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C-Terminal fusion of eGFP to the bradykinin B_2 receptor strongly affects down-regulation but not receptor internalization or signaling

Irina Kalatskaya[a], Steffen Schüssler, Cornelia Seidl, Marianne Jochum and Alexander Faussner*

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

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

Abstract

A functional comparison was made between the wild-type bradykinin B_2 receptor (B_2wt) and the chimera B_2eGFP (enhanced green-fluorescent protein fused to the C-terminus of B_2wt), both stably expressed in HEK 293 cells. There was almost no difference in terms of ligand-inducible receptor phosphorylation and internalization, signal transduction (accumulation of inositol phosphates) or expression and affinity. However, stimulation for up to 8 h with 10 μM bradykinin (BK) resulted in a strong decrease in surface receptors (by 60% within 5 h) in B_2wt, but not in B_2eGFP. When the expression levels of both constructs where comparably reduced using a weaker promoter, long-term stimulation resulted in a reduction in surface receptors for B_2wt_{low} to less than 20% within 1 h, whereas the chimera B_2eGFP_{low} still displayed 50% binding activity after 2 h. A 1-h incubation in the absence of BK resulted in a recovery of 60% of the binding in B_2wt_{low} after 1-h stimulation with BK, but of only 20% after 7-h stimulation. In contrast, B_2eGFP_{low} levels were restored to more than 70%, even after 7-h stimulation. These data indicate that although the fusion of eGFP to B_2wt does not affect its ligand-induced internalization, it strongly reduces the down-regulation, most likely by promoting receptor recycling over degradation.

Keywords: bradykinin B_2 receptor; endocytosis; G protein-coupled receptor; green fluorescent protein.

Introduction

G protein-coupled receptors (GPCRs) form the largest membrane-receptor group in the mammalian genome (Fredriksson et al., 2003). More than 40% of all commercially available drugs exert their effects via members of this group. Therefore, strong efforts have been made in academia and in pharmaceutical industry research to elucidate the mechanisms that regulate the functions of these receptors, in particular the processes that are involved in the activation or de- and resensitization of the receptors (Pierce et al., 2002; Brink et al., 2004; Maxfield and McGraw, 2004).

Our group focuses on the regulation and signaling of the bradykinin B_2 receptor (B_2wt). This receptor belongs to family A of the GPCRs (Hess et al., 1992), i.e., the rhodopsin/β-adrenergic-like receptors, and transmits signaling of the small extracellular peptides bradykinin and kallidin (Leeb-Lundberg et al., 2005) to intracellular compartments. The effects of these hormones include pain sensation, vascular permeability resulting in edema, and vasodilatation (Proud, 1988; Regoli et al., 2001; Stewart, 2004).

After stimulation with an agonist, most GPCRs sequester to intracellular compartments. One elegant tool widely applied to investigate the localization and trafficking of receptor proteins in a living cell is the expression of the receptors as fusion proteins with green-fluorescent protein (GFP) or derivatives thereof (Kallal et al., 1998; Milligan, 1999; Houle and Marceau, 2003). Although widely used, not many data are available that describe in detail whether and how joining GFP to the C-terminus of a GPCR influences its overall behavior.

Therefore, we set out to determine whether fusion to enhanced GFP (eGFP) changes the characteristics of the bradykinin B_2 receptor with respect to receptor trafficking, affinity shift and signal transduction. Moreover, experimental conditions such as receptor expression levels and short- or long-term stimulation where also investigated in detail. Taken together, the results indicate that fusion of eGFP to the C-terminus of the bradykinin B_2 receptor does not affect its ligand-induced internalization, but strongly reduces receptor down-regulation, most likely via enhancement of receptor recycling.

Results

To determine to what extent ligation of eGFP to B_2wt affects its characteristics, we investigated several parameters: ligand affinity at 4°C and 37°C, accumulation of inositol phosphates (IPs), basal and ligand-inducible phosphorylation and internalization, and receptor down-regulation. As the eGFP was fused to the C-terminus of B_2wt, this C-terminus was the main candidate domain for which function could be affected. Therefore, we also compared both constructs (B_2wt and B_2eGFP) with the B_2 receptor truncation K315stop as a control. This mutant is lacking the last 50 amino acids of the C-terminus due to insertion of a stop codon after lysine 315

[a] Present address: Centre de Recherche, #6737 Hôpital Sainte-Justine, 3175 Chemin de la Côte, Sainte-Catherine, Montréal H3T 1C5, Canada.

Table 1 Binding and functional properties of various receptor constructs.

Receptor construct	[^{3}H]BK binding			Inositol phosphate accumulation		
	B_{max}[a] (fmol/mg protein)	K_d (PAO/4°C)[b] (nM)	K_d(PAO/37°C)[b] (nM)	Basal[c] (nM)	Maximum effect[c] (nM)	EC_{50}[d] (nM)
B_2wt	10.4	3.53±0.91 (5)	11.78±1.82	1.93±0.17 (7)	12.86±1.37	0.79±0.34 (4)
B_2eGFP	9.3	4.48±0.95 (3)	10.38±0.08	1.71±0.16 (3)	15.38±2.72	1.26±0.87 (3)
B_2wt_{low}	4.4	2.02±0.22 (5)	8.05±1.10	1.73±0.12 (5)	11.39±1.64	0.67±0.02 (3)
B_2eGFP_{low}	4.0	ND	ND	ND	ND	ND
K315stop	1.5	3.79±0.22 (3)	4.65±0.60	1.56±0.16 (4)	7.86±1.69	13.10±2.75 (4)

[a]Estimated from at least three different clones in 24 wells after incubation with 200 µl of 10 nM [^{3}H]BK on ice.
[b]The dissociation constant K_d (mean±SEM) was determined in parallel at 4°C and at 37°C after pretreament of the cells with phenylarsine oxide (PAO) as described in the materials and methods section.
[c]Total inositol phosphate (IP) accumulation after 30-min incubation in buffer with inhibitors and 50 mM LiCl at 37°C with (maximal effect) and without (basal) 1 µM BK, expressed as the fold increase in initial total IP production (t=0 min). The results represent the mean±SEM of the number of experiments (given in parentheses) performed in triplicate.
[d]Calculated from duplicate incubations with 10^{-11}–10^{-5} M BK for 30 min at 37°C in the presence of 50 mM LiCl. Results are the mean±SEM of independent experiments (number indicated in parentheses). ND, not determined.

and can serve as an example for the characteristics of a mutant with an extremely truncated C-terminus.

Fusion with eGFP does not affect receptor expression or affinity

Tagging the C-terminus of the B_2wt receptor with eGFP apparently did not affect receptor expression, as B_2wt and B_2eGFP with approximately 10 pmol/mg protein were both similarly overexpressed (Table 1). The Flp-In expression system uses the transient co-expression of a recombinase to insert the gene of interest at a specific site in the genome of a host cell line. Thus, the distinctly lower expression level of truncation K315stop most likely reflects differences in posttranscriptional or posttranslational processes, such as trafficking and/or protein turnover, caused by the lack of the C-terminus that were obviously not mimicked by the fusion of eGFP to the receptor C-terminus.

When sequestration of B_2wt is inhibited by preincubation of the cells with phenylarsine oxide, it is possible to determine the equilibrium binding constant (K_d) at 4°C, as well as at 37°C (Faussner et al., 2004). B_2wt showed a shift to lower affinity when the temperature was increased from 4°C to 37°C and *vice versa*. In contrast, the mutant receptor K315stop did not exhibit significantly lower affinity at 37°C than at 4°C, indicating that the C-terminally truncated receptor is trapped in a conformation with higher affinity. However, it is not yet clear whether this is an intrinsic property of the truncation mutant or due to interactions with other intracellular proteins that have been changed by the lack of the C-terminus. The B_2eGFP chimera, however, still displayed shift behavior similar to the B_2wt (Table 1), suggesting that tagging with eGFP does not hamper the role that the C-terminus plays in the affinity shift.

B_2eGFP mediates release of total inositol phosphate levels comparable to B_2wt

When challenged with bradykinin (BK), B_2wt and B_2eGFP induced an accumulation of total inositol phosphates that was almost identical in terms of potency (EC_{50}) and efficacy (maximal effect). In contrast, the K315stop receptor mutant exhibited a reduced maximal effect – probably due to its lower expression level – and a more than 10-fold shift of the EC_{50} to lower values (Table 1), indicating that the C-terminus directly or indirectly plays a role in the coupling of the receptor to G protein $G_{q/11}$. Again this function was not affected by C-terminal fusion of the receptor to eGFP.

B_2wt and B_2eGFP display equally fast internalization

As we have shown recently using high ligand concentrations, the rate of [^{3}H]BK uptake (ligand internalization) decreases in cells overexpressing B_2wt when too many receptors are occupied (Faussner et al., 2003). Therefore, we took care to apply a [^{3}H]BK concentration below the K_d value, at which the internalization rate is largely concentration-independent, even for high expression levels.

At these low concentrations, the decrease in surface binding and the increase in internalized [^{3}H]BK for the chimeric construct B_2eGFP were similar to the rates observed for B_2wt (Figure 1A, B). For both constructs, more than 80% of total bound [^{3}H]BK could be detected inside the cells within 10 min (Figure 1C), indicating that joining of the 27-kDa eGFP protein to the C-terminus of B_2wt does not affect the interaction of this domain with the internalization machinery of the cell. Mutant receptor K315stop, however, exhibited very little internalization, not surpassing 20% after 30 min, thus confirming our results obtained in Chinese hamster ovary cells (Faussner et al., 1998) and the general importance of this receptor part for receptor internalization.

Phosphorylation patterns of B_2wt and B_2eGFP reflect their agonist-inducible internalization

B_2wt displayed distinct phosphorylation, even in the absence of a stimulus (Figure 2), as previously reported for HEK 293 cells and human fibroblasts (Blaukat et al., 2001; Kalatskaya et al., 2004). When stimulated for 5 min with a saturating concentration of 1 µM BK at 37°C, B_2wt responded with a marked increase (2.5±0.06-fold over basal, n=3) in phosphorylation. Interestingly, B_2eGFP exhibited even stronger basal phosphorylation, with bands appearing in the region of 80–110 kDa, as could

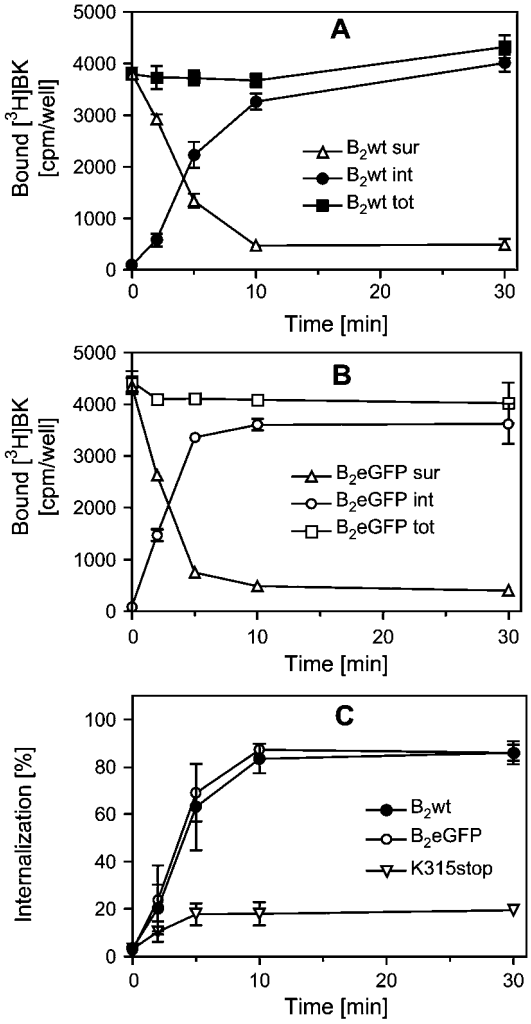

Figure 1 Internalization of [^{3}H]BK by B_2wt, chimera B_2eGFP, and truncation K315stop.
Monolayers of HEK 293 cells stably expressing B_2wt were preincubated with less than 1.5 nM [^{3}H]BK for 90 min at 4°C. The internalization was initiated by warming the plates to 37°C. At the times indicated, internalization was stopped and surface-bound and internalized [^{3}H]BK were determined as described in the materials and methods section. Results are given as the mean±SD of triplicate determinations. A representative experiment of three is shown for B_2wt (A) and the chimera B_2eGFP (B): sur, surface-bound [^{3}H]BK; int, internalized [^{3}H]BK; tot, total [^{3}H]BK. (C) Internalization as a percentage of total binding (surface bound+internalized [^{3}H]BK). All symbols show the mean±SD for at least three experiments performed in triplicate.

be expected for the larger chimera (Figure 2). Nevertheless, the fusion protein still responded to BK stimulation with a significant increase in phosphorylation (1.3±0.03-fold over basal, n=3). Mutant K315stop lost all phosphorylation sites, and therefore no basal or stimulated phosphorylation could be expected (Blaukat et al., 2001).

Stability of BK in cell culture medium

To study B_2wt down-regulation, it is necessary to stimulate the receptor over a period of several hours with an agonist. Bradykinin, however, is a very good substrate, especially for metalloproteases (Dendorfer et al., 1997; Stewart, 2004) found either on the cell membrane such as neutral endopeptidase (NEP, neprilysin) or in serum

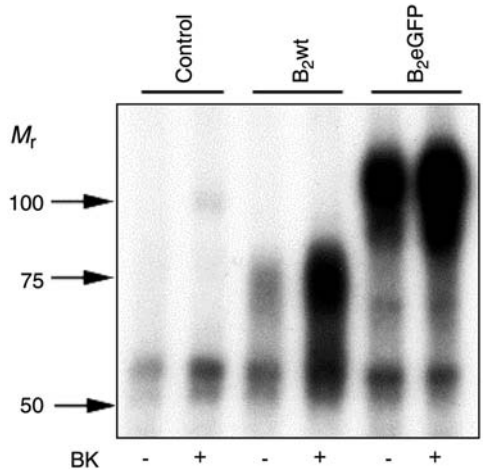

Figure 2 Agonist-induced phosphorylation of B_2wt and B_2eGFP.
HEK 293 cells expressing B_2wt or B_2eGFP were labeled for 12 h with [^{32}P]orthophosphate before stimulation with 1 μM BK for 5 min. Cells were lysed and receptor proteins were solubilized, precipitated, and visualized by autoradiography as described in the materials and methods section. Molecular size markers are indicated on the left. Mock-transfected HEK 293 cells were used as control cells. The experiment shown was repeated three times with similar results.

added to the cell culture medium (Bachvarov et al., 2001). The addition of protease inhibitors, such as 1,10-phenanthroline, to prevent proteolysis of the labeled or unlabeled BK is tolerated by cells for only short incubation times for the binding or stimulation periods (Faussner et al., 2003, 2004). However, the cells do not tolerate such additives for the longer periods necessary for down-regulation experiments. To avoid long-term exposure to these harmful protease inhibitors, we tested the stability of BK at 37°C in a cell culture medium supplemented with fetal calf serum (10% v/v) for which the proteolytic activity was strongly reduced by incubation for 2 h at 60°C. To determine the effectiveness of this pretreatment, intact BK (initially 10 μM) in the supplemented medium was tested after various times for binding to the receptor in a competition assay with [^{3}H]BK. As the full reduction in [^{3}H]BK binding to B_2wt-expressing control cells in Figure 3 reveals, there was still a saturating amount of BK left after 8 h when added to cell medium supplemented with inactivated serum, and even after 24 h, binding of 2 nM [^{3}H]BK could still be considerably inhibited. In cell culture medium supplemented with normal fetal calf serum, however, there was not enough BK left after 3 h to fully compete with 2 nM [^{3}H]BK for binding to the receptor. These data demonstrate that when higher amounts (>1 μM) of BK are used, the proteolytic activity of the cells themselves can be neglected over a period of 24 h, as long as the proteolytic activity in the cell culture medium is strongly reduced by heat inactivation. Importantly, the heat-inactivated fetal calf serum itself had no specific effects on the cells, as no difference regarding either phenotype or [^{3}H]BK binding activity was observed compared to cells incubated for 24 h with cell culture medium supplemented with normal fetal calf serum.

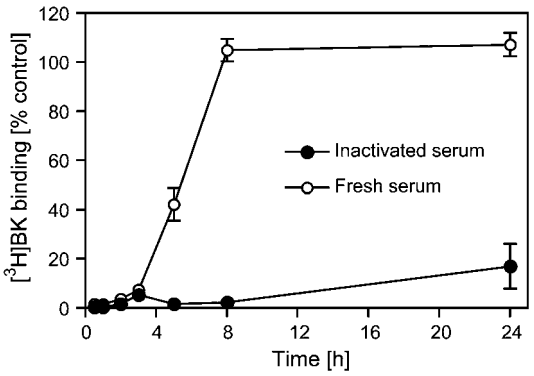

Figure 3 Stability of BK in different cell culture media. HEK 293 cells in 48-well plates were incubated with 10 μM BK in 500 μl of medium with either inactivated (treated for 2 h at 60°C) serum (●) or with fresh serum (○) for the times indicated. Thereafter, binding of 2 nM [^{3}H]BK to HEK 293 expressing B_2wt (48-well plates, 150 μl) was determined in a competition assay after addition of 10 μl of the respective media as described in the materials and methods section. Binding is expressed as a percentage of binding in control cells that received 10 μl of medium without the addition of 10 μM BK. Points represent the mean±SD of three experiments performed in duplicate (●) or of two experiments performed in triplicate (○).

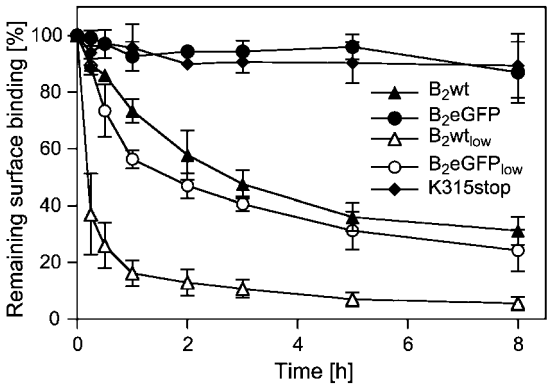

Figure 4 Time curve for the effect of BK on surface binding. HEK 293 cells stably expressing B_2wt at a high level (B_2wt, 10.4 pmol/mg protein) or at a lower level (B_2wt_{low}, 4.4 pmol/mg protein), chimera B_2eGPF at a high level (B_2eGFP, 10 pmol/mg protein) or at a lower level (B_2eGFP_{low}, 4 pmol/mg protein) or C-terminally truncated receptor mutant K315stop at a level of 1.5 pmol/mg protein were incubated with 10 μM BK in cell culture medium containing 10% inactivated fetal calf serum. At the times indicated, all bound and free unlabeled BK was removed with an acetic acid solution and the remaining specific surface binding was determined with 2 nM [^{3}H]BK at 4°C as described in the materials and methods section. Symbols represent the mean±SD for at least three experiments performed in duplicate or triplicate.

Highly expressed B_2eGFP displays no down-regulation

When monolayers of B_2wt-expressing HEK 293 cells were incubated in cell culture medium supplemented with inactivated fetal calf serum for up to 24 h with 10 μM BK, a clear decrease in surface binding could be observed for B_2wt, which after 5 h reached a plateau of <40% of the control binding measured in unstimulated cells (Figure 4). The rate of decrease strongly depended on the expression level, as indicated by another HEK 293 clone (B_2wt_{low}) expressing the same B_2wt construct under the control of the weaker promoter P_{min} (Faussner et al., 2004). The use of this weaker promoter resulted in distinctly lower expression, with 4.4 compared to 10.4 pmol/mg protein of B_2wt expression obtained under the control of the standard CMV promoter (Table 1). The construct – termed B_2wt_{low} – responds to prolonged BK stimulation with a reduction in surface binding of 90% within 2 h (Figure 4), thus stressing the influence of the receptor expression level on the outcome of a down-regulation experiment.

When challenged with BK, the chimera B_2eGFP showed no loss of surface binding at all, even after 8 h of stimulation with an initial concentration of 10 μM BK (Figure 4). Only after longer incubation periods for up to 24 h could a decrease in binding be measured (data not shown). As almost all cells expressing bradykinin B_2 receptors and variants thereof responded to prolonged exposure to BK with strong cell contraction and weakened adherence to the cell culture plate, it is not clear whether this apparent decrease in agonist binding should be considered as receptor down-regulation or rather as the result of general degeneration of the cells caused by overstimulation.

To test whether the apparent lack of B_2eGFP down-regulation during an 8-h incubation time is absolute or in part due to the strong receptor overexpression, we also generated clones expressing the chimera B_2eGFP at a distinctly lower level (4 pmol/mg protein) under the control of the promoter P_{min} (termed B_2eGFP_{low}). In this case, stimulation with BK resulted in reduced surface binding resembling that of the highly expressed B_2wt (Figure 4); thus, it was much less pronounced than the reduction observed for B_2wt_{low} expressed at a comparable level. These data indicate that fusion of eGFP to the C-terminus of the bradykinin B_2 receptor strongly reduces but does not completely block its down-regulation.

Despite its low receptor expression, the truncation mutant K315stop showed no decrease at all in agonist binding even after 8 h of stimulation with BK, suggesting that ligand-induced receptor down-regulation requires either internalization of the receptor or at least (modification of) specific sequences in the C-terminal receptor domain as a first step.

Fusion to eGFP results in enhanced recycling of the B_2 receptor

The fast internalization of [^{3}H]BK, but no (B_2eGFP) or only a slow decrease (B_2eGFP_{low}) in the cell surface-receptor number after stimulation suggests that there is significant recycling of the B_2eGFP chimera after internalization. In addition, it is of interest to know whether the reduced receptor number on the cell surface after long-term stimulation constitutes true down-regulation, i.e., inactivation/degradation of the sequestered receptors, or whether they can reappear at the cell surface to a lesser degree after removal of the stimulus.

Roscher et al. (1984) showed that after short stimulation of 15 min with a saturating concentration of BK, a recovery time of 1 h is enough to restore binding almost completely in human fibroblasts. As this recovery was

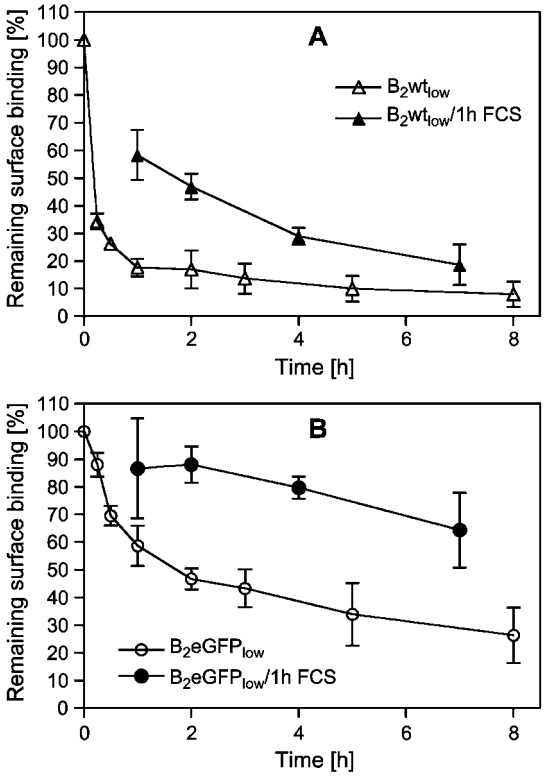

Figure 5 Receptor recovery in B_2wt_{low} and B_2eGFP_{low}. HEK 293 cells stably expressing B_2wt_{low} (A) or chimera B_2eGPF_{low} (B) were incubated for the times indicated with 10 μM BK in cell culture medium containing 10% inactivated fetal calf serum (FCS). The remaining surface binding was either determined immediately (△,○) as described in the legend to Figure 4 or the monolayers were washed and further incubated for 1 h at 37°C in medium with fresh FCS to promote degradation of residual BK and allow receptor recycling back to the cell membrane, before the remaining surface binding was determined as described above (▲, ●). Symbols represent the mean±SD for three experiments performed in duplicate.

unaffected by cycloheximide, it necessarily implied recycling of the receptors. We tested, therefore, the capability of the low-expressing cells to recover receptor binding after incubation for 1 h in the absence of BK.

Stimulation of B_2wt_{low} with a saturating concentration of 10 μM BK resulted in a fast decrease in surface binding (Figure 5A; see also Figure 4). Although stimulation for 1 h produced a decrease to <20% of the initial binding, subsequent incubation at 37°C in the absence of BK generated a recovery in binding to almost 60%, indicating that a greater proportion of the sequestered receptors was not degraded after 1 h, but could be recycled back to the cell surface. Stimulation with BK for more than 1 h strongly diminished this capability to recover, implying that degradation had overtaken recycling (Figure 5A). For the chimera B_2eGFP_{low} the situation was clearly different. The reduction in cell surface receptor number caused by stimulation with BK for up to 2 h (approx. 50%) could be nearly completely restored by incubation for 1 h in the absence of BK (Figure 5B), and even after a stimulation period of 7 h almost 70% of the receptors reappeared after 1 h without stimulus, suggesting that even during prolonged stimulation most of the chimera continues to recycle between the cell surface and intracellular compartments. This clearly indicates that the fusion of eGFP to the receptor C-terminus strongly promotes recycling of the receptor, thus overcoming its degradation. It also explains the apparently poor sequestration, i.e., the reduction in receptor number on the cell surface after stimulation, unequivocally observed for the chimera (Figure 4).

Discussion

Many GPCRs respond to stimulation by an agonist with sequestration of the receptor from the cell surface to intracellular compartments such as early endosomes. From there, they either return to the cell surface (recycling) or end up in lysosomes and are degraded (down-regulation). As this receptor endocytosis and its regulation play a pivotal role in the control of cell responsiveness to extracellular stimuli, elucidation of the mechanisms involved is a main focus of ongoing research in the GPCR field (Ferguson, 2001; Marchese et al., 2003).

One widely used tool is the generation of receptor chimeras with eGFP that allow the direct tracking of a receptor within a living cell with the help of fluorescence microscopy. For GPCRs, the fluorescent protein has been exclusively joined to the intracellular C-terminus. The current model of receptor internalization – resulting predominantly from data obtained for the β_2-adrenergic receptor (Perry and Lefkowitz, 2002) – starts with the phosphorylation of specific Ser/Thr-residues in the C-terminus through the action of second-messenger activated kinases and/or the more receptor-specific GRKs (G protein-coupled receptor kinases). Subsequently, adaptor proteins such as arrestins bind to these phosphorylated sequences and mediate the translocation of the receptor to and its internalization through clathrin-coated pits (Mousavi et al., 2004) or other less well-understood mechanisms, such as pathways involving caveolae (Haasemann et al., 1998; Roseberry and Hosey, 2001).

The almost complete absence of receptor internalization observed in the B_2 truncation mutant K315stop (Faussner et al., 1998; Figure 1C) demonstrates the relevance of the intact C-terminus for this function in B_2wt. It has also been shown for B_2wt that the phosphorylation of Ser/Thr residues is indispensable for fast sequestration (Blaukat et al., 2001). Deglycosylated B_2wt has a molecular mass of approximately 40 kDa and its C-terminus consists of only approximately 60 amino acid residues. For this reason it cannot be taken for granted that tagging the C-terminus with a 27-kDa protein would not interfere with its function, in particular with the receptor trafficking that largely depends on this domain.

On first sight, B_2eGFP displayed properties similar to those of B_2wt: it bound [^{3}H]BK with the same affinity and had a similar expression level. In addition, B_2eGFP was located almost exclusively at the membrane and was found in intracellular vesicles of various sizes only after stimulation with BK (data not shown here, but reported by Kalatskaya et al., 2004), in full agreement with other reports (Bachvarov et al., 2001; Sabourin et al., 2002; Simaan et al., 2005). The fusion protein can interact normally with its cognate G proteins, as it exhibited a low

level of basal signaling and IP accumulation could be stimulated several-fold above this basal level. Moreover, similar to B_2wt, B_2eGFP displayed a clear temperature-dependent affinity shift, indicating proper interaction at the plasma membrane with the cognate G protein and/or other cytosolic proteins (Mitchell et al., 1998; Gether, 2000). Finally, the chimera B_2eGFP displayed significant ligand-inducible phosphorylation and fast ligand internalization comparable to that of B_2wt. The properties of the truncation receptor K315stop demonstrated that the C-terminus plays an important role in all these processes, as this mutant lacking the C-terminus displayed no affinity shift, whereas its signaling exhibited a more than 10-fold shift of EC_{50} to lower values for IP accumulation (Table 1). This lower EC_{50} may also explain the apparently strong reduction in maximal effect (by 70%) found for similar truncation of the rat bradykinin B_2 receptor, as a low concentration of 10 nM BK was used to determine the maximal effect (Prado et al., 1997).

The truncation mutant K315stop showed no decrease in surface binding, even after prolonged stimulation with BK, suggesting that ligand-induced receptor down-regulation requires either internalization of the receptor or specific sequences and/or modification thereof in the C-terminal receptor domain as a first step. This, and the fact that internalization but not down-regulation remained unchanged in the presence of eGFP, indicates that these two processes are sequential and that they can be uncoupled by fusion of the receptor to eGFP. Thus, these data are in contrast to results obtained for the angiotensin AT_1 receptor, for which down-regulation was reported to be independent of ligand-inducible receptor endocytosis (Modrall et al., 2001). Two AT_1 truncation mutants, comparable to K315stop, did not internalize, but could nevertheless be down-regulated to 60% within 8 h, similar to the wild type AT_1 receptor. This may indicate different regulation and trafficking of bradykinin B_2 compared to the angiotensin AT_1 receptor.

The rate of decrease in surface binding of both B_2wt and the chimera B_2eGFP is strongly dependent on the expression levels. However, comparison of the constructs at two different expression levels clearly revealed that fusion of eGFP to the receptor C-terminus strongly reduced sequestration as an effect of long-term BK stimulation, blocking it completely at the high expression level (10 pmol/mg protein) and strongly slowing it down at the lower level (4 pmol/mg protein). As the internalization rates of B_2wt and B_2eGFP were comparable, this suggests the possibility that the B_2eGFP chimera has either a higher recycling rate than B_2wt, or that it continues to recycle, whereas B_2wt is degraded on prolonged stimulation. The results of the recovery experiment (Figure 5) point to the latter possibility, because even after 7 h of stimulation, most of the internalized B_2eGFP_{low} receptor could be restored to the cell surface, whereas this was not the case for B_2wt_{low}.

To identify the cause of the different behavior of B_2wt and the chimera B_2eGFP, several possibilities have to be taken into account. For example, our data demonstrate that the chimera B_2eGFP can interact properly with kinases involved in receptor internalization, but owing to the many more spots observed for the chimera, even in the absence of BK (data not shown), we were not able to perform two-dimensional phosphopeptide mapping of the chimera, as successfully carried out for B_2wt (Kalatskaya et al., 2004). The strong basal phosphorylation observed in our experiment with B_2eGFP may, therefore, result in part from phosphorylation of the eGFP-domain itself. Furthermore, it is conceivable that a specific phosphorylation pattern, generated during recycling of the receptor, is necessary to finally direct an internalized receptor to the degradation pathway and that this pattern cannot be created in the presence of eGFP fused to the C-terminus. Simaan et al. (2005) recently showed that proper interaction of the bradykinin B_2 receptor with β-arrestin2 is important for recycling and resensitization of the receptor after stimulation. Fusion of eGFP to the receptor C-terminus might affect this interaction in a manner that favors recycling over degradation. Of course, other processes such as ubiquitination, that are widely discussed for receptor internalization and down-regulation (Haglund et al., 2003; Shenoy and Lefkowitz, 2005), may be also involved and could be hampered – although not completely blocked – by the C-terminal presence of eGFP.

Taken together, our results demonstrate that internalization of B_2wt is most likely required for its down-regulation. However, while fusion of eGFP to the C-terminus does not affect most of the features of the receptor, such as receptor affinity shift or ligand-induced receptor internalization, it does strongly reduce receptor down-regulation through promotion of the recycling of internalized receptors over degradation. Therefore, data obtained in recovery and localization experiments with eGFP-tagged GPCRs should be interpreted with caution as to whether the results also apply to the wild-type receptor. As the presence of eGFP at the C-terminus results in uncoupling of the processes of internalization and down-regulation, the use of eGFP-fusion proteins might represent an excellent tool to identify the determinants that are decisive for recycling vs. down-regulation of an internalized receptor through, e.g., overexpression of proteins that are supposed to be involved in receptor down-regulation. This information should eventually allow the generation of compounds that therapeutically enhance or reduce the action of endogenous hormones or exogenous drugs by influencing the balance between recycling and degradation of the GPCRs that mediate their effects.

Materials and methods

Expression vector and expression system

For expression of B_2wt, we used the sequence starting with the third encoded methionine (Hess et al., 1992). For generation of the B_2eGFP chimera, the sequence for eGFP taken from the pEGFP-C1 vector (Clontech, Heidelberg, Germany) was joined to the C-terminus of B_2wt using standard PCR technology with a chimeric primer that included the start codon of eGFP and left out the B_2wt stop codon (Faussner et al., 2003). For stable construct expression, the Flp-In system (Invitrogen, Karlsruhe, Germany) was employed, whereby the vector containing the gene of interest is inserted at a specific position into the genome of a commercially available host cell line (Flp-In™ T-REx-293; Invitrogen) through the transient co-expression of a recombinase.

All receptor coding sequences were preceded at the N-terminus by a single hemagglutinin (HA)-tag (MGYPYDVPDYAGS), with the last two amino acids (GS) of the tag generated by the insertion of a BamHI site, and were cloned into the HindIII and the XhoI sites of the pcDNA5/FRT vector. HEK 293 cells were transfected using Fugene (Roche, Mannheim, Germany) according to the manufacturer's instructions and stable clones were selected using 250 μg/ml hygromycin B. The cells were cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin. For monolayer experiments requiring rinsing of the cells, poly-D-lysine-treated [0.01% in phosphate-buffered saline (PBS)] multi-well plates were used.

Binding studies: determination of affinity (K_d) shift at 4°C and at 37°C

To compare the binding affinities of the constructs at 4°C and at 37°C, receptor sequestration was inhibited by pretreatment of cell monolayers in 48-well plates with 100 μM phenylarsine oxide (PAO) for 5 min at 37°C, as recently reported (Faussner et al., 2004). Thereafter, cells were rinsed with PBS and incubated with increasing concentrations of [^{3}H]BK (0.01–30 nM) in incubation buffer (40 mM PIPES, 109 m NaCl, 5 mM KCl, 0.1% glucose, 0.05% BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, and the protease inhibitors 2 mM bacitracin, 0.8 mM 1,10-phenanthroline, and 100 μM captopril). After 30 min, the plates were placed on ice and washed with PBS either immediately (K_d at 37°C) or after an additional incubation step for 90 min on ice (K_d at 4°C). Surface-bound [^{3}H]BK was dissociated in both cases using an ice-cold dissociation solution (0.2 M acetic acid/0.5 M NaCl, pH 2.7) for 10 min and samples were then transferred to scintillation vials. The radioactivity of all samples was then determined in a β-counter after addition of scintillation fluid. Non-specific binding was measured in the presence of 5 μM unlabeled BK and subtracted from total binding to obtain the specific values.

Internalization of [^{3}H]BK

Cells seeded on 24-well plates were rinsed three times with PBS and incubated with [^{3}H]BK (<1.5 nM) in incubation buffer for 90 min on ice to reach equilibrium binding. Thereafter, [^{3}H]BK internalization was initiated by placing the plates in a water bath at 37°C. At the times indicated, the internalization process was stopped by putting the plates back on ice and washing them with ice-cold PBS. Surface-bound [^{3}H]BK was separated by treating the monolayers with ice-cold dissociation solution for 10 min on ice and was then quantitatively transferred to a scintillation vial. The remaining monolayer containing the internalized [^{3}H]BK was lysed with 0.3 M NaOH and also transferred to a scintillation vial. The radioactivity of both samples was determined in a β-counter after addition of scintillation fluid. Non-receptor-mediated [^{3}H]BK internalization and surface binding were measured in the presence of 5 μM unlabeled BK and subtracted from total binding to calculate the specific values.

Receptor phosphorylation

Confluent cells on six-well plates were washed twice with phosphate-free DMEM, incubated for 3 h at 37°C in the same medium, and labeled with 0.2 mCi/ml [^{32}P] orthophosphate for 10–12 h. After exposure to 1 μM BK for 5 min at 37°C, monolayers were solubilized in 0.5 ml of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, pH 7.5) supplemented with 0.5 mM Pefabloc SC (Roche) and 10 μM each of 1,10-phenanthroline, aprotinin, leupeptin, and pepstatin A and phosphatase inhibitors (25 mM NaF, 1 mM sodium orthovanadate, 0.3 μM okadaic acid) for 1 h at 4°C with gentle rocking. The lysate was centrifuged at 6000 *g* for 20 min at 4°C. The supernatant was incubated with 15 μl of anti-HA affinity matrix (Roche) for 4 h at 4°C. The mixture was then washed twice with RIPA buffer and once with distilled water. Electrophoresis on a 10% SDS polyacrylamide gel and electroblotting were carried out as previously described (Kalatskaya et al., 2004). The proteins of interest were identified by autoradiography.

Determination of BK stability in culture medium

To determine the stability of BK in media with differently treated fetal calf serum (with and without incubation for 2 h in a water bath at 60°C), BK was added to cell monolayers (48-well plates) in these media (500 μl) to a final concentration of 10 μM and incubated at 37°C for the times indicated. Thereafter, 10 μl of the respective medium was added to 150 μl of 2 nM [^{3}H]BK in incubation buffer and binding to cells expressing B_2wt in 48-well plates was determined as described above at 4°C. The reduction in [^{3}H]BK binding was expressed as a percentage of the binding measured in B_2wt control cells that received 10 μl of the media without addition of BK.

Receptor down-regulation and recovery

For down-regulation experiments, confluent cell monolayers in 48-well plates were incubated with 10 μM BK in 500 μl of medium containing 10% fetal calf serum that was pretreated for 2 h at 60°C to reduce its proteolytic activity (inactivated serum). At the times indicated, both bound and free unlabeled BK were removed by treating the cell monolayers immediately with ice-cold dissociation solution for 10 min and rinsing them with PBS. Alternatively, for recovery experiments the cells were first washed with 1 ml of cell culture medium at 37°C containing fresh (to promote degradation of residual BK) fetal calf serum, incubated in another 1 ml of medium for 1 h at 37°C and then treated with the dissociation solution. To measure the remaining surface binding, all cells were incubated with approximately 2 nM [^{3}H]BK in incubation buffer on ice. After 90 min, free [^{3}H]BK was removed by rinsing the cells with PBS and surface-bound [^{3}H]BK was determined after dissociation with ice-cold dissociation solution as described above. Non-specific binding was measured in the presence of 5 μM unlabeled BK.

Determination of total inositol phosphate (IP) release

Confluent monolayers in 12-well plates were incubated overnight with 0.5 ml of medium containing 10% inactivated fetal calf serum and 0.5 μCi [^{3}H]inositol. The cells were washed with PBS and preincubated on ice in incubation buffer supplemented with 50 mM LiCl with or without the addition of BK (10^{-11}–10^{-5} M). After 90 min the accumulation of IP was started by placing the cells in a water bath at 37°C. The stimulation was terminated after 30 min by exchanging the buffer for 0.75 ml of ice-cold 20 mM formic acid solution. Total IPs were then determined using AG 1-X8 anion exchange columns as previously described (Kalatskaya et al., 2004).

Protein determination

Total protein was quantified with the Micro BCA Protein assay reagent kit from Pierce (Rockford, IL, 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., San Diego, CA, USA).

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