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Inhibition of sequestration of human B_2 bradykinin receptor by phenylarsine oxide or sucrose allows determination of a receptor affinity shift and ligand dissociation in intact cells

Alexander Faussner*, Steffen Schuessler, Cornelia Seidl and Marianne Jochum

Ludwig-Maximilians-Universität München, Abteilung für Klinische Chemie und Klinische Biochemie, Nussbaumstrasse 20, D-80336 München, Germany

* Corresponding author
e-mail: alexander.faussner@med.uni-muenchen.de

Abstract

Depending on their interaction with intracellular proteins, G protein-coupled receptors (GPCR) often display different affinities for agonists at 37°C. Determining the affinity at that temperature is often difficult in intact cells as most GPCRs are internalized after activation. When sequestration of the B_2 bradykinin receptor (B_2R) was inhibited by either 0.5 M sucrose or phenylarsine oxide (PAO), a shift in the affinity was detected when the incubation temperature was raised from 4°C to 37°C or lowered from 37°C to 4°C. In contrast, binding of the antagonist [^{3}H]NPC 17731 was temperature-independent. B_2R mutants displayed different affinity shifts allowing conclusions on the role of the involved amino acids. By inhibiting receptor sequestration it was possible to determine also dissociation of [^{3}H]BK and of [^{3}H]NPC 17731 from intact cells at 37°C. Surprisingly, both dissociation rates were markedly enhanced by the addition of unlabeled ligand, most likely via prevention of reassociation of dissociated [^{3}H]ligand. This suggests that dissociated [^{3}H]ligand cannot move freely away from the receptor.

In summary, our data demonstrate that inhibition of receptor internalization either by PAO or sucrose provides an excellent method to study receptor function and the effects of mutations in intact cells.

Keywords: G protein-coupled receptor; internalization; kinin; NP*XX*Y.

Introduction

The family of the G protein-coupled receptors (GPCRs) represents one of the largest groups with more than a thousand members identified in the mammalian genome (Gether, 2000; Pierce et al., 2002). Their name derives from their ability to recruit intracellular heterotrimeric G proteins when stimulated by an extracellular agonist. They play a pivotal role in the perception of the environment because light and odor are sensed in higher organisms via members of this receptor family. They are also involved in the control of physiology and behavior as indicated by the immense chemical diversity of their endogenous ligands. Therefore deeper insight into the mechanisms by which these GPCRs are regulated and how they mediate extracellular signals through the membrane into the cell is of highest interest. Advanced knowledge of the structure/function relationship of GPCRs as well as of useful differences between the various family members is indispensable for the development of better and more specific drugs to interfere with their signal transduction.

One characteristic property of GPCRs is that most of them respond in crude membrane preparations to the addition of GTP at higher temperatures (>25°C) with a shift to lower affinity for agonists but usually not for antagonists. The extent of the shift that can be observed with different GPCRs varies markedly from no shift at all (AT_2 angiotensin receptor, Zhang and Pratt, 1996; B_1 bradykinin receptor, Faussner et al., 1999), to 3–10 fold reductions for some peptide receptors (AT_1 angiotensin receptor, Hunyady et al., 1995; B_2 bradykinin receptor, Faussner et al., 1998) to 50–100 fold, e.g. for the A_{2A} adenosine (Murphree et al., 2002) or the β-adrenergic receptor (Schwarz et al., 1986; Rasenick et al., 1994). Despite this variance and sometimes even its absence, this shift in response to GTP addition is considered a landmark of GPCRs because according to the currently accepted models of receptor regulation it reflects the coupling to (high affinity state) or uncoupling from (low affinity state) their cognate G protein(s). It has, however, been reported that not only heterotrimeric G proteins but also other intracellular proteins, e.g. small G proteins or arrestins, can influence the affinity of a GPCR (Gurevich et al., 1997; Mitchell et al., 1998).

In order to determine a shift in affinity usually (crude) membrane preparations have to be prepared and the dissociation constant K_d has to be determined with a radiolabeled agonist in the presence or absence of GTP. In many cases, the assay has to be conducted at temperatures lower than 37°C, because otherwise no shift can be detected. Alternatively, using a suitable radiolabeled antagonist, a right-shift in the competition curves of the unlabeled agonist after addition of GTP can indicate an affinity shift. The latter is applicable mostly for receptors that show a strong shift. The determination of the shift in intact cells is in many cases hampered by the fact that the majority of the GPCRs respond to agonist stimulation with rapid sequestration of the receptors from the cell surface (Koenig and Edwardson, 1997). Correct measurement of the K_d is, therefore, impossible, as binding at equilibrium is a prerequisite.

The B_2 bradykinin receptor (B_2R) belongs to the class A, the rhodopsin/β_2-adrenergic-like GPCRs (Fredriksson et al., 2003), and mediates the effects of the hormones bradykinin (BK) and kallidin (Regoli et al., 2001). These

short peptides (nine and ten amino acids, respectively) are released from their high-molecular weight precursors, the kininogens, through the action of specific enzymes, the kallikreins (Clements et al., 2001). Stimulation of the B_2R results in vasodilatation, edema formation, and induction of pain (Proud, 1988).

We have previously shown that the B_2R in intact human fibroblast as well as in crude membrane preparations when kept 4°C on ice is apparently in a high affinity state. In crude membranes this high affinity state was also quite stable at 37°C, but could be reduced to lower affinity by addition of GTP or GDP (Faussner and Roscher, 2000).

When we observed in intact cells that poorly internalizing B_2R mutants (Faussner et al., 1998) still exhibited a significant drop in surface binding activity when the temperature was raised from 4°C to 37°C, we set out to investigate whether this observation was caused by a shift in the receptor affinity and if so, how this effect could be used to study the binding behavior and signaling of internalizing wild-type receptor and receptor mutants in intact cells.

Results

Poorly internalizing B_2R mutant displays decreased binding activity at 37°C

A B_2R mutant (G327stop) lacking the C-terminal tail due to insertion of a stop codon after glycine 327 (Faussner et al., 1998) was preincubated at 4°C with [^{3}H]BK and then warmed up to 37°C in order to start ligand internalization. A clear reduction in surface binding was observed that reached a plateau within 5 min. This reduction was seen despite the poor ligand internalization, suggesting that this effect is unlikely caused by sequestration of the receptor. As the internalization rate is usually calculated as internalized [^{3}H]ligand in percentage of the combined amount of internalized and surface bound [^{3}H]ligand, a reduction in surface binding leads to an apparently increased ligand internalization (inset Figure 1) even when absolute ligand internalization (Figure 1) is slow. This drop in total binding can of course be observed only when the internalization process is started at 37°C after reaching the binding equilibrium at 4°C, and not when the [^{3}H]agonist is added directly at 37°C. Similar results were obtained with G327stop expressed in Chinese hamster ovary cells, indicating that this effect does not depend on the cell type.

Inhibition of receptor sequestration reveals reduced surface binding also in wt B_2R

Next we investigated whether the observed reduction in surface binding is due to a loss in receptor number, i.e. part of the receptors becoming inactive for binding, or due to a defined shift of the receptor affinity occurring when the incubation temperature is raised from 4°C to 37°C. Moreover, it should be clarified whether this reduction is a general property of the wt B_2R or solely of the mutant G327stop lacking the C-terminal domain. Due to the fast internalization of the wt B_2R reduction of surface binding is generally considered to be a consequence of

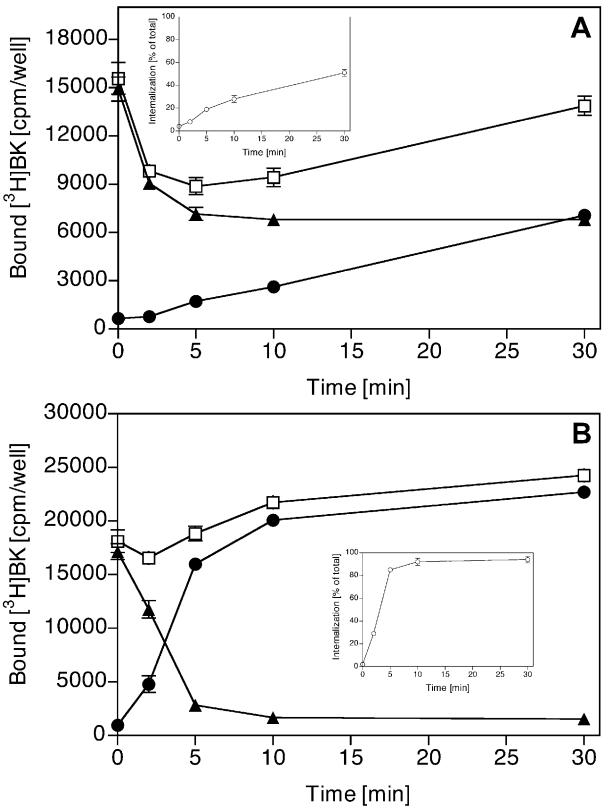

Figure 1 Binding reduction in the poorly internalizing B_2R mutant G327stop.
After preincubation with 2 nM [^{3}H]BK for 90 min on ice, HEK 293 cells stably expressing the poorly internalizing B_2R mutant G327stop (A) or, for a comparison, the wild-type B_2R (B) were placed in a water bath at 37°C. At the indicated times the amounts of surface-bound (▲) and internalized (●) [^{3}H]BK were determined as described in the materials and methods section. The insets show the internalization as calculated in percentage of total bound (□) [^{3}H]BK (surface plus internalized). Values represent the mean±SD of triplicate determinations. One representative experiment out of three is shown.

receptor sequestration. An accompanying receptor inactivation or affinity shift would be noticed only after inhibition of the receptor internalization.

Therefore, we applied two common methods to prevent B_2R sequestration: firstly, the hyperosmolaric method with addition of 0.5 M sucrose in the incubation medium (Heuser and Anderson, 1989) is supposed to disrupt the clathrin-coat of certain vesicles, thereby inhibiting receptor sequestration. This method, however, may affect also receptor sequestration which does not involve clathrin-coated pits via so far unknown mechanisms (Roseberry and Hosey, 2001).

Secondly, we used preincubation of the cells for 5 min at 37°C with 100 μM phenylarsine oxide (PAO; Hertel et al., 1985).

Both treatments effectively prevented the internalization of [^{3}H]BK (Figure 2A, B) by the wt B_2R. Less than 20% (0.5 M sucrose, Figure 2A) and even less than 10% (100 μM PAO, Figure 2B) of totally bound [^{3}H]BK became internalized after 30 min at 37°C. However, despite this inhibition of ligand internalization, a reduction in surface binding was observed with both treatments resulting in a plateau after 5–10 min. Because the PAO preincubation evoked a stronger inhibition and is easier to perform, it

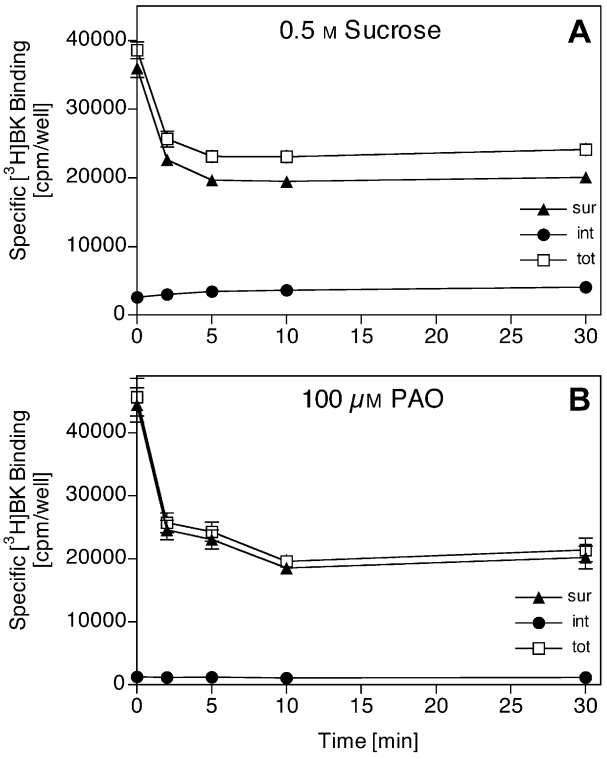

Figure 2 Inhibition of [^{3}H]BK internalization by sucrose and PAO.
HEK 293 cells with high expression of B_2Rs [10.4 pmol/mg protein; B_2R(high)] were either incubated on ice for 90 min with [^{3}H]BK in the presence of 0.5 M sucrose (panel A) or after pre-treatment with PAO (panel B). Subsequently the cells were transferred to a water bath at 37°C. The concentration of [^{3}H]BK was 12 nM and 13.4 nM, respectively. At the indicated times the amount of internalized and surface-bound [^{3}H]BK was determined as described in the materials and methods section. The determinations were performed in triplicates and are given as mean±SD. Each experiment was conducted at least three times with similar results.

became our preferred method for inhibiting receptor sequestration. Other (pre)treatments of the cells either with N-methyl-maleimide (1 mM, 5 min/37°C) or digitonin (20 μg/ml, 5 min/37°C) also strongly inhibited ligand internalization of the wt B_2R and resulted in identically reduced binding activity at 37°C.

Reduction of surface binding at 37°C is due to a reversible shift in affinity

Equilibrium binding and subsequent Scatchard analysis of the data revealed that the binding reduction as a consequence of the raise of the incubation temperature from 4°C to 37°C is not due to a loss of receptors, but is rather caused by a shift in the receptor affinity for [^{3}H]BK (Figure 3A). This shift is not an artifact provoked by increased [^{3}H]BK degradation at 37°C, because it can also be observed in the reverse direction: while the binding curves of cells incubated at 37°C indicate a lower receptor affinity (Figure 3B), the binding curves become almost identical after an additional incubation on ice to those of cells that have been placed on ice immediately. The receptor affinity at 4°C after PAO pre-treatment was identical to that of control cells which were not treated with PAO (not shown), suggesting that PAO does not affect those parts of the receptor that are directly involved in ligand binding. The temperature change influenced only the binding of the agonist [^{3}H]BK, but not that of the antagonist [^{3}H]NPC 17731 (cf. Figure 5B).

Different affinity shifts occur in wt B_2R and receptor mutants

Next we wanted to compare the temperature-induced affinity shifts of wt B_2Rs with high (10.4 pmol/mg protein) and lower (4.4 pmol/mg protein) expression with that of different receptor mutants (see Table 1). One of these mutants was the construct Y305A, in which the tyrosine in the highly conserved NP*XX*Y sequence located at the end of the 7th transmembrane segment was exchanged by an alanine. This mutant was of particular interest, because we had shown recently that it is precoupled to G protein $G_{q/11}$ even in the absence of an agonist (Kalatskaya et al., 2004). A reason for this behavior might be

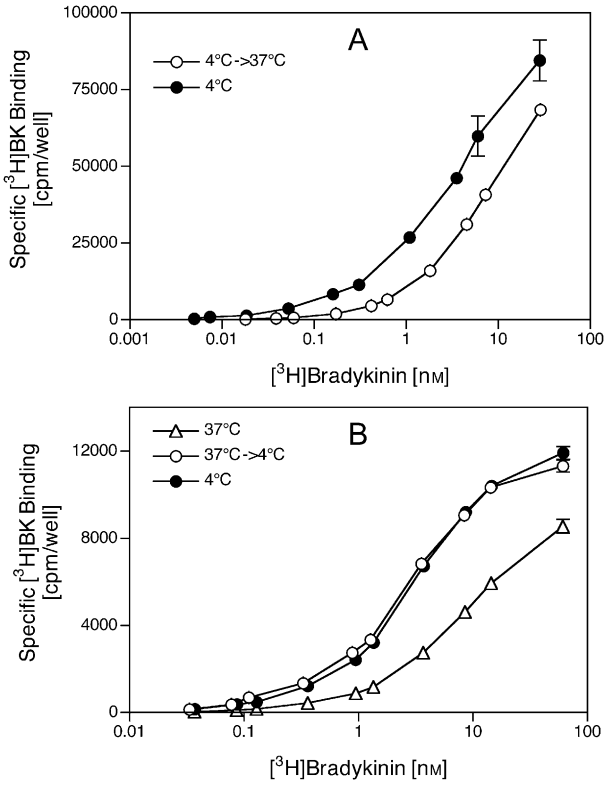

Figure 3 Equilibrium binding curves at 4°C or at 37°C, respectively, and after change of temperature.
(A) HEK 293 cells expressing B_2R(high) were incubated with increasing concentrations of [^{3}H]BK (0.03–30 nM) for 90 min on ice with (○) and without (●) additional incubation for 30 min at 37°C. Thereafter, specific binding was measured as described in 'materials and methods'. Each point represents the mean of duplicate determinations. Regression analysis of the data gave the equilibrium constants K_d=3.0±0.34 nM (4°C) and K_d=8.2 0.12 nM (4°C→37°C).
(B) HEK 293 cells expressing B_2Receptors [4.4 pmol/mg protein; B_2R(low)] were incubated with increasing concentrations of [^{3}H]BK at 37°C with (○) or without (△) by an additional incubation on ice for 90 min. Control cells (●) were incubated on ice only. Each value is the mean of duplicates. Regression analysis resulted in the following equilibrium constants K_d=2.8±0.17 nM (37°C), 9.7±0.15 nM (37°C→4°C), and 3.5±0.18 nM (4°C). Each experiment was repeated at least twice with similar results.

Table 1 Expression levels (B_{max}) and dissociation constants (K_d) of wild-type and mutant B_2 receptors at 4°C and at 37°C.

Construct	B_{max} (pmol/mg protein)	Dissociation constant K_d (nM)	
		4°C	37°C
B_2R (high)	10.4	4.71±0.99 (3)	11.77±2.77 (3)
B_2R (low)	4.4	1.99±0.28 (4)	7.56±1.28 (4)
Y305A	2.1	2.82±0.58 (5)	1.65±0.24 (5)
E177A	7.9	23.28±1.05(3)	>200 (3)
E177A/Y305A	3.2	9.64±0.55 (7)	58.23±8.44 (7)

Receptor constructs were stably expressed in HEK 293 cells. Maximal binding capacity B_{max} was estimated with 40 nM [^{3}H]BK at 4°C. The values for the dissociation constant K_d at 4°C and at 37°C were determined as described in the materials and methods section and are given as mean±SEM. The number of experiments is indicated in parentheses.

that Y305A displays a persistent high affinity conformation. The second mutant, termed E177A, was of interest as the mutated glutamate at position 177 should play a role in high affinity binding of [^{3}H]BK (Kyle et al., 1994; Jarnagin et al., 1996). The third construct carried both mutations (mutant E177A/Y305A).

The high and low expressed B_2Rs exhibited a slightly different affinity, however, in both cases a clear temperature-dependent shift was obvious (see Table 1). In contrast, mutant Y305A revealed a tendency to higher affinity at 37°C instead of exhibiting a shift to lower affinity. This result suggests that the mutation of the tyrosine in the NP*XX*Y sequence indeed induces a receptor conformation that permanently displays a high affinity.

Mutant E177A displayed a complete different behavior than construct Y305A. Already at 4°C its affinity was approximately 6 times lower than that of the wt B_2Rs suggesting that glutamate 177 is directly involved in binding. At 37°C its affinity was reduced to such an extent that it could only be estimated as being >200 nM under the assumption that the reduction in binding was not due to a loss in receptors. That this assumption is justified was indicated by the fact that the high affinity binding could almost completely be recovered by a subsequent incubation on ice (not shown), similarly as demonstrated before for the wt B_2R (cf. Figure 3). This strong affinity shift might be a combination of two independent effects, the reduced binding affinity caused by changing an amino acid involved in ligand binding and the affinity reduction due to a conformational change induced by activation of the receptor mutant. This notion is supported by the affinity shift observed for the double mutant E177A/Y305A that also exhibited a reduced affinity at 4°C as compared to the wt B_2Rs, but a distinctly lesser reduced affinity at 37°C (cf. Table 1). This was most likely because the reduction component caused by the conformational change is blocked by the second mutation of tyrosine Y305 to alanine. These results suggest that glutamate 177 is important solely for high affinity binding of BK but is not directly involved in the activation of the B_2 receptor.

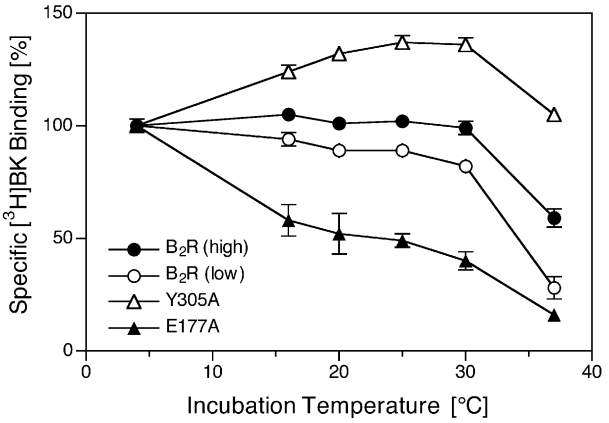

Figure 4 Temperature dependence of ligand binding by wt B_2Rs(high/low) or by the receptor mutants Y305A and E177A. PAO-pretreated cells expressing the indicated receptor constructs were incubated on ice with 1.3 nM [^{3}H]BK for at least 60 min. Subsequently, cells were warmed up to the indicated temperatures (16°C, 20°C, 25°C, 30°C, 37°C) or kept further on ice. Specific binding was determined after 30 min as described in 'materials and methods'. The results are given as mean±SD from a representative experiment performed in triplicate and expressed in percentage of the binding at 4°C. For each construct the experiment was repeated at least two times in independent experiments with similar results. (●) wt B_2R(high), (○) wt B_2R(low), (△) Y305A, (▲) E177A.

Temperature dependence of the affinity shift varies in wt B_2R and the selected mutants

We next investigated the behavior of these receptor constructs by taking a closer look at the temperature-dependence of their ligand binding (Figure 4). For this purpose the different cell clones were pre-equilibrated with [^{3}H]BK at concentrations below their K_d and then warmed up to the indicated temperatures. Mutant E177A showed an almost linear inverse correlation between increased temperature and reduction of binding, whereas both wt B_2Rs responded only at temperatures above 25°C with a strong decrease in binding. Construct Y305A displayed no reduction at all, but rather a maximum of binding at temperatures between 20°C and 30°C.

Fast dissociation at 37°C requires addition of unlabeled BK or antagonist JE049

Besides the measurement of equilibrium dissociation constants at 37°C, the inhibition of receptor sequestration also allowed the determination of the dissociation rate of [^{3}H]labeled agonist ([^{3}H]BK) or antagonist

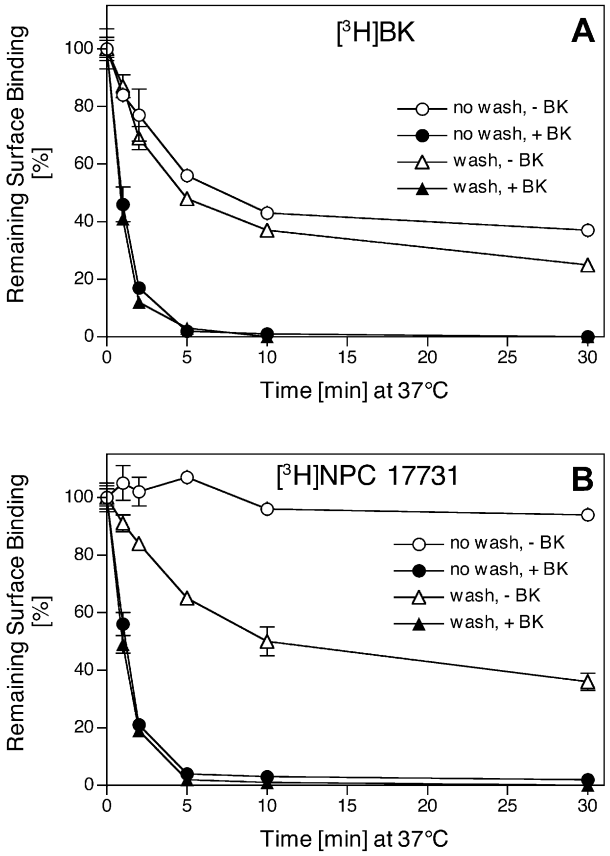

Figure 5 Dissociation of the agonist [^{3}H]BK and the antagonist [^{3}H]NPC 17731 from PAO treated cells.
PAO treated HEK 293 cells expressing wt B_2R(high) were incubated on ice for 90 min with 3 nM [^{3}H]BK or 16 nM [^{3}H]NPC 17731. Subsequently, either 1 μM unlabeled BK was added and the cells placed at 37°C (●) or the cells were washed on ice to remove unbound [^{3}H]ligand and the dissociation was started by adding 37°C warm buffer with (▲) or without (△) addition of 1 μM BK. At the indicated times the remaining surface binding was determined as described in the materials and methods section. For comparison data from cells without washing and addition of unlabeled BK (○) are shown. The values represent the mean±SD of triplicate determinations.

([^{3}H]NPC 17731) at 37°C from cell surface receptors in intact cells. The experiments were performed in two ways: after preincubation of PAO treated cells at 4°C with [^{3}H]ligand (agonist or antagonist), dissociation was started either by directly adding 1 μM unlabeled BK – in order to prevent reassociation of dissociated [^{3}H]ligand – and placing the trays in a water bath at 37°C. Alternatively, unbound [^{3}H]ligand was first removed by washing the cells at 4°C and then the dissociation was started by addition of warm incubation buffer (37°C) with or without addition of 1 μM unlabeled BK. At the indicated times remaining surface binding was determined by acetic acid treatment.

Quite surprisingly, a large difference in the dissociation rate of [^{3}H]ligand became obvious depending on whether a high surplus of unlabeled BK was added or not. In the presence of 1 μM unlabeled BK (or of the antagonist JE049, data not shown) there was a rapid dissociation of the agonist [^{3}H]BK as well as of the antagonist [^{3}H]NPC 17731 (Figure 5A, B). The dissociation did not depend on whether unbound [^{3}H]ligand had been removed before by washing the cells or the incubation was continued in its presence. In the absence of unlabeled BK, however, the dissociation rate of [^{3}H]BK was much slower and did not exceed significantly the rate that was caused by the drop in affinity (Figure 5A). The same effect was observed for the antagonist [^{3}H]NPC 17731, yet with the difference that there was no change in binding when the cells were incubated at 37°C in the continued presence of [^{3}H]NPC 17731 (Figure 5B), demonstrating that the receptor affinity for the antagonist is clearly temperature-independent.

Effect of unlabeled ligand is probably due to prevention of reassociation of dissociated [^{3}H]ligand

In Chinese hamster ovary (CHO) cells expressing high levels of B_2R a negative cooperativity of the receptors resulting in faster dissociation rates with higher receptor occupancy has been reported (Pizard et al., 1998). In order to differentiate between a fast dissociation rate caused by a negative cooperativity effect and one resulting from inhibition of rebinding of dissociated [^{3}H]ligand, PAO-treated HEK 293 cells expressing the wt B_2R(high) were incubated with 1 nM [^{3}H]BK (a concentration below the K_d at 4°C) on ice, leading to occupation of less than half of all B_2 receptors. Subsequently, unbound [^{3}H]BK was removed by a brief wash with buffer at 4°C and the cells were further incubated with increasing amounts of unlabeled BK up to 1 μM for another 10 min on ice. This procedure should have resulted in increased receptor occupancy with full occupancy at the highest concentrations of unlabeled BK and at the same time almost identical binding of [^{3}H]BK due to the very slow off-rate of [^{3}H]BK at 4°C. When these cells were placed at 37°C, dissociation of [^{3}H]BK was indeed more rapid with higher receptor occupancy. However, the maximal rates were

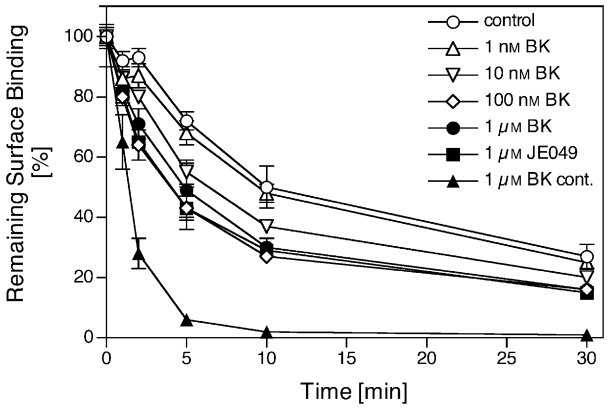

Figure 6 Dissociation of [^{3}H]BK with increased receptor occupancy.
PAO-pretreated HEK 293 cells expressing B_2R(high), were preincubated with 1.2 nM [^{3}H]BK for 90 min on ice. Thereafter the indicated amounts of unlabeled agonist BK or antagonist JE049 were added for another 10 min on ice. The cells were washed to remove unbound labeled as well as unlabeled ligand and the dissociation was started by adding warm buffer and placing the cells in a water bath at 37°C. For a comparison also the dissociation in the continued presence of 1 μM BK was performed (▲). Remaining surface binding was determined at the indicated times as described in 'materials and methods'. Values are given as the mean±SD of triplicate determinations. A second experiment was performed with similar results.

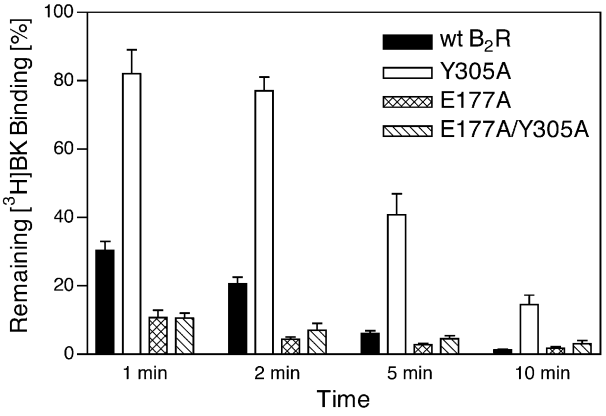

Figure 7 Dissociation rates of different receptor constructs. PAO treated HEK 293 cells expressing the indicated constructs were preincubated on ice with approximately 4 nM [^{3}H]BK for 90 min to reach equilibrium. Subsequently, the cells were washed with ice-cold PBS and placed in a water bath at 37°C after addition of 1 μM BK in incubation buffer (37°C). At the indicated times dissociation was stopped by washing the cells with ice-cold PBS and remaining surface binding was determined as described in the materials and methods section. Data are expressed as percentage of initial binding on ice and represent the mean±SEM from at least 3 independent experiments performed in duplicate. Abbreviations: wt B_2R: wild-type B_2 bradykinin receptor; Y305A: B_2 bradykinin receptor mutant with a Tyr→Ala exchange at position 305; E177A: receptor mutant with a Glu→Ala exchange at position 177; E177A/Y305A: receptor double mutant with both amino acid exchanges.

still far slower than the rate reached in the continued presence of 1 μM unlabeled BK (Figure 6). These data suggest that prevention of reassociation of [^{3}H]BK plays an important role in the fast dissociation observed in the presence of unlabeled BK. This notion was further supported by the fact that the dissociation rate enhancing effect was not specific for the agonist BK but was also found with the antagonist JE049 (Figure 5).

[^{3}H]BK dissociation rates of receptor constructs reflect their K_d at 37°C

Determination of the dissociation constants K_d (see above) yields information on the system under equilibrium conditions but does not provide information on the kinetics of ligand association or dissociation. Therefore, we also determined the dissociation rates (in the presence of 1 μM BK) of the before mentioned mutants. There was no difference in the dissociation rates between the high and the lower expressing B_2R. They are therefore included as one construct in Figure 7, and displayed a reduction of binding to approx. 25% of initial values on ice within 2 min at 37°C. Mutant E177A dropped to 10% in less than a minute, whereas mutant Y305A revealed a very slow off-rate displaying approx. 75% of the initial binding after 2 min. The double mutant E177A/Y305A showed a dissociation rate slower than E177A but distinctly faster than the wt B_2R. Thus, the dissociation rates reflected well the different equilibrium affinities determined at 37°C after 30 min (cf. Table 1) and could be used to obtain a first estimate of the affinities of a receptor construct at 37°C.

Discussion

One of the hallmarks of G protein-coupled receptors is the shift to lower affinity for agonists when guanine nucleotides such as GDP or GTP, or their poorly hydrolysable derivatives such as GTPγS, are added to membrane preparations at higher temperatures (Milligan, 2003). In the ternary complex model the high affinity state represents the receptor in its active conformation stabilized by binding to the G protein, whereas the low affinity state represents the uncoupled receptor (Gether, 2000). In the absence of guanine nucleotides, the ternary complex composed of agonist, G protein, and the receptor exhibiting high affinity is stable and dissociates only after addition of guanine nucleotides.

Despite being a hallmark of GPCRs the extent of the affinity shift can vary strongly from receptor to receptor. Whereas, for example, addition of guanine nucleotides to the β_2-adrenergic receptor leads to a shift from 1.91×10^{-9} to 1.8×10^{-5} M, i.e. a shift of almost four orders of magnitude (Rasenick et al., 1994), the B_2R in crude membrane preparations of human foreskin fibroblasts exhibited only a shift from approximately 2 to 14 nM (Faussner and Roscher, 2000). Moreover, in the AT_2 angiotensin receptor no shift at all is observed (Zhang and Pratt, 1996). In this respect it has to be mentioned that also interactions with other cytosolic proteins, such as arrestins or small G proteins, have been reported to result in high affinity binding of receptors (Gurevich et al., 1997; Mitchell et al., 1998).

In receptors that exhibit a large affinity shift this effect can be measured in competition experiments allowing a radioactive labeled antagonist to compete with increasing concentrations of unlabeled agonist. Addition of GTP then leads to a shift in the agonist displacement curve to the right side indicating the reduction in receptor affinity. For other receptors, such as the B_2R, a right-hand shift in the order of one magnitude would be difficult to determine and would require a radiolabeled high affinity antagonist. The latter, however, is currently not commercially available for the B_2R. In such a case, it is more convenient to measure the different affinities of the receptor for a radioactive agonist in the presence and absence of guanine nucleotides directly in crude membrane fractions at higher temperatures. However, especially with lysates of cells overexpressing the B_2R, we found often a reduction in the affinity of the receptor when the temperature was raised from 4°C to 37°C. This effect was independent of an addition of exogenous GTP, thus reducing the measurable change (not shown). A reason for this behavior could be that due to the overexpression of the receptors not all receptors are coupled to a G protein and, therefore, can bind only with low affinity at 37°C. Since the B_2R on ice always exhibited only one binding site with high affinity this would suggest that coupling to a G protein is not required for high affinity binding at 4°C. At this low temperature, weak interactions between side chains of the receptor and/or with membrane lipids might be strong enough to stabilize a conformation which may be maintained at 37°C only by coupling to a G protein or other cytosolic proteins. If this hypothesis holds true, then the conformation of the receptors on ice, in partic-

ular the conformation of its binding site, should be identical or very similar to the conformation of the coupled receptor at 37°C. This hypothesis is strengthened by the reversibility of the shift through an additional incubation on ice. At this low temperature it is unlikely that the high affinity binding state is obtained by the receptor via recoupling to or recruiting of a G protein or other signaling proteins. However, the possibility cannot be excluded that the receptor – similar to its ability to bind extracellularly an agonist at 4°C – is capable of interacting intracellularly with proteins at low temperatures. Further studies are therefore required to reach a final conclusion on this matter. The GTP-independent affinity reduction in membrane preparations at higher temperatures seems to be a common phenomenon of GPCRs, since most assays for a GTP effect on receptor affinity are conducted at temperatures below the physiological temperature of 37°C, i.e. at 25°C or 30°C.

There was no significant difference between the K_d values of B_2R and mutant Y305A at 4°C, although we have shown recently that the latter is precoupled to G protein $G_{q/11}$ even in the absence of an agonist (Kalatskaya et al., 2004). The high affinity of this mutant at 37°C could at first sight be explained as a consequence of this increased coupling to $G_{q/11}$. Mutant Y305A is, however, able to stimulate inositol phosphate accumulation even better than the wt B_2R (Kalatskaya et al., 2004), and therefore also should repeatedly uncouple from $G_{q/11}$. For this reason it seems that this mutation results in a receptor conformation that is not only able to couple to $G_{q/11}$ in the absence of an agonists but also persistently displays the high affinity binding that in the wt B_2R would be induced only by the coupling to a G protein. Thus, this mutant apparently combines a propensity to precouple to $G_{q/11}$ with permanent high affinity for the agonist BK.

By inhibiting receptor sequestration either through the use of a hyperosmolaric incubation buffer (0.5 M sucrose) or pretreating the cells with 100 μM PAO, we were able to measure a temperature-dependent shift in the receptor affinity in intact cells with good reproducibility. Phenylarsine oxide (PAO) acts on proteins by bridging vicinal sulfhydryl (SH)-groups of cysteins (Rokutan et al., 2000). The bridging, however, is not required for inhibition of B_2R internalization since monovalent N-methyl-maleimide could also block [^{3}H]BK internalization. Because PAO does not change the affinity of the receptor at 4°C, there should be no direct modification of the ligand binding site or the receptor. Furthermore, a cell impermeable SH-group-modifying reagent (monobromotrimethyl-ammoniobimane bromide; de Lamirande and Gagnon, 1998) was not able to block receptor sequestration (not shown) suggesting that the SH-groups which must be modified for inhibition of receptor internalization are located inside the cell. Although PAO is often applied as a nonspecific tyrosine phosphatase inhibitor (Fletcher et al., 1993; Yingst et al., 2000), the concentrations used for this purpose (<25 μM) were not sufficient to inhibit B_2R internalization. For this reason it is unlikely that tyrosine phosphatases play a role in the prevention of receptor sequestration by PAO. The fact that besides treatment with PAO and 0.5 M sucrose also pretreatment with digitonin or N-methyl maleimide allowed the determination of the temperature-dependent affinity shift with similar results indicates that this shift is independent of the method used for inhibition of receptor sequestration and, therefore, represents an intrinsic property of the receptor itself.

The presented data suggest that the shift in the affinity of the B_2R for [^{3}H]BK is not caused by unspecific physical effects of the raise in incubation temperature, such as increased membrane fluidity, but involves the activation of the receptor by an agonist at temperatures higher than 20°C (cf. Figure 4). The affinity shift is also not cell type dependent, since it was also observed in CHO cells heterologously expressing the human B_2R, as well as in human fibroblasts or in human embryonic lung fibroblasts WI-38 expressing naive B_2 kinin receptors (unpublished results). In addition, for the α_1-adrenergic receptor it has been shown that the magnitude of a similar temperature shift determined in membranes for several (partial) agonists correlates well with their functional efficacy (Weiland et al., 1979).

Moreover, we could show that the presence of a surplus of an unlabeled ligand is necessary for fast dissociation of a bound [^{3}H]ligand (cf. Figures 5 and 6). Since this is similar for agonists and antagonist and works despite inhibition of receptor sequestration, the most likely explanation for this phenomenon is as follows: the receptors can still move into some half-closed compartments on the cell surface, where dissociated [^{3}H]ligand remains close to the receptor and unlabeled ligand is necessary to prevent its reassociation. This behavior would be similar to that observed for the B_1 bradykinin receptor, which is not internalized after activation but translocates within in the plasma membrane to caveolae-related rafts on the cell surface (Sabourin et al., 2002).

In summary, using the B_2 bradykinin receptor as a model we have demonstrated in this report how inhibition of receptor sequestration by either 100 μM PAO or 0.5 M sucrose can be used to determine in intact cells the temperature-dependent affinity shift and ligand dissociation rates of G protein-coupled receptors or mutants thereof, thus providing information on the functional role of amino acids and domains in these receptors.

Materials and methods

Materials

Flp-In TREx-293 (HEK 293) cells were bought from Invitrogen (Groningen, Netherlands). [2,3-prolyl-3,4-^{3}H]bradykinin (90 Ci/mmol) and [prolyl-3,4-^{3}H]NPC17731 (48.5 Ci/mmol, no longer commercially available) were obtained from Perkin Elmer Life Sciences (Boston, USA). Bradykinin was purchased from Bachem (Heidelberg, Germany). The antagonist JE049 (formerly known as Hoe 140/Icatibant) was a generous gift from Jerini AG (Berlin, Germany). Roche (Mannheim, Germany) delivered Fugene. Poly-D-lysine hydrobromide, captopril, 1.10-phenanthroline, and bacitracin were purchased from Aldrich (Taufkirchen, Germany). Fetal calf serum, culture media, hygromycin B, and penicillin/streptomycin were delivered by PAA Laboratories (Cölbe, Germany).

Expression system and cell culture

The Flp-In system from Invitrogen was used to generate stable clones expressing the wt B_2R, the receptor truncation G327stop (stop codon after glycine 327; Faussner et al., 1998) and the point mutants Y305A (Kalatskaya et al., 2004) and E177A. All sequences started with the third encoded methionine (Hess et al., 1992) and were cloned into the *Hin*dIII and the *Xho*I sites of the pcDNA5/FRT vector. The receptor sequences were preceded at the N-terminus by a hemagglutinin-tag (MGYPYDVPDYAGS), with the last two amino acids (Gly-Ser) of the tag being created by the insertion of a *BamH*I site. Flp-In TREx-293 (HEK 293) cells were transfected with Fugene (Roche) following the instructions of the manufacturer [2 μg plasmid(s) plus 5 μl Fugene per 6-well dish]. Single stably transfected clones were obtained after selection with 250 μg/ml hygromycin B. Clones exhibiting similar high binding activities were considered to represent a single insertion of the pCDNA5/FRT vector (containing the receptor gene) at the recombinase target site (usually at least 3 out of 6 clones). Cells with twice as much binding activity were presumed to have an additional insertion of the vector (rare), clones with less binding activity were supposed to be inhomogeneous and, therefore, not further propagated. HEK 293 cells were cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin. For experiments requiring rinsing of the cells poly-D-lysine-treated multi-well plates were used.

Internalization of [^{3}H]BK

Monolayers on multi-well trays were rinsed with phosphate-buffered saline (PBS) and incubated with [^{3}H]BK in incubation buffer (40 mM PIPES, 109 mM NaCl, 5 mM KCl, 0.1% glucose, 0.05% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 containing the degradation inhibitors: 2 mM bacitracin, 0.8 mM 1.10-phenanthroline, and 100 μM captopril) for 90 min on ice in order to obtain equilibrium binding (Faussner et al., 2003). Internalization of [^{3}H]BK was started by warming the plates to 37°C in a water bath and was terminated at the indicated times by placing the plates on ice and washing the cells four times with ice-cold PBS. Thereafter, surface-bound [^{3}H]BK was dissociated with 0.2 ml of an ice-cold dissociation solution (0.2 M acetic acid/0.5 M NaCl, pH 2.7) for 10 min on ice. The supernatant was quantitatively transferred to a scintillation vial by rinsing the cells with another 0.2 ml of PBS. The remaining cell monolayer, containing the internalized [^{3}H]BK, was lysed using 0.2 ml 0.3 M NaOH and transferred with another 0.2 ml water to a scintillation vial. The amount of tritium in both samples was measured in a β-counter after addition of scintillation fluid. Nonspecific [^{3}H]BK internalization and surface binding were determined in the presence of 3 μM unlabeled BK and subtracted from total binding to obtain specific values. Internalization was expressed as the amount of internalized [^{3}H]BK in percentage of the combined amount of internalized and surface bound [^{3}H]BK.

[^{3}H]BK binding studies

For the determination of the dissociation constant K_d and receptor number B_{max} at 4°C and in particular at 37°C, receptor sequestration was inhibited by pretreatment of the cells with 100 μM phenylarsine oxide (PAO, 10 mM stock solution in DMSO) in incubation buffer without inhibitors for 5 min at 37°C. Alternatively, receptor sequestration was blocked by the continued presence of 0.5 M sucrose in the incubation buffer.

Confluent monolayers on 24-well/48-well trays with or without PAO treatment were rinsed three times with PBS and incubated with 0.3/0.2 ml of ice-cold incubation buffer containing increasing concentrations of [^{3}H]BK (10 concentrations ranging from approx. 0.01 nM to 30 nM) for at least 90 min on ice. For the determination of values at 4°C the incubation was stopped by rinsing the cells four times with ice-cold PBS. The cells were then lysed in 0.2 ml 0.3 M NaOH and transferred to a scintillation vial. For determination of the K_d at 37°C, the cells were first incubated for 90 min on ice and then warmed up in a water bath at 37°C. After 30 min the monolayers were washed with ice-cold PBS and [^{3}H]BK binding determined as described before.

For the incubation at 37°C with or without a second incubation at 4°C, the radioactive solutions were added quickly on ice and the trays then immediately placed in the water bath at 37°C. After 30 min the incubation was either stopped as described above (37°C) or continued on ice (37°C → 4°C) for 90 min. Determination of [^{3}H]BK binding activity was then performed as described above. Nonspecific binding (usually less than 5% of total binding) was determined in the presence of 3 μM of unlabeled BK and subtracted from total binding determined with [^{3}H]BK alone to calculate specific binding.

Protein determination

Protein concentrations were quantified with the Micro BCA Protein assay reagent kit from Pierce (Rockford, USA) using bovine serum albumin as standard.

Data analysis

All data analysis was performed using GraphPad Prism for Macintosh, Version 3.0a (GraphPad Software, Inc.).

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