Negative Cooperativity in the Human Bradykinin B\textsubscript{2} Receptor*  

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A human kidney bradykinin (BK) B\textsubscript{2} receptor cDNA was transfected in CHO-K1 cells to establish cell lines that express stably and at high density a receptor exhibiting B\textsubscript{2} receptor properties in terms of coupling to cell signaling effectors, desensitization, and internalization. A cell line with a density of $1.3 \times 10^6$ receptors/cell allowed us to carry out a detailed study of BK-receptor interaction over a wide range of BK concentrations. A model assuming that BK binds to two receptor affinity states (depending on guanine nucleotide-sensitive coupling) was not sufficient to account for the kinetics of BK binding. Equilibrium kinetic analysis and studies of the effects of receptor occupancy by agonists or antagonists on the kinetics of BK-receptor complex dissociation revealed features typical of negative cooperative binding. The negative cooperativity phenomenon was also observed in isolated membranes in both the presence and absence of guanine nucleotide. Thus, following the interaction with BK, B\textsubscript{2} receptor molecules likely interact with each other, resulting in an acceleration of bound ligand dissociation and a decrease in the apparent affinity of the receptor for BK. This phenomenon can participate in the desensitization process.

Bradykinin (BK)\textsuperscript{1} is involved in a variety of physiological and pathological processes, including vasodilation and control of vascular tone, ion transfer in epithelia, and pain (1). BK binds to specific receptors that have been classified into B\textsubscript{1} and B\textsubscript{2} receptors according to their relative affinities for des-Arg\textsuperscript{9}-BK and BK (2). These two types of receptors belong to the superfamily of seven-transmembrane domain receptors (3, 4). Most of the BK effects described so far are mediated by the B\textsubscript{2} receptor subtype. BK receptors are coupled through pertussis toxin-insensitive G proteins (5, 6) to at least two separate pathways of phospholipid metabolism (7–12), the hydrolysis of inositol phospholipids by phospholipase C (PLC) and the release of arachidonic acid by phospholipase A\textsubscript{2} (PLA\textsubscript{2}). PLC stimulation produces the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate is likely responsible for the release of Ca\textsuperscript{2+} from internal stores (13–15), and the physiological effects of BK are thought to be strongly dependent on its ability to mobilize [Ca\textsuperscript{2+}], BK-induced production of inositol phosphates, release of arachidonic acid, and elevation of [Ca\textsuperscript{2+}], as well as in vitro physiological responses to BK are of a smaller magnitude in cells or tissues preexposed to BK (7, 13, 16–19). Agonist-induced phosphorylation of the receptor, resulting in receptor uncoupling and modulation of receptor affinity (20, 21) and loss of cell-surface binding sites (19, 22, 23) as a consequence of internalization of the ligand-receptor complex (19, 23, 24), has been proposed to play a role in the decreased responsiveness to BK. The receptor-mediated desensitization can contribute, together with the action of kinases and the triggering of physiological counter-regulatory mechanisms, to the limitation of the physiological action of the peptide in vivo and reduce the potential therapeutic interest of agonists. After the obtaining and pharmacological characterization of a recombinant human renal BK B\textsubscript{2} receptor that was expressed at high density in CHO-K1 cells, we studied in detail the kinetics of BK interaction with the receptor. This study was performed because analysis of previous studies revealed large variations, by $>4$ orders of magnitude, in the affinity constants reported for BK (4, 21, 25). Leeb-Lundberg and Mathis (26) have demonstrated that the B\textsubscript{2} receptor can be in different affinity states depending on the presence of guanine nucleotide. The present work demonstrates, with the analysis of BK-receptor interaction over a wide range of ligand concentrations and dissociation kinetic experiments, a new property of the B\textsubscript{2} receptor, which is negative cooperativity in the binding of BK, a phenomenon described so far for a few seven-transmembrane domain receptors (27–31).

**EXPERIMENTAL PROCEDURES**  
Cloning of Human BK B\textsubscript{2} Receptor and Transfection—One μg of total RNA prepared from normal human kidney tissue (CLONTECH, Heidelberg, Germany) was reverse-transcribed and amplified (30 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1 min at 72°C) using two B\textsubscript{2} receptor-specific primers, 5’-GGATTTCTCTACACTCCATCTGGAGTCC-3’ and 5’-GGATTTCAAGCCGCTCTCTTGCTGC-3’, corresponding to the sequence of the published human fibroblast B\textsubscript{2} receptor (4) with an EcoRI site added at the 5’-end. The amplified fragment (1232 base pairs) was subcloned into the EcoRI site of pcDNA3 KS\textsuperscript{+} (Stratagene), and both strands were sequenced using [α-\textsuperscript{32}P]dATP (Amersham International, Buckinghamshire, United Kingdom) and an Amplicycle\textsuperscript{TM} sequencing kit (Perkin-Elmer, Lausanne, Switzerland). The cDNA differs in position 641 by A in place of G from the published fibroblastic sequence, but codes for the same amino acid sequence. The construct comprising 108 base pairs upstream of the initiator methionine codon and 30 base pairs downstream of the stop codon was digested with XhoI and BamHI to isolate an insert that was subcloned into corresponding sites of the eucaryotic expression vector pcDNA (Invitrogen, Leek, Netherlands). CHO-K1 cells (American Type Culture Collection, Rockville, MD) were transfected with this construct using the calcium phosphate precipitation method, and selection was done with G418 according to standard procedures (32). Cell clones expressing the recombinant receptor were then selected on the basis of their ability to bind [\textsuperscript{3}H]BK (110 Ci/mmol; NEN, LeBlanc Mesnil, France). These clones as well as parental CHO-K1 cells were grown in Ham’s F-12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.
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10\% fetal calf serum, 1 mM glutamine, 0.2 units/ml penicillin, 20 pg/ml streptomycin, and 0.5 mg/ml amphotericin B and maintained at 37 °C in a humidified water-jacketed incubator with 5% CO\textsubscript{2}. BK receptor expression was stable for at least 30 passages.

**Measurement of [\textsuperscript{3}H]BK Binding in intact Cells**—Binding assays were performed on confluent cells grown on 24- or 48-well plates (~500,000 or 250,000 cells/well, respectively). After two washes with 0.5 ml of HBSS (0.33 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.44 mM KH\textsubscript{2}PO\textsubscript{4}, 127 mM NaCl, 5 mM KCl, 20 mM NaHCO\textsubscript{3}, 5 mM glucose, 10 mM sodium acetate, 0.8 mM MgSO\textsubscript{4}, and 1.5 mM CaCl\textsubscript{2}, pH 7.4), [\textsuperscript{3}H]BK binding was performed for various times at 37 or 4 °C in 0.3 ml of HBSS containing 0.1% BSA and protease inhibitors (8 \times 10^{-5} units/ml aprotinin, 0.1 mg/ml bacitracin, 1 mM 1,10-phenanthroline, and 10^{-5} M captopril). Equilibrium studies were performed with incubation times of 1 and 16 h at 37 and 4 °C, respectively. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled BK and subtracted from total binding. It did not exceed 10\% of total binding. The dissociation of the [\textsuperscript{3}H]BK receptor complex was followed at 4 °C in the absence or presence of unlabeled BK in cells labeled with variable amounts of [\textsuperscript{3}H]BK and washed with HBSS containing 0.1% BSA.

The following BK analogues were tested in the range of 10^{-10} to 10^{-5} M for their ability to compete for 1 nM [\textsuperscript{3}H]BK binding at 37 °C: [\textsuperscript{125}I,\textsuperscript{Tyr(Me)}\textsubscript{5}]BK, [(\textsuperscript{125}I,\textsuperscript{Tyr(Me)}\textsubscript{5}]BK, HOE 140, Lys(\textsuperscript{5})-BK, des-Arg\textsubscript{9}-BK, des-Arg\textsubscript{9}-[Leu\textsubscript{8}]BK, and B<sub>2</sub>-Arg-[Hyp\textsubscript{3},D-Phe\textsubscript{7},Leu\textsubscript{8}]BK (33). The effect of these compounds on the dissociation rate of the [\textsuperscript{3}H]BK-receptor complex at 4 °C were also studied in cells prelabelled with 2 nM [\textsuperscript{3}H]BK.

The bound radioactively labeled ligand was separated from the free ligand by three successive washes with 0.5 ml of ice-cold HBSS. Cell-bound radioactivity was determined by scintigraphy (LKB 1211 Rackbeta, Wallac) either after cell lysis with 0.2 ml of 1 N NaOH or after acid washing according to Haigler et al. (34). This technique consisted of a 4-min treatment with 0.5 ml of ice-cold acid buffer (0.2 N acetic acid and 0.5 N NaOH, pH 2.5) followed by cell lysis with 1 N NaOH to dissociate the radioactivity bound to the cell surface, which is removed by acid washing, from internalized radioactivity, which is acid-resistant. Control experiments established that the sum of acid-binding plus acid-resistant binding gave values similar to those determined after simple cell lysis with NaOH.

In some experiments, cells were pretreated with unlabeled BK to induce desensitization before the determination of cell-surface [\textsuperscript{3}H]BK binding. In these experiments, free BK and cell surface-bound unlabeled BK were removed by washing with 0.5 ml of ice-cold HBSS and a 4-min treatment with 0.5 ml of ice-cold acid buffer (90 mM NaCl, 50 mM sodium citrate, and 0.2 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 5) plus two washes with 0.5 ml of ice-cold HBSS, and [\textsuperscript{3}H]BK binding was then performed for 1 h at 4 °C. In each culture plate, the protein content was determined according to the manufacturer’s instructions (Bio-Rad, Munich, Germany) in three wells using BSA as a standard to normalize the results per mg of cell protein. The specific binding determined after a 1-h incubation at 37 °C with 10^{-5} M [\textsuperscript{3}H]BK was 8.34 ± 2.50 pmol of [\textsuperscript{3}H]BK bound per mg of protein (mean ± S.D., n = 3, 10). [\textsuperscript{3}H]BK Binding in Isolated Cell Membranes—After rinsing with phosphate-buffered saline, cells grown on 75-cm\textsuperscript{2} flasks were scraped and homogenized in 5 mM Tris-HCl, 225 mM sucrose, 5 mM EDTA, and 2 mM MgCl\textsubscript{2}, pH 7.4, with a Potter-Elvehjem homogenizer in an ice bath. Protease inhibitors (leupeptin (10 μM), pepstatin (0.7 μM/ml), aprotinin (80 units/ml), and Pefabloc (0.5 mM)) were added. Cell debris were eliminated by centrifugation at 100,000 × g for 15 min at 4 °C, and the particulate fraction was resuspended at 1500 × g for 15 min in a Sorvall SS34 rotor (referred to as cell membranes) and resuspended in the same medium without sucrose and frozen at -80 °C until use.

[\textsuperscript{3}H]BK binding was performed in a medium containing 50 mM Tris-HCl, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, pH 7.4, and protease inhibitors. Bound radioactivity was separated from free ligand by filtration using Millipore HVLPO2500 filters.

**Measurement of Phospholipase C Activity**—Cells grown on 24-well plates were labeled with 4 μCi/ml [\textsuperscript{3}H]myo-inositol (10-20 Ci/mmol; Amersham International) for 20 h in Ham’s F-12 medium. Extracellular unincorporated radioactivity was then removed by three washes with 0.5 ml of HBSS containing 0.1% BSA. Incubation with test compounds or [\textsuperscript{3}H]BK binding was then performed at 37 °C in HBSS containing 0.1% BSA and protease inhibitors at 37 °C for 10 min and stopped by adding test compounds or vehicles and stopped by the removal of the incubation medium. The incubation medium was mixed with 1000 × g for 5 min, and 0.35 ml of supernatant containing the released [\textsuperscript{3}H]arachidonic acid were transferred into counting vials for liquid scintigraphy. Cell-associated radioactivity was also determined after lysis with 0.2 ml of 1 N NaOH to express results as the ratio between the medium radioactivity containing the [\textsuperscript{3}H]arachidonic acid released and the sum of the medium plus cell-associated radioactivity. The radioactivity in the medium corresponds to the sum of [\textsuperscript{3}H]arachidonic acid plus derived [\textsuperscript{3}H]-labeled metabolites.

**Measurement of Variations in Intracellular Calcium Concentration**—Cells were cultured on a thin glass microscope coverslip coated with polyornithine (150 μg/ml). Then, they were loaded with 5 μM fura-2/AM plus 0.01% pluronid in room temperature for 2 h. The glass coverslip carrying the cells was glued to the bottom of the superfusion chamber and fixed to the stage of an inverted fluorescent microscope equipped with a 40-fold magnification fluorescence objective (Nikon). Cells were continuously superfused, and fluorescence was measured with a PTI Photoscan II microfluorometer as described previously (36).

**Statistical Analysis**—Results are presented as means ± S.D. in the text, tables, and figure legends and are plotted as means ± S.E. on the figures. Comparisons between experimental conditions were performed by analysis of variance.

RESULTS

This study was performed on stable CHO-K1 cell lines transfected with BK \textsubscript{2} receptor cDNA synthesized from human kidney RNAs. No [\textsuperscript{3}H]BK binding and no BK activation of PLC and PLA\textsubscript{2} were detected in nontransfected CHO-K1 cells. One clone (hB\textsubscript{2}-r-CHO) with a high density of [\textsuperscript{3}H]BK-binding sites (1.3 × 10\textsuperscript{6} sites/cell) was used for the experiments. The recombinant receptor in this clone exhibited biochemical and pharmacological features typical of a bradykinin \textsubscript{2} receptor subtype. BK activation induced PLC and PLA\textsubscript{2} activation with EC\textsubscript{50} values of 0.22 ± 0.06 (n = 3) and 1.10 ± 0.20 (n = 3) mM and maximal stimulation of 33- and 6-fold the basal value, respectively. BK activation elevated the cytosolic calcium concentration. Also, as reported for other \textsubscript{2} receptor-expressing cells (7, 13, 19, 20, 22, 23), PLC, PLA\textsubscript{2}, and calcium responses were desensitized following BK application. This was associated with internalization of cell-surface binding sites, a highly temperature-sensitive process that occurred as complexes with BK as evidenced by an acid-salt washing technique. Using 2 × 10^{-5} M [\textsuperscript{3}H]BK, the internalization represented 62.0 ± 10.7\% of the total binding determined after a 1-h incubation at 37 °C (n = 5) and only 4.4 ± 2.7\% (n = 5) when the binding assay was performed for 16 h at 4 °C. The EC\textsubscript{50} values (BK concentration during preincubation) for the desensitization of PLC and PLA\textsubscript{2} responses to 10^{-7} M BK were roughly of 0.1 and 2 nm, respectively. Finally, [\textsuperscript{3}H]BK binding was specifically inhibited by BK receptors agonists and antagonists, and PLA\textsubscript{2} activity was selectively activated by B\textsubscript{2} receptor agonists. Table 1A presents the activity of hB\textsubscript{2}-r-CHO cells that might be related to receptor overexpression is that none of the BK-induced phenomena ([\textsuperscript{3}H]BK binding, receptor internalization rate, and activation of PLC or PLA\textsubscript{2}) were sensitive to phorbol 12-myristate 13-acetate and staurosporine, an activator and an inhibitor of protein kinase C, respectively (data not shown). It is conceivable that the
protein kinase C content in CHO cells is low compared with the receptor content and that only a small undetectable portion of the receptors could be altered by protein kinase C.

Fig. 1 illustrates a comparison of the equilibrium binding of a very wide range of [3H]BK concentrations (from $10^{-10}$ to $10^{-7}$ M) at 37 and 4 °C. The graph shows that at both temperatures, [3H]BK binds to a heterogeneous population of binding sites with differing affinities or in a negative cooperative manner, which implies that receptor affinity decreases with increased receptor occupancy (38). They also indicate that the internalization of the [3H]BK-receptor complexes that occurs at 37 °C is accompanied by a recycling of the receptor back to the cell surface, which explains why maximal binding is greater at 37 °C than at 4 °C. The fact that, at low [3H]BK concentrations, the binding was less at 37 °C than at 4 °C, and that the binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable. The binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable. The binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable. The binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable. The binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable. The binding was greater at 10 °C than at 37 °C at low [3H]BK concentrations, and the binding curves were not superimposable.
dissociation of [3H]BK-receptor complexes had at least two components with differing rates. The presence of unlabeled BK during the dissociation reaction accelerated the complex dissociation. Thus, the rate of the slowest dissociation component, which was $2.2 \pm 0.5 \times 10^{-4}$ min$^{-1}$ in the absence of unlabeled BK, increased to $6.6 \pm 1.9$ and $11.5 \pm 1.9 \times 10^{-4}$ min$^{-1}$ when 2 and 100 nM unlabeled BK, respectively, were present in the solution, indicating that the unlabeled BK accelerating effect occurs in a dose-dependent manner. The dose dependence of this effect was studied in more detail in cells previously labeled with 2 nM [3H]BK (Fig. 2b). The acceleration of [3H]BK dissociation was detected with $4 \times 10^{-10}$ M BK ($p < 0.05$) in these cells and was maximal at $1 \mu$M unlabeled BK. These observations clearly demonstrate that occupancy of free binding sites by unlabeled BK results in an accelerated dissociation of already bound [3H]BK. Furthermore, the greater the number of binding sites occupied by unlabeled BK, the faster the dissociation of the complex.

The dependence of BK-receptor dissociation on receptor occupancy was also demonstrated by the experiments depicted in Fig. 3, which shows that, in the absence of unlabeled BK, the dissociation of bound [3H]BK was faster when there was an initial occupancy of a greater number of binding sites. The dissociation rates of the slow component were $0.8 \pm 0.2$ and $3.7 \pm 1.2 \times 10^{-4}$ min$^{-1}$ with initial occupancies of $0.050 \pm 0.005$ and $0.951 \pm 0.130$ pmol of [3H]BK/mg of protein, respectively.

Table II summarizes the effects of various structural BK analogues on [3H]BK dissociation studied at 4 °C using the same experimental conditions used as for BK. The effects of these analogues on [3H]BK binding and PLC activity at 37 °C are presented in Table I to indicate their agonist or antagonist properties in hB2r-CHO cells. The data show that, like BK, the two B2 receptor agonists [Hyp$^3$,Tyr(Me)$^6$]BK and [Aib$^7$]BK accelerated the dissociation of bound [3H]BK; [Aib$^7$]BK, a strong agonist that is able to induce an increase in IP production

1.7-fold higher than the maximal response to BK, produced the same effect as BK on [3H]BK dissociation. d-Arg-[Hyp$^3$,D-Phe$^7$,Leu$^8$]BK, which behaved as a partial agonist, and the B2 receptor antagonist HOE 140, which completely inhibited BK-sensitive IP production, were also able to accelerate [3H]BK dissociation. Similar results were obtained with [d-Phe$^7$]BK, which is another B2 receptor antagonist (33). In contrast, the

[FIG. 2. Accelerated dissociation of bound [3H]BK by unlabeled BK. All experimental steps were carried out at 4 °C. They included a 20-h incubation of cells with 2 nM [3H]BK, extensive washing with HBSS containing 0.1% BSA to remove unbound [3H]BK, and a second incubation in the absence or presence of unlabeled BK with determination of the specific binding fractions that were released in the medium or remained associated with cells. The cell-associated binding is expressed as a percentage of the specific binding determined just before the dissociation reaction. a, the second incubation was performed for the different indicated times without (no addition) or with unlabeled BK (2 or 100 nM). The slope of the regression lines corresponding to cell-associated binding values at 60, 180, and 300 min was determined to estimate the dissociation rate constant given under “Results.” b, the second incubation was performed for 3 h with the indicated unlabeled BK concentrations. Values are from three independent experiments, each performed in duplicate.

[FIG. 3. Dependence of [3H]BK dissociation rate on receptor occupancy level. Experiments were conducted and data were expressed as explained in the legend to Fig. 2a, except that cells were labeled with $10^{-10}$ or $10^{-8}$ M [3H]BK, and no unlabeled BK was added during the dissociation reaction. The specific binding before dissociation was $0.050 \pm 0.005$ and $0.951 \pm 0.130$ pmol of [3H]BK/mg of protein, respectively. The dissociation rate constants given under “Results” were estimated from cell-associated binding values at 120, 180, 300, and 480 min. Values are from three independent experiments, each performed in triplicate.]
Figure 4. Accelerated dissociation of bound \(^{3}H\)BK by unlabeled BK in isolated hB\(_{2}\)-r-CHO cell membranes: effect of guanine nucleotide. Experiments were carried out at 25°C. Cell membranes were incubated for 90 min with 2 nM \(^{3}H\)BK. Then, 50-μl aliquots of the reaction mixture were transferred into 5 ml of \(^{3}H\)BK-free incubation medium with no addition or with unlabeled BK (100 nM), GTPγS (10 μM), or both. \(^{3}H\) Radioactivity bound to membranes was determined at the times indicated. After subtraction of nonspecific binding measured with cell membranes incubated with 2 nM \(^{3}H\)BK plus 2 μM unlabeled BK, values were expressed as a percentage of the specific binding determined just before the dissociation reaction. Values are from six determinations.

![Figure 4](image3)

**TABLE II**

| Analogues | conc | Cell-associated radioactivity |
|-----------|------|-----------------------------|
| None      |      | 74.8 ± 3.4 \(^{g}\)        |
| B\(_{2}\) agonists |      |                             |
| BK        | \(10^{-7}\) | 58.3 ± 1.7 \(^{g}\) |
| [Hyp\(^{3}\),Tyr(Mo\(^{8}\))]BK | \(10^{-7}\) | 56.8 ± 2.0 \(^{g}\) |
| [Alb\(^{6}\)]BK | \(10^{-5}\) | 58.5 ± 1.4 \(^{g}\) |
| B\(_{2}\) antagonists |      |                             |
| HOE 140   | \(10^{-5}\) | 58.2 ± 5.3 \(^{g}\) |
| \(d\)-Arg-[Hyp\(^{3}\),\(d\)-Phe\(^{5}\),Leu\(^{7}\)]BK | \(10^{-5}\) | 62.5 ± 4.2 \(^{g}\) |
| \(d\)-Phe\(^{5}\)BK | \(10^{-5}\) | 65.3 ± 2.5 \(^{g}\) |
| B\(_{2}\) analogues |      |                             |
| Des-Arg\(^{9}\)BK | \(10^{-5}\) | 75.5 ± 4.0 \(^{g}\) |
| Lys-des-Arg\(^{9}\)BK | \(10^{-5}\) | 78.7 ± 1.7 \(^{g}\) |
| Des-Arg\(^{9}\)-[Leu\(^{7}\)]BK | \(10^{-5}\) | 77.8 ± 2.5 \(^{g}\) |

**DISCUSSION**

This study provides, for the first time, evidence that the interaction between BK and the BK B\(_{2}\) receptor exhibits negative cooperativity. This phenomenon implies that BK binding is accompanied by a decrease in receptor affinity.

The cells used in this study were CHO-K1 cells transfected with a BK B\(_{2}\) receptor cDNA synthesized from human kidney RNAs. A cell clone (hB\(_{2}\)-r-CHO) that expresses the receptor stably at high density (1.3 × 10\(^{9}\) sites/cell) was used for this study because it allowed us to analyze the ligand-receptor interaction over a wide range of ligand concentrations.

Our data show that the specific \(^{3}H\)BK binding to hB\(_{2}\)-r-CHO cells displays heterogeneous features in terms of binding affinity (curvilinear Scatchard plots and a Hill coefficient value less than unity) and \(^{3}H\)BK dissociation rate (more than one component). Such a heterogeneity cannot be accounted for by the presence of other BK receptors in addition to the recombinant receptor. Indeed, there was no specific \(^{3}H\)BK binding, no BK activation of PLC and PLAr, and no increase in cytosolic calcium upon the application of BK in the parental nontransfected CHO-K1 cells (data not shown). Also, we can exclude that the heterogeneity in the \(^{3}H\)BK binding is related to the receptor internalization/recycling process, which was almost abolished at the temperature of 4 °C chosen for the experiments. This was further supported by the observation that \(^{3}H\)BK binding resulted also in curvilinear Scatchard plots (data not shown) in the presence of sucrase, which inhibits endocytosis by disrupting clathrin-coated vesicles (39). The use of sucrase to abolish internalization was, however, not considered appropriate in the present work because besides the suppression of internalization, sucrase also markedly reduced the capacity of hB\(_{2}\)-r-CHO cells to bind BK (data not shown), as has also been reported for the binding of growth hormone to its receptor in IM-9 cells (40).

Moreover, lowering the temperature to 4 °C has the advantage of preventing agonist-induced functional coupling of the receptor to cell signaling effectors and abolishing cellular enzymatic reactions like phosphorylation that can modify receptor structure and binding affinity (20, 21). In addition, its combination with the use of protease inhibitors prevented ligand or receptor degradation, a condition required to study the kinetics of ligand binding.

The data obtained in the equilibrium binding studies support a negative cooperativity phenomenon in BK binding. However, the curvilinear Scatchard plots and the observation of a faster \(^{3}H\)BK dissociation rate from cells prelabeled with high compared with low \(^{3}H\)BK concentrations could also be interpreted by the finding of Leeb-Lundberg and Mathis (26) that the unoccupied B\(_{2}\) receptor can exist in various states that have different affinities for BK. By using cell membranes from bovine myometrium and incubating these membranes in the absence of guanine nucleotide, a situation that is comparable to that prevailing in our intact cells incubated at 4 °C (no functional receptor coupling to cell signaling effectors), these authors demonstrated that BK binds to at least two states of the B\(_{2}\) receptor, which consist of a guanine nucleotide-insensitive low affinity state (occupied at high BK concentrations only) from which BK dissociates quickly and a guanine nucleotide-sensitive high affinity state from which BK dissociates slowly.
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The existence of a guanine nucleotide-sensitive high affinity state of the hB$_2$r-CHO cell $B_2$ receptor is suggested by the fact that at low [3H]BK concentrations, equilibrium binding was less at 37 °C than at 4 °C despite the [3H]BK accumulation through the receptor internalization/recycling process that occurred at 37 °C, but not at 4 °C. This decrease in [3H]BK binding at 37 °C is probably related to a cellular GTP effect and involves a guanine nucleotide-sensitive process that is responsible for receptor and G protein coupling to cell signaling effectors and for decreases in receptor affinity like the guanine nucleotide-induced reduction in receptor affinity described by Leeb-Lundberg and Mathis (26) in myometrial cell membranes. In addition, as in myometrial cell membranes (26), [3H]BK dissociation was increased by guanine nucleotide in cell membranes prepared from hB$_2$r-CHO. However, it should be pointed out that a model in which BK interacts in a bimolecular independent fashion with two receptor states cannot entirely account for the equilibrium kinetics observed and for the time courses of BK dissociation observed in hB$_2$r-CHO cells. Indeed, when compared with a theoretical Scatchard plot for such a model (Fig. 5), the data points occupy a rightward position in the graph, suggesting that the affinity of the high affinity state declines with increasing binding site occupancy and that of the low affinity state increases with decreasing binding site occupancy. Moreover, the slow dissociation component, reflecting [3H]BK dissociation from the high affinity receptor state, was more rapid in cells labeled with the higher concentration of [3H]BK, indicating that the affinity of this state decreased when the density of [3H]BK-receptor complexes was increased (Fig. 3). A similar observation is apparent in the study of Leeb-Lundberg and Mathis (26) with myometrial cell membranes. Therefore, these experiments suggest that a phenomenon of negative cooperativity can modulate $B_2$ receptor affinity, whether the receptor is in its guanine nucleotide-insensitive or -sensitive state. According to published studies, the dissociation constant for BK binding to the $B_2$ receptor ($K_d$) may fall between 0.003 and 50 nM, and the $B_2$ receptor may exist in one (19, 41), two (4, 17), or three affinity states (21, 26). We believe that these discrepancies result at least partly from the use of a low (4, 11, 17, 21), intermediate (21, 41, 42), or high (7, 17, 19, 21) labeled BK concentration and from a narrow (4, 11, 19, 41, 42) or wide (17, 21, 26) concentration range of labeled BK, which can prevent observation of the binding regulation by G protein and the cooperativity phenomenon. In the present study, BK binding was studied over 3 orders of magnitude of BK concentrations. This was possible because of the high density of the $B_2$ receptor in hB$_2$r-CHO cells.

The experiments that we believe demonstrate unambiguously negative cooperativity in BK binding are those following the effect of unlabeled BK and of $B_2$ antagonists on the dissociation rate of the [3H]BK-receptor complex, an effect that cannot be accounted for by inhibition of reassociation of dissociated [3H]BK under the experimental conditions used. In cells prelabeled with a fixed concentration of [3H]BK, washed to remove the unbound radioligand, and then challenged with various concentrations of unlabeled peptide, the added BK clearly accelerated the dissociation of the already bound [3H]BK. This indicates that an increase in the density of ligand-receptor complexes by unlabeled BK binding to free receptor molecules induces a decrease in receptor affinity. The rate of [3H]BK dissociation from the high affinity state (slow dissociation component) increased in parallel with the degree of free receptor occupancy by added unlabeled BK. In cells previously labeled with 2 nM [3H]BK, a significant acceleration of [3H]BK dissociation was observed with only $4 \times 10^{-10}$ M unlabeled BK, indicating that a very slight increase in receptor occupancy is sufficient to alter receptor affinity. It is of interest that when experiments were performed with cell membrane preparations, no acceleration of [3H]BK dissociation could be obtained at 4 °C even with $10^{-7}$ M unlabeled BK. However, an accelerated dissociation was observed if the temperature was increased to 25 °C, suggesting that cell membrane fluidity, very likely reduced following the cell membrane preparation, can play an important role in the phenomenon of negative cooperativity (29). The fact that accelerated dissociation could be observed with isolated cell membranes in the absence as well presence of guanine nucleotide further demonstrates that the phenomenon of negative cooperativity can modulate $B_2$ receptor affinity whether or not the receptor is coupled to G protein, as already stated.

Finally, $B_2$ receptor antagonists like BK and other $B_2$ receptor agonists were also able to accelerate the dissociation of bound [3H]BK. Since $B_2$ receptor antagonists fail to elicit any cell response and stabilize a G protein-uncoupled form of the receptor (43), this observation implies that the phenomenon of negative cooperativity can occur independently of functional receptor and G protein coupling to signaling effectors. Negative cooperativity in binding to other G protein-coupled receptors, adrenergic receptors, has also been documented by the use of antagonists (29, 31). Thus, in some receptors, including the $B_2$ receptor, antagonists apparently possess two complementary actions, which are to prevent the binding of agonist to free receptor and to facilitate the dissociation of bound agonist.

In conclusion, the high expression of a renal human $B_2$ receptor in CHO cells enabled us to characterize a negative cooperativity among $B_2$ receptors. Following their interaction with BK molecules, $B_2$ receptor molecules very likely interact with each other, leading to an acceleration of bound BK dissociation. Such a receptor-receptor interaction has been proposed for the thyrotropin receptor expressed in CHO cells, for which a progressive reduction in affinity for thyrotropin was observed with increasing numbers of receptors (27). This indicates that increasing the probability for the interaction between receptor

![Fig. 5. Binding with two independent categories of binding sites does not account for [3H]BK binding in hB$_2$r-CHO cells.](image-url)
molecules favors the phenomenon of negative cooperativity in binding. This has been proposed for receptors that are expressed endogenously to much less extent but that can form clusters or aggregate after ligand binding (44). Interaction between receptor molecules has also been suggested for other receptors. Thus, angiotensin II binding was restored following the coexpression of two single-point mutants of the angiotensin II A1 receptor that were each deficient in ligand binding capacity (45). Similarly, a restoration of carbachol-induced PI hydrolysis has been reported following the coexpression of two fragmented muscarinic m3 receptors deficient in their ability to bind the agonist and to stimulate IP production (46, 47). A protein-protein interaction within the plasma membrane may therefore cause a loss or a gain of functional properties. The present study is the first description of the involvement of negative cooperativity in the desensitization process of the human bradykinin B2 receptor. The molecular basis of this phenomenon is not yet understood for the B2 receptor.

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