Antagonist-Induced Intracellular Sequestration of Rabbit Bradykinin B₂ Receptor

Steeve Houle, Jean-François Larrivée, Magdalena Bachvarova, Johanne Bouthillier, Dimcho R. Bachvarov, François Marceau

Abstract—In a contractility assay based on the rabbit jugular vein, the structurally related drugs NPC 17731 or icatibant (1 to 3 nmol/L) were insurmountable antagonists of bradykinin (BK) B₂ receptors (B₂Rs). After ample washing (3 hours), the antagonism exerted by these peptides was not reversible. By contrast, the antagonist LF 16.0687 (30 to 100 nmol/L) was competitive and reversible. A rabbit B₂R-green fluorescent protein (B₂R-GFP) conjugate was expressed in mammalian cells. In COS-1 cells, it exhibited an affinity for [³H]BK (Kᵦ=1.61 nmol/L) similar to that of the wild-type rabbit B₂R. The stably expressed construction in HEK-293 cells was functionally active (phospholipase A₂ assay), and the antagonists mentioned above retained their respective surmountable or insurmountable behavior. Competition of [³H]BK binding to B₂R-GFP by the antagonists or BK was largely reversible after a 3-hour washout period at 0°C; at 37°C, icatibant or NPC 17731 effects were not reversible. B₂R-GFP was visualized in the plasma membranes of HEK-293 cells and rapidly internalized in response to BK. NPC 17731 or icatibant slowly translocated B₂R-GFP into cells over 24 hours, whereas LF 16.0687 had no effect on the subcellular distribution of B₂R-GFP. Cell extract immunoblotting with anti-GFP antibodies revealed a 101- to 105-kDa protein that was not significantly degraded on 24 hours of cell treatment with any of the ligands but was translocated in part to the 15 000-g pellet of the extract on treatment with BK or the noncompetitive antagonists. NPC 17731 and icatibant are noncompetitive, nonequilibrium antagonists that promote the cellular sequestration of rabbit B₂R expressed in an heterologous system. (Hypertension. 2000;35:1319-1325.)

Key Words: rabbits ■ bradykinin ■ veins ■ receptors, bradykinin

The kinin B₂ receptor (B₂R) subtype exhibits a high affinity for bradykinin (BK) and is prominent in the human cardiovascular and renal systems.¹,² The known B₂R antagonists are sequence-related peptides³,⁴ or nonpeptide compounds.⁵,⁶ The introduction of the B₂R peptide antagonist icatibant (Hoe 140; D-Arg[Hyp³, Thr⁵, D-Tic⁷, Oic⁸]-BK)¹¹ allowed a critical assessment of the role of kinin in the autoregulation of the circulation, blood pressure regulation, and the therapeutic effects of angiotensin-converting enzyme inhibitors, notably in clinical studies.¹,² The competitive status of icatibant, verified in human tissues and cells, unexpectedly varied according to the species studied. Indeed, the drug was noncompetitive and not readily reversible in a contractility assay based on the rabbit jugular vein.³ The nonequilibrium variety of antagonism was involved because receptors are apparently inactivated in a time-dependent manner in the presence of the peptide.⁸

The cloned rabbit and human B₂R molecules, although 83% identical at the amino acid level, differed in their interaction with the drug icatibant, as evidenced by Scatchard plot analysis of [³H]BK binding.⁹ Preincubation with the antagonist at 0°C removed many rabbit receptors from the reaction without changing the apparent affinity of the remaining sites.⁹ It was also recently observed that in vivo treatment of rabbits with repeated doses of icatibant over a 48-hour period drastically reduced the abundance of the B₂Rs in various tissues, including the jugular vein, as assessed by immunohistochemistry.¹⁰ Thus, a low reversibility of drug-receptor interaction may not be sufficient to account for the observed nonequilibrium behavior, because some form of antagonist-induced receptor internalization may occur in this system. In the present study, we investigated the competitive nature and reversibility of a constrained peptide structurally related to icatibant (NPC 17731, D-Arg[Hyp³, D-Hyp(E(trans-propyl))⁵, Oic⁸]-BK)¹¹ and of a novel nonpeptide antagonist (LF 16.0687)⁶ in the rabbit jugular vein; the behavior of B₂R antagonists was correlated with events posterior to receptor binding in this species, with comparison to the well-established agonist-induced internalization of the B₂R.¹²

Methods

Drugs
Icatibant and LF16.0687 (1-[(2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy]methyl]phenyl)sulfonyl]-N-[3-[(4-aminomethyl) -
phényl]-carbonylamino[proplyl]-2(S)-pyrrolidinocarbboxamide, mesylate salt) were gifts from Laboratoires Fournier SCA (Daix, France) and NPC 17731 was a gift from Dr Donald J. Kyle, Scios Inc (Sunnyvale, Calif). BK was purchased from Bachem and the other drugs from Sigma.

**Contractility Studies**

A local ethics committee approved the animal experimentation. Isometric changes of the tone of rabbit jugular vein strips were measured as reported. These studies aimed to investigate the nature and specificity of BK B₂R antagonists in the vascular smooth muscle preparation. Each tissue was subjected to the construction of 3 full cumulative concentration-response curves for BK (a B₂R agonist) or histamine in the absence of antagonist (control, time 1 hour), in the presence of an antagonist applied 30 minutes earlier (3 hours), and after ample washing (recovery, 6 hours). Contractility results are expressed as a percentage of the maximal response recorded when constructing the control curve (1 hour). Results were expressed as mean±SEM, and statistics were calculated with the use of the InStat 2.0 computer program (GraphPad Software).

**Construction and Expression of Rabbit B₂ Receptor–Green Fluorescent Protein Conjugate**

With the use of the cloned rabbit B₂R in the eukaryotic expression vector pCDAO⁹ as a template, the entire coding region of the gene (excluding the stop codon) was amplified by polymerase chain reaction (PCR) 5'-GGAGCACAAGCTTTGAAAATTCTCAACATCAC-3' and 5'-TAGATTGATCCCCGTGGTAGGCCCTGCCCAGCTCAGTCC-3' were used as PCR sense and antisense primers, respectively. These primers contained additional HindIII and BamHI sites (italicized) for the directional cloning of the rabbit B₂R coding region in the eukaryotic expression vector pEGFP-N3 (Clontech Laboratories, Inc), encoding a red-shifted variant of green fluorescent protein (GFP). Both the PCR fragment and the pEGFP-N3 vector were digested with HindIII and BamHI and ligated at 12°C overnight. The resultant vector (B₂R-GFP) contained the rabbit B₂R coding sequence fused in frame at its carboxyl terminus with the GFP and under the control of the strong cytoskeletal promoter. COS-1 cells, grown in 12-well plates until 70% confluent, were transiently transfected (48 hours) with the B₂R-GFP coding vector or the pEGFP-N3 vector with the use of the EX-Gen 500 transfection reagent (MBI Fermentas Inc) as directed. The B₂R-GFP vector was also transfected in HEK-293 cells by means of the same procedure, and stable transfectants were selected after growing the cells for 1 month in α-MEM medium supplemented with fetal bovine serum (5%), horse serum (5%), penicillin-streptomycin (1%), and gentamicin (500 μg/mL; Gibco BRL).

**Binding Assays**

The binding of [³H]BK (NEN Life Science Products; 90 Ci/mmol) to adherent intact cells was evaluated as described. Briefly, 24-well plates of cells (70% confluent for COS-1, confluent for HEK 293 cells) were washed twice with ice-cold binding medium (PBS, pH 7.4, supplemented with 0.02% sodium azide, 0.1% bovine serum albumin, 1 mM/L phenylmethylsulfonyl fluoride, and 1 μM/L captopril), and 500 μL of this medium was added to each well. To construct saturation curves, the total binding of the ligand (0.25 to 7 nM) to cells transfected with the B₂R-GFP construction or with GFP alone (as control) was determined in duplicate wells, and 300 nM/L of cold BK was added to matched wells to determine the nonspecific binding. On 90 minutes of incubation, the wells were rinsed (×3) with ice-cold PBS, the supernatant was removed, and the cells were dissolved in 1 mL of 0.1 M/L NaOH. The resulting suspension was then counted by scintillation. Binding experiments involving 2 nM/L [³H]BK and the same general conditions as outlined above were also performed on the HEK 293 stable transfectant cell line to verify antagonist binding to the fusion protein. For this purpose, plates of cells were concomitantly treated at 0°C with the cold competitors (icatibant, NPC 17731, LF16.0687, or cold BK) at various concentrations and the radioactivity in each plate determined. In other plates, the competing drugs were incubated for 30-minute periods, either in the complete culture medium at 37°C or in the ice-cold PBS-based binding buffer described above. These treatments were followed by drug washout and cell rinsing (×2) and by 3 hours of incubation in the same medium at the same temperature. The plates were then transferred on ice and the binding of 2 nM/L of [³H]BK was evaluated in the PBS-based buffer (90-minute equilibration without competitor, except for 1 μM/L BK in some wells to determine the nonspecific binding).

**Effect of BK or Antagonists on Subcellular Distribution of B₂R-GFP**

The agonist BK or 1 of the 3 antagonists studied was added to the cultured medium of the stable transfectant HEK 293 cells, and the subcellular fluorescence distribution was observed without fixation or drug washout with a BioRad 1024 confocal microscope as a function of treatment duration (×60 objective with oil immersion; emission 488 nm).

**Phospholipase A₂ Assays**

An arachidonic acid release assay was performed to evaluate the function of B₂R-GFP stably expressed in HEK 293 cells; 2.5×10⁵ cells were seeded in 2-cm² wells (24-well plates) containing 1 mL of the complete culture medium (see above). Twenty-four hours later, as the cells were 50% to 60% confluent, 0.1 μCi of [³H]arachidonic acid (NEN; specific activity 185 Ci/mmol) was added to each well. The cells were further incubated for 18 hours, then washed 3 times with Earle's balanced salt solution containing 2 mg/mL of bovine serum albumin. One milliliter of this medium was left in each well. B₂R antagonists were first added to the appropriate wells, and the agonist BK was added 30 minutes later. The plates were further incubated at 37°C for 20 minutes, at which point 500 μL of the medium from each well was recovered in 1.5 mL conical tubes and centrifuged for 5 minutes at 15 000g. Four hundred microliters of the supernatant was transferred into vials for scintillation counting of the released arachidonic acid.

**Immunoblot**

For the analysis of B₂R-GFP, 75-cm² flasks of confluent transfectant HEK 293 cells were treated with drugs (icatibant, NPC 17731, or BK, 30 mM/L of each or 100 mM/L, LF16.0687) for specific time periods. The cells were then put in boiling lysis buffer containing 10 mM/L Tris, pH 7.4, 1.0 mM/L Na₃VO₄, and 1.0% SDS. The lysates were centrifuged at 15 000g for 5 minutes and incubated for 5 minutes at 10°C. Total protein concentrations were then determined with the use of the BCA Protein Assay (Pierce). Fifteen micrograms of total protein was run on a 10% SDS-PAGE and transferred to a PVDF membrane. Other experiments involved sequential centrifugation of cell homogenates (one 75-cm² flask per drug treatment) prepared in 0.5 mL sucrose buffer (250 mM/L sucrose, 20 mM/L tricine buffer, 1 mM/L EDTA, 1 mM/L PMSF, 10 μg/mL leupeptin, 2 μg/mL pepstatin, and 10 μg/mL soybean trypsin inhibitor, pH 7.5) to document drug-induced translocation into specific cell compartments. The first pellet (600 g, 5 minutes) was discarded; the whole content of the second (15 000 g, 5 minutes) and third (150 000 g, 3 hours) pellets as well as the final supernatant were migrated and transferred as described above. Either type of blot was then incubated for 1 hour at room temperature in blocking buffer (washing buffer [10 mM/L Tris, pH 7.5, 100 mM/L NaCl, 0.1% Tween-20] containing 5% skimmed milk). The primary antibody (polyclonal anti-GFP; S-8334; dilution 1:875; Santa Cruz Biotechnologies) was added for 30 minutes 37°C in fresh blocking buffer. The membranes were washed for 30 minutes in washing buffer at room temperature before adding the secondary antibody (horseradish peroxidase–coupled goat anti-rabbit IgG; A-0545; Sigma; dilution 1:16 000) for 1 hour at room temperature in blocking buffer. The membranes were washed in washing buffer for another 30 minutes and the antibodies then revealed with the use of the Western Blot Chemoluminescence Reagent Plus (NEN), as directed.
Results

Vascular Contractility Mediated by B₂R in Rabbit Jugular Vein

The rabbit jugular vein stimulated with the B₂R agonist BK responded by a concentration-related contraction, with an EC₅₀ in the range of 3 to 8 nmol/L (Figure 1). The structurally constrained B₂R antagonist NPC 17731 (1 or 3 nmol/L) shifted the concentration curve to the right (EC₅₀ of 53.1±15.2 nmol/L at 3 nmol/L of the antagonist, P=0.004 by Mann-Whitney test) and depressed the maximum BK-induced effect (Emax of 14.8±4.6% of the control maximum at 3 nmol/L of the antagonist, P=0.002) (Figure 1, A and B). After ample washing during a 3-hour period, the antagonism exerted by NPC 17731 was only partly reversible (Figure 1, A and B). At 3-nmol/L antagonist concentration, the effect of BK after washing returned to 46.2±11.5% of the 1-hour control maximum (P=0.002), and the EC₅₀ was 12.8±3.4 nmol/L (P=0.015 when compared with the control value of 4.4±1.1 nmol/L). NPC 17731 (3 nmol/L) did not exert any antagonism on histamine-induced contraction in the rabbit jugular vein (Figure 1C). As reported previously, icatibant (3 nmol/L) was also an insurmountable antagonist of BK in the rabbit jugular vein (Figure 1D; EC₅₀ increased from 3.30±1.44 to 16.5±3.6 nmol/L in the presence of the drug, P=0.002; Emax reduced to 63.0±6.0% of the control maximal response, P=0.002). The Emax depression was essentially nonreversible on 3 hours of washing (56.3±7.1% of control, P=0.009).

By contrast, the B₂R antagonist LF 16.0687 (Reference 6) was surmountable when applied at 30 or 100 nmol/L (EC₅₀ of

Figure 1. Effect of 3 B₂R antagonists on BK-induced contraction in rabbit isolated jugular vein. NPC 17731 was also tested against histamine-induced contraction in the same preparation (C). Each tissue was subjected to construction of 3 full cumulative concentration-effect curves in absence of antagonist (×, control, 1 hour), in presence of antagonist applied 30 minutes earlier (●, antagonist, 3 hours), and after ample washing (∗, recovery, 6 hours). Values are mean±SEM (n=6 to 8). See text for statistics.
44.6±11.6 nmol/L, P=0.009, and \( E_{\text{max}} \) of 99.0±3.6, NS, at 100 nmol/L of the drug), and the antagonism was reversible on washing (\( E_{\text{max}} \) of 90.6±4.5% of the 1-hour control maximum, NS, and EC_{50} of 5.6±3.2 nmol/L, not significantly different from the control EC_{50} value of 5.1±2.0 nmol/L) (Figure 1, E and F). The apparent competitive behavior of LF allowed the calculation of a pA_{2} value (7.99±0.27) with computerized Schild plot analysis\(^8\) (the slope of the regression, 1.09, was close to unity).

**Pharmacology of B_2R-GFP Fusion Protein**

COS-1 cells transiently transfected with a GFP coding vector failed to bind \[^{3}H\]BK, whereas cells that expressed B_2R-GFP exhibited specific and apparently saturable binding (Figure 2A). The affinity estimate derived from Scatchard plot analysis (Figure 2A, inset) was \( K_{d} = 1.61 \) nmol/L, close to that of the wild-type B_2R transiently expressed in the same cell type (2.09±0.39 nmol/L).\(^9\) Stably transfected HEK 293 cells expressing B_2R-GFP bound far larger quantities of the radioligand but not in a saturable manner in the 0.25 to 7 nmol/L concentration range (Figure 2A). The irregular shape of the curve may suggest the presence of more than one state of affinity.

The potency and reversibility of antagonist or BK binding was addressed by measuring the specific binding of \[^{3}H\]BK (2 nmol/L) to the transfectant HEK 293 cells at 0°C as a function of the presence of one of the antagonists or cold BK. All 3 tested antagonists or BK could completely displace radioligand-specific binding at suitable concentrations when coincubated with \[^{3}H\]BK (Figure 2B). Receptor occupancy by cold drugs was largely reversible for drug concentrations <1 \( \mu \)mol/L on 3 hours of washing at 0°C with the binding buffer (Figure 2D), whereas the inhibition of binding in cells treated with the peptides NPC 17731 or icatibant was fully persistent after a 3-hour washout period at 37°C (Figure 2C).

In a phospholipase A_2 assay, BK (100 nmol/L) was active to release \[^{3}H\]arachidonate from the stably transfected cells, but the 3 B_2R antagonists were inactive in this respect (Figure 3A). Varying the agonist concentration showed that BK was maximally active at 1 nmol/L, but its effect was inhibited by preincubation with any of the 3 tested antagonists (Figure 3B). The nature of the antagonism observed for each drug (insurmountable for icatibant or NPC 17731 at 30 nmol/L, surmountable for LF 16.0687 at 1 \( \mu \)mol/L) was similar to that observed in the contractility assay (compare Figure 3B with Figure 1).

The B_2R-GFP fusion protein stably expressed in HEK-293 cells was mostly visualized in the plasma membrane (Figure 4). The acute addition of BK (10 to 100 nmol/L) was rapidly
(10 minutes) followed by translocation of the fluorescence into the cells (diffuse labeling close to the plasma membrane and discrete intracellular structures; Figure 4). The antagonists icatibant, NPC 17731, or LF 16.0687, used at concentrations proven to occupy the receptor fusion protein (Figures 2 and 3), exerted a modest acute (15 minutes) effect consisting of a loss of plasma membrane–associated fluorescence (Figure 4); however, after 24 hours of treatment, NPC 17731 or icatibant induced a variable loss of membrane fluorescence, and, depending on the cell population and/or the specific area in a given cell monolayer, diffuse fluorescence in the cytosol was observed (the full spectrum of these effects is illustrated in Figure 4). B2 R-GFP remained mostly associated to the plasma membrane in cells exposed to LF 16.0687 (0.1 to 1 μmol/L) for 24 hours.

Two protein bands (92 and 57 kDa) appeared on reaction with anti-GFP antibodies in the cell extract from untransfected HEK 293 cells (Figure 5A); these bands were also present in all other extracts from the same cell type and were regarded as nonspecific. HEK 293 cells transiently expressing GFP exhibited the expected ~28-kDa protein, as assessed by immunoblotting (Figure 5A). Under fluorescence microscopy, positive cells exhibited a uniform cytosolic fluorescence (not shown). Cells stably expressing B2-R-GFP exhibited a specific double band (~101 to 105 kDa). Treatments with antagonists (24 hours) or BK (30 minutes, in the presence of 1 μmol/L captopril and 3 μmol/L phosphoramidon to inhibit inactivation by serum) failed to reveal significant degradation of these bands (especially when band intensity was corrected with the nonspecific bands for loading fluctuation); notably, there was no lower-molecular-weight band suggestive of cleavage. However, ligand-induced translocation of the 101- to 105-kDa bands into a dense cell fraction (15 000 – g pellet) was observed in cells exposed to BK (10 minutes) or the peptide antagonists but not to LF16.0687 (24 hours, Figure 5B). Icatibant effect was more intense than that of NPC 17731 but small after only 3 hours of treatment (data not shown). Immunoreactive B2 R-GFP was present in the 150 000 – g pellet from all transfected and drug-treated cells but not in the corresponding supernatant (data not shown).

Discussion

The structurally related and constrained peptides NPC 17731 and icatibant are nonequilibrium antagonists of the rabbit B2R...
in living cells because their insurmountable effect at nanomolar concentrations is not reversible or is only slowly reversible (Figure 1). This durable loss of BK-induced effect is not related to toxicity or postreceptor effect on contractility because the histamine-induced responses were unaffected by NPC 17731 in the rabbit jugular vein (3 nmol/L, Figure 1C). Previous work has also established that icatibant is selective for B₂Rs in the rabbit jugular vein.⁷ Other BK sequence-related peptide antagonists, such as d-Arg-[Hyp³, d-Phe⁶, Leu⁸]BK, are highly potent and competitive in the rabbit jugular vein while retaining key structural features present in NPC 17731 and icatibant (for instance, both Arg⁴ and Arg⁶ residues).⁸ Similarly, the nonpeptide B₂R antagonists WIN 64338, FR 173657, or LF 16.0687 (Figure 1, E and F) were verified to be competitive on the basis of the rabbit vein contractility assay, but a nonpeptide mimetic of the C-terminal tripeptide of icatibant (compound 9)⁹ behaves as an insurmountable and irreversible antagonist of BK in this preparation (E.K. Dziadulewicz and F. Marceau, unpublished data). This nonequilibrium behavior is likely to be determined by both the constrained C-terminal region structurally similar in icatibant and NPC 17731 and by the fine receptor structure, as icatibant is competitive in the highly homologous human B₂Rs.⁸,⁹ Thus, an obvious opportunity exists to map the structural determinants of this pharmacological behavior in the rabbit B₂R sequence, but another report described differences in the pharmacology of the B₂R antagonists between the two species (eg, LF 16.0687 is more potent and D-Arg-[Hyp³, d-Phe⁶, Leu⁸]BK less potent at the human B₂R).³⁶

Visual monitoring of GFP-fused G-protein–coupled receptors, introduced previously for studying the cycling of the β-adrenoceptor, is usually well tolerated as far as the pharmacological properties of the fusion protein is concerned. Our B₂R-GFP construction retained a binding affinity virtually identical to that of the wild-type rabbit B₂R (Figure 2); rapid agonist-induced internalization (Figure 4) as well as

**Figure 5.** Immunoblot of HEK 293 cell extracts based on polyclonal anti-GFP antibodies. A, HEK 293 cells stably transfected with B₂R-GFP were treated for 24 hours with various drugs: control (lane 1), 1 μmol/L captopril + 3 μmol/L amastatin (lane 2), 1 μmol/L captopril + 3 μmol/L amastatin + 30 nmol/L BK (lane 3), 30 nmol/L icatibant (lane 4), 30 nmol/L NPC 17731 (lane 5), 100 nmol/L LF 16.0687 (lane 6). Extracts from untransfected HEK 293 cells (lane 7) and HEK 293 cells transiently transfected with GFP (lane 8) were also immunoblotted as controls. B, B₂-R-GFP analysis (101- to 105-kDa bands) in dense compartment (15 000-g pellet) of the stable transfecant cell lines (lanes 1 to 5) or of untransfected HEK-293 cells (lane 6). Cells were treated for 24 hours (or 10 minutes for BK) with saline vehicle (lane 1), BK (100 nmol/L, lane 2), icatibant (3), NPC 17731 (4), or LF 16.0687 (5; antagonist concentrations as in A). Results are representative of 5 experiments. Lanes 1 and 2 were replicated in the experiment shown.
from rabbits treated with this drug is not entirely consistent with the peptide-induced subcellular redistribution without significant destruction of B2-R-GFP, as assessed by our immunoblot data (Figure 5A). The discrepancy may originate from a loss of immunoreactivity of the internalized B2R in rabbit tissues or from a saturation of the receptor breakdown mechanisms in HEK 293 cells expressing B2-R-GFP at high levels. Heterologously expressed B2-R-GFP is a suitable system to test various hypotheses about the mechanism of agonist-induced sequestration, which may involve a partial agonist behavior of icatibant or NPC 17731 (although not detected in any of the functional assays applied), phosphorylation and internalization pathways common to those recruited by agonists, or a different and novel mechanism. NPC 17731 may be less effective than icatibant in this respect (Figure 5B).

Perhaps related to the present observations, by using anti-icatibant antibodies, Vio et al have shown the internalization and cytosolic distribution of icatibant in the distal nephron epithelium after a single intravenous injection of the drugs to rats. This distal epithelium is particularly rich in B2-Rs, and the authors have suggested that a form of receptor-mediated antagonist uptake was occurring. The importance of antagonist-induced receptor inactivation in pharmacotherapy is not clear, nor whether other pharmacological antagonists binding with low reversibility can stimulate receptor sequestration. However, it is interesting to note that virtually all the antagonists AT1-receptor antagonists in clinical use (or their active metabolites) exert to variable degrees the nonequilibrium, insurmountable antagonism. As for B2-R antagonists in the live rabbit, icatibant was superior in potency to any other tested reversible antagonists in hemodynamic tests in a recent study, even when these alternate antagonists were more potent than icatibant in vitro.

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