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A Nonpeptide Antagonist Reveals a Highly Glycosylated State of the Rabbit Kinin B_1 Receptor

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ABSTRACT

The inducible kinin B_1 receptor is emerging as an attractive therapeutic target for the treatment of pain and inflammation. Although many studies described its regulation at the transcriptional level, little is known about the maturation of the B_1 receptor. Using two human embryonic kidney (HEK) 293 cell lines stably expressing rabbit B_1 receptors tagged with the yellow fluorescent protein at the C terminus (B_1R-YFP) or the N-terminal myc epitope (myc-B_1R), we showed that receptors are mainly retained in a perinuclear compartment and detectable as low-glycosylated species under control conditions. Interference with the ubiquitin-proteasome pathway function (proteasome inhibitors, coexpression with dominant-negative ubiquitin) blocked B_1 receptor degradation and amplified its intracellular accumulation. A potent nonpeptide antagonist specifically increased the abundance of highly glycosylated B_1R-YFP forms at the cell surface (accessible to chymotrypsin digestion in intact cells); this compound augmented low-glycosylated receptors in brefeldin A-treated cells, supporting the hypothesis that it reaches a newly synthesized receptor in the endoplasmic reticulum. Cell-impermeant peptide or low-affinity nonpeptide B_1 receptor antagonists failed to influence the level of highly glycosylated receptors. Chemical chaperones stabilized all B_1R-YFP species and up-regulated endogenous B_1 receptors expressed at the surface of rabbit smooth muscle cells. Although myc-B_1Rs behaved similarly to B_1R-YFP in most aspects, antibody-based detection assays failed to reveal highly glycosylated species of this construct. Taken together, these results show that B_1 receptors overexpressed in HEK 293 cells are degraded by the proteasome. Furthermore, a pharmacological chaperone highlights the existence of a highly *N*-glycosylated form of the rabbit kinin B_1 receptor at the cell surface.

Bradykinin related peptides stimulate two types of mammalian G protein coupled receptors (GPCRs), termed the B_1 and B_2 receptors (Leeb-Lundberg et al., 2005); Lys-des-Arg^9-BK represents the optimal agonist sequence at the rabbit and human B_1 receptors. In contrast to the constitutive expression of the B_2 receptor in many cell types, the B_1 receptor is now widely recognized as an inducible gene product in the course of inflammatory reactions. The up-regulation of B_1 receptor gene expression under the influence of proinflammatory cytokines, lipopolysaccharide, and noxious stimuli has been shown to involve the nuclear factor-κB (Leeb-Lundberg et al., 2005). This disease-dependent expression profile and accumulating pharmacological evidence suggesting that B_1 receptor blockade may be useful for the treatment of pain and inflammatory diseases recently stimulated medicinal chemistry efforts to develop highly selective and potent peptide and nonpeptide B_1 receptor antagonists (Leeb-Lundberg et al., 2005).

The GPCR superfamily includes several hundreds of heptahelical membrane proteins that require cell surface expres-

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ABBREVIATIONS: GPCR, G protein coupled receptor; ER, endoplasmic reticulum; HEK, human embryonic kidney; MG 132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; compound 11, 2-{(2*R*)-1-[(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl}-*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}acetamide; compound A, *N*-[2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl]-2-[(2*R*)-1-(2-napthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide; NVP-SAA164, {2-(2,2-diphenylethylamino)-5-[4-(4-methylpiperazine-1-carbonyl)piperidine-1-sulfonyl]phenyl}morpholin-4-yl methanone; B_1R-YFP, B_1 receptor conjugated to yellow fluorescent protein; myc-B_1R, myc epitope conjugated B_1 receptor; B_2R-GFP, B_2 receptor conjugated to green fluorescent protein; Lys-des-Arg^9-BK, lysyl-des-arginine^9-bradykinin; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PAGE, polyacrylamide gel electrophoresis; B10352, D-Lys-Lys-[Hyp^3,CpG^5,D-Tic^7,CpG^8]des-Arg^9-BK; BK, bradykinin; B9958, Lys-Lys-[Hyp^3,CpG^5,D-Tic^7,CpG^8]des-Arg^9-BK; DMSO, dimethyl sulfoxide; SMC, smooth muscle cell; HA, hemagglutinin; ANOVA, analysis of variance.

sion to elicit the transmission of signals across the plasma membrane. Despite the diversity of their polypeptide sequences, the GPCRs retain enough structural information to fold in the endoplasmic reticulum (ER) to adopt their highly conserved multispanning conformation. Like all polypeptides that enter the ER, GPCRs are subjected to a stringent quality control that assures that only correctly folded proteins and fully assembled protein complexes are targeted to their site of action within the cell (Romisch, 2005). Misfolded proteins in the ER are selectively transported back across the ER membrane to the cytosol for degradation by the proteasomes; dislocated proteins are frequently ubiquitinated before degradation (Romisch, 2005). This process, ensuring the fidelity of gene expression at the post-transcriptional level, is crucial for the cellular functional integrity. Naturally occurring mutations in GPCRs that lead to conformationally defective proteins that are retained in the ER have been associated with human diseases such as nephrogenic diabetes insipidus (vasopressin V_2 receptor), hypogonadotropic hypogonadism (gonadotropin-releasing hormone receptor), and retinitis pigmentosa (rhodopsin) (Bernier et al., 2004). However, it was also shown that folding efficiency in the ER is a rate limiting step in the maturation and surface expression of wild-type GPCRs such as the human δ opioid receptor, the olfactory receptors, the D4 dopamine receptor, and the rat-luteinizing hormone receptor (Petäjä-Repo et al., 2001; Lu et al., 2003; Pietilä et al., 2005; Van Craenenbroeck et al., 2005). Optimization of GPCR maturation can be accomplished with pharmacological chaperones. According to this concept, selective high-affinity and liposoluble ligands can bind to inefficiently folded receptors in the ER, altering the thermodynamic equilibrium in favor of the correctly folded receptor and thus favoring the escape from the ER quality control and increasing the expression of highly glycosylated and functional receptors at the cell surface (Bernier et al., 2004). This concept is thought to be widely applicable, because increasing evidence confirms that the pharmacological rescue of wild-type and mutant receptors is often achievable when such potent hydrophobic ligands are available (Bernier et al., 2004; Van Craenenbroeck et al., 2005).

Although much is known about the factors regulating the induction of the B_1 receptor at the transcriptional level (Leeb-Lundberg et al., 2005), little information on the mechanisms involved in its maturation is available. Because the amount of endogenous B_1 receptors is often too low in primary cells for an efficient detection in immunoblots, the widely used HEK 293 cell model expressing different epitope-tagged receptors was chosen to study the maturation and cellular stability of the rabbit B_1 receptors. Our results reveal that the majority of overexpressed B_1 receptors were retained intracellularly, degraded by proteasomes, and mainly detectable as low-glycosylated forms under control conditions. Furthermore, we show that increasing the folding efficiency of B_1 receptors by treatment with high concentrations of a selective nonpeptide antagonist (acting as a pharmacological chaperone) resulted in the cell surface accumulation of highly *N*-glycosylated forms of the B_1 receptor. We also highlight that highly glycosylated N-terminally myc-tagged B_1 receptors were not detectable with a specific antibody directed against the antigenic epitope. We finally propose that these previously unappreciated highly glycosylated receptor species represent agonist-sensitive B_1 receptors.

Materials and Methods

Drugs. Anisomycin, lactacystin, brefeldin A, tunicamycin, and α-chymotrypsin were from Sigma-Aldrich (St. Louis, MO). MG 132 was from Calbiochem (San Diego, CA). Compounds 11 and A are powerful antagonists at the human and rabbit B_1 receptors but comparatively weak at the rodent receptors (Morissette et al., 2004; Fortin et al., 2005a) (gifts from Dr. D. J. Pettibone, Merck Research Laboratories, West Point, PA). NVP-SAA164 is a nonpeptide B_1 receptor antagonist with some structural similarity with the previously cited drugs (Ritchie et al., 2004) and active at the human form of the receptor but with lower affinity for B_1 receptors from some laboratory animals. Bradyzide is a nonpeptide antagonist of the related B_2 receptor active at the rabbit B_2 receptor (Dziadulewicz et al., 2000; Marceau et al., 2001).

Cells and Transfection. The expression vector coding for B_1R-YFP and myc-B_1R has been described elsewhere, as well as the pharmacological profile of these fusion proteins (Sabourin et al., 2002; Morissette et al., 2004). Two novel HEK 293 cell lines stably expressing B_1R-YFP and myc-B_1R with high density have been developed using a novel subclone of HEK 293 cells transformed with human Ad5 adenovirus DNA and obtained from Sigma-Aldrich. Stably transfected cell lines were selected and isolated as described previously (Sabourin et al., 2002) but were grown and maintained in a different culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum). The derivation of a HEK 293 cell line stably expressing the bradykinin B_2 receptor fused to green fluorescent protein (B_2R-GFP) and some of its properties have been described elsewhere; this receptor fusion protein is a high-affinity, functional receptor (Houle et al., 2000). In some experiments, the YFP (pEYFP-N1; Clontech, Mountain View, CA), the myc-B_1R, or the myc-labeled ubiquitin K48R dominant-negative (a gift from Dr. Jacques Landry, CHUQ, Québec, QC, Canada) expression vectors were transiently transfected for 48 h using the Ex-Gen 500 transfection reagent (MBI Fermentas, Flamborough, ON, Canada) as directed by the manufacturer. Transfected and untransfected HEK 293 cells were used in binding assays (24-well plates), confocal microscopy (35-mm Petri dishes), or immunoblots of tagged proteins (60-mm culture plates) and were subcultured to 70 to 90% confluence before experiments. Rabbit aortic smooth muscle cells were cultured as described previously (Fortin et al., 2003).

Immunoblotting. Total cell lysates were separated on 8% polyacrylamide gels and transferred overnight on polyvinylidene difluoride membranes. The immunodetection of the different molecular forms of tagged B_1 receptors (B_1R-YFP and myc-B_1R) and of the ubiquitin construct (myc-Ub K48R) present in total HEK 293 cell lysates was done as previously reported (Sabourin et al., 2002). The monoclonal antibodies directed against GFP (clone JL-8; Clontech) and myc (Upstate, Charlottesville, VA) were exploited under the experimental conditions described elsewhere (GFP, Sabourin et al., 2002; myc, Morissette et al., 2004). The drugs anisomycin, MG 132, lactacystin, brefeldin A, tunicamycin, α-chymotrypsin, peptide and nonpeptide ligands, or appropriate vehicles were directly added to the complete cell culture medium to the final concentrations and for the durations indicated under *Results*.

Immunofluorescence and Confocal Microscopy. The subcellular fluorescence distribution, in live HEK 293 cells and without fixation, was observed using a Bio-Rad 1024 laser beam confocal microscope (40 or 60× objective with oil immersion; GFP: emission 488 nm, detection above 510 nm; Alexa-594: emission 568 nm, detection above 585 nm; Bio-Rad, Hercules, CA). Saponin permeabilization of HEK 293 transfectants was applied for the double detection of B_1R-YFP coupled to staining with myc-labeled ubiquitin K48R with a specific monoclonal antibody against the myc tag, as previously used (Morissette et al., 2004). Fixed but nonpermeabilized HEK cells were used to detect surface myc-B_1R in immunofluorescence with the same anti-myc antibody.

Binding Assays. The binding of different peptidic and nonpeptidic ligands to B_1R-YFP stably expressed in HEK 293 cells was evaluated with a competition binding assay, as described previously (Sabourin et al., 2002), except that the concentration of [^{3}H]Lys-des-Arg^9-BK (69–80 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) was different (0.5 or 2 nM).

Phospholipase A_2 Assay. To evaluate the ability of a nonpeptide drug to antagonize the function mediated by B_1R-YFP stably expressed in HEK 293 cells, an arachidonic acid release assay was performed as reported elsewhere (Sabourin et al., 2002). Cells were pretreated with compound 11 (0.1–1000 nM) or vehicle 15 min before incubation with or without the agonist Lys-des-Arg^9-BK (10 nM) for 60 min. Untransfected HEK 293 cells were used as a negative control.

Enzymatic Deglycosylation of Total HEK 293 Lysates. A protein sample (30 μg) of HEK 293 cells stably transfected or not with B_1R-YFP or myc-B_1R (extracted as described under *Immunoblotting*) was denatured in 1× denaturation buffer (0.5% SDS, 1% β-mercaptoethanol) at 95°C for 5 min (final volume, 10 μl). The enzymatic deglycosylation was then performed in 1× reaction buffer (50 mM $NaPO_4$, pH 7.5, supplemented with 1% NP-40) in the presence of 5 units of PNGase F at 37°C for 16 h (final volume, 15 μl); the enzyme was omitted in negative controls. The reaction was stopped by transferring samples on ice and adding 5 μl of 4× SDS sample buffer. The protein samples (20 μl) were further heated 5 min at 95°C and subjected to SDS-PAGE.

Effect of α-Chymotrypsin on Surface Receptors. To test whether the form of B_1R-YFP enriched after antagonist treatment is expressed at the cell surface and binds the radioligand, HEK 293 cells stably expressing this construction were treated for 24 h with compound 11 (1 μM) in the complete culture medium, then rinsed with serum-free DMEM containing 10 μM anisomycin and further incubated for 4 h in this medium. The latter incubation period aimed at dissociating at least a part of the antagonist drug from the surface receptors while preventing the formation of novel receptors. Then, the intact cells were exposed for 10 min to bovine α-chymotrypsin (10 μM) for 10 min, washed, and submitted either to the radioligand binding assay (24-well plates) or to immunoblot (60-mm Petri dishes), as described above.

Results

Stably Transfected HEK 293 Cells Express Three Main B_1R-YFP Species. We previously reported that kinin B_1 receptor binding sites are constitutively cleared from cell surface in cultured cells at a high rate; the treatment of HEK 293 cells stably expressing B_1R-YFP with the protein synthesis inhibitor anisomycin (10 μM) or the Golgi-disrupting drug brefeldin A (18 μM) for 4 h was shown to lead to a marked decrease in cell surface natural or recombinant B_1 receptors (Fortin et al., 2003). Because the stable transfectant HEK 293 cell line exploited at this occasion expressed low levels of B_1R-YFP that were almost undetectable by immunoblots (Fortin et al., 2003), and to analyze the maturation and stability of B_1 receptors by this technique, we generated a new HEK 293 cell line stably expressing higher levels of this construct. Binding assays using a saturating concentration of [^{3}H]Lys-des-Arg^9-BK (2 nM) showed that this new stable transfectant expresses approximately 2.5-fold more receptors at the plasma membrane compared with the previously described cell line (Fig. 1A; Sabourin et al., 2002). Total cell lysates of this new transfectant subjected to SDS-PAGE and immunoblotted using a specific antibody directed against the C-terminal YFP tag revealed the presence of three main band families (Fig. 1B). A sharp band with an apparent weight of 70 kDa (black arrow) is close to the theoretical molecular mass of the native B_1R-YFP fusion protein and was thus considered as the monomeric form of the receptor. A second band with an extrapolated molecular mass of approximately 145 kDa (gray arrow) is consistent with the recently postulated homodimeric form of the B_1 receptor (Kang et al., 2005) and was detectable above the 132-kDa mass standard. Interestingly, a third intermediate band family with a mass of 100 to 105 kDa (white arrow) was consistently detectable in B_1R-YFP cell lysates. Higher molecular mass immunoreactive complexes that extended toward the top of the gel (bracket) and a YFP-sized band (25 kDa, arrowhead) were also present. The detection of YFP-sized bands in total lysates of cells expressing B_1R-YFP has previously been suggested to represent C-terminal degradation products reflecting the instability of this receptor (Fortin et al., 2003). This band pattern was not present in untransfected HEK 293 cells (Fig. 1B), showing that it specifically represents products of the single fusion protein gene. The

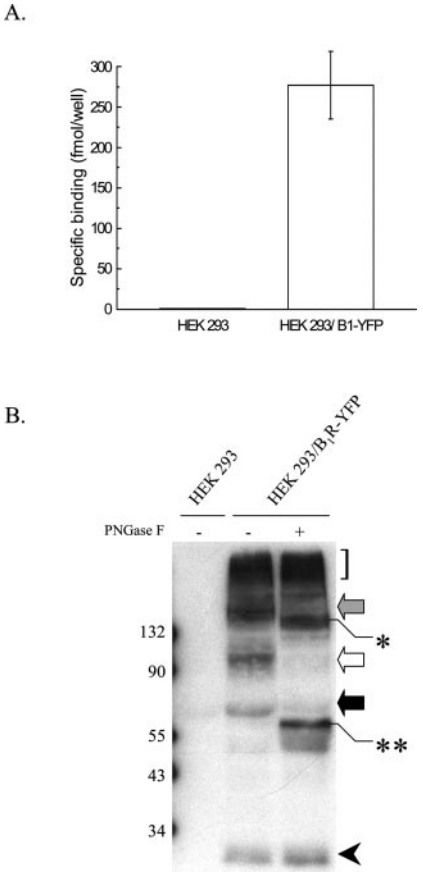

Fig. 1. A, evaluation of the specific binding of [^{3}H]Lys-des-Arg^9-BK (2 nM) to HEK 293 cells stably transfected or not with B_1R-YFP. B, identification of the B_1R-YFP molecular forms expressed in HEK 293 cells. Total lysates of HEK 293 cells stably expressing or not the B_1R-YFP fusion protein were solubilized in SDS sample buffer. A 30-μg protein sample was then incubated with PNGase F (5 units/15 μl) for 16 h at 37°C (see *Materials and Methods*). The membranes were probed with a monoclonal antibody against the YFP tag. Molecular mass markers are indicated. Symbols: bracket, high-molecular mass YFP-immunoreactive complexes; gray arrow, postulated low-glycosylated dimeric B_1R-YFP species; white arrow, highly *N*-glycosylated monomeric B_1R-YFP; black arrow, low-glycosylated monomeric B_1R-YFP; arrowhead, YFP-sized degradation product; double asterisk, deglycosylated monomeric B_1R-YFP; single asterisks, deglycosylated dimeric B_1R-YFP. Symbols are also explained under *Results*.

identities of these B_1R-YFP species were analyzed by treating the lysates with PNGase F, an enzyme that can remove all types of *N*-linked oligosaccharides from glycoproteins (Maley et al., 1989). PNGase F treatment (5 units, 16 h) converted the 70-kDa and 100- to 105-kDa species to a unique 65-kDa form (double asterisk), corresponding to the unglycosylated B_1R-YFP (Fig. 1B). These results indicate that the 100- to 105-kDa B_1R-YFP is likely to represent a highly glycosylated receptor containing *N*-glycans, whereas the 70-kDa is a low *N*-glycosylated form of this receptor. The conversion of the 145-kDa band to 135-kDa species (single asterisk) after PNGase F treatment strongly suggests that these forms represent stable dimeric B_1 receptor complexes made of low-glycosylated B_1R-YFP. Although the relative intensities of the three groups of bands in control conditions varied slightly from one experiment to another, the ones considered as low-glycosylated receptors (monomers and/or dimers) were always predominant compared with the highly glycosylated monomeric form (Fig. 1B; see also below).

Inhibition of the Proteasome Promotes the Intracellular Accumulation of B_1 Receptors. Immunoblots based on total cell lysates showed that treatment of HEK 293 cells overexpressing B_1R-YFP with the protein synthesis inhibitor anisomycin (10 μM) for 4 h led to an important decrease in B_1R-YFP gene product immunoreactivity (Fig. 2A). High molecular mass complexes, highly abundant in control conditions, appeared to be particularly unstable as they became almost undetectable after protein synthesis inhibition (Fig. 2A). In contrast, the abundance of the B_2R-GFP (101–105 kDa) or YFP (27 kDa), stably or transiently expressed in HEK 293 cells, was not influenced by the same anisomycin treatment, respectively. Proteasome inhibitors such as MG 132 and lactacystin are cell-permeable compounds that cause the accumulation of proteins that are degraded by the 20S proteasome (Fenteany et al., 1995; Lee and Goldberg, 1998). Exposition of HEK 293 cells stably expressing B_1R-YFP to the highly specific proteasome inhibitor lactacystin or to MG 132 (10 μM each; Fig. 2A) blocked B_1R-YFP degradation, leading to a rapid increase in the amount of all the detectable B_1R-YFP species. Higher molecular mass immunoreactive complexes, present in control conditions, also accumulated rapidly after proteasome inhibition (Fig. 2A). The lysosomal acidification inhibitor bafilomycin A1 (300 nM, data not shown) had no similar effect on B_1R-YFP abundance over a 4-h period. Simultaneous application of MG 132 and anisomycin stabilized the B_1R-YFP levels (Fig. 2A), showing that much of the ligand-independent degradation of B_1 receptors occurs via the proteasome pathway. MG 132 had no effect on the total cell amounts of YFP or B_2R-GFP (Fig. 2A). Confocal microscopy imaging revealed that the majority of B_1 receptors are present in a perinuclear intracellular compartment in this HEK 293 cell line (Fig. 2B). Treatment with MG 132 for 4 h (Fig. 2B) or lactacysin (data not shown) caused a marked increase in intracellular fluorescence. Radioligand binding assays confirmed that the effect of proteasome inhibition on B_1 receptors is predominantly intracellular, because only a small decrease in cell surface [^{3}H]Lys-des-Arg^9-BK binding was observed after a 4-h incubation with MG 132 (Fig. 2C). Fractionation of HEK 293-expressing GFP-tagged proteins to enrich for caveolae-related rafts, as previously applied by us (Fortin et al., 2005b), showed that all the B_1R-YFP forms are present in the low-density insoluble fractions of the gradient under control condition (data not shown). Interestingly, the incubation of the cells with MG 132 resulted in an increase of the 70-kDa and lower mass degradation product bands in the soluble fractions of the

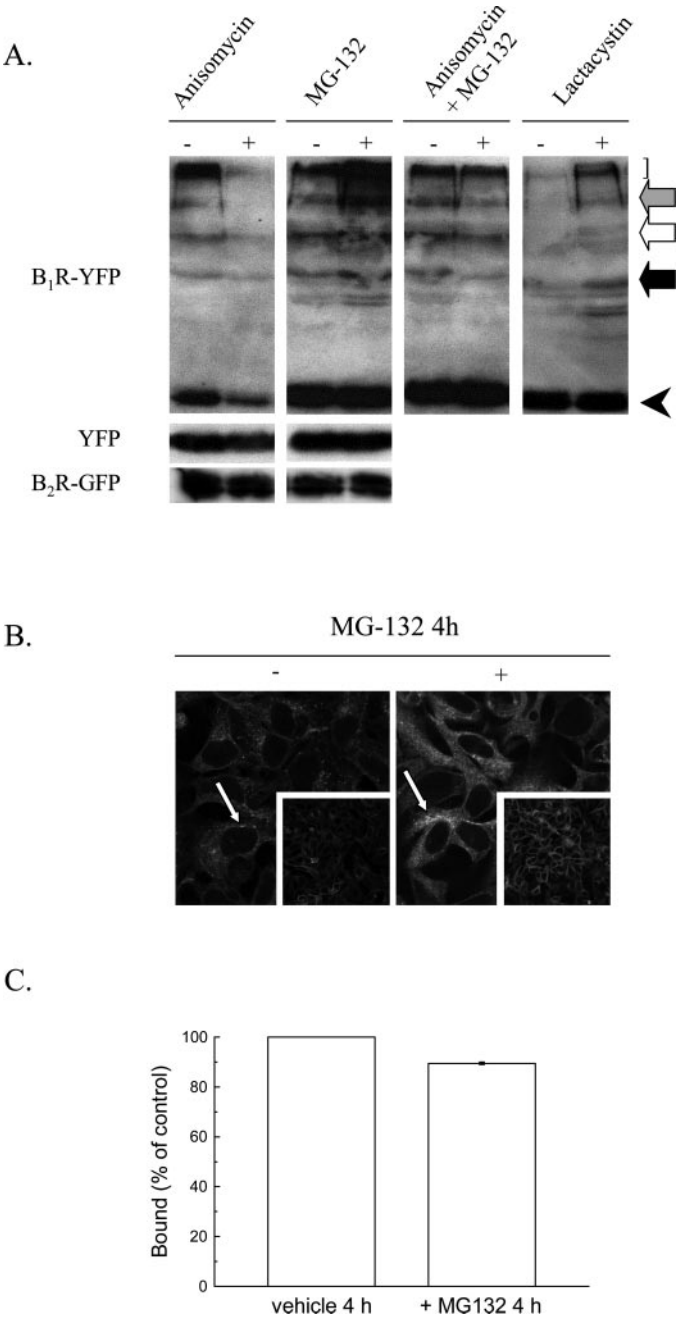

Fig. 2. Proteasome inhibitors block B_1R-YFP degradation. A, HEK 293 cells stably transfected with B_1R-YFP or B_2R-GFP, or transiently expressing YFP, were incubated for 4 h at 37°C with anisomycin (10 μM), MG 132 (10 μM), the combination of both drugs, or lactacystin (10 μM). Negative controls were treated with the appropriate vehicle (DMSO for MG 132 or saline for the other drugs). Total cellular lysates were prepared and subjected to SDS-PAGE, and immunoblots were revealed using the anti-YFP antibody. Symbols at the right of the B_1R-YFP panel as in Fig. 1. B, live cell confocal microscopy analysis of B_1R-YFP distribution after incubation with MG 132 treatment (10 μM, 4 h; magnification 120× and 60× for the inset). The selected confocal planes are halfway to the thickness of most cells. C, effect of MG 132 (10 μM, 4 h) on the number of B_1R-YFP binding sites at the surface of HEK 293 cells stably transfected with B_1R-YFP. The binding assay was performed on intact cells with a saturating dose of 1 nM [^{3}H]Lys-des-Arg^9-BK.

gradient (data not shown), a distribution similar to that of GFP (Fortin et al., 2005b), suggesting that the accumulated monomeric B_1R-YFPs may not be raft-associated. Taken together, these results support the idea that the previously reported cell surface instability of the B_1 receptor (Fortin et al., 2003) also applies to total B_1 receptors detected by immunoblots. Furthermore, these experiments suggest that the susceptibility to an intracellular proteasome-mediated degradation is an intrinsic property of B_1R-YFP overexpressed in HEK 293 cells.

Disruption of Ubiquitination by the Dominant-Negative Ubiquitin Mutant Interferes with B_1 Receptor Degradation. To investigate whether kinin B_1 receptor ubiquitination may be required for its degradation by the proteasome, we transiently coexpressed B_1R-YFP with a construct encoding a ubiquitin mutant that has the invariant lysine in position 48 mutated to arginine (K48R). The lysine 48 in ubiquitin is the site of isopeptide linkage to other ubiquitin molecules and is required for the completion of multiubiquitin chains that mark proteins for degradation (Ward et al., 1995; Yu and Kopito, 1999). Expression of ubiquitin K48R has a chain-terminating dominant-negative effect, resulting in the premature termination of ubiquitin chains and in the accumulation of "incompletely" ubiquitinated proteins that are not properly targeted for destruction by the proteasome (Ward et al., 1995; Yu and Kopito, 1999; Kolodziejski et al., 2002). The transient overexpression of B_1R-YFP promotes the formation of perinuclear accumulations readily observed as bright dots by fluorescence microscopy at a low magnification (Fig. 3A). When B_1R-YFP was transiently coexpressed with ubiquitin K48R, the proportion of cells displaying intracellular fluorescent accumulations was dramatically increased comparatively with control cells transfected with the B_1R-YFP coding vector only (Fig. 3A). Cotransfection of B_1R-YFP with the myc-tagged K48R ubiquitin and subsequent observation of the paraformaldehyde-fixed cells using confocal microscopy revealed that both proteins colocalized in these cytoplasmic aggregates (Fig. 3B). Such intracellular structures enriched in B_1R-YFP were larger but similar to those amplified after MG 132 treatment (arrows in Fig. 2B). Because some of these observations were made using live cell imaging techniques (Figs. 2B and 3A), these fluorescent aggregates did not result from fixation. Furthermore, immunoblots based on the anti-GFP antibody revealed that the K48R ubiquitin construct led to the accumulation of immunoreactive B_1R-YFP, which appeared as an ascending ladder relative to the postulated low-glycosylated monomeric fusion protein and an increase of high molecular mass aggregates; this is typical of polyubiquitinated proteins (Fig. 3C). The theoretical molecular mass of ubiquitin K48R is 8.6 kDa; the blot exploiting the anti-myc antibody confirms that the mutant ubiquitin has reacted with a myriad of higher mass proteins (Fig. 3C, bottom; Kolodziejski et al., 2002). Ubiquitin K48R had no effect on the microscopic abundance of YFP when both proteins were cotransfected (data not shown). Taken together, these results support the observations made with proteasome inhibitors and strengthened the demonstration that the proteasome, and possibly ubiquitination, are implicated in the constitutive intracellular destruction of B_1R-YFP overexpressed in HEK 293 cells.

Treatment with a High Concentration of Compound 11 Results in the Accumulation of the Highly Glycosylated B_1R-YFP Species at the Cell Surface. Similar results reported for other recombinant wild-type GPCRs transiently transfected in HEK 293 cells have been attributed to an inefficient folding, a poor export from the ER and a proteasome-associated degradation process (Petäjä-Repo et al., 2001; Lu et al., 2003; Bernier et al., 2004; Pietilä et al., 2005; Van Craenenbroeck et al., 2005). To confirm that the observed intracellular retention and degradation of the B_1R-YFP was linked to the secretory pathway, we verified whether it was possible to increase the maturation efficiency of this receptor with a pharmacological chaperone. Compound 11 has previously been shown to be a very selective and high-affinity nonpeptide antagonist of the rabbit receptor (Morissette et al., 2004). The effective competition of

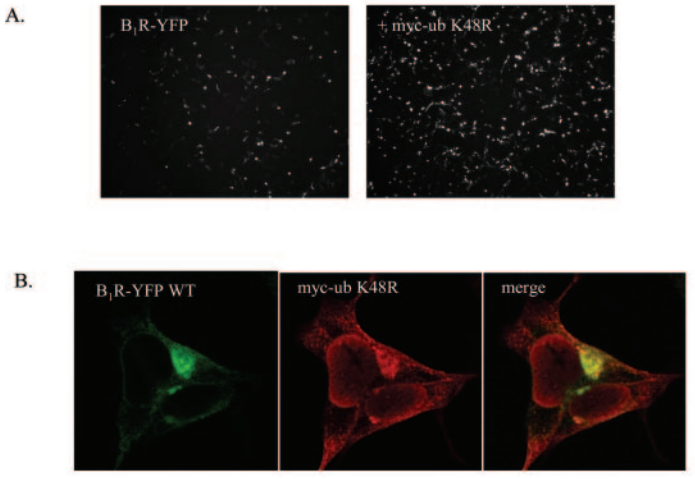

Fig. 3. Disruption of ubiquitination by a dominant-negative ubiquitin mutant (myc-Ub K48R) interferes with B_1R-YFP degradation in transiently cotransfected HEK 293 cells. A, live cell fluorescence microscopy (low magnification, 20×) of HEK 293 cells transiently cotransfected with plasmids encoding B_1R-YFP and either the ubiquitin mutant or its empty vector shows that the latter protein increases the number of YFP-positive fluorescent aggregates. The molar ratio of B_1R-YFP plasmid to the coplasmid was 1:2. B, HEK 293 cells were cotransfected with plasmids encoding B_1R-YFP and the ubiquitin K48R for 24 h. The cells were then fixed, permeabilized, reacted with an anti-myc monoclonal antibody and secondary Alexa-fluor 594-labeled antibodies, and observed using confocal microscopy (original magnification 180×; green signal from YFP, red signal from the anti-myc reaction). C, total cell lysates (30 μg) of HEK 293 cells untransfected or transfected with B_1R-YFP in the presence or absence of ubiquitin K48R were subjected to SDS-PAGE and immunoblotted with antibodies against GFP (top) or myc (bottom).

[^{3}H]Lys-des-Arg^9-BK (0.5 nM) binding to HEK 293 cells stably expressing B_1R-YFP by compound 11 (K_i = 0.92 nM) confirmed that it retains a high potency in the present cellular model (Fig. 4A). B10352 (D-Lys-Lys-[Hyp^3,CpG^5,D-Tic^7,CpG^8]des-Arg^9-BK) is a new aminopeptidase-resistant antagonist analog of the B_1 receptor antagonist of high-affinity B9958 (Gera et al., 2006). Isomerization of the N-terminal Lys residue in the latter peptide increases the stability of the antagonist by protecting it from cleavage by aminopeptidase N. The peptide antagonist B10352 and agonist Lys-des-Arg^9-BK could also displace the radioligand efficiently (Fig. 4A; K_i = 3.2 and 2.3 nM, respectively). However, bradyzide (a selective B_2 receptor antagonist) failed to compete for radioligand binding and NVP-SAA164 (a nonpeptide antagonist selective for the human B_1 receptor) exhibited very little affinity (Fig. 4A). These findings extend the previously described pharmacological profile of the B_1R-YFP stably expressed in HEK 293 cells (Sabourin et al., 2002). Unstimulated HEK 293 cells stably expressing B_1R-YFP displayed a significantly higher baseline level of arachidonate release over a 1-h period compared with untransfected cells (Fig. 4B); this observation is compatible with the previously reported high agonist-independent activity of human B_1 receptors overexpressed in the same cell type (Leeb-Lundberg et al., 2001). The agonist Lys-des-Arg^9-BK (10 nM) stimulated the release of arachidonate from HEK 293 cells stably expressing B_1R-YFP, whereas nontransfected cells were not responsive (Fig. 4B). Compound 11 (1 μM) had no direct stimulatory effect, but concentrations between 1 and 10 nM were sufficient to completely abolish the agonist-induced phospholipase A_2 activation (Fig. 4B). Taken together, these results show that the B_1R-YFP stable transfectant cell line expresses typical and functional rabbit B_1 receptors and highlight that compound 11 is a potent nonpeptide antagonist in this model.

Treatment of B_1R-YFP-expressing cells with increasing concentrations of compound 11 (0.1–1000 nM, 4 h) revealed that only the micromolar concentration of this drug can increase the expression of highly glycosylated B_1R-YFP forms (Fig. 5). A concomitant decrease in the levels of the 145-kDa forms was also consistently observed after the same treatment (Figs. 5–7). Higher molecular mass complexes, previously found to be unstable species that accumulate after interference with the proteasome function (Figs. 2 and 3), were also less abundant in compound 11-treated cells (Figs. 5–7). Treatment of cells with compound A (1 μM), a close analog of compound 11 displaying a high affinity for the rabbit B_1 receptor (Fortin et al., 2005b), produced similar results (Fig. 5). To ascertain the pharmacological properties

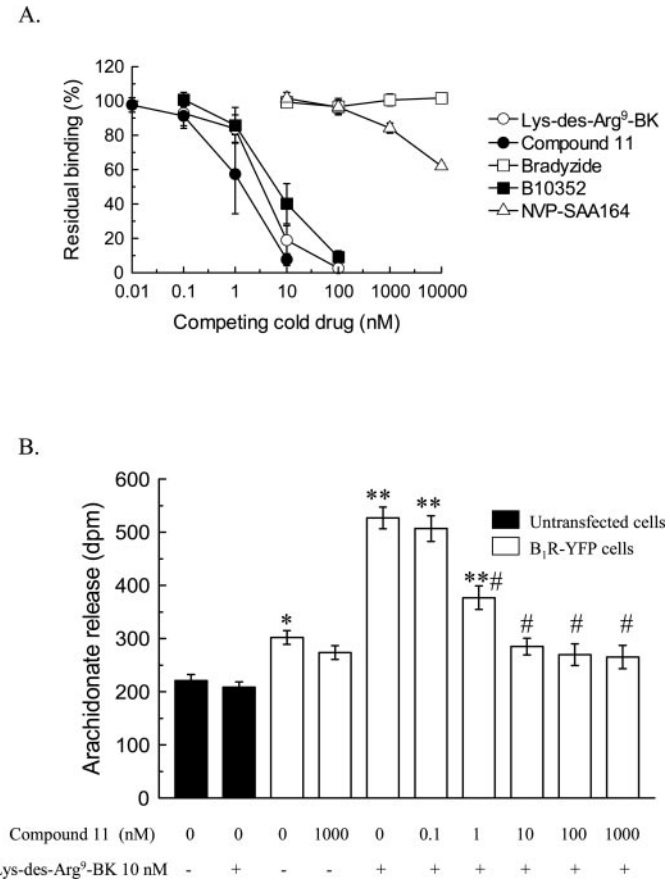

Fig. 4. Compound 11 is a highly potent nonpeptide antagonist of the B_1R-YFP stably expressed in HEK 293 cells. A, pharmacological profile of B_1R-YFP stably expressed in HEK 293 established by using competition of the binding of [^{3}H]Lys-des-Arg^9-BK (0.5 nM) by unlabeled peptide and nonpeptide ligands. B, [^{3}H]arachidonate released from untransfected HEK 293, or cells stably expressing B_1R-YFP, exposed to the agonist Lys-des-Arg^9-BK (or vehicle) in the presence or absence of various concentrations of compound 11 (0.1–1000 nM). Results are expressed as means ± S.E.M. (n = 12–18). ANOVA showed that groups were heterogeneous ($P < 10^{-4}$). Comparisons (Dunnett's test) were performed with the basal release value from nontransfected cells (*, $P < 0.05$; **, $P < 0.01$) and also with the agonist-stimulated transfected cells without antagonist for the effect of various antagonist concentrations (#, $P < 0.01$).

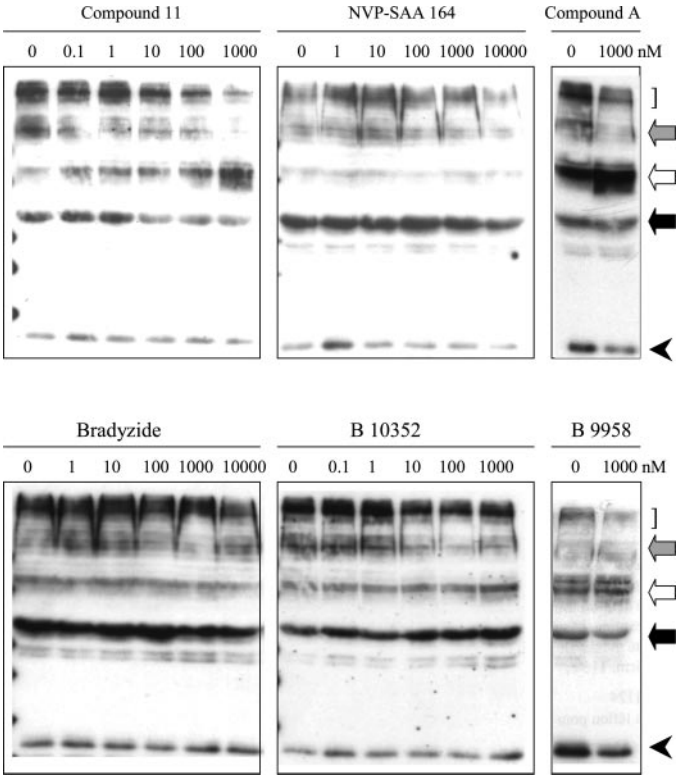

Fig. 5. High concentrations of high-affinity cell-permeable nonpeptide antagonists, which increase the abundance of highly glycosylated B_1R-YFP. HEK 293 cells stably expressing B_1R-YFP were treated for 4 h at 37°C with increasing concentrations of several peptide and nonpeptide drugs. Cells were solubilized and subjected to SDS-PAGE as described in Fig. 1. Symbols at the right as in Fig. 1.

required for this stabilizing action, we assessed the ability of membrane impermeable peptide antagonists of the rabbit B_1 receptor (charged peptides) to mimic the effect of compound 11. Both B9958 (1 μM; Fig. 5) and the more stable agent B10352 (1 μM) failed to influence the B_1R-YFP immunoblot band pattern at high concentrations (Fig. 5). The B_2 receptor antagonist bradyzide (10 μM) and the human B_1 receptor selective antagonist NVP-SAA164 (10 μM) also failed to influence the concentration of B_1R-YFP-related proteins, even if these nonpeptide drugs are both structurally related to compound 11 and A, sharing a similar phenylsulfonylamide molecular scaffold (Marceau et al., 2001; Leeb-Lundberg et al., 2005). The stabilizing effect of compound 11 is time-dependent, as exposition of cells for up to 24 h led to a stronger accumulation the highly glycosylated B_1 receptors (Fig. 6A). The observation that compound 11 had no influence on the amount of detectable B_2R-GFP expressed in HEK 293 cells further confirms that the effect of this antagonist relies on its selectivity for the rabbit B_1 receptor (Fig. 6A). Fluorescence microscopy revealed that the increased expression of the highly glycosylated B_1R-YFP species correlates with a marked change in the cellular localization of B_1R-YFP, which migrated from an intracellular to a predominant cell surface distribution after incubation with compound 11 (1 μM, 4 or 24 h). These results show that a high affinity for the rabbit B_1 receptor and a more lipophilic nonpeptide structure are required for the stabilizing effect of compound 11 on B_1R-YFP; this is compatible with a pharmacological chaperoning action promoting a more efficient maturation and cell surface expression of the receptor. Furthermore, because this drug saturates entirely cell surface receptors at low nanomolar concentrations (Fig. 4), the observed effect at 1 μM suggests that high concentrations of compound 11 are necessary to reach maturing B_1 receptors into an intracellular compartment.

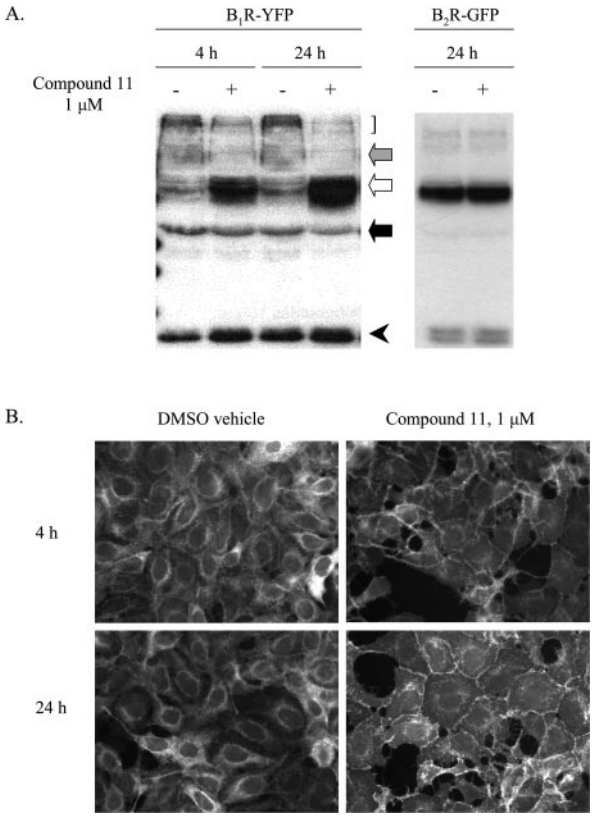

Fig. 6. The time-dependent increase in highly glycosylated species paralleled a cell-surface redistribution of B_1R-YFP. HEK 293 stably transfected cells with B_1R-YFP or B_2R-GFP were treated for 4 or 24 h with compound 11 (1 μM) at 37°C, and the cells were analyzed by immunoblots (A) or fluorescence microscopy on live cells (B, magnification 40×). Symbols at the right of B_1R-YFP panel as in Fig. 1.

Compound 11 Acts in the ER to Promote the Biogenesis of Highly Glycosylated B_1 Receptors. In an attempt to assess the site of action of compound 11, its effects on B_1R-YFP maturation were tested in cells treated or not with brefeldin A, a toxin that blocks transport of newly synthesized proteins to the plasma membrane by disrupting the Golgi apparatus (Lippincott-Schwartz et al., 1989). Upon a 4-h treatment with brefeldin A (18 μM), the immunoreactivity of both highly and low-glycosylated receptors decreased compared with the untreated control (Fig. 7A). In contrast to its selective stabilizing effect on highly glycosylated forms in control cells, compound 11 promoted the stabilization of low-glycosylated B_1R-YFP species in brefeldin A-treated cells (Fig. 7A). As already observed in other studies, the latter result confirms the fact that compound 11 can interact with B_1R-YFP in the ER to act as a pharmacological chaperone promoting the maturation of ER precursor species (Petäjä-Repo et al., 2001; Chaipatikul et al., 2003; Van Craenenbroeck et al., 2005). Inhibition of *N*-glycosylation with tunicamycin (25 μM) also led to a marked lost of highly and low-glycosylated B_1R-YFP bands, further supporting a crucial role of *N*-linked glycosylation in B_1 receptor maturation and stability. Although compound 11 was able to protect highly glycosylated B_1R-YFP from spontaneous degradation

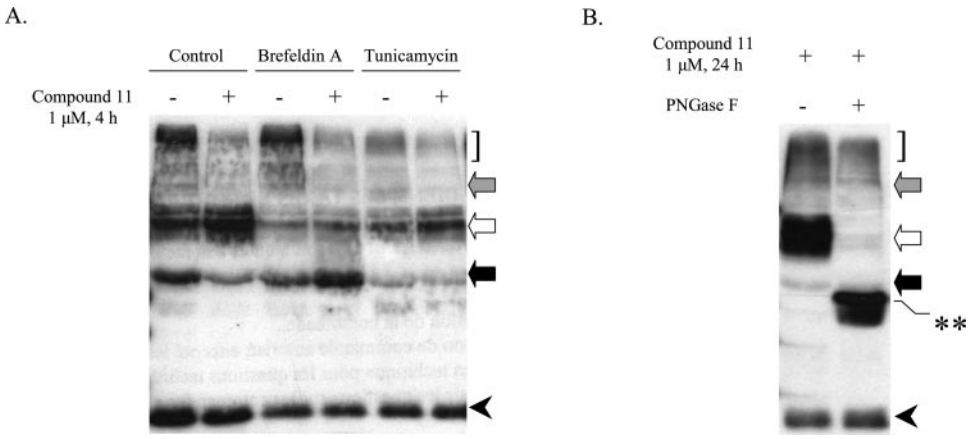

Fig. 7. A, effect of the metabolic inhibitors brefeldin A and tunicamycin on compound 11-induced stabilization of highly glycosylated B_1R-YFP. Cells were treated with the metabolic inhibitors in the presence or absence of compound 11 for 4 h at 37°C. The total cell lysates were analyzed by immunoblot as described in Fig. 1. B, effect of PNGase F digestion on B_1R-YFP species modified by compound 11 treatment. A 30-μg protein sample was incubated with PNGase F (5 units/15 μl) for 16 h at 37°C (see *Materials and Methods*). Symbols at the right as in Fig. 1.

in tunicamycin-treated cells, its lack of effect on low-glycosylated species suggests that its action as a pharmacological chaperone is intimately linked to the early addition of *N*-linked oligosaccharides on the receptor (Fig. 7A). We finally confirmed that the B_1R-YFP species that accumulates in cells incubated with compound 11 are fully glycosylated receptors by showing that complete deglycosylation with PNGase converted all the stabilized forms to unglycosylated receptors (Fig. 7B).

Highly Glycosylated B_1R-YFP Species Are Sensitive to the Agonist Lys-des-Arg^9-BK. To verify whether the stabilized highly glycosylated B_1 receptors were able to bind the agonist, we checked the influence of Lys-des-Arg^9-BK on the multiple B_1R-YFP species detected by immunoblot under basal conditions. A treatment with a high concentration of Lys-des-Arg^9-BK (1 μM, 2–4 h) was associated with a time-dependent attenuation of the highly glycosylated B_1R-YFP band signal, with the reinforcement of lower molecular mass bands (Fig. 8). This phenomenon may represent a mitigated form of down-regulation of the receptor after prolonged exposition to the agonist or a manifestation of the previously reported agonist-dependent translocation of B_1R-YFP in a phenomenon where detergent-resistant membrane microdomains reorganize (Sabourin et al., 2002); further work is required to distinguish between these possibilities.

To support the idea that highly glycosylated B_1R-YFP species represent a relevant agonist-sensitive B_1 receptor, we verified whether it was possible to associate the cell surface expression of these immunoreactive receptor forms with the presence of a specific [^{3}H]Lys-des-Arg^9-BK binding site. Cells stably expressing B_1R-YFP were pretreated with compound 11 (1 μM) for 24 h to enrich the highly glycosylated receptor species and then subjected to a 4-h washout with serum-free medium containing the protein synthesis inhibitor anisomycin (10 μM). This procedure, designed to dissociate at least a fraction of compound 11 from the antagonist-stabilized receptors and to prevent the emergence of newly synthesized receptors, led to a 4.4-fold increase in the number of specific [^{3}H]Lys-des-Arg^9-BK binding sites detectable at the cell surface (6.1 and 26.5 fmol/well without and after washout, respectively; Fig. 8B); this partial recovery of the binding capacity from occupancy by the high-affinity antagonist was observed while a unique, highly glycosylated B_1R-YFP species was detected by immunoblot; other immunoreactive forms were cleared after protein synthesis inhibition (Fig. 8B) as previously observed for nontreated cells (Fig. 2A). Acute extracellular treatment with α-chymotrypsin (10 μM, 10 min) to degrade cell surface receptors accessible to the protease in intact cells led to a decrease in the detectable amount of highly glycosylated B_1R-YFP that paralleled a marked fall in the number of specific [^{3}H]Lys-des-Arg^9-BK binding sites at the cell surface. The sensitivity of the highly glycosylated species to α-chymotrypsin is in agreement with their cell surface localization as observed in microscopy (Fig. 6B). The formation of a YFP-sized product after α-chymotrypsin treatment (Fig. 8B) also confirms that this band, detectable in untreated cells (Fig. 1B), can be produced as a C-terminal proteolytic degradation product of the fusion protein. Taken together, these data further support the idea that highly glycosylated antagonist-stabilized forms of the B_1R-YFP construct represent cell surface binding sites for the natural B_1 receptor agonist Lys-des-Arg^9-BK.

Chemical Chaperones Increased Recombinant and Wild-Type B_1 Receptor Expression. It is now well recognized that several chemicals such as glycerol, dimethyl sul-

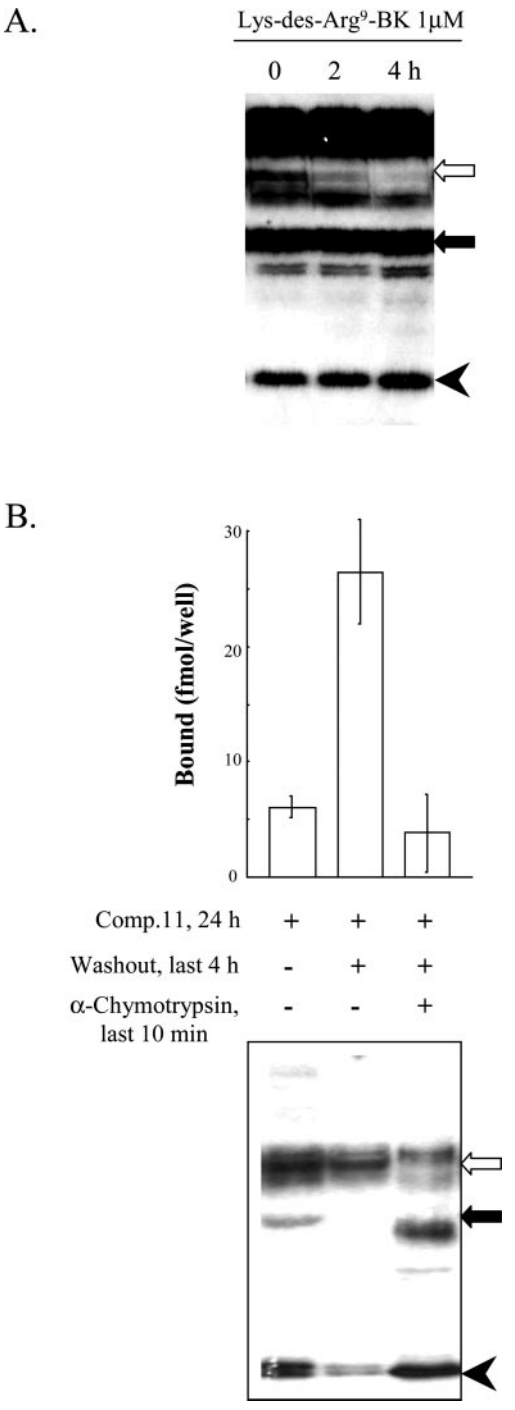

Fig. 8. A, effect of the agonist Lys-des-Arg^9-BK on the different molecular forms of the B_1R-YFP. Cells were treated for 2 or 4 h with 1 μM agonist in the complete culture medium; total cell lysates were prepared, subjected to SDS-PAGE, immunoblotted, and revealed using the anti-GFP antibody. B, top, specific binding of [^{3}H]Lys-des-Arg^9-BK to HEK 293/B_1R-YFP cells pretreated with compound 11 (1 μM) for 24 h and subsequently washed or not with serum-free culture medium supplemented with anisomycin (10 μM) for 4 h. The specific [^{3}H]Lys-des-Arg^9-BK binding to washed cells after α-chymotrypsin (10 μM) for 10 min was also evaluated. Bottom, effect of the 4-h washout in the presence of anisomycin (10 μM) and of the α-chymotrypsin treatment (10 μM, 10 min) on the B_1R-YFP species detectable in total cell lysate of HEK 293/B_1R-YFP cells pretreated with compound 11 (1 μM) for 24 h.

foxide (DMSO), and trimethylamine-*N*-oxide can act as chemical chaperones that assist the folding of wild-type and mutant proteins, including GPCRs, allowing them to escape the ER quality control system and achieve a functional state (Morello et al., 2000; Van Craenenbroeck et al., 2005). Therefore, we investigated the effects of the two chemical chaperones DMSO and glycerol on the detectable immunoreactive levels of the B_1R-YFP stably expressed in HEK 293 cells or the plasma membrane expression of wild-type rabbit B_1 receptors expressed at physiological densities by rabbit aortic SMCs. The B_1 receptor expressing cells were treated with various concentrations of DMSO and glycerol for 24 h in the complete culture medium. Both chemical chaperones strongly increased the amount of highly and low-glycosylated B_1R-YFP species, confirming that recombinant B_1 receptors were sensible to the protein stabilizing action of these chemicals (Fig. 9A). Chemical chaperones also significantly upregulated the number of cell surface B_1 receptors expressed by rabbit SMCs (Fig. 9B). These results suggest that the folding efficiency, a limiting step in the biosynthesis of B_1 receptors overexpressed in HEK 293 cells, is an intrinsic property of these receptors that can be extrapolated to more physiologically relevant cellular models.

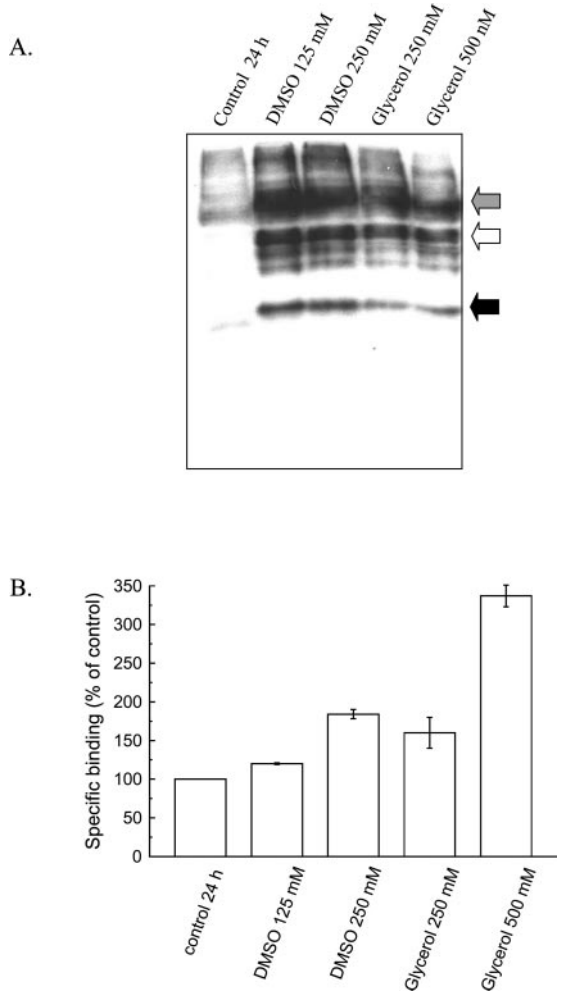

Fig. 9. Effect of chemical chaperones on B_1R-YFP and wild-type B_1Rs expressed by rabbit SMCs. The chemical chaperones DMSO or glycerol were added to the complete culture medium of HEK 293 cells stably expressing B_1R-YFP (A) or rabbit SMCs (B) for 24 h, and their effect on the abundance of the detectable receptor levels was evaluated by immunoblots (A) or using a binding assay (0.5 nM [^{3}H]Lys-des-Arg^9-BK) (B).

Intracellularly Retained and Low-Glycosylated myc-B_1R Species Are Stabilized in the ER by Compound 11, but Are Not Detectable as Highly Glycosylated Forms. The important effect of compound 11 on B_1R-YFP maturation contrasts with our recent report showing that treatment of HEK 293 cells transiently expressing an N-terminally myc-tagged B_1 receptor with this drug results in a relatively small stabilization cell surface receptors, as evaluated by cytofluorometry (1 μM, 24 h; Morissette et al., 2004). To confirm previous observations, we first verified the effect of compound 11 by fluorescence microscopy on nonpermeabilized and fixed HEK 293 cells transiently transfected with the myc-B_1R construct. This experiment essentially corroborated our previous conclusions, because compound 11 treatment led to a very modest increase in the detected myc-B_1R cell surface labeling; untransfected HEK 293 cells exhibited no significant fluorescent signal (data not shown).

We next examined the effect of compound 11 on the global cellular distribution of myc-B_1Rs in permeabilized cells stably expressing myc-B_1Rs. As observed with the YFP-tagged construct, myc-labeled receptors were highly enriched in a perinuclear compartment under basal conditions; only a faint nuclear labeling was detectable in control untransfected cells (Fig. 10A). Although a treatment with compound 11 also resulted in a marked loss of the myc-B_1R perinuclear labeling, this effect was not associated with the concomitant enrichment of the receptors at the cell surface (Fig. 10A). Total cell lysates of myc-B_1R expressing cells subjected to SDS-PAGE and immunoblotted with a monoclonal antibody specific for the myc tag revealed the presence of specific bands of 39 to 43 kDa (black arrow) and 92 kDa (gray arrow), probably representing low-glycosylated monomeric and dimeric receptor species, respectively (Fig. 10B). This pattern closely resembles to the one described for N-terminally hemagglutinin (HA) or FLAG-tagged human B_1 receptors (Kang et al., 2004, 2005). Three other bands with a molecular mass of 55, 63, and 71 kDa were also detectable (Fig. 10B). Treatment with PNGase F showed that most of the immunoreactive species were resistant to this enzyme and were thus not made of *N*-linked oligosaccharides (Fig. 10C). The 43-kDa myc-B_1R form was the only form clearly sensitive to PNGase F, but its deglycosylation only produced a faint band of 35 kDa (Fig. 10C). Treatment with the proteasome inhibitor MG 132 led to a strong accumulation of not only low-glycosylated receptors but of the other species. Taken together, these data show that the myc-B_1R behaved like B_1R-YFP under control conditions because it is retained in a perinuclear compartment, present as low or unglycosylated receptors, and extensively degraded by the proteasome pathway. They further suggest that these observations are typical of B_1 receptors overexpressed in HEK 293 cells independently of the tag position. The exact nature of the 55-, 63-, and 71-kDa receptor forms remain unknown, but the constant shift of 8 kDa and their rapid accumulation after proteasome inhibition suggest that they might represent ubiquitinated receptors. The effect of compound 11 (1 μM, 4–24 h) on myc-B_1Rs in immunoblots was consistent with cell imaging (Fig. 10A) because it led to a sharp decrease in the abundance of low-glycosylated receptor species (Fig. 10B); however, no highly glycosylated receptor forms were produced. The stabilizing effect of compound 11

on myc-B_1R low-glycosylated forms in brefeldin A-treated cells further highlights that myc-B_1Rs are unstable in the ER and respond like B_1R-YFP to the pharmacological chaperone in these conditions (Fig. 10B).

Other studies reported that the level of *N*-glycosylation between a FLAG- and HA-tagged human B_1 receptors was different (Kang et al., 2005). Furthermore, these investigators suggested that interference between the N-terminal tag and the *N*-glycosylation of the receptor or a difference in sensitivity of the antibodies used for this type of glycosylation represent plausible hypotheses to explain these differences in the detected *N*-glycosylation states (Kang et al., 2005). As observed for other antigens (Cao and Karsten, 2002), it is also possible that the hitherto unappreciated extensive oligosaccharide arborization of highly glycosylated B_1 receptors interferes with the recognition of the peptide epitope by the monoclonal antibody directed against it; this would explain why highly glycosylated and agonist-sensitive N-terminally tagged B_1 receptors have potentially been underestimated in HEK 293 cells (Fig. 10; Morissette et al., 2004; Kang et al., 2005). To verify the latter hypothesis, total cell lysates from the myc-B_1R stable transfectant, treated or not with compound 11 (1 μM, 24 h), were deglycosylated with PNGase F and subjected to SDS-PAGE; immunoblots showed that no myc-labeled receptors were uncovered by this strategy (Fig. 10C).

Discussion

The B_1 receptor, inducible under inflammatory conditions (Leeb-Lundberg et al., 2005), has also been previously shown to be rapidly degraded in a ligand-independent manner (Fortin et al., 2003). The discovery of this receptor in rabbit blood vessels was followed by the demonstration that rapid transcription and de novo protein synthesis were required after tissue isolation for the development of the B_1 receptor-mediated contractile response (Leeb-Lundberg et al., 2005). Further studies using brefeldin A and tunicamycin also highlighted a crucial role of protein maturation and *N*-glycosylation in B_1 receptor agonist-induced responses in the rabbit aorta (Audet et al., 1994). The cloning of the human B_1 receptors and that from several other mammalian species showed that three consensus *N*-glycosylation sites are absolutely conserved in extracellular domains (Menke et al., 1994; Leeb-Lundberg et al., 2005); however, rather low molecular mass values for the recombinant B_1 receptor in immunoblot experiments were commonly cited (38–40 kDa; Hess et al., 1996; Blaukat et al., 1999; Kang et al., 2004, 2005), suggesting a low glycosylation state.

The basal intracellular and perinuclear localization of B_1 receptor constructions in the presently exploited HEK 293 cell lines is further correlated with an enrichment of low-glycosylation receptor species and high molecular mass aggregates detected by immunoblots. Previous studies also observed such an imbalance between cell surface and intracellular localization (Hess et al., 1996; Blaukat et al., 1999; Lamb et al., 2002) and reported the detection of high molecular mass complexes containing B_1 receptors in HEK 293 cells (Kang et al., 2004, 2005). Our data suggest that overexpressed B_1 receptor molecules are unstable in HEK 293 cells and prone to an inefficient maturation process with secondary accumulation and degradation of biosynthetic low-glycosylated precursors by a proteasome-dependent pathway. The accumulation of higher molecular mass complexes of a protein on immunoblots after proteasome inhibition is often interpreted as the result of multiubiquitin conjugation (Ward et al., 1995; Yu and Kopito, 1999; Petäjä-Repo et al., 2001) and cytosolic protein aggregation (Lu et al., 2003; Romisch, 2005). However, the present results do not firmly

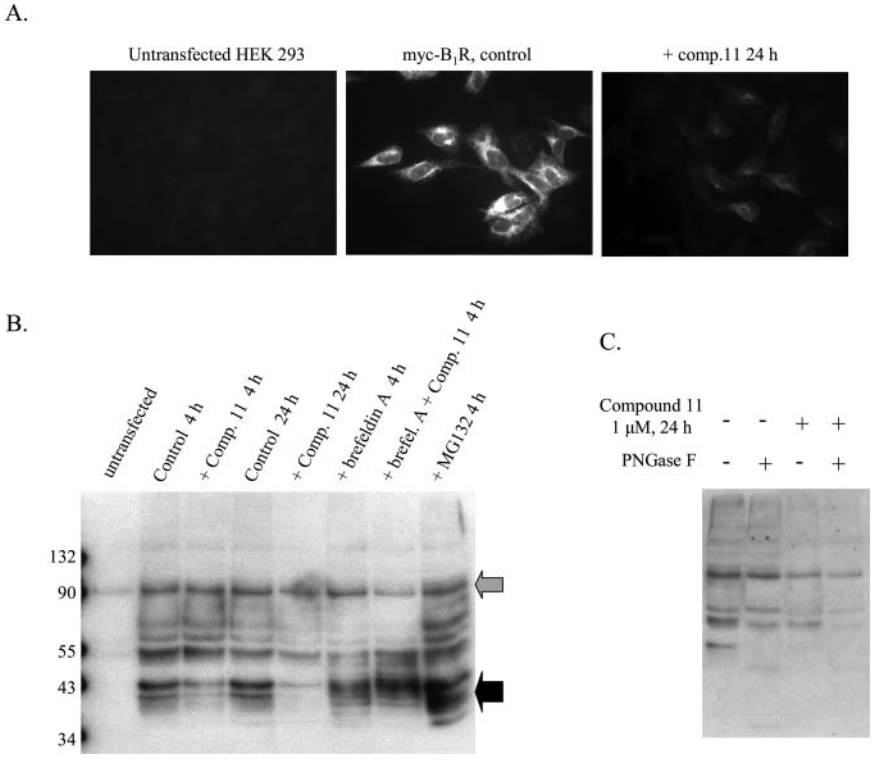

Fig. 10. Effect of compound 11 on the cellular distribution and abundance of the myc-B_1R construct. A, HEK 293 cells expressing myc-B_1Rs or not, optionally treated with compound 11 (1 μM) for 24 h, were fixed, permeabilized, and labeled with a monoclonal antibody directed against the antigenic tag to assess the effect of the pharmacological chaperone by fluorescence microscopy. Magnification, 40×. B, total lysates of cells treated for 4 or 24 h with compound 11 (1 μM), brefeldin A (18 μM), or MG 132 (10 μM) were subjected to SDS-PAGE and immunoblotted with the anti-myc antibody to verify the effect of the drugs on myc-B_1R molecular species. C, the impact of *N*-oligosaccharides removal using PNGase F on the detected myc-B_1R species was evaluated by immunoblots of total lysates of HEK 293 cells treated or not with compound 11 (1 μM) for 24 h.

demonstrate that B_1 receptors accumulated after interference with the proteasomal function are ubiquitinated and that ubiquitin is the targeting signal for degradation.

One of the most original findings of this study is that a micromolar concentration of a high-affinity nonpeptide antagonist of the rabbit B_1 receptor profoundly modifies the subcellular distribution and immunoblot pattern compared with control conditions. Compound 11-induced enrichment of B_1R-YFP at the cell surface paralleled exactly the stabilization of highly glycosylated forms of the receptor; this receptor form bound the radioligand and was accessible to extracellular chymotrypsin. Compound 11 acts with selectivity as a pharmacological chaperone intracellularly to increase the folding efficiency of maturating B_1 receptors and favoring the cell surface expression of highly glycosylated receptors. Similar effects, observed for other nonpeptide lipophilic drugs having a high affinity and selectivity for various wild-type and mutant GPCRs, tend to show that the pharmacological chaperoning concept may be widely applicable (Bernier et al., 2004; Van Craenenbroeck et al., 2005). The apparent decrease in the level of higher mass complexes after compound 11 treatment supports a less pronounced degradation of B_1R-YFP in favor of maturation. Although we have no molecular proof that the 145-kDa B_1R-YFP band represents the recently described homodimeric form of the receptor, its molecular mass is consistent with the reported properties of the dimer (Kang et al., 2005). The modest effect of PNGase F treatment on the 145-kDa mass of this band is also compatible with its formation from two low-glycosylated receptors, and the decline of this receptor form after compound 11 treatment is consistent with a pharmacological chaperoning action favoring highly glycosylated receptors biosynthesis. These results are also in agreement with the emerging idea that oligomeric complexes of B_1 receptors and other GPCRs might assemble early in their biosynthesis (Bulenger et al., 2005; Kang et al., 2005).

The observation that cell-impermeable agonist Lys-des-Arg^9-BK can specifically bind to and influence the distribution of the B_1R-YFP highly glycosylated band family in immunoblots further supports that this form of the receptor is expressed at the plasma membrane, relevant and functional. Furthermore, the latter result is in agreement with those obtained with another HEK 293 cell line expressing the same fusion protein at a lower level, for which the predominant cell surface distribution correlated with a faint reaction at ~100 kDa in immunoblots and a high affinity and responsiveness to the agonist Lys-des-Arg^9-BK (Sabourin et al., 2002; Fortin et al., 2003). Whether the agonist effect observed in immunoblots is related to the previously described agonist-dependent translocation of the receptor remains to be determined (Sabourin et al., 2002). Interestingly, other studies focusing on low *N*-glycosylated B_1 receptor species expressed in HEK 293 cells failed to detect any effect of the agonist on these receptor forms that may be intracellular by immunoblots (Lamb et al., 2002; Kang et al., 2005). The sensitivity of endogenous receptors to chemical chaperones further support the contention that the findings made with the YFP construct might also apply to more physiologically relevant cell systems.

Previous results showed that two different N-terminally epitope-tagged B_1 receptors, also expressed in HEK 293 cells and isolated by immunoprecipitation, were much less extensively glycosylated (~8 kDa of *N*-linked glycans; Kang et al., 2004, 2005). In fact, the glycosylation state of those N-terminally HA and FLAG constructs seems very similar to the forms that emerged from the present work as low-glycosylated receptors (Kang et al., 2004, 2005). Interestingly, the influence of N-terminal tags on B_1 receptor apparent glycosylation states was also proposed (Kang et al., 2005). In line with the latter observations, we show that N-terminally, myc-B_1Rs behave differently from the B_1R-YFP after incubation with a pharmacological chaperone because no cell surface enrichment or fully glycosylated forms of this construct are detectable by immunofluorescence or immunoblots with an antibody directed against the antigenic tag (Fig. 10; Morissette et al., 2004). Our experiments suggest that epitope masking by *N*-glycosylation is not likely to explain the lack of immunoreactivity for an antagonist-stabilized highly glycosylated receptor form. Because our results clearly showed that myc-B_1Rs, like B_1R-YFP, are mostly retained within cells, mostly unglycosylated, degraded by the proteasome, and highly sensible to a pharmacological chaperone, it is not clear why their antibody-based detection became impossible in post-ER compartments or at the cell surface. In a previous report, the systematic comparison of an N- and C-terminally myc-tagged vasopressin V_2 receptors demonstrated that the presence of the antigenic epitope on the N-terminal end greatly impaired the detection of highly glycosylated V_2 receptor species (Andersen-Beckh et al., 1999). The first possibility could be that N-terminally tags negatively influence the maturation and glycosylation of B_1 receptors. Previous studies have highlighted that the N terminus of receptors plays a significant role in promoting normal cell surface expression of receptors. For example, truncation of the initial amino acids from the N terminus of the endothelin B receptor or the rat corticotrophin-releasing factor receptor 1 decreased their expression severalfold (Kochl et al., 2002; Alken et al., 2005). The myc-B_1R construct has previously been shown to possess a high affinity but relatively low capacity for the agonist Lys-des-Arg^9-BK and is thus present at the cell surface (Morissette et al., 2004). A second plausible explanation could be that maturation of B_1 receptors requires the cleavage of its N terminus by a protease; such a process would result in the impossibility to detect the tag with a specific antibody. Figure 7B supports this hypothesis because the appearance of lower molecular mass products relative to the unglycosylated monomer, after deglycosylation of the antagonist-stabilized B_1R-YFP species, reveals that the heterogeneity in the apparent molecular mass of the highly glycosylated forms does not result solely from a different *N*-glycosylation state but also possibly from the N-terminal degradation of B_1R-YFP. The cell surface highly glycosylated form of a sphingosine-1-phosphate GPCR was shown to be an N-terminally truncated form of the receptor that had undergone complex oligosaccharide modification at the Golgi (Kohno and Igarashi, 2003).

In conclusion, within the limitations of the main molecular model (YFP being a large molecule added to the C terminus of the receptor in B_1R-YFP), this study reveals that the agonist-sensitive rabbit kinin B_1 receptor is a highly glycosylated protein. Because the three putative *N*-glycosylation sites are conserved in all the known mammalian sequences of the B_1 receptor, it is most probable that this glycosylation state also exists for the human B_1 receptor. We propose that

the low basal abundance of highly glycosylated B_1 receptors in overexpression models and the impossibility to detect it using N-terminally labeled constructs might explain why these species remained unappreciated. Thus, previous molecular studies exploiting N-terminally tagged receptors (Kang et al., 2004, 2005) potentially drew incomplete molecular portraits of the B_1 receptor. The present results, generated with a nonphysiological overexpression cell model, also illustrate for the first time the use of B_1 receptor lipophilic antagonists as pharmacological chaperones and explain why more physiologically relevant responses mediated by this dynamically regulated GPCR are highly sensitive to the *N*-glycosylation inhibitor tunicamycin.

Acknowledgments

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