Interruption of the ionic lock in the bradykinin B₂ receptor results in constitutive internalization and turns several antagonists into strong agonists

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Abstract

The DRY motif with the highly conserved R3.50 is a hallmark of family A GPCRs. The crystal structure of rhodopsin revealed a salt bridge between R135$^{3.50}$ and another conserved residue, E247$^{6.30}$, in helix 6. This ionic lock was shown to maintain rhodopsin in its inactive state. So far, little information is available on how interruption of this ionic bond affects signaling properties of non-rhodopsin GPCRs, as the focus has been on mutations of R3.50, although this residue is indispensable for G protein activation. To investigate the importance of an ionic lock for overall receptor activity in a non-rhodopsin GPCR, we mutated R128$^{3.50}$ and E238$^{6.30}$ in the bradykinin (BK) B$_2$ receptor (B$_2$R) and stably expressed the constructs in HEK293 cells. As expected, mutation of R3.50 resulted in lack of G protein activation. In addition, this mutation led to considerable constitutive receptor internalization. Mutation of E6.30 (mutants E6.30A, E6.30R) also caused strong constitutive internalization. Most intriguingly, however, although the two E6.30 mutants displayed no increased basal phosphatidyl inositol hydrolysis, they gave a response to three different B$_2$R antagonists that was almost comparable to that obtained with BK. In contrast, swapping of R3.50 and E6.30, thus allowing the formation of an inverse ionic bond, resulted in rescue of the wild type phenotype. These findings demonstrate for the first time that interruption of the ionic lock in a family A GPCR can have distinctly different effects on receptor internalization and G protein stimulation, shedding new light on its role in the activation process.
Introduction

G protein-coupled receptors (GPCRs) represent the largest superfamily of transmembrane receptors in the human genome. 25-50% of all marketed drugs act directly or indirectly via members of this family. Nonetheless, their potential for future drug development is not at all fully utilized as an in detail elucidation of GPCR regulatory mechanisms, in particular with regard to their receptor specificity, is still lacking (Vassilatis et al., 2003; Jacoby et al., 2006; Overington et al., 2006). Rhodopsin-like family A GPCRs are characterized by a few highly conserved amino acid motifs (Figure 1) that are assumed to play a crucial role for structure and/or function (Nygaard et al., 2009; Salon et al., 2011). The dark-state crystal structure of bovine rhodopsin (PDB: 1U19) displayed a salt bridge between arginine 3.50 \(R3.50\), denoted according to the Ballesteros/Weinstein numbering (Ballesteros et al., 1998) of the E/DRY motif at the cytosolic end of transmembrane domain 3 (TM3) and glutamate 6.30 (E6.30) in helix 6 (Palczewski et al., 2000). This interaction became also known as “ionic lock” and has been reported to maintain the inactive rhodopsin conformation by connecting TM3 and TM6 (Teller et al., 2001; Okada et al., 2004; Vogel et al., 2008). R3.50 is the most conserved residue in family A GPCRs (96%), in which an acidic residue in position 6.30 is also quite common (32%) (Mirzadegan et al., 2003; Springael et al., 2007). However, regarding the latter, there are large differences amongst subfamilies: It is found in almost all amine GPCRs (> 90% E/D6.30), but occurs rarely in peptide GPCRs (< 7%). Surprisingly little information is available on the importance of the ionic lock itself for the overall activity status in these non-rhodopsin GPCRs. This is mainly due to the fact that most investigations focused on R3.50 in the DRY motif instead of on the acidic residue in position 6.30 (Scheer et al., 1996; Ballesteros et al., 1998; Rovati et al., 2007). R3.50, however, is \textit{per se} indispensable for G protein activation (Rovati et al., 2007; Schneider et al., 2010). Therefore, unfortunately, mutation of R3.50 and G protein-
mediated processes are not suitable to determine the consequences of a disruption of the ionic lock on the receptor activity status. In contrast, receptor internalization is also an important process of most activated non-rhodopsin GPCRs and has been reported to be G protein-independent (Shenoy and Lefkowitz, 2005; Shukla et al., 2011). Thus, it is astonishing that the impact of a mutation of R3.50 and the disruption of the ionic lock on this important regulatory mechanism has not been investigated yet.

The human bradykinin B₂ receptor (B₂R) is a member of the family A GPCRs. Moreover, it is one of the very few peptide GPCRs with an acidic glutamate in position 6.30 and thus suited for studying the ionic lock in a non-rhodopsin GPCR. The B₂R is ubiquitously expressed in almost all cells and tissues and plays an important role in a variety of physiological processes comprising vasodilatation, oedema formation, natriuresis and hyperalgesia (Leeb-Lundberg et al., 2005). Its stimulation results in activation of the G proteins G_q/11 and G_i, and of G protein-dependent MAPK cascades (Blaukat et al., 2000; Leschner et al., 2011). Ligand-mediated receptor internalization is important for the regulation of B₂R signaling. It is initiated by phosphorylation of serine/threonine residues in the B₂R C-terminus, which leads to the recruitment of β-arrestins and ends with the sequestration of the receptor into intracellular compartments (Leeb-Lundberg et al., 2005).

In recent years it has become clear that multiple conformations of a single GPCR exist, which are coupled in different ways to the various effects controlled by the respective receptor (Rosenbaum et al., 2009; Salon et al., 2011) and may depend on the type of ligand bound. Therefore, we hypothesized that the ionic lock and the surrounding amino acid network might play distinct roles in G protein activation and receptor internalization. We used the B₂R to test our hypothesis and were able to show for the first time that interruption of the ionic lock differentially affects receptor internalization and G protein-mediated signaling. Moreover,
disrupting the ionic lock by mutating only one single amino acid (E6.30) completely altered the impact of several antagonists on these processes. Our findings argue for a multistep process of GPCR activation, where each step might have a different sensitivity for agonists and antagonists. This new insight into GPCR activation might contribute to a more efficient drug design.
Materials and Methods

Materials. Flp-In™ TREx-293 (HEK293) cells and Opti-MEM I serum-free medium were obtained from Invitrogen (Karlsruhe, Germany). [2,3-prolyl-3,4-3H]bradykinin (80 Ci/mmol), myo-[2-3H]inositol (22 Ci/mmol) and [prolyl-3,4-3H]NPC17731 (48.5 Ci/mmol) were from Perkin Elmer Life Sciences (Boston, MA, USA). [32P]phosphoric acid was delivered by Hartmann Analytic (Braunschweig, Germany). Bradykinin was purchased from Bachem (Heidelberg, Germany). B9430, icatibant and JSM10292 were generous gifts from Dr. L. Gera and J. Stewart (Denver, CO, USA) and Jerini (Berlin, Germany), respectively. Roche (Mannheim, Germany) delivered FuGeneHD, complete mini EDTA-free protease inhibitor tablets and the rat monoclonal anti-hemagglutinin (HA)-peroxidase high affinity antibody (3F10). EZview red anti-HA affinity gel, poly-D-lysine, captopril, 1.10-phenanthroline and bacitracin were purchased from Sigma-Aldrich (Taufkirchen, Germany). Fetal calf serum, DMEM, penicillin/streptomycin and hygromycin B were obtained from PAA Laboratories (Coelbe, Germany). Primers were synthesized by MWG-Biotech (Ebersberg, Germany) and delivered desalted and lyophilized.

Gene mutagenesis, expression and cell culture. Standard PCR techniques with accordingly designed primers and the bradykinin B2 receptor (B2R) gene as template, were used to generate point-mutants of the B2R. The coding sequences of the B2Rwt and the mutants started with the third encoded Met (Hess et al., 1992) and were cloned into the HindIII and XhoI sites of the pcDNA5/FRT/TO vector (Invitrogen). At the N-terminus, a single hemagglutinin-tag (MGYPYDVPDYAGS), with the last two amino acids (Gly-Ser) of the tag deriving from the insertion of a BamHI restriction site, preceded the receptor sequences. For stable, inducible expression of the constructs, we used the Flp-In TREx-293 system from Invitrogen, in which the
vector containing the gene of interest is inserted at a unique locus into the genome of the special host cell line Flp-In™ TREx-293 (HEK293) through the transient expression of recombinase pOG44. HEK293 cells, cultivated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin, were transfected using the transfection reagent FuGeneHD following the instructions of the manufacturer. Single stably expressing clones resulted from selection with 250 µg/ml hygromycin B. Receptor expression in these cells was induced by addition of 0.5 µg/ml tetracycline 1-2 days before the experiment. For experiments requiring repeated rinsing of the cells, poly-D-lysine-treated (0.01% in PBS) cell culture dishes were used to ensure adherence.

**Equilibrium binding experiments at 4°C and 37°C.** The dissociation constant (K_d) was determined with [3H]BK as described previously (Faussner et al., 2004). For determination of the equilibrium binding affinity constant at 37°C, receptor sequestration was inhibited by pre-treatment of the cells with 100 µM phenylarsine oxide (PAO) for 5 min at 37°C (Faussner et al., 2004). Cell monolayers in 48-wells were incubated either on ice for 90 min or at 37°C for 30 min 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) with degradation inhibitors (2 mM bacitracin, 0.8 mM 1.10-phenanthroline, and 100 µM captopril) containing increasing concentrations of up to 30 nM [3H]BK. Thereafter, cells were rinsed, bound [3H]BK dissociated by a 10 min incubation with 0.2 ml of an ice-cold dissociation solution (0.2 M acetic acid/0.5 M NaCl, pH 2.7), transferred to a scintillation vial and counted in a β-counter after addition of scintillation fluid. Nonspecific binding was determined with a 1000-fold excess of unlabeled BK and subtracted from total binding to give receptor specific binding.
[\textsuperscript{3}H]BK dissociation. To inhibit receptor sequestration, monolayers stably expressing the indicated constructs were pre-treated with 100 µM PAO in incubation buffer for 5 min at 37°C and then incubated with 2 - 4 nM [\textsuperscript{3}H]BK on ice for 90 min. Thereafter, cells were thoroughly washed with ice-cold PBS and incubated with 1 µM BK in pre-warmed incubation buffer at 37°C. At the indicated times [\textsuperscript{3}H]BK dissociation was stopped by removing the supernatant. Remaining surface bound [\textsuperscript{3}H]BK was dissociated and determined with ice-cold dissociation solution as described above.

Determination of receptor distribution with [\textsuperscript{3}H]JSM10292 (Faussner et al., 2012). Confluent cells in 24- or 48-wells were incubated on ice for 3 h in incubation buffer with degradation inhibitors containing 30 nM of the cell membrane-permeant antagonist [\textsuperscript{3}H]JSM10292. Nonspecific binding was determined either with a 1000-fold excess of unlabeled JSM10292 (calculated specific binding comprises intracellular and surface receptors) or BK (calculated specific binding covers only surface receptors). The amount of intracellular receptors is calculated as the difference between specific binding obtained with JSM10292 and that obtained with BK.

Determination of total inositol phosphate (IP) accumulation. Monolayers of stably transfected HEK293 cells on 12-wells were incubated overnight with 0.5 ml complete medium containing 1 µCi [\textsuperscript{3}H]inositol/ml. The cells were washed twice with PBS and pre-incubated for 90 min on ice in incubation buffer supplemented with 50 mM LiCl with or without addition of the indicated concentration(s) of (pseudo)peptide. Stimulation was started by placing the cells in a water bath at 37 °C and continued for 30 min. The accumulation of IPs was terminated by exchanging the buffer for 0.75 ml of ice-cold 20 mM formic acid solution. After 30 min on ice
another 0.75 ml of formic acid solution, followed by 0.2 ml of a 3% ammonium hydroxide solution, were added. The mixture was applied to AG 1-X8 anion exchange columns (Bio-Rad, Munich, Germany; 2 ml volume). The columns were washed with 1 ml of 1.8% ammonium hydroxide and 9 ml of 60 mM sodium formate/5 mM tetraborate buffer, followed by 0.5 ml of 4 M ammonium formate/0.2 M formic acid. Total IPs were finally eluted in 2 ml of the latter buffer and counted in a β-counter after addition of scintillation liquid.

[^3H]BK and[^3H]NPC17331 internalization. [^3H]ligand internalization was determined as described recently (Faussner et al., 2009). In brief, cells on 24-well plates were incubated with 0.2 ml of 2 nM[^3H]BK or 2.5 nM[^3H]NPC17331 in incubation buffer for 90 min on ice in order to obtain equilibrium binding.[^3H]ligand internalization was started by placing the plates in a water bath at 37°C. The internalization process was stopped at the indicated times by putting the plates back on ice and rinsing the cells with ice-cold PBS. Surface-bound[^3H]BK or[^3H]NPC17331 was dissociated by incubating the cells for 10 min with 0.2 ml of ice-cold dissociation solution. The remaining cell monolayer with internalized[^3H]ligand was lysed in 0.2 ml 0.3 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[^3H]ligand surface binding and internalization were determined in the presence of 5 µM unlabeled BK and subtracted from total binding to calculate the specific values. Internalization was expressed as amount of internalized[^3H]ligand as a percentage of the combined amounts of internalized and surface bound[^3H]ligand.

Biotinylation protection assay (Feierler et al., 2011). Confluent cell monolayers were incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 min at
4°C. After rinsing twice with ice-cold Tris-buffered saline (TBS) to quench the biotinylation reaction, cells were equilibrated in Opti-MEM I (Invitrogen) for 30 min at 37°C and further incubated in absence or presence of 10 μM bradykinin or 5 μM icatibant. After rinsing with ice-cold TBS, cells were stripped with 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH and 1% FCS for 30 min at 4°C, wherever indicated. Glutathione was quenched with 50 mM iodoacetamide and 1% BSA for 20 min at 4°C. All cells were washed twice with ice-cold PBS and lysed with extraction buffer, pH 7.4, containing 0.1% Triton X-100, 10 mM Tris-HCl, 150 mM NaCl, 25 mM KCl as well as a protease inhibitor cocktail (complete Mini, EDTA-free; Roche, Mannheim, Germany). Lysates were centrifuged for 15 min with 14000 rpm at 4°C in a microfuge. The supernatant was added to 20 μl EZview red anti-HA affinity gel pre-equilibrated with extraction buffer, and incubated under gentle mixing for 1 hour at 4°C. Subsequently, the matrix was washed extensively with extraction buffer and denatured with NuPAGE LDS sample buffer (Invitrogen) for 5 min at 95°C without reducing agents. Proteins were fractionated by SDS polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, which were blocked for 1 h in TBS containing 1% Tween 20 and 5% milk powder. Biotinylated proteins were detected by the Vectastain avidin-biotinylated enzyme complex immunoperoxidase reagent (Vector Laboratories, Burlingame, CA, USA) and developed with Western blot Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA, USA).

**Receptor phosphorylation.** Stably transfected cells on 6-well plates were washed twice with phosphate-free DMEM, incubated therein for 1 h at 37 °C and labeled with 0.2 mCi/ml \(^{32}\text{P}\)orthophosphate for 1 h. After 5 min of stimulation with 1 μM BK at 37 °C, cells were scraped in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (complete Mini, EDTA-free; Roche,
Mannheim, Germany) and centrifuged at 14000 rpm for 15 min at 4°C. Immunoprecipitation of HA-tagged receptor proteins was performed by incubation of the supernatant with 15 μl EZview red anti-HA affinity gel for 1 h at 4°C. After washing the matrix in three steps with ice-cold lysis buffer and addition of 30 μl of 1x NuPAGE LDS Sample Buffer (Invitrogen) containing 0.1 M DTT, immunocomplexes were dissociated at 95°C for 10 min. Proteins were separated as described above on a 4 - 12% SDS-PAGE gel. Receptor phosphorylation was detected by autoradiography.

**Protein determination.** Total protein was quantified with the Micro BCA Protein assay reagent kit from Pierce (Rockford, IL, USA) using BSA as standard.

**Homology modeling.** Sequence alignment of the human B2R with the bovine rhodopsin performed by ClustalW (Saitou and Nei, 1987; Chenna et al., 2003) showed 19% of identical amino acids and 34% of similar amino acids. In the end of TM3 and the beginning of TM6 with E6.30238, 32% of the residues are identical and 47% are similar. The human B2R model was obtained by using the homology modeling tool of the Molecular Operating Environment (MOE) V2010.11 (Chemical Computing Group, Inc.) with the following settings: 10 main chain models each with 3 side chain samples at a temperature of 300 K using the amber99 force field (Wang et al., 2000) were built, resulting in 30 intermediate models with an RMS gradient of 1. Out of these 30 models, the final homology model was built by applying refinement protocols with an RMS gradient of 0.5. Subsequently, side chain positions were refined to optimize the protein geometry taking into account typical dihedral angle distributions. Models for mutant B2R were generated using the mutation tool of MOE (Chemical Computing Group, Inc.).
Data analysis. All experiments were performed at least three times in duplicates or triplicates and results are given as the mean ± SEM unless otherwise indicated. Data analysis was performed using GraphPad Prism for Macintosh, Version 4.0c (GraphPad Software, Inc., San Diego, CA, USA).
Results

To determine the biochemical and functional role of the two conserved residues R3.50 and E6.30 in bradykinin B\textsubscript{2} receptor (B\textsubscript{2}R) regulation, several receptor mutants were generated: (i) by single mutation of the respective residues in the B\textsubscript{2}R wild type (B\textsubscript{2}R\textsubscript{wt}), designated R3.50X or E6.30X, (ii) by combined charge-neutralizing mutations of both corresponding residues to alanines (R3.50A/E6.30A) and (iii) by mutual swapping of the respective amino acids R3.50 and E6.30 (R3.50E/E6.30R). All receptor mutants were stably expressed in HEK293 cells and their phenotypes characterized with regard to surface receptor expression, their affinity state at 4°C and 37°C, their capacity to induce phosphatidyl inositol (PI) hydrolysis, basal and ligand-induced internalization, their phosphorylation pattern, and receptor distribution.

Use of different promoters to obtain comparable surface expression. We have shown recently for the B\textsubscript{2}R\textsubscript{wt} that high overexpression [\textgreater{} 10 pmol/mg protein under the control of the CMV (cytomegalovirus) promoter] turned ligands such as icatibant or B9430, known as antagonists in endogenously B\textsubscript{2}R expressing cells, into partial agonists. These effects were not observed when the B\textsubscript{2}R\textsubscript{wt} was expressed at a lower level (< 5 pmol/mg protein) as obtained by the P\textsubscript{min} promoter that consists of only the last 51 nucleotides of the CMV promoter (Faussner et al., 2009). To ensure comparable surface expression levels (B\textsubscript{max}) for wild type and mutant receptor constructs, mutant receptors with their generally lower surface binding were still expressed under the control of the strong CMV promoter. Thus, similar stable expression levels were obtained for all constructs (Table 1).

Single E6.30 mutants display high affinity states at 37°C. Binding studies at 4°C revealed no significant differences between wild type and mutant receptor constructs with regard to their
binding affinity $K_d$ (Table 1). However, unless respective mutations affect directly the binding site, equilibrium binding with $[^{3}\text{H}]\text{BK}$ at 4°C in our experience always results in comparable high affinity binding for $B_2R$ constructs (Faussner et al., 2009) and thus is not really meaningful for determination of the affinity state. With receptor internalization blocked by pre-treatment with 100 µM phenylarsine oxide (PAO), it is possible to get valuable information on the affinity state of a construct at 37°C in intact cells by determination of the dissociation rate of $[^{3}\text{H}]\text{BK}$. A well described example for a constitutively active $B_2R$ mutant in a permanent high affinity state is construct N3.35113A (Marie et al., 1999). Accordingly, the $[^{3}\text{H}]\text{BK}$ dissociation observed for this mutant was much slower than that found for the $B_2R_{wt}$, which shows a strong shift to lower affinity at 37°C (Faussner et al., 2004): after 10 min at 37°C N3.35113A had still more than 60% of the initial $[^{3}\text{H}]\text{BK}$ bound, whereas $B_2R_{wt}$ already after 5 min had only 5% left (Figure 2). Mutations of R3.50 resulted in dissociation rates that were comparable to those determined for the $B_2R_{wt}$, indicating a low affinity state for these constructs at 37°C. In strong contrast, both single mutations of E6.30 suggested a high affinity state, with E6.30R displaying an even significantly slower $[^{3}\text{H}]\text{BK}$ dissociation than mutant E6.30A, comparable to that of N3.35113A (Figure 2). This high affinity state of the E6.30 mutants depended, however, strongly on the presence of R3.50, as both double mutants, R3.50A/E6.30A and R3.50E/E6.30R, displayed fast $[^{3}\text{H}]\text{BK}$ dissociation, indicating a low-affinity state. These differences between the dissociation rates obtained for the various constructs at 37°C were quite well supported by the $K_d$-values of the constructs measured at 37°C (Table 1). $K_d$-values were obtained after inhibition of receptor internalization by PAO pre-treatment, but were less reproducible and therefore less reliable for determination of the affinity state than the dissociation data.
Basal and stimulated phosphatidyl inositol (PI) hydrolysis. B2Rwt responded to challenge with 1 µM BK with a 12-fold increase in the accumulation of total inositol phosphates (IPs) as compared to basal levels (Figure 3A). Substitution of R3.50 with small neutral or negatively charged residues, such as alanine, histidine or glutamate, abolished PI hydrolysis upon BK stimulation, highlighting the crucial role of R3.50 for G protein activation also in the B2R (Figure 3A, Table 1). BK stimulation of construct E6.30A, in contrast, resulted in an almost 14-fold higher IP accumulation as compared to basal levels. Since basal phospholipase C β (PLCβ) activity was not significantly elevated in E6.30A expressing cells (Figure 3A), this construct was not constitutively active with regard to G protein-mediated signal activity, although it displays a high affinity state at 37°C (Figure 2 and Table 1). However, it has been shown that besides constitutively active receptor mutants there are constructs that display normal basal activity but give strong responses to weak partial agonists and therefore are considered semi-active (Ballesteros et al., 2001; Fritze et al., 2003). In line with the idea of semi-activity, we have shown previously that some B2R mutants respond with robust PI hydrolysis to the antagonists icatibant and B9430 (Faussner et al., 2009). As depicted in figure 3A, in E6.30A and E6.30R expressing cells, icatibant displayed the same efficacy as BK, suggesting semi-active conformations for these mutants. As demonstrated by the dose-response curves shown in figure 3B all the antagonists, B9430, icatibant and even the small molecule antagonist JSM10292 (Gibson et al., 2009), stimulated mutant E6.30R with practically identical potency and efficacy as BK. In cells expressing mutant E6.30A, all the tested antagonists (icatibant and JSM10292) also behaved as agonists, however with slightly lower potency as compared to the stimulation observed with the agonist BK (Supplemental Figure 1). Thus, mutation of E6.30 to an arginine or to an alanine turned all the tested B2R ligands into strong agonists. In contrast, mutation of the two amino acids and potential interaction partners of R3.50 in the DRY motif, D3.49 or Y3.51,
to alanines, did not change BK induced PI hydrolysis at all as compared to B2Rwt (data not shown). Unsurprisingly, mutant R3.50A, which did not even respond to BK stimulation, was incapable to generate an IP signal upon challenge with the antagonist icatibant, and double substitution of R3.50 and E6.30 with alanines created the same phenotype as the single R3.50A mutation (Figure 3A). Interestingly, mutual swapping of R3.50 and E6.30 (R3.50E/E6.30R) induced a clear tendency to BK stimulated PI hydrolysis, which, however, did not turn significant (Figure 3A).

**Negative charge at position 3.50 disturbs B2R internalization.** After stimulation with BK the B2R gets rapidly internalized (Leeb-Lundberg et al., 2005). To functionally characterize the effects of disruption of the ionic lock by either mutating R3.50 or E6.30, changes in [3H]BK uptake were determined over a short time period of 30 min, for which a similar fate of receptor and ligand can be assumed.

After 10 min B2Rwt expressing cells had internalized almost 90% of the specifically bound [3H]BK to acetic acid resistant compartments (Figure 4A). Charge-neutralizing mutation of R3.50 to alanine had no significant negative effect, indicating that G protein activation is not a prerequisite for [3H]BK internalization. Only replacement of the positively charged R3.50 with negatively charged residues, such as glutamate or aspartate, significantly reduced the internalization rate (Figure 4A). This was not a direct effect of a negatively charged amino acid in position 3.50 on the internalization mechanism per se as fast internalization could be rescued by an additional mutation of either D3.49 to an arginine or E6.30 to an alanine or an arginine (Figure 4C). Accordingly, the respective single mutations of E6.30 also had no effect on [3H]BK internalization (Figure 4B).
R3.50A and E6.30A/R internalize antagonist \[^3^H\text{NPC17331}\]. Charge neutralization or charge reversal of E6.30 resulted in strong PI hydrolysis even in response to B2R antagonists (see Figure 3), indicating a semi-active conformation of these mutants with regard to G protein interaction. Our next goals were to determine, (i) whether this was limited to G protein activation or also the case with regard to receptor internalization and (ii) whether this was due to interruption of the ionic lock in general or only caused by the point mutation of E6.30.

In contrast to B2Rwt, which did not internalize the antagonist \[^3^H\text{NPC17331}\] (about 10% internalization after 10 min at 37°C), in R3.50A mutant cells about 25% of specifically bound \[^3^H\text{NPC17331}\] were internalized after 10 min at 37°C (Figure 5). Mutant receptors E6.30A and E6.30R showed even stronger antagonist internalization (approx. 60% within 10 min). Simultaneous mutation of both residues R3.50 and E6.30 to alanines, created receptors strongly internalizing \[^3^H\text{NPC17331}\] comparable to mutant E6.30A. In contrast, double mutation by mutual swapping of R3.50 and E6.30 restored the phenotype of B2Rwt, as mutant R3.50E/E6.30R only slightly internalized \[^3^H\text{NPC17331}\] (about 17% internalization after 10 min at 37°C). The results show that abolishing ionic lock formation by mutating either of the interacting residues results in increased internalization of the antagonist \[^3^H\text{NPC17331}\].

**Biotinylation protection assay reveals constitutive internalization.** The observed uptake of \[^3^H\text{NPC17331}\] could be due to either ligand-induced receptor internalization (partial agonist effect) or result from ligand-independent constitutive receptor internalization. To distinguish between these two possibilities a biotinylation protection assay (BPA) was performed (Feierler et al., 2011). Cell surface receptors were labeled at 4°C with a membrane-impermeable biotin-derivative containing a disulfide linker. Receptors that had been internalized thereafter at 37°C in a ligand-dependent or -independent manner, were selectively identified by immunoprecipitation.
and Western blot analysis: Due to their localization within intracellular compartments as a consequence of endocytosis, their biotin-label would have been protected from cleavage by the extracellular treatment with reducing glutathione.

B2Rwt got prominently internalized only upon BK stimulation (Figure 6), demonstrating that B2Rwt internalization is strictly dependent on agonist stimulation. Breaking the ionic lock by mutating the single residues to alanines (R3.50A, E6.30A) generated receptors that internalized from the cell surface in a constitutive manner, i.e. in the absence of any agonist. Addition of BK augmented this internalization (significantly for R3.50A, as a tendency only for E6.30A) to a similar level as observed for the B2Rwt. Simultaneous mutation of R3.50 and E6.30 to alanines (R3.50A/E6.30A) resulted in a construct showing maximal constitutive internalization that could no longer be increased by addition of BK. Intriguingly, any ionic lock between position 3.50 and position 6.30 is sufficient to keep B2R internalization fully agonist-dependent, since mutually swapping both residues (R3.50E/E6.30R) completely abolished ligand-independent, constitutive internalization. Regarding receptor internalization, icatibant behaved as a “neutral antagonist”, since it did in all cases neither enhance nor inhibit (constitutive) receptor internalization (Figure 6).

**E6.30 mutation significantly increases basal receptor phosphorylation.** According to the widely accepted model of GPCR trafficking, receptor phosphorylation mostly by GPCR kinases (GRKs) is considered a major requirement for internalization. Thus, we next investigated whether the ligand-dependent and -independent internalization behavior of the various B2R constructs is also reflected in their respective phosphorylation patterns.

The B2Rwt was basally phosphorylated and reacted to BK stimulation with an approx. 3-fold increase in phosphorylation intensity (Figure 7), in agreement with previous publications.
Double mutant R3.50A/E6.30A is largely located intracellularly. We previously characterized a novel cell membrane-permeant small molecule, JSM10292, which, $^3$H-labeled,
allows the differentiation between surface and intracellularly located wild type and mutant B₂Rs, as long as they are binding-competent (Faussner et al., 2012). Unlike the B₂Rwt, which, unstimulated, is located mostly at the cell surface (approx. 80%), > 50% of the single mutant receptor constructs, R3.50A and E6.30A, were found to be located intracellularly (Table 2). Strikingly, double mutation of both residues to alanines (R3.50A/E6.30A) lead to a strong intracellular localization of about 70%, whereas mutual exchange of both highly conserved residues (R3.50E/E6.30R) generated a wild type-like surface localization (Table 2).
Discussion

Homology modeling of the B₂R, based on the bovine rhodopsin structure as a template, displayed a salt bridge between R3.50 and E6.30 that connects TM3 with TM6 (Figure 8A), thus stabilizing the inactive conformation. An additional network of hydrogen bonds around R3.50 and E6.30 involving the side chains of D3.49 and T6.34, and the carbonyl oxygens of A6.33 and E6.30, supports the ionic lock in this B₂R model. All these residues can be found in identical positions in many other family A GPCRs suggesting similar structural networks.

Function of the DRY motif with R3.50. In the B₂R, a single substitution of R3.50 with other amino acids (A, H, D, E) completely abolished the receptor’s ability to induce phosphatidyl inositol (PI) hydrolysis (see Table 1), arachidonic acid release (shown for R3.50A, Supplemental Figure 2) and ERK1/2 phosphorylation (Supplemental Figure 3). This emphasizes the significance of this residue for productive G protein interaction, that has also been observed for almost all other family A GPCRs investigated so far (Scheer et al., 1996; Ballesteros et al., 2001).

For GPCRs that become phosphorylated by GRK2/3 during their desensitization it has been proposed that these GRKs are recruited by attaching with their pleckstrin homology domains to βγ-subunits that become available upon G protein activation (Willetts et al., 2003). This mechanism is obviously not the only plausible one, as all signaling-incompetent R3.50 mutants got phosphorylated (shown for R3.50A, see Figure 7) and internalized similarly to the B₂Rwt (see Figure 4). Fast internalization has also been reported for a G protein activation-incompetent R3.50A mutant of the type 1 angiotensin receptor (AT₁R) (Gaborik et al., 2003). Disruption of the ionic bond might therefore, result in a conformation that directly interacts with GRK2/3. Such a direct interaction that was primarily dependent on an intact helix 8 of the receptor has been demonstrated for wild type and truncated B₂R constructs (Feierler et al., 2011).
GPCRs can activate mitogen-activated protein kinase (MAPK) cascades G protein dependently and/or via the recruitment of β-arrestins (Shenoy and Lefkowitz, 2005). Our data support a predominantly G protein-dependent MAPK activation by the B₂R, since the PI hydrolysis-incompetent mutant R3.50A becomes internalized rapidly after BK stimulation (see Figure 4) and therefore should interact with β-arrestins (as also indicated by translocation studies with eYFP-β-arrestin 1 and 2, data not shown), but nevertheless does not induce ERK1/2 phosphorylation (Supplemental Figure 3). In contrast, the AT₁R DRY mutant was still able to elicit ERK1/2 phosphorylation in a β-arrestin dependent way as demonstrated by siRNA-knockdown experiments (Wei et al., 2003). Thus, whether an interaction with β-arrestins results not only in internalization but also in ERK1/2 activation might depend on additional factors e.g. a receptor specific GRK-generated phosphorylation pattern (Nobles et al., 2011).

Whereas for other GPCRs residue D3.49 was reported to play an important role in G protein-dependent signaling (Scheer et al., 1996; Rasmussen et al., 1999), we did not observe any significant differences between mutant D3.49A and the B₂Rwt with regard to PI hydrolysis, ligand-mediated internalization, or affinity state at 37°C (data not shown). This stresses the importance of the structural context of these highly conserved residues as they apparently not do play necessarily the same roles in different family A GPCRs.

**Function of E6.30 in the activation process.** Charge neutralization or reversal of E6.30 resulted in B₂R constructs that responded with strong PI hydrolysis, as well as ERK1/2 phosphorylation (see Supplemental Figure 3, not shown for E6.30R) not only to the agonist BK, but also to the antagonists B9430, icatibant or JSM10292 (see Figure 3 and Supplemental Figure 1). Contrary to the β₂ adrenergic receptor, where E6.30A mutation elevated basal receptor activity (Ballesteros et al., 2001), or the 5-Hydroxytryptamine 2A (5-HT2A) receptor, in which E6.30R mutation evoked a high constitutive activity (Shapiro et al., 2002), the analogue B₂R mutants showed no
or only minor increases in basal IP generation (see Figure 3) or MAPK stimulation (see Supplemental Figure 3). However, they apparently adapted a highly sensitive conformation - as also indicated by their high affinity state at 37°C (see Figure 2 and Table 1) - that could be easily activated by binding of all kinds of ligands. The data also demonstrate that a high affinity state of a GPCR does not necessarily imply constitutive activity. Similarly, E6.30 mutations in the thromboxane prostanoid receptor resulted in more efficient agonist-induced signaling without any increase in basal activity (Ambrosio et al., 2010). Moreover for the B2R, even the small molecule compound JSM10292, that so far had displayed no partial agonistic activity (Faussner et al., 2012), becomes a full agonist in mutant E6.30R. This might be explained by assuming a semi-active conformation due to repulsion of the two positively charged arginines (Figure 8C) that imitates in a way a structural change - TM6 moving away from TM3 – that has been described as part of the activation process (Ballesteros et al., 2001; Springael et al., 2007; Rasmussen et al., 2011). In addition, mutation E6.30A/R resulted in strong uptake of the antagonist [3H]NPC17331, increased basal phosphorylation, constitutive internalization and consequently considerable intracellular localization. These data strengthen the idea that the E6.30 mutants adopt a conformation that constitutively interacts with GRKs and β-arrestins, but not with G proteins as they display no significant agonist-independent PI hydrolysis.

Is there an ionic lock in the B2R?

Although homology modeling indicated an ionic lock (Figure 8A) in the B2R, its presence would set the B2R quite apart from other peptide GPCRs as most of them (> 90%) cannot form an ionic lock due to the lack of an acidic residue in position 6.30 (Mirzadegan et al., 2003). Without R3.50 and E6.30 actually interacting, effects of single mutations should be rather different and in most cases additive in the double mutant. In contrast, if they form an ionic lock, the effects of the single mutations in theory should give identical results. Both mutants, R3.50A and E6.30A
internalized the antagonist [3H]NPC17331, both indicated constitutive internalization that could be increased by BK but not icatibant, and both showed elevated basal phosphorylation and similar intracellular localization. As the double mutation R3.50A/E6.30A resulted in effects that were at least as strong as those of the single mutations, but not significantly higher or additive, the formation of an ionic lock in the B2R in its inactive state is feasible. The fact, that all the observed effects were stronger in the E6.30 mutants than in mutant R3.50A might be explained by residue R142 in the second intracellular loop acting as a compensatory interaction partner for E6.30 (Figure 8B). The strongest indication for an ionic bond in the B2R is the rescue of wild type behavior by swapping the respective arginine and glutamate (R3.50E/E6.30R), thus generating an inverse ionic lock (Figure 8D). 5-HT2A receptor activation seems to follow a similar pattern, since analogue swapping of residues 3.50 and 6.30 abolished an increase in basal receptor signaling activity (Shapiro et al., 2002). Regarding basal phosphorylation and constitutive internalization, however, a clear trend towards an additive effect was seen in the double mutant (e.g. the intracellular localization), suggesting that both residues function also via other interaction partners outside of the ionic bond. The importance of the respective microenvironment of R3.50 and E6.30 is also indicated by a report that the constitutive activity of the human histamine H4 receptor, which contains an alanine in position 6.30, cannot be simply reduced by reconstituting an ionic lock generating a mutant A6.30E (Schneider et al., 2010).

Conclusions. Our results indicate that in the inactive B2R, R3.50 and E6.30 form an ionic lock. We show for the first time that the ionic lock in a family A GPCR can play different roles when comparing G protein activation with the interaction with GRKs and arrestins in the process of receptor internalization: Its disruption in the B2R resulted in constitutive internalization with consequently strong intracellular localization of the constructs, but did not change the character of the tested B2R antagonists in this regard. In contrast, mutation of E6.30 did not result in
constitutive G protein activation but turned all tested B₂R antagonists into strong agonists. This suggests a multistep process of B₂R activation, in which the disruption of the salt bridge between R3.50 and E6.30 plays a pivotal but differential role with regard to the different processes of G protein activation and receptor internalization. Thus, our findings strengthen current concepts of biased agonism and functional selectivity. Different ligand-induced conformational changes and subsequent signaling has also been suggested for several GPCRs (Ahn et al., 2004; Kobilka and Deupi, 2007; Rosenbaum et al., 2009), highlighting the structural flexibility of GPCRs and the dynamic nature of their activation process. Similarly, multiple structurally different ligands are known to stabilize distinct conformational states of the β₂ adrenergic receptor eliciting differential cellular responses (Bhattacharya et al., 2008; Kahsai et al., 2011). Deeper insight into the multistep mechanism of GPCR activation, as provided by our study, and the generation of differentially active mutants might help in the development and screening of new specific biased agonists and antagonists for optimized therapeutic intervention.
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Authorship Contributions

Participated in research design: Wennerberg, Leschner, Faussner

Conducted Experiments: Leschner, Wennerberg, Faussner, Feierler, Welte, Kalatskaya

Contributed new reagents or analytic tools: Wolber

Performed data analysis: Leschner, Wennerberg, Faussner, Bermudez, Wolber, Feierler, Welte, Kalatskaya

Wrote or contributed to the writing of the manuscript: Leschner, Wennerberg, Faussner, Wolber
References


Footnotes

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1Jasmin Leschner and Goeran Wennerberg contributed equally to the work
Legends for Figures

**Figure 1. Schematic two-dimensional representation of a family A GPCR.** Highly conserved residues are shown as white circles and the one letter code for amino acids in black. The two residues involved in the formation of the “ionic lock” and residues T6.34 and R142 (see discussion) are depicted in black or grey circles, respectively, with white letters. The Ballesteros/Weinstein numbering (Ballesteros et al., 1998) has been used for identification of residues.

**Figure 2. [³H]BK dissociation.** After pre-treatment with phenylarsine oxide, cells stably expressing the indicated constructs were incubated with 2 – 4 nM [³H]BK on ice for 90 min. Subsequently cells were rinsed at 4°C and incubated with 1 µM BK in incubation buffer at 37°C. At the indicated times [³H]BK dissociation was stopped and remaining surface bound [³H]BK was determined as described in “Materials and Methods”. Data are shown as percentage of initial binding at 4°C. Columns represent the mean ± SEM (n=3-11). (One-way ANOVA with Newman-Keuls Multiple Comparison Test: **P < 0.001; ***P < 0.001).

**Figure 3. Inositol phosphate (IP) accumulation. (A) Basal and stimulated IP accumulation.** Cells expressing the indicated receptor constructs were pre-incubated overnight with [³H]inositol. IP accumulation in the presence of 50 mM LiCl after incubation for 30 min at 37°C with 1 µM of BK or icatibant was determined as described in “Materials and Methods”. Each column represents the mean ± SEM (n=3-7). The results are presented as fold increase over the IP content of identically treated control cells at 4°C. (One-way ANOVA with Newman-Keuls Multiple Comparison Test: ***P < 0.001). (B) Dose-dependent IP accumulation of mutant
E6.30R expressing cells in response to B$_2$R agonist and antagonists. HEK293 cells stably expressing mutant E6.30R and pre-incubated overnight with [$^3$H]inositol, were stimulated with the indicated concentrations of BK, B9430, icatibant or JSM10292 for 30 min at 37°C. Shown is a representative experiment that was repeated three times with similar results.

**Figure 4. Internalization of [$^3$H]BK.** Cells expressing B$_2$Rwt, R3.50X (X=A,E,D) and E6.30X (X=A,E) were pre-incubated with 2 nM [$^3$H]BK for 90 min on ice. Internalization was started by warming the plates to 37°C. After the indicated times, surface-bound and internalized [$^3$H]BK were determined by acetic acid treatment as described in “Materials and Methods”. Internalization is given as percentage of total bound [$^3$H]BK (surface plus internalized [$^3$H]BK). Points represent means ± SEM (n=3-5). Curves of R3.50E and R3.50D were significantly different from B$_2$Rwt, and R3.50A as well. ( *$P < 0.05$; one-way ANOVA with Newman-Keuls Multiple Comparison Test).

**Figure 5. Comparison of [$^3$H]NPC17331 and [$^3$H]BK internalization.** HEK293 cells stably expressing the indicated constructs were pre-incubated with 2 nM [$^3$H]BK or [$^3$H]NPC17331 for 90 min on ice. Internalization was induced by warming the plates to 37 °C. After 10 min, surface-bound and internalized [$^3$H]BK or [$^3$H]NPC17331 were determined by acetic acid treatment as described in “Materials and Methods”. Internalization is presented as percentage of total bound [$^3$H]ligand (surface plus internalized [$^3$H]BK or [$^3$H]NPC17331). Columns represent means ± SEM of three experiments performed in triplicate.

**Figure 6. Receptor internalization determined by biotinylation protection assay.** (A) HEK293 cells stably expressing the indicated N-terminally HA-tagged constructs were labeled at
4°C with biotin reagent containing a reducible disulfide linker. Cells were either stripped directly with 50 mM glutathione at 4°C (Strip), or incubated in the absence (-) or presence of 10 μM BK or 5 μM icatibant (icat.) for 1 h at 37 ºC and then stripped at 4°C. Immunoprecipitation of receptors and detection of their biotinylation status by Western blot analysis was performed as described in “Materials and Methods”. The blots shown are representative for three experiments. (B) Biotinylated receptors were quantified with ImageJ as described in “Materials and Methods” and normalized to the amount of biotinylated receptors obtained upon BK stimulation that served as a reference for maximal response (=100%). (One-way ANOVA with Newman-Keuls Multiple Comparison Test: *** P < 0.001).

Figure 7. Receptor phosphorylation. (A) Receptor expressing cells on 6-well plates were labeled with [32P]orthophosphate and stimulated with 1 μM BK for 5 min at 37°C. HA-tagged receptors were immunoprecipitated as described in “Materials and Methods” and separated by SDS-PAGE. Receptor phosphorylation was detected by autoradiography (upper panel) and receptor expression levels (lower panel) are shown as a control. The blot depicted is representative (n=4-5). (B) Amounts of phosphorylated receptors were quantified with ImageJ as described in “Materials and Methods” and normalized to BK-stimulated B2Rwt that served as positive control and reference for maximum phosphorylation (=100%). [t-test: basal versus BK (5 min): *** P < 0.001, ** P < 0.01, * P < 0.1; basal (B2Rwt) versus basal (receptor mutants): # P < 0.1]

**R3.50A**: Disruption of the ionic lock might result in interaction of E6.30^{238} with R142 through a newly formed salt bridge. (C) **Mutant E6.30R**: mutation might generate repulsion due to identical charges between TM6 with E6.30^{238}R and TM3 with R3.50^{128}. (D) **Mutant R3.50E/E6.30R**: Double mutation allows for formation of an ‘inverse ionic lock’ connecting TM3 (R3.50^{128}E) and TM6 (E6.30^{238}R).
### Tables

**Table 1. [³H]BK binding data, basal and BK-induced IP accumulation.**

<table>
<thead>
<tr>
<th>Receptor construct</th>
<th>Bmax a [pmol/mg protein]</th>
<th>Kd (4°C) [nM]</th>
<th>Kd (37°C) [nM]</th>
<th>Basal b [x-fold over basal at 4°C]</th>
<th>Maximal effect b [x-fold over basal at 4°C]</th>
<th>EC50 c [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2Rwt</td>
<td>4.5</td>
<td>2.02±0.22 (5)</td>
<td>8.14±1.19 (8)</td>
<td>1.69±0.09 (4)</td>
<td>12.11±1.22 (4)</td>
<td>0.67±0.22</td>
</tr>
<tr>
<td>R3.50A</td>
<td>3.9</td>
<td>2.09±0.17 (6)</td>
<td>5.53±0.64 (6)</td>
<td>1.30±0.26 (3)</td>
<td>2.12±0.22 (3)</td>
<td>n.a. d</td>
</tr>
<tr>
<td>R3.50D</td>
<td>4.2</td>
<td>n.d. e</td>
<td>n.d. e</td>
<td>2.8 (1)</td>
<td>2.4 (1)</td>
<td>n.a. d</td>
</tr>
<tr>
<td>R3.50E</td>
<td>n.d. e</td>
<td>7.22 (1)</td>
<td>1.47±0.40 (3)</td>
<td>1.87±0.55 (3)</td>
<td>n.a. d</td>
<td></td>
</tr>
<tr>
<td>R3.50H</td>
<td>2.4</td>
<td>n.d. e</td>
<td>n.d. e</td>
<td>1.72±0.32 (4)</td>
<td>1.80±0.29 (4)</td>
<td>n.a. d</td>
</tr>
<tr>
<td>E6.30A</td>
<td>3.4</td>
<td>1.10±0.19 (5)</td>
<td>2.17±0.39 (5)</td>
<td>2.09±0.02 (3)</td>
<td>13.79±1.66 (3)</td>
<td>2.65±0.73</td>
</tr>
<tr>
<td>E6.30R</td>
<td>4.8</td>
<td>1.00±0.10 (3)</td>
<td>1.44/1.01 (2)</td>
<td>2.33±0.52 (3)</td>
<td>6.03±1.10 (3)</td>
<td>0.64±0.24</td>
</tr>
<tr>
<td>R3.50A/E6.30A</td>
<td>2.4</td>
<td>1.87±0.46 (3)</td>
<td>6.14±1.51 (5)</td>
<td>1.71±0.62 (3)</td>
<td>1.9±0.71 (3)</td>
<td>n.a. d</td>
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<tr>
<td>R3.50E/E6.30R</td>
<td>4.5</td>
<td>2.88±0.42 (3)</td>
<td>12.17±0.64 (3)</td>
<td>1.61±0.36 (3)</td>
<td>3.44±1.15 (3)</td>
<td>n.a. d</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM of n independent experiments (numbers in brackets)

- **a** Estimated with approximately 30 nM [³H]BK at 4°C.
- **b** Total IP accumulation after 30 min of incubation in buffer with inhibitors and 50 mM LiCl at 37°C without (basal) and with (maximal effect) 1 µM BK, expressed as fold increase of initial total IP content (t=0 min).
- **c** Calculated from incubations in duplicates with 10⁻¹²-10⁻⁵ M BK for 30 min at 37°C in the presence of 50 mM LiCl.
- **d** not applicable
- **e** not determined
Table 2. Receptor distribution.

<table>
<thead>
<tr>
<th>Receptor construct</th>
<th>Intracellularly located receptors [% of total]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2Rwt</td>
<td>18±3 (7)</td>
</tr>
<tr>
<td>R3.50A</td>
<td>64±6 (3)</td>
</tr>
<tr>
<td>E6.30A</td>
<td>57±6 (3)</td>
</tr>
<tr>
<td>R3.50A/E6.30A</td>
<td>71±4 (4)</td>
</tr>
<tr>
<td>R3.50E/E6.30R</td>
<td>24±7 (5)</td>
</tr>
</tbody>
</table>

HEK293 cells expressing the indicated construct were incubated for 3 h at 4°C with 30 nM of the cell membrane-permeant B2R antagonist [3H]JSM10292 in the absence or presence of either 30 µM unlabeled JSM10292 (specific surface and intracellular binding) or 30 µM BK (specific surface binding only). The amount of intracellular receptor binding is given as mean ± SEM and as percentage of total receptor binding (intracellular plus surface). The number of independent experiments performed in triplicates is given in parentheses.
Figure 3

A

B

Inositol phosphate accumulation [fold basal (t=0 min)]
Figure 4
Figure 5
Figure 6

(A) Western blots showing the internalization of B2Rwt, R3.50A, E6.30A, R3.50A/E6.30A, and R3.50E/E6.30R under conditions of 4°C and 37°C. The blots were performed in the presence or absence of glutathione (strip) and with different treatments (BK, icat.).

(B) Bar graph depicting the internalization of B2Rwt, R3.50A, E6.30A, R3.50A/E6.30A, and R3.50E/E6.30R under different conditions. The graph shows the percentage of internalization compared to the maximum. Statistical significance is indicated by ***.