Bradykinin recognizes different molecular forms of the B2 kinin receptor in the presence and absence of guanine nucleotides

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We have previously reported that [³H]bradykinin ([³H]BK) identifies high- and low-affinity B2 kinin receptor sites in bovine myometrial membranes which are sensitive and insensitive respectively to guanine nucleotides. Here we show that these receptor-binding sites are solubilized by the detergent CHAPS. Equilibrium binding in soluble preparations revealed that [³H]BK identified a maximal number of binding sites ($B_{\max}$) of 1119 ± 160 fmol/mg of protein, with an equilibrium dissociation constant (K_D) of 314 ± 70 pM and with a typical B2 kinin receptor specificity. Dissociation of equilibrium binding was biphasic. In the presence of the GTP analogue guanosine 5'-[β -imido]triphosphate (Gpp[NH]p), [³H]BK bound to the soluble receptors with a K_D of 929 ± 129 pM and a $B_{\max}$ of 706 ± 38 fmol/mg of protein. The Gpp(NH)p-promoted decrease in the apparent affinity and $B_{\max}$, which was half-maximal at $0.5 \mu\text{M}$, was due at least in part to an increase in the dissociation rate of the slowly dissociating component of the equilibrium binding. Recoveries of guanine-nucleotide-sensitivity and of rapidly and slowly dissociating binding components were essentially identical, whether or not the receptor had been occupied by an agonist before solubilization. Sucrose-density-gradient sedimentation profiles revealed that [³H]BK recognized two different molecular forms of the receptor in the absence or presence of guanine nucleotides. These results provide for the first time direct evidence that guanine nucleotides promote a change in the structure of the B2 kinin-receptor complex. We propose that this structural change is due to dissociation of a guanine-nucleotide-regulatory (G-)protein.

INTRODUCTION

Bradykinin (BK) and other kinin peptides have been implicated in a number of pathophysiological conditions. The pharmacological actions of these peptides, which include increased vascular permeability, vasodilatation, pain and smooth-muscle contraction, have led investigators to believe that these peptides serve as important inflammatory mediators [1,2]. Depending on the relative order of potency of BK and its des-Arg⁹ analogue, kinin receptors have been divided into two main subtypes, named B1 and B2 [1]. Several physiological studies using specific B2-kinin-receptor antagonists as well as direct radioligand binding studies now suggest that heterogeneity also exist among B2 kinin receptors [3–6]. At the cellular level, B2-kinin-receptor-mediated responses include stimulation of inositol phospholipid turnover and release of arachidonic acid metabolites via stimulation of phospholipase C and phospholipase A₂ activity respectively, as well as stimulation of plasma-membrane Ca²⁺ flux [7–15].

Specific guanine-nucleotide-regulatory proteins (G-proteins) have been implicated in receptor-mediated stimulation of both phospholipase C and phospholipase A₂ [16–18]. However, the nature of these G-proteins is still unknown. Even though most kinin-promoted cellular responses are insensitive to pertussis toxin, indicating that they do not require a G-protein of the G_i/G_o class [9,11,13], evidence has been presented supporting the involvement of G-proteins specifically in the actions of kinins [11,13]. In a previous report we showed that bovine myometrial membranes contain high- and low-affinity B2-kinin-receptor-like binding sites for [³H]BK with different sensitivity to guanine nucleotides, and we proposed that these two sites represent

distinct subpopulations of B2 kinin receptors [19]. High-affinity [³H]BK binding specifically involved the sequential formation of at least three binding states with decreasing dissociation rates for [³H]BK. Guanine nucleotides specifically prevented the formation of the most slowly dissociating binding state. This state may be analogous to the agonist-stabilized high-affinity form of several adenylate cyclase-coupled receptors which can be converted into a low-affinity form in the presence of guanine nucleotides. In those systems, the high-affinity form of the receptors have been documented to be a complex of agonist, receptor and G-protein [20–26].

Here we demonstrate that the bovine myometrial B2 kinin receptors can be solubilized in a guanine-nucleotide-sensitive form after treatment of membranes with the detergent CHAPS. The recovery of guanine-nucleotide-sensitivity in our system was independent of agonist occupancy before solubilization. Sucrose-density-gradient sedimentation of soluble preparations revealed that [³H]BK recognized two distinct molecular forms of the high-affinity B2 kinin receptor in the absence or presence of guanine nucleotides. We propose that the different [³H]BK binding states of the high-affinity B2 kinin receptor in bovine myometrial membranes have different molecular forms and that these forms represent receptors that are coupled or uncoupled to a G-protein.

EXPERIMENTAL

Materials

Freshly excised uteri from pregnant cows were obtained from All-State Packing Co., San Antonio, TX, U.S.A. [2,3-prolyl-

Abbreviations used: BK, bradykinin; G-protein, guanine-nucleotide-regulatory protein; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate; App[NH]p, adenosine 5'-[β -imido]triphosphate; IC₅₀, concentration causing 50% inhibition.

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$^3\text{H}(\text{n})\text{BK}$ (79–89 Ci/mmol) was purchased from NEN Research Products (Boston, MA, U.S.A.). BK, Lys-BK, and des-Arg⁹-BK were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [D-Arg⁹-Hyp²-D-Phe⁷] $^3\text{H}\text{BK}$ was synthesized by Dr. R. Vavrek and generously donated by Dr. R. Vavrek and Dr. J. M. Stewart (Denver, CO, U.S.A.). The membrane detergent CHAPS was purchased from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.) or Sigma Chemical Co. Guanosine 5'-[$\beta\gamma$ -imido]triphosphate and adenosine 5'-[$\beta\gamma$ -imido]triphosphate (Gpp[NH]p and App[NH]p) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Sephadex G-50 and catalase were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). The protein assay reagent was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Captopril (SQ14,255) and teprotide (SQ20,881) were kindly given by Dr. S. J. Luciana of Squibb Pharmaceuticals (Princeton, NJ, U.S.A.). Most other chemicals and reagents were from Sigma Chemical Co.

Preparation of bovine myometrial membranes

The membrane fraction from bovine uterine myometrium was prepared as previously described [19], the procedure described by Fredrick *et al.* [27] being used. The membranes were frozen and stored in 25 mM-Tes (pH 6.8)/0.5 mM-EDTA/1 mM-MgCl₂/1 mM-1,10-phenanthroline in aliquots at a concentration of 15–25 mg of protein/ml at –80 °C until used.

Solubilization of bovine myometrial B2 kinin receptors

Aliquots of the frozen membrane suspension were thawed and diluted 5–10-fold with ice-cold buffer A [25 mM-Tes (pH 6.8)/0.5 mM EDTA/1 mM-1,10-phenanthroline/bacitracin (140 $\mu\text{g}/\text{ml}$)/1 μM -SQ20,881 (or 1 μM -SQ14,225/1 mM-dithiothreitol)]. CHAPS was added to the membrane suspension to a concentration of 3–5 mM, and the suspension was stirred vigorously on ice for 30 min. The suspension was centrifuged in a fixed-angle rotor at 4 °C for either 90 min at 46000 *g* or 60 min at 100000 *g*. The clear supernatant was carefully collected. Sufficient MgCl₂ was then added to the supernatant to give a final concentration of 1 mM.

Radioligand binding assays

All soluble samples were assayed in Buffer A in the presence of 1 mM-MgCl₂ and 2 mM-CHAPS. Equilibrium binding assays were performed in polypropylene tubes (12 mm $\times$ 75 mm) in a total volume of 0.5 ml and incubated in a shaking waterbath for 60 min at 25 °C. Assays were initiated by addition of 50 μl of [^3H]BK to a 450 μl mixture of soluble preparation with or without 1 μM -BK included (for determination of non-specific binding). Some assay mixtures also contained various concentrations of nucleotides and other kinin peptides as described in the Figure legends. The assays were terminated by separating bound and free radioligand by gel-filtration chromatography at 4 °C on columns (Kontes Disposaflex) containing Sephadex G-50 (0.8 cm $\times$ 11 cm). Columns were equilibrated with 4 ml of buffer B [buffer A including 1 mM-CHAPS, 1 mM-MgCl₂ and BSA (1 mg/ml)]. Aliquots (0.5 ml) were applied to the columns and the bound [^3H]BK was eluted in 1.0 ml with buffer B directly into 20 ml polyethylene scintillation vials and counted for radioactivity in a scintillation cocktail using a Beckman LS-230 or LS-5000TD scintillation counter. Columns were calibrated with a mixture of [^3H]BK and Blue Dextran 2000 to determine the void volume and included volume. Columns were regenerated after each use with 2 M-KCl to remove all traces of residual free [^3H]BK, followed by copious rinsing with 25 mM-Tris, pH 7.4. Specific binding was defined as the difference in the amount of radioactivity in the absence (total binding) and presence of 1 μM -

BK (non-specific binding). All [^3H]BK binding activities were computed from d.p.m. values.

Sucrose-density-gradient fractionation

Aliquots of the thawed membrane suspension were diluted 1:5 in buffer A including 1 mM-MgCl₂ to a total volume of 10 ml. The membrane suspension was then split into two equal portions. Sufficient Gpp[NH]p was then added to one portion to make a final concentration of 50 μM , and this portion was designated '+ Gpp[NH]p'; the other portion was designated 'Control'. Sufficient BK was then added to both portions to make a final concentration of 10 μM , followed by incubation at 25 °C for 20 min. Both membrane fractions were then centrifuged at 46000 *g* for 20 min at 4 °C. Each pellet was resuspended separately in buffer A, and sufficient CHAPS was added to the suspensions to give a final concentration of 5 mM (2 ml total volume). The '+ Gpp[NH]p' sample received additional Gpp[NH]p to maintain a concentration of 50 μM . Solubilization was then carried out as described above.

Polyallomer centrifuge tubes (Seton; 14 mm $\times$ 89 mm) were made wettable by curing with a commercial chromic acid/H₂SO₄ cleaning solution [28]. Sucrose solutions (15%, w/v) were made up in buffer A including 1 mM-MgCl₂ and 5 mM-CHAPS, with and without 50 μM -Gpp[NH]p; 5% sucrose solutions were made by dilution of the 15% sucrose solutions in the same buffer, with and without 50 μM -Gpp[NH]p. Linear sucrose gradients, 5–15%, in the absence and presence of 50 μM -Gpp[NH]p, were then poured to a total volume of 11.0 ml in the cured tubes by using a two-chamber gradient mixer (1.6 cm-diameter chambers) and a Pharmacia P-3 peristaltic pump. '+ Gpp[NH]p' and 'Control' samples (0.5 ml), both including catalase (0.27 mg), were layered on to the appropriate gradient. The loaded gradients were centrifuged overnight in a Beckman rotor type SW-41 Ti at 39000 rev./min for 17 h at 4 °C on a Beckman L5-65B Ultracentrifuge.

The gradients were fractionated manually at 4 °C by piercing the bottom of the gradient tube and collecting 0.25 ml fractions (average of 45.5 fractions/gradient). Aliquots (0.03 ml) were removed for analysis of catalase activity [29] and sucrose concentrations. The latter were determined by using a Schuco clinical refractometer to obtain the refractive index, and published tables were used to calculate the correlative percentage of sucrose [30]. The remaining fraction volume (0.22 ml) was diluted to 0.5 ml with buffer A including 1 mM-MgCl₂ and 2.5 mM-CHAPS. The diluted fractions were chromatographed on Sephadex G-50 columns equilibrated with the above buffer, as described under 'Radioligand binding assays' above, to remove BK and Gpp[NH]p. Assay of specific [^3H]BK binding in each fraction was carried out in buffer A including 2 mM-CHAPS and 1 mM-MgCl₂ essentially as described under 'Radioligand binding assays' above.

Protein determinations

Proteins were determined using Bio-Rad's adaptation of Bradford's method [31], with BSA (Sigma A-6003) as standard.

Data analysis

Saturation and competition curves were analysed by the non-linear least-squares curve-fitting computer program LIGAND [32].

RESULTS

[^3H]BK binding to soluble preparations of bovine myometrium

Treatment of bovine myometrial membranes with the detergent CHAPS effectively solubilized [^3H]BK binding from the mem-

Fig. 1. Saturation binding isotherms for [³H]BK in soluble bovine myometrial B2-kinin-receptor preparations in the absence and presence of Gpp[NH]p

Increasing concentrations of [³H]BK were incubated with soluble preparations (200–400 μg of protein/0.5 ml of assay volume), and the binding assays were performed as described in the Experimental section. Symbols: ●, ■, control (●, total; ■, non-specific); ○, □, +100 μM-Gpp[NH]p (○, total; □, non-specific). Each point is the average for duplicate determinations. The graphs are representations of single experiments which were repeated three to four times with comparable results. The specific binding parameters obtained using the non-linear curve-fitting program LIGAND [32] were: control: $K_D = 334$ pM, $B_{max} = 798.4$ fmol/mg of protein; +100 μM-Gpp[NH]p: $K_D = 952$ pM, $B_{max} = 336.7$ fmol/mg of protein.

Fig. 2. Competition of specific [³H]BK binding with kinin peptides in soluble bovine myometrial B2-kinin-receptor preparations

A constant concentration of [³H]BK (approx. 5 nM) was incubated with soluble preparations and with increasing concentrations of kinin peptides as shown, and the binding assays were performed as described in the Experimental section. Symbols: ●, BK; ▲, [D-Arg⁰-Hyp²-D-Phe⁷]BK; ■, Lys-BK; ▼, des-Arg⁰-BK. The results are presented as ‘% of control’ where control refers to specific [³H]BK binding in soluble preparations (200–400 μg of protein/0.5 ml of assay volume) as determined in the presence of 1 μM-BK; ‘100% specific binding’ represents 789 fmol of [³H]BK bound/mg of protein. Each assay point is the average of duplicate determinations and the result is the average ± S.E.M. of three experiments.

Table 1. Inhibition constants (K_i) for various kinin peptides on [³H]BK binding to particulate and soluble preparations of bovine myometrium

Inhibition constants were determined by the non-linear curve-fitting computer program LIGAND [32]. K_i values for soluble preparations were determined by using the competition curves shown in Fig. 2. K_i values for particulate preparations were determined by using the competition curves described in [19]. The values in parentheses indicate the numbers of experiments. Values from those series with three experiments are expressed as the mean ± S.E.M. Values from those series with two experiments are expressed as the mean; the values differed by < 10 % within each series.

Kinin peptide	K_i (pM)	
	Particulate	Soluble
Agonists		
BK	8.4 (2)	312 ± 70 (3)
Lys-BK	1.9 (2)	56 ± 7 (3)
Tyr-BK	83 (2)	—
Des-Arg ⁰ -BK	> 1 × 10 ⁷ (2)	> 1 × 10 ⁷ (2)
Antagonist		
[D-Arg ⁰ -Hyp ² -D-Phe ⁷]BK	332 (2)	3400 ± 680 (3)

Fig. 3. Association kinetics of specific [³H]BK binding to soluble bovine myometrial B2-kinin-receptor preparations

A soluble preparation was incubated in one large assay volume (approx. 16 ml) with approx. 6 nM-[³H]BK. At the indicated time intervals, aliquots (0.5 ml) were removed and the amount of bound radioligand was determined by Sephadex G-50 chromatography as described in the Experimental section. Non-specific binding was assayed separately; it was maximal at the first time point (0.5 min) and represented 2–5 % of total binding. Each assay point is the average of duplicate determinations and the result is representative of two experiments whose results differed by less than 10 %.

branes. At 5 mM-CHAPS and a detergent/protein ratio of 1.25:1, approx. 60 % of the receptors present in the membranes were solubilized. Higher detergent/protein ratios resulted in greater amounts of protein being solubilized without increases in [³H]BK binding activity. In contrast, lower detergent/protein ratios resulted in considerably lower yield. Furthermore, the overall recovery of [³H]BK binding after solubilization was not influenced by agonist occupancy of the receptor. As shown in Fig. 1, [³H]BK binding to soluble preparations was saturable and of high affinity. Non-linear regression analysis of saturation curves revealed an equilibrium dissociation constant (K_D) of 314 ± 70 pM (mean ± S.E.M., $n = 4$) and a maximum number of binding sites (B_{max}) of 1119 ± 160 fmol/mg of protein. The specific activity of the [³H]BK binding in soluble preparations was considerably higher than that in particulate preparations (476 ± 48 fmol/mg

Fig. 4. Dissociation kinetics of specific [3 H]BK binding to soluble bovine myometrial B2-kinin-receptor preparations in the absence or presence of Gpp[NH]p

(a) Membrane preparations were incubated for 90 min at 25 °C with approx. 5 nM-[3 H]BK. After incubation, membranes were centrifuged and then solubilized as described in the Experimental section. The soluble preparation was then divided into two aliquots, to one of which was added 100 μ M-Gpp[NH]p. Both aliquots were then incubated in the presence of 1 μ M-BK at 25 °C. At the indicated time intervals, samples were taken and amount of bound radioligand was determined by Sephadex G-50 chromatography. Symbols: ○, +100 μ M-Gpp[NH]p; ●, -100 μ M-Gpp[NH]p. The results are presented as '% of control', where control refers to binding immediately after addition of BK (161.3 $\pm$ 25.8 fmol; n = 4). The assay for each point was performed in duplicate, and the result is the average $\pm$ S.E.M. for four experiments. (b) Membranes were first solubilized as described in the Experimental section, and then incubated with approx. 5 nM-[3 H]BK for 90 min at 25 °C. The preparation was divided into two aliquots, which were treated as described above. The results are described as '% of control', where control refers to specific [3 H]BK binding. Total/non-specific [3 H]BK binding is 169.8 $\pm$ 7.4/4.8 $\pm$ 1.1 fmol (n = 4). The assay for each point was performed in duplicate, and the result is the average $\pm$ S.E.M. for four experiments.

of protein), indicating that CHAPS at the concentration used was slightly selective for extracting [3 H]BK binding activity as compared with total protein.

Binding of [3 H]BK to solubilized preparations displayed a typical B2 kinin specificity. The order of potency for a series of kinin agonists and an antagonist in competing for [3 H]BK binding was Lys-BK > BK > [D-Arg⁰-Hyp²-D-Phe⁷]BK $\gg$ des-Arg⁹-BK (Fig. 2). This order of potency was identical with that observed for [3 H]BK binding in particulate

Fig. 5. Effect of Gpp[NH]p on equilibrium binding of [3 H]BK to soluble bovine myometrial B2-kinin-receptor preparations

Soluble preparations were incubated with [3 H]BK at 5.5 nM (○, ●), 0.19 nM (■, □), 0.11 nM (▼) and 0.05 nM (▲) in the absence or presence of various concentrations of Gpp[NH]p (closed symbols) and App[NH]p (open symbols). Assays were performed essentially as described in Fig. 2; 100 % specific [3 H]BK binding is as shown in Fig. 2. The assay for each point was performed in duplicate and the result is representative of two experiments whose results differed by less than 10 %.

preparations of bovine myometrium [19], and demonstrates that B2 kinin receptors were solubilized. The K_i values calculated from the competition curves of kinin peptides for [3 H]BK binding in soluble and particulate preparations are shown in Table 1.

Fig. 3 shows that, at 25 °C, association of [3 H]BK with the soluble binding sites was rapid and reached an essentially maximal value at the first time point measured (0.5 min). The Figure also shows that, under these conditions, binding was stable for at least 60 min. It should be noted that the rate of association is exaggerated, since some association will occur during the time (approx. 2 min) it takes for the sample to enter the Sephadex G-50 column used to separate bound from free [3 H]BK.

Fig. 4(a) shows that, after a 60 min equilibration period with the soluble preparation at 25 °C, dissociation of [3 H]BK was biphasic. At 4 nM, approx. 50 % of the binding dissociated rapidly, whereas the residual binding dissociated considerably more slowly. Again, as described above, the actual rates may be slightly exaggerated, owing to the conditions used for separating bound and free [3 H]BK. Fig. 4(b) shows that [3 H]BK which had been equilibrated with the binding sites before solubilization dissociated with kinetics very similar to those observed above. These results indicate that the relative yield of the two binding components observed in kinetic experiments after solubilization was not influenced significantly by agonist occupancy.

Effect of Gpp[NH]p on [3 H]BK binding to soluble preparations of bovine myometrium

Soluble [3 H]BK activity was sensitive to guanine nucleotides. As shown in Fig. 1, saturation binding curves revealed that addition of 100 μ M-Gpp[NH]p significantly decreased both the affinity and the $B_{\max}$ of [3 H]BK binding to soluble preparations as compared with control experiments. Binding curves in the presence of Gpp[NH]p were monophasic, with a K_D of 929 $\pm$ 129 pM (mean $\pm$ S.E.M., n = 3) and a $B_{\max}$ of 706 $\pm$ 38 fmol/mg of protein.

The effect of Gpp[NH]p on [3 H]BK binding was at least in part due to an increase in the dissociation rate of the radioligand. Fig.

Fig. 6. Sucrose density gradient sedimentation profiles of soluble bovine myometrial B2 kinin receptors in the absence or presence of Gpp[NH]p

Soluble preparations were prepared in the absence ('Control', ●) or presence of 50 μ M-Gpp[NH]p ('+Gpp[NH]p', ○) as described in the Experimental section. A 0.5 ml aliquot (approx. 300 fmol of specific [3 H]BK binding activity) of the appropriate sample including catalase was placed on top of a 5–15% linear sucrose gradient made either in the absence or presence of 50 μ M-Gpp[NH]p; centrifugation was in an SW41 rotor at 39000 rev./min for 17 h at 4 °C. Each fraction was chromatographed on a G-50 column and then assayed in the absence of Gpp[NH]p as described in the Experimental section. The results are presented as '% of maximum', where maximum represents specific binding in the peak fraction of individual experiments. Total/non-specific binding in the peak fraction of each of the samples was: 'Control', $1195 \pm 248/111 \pm 11$ d.p.m.; '+Gpp[NH]p', $1700 \pm 426/171 \pm 26$ d.p.m. Catalase was recovered at $11.75 \pm 0.03\%$ sucrose ($n = 6$), 'Control' at $9.9 \pm 0.01\%$ sucrose ($n = 3$) and '+Gpp[NH]p' at $8.8 \pm 0.04\%$ sucrose ($n = 3$). Each assay point represents the average $\pm$ S.E.M. of single determinations from three separate experiments. Some points have error bars smaller than the symbol itself.

4(a) shows that, in the presence of Gpp[NH]p, dissociation of [3 H]BK after equilibration of binding for 60 min at 25 °C was still biphasic. At 4 nM, approx. 50% of the binding still dissociated at a relatively high rate. However, the slower phase dissociated with a rate that was considerably higher than in control experiments. Gpp[NH]p had essentially the same effect on [3 H]BK dissociation from binding sites that had been labelled with the radioligand before solubilization (Fig. 4b).

As shown in Fig. 5, the effect of Gpp[NH]p on soluble [3 H]BK binding activity was dose-dependent and more pronounced at lower than at higher concentrations of [3 H]BK. At 0.05–0.19 nM-[3 H]BK, Gpp[NH]p inhibited binding approx. 35% with an IC_{50} (concn. causing 50% inhibition) of 0.5 μ M. On the other hand, at 5.5 nM-[3 H]BK, Gpp[NH]p inhibited binding only 10%. Fig. 5 also shows that the effect appeared to be specific for guanine nucleotides, as App[NH]p at 100 μ M had no significant effect on the binding at either low or high [3 H]BK concentration.

Sucrose-density-gradient sedimentation of [3 H]BK binding activity in soluble preparations in the presence or absence of Gpp[NH]p

In order to determine whether the effect of Gpp[NH]p on the [3 H]BK binding characteristics is correlated with a molecular change in the receptors, soluble preparations were analysed by

sucrose-density-gradient sedimentation. Membrane-bound receptors were first prelabelled with BK in the presence or absence of 50 μ M-Gpp[NH]p and then extracted with CHAPS. The soluble preparations were then applied to 5–15% linear sucrose gradients. Each fraction was then assayed for [3 H]BK binding activity. Gpp[NH]p was maintained at a concentration of 50 μ M in the appropriate sample until gradient fractionation, but was excluded from the subsequent assays. Fig. 6 shows that the sedimentation rate of the [3 H]BK binding sites was different in the absence or presence of Gpp[NH]p. Both macromolecular species sedimented behind catalase. In the absence of Gpp[NH]p, binding activity migrated as a relatively larger species ($9.9 \pm 0.01\%$ sucrose, mean $\pm$ S.E.M., $n = 3$), whereas in the presence of Gpp[NH]p, binding activity migrated as a relatively smaller species ($8.8 \pm 0.04\%$ sucrose, $n = 3$). Thus different [3 H]BK binding species were observed in the presence and absence of Gpp[NH]p. The apparent decrease in the molecular size of the binding species was paralleled by a change in the dissociation kinetics for [3 H]BK.

DISCUSSION

We have shown that: (1) the detergent CHAPS solubilizes B2 kinin receptor sites from bovine myometrial membranes; (2) [3 H]BK binding to soluble B2 kinin receptors is sensitive to guanine nucleotides; (3) retention of guanine-nucleotide-sensitivity does not depend on agonist occupancy of receptors before or during solubilization; and (4) guanine nucleotides promote a decrease in the sedimentation rate of soluble B2 kinin receptors as determined by sucrose-density-gradient sedimentation. These results show that the guanine-nucleotide-promoted change in the [3 H]BK binding characteristics is directly associated with a structural change in the B2 kinin receptor, and the results support our previous contention that at least some B2 kinin receptors in bovine myometrial membranes are coupled to (a) G-protein(s).

B2 kinin receptors in particulate and CHAPS-solubilized preparations of bovine myometrium have been studied by others using 125 I-Tyr-BK [33–35]. These studies focused primarily on the pharmacological characteristics of the receptors and did not address either receptor heterogeneity or guanine-nucleotide-sensitivity. On the basis of our previous studies in this tissue, we proposed that [3 H]BK identifies two subpopulations of B2-kinin-receptor sites: a low-affinity receptor site, which is apparently not sensitive to guanine nucleotides, and a high-affinity receptor site, which is sensitive to guanine nucleotides [19]. We found that binding of [3 H]BK to the guanine-nucleotide-sensitive high-affinity receptor sites is complex and involves a time-dependent decrease in the dissociation rate for [3 H]BK. This decrease is blocked by guanine nucleotides.

After equilibrium binding to soluble preparations in the absence of guanine nucleotides, the dissociation of [3 H]BK was heterogeneous. Two dissociation phases were clearly distinguishable under these conditions, indicating that either two subpopulations of binding sites are present or [3 H]BK dissociates with two rates from a single site. If two subpopulations of binding sites are present, they must have similar equilibrium dissociation constants, since saturation curves under the same conditions were homogeneous.

After equilibrium binding in the presence of guanine nucleotides, the dissociation of [3 H]BK was also heterogeneous. The rate of the fast dissociation phase observed under these conditions was similar to the rate of the fast phase observed in the absence of guanine nucleotides. In contrast, the guanine nucleotides increased the rate of the slow dissociation phase to an intermediate rate. These results indicate that guanine nucleotides

influenced specifically the rate of the slow phase. The relative amount of the fast dissociation phase was not significantly different in the presence or absence of guanine nucleotides. Thus our results do not support a guanine-nucleotide-promoted conversion of the slowly dissociating component of the binding to the rapidly dissociating component. Rather, the guanine nucleotides seem to specifically alter the slowly dissociating component by increasing its rate of agonist dissociation.

As described in Fig. 5, the Gpp[NH]p dose-response curves revealed that the maximal level of inhibition of the total specific [3 H]BK binding by guanine nucleotides was approx. 35%, indicating that only a portion of the binding was guanine-nucleotide-sensitive. Furthermore, the level of inhibition of binding by guanine nucleotides depended upon the [3 H]BK concentration used, such that that the maximal inhibition (35%) was only observed using [3 H]BK concentrations close to, or below, the apparent K_D , and at a concentration (5.5 nM) well above the apparent K_D , the inhibition was significantly less (10%). These results show that, upon addition of guanine nucleotides, there is a specific portion of the binding that is detectable only at relatively high [3 H]BK concentrations. Thus guanine nucleotides probably modulate the affinity of [3 H]BK binding. As described below, an effect on the affinity by guanine nucleotides was confirmed in saturation binding experiments.

Guanine nucleotides promoted a 3-fold increase in the apparent K_D for [3 H]BK and a decrease in the maximal number of binding sites recognized by the highest [3 H]BK concentration used in soluble preparations. The increase in the K_D is consistent with an increase in the dissociation rate of [3 H]BK after guanine nucleotide treatment. Two explanations can be offered for the apparent loss of binding after guanine nucleotide treatment. Either guanine nucleotides decrease the number of binding sites present in the soluble preparation or guanine nucleotides decrease the affinity so that binding to some sites is too low to be detected even with the maximum [3 H]BK concentration used. The following observations are relevant in determining which explanation may be more likely: (1) in contrast with soluble preparations, our previous studies showed that guanine nucleotides did not decrease binding in particulate preparations [19]; (2) as shown in Table 1, solubilization resulted in an approx. 50-fold decrease in the overall affinity of all the agonists tested; and (3) as shown in Fig. 1, the addition of guanine nucleotides to the soluble preparation resulted in a decrease in the apparent affinity of the detectable binding. These observations lead us to believe that the apparent loss of a portion of the binding in response to guanine nucleotides in soluble preparations is actually due to a decrease in the binding affinity rather than a real loss of binding sites.

The slopes of the competition curves for the agonists BK, Lys-BK, and the antagonist [D-Arg⁰-Hyp²-D-Phe⁷]BK on [3 H]BK binding to soluble preparations were all close to unity. These results are in partial contrast with those observed for [3 H]BK binding to particulate preparations, where the slope of the competition curve for Lys-BK was clearly less than unity. The reason for the shallow competition curves for Lys-BK may be that it is better than [3 H]BK at discriminating between the two subpopulations of binding sites. This discriminatory property may be lost when the receptors are moved from their native membrane-bound form to their soluble form.

Sucrose-density-gradient sedimentation profiles showed that [3 H]BK bound to at least two distinct molecular forms of the soluble bovine myometrial B2-kinin-receptor-binding sites; one faster-sedimenting form was seen when receptors were prepared in the absence of guanine nucleotides, and another slower-sedimenting form was seen when receptors were prepared in the presence of guanine nucleotides. These results indicate that the guanine-nucleotide-promoted increase in the dissociation rate of

[3 H]BK from a slow rate to an intermediate rate is paralleled by a change in the molecular structure of the guanine-nucleotide-sensitive B2 kinin receptor from an apparently larger form to an apparently smaller form. This structural change may be a consequence of a guanine-nucleotide-promoted conversion of the receptor from a larger G-protein-coupled form to a smaller G-protein-uncoupled form in a manner analogous to that described for adenylate cyclase-coupled receptors [20–26]. The relationship between the [3 H]BK binding component that dissociates rapidly and the two receptor forms observed on sucrose gradients is unclear. Either this binding component is integral to the binding site carried on the two receptor forms observed or it is carried on a distinct receptor species. Careful inspection of the sedimentation profiles show that some heterogeneity is present at the high-density end of the peaks in both the untreated and treated samples. This heterogeneity suggests the presence of other binding species in the soluble preparation. Owing to the low resolution of these other binding species, assessment of their guanine-nucleotide-sensitivity would first require their partial purification.

We have shown that solubilization of bovine myometrial membranes with CHAPS results in extraction of B2-kinin-receptor sites. Our results indicate that there are two [3 H]BK binding components in soluble preparations which are similar in terms of affinity for [3 H]BK, but different in terms of [3 H]BK dissociation kinetics. The binding component which dissociated rapidly was not modulated by guanine nucleotides, whereas the binding component which dissociated slowly was modulated by guanine nucleotides. The guanine-nucleotide modulation was observed as a change in the dissociation rate of [3 H]BK from a low rate to an intermediate rate. [3 H]BK recognized two major receptor forms on sucrose density gradients; one form was observed in the absence of guanine nucleotides and the other in the presence of guanine nucleotides. We propose that the guanine-nucleotide-sensitive component is carried by the major species observed on the gradients. We further propose that the apparently larger form constitutes a G-protein-coupled form of the receptor from which at least a portion of the [3 H]BK dissociates with a low rate and the apparently smaller form constitutes a G-protein-uncoupled form of the receptor from which at least a portion of the [3 H]BK dissociates with an intermediate rate. These results strongly suggest that at least some bovine myometrial B2 kinin receptors are coupled to G-proteins.

This work was supported by grants from the American Heart Association, Texas Affiliate (87G365), the Robert A. Welch Foundation (AQ 1087) and the National Institutes of Health (GM 41659) to L.M.F.L.-L. We thank M. Tropea and M. Lorenzetti for expert technical assistance. We also wish to thank Dr. J. M. Stewart and Dr. R. J. Vavrek for kindly donating [D-Arg⁰-Hyp²-D-Phe⁷]bradykinin and Dr. S. J. Lucania for kindly donating captopril (SQ14,225) and teprotide (SQ20,881).

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Received 6 June 1990/12 November 1990; accepted 18 November 1990