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Agonist-Induced Translocation of the Kinin B_1 Receptor to Caveolae-Related Rafts

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ABSTRACT

The kallikrein-kinin system, activated during inflammatory conditions and the regulation of specific cardiovascular and renal functions, includes two G protein-coupled receptors for bradykinin (BK)-related peptides. The B_1 receptor (B_1R) subtype is not believed to undergo agonist-induced phosphorylation and endocytosis. A conjugate made of the rabbit B_1R fused with the yellow variant of green fluorescent protein (YFP) was expressed in mammalian cells. In COS-1 or human embryonic kidney (HEK) 293 cells, the construction exhibited a nanomolar affinity for the agonist radioligand [^{3}H]Lys-des-Arg^9-BK or the antagonist ligand [^{3}H]Lys-[Leu^8]des-Arg^9-BK and a pharmacological profile virtually identical to that of wild-type B_1R. Lys-des-Arg^9-BK stimulation of HEK 293 cells stably expressing B_1R-YFP but not stimulation of untransfected cells released [^{3}H]arachidonate in a phospholipase A_2 assay. B_1R-YFP was visualized as a continuous labeling of the plasma membranes in stably transfected HEK 293 cells (confocal microscopy). Addition of Lys-des-Arg^9-BK (1–100 nM) rapidly concentrated the receptor-associated fluorescence into multiple aggregates that remained associated with the plasma membrane (no significant internalization) and colocalized with caveolin-1. This reaction was slowly reversible upon agonist washing at 37°C and prevented pretreatment with a B_1R antagonist. β-Cyclodextrin treatment, which extracts cholesterol from membranes and disrupts caveolae-related rafts, prevented agonist-induced redistribution of B_1R-YFP but not the PLA_2 activation mediated by this receptor. The agonist radioligand copurified with caveolin-1 to a greater extent than the tritiated antagonist in buoyant fractions of HEK 293 cells treated with the ligands. Agonist-induced cellular translocation of the kinin B_1R to caveolae-related rafts without endocytosis is a novel variation on the theme of G protein-coupled receptor adaptation.

The kallikrein-kinin system includes two homolog G protein coupled receptors (GPCRs), the widely distributed B_2 receptor (B_2R), and the strongly regulated B_1 receptor (B_1R) (Marceau et al., 1998). Several findings support B_1R importance in late inflammatory events: it is selectively stimulated by a class of abundant kinin metabolites, Lys-des-Arg^9-BK or des-Arg^9-BK but not efficiently by the native kinins Lys-BK or BK. The B_1R is inducible after some types of tissue injury. The regulation of the two receptor subtypes differs at the protein level: the B_1R is not importantly internalized after agonist stimulation, relative to the B_2R (Faussner et al., 1998; Zhou et al., 2000). Accordingly, the B_1R fails to undergo ligand-induced phosphorylation, whereas the B_2R is phosphorylated in comparative experiments based on Sf9 cells (Blaukat et al., 1999). The B_1R is more resistant to functional desensitization than the B_2R in cell types that coexpress both receptor subtypes (reviewed by Marceau and Bachvarov, 1998). Another recently documented difference between the two kinin receptor subtypes is the higher agonist-independent basal signaling conferred to cells transfected with the B_1R, when corrected for receptor density (Leeb-Lundberg et al., 2001). This observation may suggest that gene transcription is sufficient for the B_1R function, and that signaling is perhaps independent of the agonist stimulation. On the other hand, the currently available peptide B_1R antagonists did not exhibit significant inverse agonist activity (Leeb-Lundberg et al., 2001). This finding suggests, rather, that the endogenous B_1R agonist(s) are present and important in pathological models where such neutral antagonists (e.g., [Leu^8]des-Arg^9-BK) exert antiinflammatory, antishock, and analgesic effects (Cruwys et al., 1994; McLean et al., 1999; Bélichard et al., 2000). Analytical biochemistry supports the existence of pharmacologically relevant concentration of des-Arg^9-kinins in inflammatory models (Blais et al., 2000).

We have recently reported the construction and properties of a rabbit B_2R-green fluorescent protein (GFP) conjugate allowing to study ligand-induced cellular endocytosis, recycling and down-regulation (Houle et al., 2000; Bachvarov et al., 2001). We report here a similar construction based on a the rabbit B_1R fusion protein. Our primary aim was to verify

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ABBREVIATIONS: GPCR, G protein coupled receptor; B_1R, B_1 receptor; B_2R, B_2 receptor; BK, bradykinin; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2; BSA, bovine serum albumin; WT, wild-type.

whether agonist stimulation of the B_1R would promote endocytosis or another form of subcellular redistribution of the ligand-receptor complex.

Materials and Methods

Construction and Expression of the Rabbit B_1 Receptor-Yellow Fluorescent Conjugate. Using genomic rabbit liver DNA as a template, the entire intronless coding region of the B_1R gene (excluding the stop codon) was amplified by polymerase chain reaction. 5′-ATAAAAGCTTATGGCCTCACAGGGCCCCCTG-3′ and 5′-ATAAGGATCCGCATTCCGCCAGAAACCCCAGAGC-3′ were used as sense and antisense polymerase chain reaction primers, respectively. These primers, derived from the rabbit receptor sequence published by MacNeil et al. (1995), contain additional *Hin*dIII and *Bam*HI sites (underlined), respectively, needed for the directional cloning of the rabbit B_1R coding region in the eukaryotic expression vector pEYFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA), encoding a variant of GFP. Both the polymerase chain reaction fragment and the pEYFP-N1 vector were digested with *Hin*dIII and *Bam*HI and ligated at 12°C overnight. The resultant vector (B_1R-YFP) contained the rabbit B_1R coding sequence fused in frame at its carboxyl terminus with the YFP. The directional cloning of the rabbit wild-type (WT) B_1R coding region in the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA) has been described elsewhere (Larrivée et al., 2000).

Cell Transfection and Binding Assay to the Recombinant Rabbit B_1 Receptors. A binding assay to WT or variant rabbit B_1Rs expressed in intact cells was conducted as described previously (Larrivée et al., 2000). Briefly, the ligands were the agonist [^{3}H]Lys-des-Arg^9-BK (80–105 Ci/mmol; [^{3}H]des-Arg^{10}-kallidin; PerkinElmer Life Sciences, Boston, MA) or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (90 Ci/mmol; [^{3}H][Leu^9]des-Arg^{10}-kallidin; PerkinElmer). COS-1 cells were seeded at a high density in 24-well plates (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics; Invitrogen, Carlsbad, CA). After 24 h, the cells (70 to 80% confluent) were transiently transfected with the expression vectors described above using the Ex-Gen 500 transfection reagent (MBI Fermentas Inc., Flamborough, Canada) as directed by the manufacturer. Some untransfected or mock-transfected (pEYFP-N1 vector coding for YFP) cells were used in control experiments. After an additional 48-h culture period, cells were used for the binding assay. The B_1R-YFP vector was also transfected in HEK 293 cells using the same procedure, and stable transfectants were selected after growing the cells for 1 month in α-minimal essential medium supplemented with fetal bovine serum (5%), horse serum (5%), penicillin-streptomycin (1%), and geneticin (500 μg/ml; Invitrogen). These cells, grown until confluence in 24-well plates, were also used for radioligand binding and other assays.

In all binding assays, the cells were washed twice with the binding medium [consisting of Medium 199 supplemented with 0.1% BSA, 1 μM amastatin, 1 μM captopril, 1 μM phosphoramidon (Sigma) and sodium azide 0.02%, w/v] and filled with 0.5 ml of prewarmed (37°C) binding medium. The B_1 receptor ligands (0.125–4 nM) and cold competing peptides (1 μM concentrations of the agonist Lys-des-Arg^9-BK or the antagonist Lys-[Leu^8]des-Arg^9-BK, used for the agonist or antagonist ligand, respectively, for the determination of nonspecific binding) were added to the wells. Separate protocols dealt with the effect of cell coincubation with a panel of cold BK-related peptides to establish the pharmacological profile of the B_1R-YFP construction using agonist radioligand competition. After 60 min of incubation at 37°C, each well was washed three times with 2 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4. One milliliter of 0.1 N NaOH was finally added to dissolve the cells. Radioactivity in the resulting suspension was determined by scintillation counting (5 min per vial). The parameters of the Scatchard plots were calculated using a computer program (Tallarida and Murray, 1987). The kinetics of association (at 0 or 37°C) and of dissociation (either at 0 or 37°C) after association at 37°C was determined for both the agonist and antagonist radioligands using the binding medium formulation described above with the omission of NaN_3.

Phospholipase A_2 Assays. An arachidonic acid release assay was performed to evaluate the function of B_1R-GFP stably expressed in HEK 293 cells. Cells (2.5×10^5) were seeded in 2-cm^2 wells (24-well plates) containing 1 ml of the complete culture medium (see above). Twenty-four hours later, when the cells were 50 to 60% confluent, 0.1 μCi of [^{3}H]arachidonic acid (specific activity, 185 Ci/mmol; PerkinElmer) was added to each well. The cells were further incubated for 18 h, then washed three times with Earle's balanced salt solution containing 2 mg/ml of BSA. One milliliter of this medium was left in each well. A B_1R antagonist was optionally added to the appropriate wells and the agonist Lys-des-Arg^9-BK or vehicle was added 30 min later. The plates were further incubated at 37°C for 30 min, at which point 500 μl of the medium from each well was recovered in 1.5-ml conical tubes and centrifuged for 5 min at 15,000*g*. Supernatants (400 μl) were transferred in vials for scintillation counting of the released arachidonate. A variant of the assay

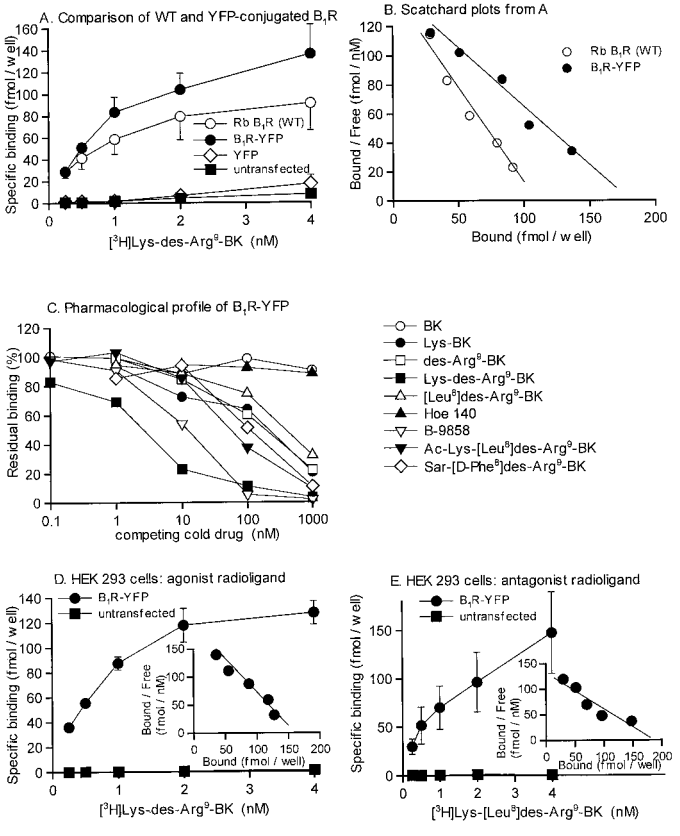

Fig. 1. Specific binding of the agonist B_1R radioligand [^{3}H]Lys-des-Arg^9-BK (A-D) or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (E) to cells expressing various recombinant proteins. A, COS-1 cells were transiently transfected with vectors coding for rabbit wild-type B_1R [Rb B_1R (WT)], its conjugate with YFP (B_1R-YFP), YFP alone (mock transfection) or were not transfected. B, Scatchard plot analysis of the averaged binding data of A involving the two receptor proteins. C, pharmacological profile of B_1R-YFP established using competition of the binding of [^{3}H]Lys-des-Arg^9-BK (1 nM) by a panel of cold peptides in COS-1 cells transiently transfected with the B_1R-YFP coding vector. D, binding of the agonist radioligand to untransfected HEK 293 cells or to cells stably expressing B_1R-YFP. E, binding of the antagonist radioligand to untransfected HEK 293 cells or to cells stably expressing B_1R-YFP. In D and E, the insets represent the Scatchard plots derived from the averaged data based on transfected cells. In A, C, D, and E, values are the means ± S.E.M. of three separate experiments with duplicate observations (except for the wild-type B_1R in A, where $n = 4$). The error bars have been omitted in C for the sake of clarity.

was performed to evaluate the effect of β-cyclodextrin on the function of B_1R-YFP stably expressed in HEK 293 cells; 6.0×10^5 cells were seeded in 5-cm^2 wells (12-well plates) containing 1 ml of the complete culture medium. To wells containing subconfluent cells, 0.1 μCi of [^{3}H]arachidonic acid was added, the cells were further incubated for 18 h and washed with Earle's balanced salt solution/BSA, as described above. Ten min later, β-cyclodextrin (10 mM) was added in the appropriate wells, which were further incubated at 37°C for 50 min (this treatment depletes ~50% of membrane cholesterol; Parpal et al., 2001). At this point, Lys-des-Arg^9-BK (10 nM) was added in the appropriate wells. Thirty minutes later, 500 μl of the medium was recovered and processed as described above for the determination of arachidonate release.

Effect of an Agonist Treatment on the Subcellular Distribution of B_1R-YFP. The agonist Lys-des-Arg^9-BK, alone or combined with other drugs, was added to the culture medium of HEK 293 cells stably expressing B_1R-YFP, and the subcellular fluorescence distribution generally observed without fixation or drug washout (unless otherwise indicated) using a BioRad 1024 confocal microscope as a function of treatment duration (60× objective with oil immersion; emission, 488 nm; detection above 510 nm). Colocalization experiments were based on the same type of cells stimulated or not with the agonist, and then washed (PBS), fixed (paraformaldehyde 1% for 20 min, followed by washing and neutralization with 0.1 M glycine in PBS for 10 min), and permeabilized (0.5% Triton X-100 in PBS for 5 min, followed by washing). The cells were washed again with 0.1% BSA in PBS, incubated with SuperBlock Blocking buffer in PBS (BioLynx) for 45 min, stained with the primary antibody (anti-caveolin-1 monoclonal, clone 2234, dilution 1/100, 90-min incubation; Transduction Laboratories). This staining was revealed using goat anti-mouse IgG labeled with Alexa Fluor 594 (dilution 1/1000; red fluorescence detected above 585 nm when excited at 568 nm; Molecular Probes, Eugene, OR). After ample washing, cells were observed using the confocal microscope.

Cell Fractionation. To analyze radioligand and receptor redistribution to buoyant cell fractions, HEK 293 cells stably expressing B_1R-YFP (five 75-cm^2 flasks in each experimental group) were stimulated for 30 min with either the agonist [^{3}H]Lys-des-Arg^9-BK or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (1 nM each), supplemented or not by an excess of cold peptide (1 μM), at 37°C in the binding assay described above but without sodium azide. Cell fractionation was performed entirely at 4°C. The medium was removed, the cells were washed twice with cold PBS pH 7.5, lyzed and scraped with Na_2CO_3 500 mM, pH 11, containing the protease inhibitor cocktail Complete Mini (Roche Molecular Biochemicals, Indianapolis, IN) used as directed (0.4 ml of buffer per flask, total of 2 ml). The cellular material recovered from scraping was homogenized (150 strokes in a glass-glass pestle; Ishizaka et al., 1998) and sonicated (6 × 15 s). The rest of the separation was adapted from Smart et al. (1995) with some modifications. Briefly, the suspension was mixed with 0.164 ml of solution A (0.25 M sucrose, 1 mM EDTA, 20 mM Tris, pH 7.8) and 1.84 ml of 50% OptiPrep (Invitrogen) diluted in solution B (0.25 M sucrose, 6 mM EDTA, 120 mM Tris, pH 7.8). The mixture is placed at the bottom of a tube later filled with a discontinuous linear gradient of OptiPrep (20 to 10% in buffer A). This tube is centrifuged for 90 min at 52,000*g*. At the end, 12 1-ml fractions are collected and the radioactivity is determined in 10 μl of each. The top five fractions are mixed again with 50% OptiPrep and placed at the bottom of another tube. OptiPrep (5%) in buffer A (2 ml) is layered over the mixture (10 ml) and the final centrifugation (52,000*g*, 90 min) is performed. One-milliliter fractions are collected from the top down for the determination of radioactivity (scintillation counting of one half of each fraction) and the caveolin-1 content (immunoblot). To determine caveolin-1, 10 μl of each fraction was run on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (immunoblot technique as in Bachvarov et al., 2001). The blots were revealed with a primary anti-caveolin-1 antibody (polyclonal, dilution 1/750; Santa-Cruz Biotechnologies, Santa Cruz, CA) and a secondary antibody (horseradish peroxidase-conjugated, preadsorbed goat anti-rabbit IgG, dilution 1/16,000; Santa Cruz Biotechnologies).

Results

[^{3}H]Lys-des-Arg^9-BK Binding to Rabbit B_1R Fluorescent Conjugates. COS-1 cells transiently transfected with a YFP coding vector (sham transfection) or untransfected cells bound very little [^{3}H]Lys-des-Arg^9-BK, whereas cells that expressed either wild-type (WT) rabbit B_1R or its fluorescent conjugate B_1R-YFP exhibited specific and saturable binding (Fig. 1A). The affinity estimates derived from Scatchard plot

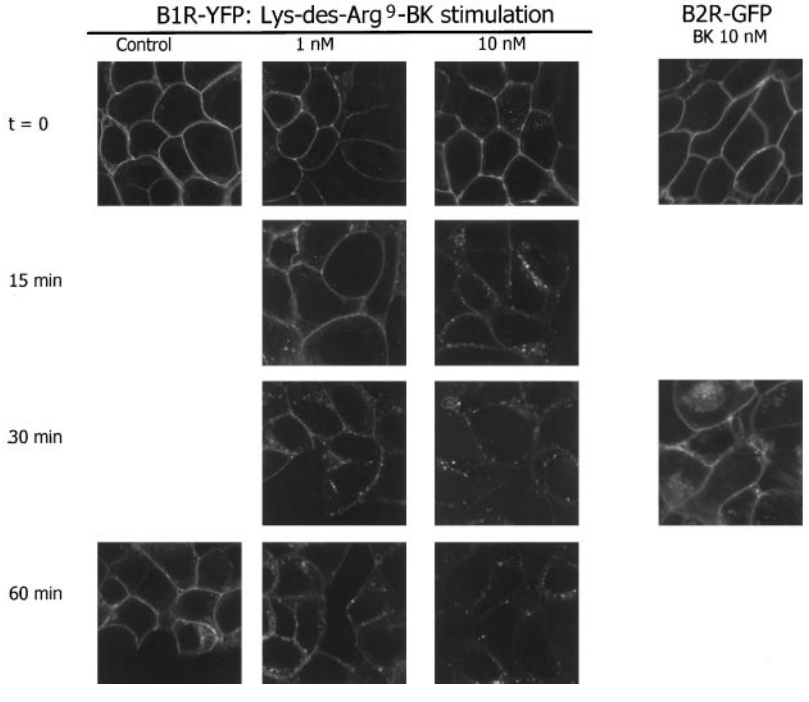

Fig. 2. Subcellular localization of the B_1R-YFP fusion protein in stably transfected HEK 293 cells maintained in the complete culture medium and treated with cycloheximide (71 μM), the indicated concentration of the agonist Lys-des-Arg^9-BK for definite time periods (magnification, 1800×). The selected confocal planes are halfway to the thickness of most cells. Results were verified during at least 2 separate days of experiments in multiple microscopic fields. For comparison, BK-induced endocytosis is illustrated in other HEK 293 cells stably expressing B_2R-GFP.

analysis (Fig. 1B) were close to each other (K_D = 0.73 and 1.26 nM, respectively; 95% confidence limits 0.55–1.08 and 0.96–1.83, respectively). These values are similar to previously reported estimates in COS-1 cells for the wild-type receptor (Larrivée et al., 2000). The B_{max} estimates understandably varied in the separate transient transfections shown in Fig. 1A (106 ± 5.4 and 179 ± 10 fmol/well, respectively).

The pharmacological profile of the B_1R-YFP fusion protein stably expressed in HEK 293 cells was investigated by the competition of [^{3}H]Lys-des-Arg^9-BK (1 nM) binding to cells by a panel of cold BK-related peptides (Fig. 1C). The cold agonist peptides displaced the radioligand with the following order of potency: Lys-des-Arg^9-BK > Sar-[D-Phe^8]des-Arg^9-BK >des-Arg^9-BK ≈ Lys-BK ≫ BK. The antagonist peptides B-9858 and Ac-Lys-[Leu^8]des-Arg^9-BK were the most potent to displace the tritiated agonist, while [Leu^8]des-Arg^9-BK was less active and Hoe 140, essentially inactive. Altogether, these binding competition data are compatible with a rabbit B_1R pharmacological profile (MacNeil et al., 1995; Marceau et al., 1998).

HEK 293 cells stably expressing B_1R-YFP were derived from geneticin-treated transfected cells; these cells exhibited a saturable binding site for the agonist radioligand [^{3}H]Lys-des-Arg^9-BK [Fig. 1D; inset, Scatchard plot; derived K_D = 0.94 nM (95% confidence limits, 0.72–1.37 nM); B_{max} = 166 ± 8 fmol/well]. The cells also bound the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK in separate experiments (Fig. 1E; inset, Scatchard plot; derived K_D = 1.36 nM, B_{max} = 182 ± 24 fmol/well). Untransfected HEK 293 cells essentially bound no radioligand (Fig. 1, C and D). Nonspecific binding of radioligands amounted to less than 10% of the specific binding to the two forms of B_1Rs in the experiments reported above.

Effect of an Agonist Treatment on the Subcellular Distribution of B_1R-YFP. HEK 293 cells that stably expressed B_1R-YFP exhibited a mostly membrane-associated fluorescence in the resting state (Fig. 2). Addition of Lys-des-Arg^9-BK (1–10 nM) rapidly concentrated the receptor-associated fluorescence into multiple aggregates that remained associated with or close to the plasma membrane (Fig. 2). A higher concentration of the B_1R agonist (100 nM) produced results indistinguishable from the effects of the 10 nM concentration level (data not shown). This agonist-induced distribution of the receptor fluorescence is morphologically different from the intracellular fluorescent labeling caused by agonist-induced endocytosis, as illustrated in the same type of cells expressing the construction B_2R-GFP stimulated with the cognate agonist BK (10 nM, 30 min; Fig. 2, right column). The agonist-induced cellular redistribution of B_1R-YFP was prevented by treatment with the B_1R antagonist Ac-Lys-[Leu^8]des-Arg^9-BK (Drapeau et al., 1993) but not with the B_2R antagonist icatibant (Hoe 140) (Fig. 3). The acetylated form of the antagonist peptide is used in these experiments because this chemical modification confers resistance to peptidase(s) from serum (Drapeau et al., 1993). Cold temperature (0°C) inhibited agonist-induced redistribution of B_1R-YFP by the agonist Lys-des-Arg^9-BK (10 nM, 30 min; Fig. 3).

The reversibility of agonist-induced subcellular redistribution of B_1R-YFP was tested by washing cells exposed to Lys-des-Arg^9-BK (10 nM, 30 min, 37°C) with serum-free medium, and further incubating them for 4 h (Fig. 4, top microphotographs). The agonist-induced translocation of receptors was largely reversible in cells maintained at 37°C during the 4-h washout period (return of the continuous membrane fluorescence), whereas it remained stable after incubation at 0°C (Fig. 4, top). Unstimulated control cells conserved the predominant membrane fluorescence labeling, whether incubated for 4 h at 37 or 0°C.

The kinetics of [^{3}H]Lys-des-Arg^9-BK association at 0 or 37°C to wells of HEK 293 cells stably expressing B_1R-YFP is shown in the lower left of Fig. 4. The radioligand binding was essentially complete after 60 min at 37°C, but was extremely slow at 0° (incomplete after 2 h), as reported for the rabbit wild-type B_1R expressed by smooth muscle cells (Levesque et al., 1995). After ligand association at 37°C, some wells were washed three times with PBS at time 60 min, and further incubated in the azide-free binding medium for 4 h either at

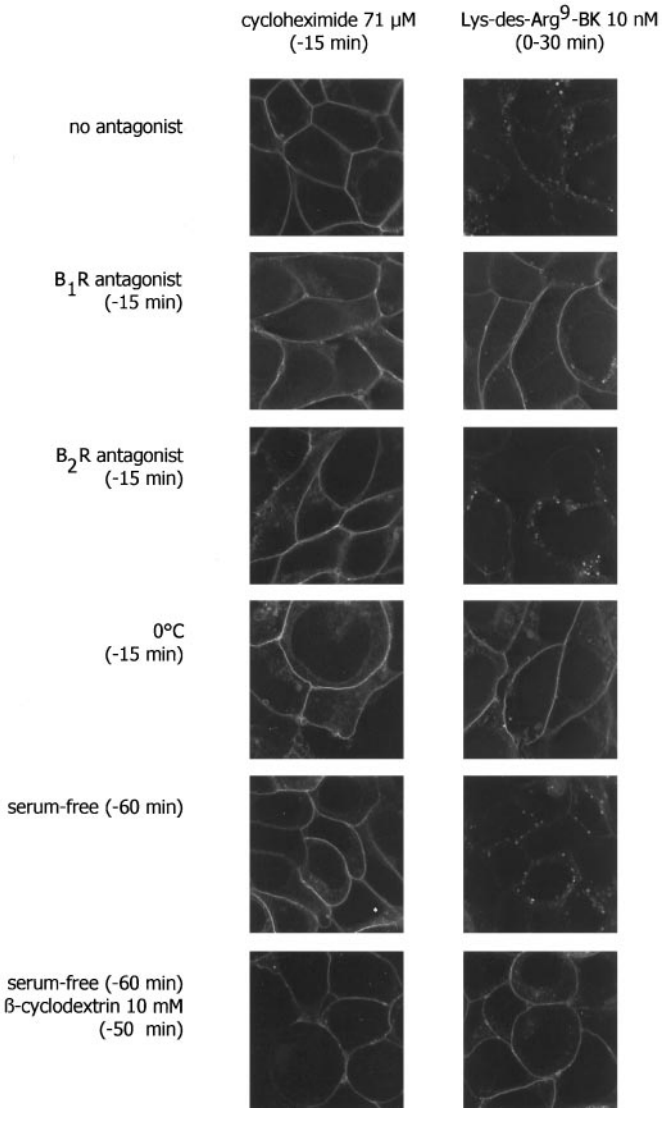

Fig. 3. Effect of drug or temperature treatments (applied 15 min before agonist) on Lys-des-Arg^9-BK (10 nM)-induced internalization (assessed at 30 min) in HEK 293 cells stably expressing B_1R-YFP and pretreated with cycloheximide (71 μM). The treatments consisted of Ac-Lys-[Leu^8]des-Arg^9-BK (B_1R antagonist; 1 μM), Hoe 140 (B_2R antagonist; 1 μM), incubation at 0°C in complete culture medium, or β-cyclodextrin (10 mM) in serum-free medium. Presentation as in Fig. 2. Results were verified during at least 2 separate days of experiments in multiple microscopic fields.

37 or 0°C. Most of the agonist radioligand specifically bound to cells was released at 37°C during the washout period, but the cells retained essentially all the radioligand if incubated on ice (Fig. 4). The same studies applied to the same concentration of the antagonist version of the radioligand documented similar findings, with quantitative differences: association was more rapid than for the agonist, but far from complete after 2 h; the dissociation at 37°C was noticeably slower. The low reversibility of radioligand binding at 0°C is a prerequisite for the cell fractionation scheme applied (see below).

PLA_2 Assays. The agonist Lys-des-Arg^9-BK increased arachidonate release from HEK 293 cells stably expressing B_1R-YFP, with an EC_{50} of 0.60 nM, whereas non transfected cells were not responsive to 100 nM the agonist (Fig. 5A). These results support that B_1R-YFP is a functional receptor. The analog Ac-Lys-[Leu^8]des-Arg^9-BK (1 μM) had no direct effect in the absence of the agonist but shifted the concentration-effect curve of Lys-des-Arg^9-BK to the right without depressing the maximal effect (Fig. 5A), supportive of a competitive antagonist behavior.

Effect of β-Cyclodextrin Treatment on B_1R-YFP Distribution and Function. β-Cyclodextrin treatment applied in serum-free culture medium extracts cholesterol from membranes and disrupts caveolae in many experimental systems (Parpal et al., 2001). The agonist-induced cellular redistribution of B_1R-YFP in cells maintained in serum-free medium for 60 min was readily observed using confocal microscopy (Fig. 3, bottom). However, addition of β-cyclodextrin (10 mM, last 50 min) before the agonist strikingly inhibited the effect of the agonist. To determine whether the inhibition of B_1R translocation also inhibited receptor function, the β-cyclodextrin treatment was adapted to the PLA_2 assay. The treatment alone significantly stimulated the basal [^{3}H]arachidonate release (Fig. 5B); however, β-cyclodextrin did not prevent further stimulation of PLA_2 by Lys-des-Arg^9-BK (Fig. 5B).

Cell Fractionation. The fractionation scheme applied was designed to recover intact caveolae and similar cholesterol-rich rafts from B_1R-YFP expressing HEK 293 cells pretreated with either the agonist [^{3}H]Lys-des-Arg^9-BK or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (1 nM each, 30 min, 37°C) in the serum-free binding buffer without sodium azide. It was found that more of the bound agonist comigrated (floatation) with the marker caveolin-1 than the bound antagonist in the fractions from the final ultra-centrifugation (Fig. 6; simultaneous determinations on the same lot of cells). Matched experiments run in the presence of an excess (1 μM) of the cold agonist or antagonist showed that most of the radioactivity (>90%) bound to caveolin-rich fractions represents specific binding sites (data not shown).

Colocalization of Caveolin-1 and B_1R-YFP. HEK 293 cells stably expressing B_1R-YFP, stimulated or not with the agonist Lys-des-Arg^9-BK (1 nM, 30 min), were fixed and permeabilized before staining with an anti-caveolin-1 monoclonal antibody. Conventionally, Fig. 7 shows YFP-associated fluorescence as green (precise hue best appreciated in cells not exposed to the primary antibody), and caveolin-associated fluorescence as red. Despite a certain loss of definition due to fixation/permeabilization, the receptor-associated fluorescence is distributed over plasma membrane in resting cells, whereas caveolin-1 is concentrated in discrete structures associated to membranes. In agonist-stimulated cells, the distribution of receptor-associated fluorescence is more restricted and colocalized to that of caveolin-1, as indicated by the yellowish color.

Discussion

As observed in many other experimental systems based on the fusion of a GPCR to GFP variants (Milligan, 1999), the fusion protein B_1R-YFP retains the pharmacological profile of the wild-type receptor in an excellent manner (affinity, binding competition assay with a panel of BK-related pep-

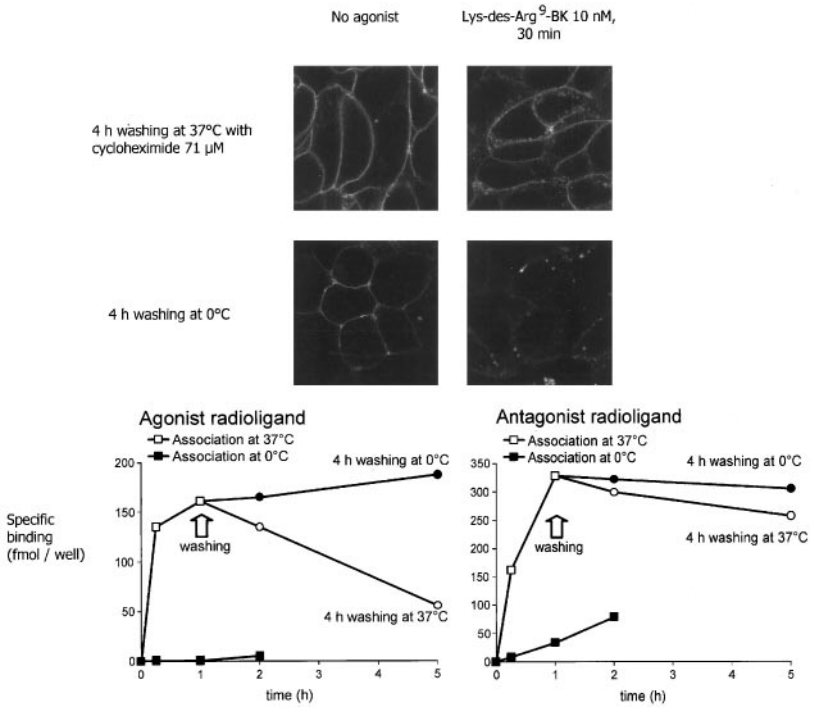

Fig. 4. Reversibility of agonist action on B_1R-YFP as a function of temperature in HEK 293 cells stably expressing the receptor fusion protein. Top, cells were exposed or not to Lys-des-Arg^9-BK (30 min, 10 nM, 37°C), then washed with serum-free α-minimal essential medium and further incubated at either 0° or 37°C for 4 h. Agonist-induced redistribution of fluorescence is largely reversible at 37°C but not at 0°C. Presentation as in Fig. 2. Bottom, left, association kinetics of 2 nM [^{3}H]Lys-des-Arg^9-BK to cells at 0 or 37°C. The association reaction at 37°C was followed by washing at time 60 min and further incubation for 4 h at either 0 or 37°C. Bottom, right: the same association-dissociation experiments were performed in other cells using the antagonist radioligand [^{3}H]Lys-[Leu^8]des-Arg^9-BK (2 nM). See *Results* for details.

tides, function in the PLA_2 assay). An additional common feature of the wild-type rabbit B_1R and B_1R-YFP is the exceptionally slow radioligand association at 0°C in intact cells (Fig. 4; Levesque et al., 1995). A further functional cellular response mediated by the fusion protein is agonist-induced redistribution of B_1R-YFP, a temperature-dependent effect occurring at low concentrations of Lys-des-Arg^9-BK (1 nM; Fig. 2) and prevented by a typical B_1R antagonist Ac-Lys-[Leu^8]des-Arg^9-BK but not by the B_2R antagonist icatibant (Fig. 3).

As mentioned above, several teams of investigators have established that the human B_1R is neither phosphorylated nor importantly internalized after agonist stimulation. These results do not exclude agonist-induced redistribution at the level of the plasma membrane, and the confocal microscopy experiments support the latter hypothesis (Figs. 2–4). The translocation of the B_1R-YFP membrane fluorescence is different from that of other GPCRs known to undergo agonist-induced endocytosis, as shown with B_2R-GFP expressed in the same cell type (Fig. 2). Indeed, the loss of membrane fluorescence is associated with an increased intracellular labeling of ill-defined structures in the case of B_2R-GFP (Bachvarov et al., 2001; Fig. 2), whereas the intracellular labeling is not convincingly stronger in stimulated cells than in control cells expressing B_1R-YFP. The discrete structures in which B_1R-YFP concentrates upon agonist stimulation may be small intracellular invaginations of the plasma membrane, consistent with the anatomical definition of caveolae (Schlegel and Lisanti, 2001). Caveolae are one of the cholesterol-enriched microdomains of the plasma membrane involved both in signaling and functional down-regulation functions (Schlegel and Lisanti, 2001). A treatment documented to deplete cholesterol from the membrane of cultured cells based on β-cyclodextrin was highly effective to prevent the translocation of B_1R-YFP into membrane-associated aggregates (Fig. 3), supporting the identity of these aggregates with caveolae. However, there are other type(s) of cholesterol rich membrane rafts that are also likely to be disrupted by β-cyclodextrin treatment and assume different roles. For instance, specific G proteins are differentially distributed between caveolae and lipid rafts that contain glycosylphosphatidylinositol (Oh and Schnitzer, 2001). The translocation of B_1R-YFP to caveolae is further supported by colocalization of caveolin-1 and of the receptor-associated fluorescence in agonist-stimulated cells (Fig. 7).

Strong evidence of receptor-ligand complex presence in caveolin-1 rich fractions comes from the cell fractionation experiment (Fig. 6). Cell bound tritiated agonist copurified with the marker caveolin-1 in the cell fractionation scheme applied, whereas the antagonist, itself ineffective to translocate the receptors based on microscopy, was less abundant in these fractions, but also present in other membrane fractions (Fig. 6). Cold temperature was an effective inhibitor of both the association of B_1R-YFP to caveolae-related rafts (Fig. 3)

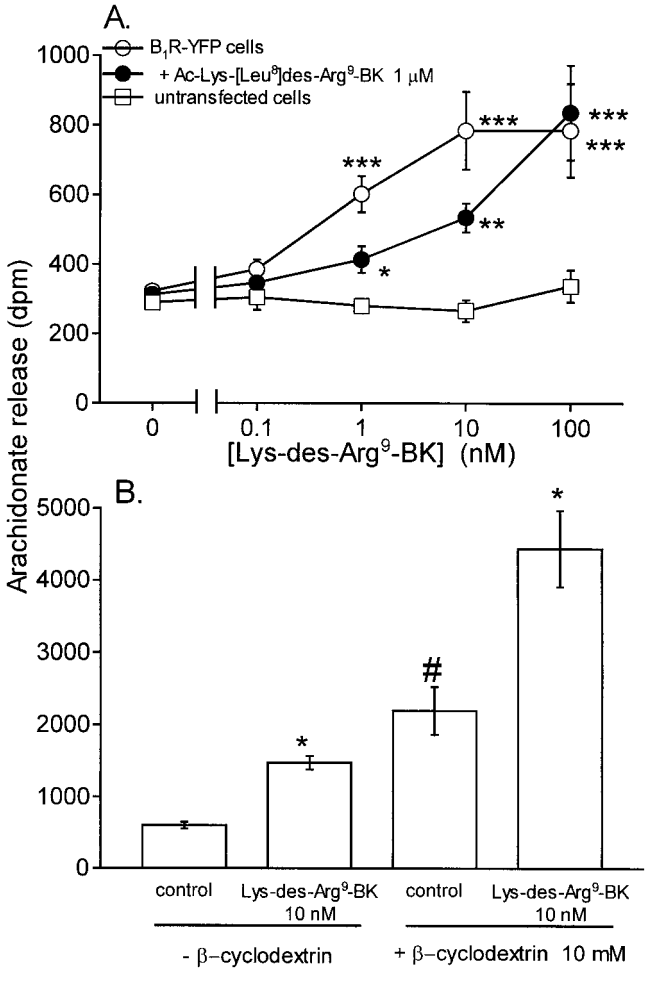

Fig. 5. A, [^{3}H]arachidonate released by untransfected HEK 293 cells or cells stably expressing B_1R-YFP and exposed to peptides for 30 min. Results are expressed as means ± S.E.M. (n = 12, except for controls without agonist, where n = 16–24). Only values from B_1R-YFP expressing cells were statistically heterogeneous (Kruskall-Wallis test, $P < 10^{-4}$). Mann-Whitney test was applied to compare agonist-stimulated cells with their appropriate controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). B, [^{3}H]arachidonate released by HEK 293 cells stably expressing B_1R-YFP as modified by pretreatment with β-cyclodextrin (50 min, 10 mM). Cells were further stimulated with the B_1R agonist Lys-des-Arg^9-BK for 30 min. Results are expressed as means ± S.E.M. (n = 8). *, $P < 0.05$ relative to respective control values from cell wells treated or not with β-cyclodextrin by Mann-Whitney test; #, $P < 0.05$, comparison of the effect of β-cyclodextrin on the basal release.

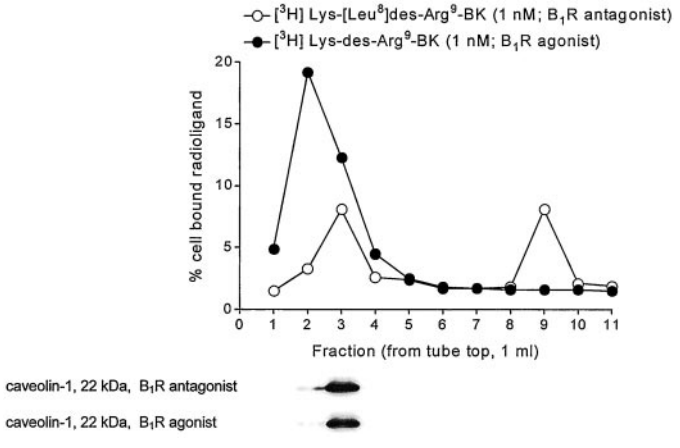

Fig. 6. Comigration of radioactivity and caveolin-1 in buoyant cell fractions from the final density gradient centrifugation the procedure applied to recover caveolae-related rafts (see text for details). HEK 293 cells stably expressing B_1R-YFP were extracted after a treatment with the agonist [^{3}H]Lys-des-Arg^9-BK or the antagonist [^{3}H]Lys-[Leu^8]des-Arg^9-BK (1 nM each) at 37°C (30 min). The caveolin-1 immunoblots (bottom) were from the same fractions. Total cell bound radioligand amounted to 1.64 or 1.96 pmol per five flasks of cells exposed to the agonist or to the antagonist, respectively.

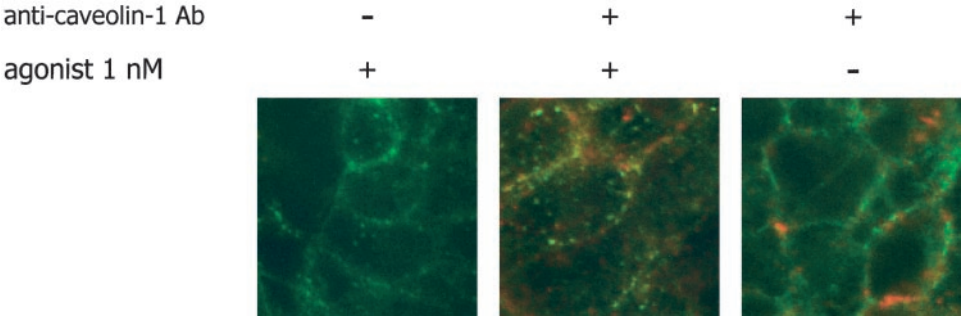

Fig. 7. Colocalization of B_1R-YFP (green) and caveolin-1 (red) in HEK 293 cells stimulated with Lys-des-Arg^9-BK (1 nM, 30 min). The left shows the agonist-induced translocation in the absence of anti-caveolin-1 antibody. See *Results* for analysis.

and of its dissociation (Fig. 4). Because the agonist radioligand association is very slow at 0°C (Fig. 4), the effect of cold temperature on B_1R-YFP translocation (Fig. 3) may be dependent on inhibition of ligand-receptor complex formation rather than of complex migration to caveolae-related rafts. However, the stability of the radioligand-receptor complexes at 0°C after association at 37°C for both the agonist or antagonist ligand versions (Fig. 4) allowed cell fractionation without major loss of specific binding. Ligand-independent spontaneous B_1R signaling, postulated to be strong (Leeb-Lundberg et al., 2001), may be associated with some presence of the antagonist radioligand in caveolin-1 rich membrane fractions, because the currently available peptide antagonists are not inverse agonists (see above).

The β-cyclodextrin treatment increased both basal and stimulated PLA_2 activity in these cells (Fig. 5B), an unexpected result of unclear significance. However, the treatment failed to inhibit the effect of the B_1R agonist, which remained approximately constant if expressed as a proportion of the basal arachidonate release (Fig. 5B). These preliminary results suggest that translocation to caveolae-related rafts is not required for this cellular response. Pike and Miller (1998) have reported that cholesterol depletion inhibit BK-induced phospholipase C activity in A431 cells. Other investigators have found that recombinant human B_1Rs are desensitized for long periods of time by the agonist Lys-des-Arg^9-BK when certain cellular responses were considered (e.g., intracellular calcium increase), but that did not apply to phosphoinositide turnover, which continued unabated; these events occurred without receptor internalization (Zhou et al., 2000). More work will be needed to determine whether translocation of B_1Rs to caveolae-related rafts is a functional down-regulation mechanism for specific cell effects, as is the case for some Gα proteins (Murthy and Makhlouf, 2000), and whether cyclodextrin-induced delocalization of phosphatidylinositol biphosphate from cholesterol-rich membrane microdomains (Pike and Miller, 1998) actually influences coupling between B_1Rs and phospholipase C.

The B_1R amino acid sequence is most highly related to those of the kinin B_2R and the angiotensin AT_1 (AT_1R) and AT_2 receptors (Menke et al., 1994). Caveolin-1, the major structural protein associated with caveolae, has been shown to coimmunoprecipitate with the agonist-stimulated human AT_1R (Ishizaka et al., 1998). Early events after agonist stimulation of the B_2R in DDT1 MF-2 or A431 cells include redistribution of B_2R to caveolae and the formation of endocytic vesicles that are not clathrin-coated (de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998). However, for both the activated AT_1R and B_2R, translocation to caveolae is not the final or dominant fate of the receptor, because massive endocytosis is documented (notably using GFP fusion proteins in each case: Chen et al., 2000; Bachvarov et al., 2001; Fig. 2). The specificity of the B_1R may reside in the fact that concentration into caveolae is the only type of agonist-induced translocation, thus illustrating a novel variation on the theme of GPCR adaptation.

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