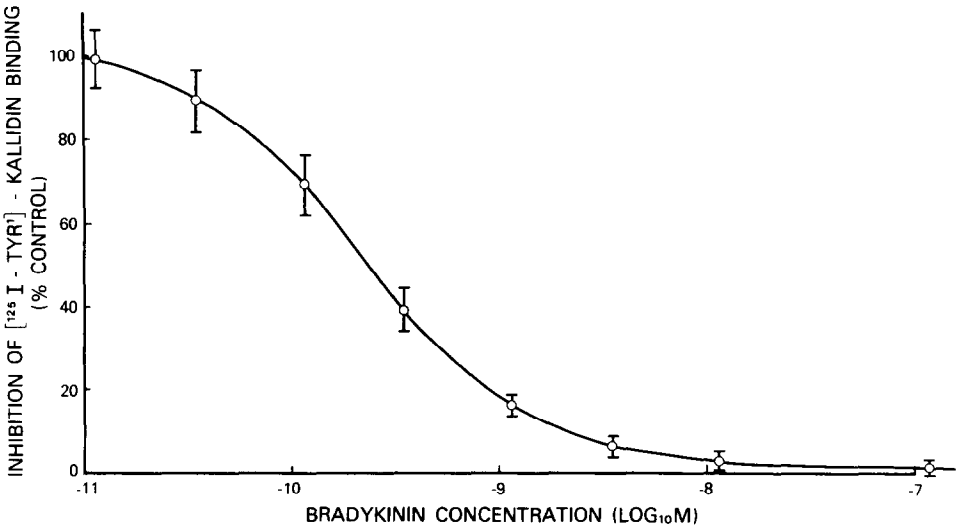

Fig. 3. Inhibition of [^{125}I-Tyr^1]kallidin binding to the 3 mM CHAPS extract of particulate bovine uterine myometrium. Serial dilutions of unlabeled bradykinin were incubated with [^{125}I-Tyr^1]kallidin and the extract as described in Methods. The amounts of [^{125}I-Tyr^1]kallidin bound in the presence of increasing amounts of unlabeled bradykinin are expressed as percentages of the maximum amount of [^{125}I-Tyr^1]kallidin binding (measured in the absence of bradykinin). All data were corrected for the amount of radioactivity bound in the presence of excess unlabeled bradykinin (5.0 μg).

binding kinins may be present. For these reasons, the most accurate determination of kinin binding specificity of a BK receptor will probably be obtained in the presence of competitive inhibitors of all non-receptor kinin binding proteins, or through the use of a receptor preparation that has been purified to separate it from these non-receptor sites. All biologically active BK analogs would be expected to bind to a purified receptor. However, analogs with low biological potency may inhibit T1K binding to a purified receptor to a greater extent than expected from biological assay data because non-receptor binding sites would no longer complicate the results.

Figure 1 shows that BK binding activity was solubilized by treatment of a particulate fraction from uterine myometrium with various CHAPS concentrations. CHAPS at a concentration of 3 mM appeared optimal for the extraction of BK binding activity. Extraction with 3 mM CHAPS resulted in solubilization of 20% of the total protein concomitant with an 80% decrease in T1K binding in the particulate fraction. As much as 45% of the saturable binding of T1K in the original particulate fraction could be recovered in the 3 mM CHAPS extract (unpublished observation). With the solubilization conditions reported here, CHAPS concentrations above 3 mM solubilized increased amounts of protein, yet the binding activity of these extracts was diminished (Fig. 1). Simonds *et al.* [19] observed a similar phenomenon while using CHAPS to solubilize opiate receptors from neuroblastoma-glioma hybrid cells, NG108–15. They determined that CHAPS above 2 mM interferes with opiate binding by the soluble receptor. However, Liscia *et al.* [20] reported that optimal prolactin receptor binding occurs when assay conditions include at least 6 mM CHAPS. CHAPS concentrations greater than 3 mM interfered with the assay for soluble BK binding activity. The mechanisms for this interference are not known. Receptor-subunit dissociation, denaturation of the receptor or binding subunit, and/or a peptide–detergent interaction may be occurring at higher CHAPS concentrations. The specificity of the CHAPS-solubilized kinin binding activity for three ^{125}I-labeled kinin analogs (Fig. 2) paralleled the biological potencies of these peptides, i.e. saturable binding of T1K > [^{125}I-Tyr^{8}]BK >> [^{125}I-Tyr^{5}]BK. This ^{125}I-labeled kinin binding specificity is also similar to that obtained for the particulate receptor-like binding activity [3]. Binding affinity of the solubilized kinin binding activity, determined by inhibition of the T1K binding with unlabeled BK (Fig. 3), yielded a K_{dissoc} of 2.25×10^{-10} M. This result is comparable to the K_{dissoc} found for the particulate receptor-like binding activity, 1.65×10^{-10} M (Table 1). Bruns *et al.* [21] reported that 8 mM CHAPS solubilizes a saturable [^{3}H]BK binding activity from guinea pig ileum that they referred to as a BK receptor. However, these investigators did not report on the kinin binding affinity or specificity of this BK binding activity. Also, the optimal CHAPS concentration for solubilization of this binding activity was not presented.

The soluble BK binding activity described in this report exhibited a binding specificity for three ^{125}I-labeled kinins and a BK binding affinity similar to those of the particulate BK receptor-like binding activity. However, these properties do not prove that the solubilized binding activity is the B2 receptor. Purification and characterization of B2 receptor-like binders should contribute to the development of specific B2 BK receptor antagonists. The application of these inhibitors will ultimately help to unravel the physiological and pathological roles of kinins.

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