**Mutation of Tyrosine in the Conserved NPXXY Sequence Leads to Constitutive Phosphorylation and Internalization, but Not Signaling, of the Human B_2 Bradykinin Receptor**

Received for publication, February 18, 2004, and in revised form, May 12, 2004

Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M401796200

Irina Kalatskaya‡, Steffen Schüßler‡, Andree Blaukat§, Werner Müller-Esterl¶, Marianne Jochum‡, David Proud†, and Alexander Faussner**

From the ‡Abteilung für Klinische Chemie und Klinische Biochemie, Ludwig-Maximilians-Universität, Nussbaumstrasse 20, D-80336 München, Germany, the §Institut für Pharmakologie, Universität Heidelberg, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany, the ¶Institute for Biochemistry II, University of Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany, and the †Department of Physiology & Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Although the G protein-coupled receptors (GPCRs) share a similar seven-transmembrane domain structure, only a limited number of amino acid residues is conserved in their protein sequences. One of the most highly conserved sequences is the NPXXY motif located at the cytosolic end of the transmembrane region-7 of many GPCRs, particularly of those belonging to the family of the rhodopsin/β-adrenergic-like receptors. Exchange of Tyr^305 in the corresponding NPLVY sequence of the bradykinin B_2 receptor (B_2R) for Ala resulted in a mutant, termed Y305A, that internalized [³H]bradykinin (BK) almost as rapidly as the wild-type (wt) B_2R. However, receptor sequestration of the mutant after stimulation with BK was clearly reduced relative to the wt B_2R. Confocal fluorescence microscopy revealed that, in contrast to the B_2R-enhanced green fluorescent protein chimera, the Y305A-enhanced green fluorescent protein chimera was predominantly located intracellularly even in the absence of BK. Two-dimensional phosphopeptide analysis showed that the mutant Y305A constitutively exhibited a phosphorylation pattern similar to that of the BK-stimulated wt B_2R. Ligand-independent Y305A internalization was demonstrated by the uptake of rhodamine-labeled antibodies directed to a tag sequence at the N terminus of the mutant receptor. Co-immunoprecipitation revealed that Y305A is precoupled to G_αq/11 without activating the G protein because the basal accumulation rate of inositol phosphate was unchanged as compared with wt B_2R. We conclude, therefore, that the Y305A mutation of B_2R induces a receptor conformation which is prone to ligand-independent phosphorylation and internalization. The mutated receptor binds to, but does not activate, its cognate heterotrimeric G protein G_αq/11, thereby limiting the extent of ligand-independent receptor internalization.

This work was supported by Deutsche Forschungsgemeinschaft. Grant FA 288/3-1 (to A. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Ludwig-Maximilians-Universit"at Muenchen, Abt. Klinische Chemie und Klinische Biochemie, Nussbaumstr. 20, D-80336 Muenchen, Germany. Tel.: 49-89-5160-2662; Fax: 49-89-5160-4740; E-mail: alexander.faussner@med.uni-muenchen.de.

G protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, represent one of the largest classes of membrane receptors in the mammalian genome (1). They are involved in all aspects of interaction with, and perception of, the environment, including sight, smell, and taste. Such receptors also play a vital role in the control of physiology and behavior, as evidenced by the immense chemical diversity of their endogenous and exogenous ligands. These receptors are named based on their ability to bind to and activate intracellular heterotrimeric G proteins when stimulated by an extracellular agonist. The A family of rhodopsin/β-adrenergic-like receptors is the largest and most well studied of all GPCR families (2). Although the members of this family do not share a high overall sequence identity, they have a characteristic pattern of a few highly conserved residues and motifs in homologous positions (most of them located in the transmembrane domains) that are not present in the other GPCR families. Given that a high degree of conservation suggests that a residue or segment might play a pivotal structural, functional, or regulatory role in the receptor actions, these residues and motifs have been examined by mutagenesis studies in many GPCRs. One of the most highly conserved motifs, together with the E/DRY sequence at the cytosolic end of transmembrane domain-3, is the NPXY sequence (where X usually represents a hydrophobic residue and N is rarely exchanged against D) located at the C terminus of transmembrane domain-7. The results of these mutagenesis studies implicate this motif in the signaling, sequestration, and internalization of many GPCRs (3–13).

A prominent member of the family A of GPCRs is the mammalian type-2 bradykinin receptor (B_2R), which mediates the effects of bradykinin (BK) and kallidin (14). These noma- and decapetides, respectively, are released from their high molecular weight precursors, the kininogens, through the action of the kallikreins. Effects of kinins via the B_2R include vasodilation, edema formation, and pain sensation (15, 16). As with any other highly potent peptides, the concentration of BK is strictly controlled by rapid degradation involving enzymes such as kinases, aprotinin, and metals.
as aminopeptidases, neutral endopeptidase, and angiotensin I converting enzymes. Inhibitors of the latter belong to the most important drugs in the treatment of heart disorders. Although the internalization and desensitization of B₂R and the involvement of serine/threonine residues in the C terminus and in other intracellular domains have been studied in great detail (17), nothing is known, thus far, about the role of its NPXXY motif in desensitization processes.

Here we have used a complementary multi-assay approach measuring internalization of radiolabeled ligands as well as receptor sequestration (relative changes in the number of surface receptors after stimulation with unliganded agonist), performing total phosphorylation and phosphopeptide mapping, using eGFP-receptor fusion proteins and fluorescent-labeled antibodies directed against a N-terminal receptor tag, to determine the role of the NPLVY sequence and in particular of the critical tyrosine residue in the regulation of wt B₂R.

Our results suggest that Tyr₇⁵₃ (Tyr 7.53 according to the generalized numbering scheme (Ref. 18)) plays an important role in keeping the receptor in an inactive uncoupled state, and that Tyr₃₀₅ (Tyr 7.53 according to the generalized numbering scheme (Ref. 18)) plays an important role in receptor activation by agonists.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phosphate-free Dulbecco’s modified Eagle’s medium (DMEM), sodium orthovanadate, aprotinin, leupeptin, peptatin A, and poly-L-lysine hydrobromide were from Sigma (Taufkirchen, Germany); pyrrolidine-2,3-dithiol (10 mM in dioxane); [³²P]orthophosphate (500 mCi/ml) from ICN (Eschwege, Germany); okadaic acid from Calbiochem (Bad Soden, Germany); cellulose thin layer chromatography (TLC) plates, 1.10-phenanthroline, and Pefabloc SC from Merck (Germany); GFP/EGFP, 6 protein G-agarose, anti-β-antibody, rhodamine-labeled anti-HA antibody, and anti-HA affinity matrix from Roche (Roche) following the instructions of the manufacturer, and Flp-In TREx-293 (HEK293) cells was performed using FuGENE/6-well dish. Single stably transfected clones with eGFP (enhanced green fluorescent protein) joined to the C terminus of B₂R with omission of the stop codon. The Flp-In system from Invitrogen (Groningen, The Netherlands) and delivered desalted and lyophilized. JEO49 was a generous gift from Jerini AG (Berlin, Germany).

**Mutations, Expression System, and Cell Culture (19)—**The wild-type (wt) B₂R gene and all other constructs started with the third encoded cysteine (C) at position 20, which, until recently, was assumed to be the start codon. Site-directed mutagenesis of B₂R was performed with standard PCR methodology using appropriate primers and confirmed by sequence analysis (Medigenomix, Martinsried, Germany). Similar methods were used for the construction of chimera with GFP (enhanced green fluorescent protein) and the tagged receptor was electrophoresed onto 0.45-μm nitrocellulose.

**Receptor Phosphorylation**—Receptor phosphorylation was measured as incorporation of [³²P]orthophosphate for 1 h at 37 °C. The internalization process was stopped by rinsing the plates with ice-cold PBS. The cell monolayers were then lysed in 3.0 ml of 0.3 M NaOH and transferred to a scintillation vial by rinsing the cells with another 0.2 ml of 2 M NaOH and transferred to a scintillation vial. The radioactivity of both samples was determined in a β-counter after addition of scintillation fluid. Non-receptor-mediated [³²P]orthophosphate internalization and surface binding was determined in the presence of 5 μM unlabeled BK and subtracted from total binding. Internalization was expressed as intracellular [³²P]orthophosphate in percentage of the combined amounts of internalized and surface-bound [³²P]orthophosphate.

**Measurement of Total Inositol Phosphate Release**—Cell monolayers at 80% confluence in 12-well dishes were labeled with 0.5 μM of [³²P]orthophosphate in DMEM containing 2 mM glycine, 2% fetal calf serum and penicillin/streptomycin. The monolayers were then placed on ice, rinsed three times with ice-cold PBS (pH 7.2), and preincubated with the indicated concentration of BK in the presence of 50 μM LiCl for at least 90 min. The stimulation was started by placing the tray in a water bath at 37 °C for 30 min and terminated by exchanging the buffer with 0.75 ml of ice-cold 20 mM formic acid, keeping the tray on ice for an additional 30 min. Subsequently, the supernatant was combined with another 0.75 ml of 20 mM formic acid and, after alkalization with 0.2 ml of 3% ammonium hydroxide solution, was applied to an AG 1-X8 anion exchange column to which the radioactive sample was added. The column was washed with 1 ml of 1.8% ammonium hydroxide, followed by 9 ml of 5 mM tetraborate, 60 mM sodium hydroxide, 20 mM of 3% ammonium hydroxide, formic acid, 0.2 M formic acid. The radioactivity was determined in a β-counter after addition of scintillation fluid.

**Immunoprecipitation and Western Blotting**—Cells were washed once with PBS and solubilized in 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) including 0.5 mM Pefabloc SC and 10 μg each 1.10-phenanthroline, aprotinin, leupeptin, and pepstatin A at 45 min with gentle rocking. The lysate was centrifuged at 6.240 × g for 20 min at 4 °C. The supernatant (0.5 ml with ~1.5 mg of total protein) was incubated with 35 μl of protein G-agarose and 2.5 μl of antiserum AS45 (21, 22) for 3 h at 4 °C. The mixture was then washed twice with 2 ml of ice-cold 1% ammonium hydroxide, followed by 9 ml of 5% tetraborate, 60 mM sodium hydroxide, 20 mM of 3% ammonium formate, 0.2 M formic acid. The radioactivity was determined in a β-counter after addition of scintillation fluid.

**Role of Tyrosine in NPXXY Sequence of Human B₂ Receptor**

**Internalization of [³²P]Bradynhin—**Monolayers on 12-well/24-well plates were rinsed three times with PBS and incubated with 0.20-0.15 μl of [³²P]BK in incubation buffer (40 mM PIPES, 109 mM NaCl, 5 mM KCl, 0.1% glucose, 0.05% bovine serum albumin, 2 mM CuCl₂, 1 mM MgCl₂, pH 7 containing 2 mM bacitracin, 0.8 mM 1.10-phenanthroline, 100 μM captopril) for 90 min on ice to reach equilibrium binding. [³²P]BK internalization was started by placing the plates in a water bath at 37 °C. The internalization process was stopped at the indicated time by putting the plates on ice and washing the cells four times with ice-cold PBS. Thereafter, the cell monolayers were treated with 0.2 ml of an ice-cold dissociation solution (0.2 mM acetic acid, 0.5 mM NaCl, pH 2.7) for 10 min on ice to induce dissociation of surface-bound [³²P]BK. The supernatant with formerly surface-bound [³²P]BK was quantitatively transferred to a scintillation vial by rinsing the cells with the remaining 0.2 ml of PBS. The remaining monolayer, containing the internalized [³²P]BK, was lysed using 0.2 ml of 0.3 M NaOH and transferred with another 0.2 ml of water to a scintillation vial. The radioactivity of both samples was determined in the presence of 5 μM unlabeled BK and subtracted from total binding. Internalization was expressed as intracellular [³²P]orthophosphate in percentage of the combined amounts of internalized and surface-bound [³²P]BK.
TABLE I

| Construct (promoter) | $K_D$ (nM) | $B_{	ext{max}}$ pmol/mg protein |
|----------------------|------------|---------------------------------|
| $B_R$ (CMV)          | 4.71 ± 0.99 | 10.4                            |
| $B_R$ (P$_{\text{MINCMV}}$) | 1.87 ± 0.36 | 4.4                             |
| Y305A (CMV)         | 2.82 ± 0.58 | 2.1                             |
| Y305F (CMV)         | 4.68 ± 0.58 | 12.5                            |

Data Analysis—All data analysis was performed using GraphPad Prism for Macintosh, version 3.0a (GraphPad Software, Inc.).

RESULTS

Mutation of Tyr$_{305}$ Does Not Affect Ligand Affinity of $B_R$—The NPLVY sequence located in the C-terminal portion of transmembrane domain-7 of $B_R$ (positions 301–305) represents the highly conserved NPYXXY motif of family A-type GPCRs. To determine the structural and functional significance of this motif in $B_R$, Tyr$_{305}$ was replaced by Ala (mutant receptor Y305A) and more conservatively by Phe (Y305F). Both mutants and the wt $B_R$ were stably expressed in HEK293 cells using the Flp-In system. Because, in this expression system, the gene of interest is inserted in the identical position in the genome of the host cell through the transient action of a recombinase, we varied expression levels by using a vector with either the original cytomegalovirus (CMV) promoter for higher expression, or the minimal CMV promoter (P$_{\text{MINCMV}}$) for lower expression levels. Maximal binding capacities of the stable clones were estimated by incubation on ice with a saturating concentration of $^{[35S]}$GTP-$\beta$-$S$ (10 nM). Mutant receptor Y305A exhibited considerably less surface binding (2.1 pmol/mg of protein) than either wt $B_R$ (10.4 pmol/mg of protein) or mutant Y305F (12.5 pmol/mg of protein). Expression of the $B_R$ under the control of the P$_{\text{MINCMV}}$ promoter (4.4 pmol/mg of protein) was closer to the levels of the Y305A mutant expressed under the control of the CMV promoter. It should, however, be mentioned that even cells with a low expression profile produced copy numbers of $B_R$ that were consistently higher than those observed in native cells, e.g. human foreskin fibroblasts. The differences observed in surface expression among the constructs using the Flp-In system (despite using the identical promoter) are likely caused by differences in cellular trafficking and transportation of the newly synthesized proteins. The affinities of $B_R$, whether expressed at high or low levels, and of the two mutant receptors did not differ markedly (see Table I), indicating that the mutations do not directly affect the ligand binding site.

Exchange of Tyr$_{305}$ with Phe or Ala Does Not Affect Internalization of $^{[35S]}$GTP-$\beta$-$S$—Replacement of the Tyr residue with Ala in the NPYXXY motif of the $\beta_2$-adrenergic receptor led to complete loss of receptor internalization (13). In other receptors such as the $\alpha_1A$, the analogous mutation did not significantly reduce the uptake of the cognate ligand angiotensin-II (12). In the human $B_R$, exchange of Tyr$_{305}$ with Phe or Ala did not reduce the internalization rate of $^{[35S]}$GTP-$\beta$-$S$ by the mutant receptors Y305F and Y305A, respectively, as compared with the highly expressed wt $B_R$ (Fig. 1). Whereas the Y305F behaved similarly to the wt receptor (plateau at ~20% after 10 min), the reduction of surface binding was less pronounced for Y305A (plateau at 40% of initial surface binding) indicating impaired receptor sequestration but unchanged ligand internalization of the latter mutant (Fig. 1, inset).
Role of Tyrosine in NPXXY Sequence of Human B₂ Receptor

Fig. 1. Internalization of [³H]BK by wild-type and mutant B₂R in HEK293 cells. Monolayers of stably transfected HEK293 cells were incubated with 1 nM [³H]BK for 90 min on ice. The internalization was started by warming the plates in a water bath at 37 °C. At the indicated times, the internalization process was stopped, and surface binding and internalized ligand were determined. The surface profile is depicted in the inset. ○, wild-type B₂R; ⌐, Y305A; △, Y305F. All values are the mean ± S.E. from at least three experiments performed in triplicate.

Fig. 2. Sequestration of high and low expressing wild-type and mutant B₂R. Cells were preincubated with 1 μM BK on ice for 90 min. Receptor internalization was started by warming the cells to 37 °C in a water bath. At the indicated times, cells were set back on ice and treated with an acidic acid solution to remove unlabel BK. Remaining surface binding was determined with 2 nM [³H]BK at 4 °C. ○, low expressing wt B₂R; △, high expressing wt B₂R; ⌐, mutant Y305A. All values are the mean ± S.E. from at least three experiments each performed in triplicate.

Mutant Receptor Y305A Is Characterized by Attenuated Sequestration—We have recently reported that cells strongly overexpressing B₂R respond to a saturating concentration of BK (1 μM) only with poor sequestration of the receptors from the cell surface (also shown in Fig. 2), probably because of an overload of the internalization machinery (19). For the sequestration assay, therefore, we used the lower expressing wt B₂R (PCMVmin), which had an expression level closer to that of Y305A. This low expressing B₂R clone responded with a strong decrease in surface binding (more than 75% within 10 min) when activated by 1 μM BK. By contrast, the Y305A mutant, which was expressed at even lower levels, dropped only to a plateau at 50% after 5 min (Fig. 2). The same tendency could be seen in long term down-regulation experiments (stimulation in culture medium with 10 μM BK in the presence of protease inhibitors but absence of fetal calf serum), where surface binding of the high expressing B₂R clone was reduced to approximately 20% after 24 h of stimulation (data not shown). In contrast, the low expressing B₂R clone had already reached this level after 30 min in the presence of 1 μM BK (cf. Fig. 2).

Conversely, the Y305A mutant still retained 40–60% of the initial surface binding even after 24 h of incubation (data not shown), indicating significantly impaired sequestration, probably because of increased receptor recycling and/or mobilization of intracellular pools of spare receptors. It should be mentioned that cells expressing either wt or mutant B₂R responded to this prolonged stimulation with cell contraction and weakened adherence, indicating that they do not tolerate persistent receptor activation well, whereas control cells exposed to the same medium without BK retained a normal appearance.

Mutant Receptors Stimulate Release of Total Inositol Phosphates and Do Not Exhibit Constitutive Activity—Next, we investigated the effects of the mutations on the main signaling pathway of the B₂R, namely the activation of phospholipase C. To this purpose, we determined the accumulation of total IP without (basal activity) and after stimulation with 1 μM bradykinin (stimulated activity) for 30 min in the presence of 50 mM LiCl and expressed it as x-fold increase over IP levels at the beginning of the assay (see Table I). Both wt and mutant kinin receptors showed a low level of agonist-independent signaling, indicating that the mutations do not produce constitutively active receptors, as was reported for an analogous Tyr mutant of the 5HT₂C serotonin receptor (6). Wild-type B₂R as well as Y305A and Y305F were able to stimulate a severalfold increase above basal level with similar EC₅₀ values (see Table I), demonstrating significant coupling to Gₛ for all three kinin receptor variants. We did not observe a linear correlation between receptor number and IP response for transfections with the wild-type receptor, i.e. despite grossly different expression levels (10.4 pmol versus 4.4 pmol of receptor/mg of protein), we failed to find a significant difference in the corresponding ligand-induced IP accumulation (12.68 ± 1.37-fold versus 9.31 ± 1.69-fold). Of note, the Y305A mutant exposed a much lower fraction of active receptors, as was reported for an analogous Tyr mutant of the 5HT₂C serotonin receptor (6).

Wild-type and Mutant Receptors Show Similar Expression Levels—Immunoprecipitation of the epitope-tagged receptors followed by Western blot analysis revealed a major diffuse band in the region of 50–80 kDa for all constructs (Fig. 3). In addition, low molecular mass bands at 37 and 40 kDa and a high molecular mass band at 100–150 kDa were detected (data not shown). Despite the divergent levels of ligand surface binding in intact cells, the expression levels of wt B₂R and Y305A were comparable, suggesting that the mutant receptor is either not completely accessible for the ligand because of an intracellular localization or that some of the mutant receptors in the plasma membrane display very low affinity for [³H]BK.

Fig. 3. Immunoprecipitation of wild-type and mutant B₂R. Wild-type and mutant receptors containing an N-terminal HA tag were immunoprecipitated from the lysates of confluent 10-cm cell culture dish, using antibody AS346 directed to the receptor C terminus. The precipitated proteins were separated by reducing SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-HA. Relative molecular masses determined with standard proteins are indicated on the left. The blot shown is representative of three experiments with similar results.
Chimeric Receptor Proteins Are Differentially Distributed—
To determine cellular localization of wt and mutant receptors, chimeric constructs were generated with eGFP joined to the C terminus of wt B2R (B2R-eGFP) or mutant receptors (Y305A-eGFP and Y305F-eGFP). All eGFP constructs exhibited expression levels and internalization rates of [3H]BK that were similar to their nonfused homologues (data not shown). B2R-eGFP was located almost exclusively at the membrane and, in agreement with previous reports (24), was found in intracellular vesicles of various sizes only after stimulation with BK (Fig. 4). By contrast, the Y305A mutant was found predominantly inside the cell even in the absence of BK. Stimulation with BK induced minor changes in the intracellular distribution with appearance of Y305A in larger vesicles. The mutant Y305F also displayed some intracellular localization in the absence of BK but, in contrast to Y305A, was mainly located at the plasma membrane and responded to BK exposure similarly to wt B2R, i.e. with a translocation from the plasma membrane to intracellular vesicles (Fig. 4). Two plausible explanations for these observations can be suggested. Either, following de novo synthesis, most of the Y305A proteins fail to reach the surface because of misguided trafficking, and/or the mutation causes the constitutive internalization of Y305 despite the fact that this mutant does not show constitutive activity. Wild-type B2R and Y305A Differ by Their Phosphorylation Patterns—Ligand-induced phosphorylation of Ser/Thr residues in the C terminus has been shown to be a prerequisite for internalization of B2R and other receptors (17, 25). The B2R displayed distinct phosphorylation even in the absence of a stimulus (Fig. 5), as has been reported before in human fibroblasts (21). When stimulated for 5 min with a saturating concentration of 1 μM BK at 37 °C, however, the wt B2R responded with a marked increase (2.5 ± 0.5-fold over basal) in phosphorylation. The Y305A mutant also exhibited basal phosphorylation but, in contrast to B2R, responded with much less (in some experiments with no) additional phosphorylation when challenged with BK (Fig. 5). At first sight, this result suggested that the Y305A mutant is resistant to agonist-induced phosphorylation, as has been reported for the analogous construct Y326A of the β2-adrenergic receptor (11). We cannot, however, exclude the possibility that surplus nonphosphorylated intracellular Y305A receptors can compete with phosphorylated surface receptors in the immunoprecipitation, resulting in a relatively weak phosphorylation signal. The Y305F mutant took up
an intermediate position between wt B2R and Y305A showing agonist-induced phosphorylation, with a slightly increased level of basal phosphorylation (Fig. 5).

Two-dimensional Phosphopeptide Mapping Reveals Constitutive Phosphorylation of Y305A—As determination of total phosphorylation does not reveal whether there are changes in the phosphorylation of single Ser/Thr residues, we performed two-dimensional phosphopeptide mapping. As previously reported for human fibroblasts, the unstimulated B2R in HEK293 cells showed basal phosphorylation of Ser\(^{348}\) (Ser(P)\(^{348}\)) contained in peptide 1 (Fig. 6, upper panel, left). Activation with 1 \(\mu M\) BK resulted in appearance of three additional spots which correspond to peptides comprising Ser(P)\(^{339}\), Ser(P)\(^{348}\), and Thr(P)\(^{342}\), as their relative intensities and distribution were in full agreement with the results reported for human fibroblasts (21). Surprisingly, however, even without stimulation, the Y305A mutant exhibited a phosphorylation pattern similar to that observed in the wt B2R only after ligand stimulation (Fig. 6, lower panel, left). This clearly indicated that the Y305A mutant was, at least in part, constitutively phosphorylated on Ser/Thr residues other than Ser\(^{348}\). Similar to our findings for total phosphorylation, the Y305F mutant showed a phosphopeptide map that was intermediate between wt B2R and Y305A, suggesting that the phenyl group of Phe can at least partially mimic the effects of the 4-hydroxyphenyl group of Tyr\(^{305}\), whereas the aliphatic side chain of Ala cannot (data not shown).

Uptake of Rhodamine-labeled Antibody Reveals Constitutive Internalization of Y305A—The observed constitutive phosphorylation of mutant Y305A led us to hypothesize that the predominant intracellular localization may be a consequence of ongoing agonist-independent internalization. To test this idea, we incubated HEK293 cells stably expressing wt B2R or Y305F with a rhodamine-labeled antibody directed to their N-terminal HA tag for 1 h at 37 °C. In cells expressing wt B2R, the rhodamine staining was seen almost exclusively at the cell surface (Fig. 7) and could be found inside the cell only after additional stimulation with BK (data not shown). The Y305A mutant, in contrast, showed almost no staining of the plasma membrane but, rather, a significant translocation of the labeled antibody probe into intracellular compartments within 1 h (Fig. 7), suggesting that this mutant internalized spontaneously from the cell surface. The distribution of antibody staining for mutant Y305F resembled that of the wt B2R. Co-incubation of Y305A with labeled antibody and antagonist JE049 (formerly also known as HOE140/Levatibant) resulted in distinctly weaker overall staining, with the majority of staining being associated with the plasma membrane. JE049, thus, acts like an inverse agonist reducing the spontaneous receptor internalization of Y305A. Another antagonist NPC17731 also appeared to function as an inverse agonist, because we did not observe internalization of \(^{3}H\)NPC17731 (PerkinElmer) as would have been expected for a neutral antagonist binding to a constitutively internalizing receptor (data not shown).

Unstimulated Y305A Couples to \(G_{q11}\) without Activating \(IR\)—Given that our data thus far suggest that the mutant Y305A receptors at the plasma membrane are constitutively phosphorylated and internalized, the issue arises as to why significant amounts of Y305A are still detected on the cell surface? As Y305A does not display increased basal IP accumulation, we speculated that the mutant receptor may couple to G protein \(G_{q11}\) without promoting guanine nucleotide exchange. To test this notion, we determined whether wt B2R or mutant Y305A co-immunoprecipitated with \(G_{q11}\), i.e. the major effector G protein of kinin receptors. Immunoprecipitation of B2R from unstimulated, highly expressing cells brought down small quantities of \(G_{q11}\) similar to those observed in control cells (Fig. 8). By contrast, immunoprecipitates of Y305A from unstimulated cells revealed a strong signal for \(G_{q11}\). This finding was obtained despite the fact that considerably less mutant receptor was applied to the gel, most likely because we used crude membrane preparations for the extract that bear much less mutant than wt receptor. To monitor basal guanine nucleotide exchange, we used a \(^{35}S\)GTP\(^{\gamma}\)S binding assay, which gave almost identical values for basal binding at 30 °C for wt B2R, regardless of the expression level, as well as for Y305A (Fig. 9), suggesting that the mutant receptor, despite being tightly coupled to \(G_{q11}\), does not significantly enhance guanine nucleotide exchange in the absence of an agonist. This finding is in agreement with our data from IP accumulation assays (see Table 1). We also observed that 1 \(\mu M\) BK induced a moderate increase in \(^{35}S\)GTP\(^{\gamma}\)S binding (28–39%) both for high and low expressing wt B2R and for Tyr\(^{305}\) probably because \(G_{q11}\) represents only a minor fraction of total G proteins contributing basal \(^{35}S\)GTP\(^{\gamma}\)S binding (28). Given that unstimulated Y305A binds to its G protein without activating it, we suggest that recruitment of \(G_{q11}\) may (partially) block the access of the internalization machinery to the phosphorylated C-tail of the mutant receptor, thereby limiting ligand-independent internalization of the mutant receptor.

DISCUSSION

The NPXXY motif located at the interface of transmembrane domain-7 and the cytosolic tail is one of the hallmarks for the family A of GPCRs. Because conservation of the pentapeptide sequence implies an important structural or functional role, this motif has been examined by mutagenesis studies in several GPCRs. These mutations affected receptor affinity (5, 7, 8), signaling (3, 5, 6, 9), sequestration (4, 13), and internalization of GPCRs (12) but to quite different extents, depending on the receptor under study. This may, in part, be reflective of the microenvironment surrounding this motif in the respective receptor sequence, the assays applied, or the cells used for ex-
pression. One of the first functions claimed for a NPXXY sequence in GPCRs was that of an endocytotic motif. This hypothesis was based on the observation that a similar sequence, NPXXY, plays a fundamental role in the internalization of the low density lipoprotein receptor because replacement of Tyr by an Ala residue abrogated the internalization capacity of the receptor (26). Support for a similar role in GPCRs was provided by the complete loss of internalization in the corresponding β2-adrenergic receptor mutant Y326A (13). In most other GPCRs, however, such as the angiotensin-II receptor AT1a (12) or the neurokinin NK1 receptor (9), a corresponding mutation had little or no effect on receptor internalization.

To monitor ligand-induced receptor translocation from the surface to the interior of a cell, two assays can be employed that both have their advantages and disadvantages. In the sequestration assay, cells are stimulated at 37 °C for various times with a saturating concentration of unlabeled agonist, and then a binding assay is performed at 4 °C with a radiolabeled, non-membrane-permeable agonist or antagonist to assess the remaining surface receptor expression. In this assay, receptor sequestration is expressed as the amount of remaining receptors on the cell surface of treated cells as a percentage of that initially present on unexposed cells. The ligand internalization assay is based on the assumption that the receptor-bound ligand and the receptor itself share, at least for a certain initial time frame, the same fate, i.e. the same cellular localization. Consequently, cells are incubated immediately with radiolabeled ligand at 37 °C (optionally after reaching equilibrium binding at 4 °C). At various times the amounts of surface-bound, and of internalized, ligand are determined separately at low pH. In this approach, internalization is expressed as amount of internalized ligand as a percentage of the total bound ligand (the sum of internalized and surface-bound ligand). As previously reported, the usage of the sequestration assay for cells that highly overexpress B2R may grossly underestimate “true” receptor sequestration, presumably because of an overload of the internalization machinery (19). The ligand internalization assay, in contrast, can be applied even under these conditions, provided appropriate low concentrations of radiolabeled agonist are used to avoid saturation of the internalization machinery. This method does not, however, differentiate between ligand internalization caused by ligand-promoted receptor translocation and that occurring via constitutive receptor translocation, because, in both cases, rapid ligand transport into intracellular compartments is observed. As our data with the mutant Y305A show, this is not just a theoretical problem.

At first sight, Y305A displays properties similar to those of wt B2R; it binds [3H]BK with the same affinity, it internalizes the agonist at a similar rate, and it induces cellular responses at similar EC50 (see Table I). By contrast, despite its relatively low surface expression, Y305A shows markedly reduced sequestration, a property previously noted to an even greater extent (i.e. no sequestration at all) for the corresponding β2-adrenergic receptor mutant (13). This dichotomous behavior, that is rapid ligand internalization and attenuated receptor sequestration, provided the first evidence that the Y305A mutant undergoes constitutive internalization. This conclusion was underlined by the demonstration of similar expression levels of wt B2R and Y305A despite markedly lower surface binding capacity of the latter. Hence a majority of mutant receptors were either not accessible to the hydrophilic ligand,
because of an intracellular location, or a major fraction of Y305A had very low affinity for the ligand. Usage of chimeric constructs showed that Y305A-eGFP fusion protein was, indeed, predominantly located inside the cell, even in the absence of a stimulus. Final proof that Y305A sponta- neously internalized came from the finding that rhodamine-labeled antibodies to the N-terminally tagged receptor were rapidly internalized by Y305A, but not by wt B2R. Two antagonists blocked this ongoing internalization, i.e. Y305A-mediated uptake of rhodamine-labeled antibody was markedly halted by JE049/ HOE140, and there was no significant internalization of the radiolabeled antagonist [3H]NPC17731 by Y305A (data not shown). This suggests that these ligands act as inverse agonists reducing the spontaneous internalization of Y305A.

Our initial studies of agonist-induced phosphorylation of Y305A suggested that this mutant is phosphorylation-resistant, as has previously been reported for the corresponding β2-adrenergic receptor mutant (11). Phosphopeptide analysis, however, suggested that this apparent resistance might be because the accessible mutant receptors on the plasma membrane are already phosphorylated on Ser/Thr residues in their basal state. This ligand-independent phosphorylation would also explain the enhanced spontaneous internalization of this mutant. Further support for the theory that the spontaneous internalization is indeed caused by ligand-independent phosphorylation is provided by the lack of spontaneous as well as agonist-induced internalization of a Y305A mutant in which the critical Ser/Thr residues had been replaced by Ala (results not shown). Hence the NPXXY sequence appears not to serve...
as an endocytotic motif interacting directly with the internalization apparatus, but, rather, is involved in the phosphorylation of Ser/Thr residues of the receptor tail.

We considered whether our data could be explained in the context of the two-state model in which an equilibrium exists between the receptor in its inactive and its active state (27). In the absence of agonist, one would expect this equilibrium to be in favor of the inactive state of wt B2R, whereas it would be shifted markedly in favor of the active state in the Y305A mutant, explaining the enhanced internalization. Binding of an antagonist also would promote the inactive state, attenuating internalization, as observed in our experiments. This model, however, is inconsistent with the lack of constitutive basal IP accumulation and of constitutive guanine nucleotide exchange. We, therefore, propose an alternative model in which the NPXXY sequence plays an important role in the interaction of the receptor with the G protein. We postulate that mutation Y305A induces a semi-active receptor conformation that is prone to phosphorylation and consequently to internalization, and that is also able to couple to the G protein, without, however, significantly activating it. Accordingly, the affinity of Y305A for G_{q,11} and/or the probability of a spontaneous ligand-independent activation of bound G_{q,11} would determine whether the internalization machinery gets access to the (already phosphorylated) C terminus resulting in the internalization of the receptor. Our hypothetical model predicts that there would be little or no increased basal IP accumulation, but stimulation with an agonist would nevertheless lead to robust phospholipase C activation and rapid receptor internalization. The fact that Y305A gives a stronger ligand-induced IP response than wt B2R may be explained in our model by assuming that, following ligand-dependent receptor activation and G_{q,11} dissociation, the precoupled semi-active Y305A conformation is more rapidly restored than the corresponding wt B2R form, allowing for an increased G protein turnover. Additionally, a reduced desensitization rate and/or a higher recycling rate of Y305A may contribute to the observed increase in IP accumulation. Inverse agonists could exert their inhibiting effect on the constitutive internalization either by converting Y305A back to an inactive conformation or, alternatively, through stabilization of the complex of Y305A with inactive G_{q,11}. Our finding that mutant Y305A, unlike wt B2R, is coupled to G_{q,11} even in the absence of a stimulus is consistent with this latter model.

The various phenotypes observed in the corresponding Tyr mutants of other GPCRs could be explained, according to our model, on the basis either of different affinities of these mutants for their cognate G proteins and/or by different degrees of spontaneous activation of these mutants, e.g. Y305A mutation of the neurokinin 1 receptor generated a mutant receptor, NK1-Y305A, that was primarily located intracellularly, even in its unstimulated state, whereas only minimal amounts of mutant were found on the cell surface (9). Moreover, the relative distribution (intracellular versus cell surface) of NK1-Y305A did not vary, regardless of whether it was incubated with agonist or not, implying deficient receptor sequestration. The mutant receptor, however, internalized radiolabeled ligand at a rate similar to that of the corresponding wt receptor. These data could be reconciled, according to our model, by assuming that NK1-Y305A forms a less stable complex with G_{q,11} than, e.g., the Y305A mutant of B2R, making the phosphorylated C terminus of NK1-Y305A more accessible to the internalization machinery and thereby promoting constitutive internalization. Assumption of a high affinity of the respective β2-adrenergic receptor mutant Y326A for G_{s}, combined with the observed inability of this mutant to activate the G protein, would explain the lack of sequestration and resistance to phosphorylation, as well as the fact that this mutant is located preferentially in the plasma membrane (10, 11, 13). As the binding of the G protein should nevertheless be reversible, the reported slightly increased intracellular localization (10) as well as the fact that overexpression of GPCR kinases or β-arrestins were able to rescue sequestration of this mutant (11) would fit our model.

In summary, our results demonstrate that the tyrosine residue of the NPXXY motif present in the human bradykinin B2 receptor plays a decisive role in the regulation of activation, phosphorylation, internalization, and thus sequestration of the receptor, presumably by controlling the affinity and activation capacity for the cognate G protein and by controlling access of the phosphorylation as well as internalization machinery.

Acknowledgments—We give our thanks to Drs. Petra Kamertisch and Ulrich Pohl for their support with confocal microscopy.

REFERENCES

1. Gether, U. (2000) Endocrin. Rev. 21, 90–113
2. Frederikson, R., Lagerstroem, M. C., Lundin, L.-G., and Schiødt, H. B. (2003) Mol. Pharmacol. 63, 1256–1272
3. Fritz, O., Filipiak, S., Kuiksa, V., Paleczewski, K., Hofmann, K. P., and Ernst, O. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2290–2295
4. Bouley, R., Sun, T.-X., Chenard, M., McLaughlin, M., McKee, M., Lin, H. Y., Brown, D., and Aussiello, D. A. (2003) Am. J. Physiol. Cell Physiol. 285, C750–C762
5. Prud’homme, C., Viisiers, I., Ebersole, B. J., Weinstein, H., and Sealfon, S. C. (2002) J. Biol. Chem. 277, 36577–36584
6. Rosendorff, A., Ebersole, B. J., and Sealfon, S. C. (2000) Mol. Brain Res. 84, 90–96
7. Hukovic, N., Panetta, R., Kumar, U., Rocheville, M., and Patel, Y. C. (1998) J. Biol. Chem. 273, 21416–21422
8. Wang, J., Zheng, J., Anderson, J. L., and Toews, M. L. (1997) Mol. Pharmacol. 52, 306–313
9. Bohn, S. K., Khitin, L. M., Smeekens, S. P. G., Grady, E. F., Payan, D. G., and Bunnell, N. W. (1997) J. Biol. Chem. 272, 2363–2372
10. Cahilando, A. M., Krasel, C., and Lohse, M. J. (1998) Eur. J. Pharmacol. 353, 243–250
11. Ferguson, S. G. S., Menard, L., Barak, I. S., Koch, W. J., Colapietro, A. M., and Caren, M. G. (1995) J. Biol. Chem. 270, 27475–27480
12. Huszady, I., Bor, M., Baulk, A. J., Balla, T., and Catt, K. J. (1995) J. Biol. Chem. 270, 16602–16609
13. Barak, I. S., Tiberi, M., Friedman, N. J., Kwatra, M. M., Lefkowitz, R. J., and Caren, M. G. (1994) J. Biol. Chem. 269, 2790–2795
14. Regoli, D., and Barabe, L. (1988) Methods Enzymol. 163, 210–230
15. Proud, D., and Kaplan, A. P. (1988) Annu. Rev. Immunol. 6, 4–83
16. Elhers, K. D., Figueroa, C. D., and worthy, K. (1992) Pharmacol. Rev. 44, 1–80
17. Blacka, A., Pizard, A., Breit, P., Alhenc-Gelas, F., Muller-Esterl, W., and Dikic, I. (2001) J. Biol. Chem. 276, 40341–40440
18. Ballerstedt, J. A., and Weinstein, H. (1995) Methods Neurosci. 25, 366–428
19. Faussner, A., Bauer, A., Kalatskaya, I., Chochom, M., and Fritz, H. (2003) Am. J. Physiol. 284, H1909–H1916
20. Hess, J. F., and Barabe, L. (1996) Methods Enzymol. 266, 9442–9446
21. Blacka, A., Abdalla, S., Lohse, M. J., and Muller-Esterl, W. (1996) J. Biol. Chem. 271, 32366–32374
22. Boyle, W. J., van der Geer, P., and Hanter, T. (1991) Methods Enzymol. 201, 110–149
23. Faussner, A., Heinz-Erian, P., Klier, C., and Rescher, A. A. (1991) J. Biol. Chem. 266, 9442–9446
24. Sahourin, T., Bastien, L., Bachvarov, D. R., and Marceau, F. (2002) Mol. Pharmacol. 61, 546–553
25. Marchese, A., Chen, C., Kim, Y. M., and Benovic, J. L. (2003) Trends Biochem. Sci. 28, 369–376
26. Towbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) Annu. Rev. Cell Biol. 9, 129–161
27. Samama, P., Cotechiza, S., Costa, T., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 4625–4638
28. Milligan G. (2003) Trends Pharmacol. Sci. 24, 87–90
Mutation of Tyrosine in the Conserved NPXXY Sequence Leads to Constitutive Phosphorylation and Internalization, but Not Signaling, of the Human B<sub>2</sub> Bradykinin Receptor

Irina Kalatskaya, Steffen Schüssler, Andree Blaukat, Werner Müller-Esterl, Marianne Jochum, David Proud and Alexander Faussner

*J. Biol. Chem.* 2004, 279:31268-31276.
doi: 10.1074/jbc.M401796200 originally published online May 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401796200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 14 of which can be accessed free at http://www.jbc.org/content/279/30/31268.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on July 25, 2018