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Kinins Promote B_2 Receptor Endocytosis and Delay Constitutive B_1 Receptor Endocytosis

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ABSTRACT

Upon sustained insult, kinins are released and many kinin responses, such as inflammatory pain, adapt from a B_2 receptor (B_2R) type in the acute phase to a B_1 receptor (B_1R) type in the chronic phase. In this study, we show that kinins modulate receptor endocytosis to rapidly decrease B_2R and increase B_1R on the cell surface. B_2Rs, which require agonist for activity, are stable plasma membrane components without agonist but recruit β-arrestin 2, internalize in a clathrin-dependent manner, and recycle rapidly upon agonist treatment. In contrast, B_1Rs, which are inducible and constitutively active, constitutively internalize without agonist via a clathrin-dependent pathway, do not recruit β-arrestin 2, bind G protein-coupled receptor sorting protein, and target lysosomes for degradation. Agonist delays B_1R endocytosis, thus transiently stabilizing the receptor. Most of the receptor trafficking phenotypes are transplantable from one receptor to the other through exchange of the C-terminal receptor tails, indicating that the tails contain epitopes that are important for the binding of protein partners that participate in the endocytic and postendocytic receptor choices. It is noteworthy that the agonist delay of B_1R endocytosis is not transplanted to the B_2R via the B_1R tail, suggesting that this property of the B_1R requires another domain. These events provide a rapid kinin-dependent mechanism for 1) regulating the constitutive B_1R activity and 2) shifting the balance of accessible receptors in favor of B_1R.

Kinins are proinflammatory peptides that are formed extracellularly in response to pathological insult and act through two G protein-coupled receptors (GPCR), named B_1 (B_1R) and B_2 (B_2R) (Leeb-Lundberg et al., 2005). Through these receptors, kinins stimulate multiple inflammatory responses such as vasodilatation, vascular permeability, hyperalgesia, and pain. Sustained insult and various pathological conditions cause many kinin responses to adapt from a B_2 type in the acute phase to a B_1 type in the chronic phase (Dray and Perkins, 1993; Ongali et al., 2003).

GPCR signaling is tightly regulated at several steps in the receptor life cycle, ranging from receptor gene transcription to degradation of the mature receptor. At the cell surface, activation of the receptor by agonist is often followed by receptor phosphorylation via a G protein-coupled receptor kinase and subsequent arrestin binding to the receptor, which results in homologous desensitization (i.e., uncoupling of the receptor from the G protein and thereby from downstream signal transduction pathways) (Lefkowitz et al., 1998). Many GPCRs also subsequently undergo endocytosis through either clathrin-coated structures or lipid rafts, such as caveolae (Ferguson, 2001; Chini and Parenti, 2004). After endocytosis, GPCRs are either recycled back to the plasma membrane, leading to functional resensitization; remain internalized through storage, leading to desensitization; or are targeted to lysosomes for degradation, leading to functional down-regulation (von Zastrow, 2003). The postendocytic fate of a GPCR is determined via direct interactions of the receptor with various protein partners such as ERM-binding phosphoprotein 50/Na^+-H^+ exchanger regulatory factor, *N*-ethyl maleimide-sensitive factor, sorting nexin 1, GPCR-associated sorting protein (GASP), and lipid membrane components

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ABBREVIATIONS: GPCR, G protein-coupled receptors; B_1R, B_1 receptor; B_2R, B_2 receptor; GASP, GPCR-associated sorting protein; HEK, human embryonic kidney; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; APC, allophycocyanin; TBS, Tris-buffered saline; DAKD, desArg^{10}kallidin; BK, bradykinin; GST, glutathione transferase; BPA, biotinylation protection assay; PNGase, peptide *N*-glycosidase F.

(Hall et al., 1998; Cao et al., 1999; Whistler et al., 2002; Xiang and Kobilka, 2003; Heydorn et al., 2004). The responsiveness of a GPCR signaling system is, to a large degree, determined by these regulatory events.

Kinin receptor adaptation is explained, at least in part, by the induction of B_1R expression by pro-inflammatory cytokines such as interleukin-1β (Bachvarov et al., 1998), which is observed in cellular systems (Phagoo et al., 1999; Bengtsson et al., 2006). This induction may function to convert kinin signaling from a transient one to a sustained one because B_1R is constitutively active (Leeb-Lundberg et al., 2001). Furthermore, B_1R is not phosphorylated either basally or in response to agonist (Blaukat et al., 1999) and desensitizes only marginally upon further stimulation by the agonist (Mathis et al., 1996). On the other hand, B_2R is subject to both basal and agonist-promoted phosphorylation (Blaukat et al., 1999, 2001) and desensitizes rapidly upon agonist stimulation (Mathis et al., 1996).

Kinins decrease the availability of cell surface B_2R and increase the availability of B_1R in some cell systems (Phagoo et al., 1999; Bengtsson et al., 2006), which may contribute to kinin receptor signal adaptation. The details of this regulation are not well understood, but some studies have suggested it may involve changes in receptor trafficking. Most trafficking studies of B_2R and B_1R have used bound radiolabeled agonist to monitor the receptors (Munoz and Leeb-Lundberg, 1992; Munoz et al., 1993; Faussner et al., 1998; Pizard et al., 1999; Lamb et al., 2001). In a few cases, receptors fused in their C-terminal domains to fluorescent proteins have also been used for this purpose (Bachvarov et al., 2001; Lamb et al., 2001; Sabourin et al., 2002). The former approach is restricted to monitoring only agonist-promoted changes, and is sensitive to changes in agonist affinity, whereas the latter approach requires receptor constructs drastically modified in their C-terminal domain, which can be critical for the postendocytic fate of the receptor (Cao et al., 1999; Finn and Whistler, 2001; Whistler et al., 2002; Gaborik and Hunyady, 2004; Heydorn et al., 2004; Bartlett et al., 2005). Nevertheless, these studies have proposed that B_2R is rapidly and almost completely internalized by the agonist, whereas B_1R is much less or differently affected by the agonist in this regard.

Our aim with this study was to use a representative cellular model with minimally modified receptors to determine the role of cellular trafficking in the rapid and inverse kinin regulation of B_2R and B_1R. HEK293 cells were chosen as a model system because this cell line is well characterized, easy to use and manipulate, has been shown to be a valuable tool in the trafficking studies of other model GPCR systems, and allows for the independent study of the trafficking of each receptor in the absence of regulation of de novo receptor synthesis.

Materials and Methods

Cell Culture and DNA Constructs. IMR90 human embryonic lung fibroblast cells (American Type Culture Collection, Manassas, VA) were grown in minimum essential medium containing 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) in 5% CO_2 at 37°C. HEK293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS in 10% CO_2 at 37°C. The human B_2R and B_1R cDNA were subcloned into a pcDNA3.1 vector containing a Zeocin selection marker. An N-terminal artificial signal sequence, as described previously (Whistler et al., 2002), and the FLAG sequence tag were added in series to make the B_2R and B_1R constructs named SFB2 and SFB1, respectively. Receptor constructs with exchanged C-terminal tails starting from the first amino acid after the NP*XX*Y motif (B1CB2 and B2CB1) and B_1R truncated after residue Phe^{319} (B1Stop320), which is located seven residues beyond the NP*XX*Y motif, were created by PCR. GFP-β-arrestin 2 was kindly provided by Dr. Marc Caron (Duke University Medical Center, Durham, NC) (Barak et al., 1997). The cells were transfected using the calcium phosphate precipitate method. Single colonies were then chosen and propagated in the presence of selection-containing media to generate clonal stable cell lines.

Radioligand Binding Assay. Assays were performed on particulate preparations and on intact cells using [^{3}H]BK and [^{3}H]DAKD as described previously (Leeb et al., 1997; Phagoo et al., 1999). Pretreatment of intact cells with agonist at 37°C was followed by a 6-min wash with ice-cold 50 mM glycine-HCl, pH 3, and two washes in ice-cold phosphate-buffered saline (PBS) to remove the agonist.

Phosphoinositide Hydrolysis. Confluent cells were labeled with 0.4 μCi/well [*myo*-^{3}H]inositol for 16 to 20 h in inositol-free DMEM containing 0.5% bovine serum albumin in 48-well dishes, washed in Leibovitz's L-15 medium, pH 7.4, containing 10 mM LiCl, and then stimulated with increasing concentrations of agonist for 30 min at 37°C. The cells were then lysed with 100 mM formic acid, and the lysate was mixed with anion exchange resin in a 48-well plate with a small hole in the bottom of each well. The resin was then washed sequentially with water and 60 mM ammonium formate by aspiration. Inositol phosphates were eluted from the resin with 1 M ammonium formate and counted for radioactivity in a scintillation counter (LS6000; Beckman Coulter, Fullerton, CA).

FACS Analysis. Confluent cells in 100-mm dishes were treated with and without agonist in DMEM and 10% FBS. The cells were then trypsinized and washed with ice-cold PBS plus Ca^{2+}/Mg^{2+}. Cells were then resuspended in 100 μl of ice-cold PBS plus Ca^{2+}/Mg^{2+} with 50% FBS, 0.4 μg of M1 anti-FLAG antibody conjugated with APC using a commercial kit (Prozyme, San Leandro, CA), 1 μg of mouse IgG1 κ (MOPC 21), incubated for 20 min at 4°C, which was followed by two washes in PBS plus Ca^{2+}/Mg^{2+}. The cell pellet was finally resuspended in 1 ml of PBS plus Ca^{2+}/Mg^{2+} with 50% FBS. Cells (~20,000) were counted by FACS, and each receptor-positive cell line was gated against untransfected HEK293 cells to reduce background staining. Mean fluorescence was calculated for the gated signal-positive population of untreated and agonist-treated cells.

Biotinylation Protection Assay. Confluent cells were washed twice with ice-cold PBS and then incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-SS-biotin (Pierce) in PBS for 30 min at 4°C with gentle agitation. The cells were then washed twice with Tris-buffered saline (TBS) to quench the biotinylation reaction and returned to DMEM and 10% FBS for treatments. Cells were allowed to re-equilibrate to growth conditions for 30 min at 37°C before further experimentation. Cells labeled Total and Strip in the figures were left on ice in TBS. At 37°C, cells were treated without (NT) or with 1 μM desArg^{10}kallidin (DAKD) or 1 μM bradykinin (BK) (AG) for the indicated intervals and washed twice with ice-cold TBS. The remaining cell surface-biotinylated receptors on the cells together with those cells designated Strip were stripped with 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, and 1% FBS for 30 min at 4°C. The glutathione was then quenched using a 20-min wash with PBS containing 50 mM iodoacetamide and 1% bovine serum albumin. Cells were then extracted in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 25 mM KCl containing a complete protease inhibitor cocktail (Roche), and cell debris was removed by centrifugation at 10,000*g* for 10 min at 4°C. Receptors were immunoprecipitated in the extraction buffer by incubating in anti-FLAG M2 affinity resin (Sigma) overnight at 4°C. The precipitates were washed exten-

sively and sequentially in the extraction buffer and in 10 mM Tris-HCl, pH 7.4. The receptors were then deglycosylated for 2 h at 37°C with PNGase F (New England Biolabs, Ipswich, MA). Proteins were denatured in SDS sample buffer without reducing agent, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and the membrane was blocked for at least 1 h in TBS containing 0.1% Tween 20 and 10% nonfat milk. Biotinylated proteins were visualized by incubating with the Vectastain avidin-biotinylated enzyme complex immuno-peroxidase reagent (Vector Laboratories, Burlingame, CA) followed by development with ECL reagents according to the manufacturer's instructions (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Biotinylation Degradation Assay. Confluent cells were washed twice with ice-cold PBS and then incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-SS-biotin in PBS for 30 min at 4°C with gentle agitation. The cells were then washed twice with TBS to quench the biotinylation reaction and returned to DMEM and 10% FBS. Cells were then left on ice (Total) or returned to 37°C for 30 min after which they were incubated without (NT) or with 1 μM DAKD or BK (AG) for 120 min at 37°C. The receptors were then extracted, immunoprecipitated, and detected as described above under *Biotinylation Protection Assay*.

Fluorescence Microscopy. Cells were propagated in growth media on glass coverslips to 50% confluence and then treated in one of three ways. For whole-cell receptor distribution experiments, cells were incubated without and with agonist or antagonist for 30 min at 37°C. For cell surface receptor redistribution experiments, cells were incubated in media containing 3.5 μg/ml primary mouse anti-FLAG M1 monoclonal antibody (Sigma) for 30 min at 37°C to label surface receptors. The cells were then incubated without agonist or antagonist or a combination of the two for an additional 30 min at 37°C. For receptor recycling experiments, cells were incubated as described immediately above followed by stripping of remaining cell surface M1 antibody by depleting the media of Ca^{2+} with PBS containing 0.1% EDTA at room temperature. The cells were then further incubated without and with agonist or antagonist for an additional 30 min at 37°C. In all experiments, cells were then fixed using 3.5% formaldehyde in PBS and permeabilized using 0.1% Triton X-100. For visualization of receptors, fixed specimens were incubated with Alexa488-labeled anti-mouse IgG2b antibody (Invitrogen) to detect FLAG M1 antibodies bound to the tagged receptors. For colocalization of FLAG-tagged receptors with LAMP 1 and LAMP 2, primary H4A3 and H4B4 mouse IgG1 (Developmental Studies Hybridoma Data Bank) antibodies were incubated with secondary Alexa568-labeled anti-mouse IgG_1 antibody (Invitrogen), to detect LAMP 1 and LAMP 2, along with secondary Alexa488-labeled anti-mouse IgG2b, to detect FLAG M1 antibodies bound to receptors. Images were collected by confocal microscopy using a Nikon Eclipse confocal microscope. Quantification of colocalized fluorescence was done using the Imaris software (Bitplane AG, Zurich, Switzerland).

β-Arrestin Recruitment. Cells stably expressing GFP-β-arrestin 2 were transiently transfected with SFB1, SFB2, SFB1CB2, or SFB2CB1. Transfected cells were plated on polylysine-treated glass coverslips in six-well dishes for 24 h before assay. The cells were then incubated in growth media without and with agonist for 5 min at 37°C. Cells were then fixed in PBS containing 3.5% formaldehyde for 30 min followed by primary staining with M1 anti-FLAG antibodies and secondary staining with Alexa568-labeled anti-mouse IgG1 antibody. Images were then collected by confocal microscopy using a confocal microscope (Eclipse; Nikon, Tokyo, Japan).

In Vitro Affinity Assay of GST Fusion Proteins and GASP. B_1R and B_2R receptor C-terminal tails starting after the NP*X*Y*Y* motif were cloned into the pGEX4t1 vector and C-terminal tail-GST fusion proteins were produced and attached to glutathione-Sepharose (Sigma, St. Louis, MO). Full-length GASP was in vitro-translated using the TnT quick-coupled transcription/translation systems (Promega, Madison, WI) with ^{35}S-labeled methionine. Fusion proteins and radiolabeled probe were mixed in a wash solution containing 0.1% Triton X-100 and incubated for 1 h at room temperature. Glutathione resin was washed several times followed by denaturation by boiling in SDS sample buffer and proteins fractionated by SDS-polyacrylamide gel electrophoresis. Protein concentrations of receptor tails and controls were estimated by Coomassie staining of the gel, and pulled down probe was visualized by autoradiography.

Coimmunoprecipitation of Receptor and GASP. Confluent cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, and 10 mM Na_2HPO_4) with complete protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were cleared by centrifugation at 14,000*g* for 15 min at 4°C. An aliquot of the cleared lysates was withdrawn from each sample to compare the GASP concentration by immunoblotting as described below. The rest of the cleared lysate was immunoprecipitated using 15 μl of M2 anti-FLAG affinity resin (Sigma) for 1 h at 4°C, washed extensively with 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 25 mM KCl containing a complete protease inhibitor cocktail (Roche), and deglycosylated with PNGase F (New England Biolabs) for 2 h. Precipitates were resolved on a 4 to 20% gradient Tris-HCl precast gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride membrane. The blots were cut below the 75-kDa marker band to separately immunoblot for either receptor (lower blot) or GASP (upper blot). GASP blots were incubated for 2 h at room temperature with rabbit anti-GASP (1:1000) and for 1 h at room temperature with HRP-conjugated anti-rabbit antibody (New England Biolabs) (1:4000), then visualized with ECL Plus (GE Healthcare). Receptor blots were incubated for 2 h with biotinylated FLAG M2 antibody (1:250) (Sigma) and then visualized with streptavidin overlay (Vectastain ABC reagents; Vector Laboratories) and ECL Plus.

Data Analysis. Data were analyzed by the Student's *t* test as indicated.

Results

Effect of Kinin Agonists on Cell Surface Availability of Kinin Receptors. Human embryonic lung fibroblast IMR90 cells express B_2R and B_1R at 1283 and 15 fmol/mg of protein, respectively (Phagoo et al., 1999). Treatment of these cells with a 0.1 μM concentration of the cognate B_2R ligand BK at 37°C led within 30 min to a 78% decrease in the amount of B_2R available on the cell surface, as determined by radioligand binding, and this effect was maintained at 120 min of treatment (Fig. 1A). In contrast, treatment with a 0.1 μM concentration of the cognate B_1R ligand DAKD led within the same time period to an 81% increase in the number of available B_1R, which was also maintained at 120 min of treatment (Fig. 1A). Thus, kinins rapidly and inversely regulate the cell surface availability of native B_2R and B_1R as reported previously (Phagoo et al., 1999; Bengtsson et al., 2006).

To address the mechanism underlying this rapid inverse regulation, we generated HEK293 cell clones stably expressing FLAG-tagged human B_2R or B_1R at 1921 ± 496 and 343 ± 40 fmol/mg of protein ($n = 3$), respectively. These cells bound and responded to their respective agonist ligands with affinities and potencies similar to those of their wild-type counterparts (Table 1). FACS analysis with APC-conjugated M1 FLAG antibodies revealed that BK and DAKD treatment of these cells for 30 min at 37°C led to a 44% decrease and a 16% increase in the amount of B_2R and B_1R available at the cell surface, respectively (Fig. 1B). The lower percentage increase in B_1R in the stable HEK293 cells compared with the IMR90 cells is probably due in part to the relatively

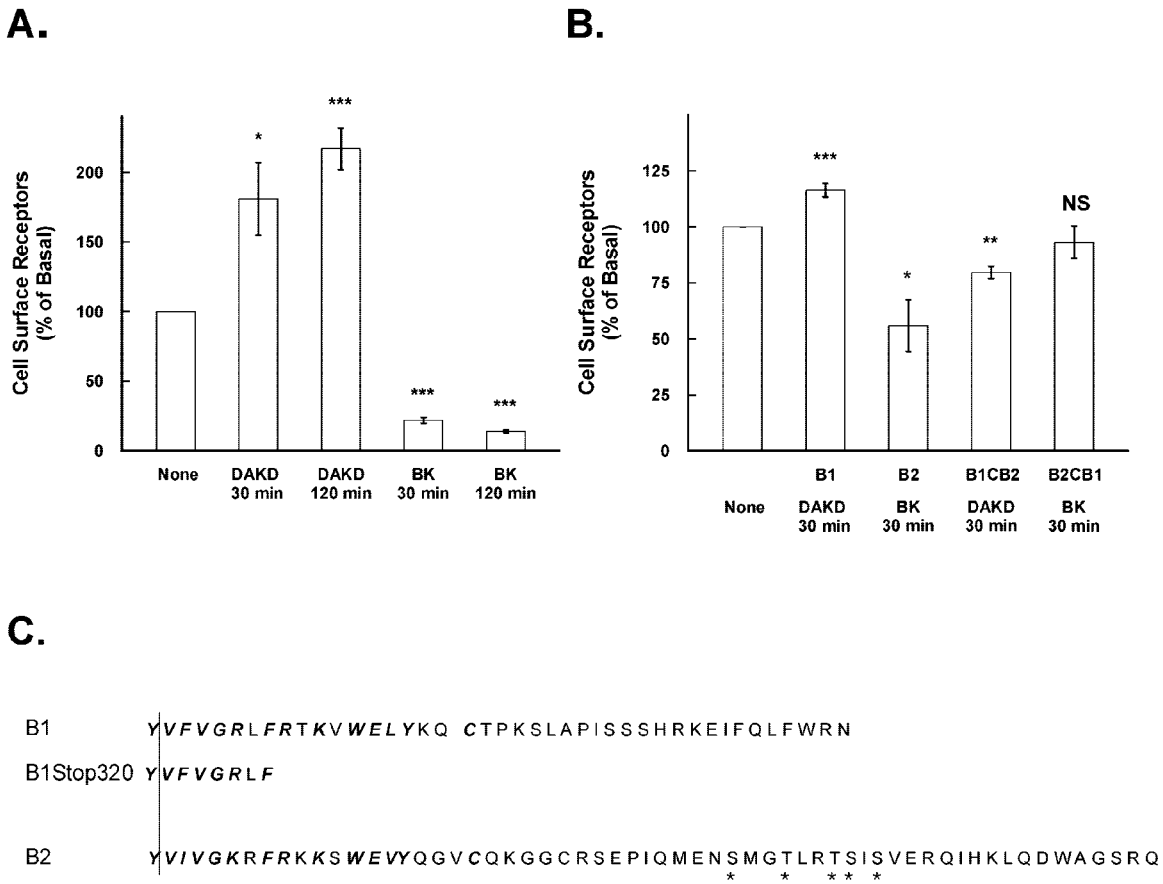

Fig. 1. Effects of kinins on cell surface receptors expression. A, IMR90 cells were treated with 0.1 μM BK or 0.1 μM DAKD for 30 min and 120 min at 37°C, washed with low-pH buffer at 4°C, and then assayed for specific [^{3}H]BK or [^{3}H]DAKD binding at 4°C as described under *Materials and Methods*. The results are shown as percentage of basal, where 100% basal is the specific binding in the absence of agonist treatment. Data are presented as mean ± S.E. of at least five independent experiments. Comparison with the absence of agonist treatment: *, $p < 0.05$; ***, $p < 0.001$. B, HEK293 cells stably expressing B_1R, B_2R, B1CB2, and B2CB1 were treated with 1 μM BK (B_2R, B2CB1) or 1 μM DAKD (B_1R, B1CB2) for 30 min at 37°C. Cells (~20,000) were then counted by FACS using APC-conjugated M1 anti-FLAG antibodies as described under *Materials and Methods*. The results are shown as percentage of basal, where 100% basal refers to the mean fluorescence intensity in the absence of agonist. Data are presented as mean ± S.E. of three independent experiments. Comparison with the absence of agonist treatment: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; NS, not significant. C, amino acid sequences of the C-terminal domains of the human B_1R, B1stop320, B_2R. Indicated is the predicted junction of the seventh transmembrane domain and the C-terminal domain (vertical line), conserved residues between the receptor subtypes (bold italics), and residues shown to be phosphorylated in vivo (Blaukat et al., 2001) (star).

higher amount of receptors expressed in the former cells. Nevertheless, stable HEK293 cells reflect the IMR90 cells concerning agonist-induced changes in surface receptor number. We thus used these cell lines to investigate the molecular mechanism whereby agonist ligand regulates these receptors.

Receptor-Mediated β-Arrestin Recruitment. β-Arrestin is a key GPCR regulatory protein that is rapidly recruited from the cytosol to the plasma membrane receptor upon agonist stimulation and involved in both receptor desensitization and internalization. Agonist stimulation of B_2R led to β-arrestin 2 recruitment to the plasma membrane within 5 min (Fig. 2, B2 NT, AG). In contrast, no apparent effect on β-arrestin 2 distribution was observed either without or with agonist stimulation of B_1R for the same time period (Fig. 2, B1 NT, AG). These results are consistent with the different agonist regulation of these receptors, where B_2R is rapidly phosphorylated at specific serines and threonines in the C-terminal tail (Fig. 1C) (Blaukat et al., 1999, 2001) and desensitized by the agonist (Mathis et al., 1996), whereas B_1R is apparently not phosphorylated (Fig. 1C) (Blaukat et al., 1999) and desensitizes very slowly in response to agonist (Mathis et al., 1996), as well as being constitutively active (Leeb-Lundberg et al., 2001).

TABLE 1

Kinin agonist affinity and potency on cells stably expressing SFB2, SFB1, WTB2, and WTB1

K_D values were obtained by nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) of equilibrium radioligand binding isotherms on particulate preparations using [^{3}H]BK for SFB2 and WTB2 and [^{3}H]DAKD for SFB1 and WTB1. EC_{50} values were obtained by nonlinear regression analysis (Prism) of dose response curves on phosphoinositide hydrolysis in intact cells using BK for SFB2 and WTB2 and DAKD for SFB1 and WTB1.

Receptor	K_D[a]	EC_{50}[b]
	nM	
SFB2	0.13 ± 0.02	5.06 ± 0.25
SFB1	0.14 ± 0.03	1.32 ± 0.37
WTB2	0.14 ± 0.02[a]	3.12 ± 0.15[a]
WTB1	0.27 ± 0.11[a]	1.28 ± 0.06[a]

[a] Data obtained from Lamb et al. (2001).

Identification of the Receptor Domain Responsible for Differential Endocytic Trafficking of Receptors. The receptor C-terminal tail is important in the trafficking of many GPCRs. We thus examined whether the C-terminal

tails of the B_1R and the B_2R conferred trafficking specificity (Fig. 1C). Receptors were generated in which the tail of the B_1R was transplanted to the B_2R (B2CB1) and the tail of the B_2R was transplanted to the B_1R (B1CB2). This exchange does not affect the agonist specificity of the receptors (Leeb-Lundberg et al., 2001; Kang and Leeb-Lundberg, 2002). We then examined the effects of the tail exchange on β-arrestin 2 recruitment and agonist-induced changes in cell surface receptor number. Substitution of the B_2R tail in B_1R created a receptor (B1CB2) that rapidly recruited β-arrestin 2 in response to DAKD (Fig. 2, B1CB2 NT, AG) in contrast to the B_1R. These data suggest that the B_2R tail contains an epitope important for β-arrestin recruitment. This epitope is most likely the phosphorylation of specific serines and threonines in the B_2R tail, which is lacking in the B_1R tail (Fig. 1C). Figure 1B shows that DAKD decreased the cell surface availability of B1CB2 by 20%, making it significantly different from B_1R in which DAKD increased cell surface availability ($p = 0.0003$). Substitution of the B_1R tail in B_2R created a receptor (B2CB1) that was unable to recruit β-arrestin 2 in response to BK (Fig. 2, B2CB1 NT, AG). BK had no effect on the availability of B2CB1 making it significantly different from B_2R in which BK decreased cell surface availability ($p = 0.03$). However, the C-terminal tail alone was not enough to transfer the B_1R phenotype of agonist-promoted increase in receptor availability to the B_2R.

Whole-Cell Distribution of Receptors. We next examined the distribution of the receptors and the tail swaps in

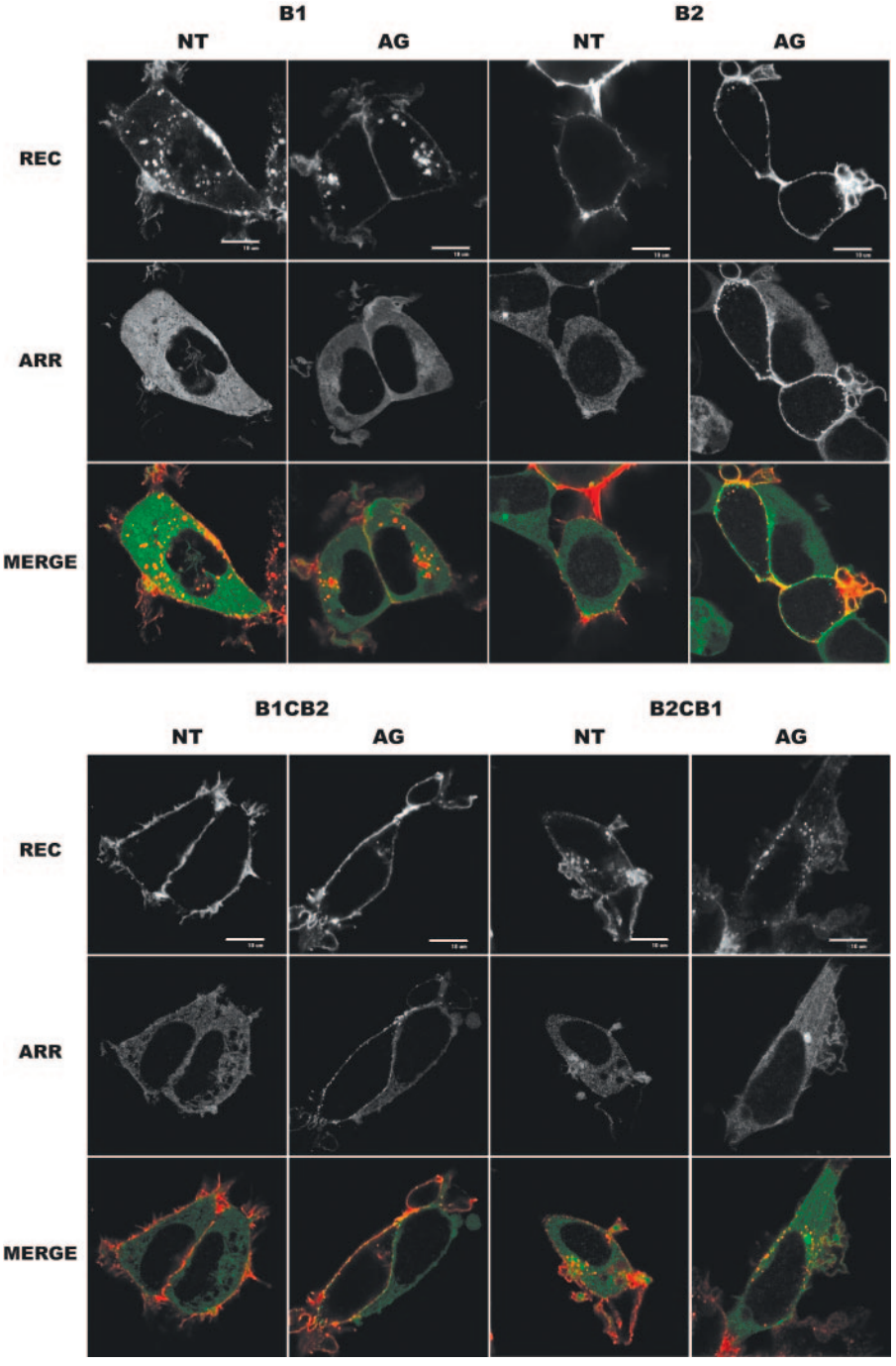

Fig. 2. Effects of kinins on receptor-mediated β-arrestin recruitment. Cells stably expressing GFP-β-arrestin 2 and transiently transfected to express B_1R, B_2R, B1CB2, and B2CB1 were incubated in the absence (NT) and presence of 1 μM receptor-specific agonist (AG) for 5 min at 37°C as indicated. Cells were then stained with primary mouse M1 anti-FLAG antibodies and secondary ALEXA488-labeled anti-mouse antibody as described under *Materials and Methods*. Individual images of receptors (REC) and β-arrestin (ARR) and their merged images (MERGE) were collected with a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. Bar, 10 μm.

the presence and absence of ligand. The whole-cell distribution of each receptor was analyzed by confocal fluorescence microscopy of formaldehyde-fixed and permeabilized cells. Under these conditions, the vast majority of B_2R were located in the plasma membrane in the absence of agonist (Fig. 3A, B2 NT). On the other hand, treatment with 1 μM BK (B2 AG) for 30 min at 37°C promoted the redistribution of receptors to intracellular compartments, although some plasma membrane staining was still observed. Treatment with a 1 μM concentration of the B_2R-specific antagonist icatibant alone had little effect on B_2R distribution (data not shown). In contrast to B_2R, a disperse pattern of B_1R distribution was observed even in the absence of agonist, with receptors localized primarily intracellularly, in both a tubular network and in distinct puncta, and to a lesser degree in the plasma membrane (Fig. 3A, B1 NT). No obvious change in receptor distribution was observed after treatment with 1 μM DAKD (B1 AG) or the B_1R-specific antagonist des-Arg^{10}-icatibant (data not shown). Thus, the steady-state distribution of B_2R and B_1R are different, with a much greater intracellular pool of B_1R. The tubular network may represent slow B_1R maturation, whereas the distinct puncta may represent constitutive B_1R endocytosis (see below). B1CB2 exhibited both basal and agonist-promoted distribution patterns (Fig. 3A, B1CB2 NT, AG) that were very similar to the B_2R. Likewise, the B2CB1 distribution patterns in both the presence and absence of agonist (B2CB1 NT, AG) were very similar to B_1R. Thus, the receptor C-terminal tails seem to be involved in both receptor maturation and endocytosis.

Constitutive and Kinin-Dependent Endocytosis of Receptors. To specifically address receptor endocytosis without the interference of receptor maturation, we used two assays that specifically monitor the fate of the plasma membrane pools of receptors. The first assay was an immunofluorescence confocal microscopy assay in which live cells are "fed" primary antibody to label surface receptors. The second assay was a quantitative biochemical assay, the biotinylation protection assay (BPA). The immunofluorescence assay takes advantage of the extracellular FLAG epitope tag on the receptors to monitor selectively the movement of cell surface receptors. Live cells were first incubated with M1 anti-FLAG antibody in the absence (NT) or presence of agonist (AG) for 30 min at 37°C. The cells were then fixed, permeabilized, and incubated with secondary antibody to detect receptor-antibody complexes. In the absence of agonist, B_2R were located almost exclusively in the plasma membrane (Fig. 3B, B2 NT). Incubation with agonist at 1 μM promoted endocytosis of the B_2R-antibody complexes (B2 AG), which was blocked by coincubation with a 5-fold excess (5 μM) of antagonist (data not shown). On the other hand, spontaneous agonist-independent endocytosis of the B_1R-antibody complexes was found to occur (Fig. 3B, B1 NT). No apparent change in B_1R distribution was observed when cells were treated with the agonist at 1 μM either without (B1 AG) or with a 5-fold excess (5 μM) of

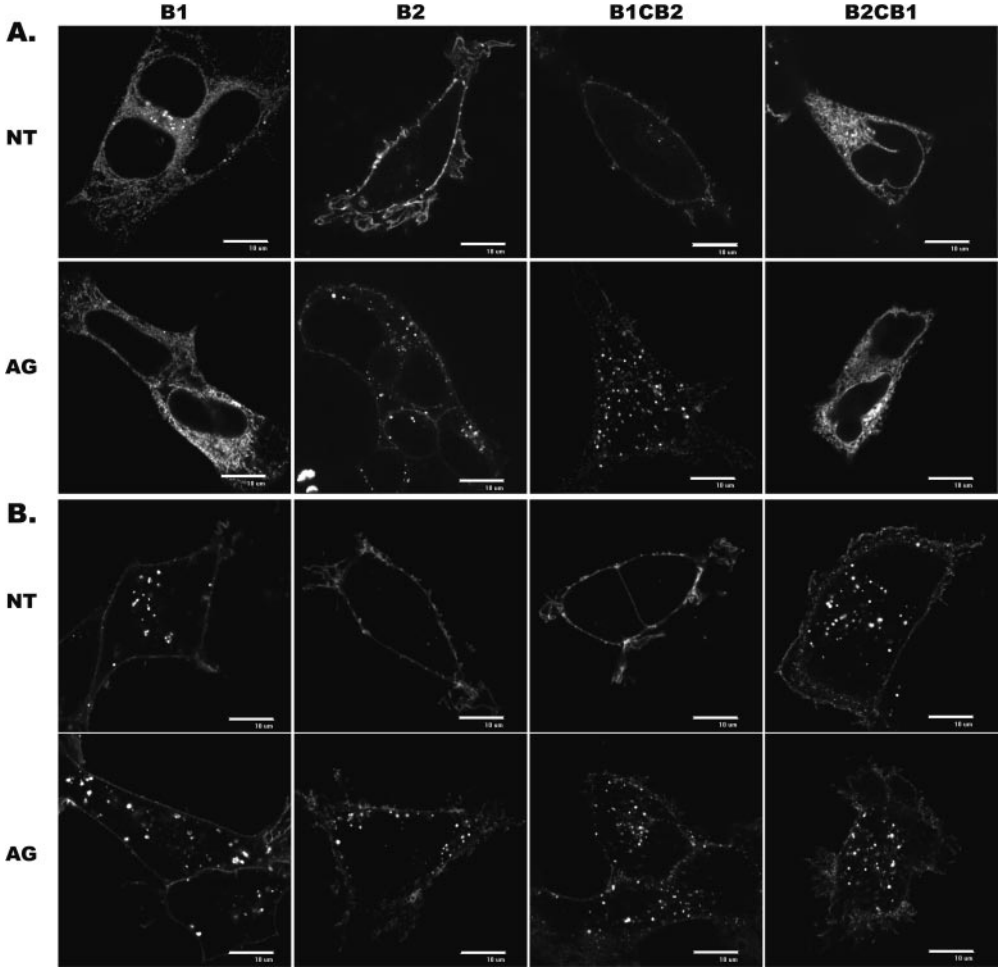

Fig. 3. Whole-cell receptor distribution and constitutive and kinin-dependent receptor endocytosis as determined using confocal microscopy. A, cells stably expressing B_2R, B_1R, B2CB1, and B1CB2 were incubated in the absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 30 min at 37°C as indicated. Cells were then fixed and permeabilized before incubation with primary mouse M1 anti-FLAG antibody and secondary ALEXA488-labeled anti-mouse antibody as described under *Materials and Methods*. B, cells stably expressing B_2R, B_1R, B2CB1, and B1CB2 were incubated with primary mouse M1 anti-FLAG antibody followed by incubation in the absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 30 min at 37°C as indicated. Cells were then fixed, permeabilized, and stained with secondary ALEXA488-coupled anti-mouse antibody as described under *Materials and Methods*. In both A and B, images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. Bar, 10 μm.

antagonist (data not shown). It is noteworthy that the tubular distribution of B_1R that was seen in Fig. 3A was absent using this protocol, which strongly argues that the intracellular pool of B_1R is a combination of both slow receptor maturation and constitutive receptor endocytosis.

In the BPA, cell surface receptors were first selectively labeled with a membrane-impermeable biotin analog. Internalized receptors were then selectively monitored by their protection from stripping by a membrane-impermeable reducing agent. This assay revealed internalization of B_2R only in the presence of agonist (Fig. 4A, compare B2 NT 30 min and B2 AG 30 min), whereas B_1R internalization occurred both in the absence and presence of agonist (Fig. 4A, compare B1 NT 30 min and B1 AG 30 min). These data confirm the results obtained using the immunofluorescence microscopy assay (Fig. 3). It is noteworthy that detailed densitometric analysis of the BPA revealed that significantly less B_1R (43%, $p = 0.02$) was internalized in the presence of agonist than in the absence of agonist at 30 min (Fig. 4B). Thus, the B_1R is subject to constitutive endocytosis but the agonist delays this event. In contrast, the B_2R resides in the plasma membrane until it is exposed to agonist, at which point it rapidly internalizes. The relatively low amount of internalized B_2R recovered in this assay may be explained in part by the extraction conditions used. However, other factors are probably also involved, because B1CB2, which behaved similarly to B_2R, was significantly easier to extract.

B1CB2 was located exclusively in the plasma membrane in the absence of agonist, whereas incubation with agonist led to rapid endocytosis of the construct as evidenced by both the immunofluorescence assay (Fig. 3B, B1CB2 NT, AG) and BPA (Fig. 4, A and B). Thus, B1CB2 gains the endocytic features of B_2R, which is consistent with introduction of both agonist-promoted β-arrestin recruitment (Fig. 2) and agonist-promoted decrease in the cell surface receptors (Fig. 1B). The immunofluorescence assay revealed that B2CB1 is constitutively internalized (Fig. 3B). This constitutive endocytosis, like that for the B_1R, apparently proceeds by a β-arrestin 2-independent mechanism, because this receptor does not recruit β-arrestin 2 in either the presence or the absence of agonist (Fig. 2). The low level of expression of this construct in the stable cell lines prohibited BPA analysis. We were thus unable to determine whether agonist decreased receptor endocytosis of the B2CB1, as it does for the B_1R (Fig. 4). However, the failure of BK to increase the number of accessible cell surface B2CB1 (Fig. 1B) suggests that the agonist does not delay the endocytosis of this receptor.

Inhibition of Receptor Endocytosis by Hyperosmotic Sucrose Treatment. Treatment of cells with hyperosmotic sucrose treatment has been shown to yield abnormal clathrin polymerization resulting in empty microcages in the membrane (Heuser and Anderson, 1989). Pretreatment of cells stably expressing B_1R with 0.4 M sucrose for 60 min led to a 31% increase in the number of available B_1R on the cell surface (Fig. 5A). Confocal microscopy of cells incubated with primary antibody during the last 30 min of the sucrose treatment showed that the increase in B_1R was due to inhibition of constitutive receptor endocytosis (Fig. 5B). On the other hand, treatment with 5 mM methyl-β-cyclodextrin, which depletes the cells of cholesterol and perturbs lipid rafts, had no apparent effect on B_1R endocytosis (data not shown). Hyperosmotic sucrose treatment had no effect on the number of cell surface B_2R (Fig. 5A) or their localization in the plasma membrane (Fig. 5B). However, sucrose completely inhibited the agonist-promoted B_2R endocytosis (Fig. 5B). Thus, both constitutive B_1R and agonist-promoted B_2R endocytosis proceed through a clathrin-dependent pathway.

Postendocytic Sorting and Stability of Receptors. We next examined the postendocytic sorting of the B_1R and B_2R. First, a modification of the immunofluorescence assay described above in Fig. 3B was used to monitor the postendocytic trafficking profiles of the internalized receptors. To monitor receptor recycling, receptors were first allowed to internalize in the presence of the M1 anti-FLAG antibody and in the presence or absence of agonist for 30 min as described in Fig. 3B. Because the M1 antibody is dependent

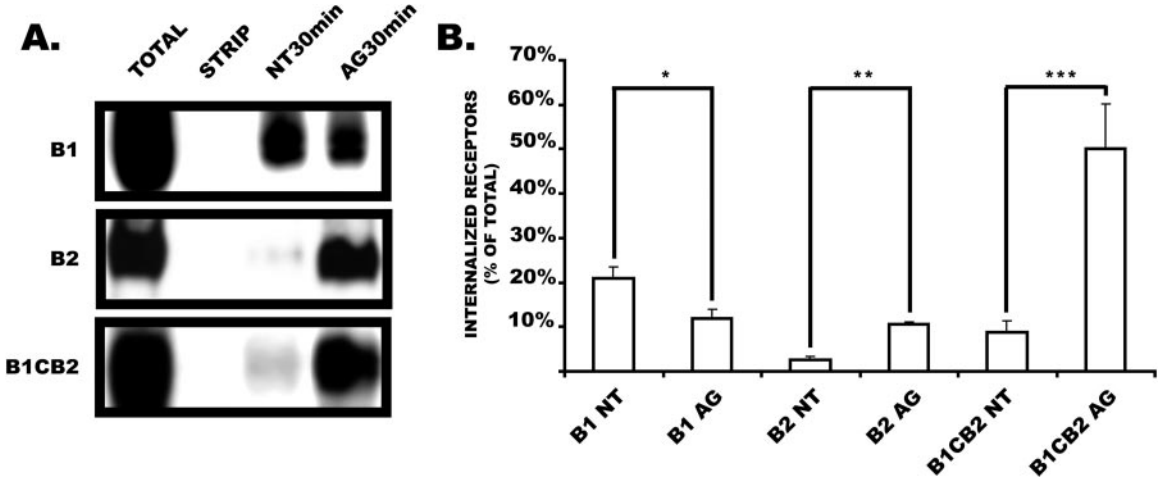

Fig. 4. Constitutive and kinin-dependent receptor endocytosis as determined by BPA. A, cells stably expressing B_2R, B_1R, and B1CB2 were first labeled at 4°C with a biotin reagent containing a reducible linker (TOTAL). The cells were then either stripped directly of remaining surface biotin (STRIP) or incubated in absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 30 min at 37°C as indicated and then stripped. The receptors were then immunoprecipitated as described for biotinylation protection assay under *Materials and Methods*. Blots were visualized by chemiluminescence in a Fluoromax Image Analyzer using Quantity One software (Bio-Rad Laboratories). B, densitometric analysis of the results in A. The results are shown as percentage of total where 100% total refers to the density of biotinylated cell surface receptors at 4°C in the absence of agonist (TOTAL). Data are presented as mean ± S.E. from three independent experiments. Comparison between the absence and presence of agonist: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

on calcium ions for binding to the FLAG epitope, any antibody remaining on the cell surface, either nonspecifically or receptor-bound, was then stripped by washing with EDTA, which also removes any residual agonist. As a result, only internalized receptors remain bound to the antibody. Any return of internalized receptor-antibody complexes to the plasma membrane can then be monitored as described previously by Finn and Whistler (2001) and Bartlett et al. (2005). According to this protocol, a protected pool of intracellular B_2R-antibody complexes was observed primarily only after agonist treatment (Fig. 6, compare B2NT STRIP with B2 AG STRIP), whereas protected B_1R-antibody complexes were observed both without and with agonist treatment (B1 NT STRIP, AG STRIP) confirming the significant constitutive endocytosis of B_1R. The pool of B_2R that was internalized by agonist rapidly recycled to the plasma membrane after further incubation in the absence of agonist for 30 min (Fig. 6, B2 AG STRIP NT). In contrast, the internalized pool of B_1R remained intracellular after further incubation in the absence of agonist (B1 AG STRIP NT).

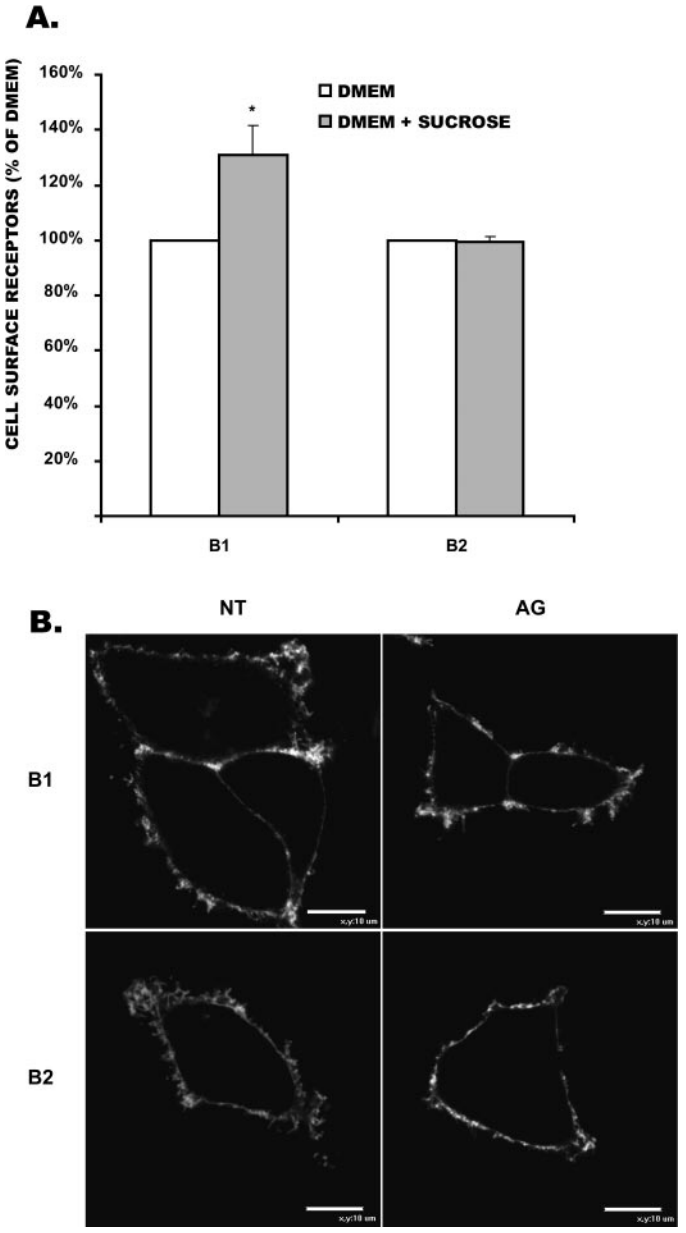

Fig. 5. Inhibition of receptor endocytosis by hyperosmotic sucrose treatment. A, HEK293 cells stably expressing B_1R or B_2R were treated without or with 0.4 M sucrose in DMEM for 60 min at 37°C and then assayed for specific [^{3}H]DAKD or [^{3}H]BK binding at 4°C as described under *Materials and Methods.* The results are shown as percentage of Basal where 100% Basal is the specific binding in the absence of sucrose treatment. Data are presented as mean ± S.E. of at least three independent experiments. Comparison with the absence of sucrose treatment: *, $p < 0.05$. B, cells stably expressing B_2R and B1R were treated with 0.4 M sucrose in DMEM for 60 min at 37°C. During the last 30 min of the 60-min incubation, the cells were also incubated with primary mouse M1 anti-FLAG antibody without (NT) and with 1 μM receptor-specific agonist (AG). Cells were then fixed, permeabilized, and stained with secondary ALEXA488-coupled anti-mouse antibody as described under *Materials and Methods.* In both A and B, images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. Bar, 10 μm.

To determine whether B_1R enters a degrading pathway, we first used the BPA, which selectively monitors the stability of internalized receptors. Clearly, the internalized B_1R were significantly degraded between the 30- and 120-min time points in both the absence (Fig. 7, NT) ($p < 0.05$) and presence of agonist (AG) ($p < 0.01$). The degrading phenotype of B_1R was further investigated by colocalization of receptors with LAMP 1 and LAMP 2, which are markers for the end-stage compartments of the degrading pathway. B_1R showed an overlap with LAMP 1 and LAMP 2 at 120 min both without (Fig. 8, *B1 NT*) (29%, $n \geq 25$ cells) and with agonist treatment (B1 AG) (25%, $n \geq 25$ cells) that was much higher than for B_2R without (B2 NT) (0.01%, $n \geq 25$ cells, $p < 0.001$) and with agonist (B2 AG) (0.66%, $n \geq 25$ cells, $p < 0.001$) at this time point. Together these data demonstrate that the B_2R is targeted to a recycling pathway, whereas the B_1R is targeted to a degrading pathway.

Because the C-terminal tails conferred the endocytic properties of the receptors, we next examined whether they were involved in postendocytic receptor sorting. The B1CB2 receptor recycled to the plasma membrane upon agonist removal (Fig. 6, B1CB2 AG STRIP NT) and exhibited limited overlap with LAMP 1 and 2 without (Fig. 8, B1CB2 NT) (0.14%, $n \geq 25$ cells) and with agonist (B1CB2 AG) (0.57%, $n \geq 25$ cells). Furthermore, a B_1R truncated of its C-terminal tail (B1Stop320) internalized constitutively (Fig. 6, B1STOP320 NT) but did not recycle after endocytosis (B1STOP320 NT STRIP AG). In addition, the B2CB1 receptor did not recycle back to the plasma membrane (Fig. 6, B2CB1 AG STRIP NT) and showed overlap with the lysosomal markers without (Fig. 8, B2CB1 NT) (13%, $n \geq 25$ cells, $p < 0.001$) and with agonist (B1CB2 AG) (20%, $n \geq 25$ cells, $p < 0.001$) that was significantly higher than both B_2R and B1CB2. Thus, the C-terminal tails of these receptors contain epitopes that participate in the postendocytic sorting of the receptors.

Interaction of Receptors with GASP. GASP is one candidate molecule responsible for preferentially targeting B_1R to the lysosomal degrading pathway as opposed to the plasma membrane recycling pathway. We have previously established that this protein specifically interacts with the intracellular C-terminal tail of some GPCRs targeted for destruction by lysosomal proteolysis (Whistler et al., 2002; Bartlett et al., 2005). Because the B_1R was degraded after its endocytosis whereas the B_2R was not, we examined whether GASP binds specifically to the former receptor.

A difference in GASP1 binding to B_1R and B_2R was first evaluated using in vitro affinity assays with in vitro-translated GASP1 and GST fusion proteins corresponding to the

C-terminal tails of B_2R and B_1R beyond the NP*X*X*Y* motif. GASP1 showed at least a 5-fold higher affinity for the B_1R tail than for the B_2R tail (Fig. 9A). Furthermore, the binding of GASP1 to the B_1R tail was directly dependent on the concentration of the tail in the in vitro affinity assays. The binding preference of GASP1 for B_1R was also evaluated in vivo in HEK293 cells stably expressing each receptor. Immunoprecipitation of receptors with M1 anti-FLAG antibodies revealed that endogenous GASP1 bound to the B_1R but not the B_2R (Fig. 9B). Thus, GASP1 exhibits a clear preference for B_1R over B_2R. Substituting the B_2R C-terminal tail in the B1CB2 receptor decreased the binding of GASP1 by 50% (Fig.

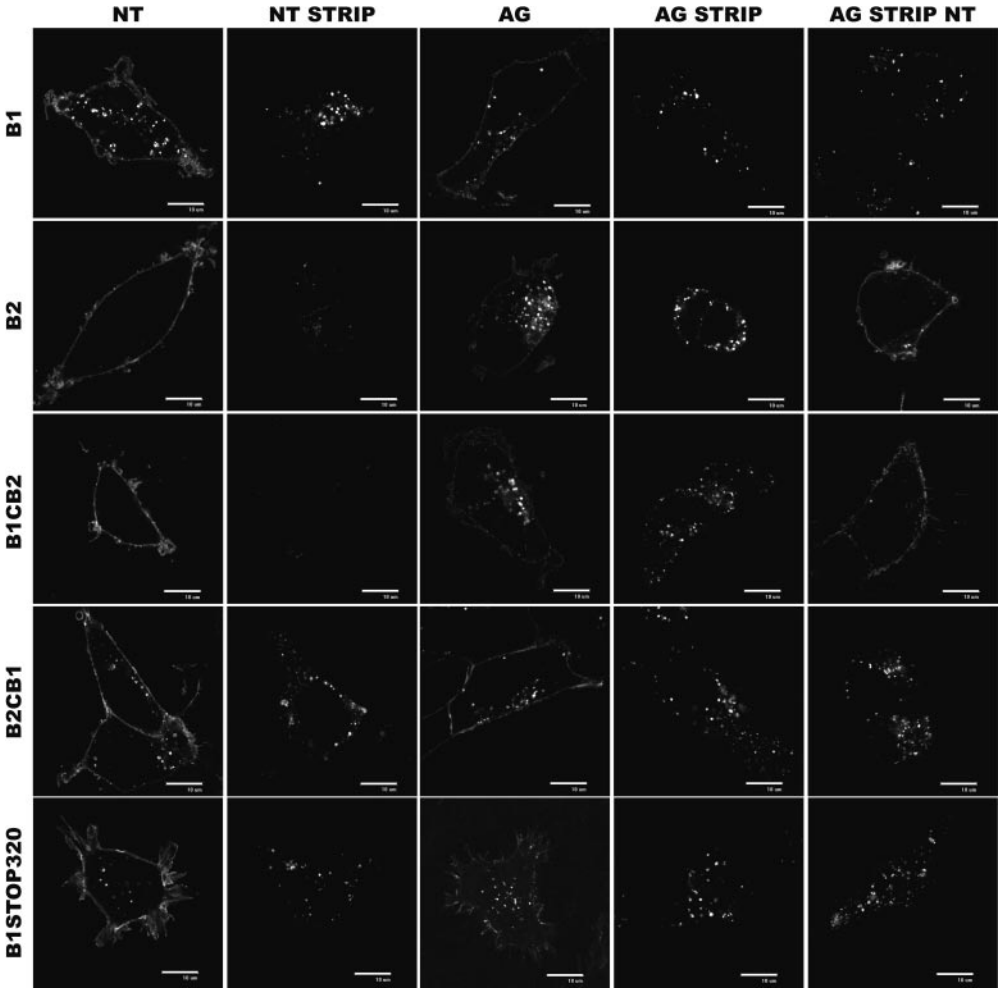

Fig. 6. Postendocytic sorting of receptors as determined by confocal microscopy. Cells stably expressing B_2R, B_1R, B1CB2, B2CB1, and B1Stop320 were incubated with primary mouse M1 anti-FLAG antibody for 30 min followed by further incubation in the absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 30 min at 37°C, upon which they were washed with EDTA-containing medium (NT STRIP, AG STRIP) to strip the surface of remaining antibody. The cells treated with agonist and stripped were then incubated in medium for 30 min (AG STRIP NT). Cells were then fixed, permeabilized, and stained with secondary ALEXA488-labeled anti-mouse antibody as described under *Materials and Methods.* Images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50 μm zoom. Bar, 10 μm.

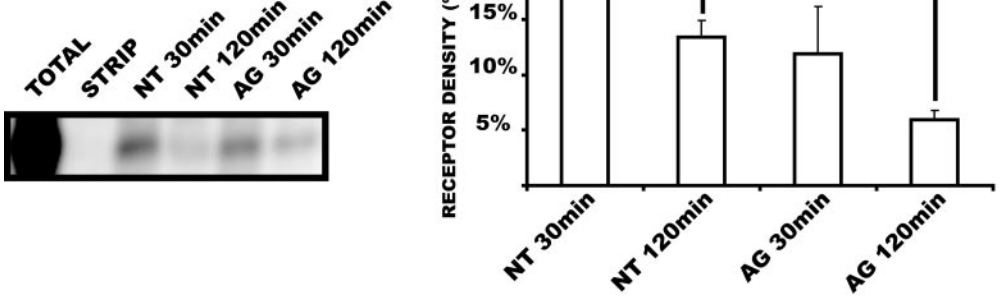

Fig. 7. Postendocytic degradation of intracellular B_1 receptors. A, cells stably expressing B_1R were first labeled at 4°C with a biotin reagent (TOTAL). The cells were then either stripped directly of remaining surface biotin (STRIP) or incubated in absence (NT) or presence of 1 μM DAKD (AG) for 30 and 120 min at 37°C as indicated as indicated and then stripped. The receptors were then immunoprecipitated as described for biotinylation protection assay under *Materials and Methods.* Blots were visualized by chemiluminescence in a Fluoromax Image Analyzer using Quantity One software (Bio-Rad Laboratories). B, densitometric analysis of the results in A. The results are shown as percentage of Total where 100% total refers to the density of biotinylated cell surface receptors at 4°C in the absence of agonist (TOTAL). Data are presented as mean ± S.E. from three independent experiments. Comparison between the absence and presence of agonist: *, $p < 0.05$; **, $p < 0.01$.

9B). Thus, GASP1 binding to B_1R involves the C-terminal tail of the receptor, but another epitope(s) also seems to be involved. Such multiepitopal binding seems to common for several GPCR effector proteins, including β-arrestins.

Effect of Kinins on Total Receptor Stability. The above results suggest that kinins increase available cell surface B_1R by delaying constitutive receptor endocytosis and consequently delay their degradation. In contrast, kinins acutely decrease available B_2R by promoting endocytosis, but they do not promote down-regulation of this receptor because they enter an endocytic recycling pathway. Thus, kinins would be expected to increase the stability of the total cell

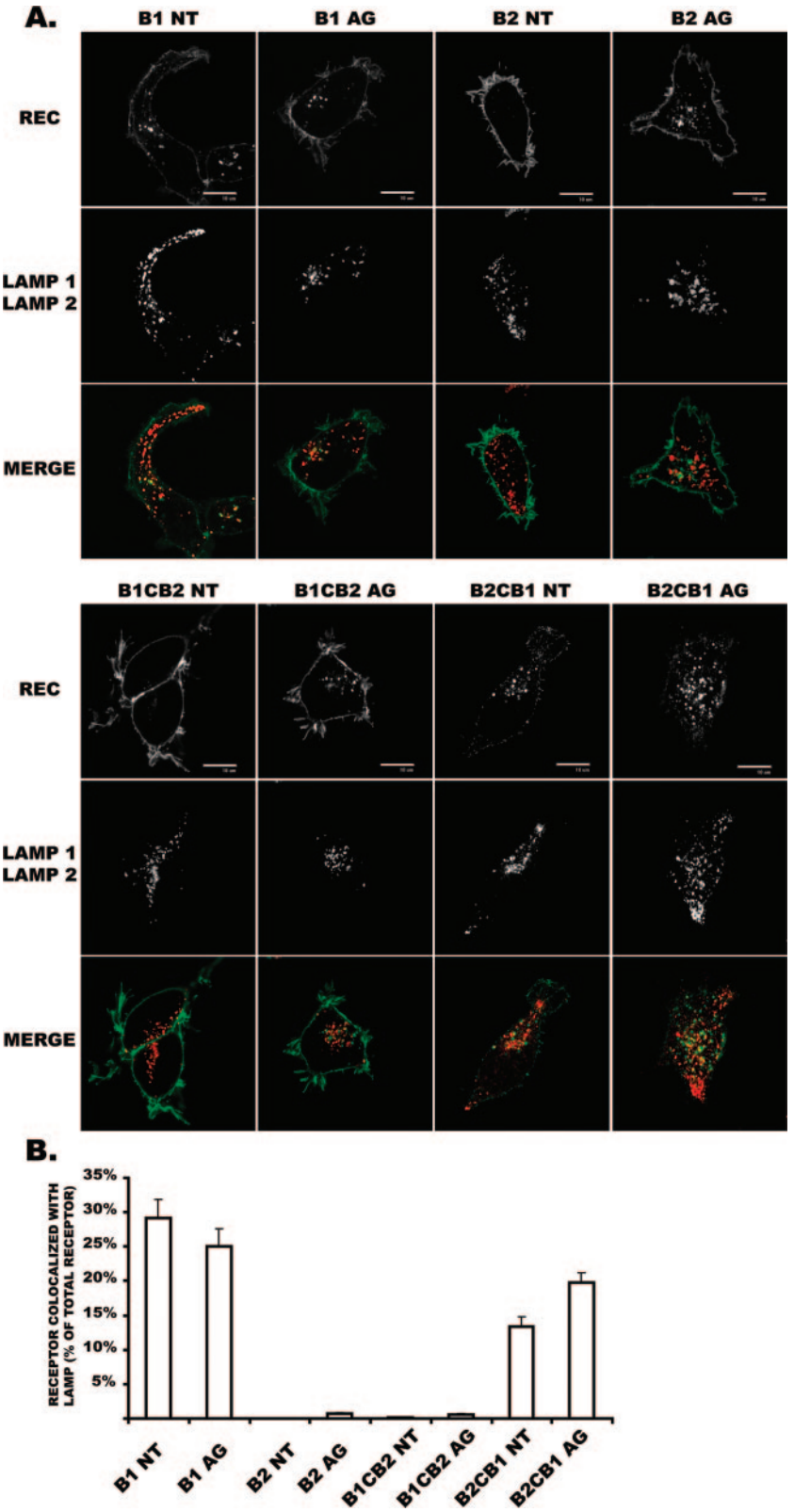

Fig. 8. Colocalization of receptors with lysosomal markers as determined by confocal microscopy. Cells stably expressing B_2R, B_1R, B2CB1, and B1CB2 were incubated with primary mouse M1 anti-FLAG antibody in absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 60 min at 37°C as indicated. Cells were then fixed, permeabilized, and stained with primary mouse anti-lamp 1 and anti-lamp 2 antibodies and then specifically stained with secondary ALEXA568-labeled anti-mouse antibody for LAMP 1 and LAMP 2 (LAMP 1 LAMP 2) and secondary ALEXA488-labeled anti-mouse antibody for receptors (REC). Images collected using a Nikon Eclipse confocal microscope, 60x objective, 50 μm zoom. Bar, 10 μm. B, quantification of colocalized receptors with LAMP 1 and 2 (MERGE). The results are shown as percentage of total receptors. Data are presented as mean ± S.E. from at least 25 different cells.

surface pool of B_1R. To examine this hypothesis, a biotin degradation assay was used, which monitors the stability of the total pool of mature cell surface receptors. At 120 min, 29 ± 4% of the B_1R originating on the plasma membrane remained intact (Fig. 10, NT120min), whereas the presence of agonist significantly increased this amount to 56 ± 14% at this time point ($p = 0.03$) (AG120min). B1CB2 was more stable than B_1R at 120 min, and agonist did not have any significant effect on the B1CB2 receptor stability. The stability of B2CB1 at 120 min was similar to B_1R but agonist did not have any effect on the B2CB1 receptor stability, which is consistent with the failure of the B_1R C-terminal tail to transplant the agonist delay of constitutive endocytosis to B_2R. Agonist was unable to delay the degradation of B1Stop320. On the other hand, truncation of the B_1R C-terminal tail led to a significant decrease in the degradation of the receptor. Thus, the B_1R tail contains an epitope that participates in targeting the receptor for degradation but additional epitopes are likely to be involved as well.

Discussion

Here, we used a HEK293 cell model to elucidate the trafficking mechanism underlying the relatively rapid kinin-promoted decrease of B_2R and increase of B_1R on the cell surface seen in native cells. In our model, B_2R are relatively stable cell surface receptor components in the absence of agonist but translocate rapidly and in a clathrin-dependent manner to intracellular endocytic vesicles in response to agonist stimulation with the cognate agonist ligand BK and recycle back to the plasma membrane. In contrast, B_1R are subject to constitutive endocytosis, also via a clathrin-dependent pathway, and target lysosomes for degradation. Binding of the cognate B_1R agonist DAKD inhibits constitutive B_1R endocytosis thereby decreasing the rate of spontaneous B_1R clearance from the cell surface and delaying degradation. This regulation is also consistent with the agonist-dependent and constitutive activities of these receptors and the pattern of inflammatory kinin signaling during sustained pathological insult in vivo, which adapts from a B_2-type to a B_1-type during the course of the insult. Furthermore, delaying spontaneous endocytosis to temporarily increase the steady-state level of receptors on the cell surface presents a novel mechanism by which some agonists may act to promote GPCR signaling.

B_2R is expressed almost exclusively in the plasma membrane, whereas B_1R is retained to a large degree intracellularly. The tubular shape of the receptor-retaining intracellular structures suggests a membrane-bound localization of the receptor rather than a diffuse cytosolic localization, which presumably reflects the biosynthetic pathway. PNGase treat-

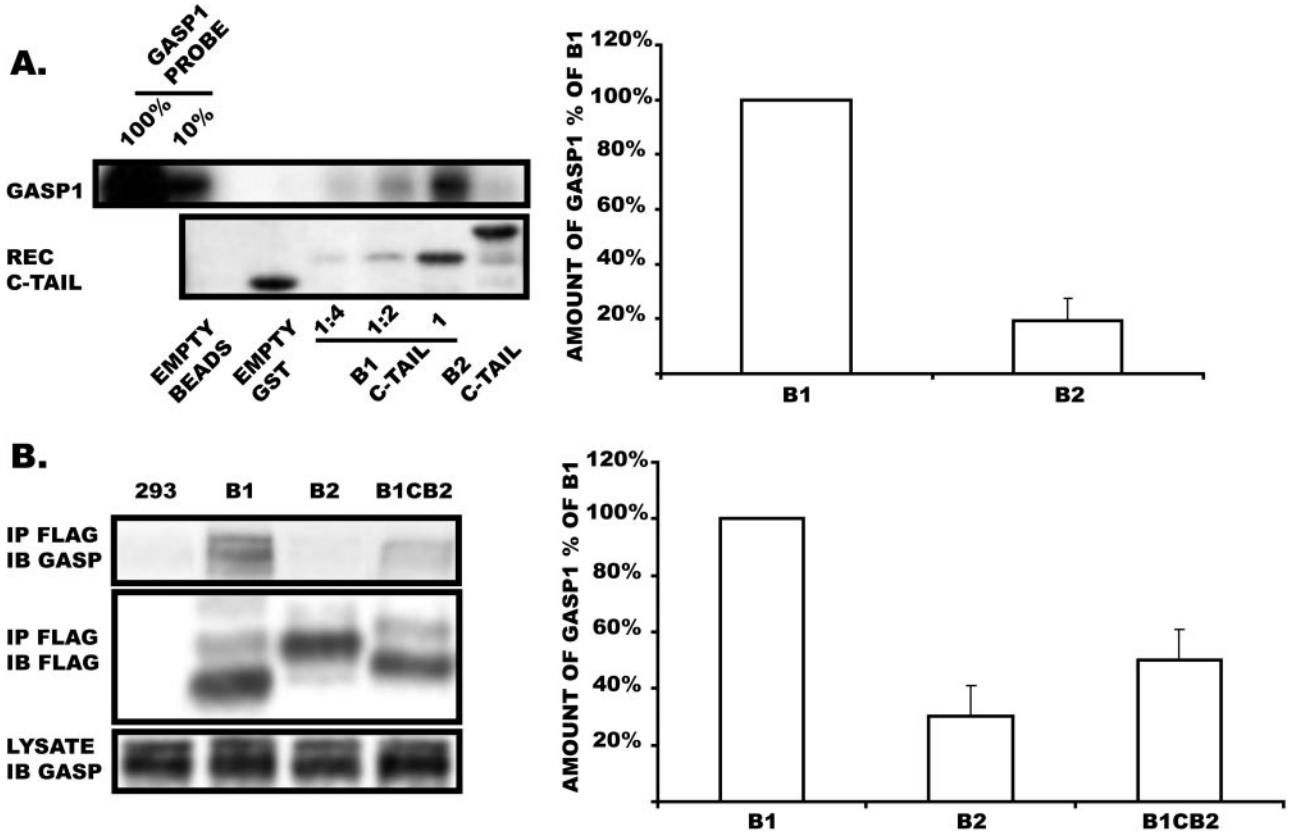

Fig. 9. Binding of GASP to receptors. A, in vitro-translated and radioactively labeled GASP1 was incubated with GST-fusion constructs of the B_1R and B_2R C-terminal tails as described under *Materials and Methods.* The receptor tails were quantified by Coomassie staining and GASP1 by immunoblotting. The relative amounts of pulled-down GASP1 by the receptor tails were quantified by densitometric analysis. B, cells stably expressing B_1R, B_2R, and B1CB2 were immunoprecipitated with M2 anti-FLAG affinity resin as described under *Materials and Methods.* Nontransfected HEK293 were used as negative control. The receptors and GASP1 were quantified by immunoblotting. The relative amounts of pulled-down GASP1 by the receptors were quantified by densitometric analysis. In *A* and *B*, the results are presented as percentage of B1 where 100% B1 refers to the amount of GASP1 pulled down with the B_1R. In B, the results were normalized to the amount of GASP1 in the lysate. Data are presented as mean ± S.E. from three independent experiments.

ment of biotinylated cell surface B_1R led to the conversion of higher molecular mass receptor species to a relatively homogeneous species of approximately 35 kDa consistent with the idea that the cell surface receptor is glycosylated as suggested previously (Fortin et al., 2006). The difference in distribution of B_1R and B_2R was apparently determined entirely by the C-terminal tail. Thus, the B_2R C-terminal tail may be equipped with a plasma membrane targeting motif or the B_1R tail with an intracellular retention motif. Whether or not the intracellularly retained B_1R species can be recruited to the plasma membrane is unclear. However, considering the rapid spontaneous endocytosis of the receptor once it reaches the cell surface and the postendocytic targeting of the receptor for destruction described here, the retained receptor pool could possibly be viewed as a deposit to ensure that a steady flow of receptors reach maturation once expression of the receptor has been induced by a pro-inflammatory stimuli.

The rapid agonist-promoted recruitment of β-arrestin by B_2R, clathrin-dependent endocytosis, and the ability of this receptor to recycle after agonist-promoted endocytosis are consistent with previous studies. Indeed, agonist exposure of the B_2R leads, within minutes, to internalization of the receptor protein, which has been determined by immunoelectron microscopy (Haasemann et al., 1998), fluorescence microscopy (Bachvarov et al., 2001), the appearance of acid-resistant radiolabeled agonist binding (Munoz and Leeb-Lundberg, 1992), and the reduction in the number of cell surface binding sites, which rapidly reappear on the cell surface upon agonist removal (Munoz et al., 1993). The fact that the internalized agonist reaches a steady-state level over time that is higher than can be explained by the total number of cell surface binding sites (Lamb et al., 2001) further supports our finding that the internalized B_2R recycles to the plasma membrane.

Unlike the B_2R, the plasma membrane B_1R is unstable in that it is subject to high spontaneous endocytosis and degradation. This observation is consistent with the inducible expression and high agonist-independent activity reported for this receptor (Leeb-Lundberg et al., 2001). In other words, the functional properties of this receptor necessitate a tight regulation at every level from transcription, through maturation and signaling, and ending in lysosomal targeting and destruction. Our results are also consistent with the rapid and spontaneous clearance of the B_1R response in rabbit aortic rings (Fortin et al., 2003). On the other hand, our observation apparently contradicts the results from the same group using a C-terminally GFP-tagged B_1R, which showed neither spontaneous nor agonist-promoted internalization, even though the receptor was reported to relocate on the plasma membrane in response to agonist (Sabourin et al., 2002). It is important to realize that the C-terminal tail of the receptor has been shown to be crucial for the trafficking

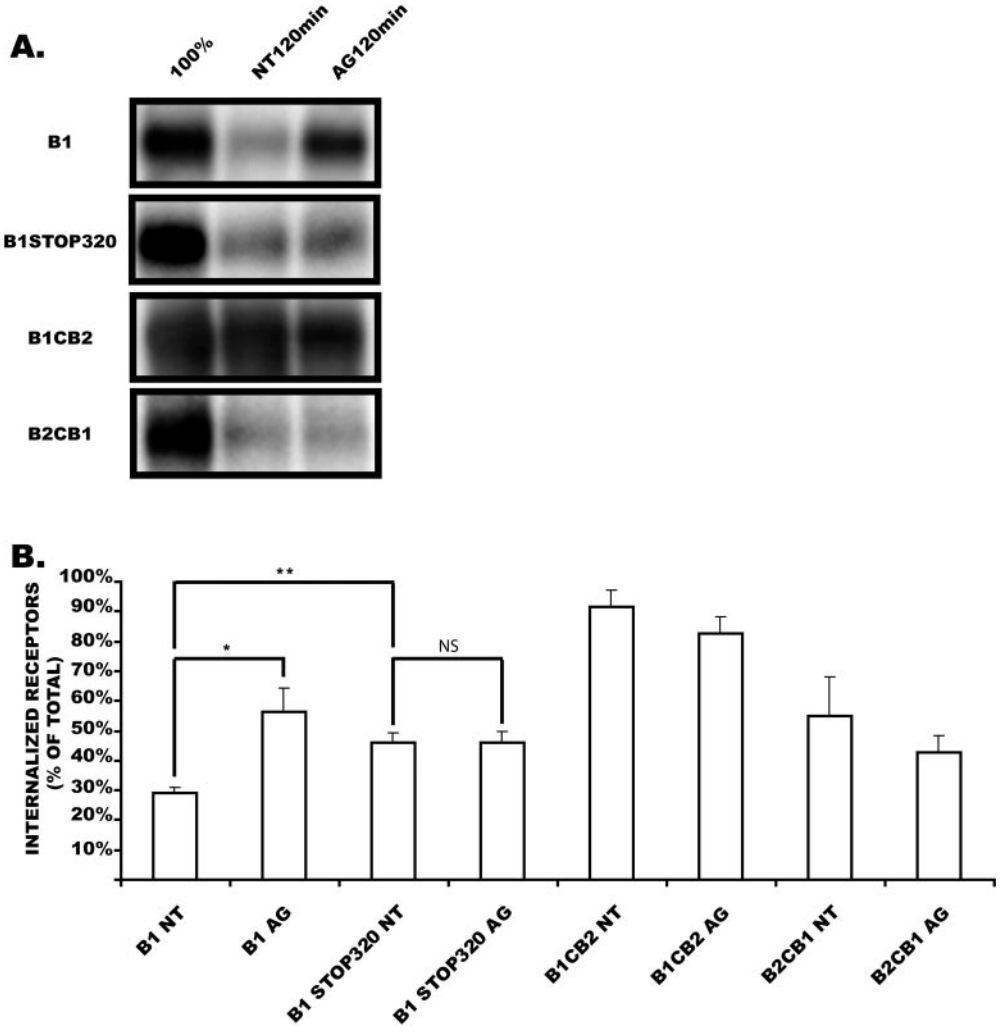

Fig. 10. Postendocytic degradation of total cell surface receptors. A, cells stably expressing B_1R, B1stop320, B1CB2, or B2CB1 were first labeled at 4°C with a biotin reagent containing a reducible linker (*TOTAL*). The cells were then incubated in absence (NT) or presence of 1 μM receptor-specific agonist (AG) for 120 min at 37°C as indicated. The receptors were then immunoprecipitated as described for biotinylation degradation assay under *Materials and Methods*. Blots were visualized by chemiluminescence in a Fluoromax Image Analyzer using Quantity One software (Bio-Rad Laboratories). B, densitometric analysis of the results in A. The results are shown as percentage of total, where 100% total refers to the density of biotinylated cell surface receptors at 4°C in the absence of agonist (TOTAL). Data are presented as mean ± S.E. from three independent experiments. Comparison between the absence and presence of agonist: *, $p < 0.05$; NS, not significant.

properties of a number of GPCR (von Zastrow, 2003; Heydorn et al., 2004; Terrillon et al., 2004), which may be particularly apparent with spontaneous endocytosis (Waldhoer et al., 2003).

Previous studies have shown that the kinetics and amount of agonist internalization of the B_1R, as determined by the appearance of acid-resistant DAKD binding, is very slow and low, which is opposite to the B_2R, through which acid-resistant BK binding occurs very rapidly (Faussner et al., 1998; Lamb et al., 2001). This, at first, would seem to support a model of a noninternalizing receptor. However, as we present here, agonist binding to the B_1R in fact retards the rapid spontaneous internalization of this receptor, thereby temporarily stabilizing the receptor on the cell surface. The sucrose sensitivity of both constitutive and agonist-dependent B_1R endocytosis suggests a common endocytic pathway under the two conditions but that the agonist promotes a receptor conformation that has lower affinity for the endocytic machinery. Thus, the combined effect of spontaneous surface clearance of the B_1R and the retardation of the clearance by the agonist may then be viewed as a coincidence detector that would only allow optimal receptor signal transduction at the precise right moment.

The B_2R phenotype of agonist-dependent β-arrestin 2 recruitment and sucrose-sensitive internalization support an internalization pathway well in line with a majority of the GPCR studied to date. On the other hand, the B_1R still lacks an identified adaptor protein that links it to the internalization machinery. Several receptors, such as GluR1 (Dale et al., 2001) and PAR-1 (Paing et al., 2002) have been reported to internalize by a clathrin-dependent but β-arrestin-independent mechanism. The mechanisms underlying B_1R internalization, therefore, need further investigation for us to understand the regulation of receptor level and hence tissue responsiveness to receptor agonists.

Because most of the trafficking phenotypes of B_2R and B_1R were transplantable by C-terminal tail exchange, this domain must be important for the unique trafficking protein interactions. Substitution of the B_1R tail in B_2R yielded a loss of β-arrestin 2 binding, which may be explained by the apparent absence of phosphorylation of this tail (Blaukat et al., 1999), in contrast to the B_2R tail, which is phosphorylated (Blaukat et al., 2001). The importance of the B_2R C-terminal tail for agonist-promoted endocytosis was confirmed when substituted in B_1R. In contrast to B_2R, the B_1R tail conferred constitutive endocytosis to B_2R, a process that must then be β-arrestin 2-independent. The B_1R tail was unable to transplant the agonist delay in constitutive endocytosis to the B_2R. However, B_1R tail truncation also lost the agonist delay suggesting that this delay in endocytosis involves a fine interplay between the C-terminal tail and other domains in the receptor.

The receptor tails are also important for the postendocytic choices of the receptors. Lysosomal B_1R targeting may depend in part on GASP binding, which showed higher affinity for the B_1R tail than the B_2R tail both in vitro and in vivo. These results are consistent with the fact that substitution of the B_1R tail in B_2R prevented the receptor from recycling and promoted degradation. Furthermore, truncation of the B_1R tail decreased the rate of receptor degradation but did not abolish it, indicating that additional domains are involved. Therefore, the B_2R tail either contains an epitope important for recycling or is able to mask an epitope in B1R that is important for degradation.

The combined events of kinin-promoted rapid B_2R desensitization and internalization, together with the recruiting effect of kinins and their limited desensitizing potential on the induced B_1R, provide a mechanism for the inverse kinin regulation of these receptors observed in cultured cells and agree well with the prevailing paradigm of an acute inflammatory B_2-type response that over time shifts toward a chronic B_1-type response. Chronic phase B_1R signaling has attracted a lot of attention as a drug target for chronic pain management. Compounds have been, and are being, screened for their ability to block the ligand-independent signal transduction of this receptor, so called inverse agonists, but no candidate drugs are of yet available. Our data in the present study favor an increased rate of B_1R clearance as a means to reduce B_1R activity. Hence, we suggest screening for endocytic agonist properties rather than signal transduction inverse agonist properties as a novel approach to evaluate "antagonism" at B_1R.

Acknowledgments

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