

Guanosine Nucleotides Regulate B2 Kinin Receptor Affinity of Agonists But Not of Antagonists: Discussion of a Model Proposing Receptor Precoupling to G Protein

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The effect of nucleotides on binding of the B2 kinin (BK) receptor agonist [³H]BK and the antagonist [³H]NPC17731 to particulate fractions of human foreskin fibroblasts was studied. At 0 °C, particulate fractions exhibited a single class of binding sites with a K_d of 2.3 nM for [³H]BK and a K_d of 3.8 nM for the antagonist [³H]NPC17731. Incubation with radioligands at 37 °C for 5 min gave a reduction of agonist, as well as antagonist, binding that was between 0–40% depending on the preparation, even in the absence of guanosine nucleotides. As shown by Scatchard analysis, this reduction in specific binding was due to a shift in the affinity of at least a fraction of the receptors. The presence at 37 °C of the guanine nucleotides GTP, GDP and their poorly hydrolyzable analogs left [³H]-NPC17731 binding unaffected, but reduced the receptor affinity for [³H]BK to a K_d of about 15 nM. The maximal number of receptors, however, was unchanged. This affinity change was strongly dependent on the presence of bivalent cations, in particular Mg²⁺. It was reversed by incubation at 0 °C. The rank order of the guanosine nucleotides for [³H]BK binding reduction was GTP[γ S] = Gpp[NH]p > GTP = GDP > GDP[β S]. GMP, ATP, ADP and AMP showed no influence on agonist binding. A model for the interaction of the B2 kinin receptor with G proteins is discussed.

Key words: Bradykinin / G protein coupling.

Introduction

The nonapeptide bradykinin (BK) is released from high molecular weight precursors, the kininogens, *via* the action of proteolytic enzymes known as kallikreins. Generation of BK and or lysyl-BK (kallidin) frequently occurs in response to tissue injury or inflammation, where these

peptides can exert a variety of biological effects, including vasodilatation, increased vascular permeability, stimulation of pain and hyperalgesia, and enhanced cell proliferation (Proud and Kaplan, 1988; Bhoola *et al.*, 1992). According to pharmacological criteria, these effects are mediated by at least two different receptors, B1 and B2 receptors (Regoli and Barabe, 1988; Farmer and Burch, 1992). Radioligand binding studies proved the existence of B2 receptors in a variety of tissues and cell lines (Bathon and Proud, 1991). Recently the rat, mouse and human B2 kinin receptors, as well as the human B1 kinin receptor, have been cloned (McEachern *et al.*, 1991; Hess *et al.*, 1992; Menke *et al.*, 1994; Webb *et al.*, 1994) and, based on sequence homology, were grouped into the superfamily of the G protein-coupled receptors (GPCRs). These findings were in agreement with previous reports, that BK, *via* the B2 receptor, is capable of activating phospholipase A and/or C and of inhibiting adenylate cyclase *via* signal transduction pathways that utilize guanosine nucleotide regulatory proteins (Liebmann and Reissmann, 1990; Gutowski *et al.*, 1991; Liebmann *et al.*, 1991; McEachern *et al.*, 1991; Tippmer *et al.*, 1994). However, both the subtype of the G protein(s) involved, as well as the effect of guanine nucleotides on [³H]BK binding sites, are not completely understood. It has been reported that in human fibroblasts (Etscheid and Villereal, 1989) and in bovine aortic endothelial cells (Keravis *et al.*, 1991) addition of guanosine nucleotides may induce a reduction of B2 kinin receptor number. In other studies, a change in affinity of B2 kinin receptors (Burch and Axelrod, 1987; Leeb-Lundberg and Mathis, 1990; Liao and Homcy, 1993) or changes in both affinity and receptor number (Mathis and Leeb-Lundberg, 1991) were reported. By contrast, guanosine nucleotides were reported to inhibit [³H]BK binding to the human B2 kinin receptor expressed in COS-7 cells (Hess *et al.*, 1992), while no effect of these nucleotides was observed on BK binding to receptors in guinea pig lung (Trifillieff *et al.*, 1994).

Cultured human foreskin fibroblasts express high numbers of B2 kinin receptor, which, when occupied by agonist, are subject to strict regulatory control *via* internalization and down-regulation (Roscher *et al.*, 1984). Although G proteins appear to couple these receptors to the synthesis of eicosanoids, thereby accumulating cAMP and the release of inositol phosphates (Roscher *et al.*, 1983; Burch and Axelrod, 1987; Dengler *et al.*, 1990), little is known regarding the exact mechanism of the interaction between the B2 kinin receptor and these G proteins. In this study, we compared the effect of guanine nucleotides on B2 kinin receptor agonist and antagonist binding to partic-

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ulate fractions of human foreskin fibroblasts at 0 °C and at 37 °C.

We were able to demonstrate that at 37 °C the guanosine nucleotides GTP and GDP, and their hydrolysis-resistant analogs, induce a reduction in affinity without a concomitant loss of receptor number in the presence of agonist, but not antagonist. This affinity reduction was reversible by incubation on ice, indicating that at 0 °C the B2 kinin receptor is always in a high-affinity state and therefore presumably coupled to a G protein.

Results

[³H]BK Binding to Particulate Fractions of Human Foreskin Fibroblasts at 37 °C in the Absence and Presence of Gpp[NH]p

Preliminary experiments with particulate fractions showed that binding of [³H]BK, even in the absence of exogenous guanosine nucleotides, rapidly decreased in some preparations with time at 37 °C, but was stable in all preparations at 0 °C for at least three hours. Therefore, for experimental convenience, all incubations were prepared on ice and kept on ice for up to two hours until being used for studies at 37 °C. [³H]BK binding activity measured after a 90 min incubation on ice was referred to as the maximum binding capacity of the preparation used.

In Figure 1 the time course of specific [³H]BK binding at 37 °C to a particulate fraction was followed in the absence and presence of 100 μM Gpp[NH]p. Two concentrations (4 nM and 0.35 nM) of the radioligand were used. Maximum [³H]BK binding measured with these concentrations by incubation for 90 min on ice is shown in the graph at t = 0.

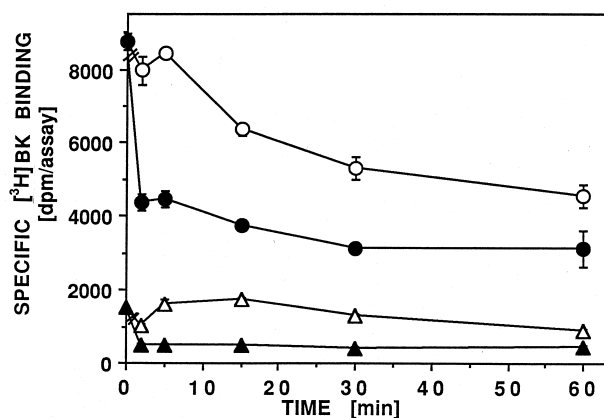


Fig. 1 Time Curve of [³H]BK Binding to Particulate Fractions in the Presence and Absence of Gpp[NH]p.

Aliquots of a particulate fraction were incubated with 0.35 nM (Δ, ▲) and 4 nM (○, ●) [³H]BK in the absence (Δ, ○) or presence (▲, ●) of 100 μM Gpp[NH]p at 37 °C for the indicated times or at 0 °C for 90 min (t = 0). Thereafter specific binding was measured as described under Materials and Methods. Values represent the mean of triplicate determinations. Three other experiments gave similar results, with the exemption to the difference between binding at 0 °C after 90 min and 37 °C at 5 min.

In the absence of Gpp[NH]p, specific [³H]BK binding activity measured at 37 °C reached a maximum value for both concentrations after 5 min that declined with time. In the presence of 100 μM Gpp[NH]p, the binding activity for both [³H]BK concentrations rapidly dropped within 2 min at 37 °C, reaching a plateau that remained almost unchanged for the subsequent 60 min. This indicates that association and binding reduction induced by the presence of Gpp[NH]p had already reached an equilibrium 2 min after warming up to 37 °C. Therefore, all subsequent incubations at 37 °C were performed for 5 min, since at this time point the reduction of specific [³H]BK binding by GTP-analogs was already completed, and problems with the spontaneous reduction of binding activity could also be avoided.

Effect of Mg²⁺ on Specific B2 Kinin Receptor Binding in the Presence and Absence of 100 μM Gpp[NH]p

In the absence of Gpp[NH]p [³H]BK binding activity reached a maximum at about 2 mM Mg²⁺ (Figure 2). Higher concentrations of the divalent cation resulted in a slight reduction of [³H]BK binding. In the presence of 100 μM Gpp[NH]p a continuing reduction of [³H]BK binding with increasing Mg²⁺ concentrations was observed. In the absence of Mg²⁺ only a minor difference (or none in some experiments) between [³H]BK binding activity in the presence and absence of Gpp[NH]p was obtained. A maximum decline in binding activity induced by Gpp[NH]p was observed at Mg²⁺ concentrations higher than 10 mM (Figure 2, inset). Therefore, all assays were performed in the presence of 25 mM MgCl₂ unless otherwise indicated. The divalent cation Ca²⁺ similarly supported the reduction of binding induced by Gpp[NH]p (not shown).

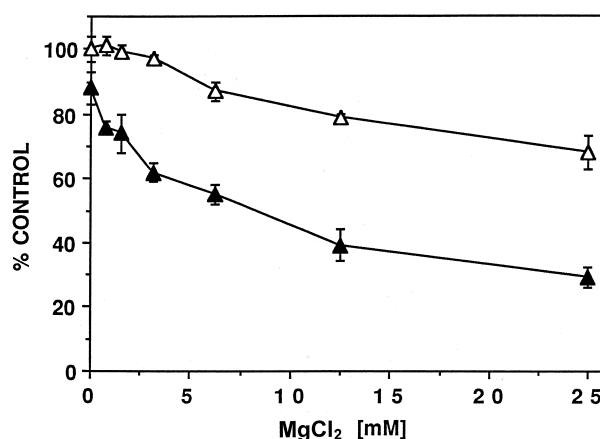


Fig. 2 Influence of Mg²⁺ on [³H]BK Binding in the Absence and Presence of Gpp[NH]p.

Aliquots of a particulate fractions were incubated with 1.2 nM [³H]BK and the indicated concentrations of Mg²⁺ added as chloride salt, for 5 min at 37 °C in the absence (Δ) or presence (▲) of 100 μM Gpp[NH]p. Subsequently, specific binding was measured as described under Materials and Methods. Values represent the mean of triplicate determinations. A representative experiment out of four is shown.

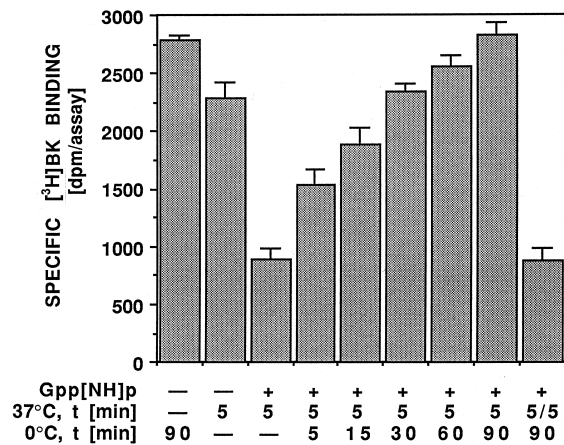


Fig. 3 [^3H]BK Binding Activity at 0°C and 37°C in the Presence and Absence of Gpp[NH]p.

Aliquots of a particulate fraction were incubated with 2.1 nM [^3H]BK in the absence (-) or presence (+) of 100 μM Gpp[NH]p at 0°C for 90 min or at 37°C for 5 min followed by an incubation at 0°C for the time indicated. In the incubation depicted on the last column reaction vials were again incubated at 37°C for 5 min. Thereafter specific binding was measured as described under Materials and Methods. Values represent the mean of triplicate determinations. Another experiment gave similar results.

Effect of Gpp[NH]p on [^3H]BK Binding Activity at 0°C and 37°C

In the experiment shown in Figure 3 [^3H]BK binding activity, measured after an incubation for 5 min at 37°C, was reduced to about 80% of the value obtained after an incubation for 90 min at 0°C. This difference between binding activity at 0°C and 37°C, however, was very variable from one assay to another. Some preparations showed absolutely no difference whereas others revealed at 37°C only 60% of the binding activity as compared to that obtained at 0°C (not shown). Technical reasons such as the temperature during the preparation, the volume of buffer the cells were suspended in, the time of sonification or centrifugation, the protein amount in the assay, or the influence of reducing agents (addition of dithiothreitol and mercaptoethanol) were excluded as the cause for this interassay variability (not shown). The presence of 100 μM Gpp[NH]p at 37°C led to a further reduction of [^3H]BK binding to about 30% of the control value obtained at 0°C. Interestingly, both reducing effects on [^3H]BK binding, the one in the absence (not shown) and the one in the presence of Gpp[NH]p, were slowly reversed by a subsequent incubation at 0°C. The full complement of receptor binding activity was restored within 90 min (Figure 3) at 0°C. A second incubation at 37°C again induced a reduction in [^3H]BK binding that was similar to the initial effect (Figure 3, last column).

Reduction of [^3H]BK Binding by Various Nucleotides

Increasing concentrations of various nucleotides were tested for their capability to reduce [^3H]BK binding in par-

ticulate fractions (Figure 4). Increasing concentrations of all the guanosine nucleotides tested reduced [^3H]BK binding in a dose-dependent fashion. The poorly hydrolyzable nucleotides GTP[γS] and Gpp[NH]p were most efficient with a half-maximal effect at concentrations less than 1 μM , followed by GTP and GDP with a half-maximal effect at about 3 μM . The poorly hydrolyzable compound GDP[βS] gave this effect only at concentrations higher than 10 μM . Maximal reduction was comparable for all nucleotides tested in this experiment, reaching about 30% of the control value obtained in the absence of nucleotides. No reduction was observed by addition of adenosine nucleotides (ATP, ADP, AMP) or GMP even at high concentrations of 1 mM (not shown). Since we found no differences in any of our experiments between GTP[γS] and Gpp[NH]p in terms of reduction of [^3H]BK binding, we used Gpp[NH]p for the subsequent experiments.

Equilibrium Analysis of [^3H]BK (Agonist) and [^3H]NPC17731 (Antagonist) Binding to Particulate Fractions: Effect of Gpp[NH]p

Equilibrium binding of the B2 kinin receptor agonist [^3H]BK to aliquots of a particulate fraction as a function of [^3H]BK concentration is shown in Figure 5 A. At 0°C Gpp[NH]p had no effect on [^3H]BK binding (not shown). The binding curves were steep in the absence of Gpp[NH]p at 0°C as well as at 37°C, reaching a plateau at [^3H]BK concentrations higher than 15 nM. In the presence of Gpp[NH]p at 37°C the binding curve was less steep. Scatchard analysis of these data revealed different receptor affinities, but no difference in maximal binding capacity (Figure 5 A, inset). Only a single category of binding sites with a K_d of 2.1 nM was found for [^3H]BK binding at 0°C. At 37°C, in the absence of Gpp[NH]p, the Scatchard curve was more complex indicating the presence of more than one binding site.

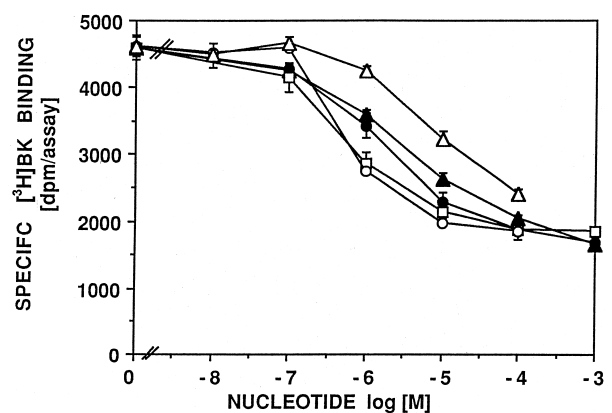


Fig. 4 Inhibition of Specific [^3H]BK Binding by Guanosine Nucleotides.

Aliquots of a particulate fraction were incubated with 2 nM [^3H]BK and the indicated concentrations of guanosine nucleotides for 5 min at 37°C. Thereafter specific binding was measured as described under Materials and Methods. Values represent the mean of triplicate determinations. (○) GTP[γS], (□) Gpp[NH]p, (●) GTP, (▲) GDP, (△) GDP[βS]. For each nucleotide at least one other experiment was performed with similar results.

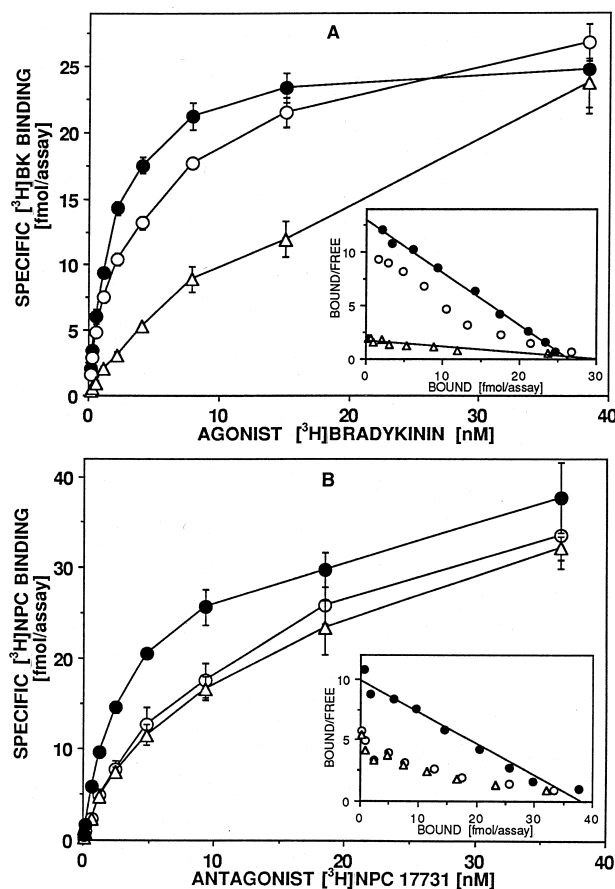


Fig. 5 Equilibrium Binding of Agonist [^3H]BK and Antagonist [^3H]NPC17731 as a Function of Ligand Concentration.

(A) Aliquots of a particulate fraction were incubated with increasing concentrations of [^3H]BK (0.17–38.4 nM) for 5 min at 37°C (○, △) or for at least 90 min at 0°C (●) in the absence (○, ●) or presence (△) of 100 μM Gpp[NH]p. Thereafter specific binding was measured as described under Materials and Methods. Each point represents the mean of triplicate determinations. Inset: Scatchard plot of the data. The lines for the data at 0°C in the absence of Gpp[NH]p and at 37°C in the presence of Gpp[NH]p were computed by linear regression analysis. The equilibrium dissociation constants estimated from the slopes ($-1/K_d$) were 2.0 ± 0.5 nM for binding at 0°C and 13.8 ± 1.1 nM for binding at 37°C in the presence of 100 μM Gpp[NH]p. The average values including other experiments gave a K_d of 2.3 ± 0.4 nM (mean \pm S.E., $n = 4$) for binding at 0°C and 14.6 ± 4 nM (mean \pm S.E., $n = 3$) for binding at 37°C in the presence of 100 μM Gpp[NH]p or GTP[γS] ($n = 3$). (B) A similar experiment is shown with increasing concentrations of antagonist NPC17731 (0.05–36.4 nM). Symbols have the same meaning as described in (A). Inset: Scatchard plot of the data in (B). The equilibrium dissociation constant estimated from the slope ($-1/K_d$) was 3.8 ± 0.12 nM. A second experiment gave similar results. Scatchard analysis using the Enzfitter program did not give reasonable results for agonist and antagonist binding at 37°C in the absence of Gpp[NH]p, when proposing one binding site or two binding sites with different affinities.

In the presence of 100 μM Gpp[NH]p, however, apparently only a single binding site with an affinity of about 15 nM was observed at 37°C.

Equilibrium binding isotherms of the B2 kinin receptor antagonist [^3H]NPC17731 at 0°C and 37°C in the pres-

ence and absence of 100 μM Gpp[NH]p are shown in Figure 5 B. The binding curve obtained at 0°C is distinctly steeper than the curves obtained at 37°C, which exhibited a similar course regardless of the presence of 100 μM Gpp[NH]p. The Scatchard analysis of these data revealed only a single binding site for antagonist binding at 0°C with an apparent K_d of 3.8 nM. At 37°C, however, the Scatchard plot exhibits a bound curve in the absence of Gpp[NH]p, indicating more than one binding site. In addition, this curve was almost identical to the one obtained at 37°C in the presence of Gpp[NH]p, confirming that antagonist binding to B2 kinin receptors is not affected by guanosine nucleotides.

Dissociation Kinetics of [^3H]BK in the Absence and Presence of 100 μM Gpp[NH]p at 37°C

In order to test whether the reduction of B2 kinin receptor affinity by guanosine nucleotides is due to an increase in the dissociation rate, particulate preparations were incubated with [^3H]BK for 5 min at 37°C in the presence and absence of Gpp[NH]p. Dissociation was then induced by addition of unlabeled BK (Figure 6). [^3H]BK dissociated rapidly in the presence and absence of Gpp[NH]p, whereas control binding remained almost unchanged. The semi-logarithmic plot of the data, normalized to the initial value as 100% (Figure 6, inset), however, clearly shows that the dissociation is faster in the presence than in the absence of Gpp[NH]p.

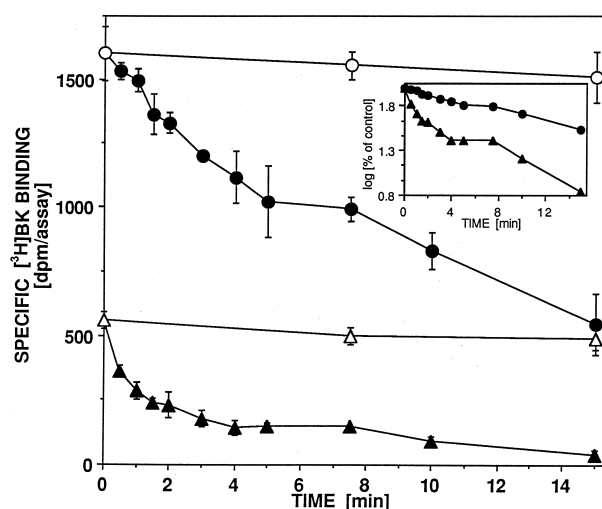


Fig. 6 Dissociation Kinetics of [^3H]BK in the Absence and Presence of Gpp[NH]p.

Aliquots of a particulate fraction were incubated with 2.1 nM [^3H]BK in the absence (○, ●) or presence (△, ▲) of 100 μM Gpp[NH]p for 5 min at 37°C. Dissociation (●, ▲) at 37°C was initiated by addition of unlabeled BK (5 μM final concentration). Specific binding was measured at the indicated times as described under Materials and Methods. Control values without addition of BK are also shown (○, △). Values represent the mean of triplicate determinations. Inset: Semi-logarithmic plot of the dissociation data, normalized to the control values at $t = 0$, using the same symbols.

Discussion

Depending on the tissue source or cell line under study BK is reported to stimulate, or to inhibit, a variety of signal transduction pathways. The role of G proteins, however, in the variety of BK signal cascades is not fully elucidated. Human foreskin fibroblasts expressing high numbers of B2 kinin receptor have already been shown to be an excellent tool to study the regulation and the properties of the human B2 kinin receptor, as well as its signal transduction pathways (Roscher *et al.*, 1983, 1984; Dengler *et al.*, 1990; Fausser *et al.*, 1991). Therefore, we decided to use this cell system to investigate the regulatory role of guanosine nucleotides in the binding of agonist and antagonist to B2 kinin receptors in particulate fractions of human foreskin fibroblasts.

In the presence of GTP, GDP, and their poorly hydrolyzable analogs there was a reduction in agonist ($[^3\text{H}]\text{BK}$), but not antagonist ($[^3\text{H}]\text{NPC17731}$) binding affinity in particulate fractions of human foreskin fibroblasts at 37 °C.

This effect was dependent on the presence of divalent cations, particularly Mg^{2+} . In the absence of Mg^{2+} there was only a minimal reduction of binding activity upon addition of guanosine nucleotides. This dependence on divalent cations may explain the observed lack of reduction of $[^3\text{H}]\text{BK}$ binding to guinea pig lung membranes by GTP analogs (Trifillieff *et al.*, 1994), since Mg^{2+} was not included in the incubation buffer in these studies. As suggested by Keravis *et al.* (1991), the regulation of receptors by G proteins may vary depending on differences in species or tissues. They observed an inhibition of BK binding to B2 kinin receptors in membranes of bovine endothelial cells by GTP, GDP, GTP[γS], and GDP[βS]. This inhibitory effect, however, did not depend on the presence of Mg^{2+} . Indeed, in contrast to our results in fibroblasts, the described effect was almost completely blocked by addition of 5 mM MgCl_2 or 5 mM CaCl_2 . In particulate fractions of fibroblasts we found only a single binding site with a K_d of 2.3 nM in the presence of 25 mM Mg^{2+} at 0 °C. As already described for other B2 kinin receptor assays, Mg^{2+} (25 mM) reduced agonist affinity, since we obtained a higher K_d of 0.7 nM at 0 °C in the absence of Mg^{2+} (not shown).

A spontaneous reduction of binding activity was observed for agonist and antagonist upon incubation at 37 °C. Scatchard analysis of equilibrium binding curves revealed that the reduction in binding activity is the consequence of a change in the affinity of at least a part of the receptors and is not due to a decrease in receptor number. Thus, one can assume that under certain conditions B2 kinin receptors in particulate fractions can change their affinity even in the absence of exogenous guanosine nucleotides. Addition of GTP derivatives led to a further reduction of agonist binding but not antagonist binding. Scatchard analysis of equilibrium binding of agonist ($[^3\text{H}]\text{BK}$) and antagonist ($[^3\text{H}]\text{NPC17731}$) binding revealed that at 0 °C there is only one single category of binding sites with a dissociation constant (K_d) of 2.3 ± 0.4 nM (mean \pm S. E., $n = 4$) for $[^3\text{H}]\text{BK}$ and a K_d of $3.8/5.2$ nM ($n = 2$)

for $[^3\text{H}]\text{NPC17731}$. In membranes of bovine aortic endothelial cells the existence of a high-affinity B2 binding site has been reported for BK at 0 °C, that even at this temperature in the presence of 100 μM Gpp[NH]p is changed to a low-affinity site by incubation for 90 min (Liao and Homcy, 1993). Our results with foreskin fibroblasts are completely different, since incubation at 0 °C actually reversed a prior change in affinity induced at 37 °C (Figure 3). This different influence of the temperature on B2 kinin receptor affinity reduction by guanosine nucleotides may reflect a species-specific difference or might be due to coupling of the B2 kinin receptor to different G proteins in different cell types.

The binding reduction observed for agonist and antagonist binding after an increase of temperature from 0 °C to 37 °C seems to be due to the change in the affinity of a part of the receptors, as can be seen from the Scatchard plots. In the presence of GTP[γS] or Gpp[NH]p at 37 °C only a low-affinity site for the agonist with a K_d of 14.6 ± 4 nM (mean \pm S. E., $n = 3$) was seen. Dissociation kinetics demonstrate that this reduction of receptor affinity by guanosine nucleotides is due to an increase of the dissociation rate for $[^3\text{H}]\text{BK}$ (Figure 6), as also suggested by other reports (Leeb-Lundberg and Mathis, 1990). These results clearly indicate that in human foreskin fibroblasts G proteins influence the affinity but not the number of B2 kinin receptors in particulate fractions. This is in contrast to previous results reported by others (Etscheid and Villeval, 1989) who observed a 45% reduction in B2 kinin receptor number in the presence of 100 μM GTP[γS] in human foreskin fibroblasts.

GTP, GDP and their poorly hydrolyzable analogs GTP[γS], Gpp[NH]p and GDP[βS] were all capable of reducing $[^3\text{H}]\text{BK}$ binding in particulate fractions; the poorly hydrolyzable analogs GTP[γS] and Gpp[NH]p showed highest potencies with IC_{50} values around 1 μM , followed by GTP and GDP (IC_{50} about 3 μM) and GDP[βS] (IC_{50} about 10 μM). GMP and adenosine nucleotides (ATP, ADP, AMP) showed no effect (not shown). Similar potencies of these nucleotides have been reported for the reduction of B2 kinin receptors in bovine endothelial cells (Keravis *et al.*, 1991), for the β -adrenergic receptor (Lefkowitz *et al.*, 1976), and for the α -adrenergic receptor (Rouot *et al.*, 1980). The observation that not only GTP but also GDP exerts a strong effect on agonist binding to B2 kinin receptors has important implications when considering any hypothesis for the interaction of the B2 kinin receptor with G proteins.

Results derived from dissociation studies in the presence and absence of Gpp[NH]p (Leeb-Lundberg and Mathis, 1990) led to the suggestion that the B2 kinin receptor may exist in at least two different states that display different affinities depending on coupling to a G protein. In bovine myometrium, the B2 kinin receptor has been suggested to exhibit a high-affinity binding site when coupled to a G protein, and a low-affinity binding site when uncoupled. Building on this assumption, the similar effects of GTP and GDP in our current study may be explained by the

existence of an intermediate ternary complex of agonist, receptor and G protein, that is only stable in the absence of GTP and GDP as suggested by others (De Lean *et al.*, 1980; Haga and Haga, 1987; Keravis *et al.*, 1991). Since GTP and GDP are almost completely lost during the preparation of the particulate fraction, the receptor should remain coupled to the G protein while exhibiting high affinity for the agonist. Under these circumstances our results would imply that GTP or GDP are able to bind to the G protein in this ternary complex only in the presence of an agonist and thereby promote dissociation of the ternary complex. One would also expect neither binding of GTP/GDP to the ternary complex at 0 °C nor dissociation of this complex, since receptor affinity was not influenced at this temperature.

At 0 °C the agonist binds to B2 kinin receptors only with high affinity. The same appears to be the case for antagonist binding. This, however, can be only deduced indirectly by the fact that an increase of temperature from 0 °C to 37 °C leads to lower affinities for antagonist binding (Figure 5 B) without apparent loss in receptor number. Several other experiments using antagonist concentrations close to 3 nM at 37 °C gave variable binding reductions, ranging between 0% and 40% as compared to values obtained at 0 °C. Experiments were performed with prior incubation of particulate fractions with 0.5 nM unlabeled BK in the absence (mainly high-affinity state) and presence of 100 μM Gpp[NH]p for 5 min (to induce the low-affinity state). Subsequent assays for 5 min at 37 °C with 8 nM [³H]NPC17731 resulted in almost identical antagonist binding activities in both pretreatment groups (not shown). This may suggest that antagonist binding is capable of reversing the affinity reduction, induced by an agonist in the presence of guanosine nucleotides, or that a B2 kinin receptor after dissociation of the agonist immediately returns to its high-affinity conformation. A third explanation taking into account the different consequences of mutations on agonist and antagonist binding (Nardone and Hogan, 1994) may be that the antagonist is not able to differentiate between the high- and the low-affinity states.

Taken together these results are best explained by a model (Figure 7) in which B2 kinin receptors (R) are pre-coupled to G proteins (G) with an empty guanosine nucleotide binding site in the absence of agonist (B) as well as in the presence of antagonist (N). The preceding release of GDP from this receptor-G protein-complex should be fast, not depending on the presence of an agonist. This proposal is also supported by the results of Mathis and Leeb-Lundberg (1991) showing a sensitivity to Gpp[NH]p of soluble B2 kinin receptors of bovine myometrium even when the receptors have been solubilized in the absence of agonist. We have previously shown that gel filtration of B2 kinin receptors solubilized from human foreskin fibroblasts (Faussner *et al.*, 1991) gave an apparent molecular mass for the B2 kinin receptor that was much higher (approximately 250 kDa) than would be expected by the calculated molecular mass of 41 kDa deduced from cDNA analysis (Hess *et al.*, 1992) or by the molecular mass of

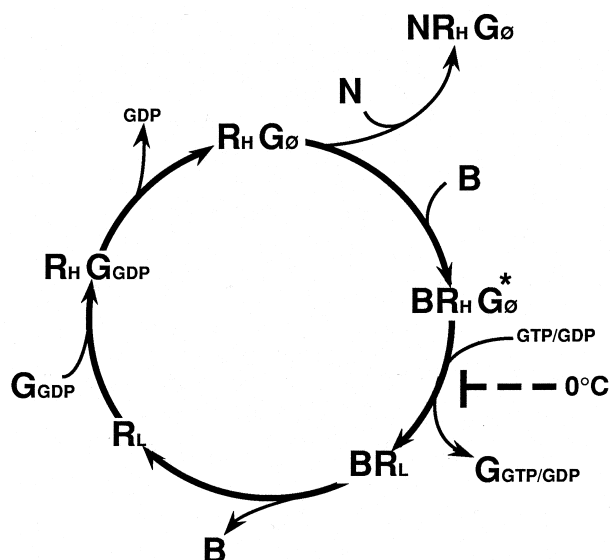


Fig. 7 Proposed Model for the Interaction of the B2 Kinin Receptor with a G Protein.

B, agonist; N, antagonist; R_H, high-affinity receptor; R_L, low-affinity receptor; G₀, G protein without and inaccessible for guanosine nucleotide; G₀^{*}, G protein without bound, but accessible for guanosine nucleotide. The model is explained in the Discussion section.

about 68 kDa that has been found by immunoprecipitation and affinity cross-linking (Abd Alla *et al.*, 1993). This fact may well be due to co-solubilization of coupled G protein(s). According to the model in Figure 7, one could speculate that the major task of an agonist is to open the receptor-coupled G protein for binding of either GTP or GDP. Only binding of GTP would relay a signal; however, due to the prevalence of this guanosine nucleotide in the cell this is almost always the case. Furthermore, opening of the G proteins for guanosine nucleotides by agonists should happen only at 37 °C, but not at 0 °C, and should be blocked by occupation of the receptor with antagonist. Therefore, one can assume that in the absence of an agonist and in the presence of an antagonist, or at 0 °C, a complex is formed consisting of G proteins having no guanosine nucleotide bound and of B2 kinin receptors exhibiting a high-affinity binding for agonist as well as for antagonists. This would explain the co-solubilization of B2 kinin receptors and G proteins. A recent publication describing that antagonists like NPC17731 and Hoe140 may act as inverse agonists in rat myometrial cells supposed that the antagonists bind to and stabilize a G protein uncoupled state of the B2 kinin receptor (Leeb-Lundberg *et al.*, 1994). It should therefore be a low-affinity binding state, always provided that the antagonist is able to discriminate between high- and low-affinity states. In our opinion, however, these data do not demonstrate experimentally that the antagonists preferentially bind to the G protein uncoupled state. The reported fact that antagonists may act as inverse agonists could also be explained by the assumption of a spontaneous opening of the G protein for GTP in the coupled, unoccupied receptor state,

leading to the dissociation of an activated G protein. If occupation of the receptor with an antagonist reduces this spontaneous activation of the coupled G protein, this antagonist would behave like an inverse agonist.

Transfer of the C-terminus of the internalizing B2 kinin receptor to the normally not internalizing B1 kinin receptor resulted in almost completed transfer of the internalization pattern, *i. e.* the receptor chimera responded to stimulation with a B1 kinin receptor agonist with fast receptor internalization (Faussner *et al.*, 1998), despite the fact that the two receptors have only an overall homology of 36% and even less when comparing only the intracellular domains. This result could be easily explained assuming that the internalization process does not require a specific interaction of the remaining part of the receptor with its C-terminus but requires only access of the internalization machinery to the C-terminus. In the proposed model of a precoupled receptor this access, however, would sterically be blocked by the G protein, thus allowing receptor internalization only after agonist stimulation and following activation/release of the blocking G protein.

Our model shown in Figure 7 contrasts somewhat to models developed for other G protein-coupled receptors (*e. g.* the muscarinic receptor, where coupling to G proteins is supposed to be a consequence of agonist binding; De Lean *et al.*, 1980; Haga and Haga, 1987; Freissmuth *et al.*, 1989). Nonetheless, since the G protein-coupled receptors apparently not even share the same seven-transmembrane domain structure as indicated by the fact that the B2 kinin receptor in particular apparently does not have three but only two internal loops (Quitterer *et al.*, 1999) and that they are composed of internal loops and C-termini of very different amino acid composition and length, their regulation by G proteins may also well be very different.

Materials and Methods

Materials

[2,3-prolyl-3,4-³H]bradykinin (108 Ci/mmol) and [prolyl-3,4-³H]-NPC17731 (48.5 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA, USA). All nucleotides were from Boehringer Mannheim (Mannheim, Germany). Unlabeled BK and all other reagents were from Sigma (Deisenhofen, Germany). Hoe140 was a gift from Dr. B. Schoelkens (Hoechst, Frankfurt, Germany).

Cell Culture

Two lines of foreskin fibroblasts (# 7241 and #1634) were purchased from ATCC (Rockville, MD, USA). Cells were used between the 5th and 19th passage. Stock cultures were grown in Eagle's basal medium supplemented with Earle's salts, 10% fetal calf serum, and 2 mM glutamine, as described previously (Faussner *et al.*, 1991). For all experiments confluent stock cultures were trypsinized and split 1:5–1:8 on tissue culture dishes (100 or 145 mm diameter). Medium was changed after 4–7 days and subcultures were used upon reaching confluence usually 7 to 14 days after seeding.

Preparation of Particulate Fibroblast Fractions

Confluent cell monolayers were washed with a hypotonic solution (H₂O) at room temperature and scraped off in ice-cold 20 mM PIPES-buffer, adjusted to pH 6.8 with KOH, containing 2 mM bacitracin and 25 mM MgCl₂ (PBM-buffer), and disrupted by sonification. Thereafter, the homogenate was centrifuged at 4 °C at 30000 *g* for 15 min. For radioligand binding studies the pellets were resuspended by sonification in ice-cold PBM-buffer (unless otherwise indicated).

Binding Assay with [³H]BK or [³H]NPC17731

Binding of [³H]BK and [³H]NPC17731 to homogenates and particulate fractions of fibroblasts was performed as described previously (Faussner *et al.*, 1991) with slight modifications. 100 or 200 μl of an ice-cold suspension of a particulate fraction, corresponding to 15–45 μg protein, were added to reaction vials placed on ice. Nucleotides and other agents were added as 100-fold concentrated solutions, while MgCl₂ × 6H₂O and CaCl₂ × 2H₂O were added as 50-fold concentrated solutions, as indicated in the Figure legends. A volume equal to that of the particulate fraction of PBM-buffer was added containing appropriate concentrations of [³H]BK or [³H]NPC17731 without (for determination of total binding), or with 5 μM unlabeled BK or 5 μM Hoe140 (in antagonist studies) for determination of nonspecific binding. For binding studies at 0 °C, the incubation mixture was shaken and further incubated for at least 90 min on ice. Binding studies at 37 °C were performed by transferring the ice-cold incubation mixture to a water bath. Incubations were then continued for 5 min unless otherwise indicated. Separation of protein-bound ligand from free ligand was obtained by rapid filtration through glass fiber filters (Whatman GF/C), presoaked in ice-cold 0.3% polyethylenimine. The filters were rapidly washed with ice-cold 14% isopropyl alcohol (3 × 4 ml), using a filtration device (Hoeffer Instruments, San Francisco, CA, USA) and the filter-bound radioactivity was determined after addition of 3 ml scintillation liquid. Specific binding was calculated as the difference between total and nonspecific binding. In typical experiments using [³H]BK at a concentration of approximately 2 nM, nonspecific binding was less than 20% of total binding; that of [³H]NPC17731 at a concentration of 4 nM was less than 30%. Data calculation and best curve fits were made using the Enzfitter program from Biosoft (Ferguson, MO, USA).

Protein Determination

Concentrations of protein were measured according to the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

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