A Novel Nonpeptide Antagonist of the Kinin B₁ Receptor: Effects at the Rabbit Receptor

Guillaume Morissette, Jean-Philippe Fortin, Sophie Otis, Johanne Bouthillier, and François Marceau

Centre Hospitalier Universitaire de Québec, Centre de recherche, Québec, Canada

Received May 11, 2004; accepted July 26, 2004

ABSTRACT

The kinin B₁ receptor (B₁R) has attracted interest as a potential therapeutic target because this inducible G protein-coupled receptor is involved in sustained inflammation and inflammatory pain production. Compound 11 (2-{(2(R)-1-[[3,4-dichlorophenyl]sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]-N-[2-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl}acetamide) is a high-affinity nonpeptide antagonist for the human B₁R, but it is potent at the rabbit B₁R as well: its Kᵢ value for the inhibition of [³H]Lys-des-Arg⁹-BK (bradykinin) binding to a novel myc-labeled rabbit B₁R expressed in COS-1 is 22 pM. In contractility tests (organ bath pharmacology), we found that compound 11 is an apparently surmountable antagonist of des-Arg⁹-BK- or Lys-des-Arg⁹-BK-induced contraction of the rabbit isolated aorta (pA₂ values of 10.6 ± 0.14 and 10.4 ± 0.12, respectively). It did not influence contractions induced by angiotensin II in the rabbit aorta or by BK or histamine in the jugular vein, but it suppressed the prostaglandin-mediated relaxant effect of des-Arg⁹-BK on the rabbit isolated mesenteric artery. Compound 11 (1 nM) inhibited both the phosphorylation of the extracellular signal-regulated kinase1/2 mitogen-activated protein kinases induced by Lys-des-Arg⁹-BK in serum-starved rabbit aortic smooth muscle cells and the agonist-induced translocation of the fusion protein B₁R-yellow fluorescent protein expressed in human embryonic kidney (HEK) 293 cells. Compound 11 does not importantly modify the expression of myc-B₁R over 24 h in HEK 293 cells (no detectable action as "pharmacological chaperone"). The present results support that compound 11 is a potent and highly selective antagonist suitable for further investigations of the role of the kinin B₁R in models of inflammation, pain, and sepsis based on the rabbit.

The kinin B₁ receptor (B₁R) is a G protein-coupled receptor selectively stimulated by sequences related to bradykinin (BK) but not by BK itself. Instead, des-Arg⁹-BK, Lys-BK (kallidin) and Lys-des-Arg⁹-BK (des-Arg⁹-kallidin) stimulate the human and rabbit recombinant B₁Rs, the last peptide with the highest affinity (Menke et al., 1994; MacNeil et al., 1995). The B₁R is also strongly regulated by inflammatory conditions, to the point of being largely inducible in several experimental systems (Marceau et al., 1998). The kinin B₁R, expressed by vascular and epithelial cells, fibroblasts, some leukocytes, and sensory and other neurons

(Studentship award to J.-P.F.).

This study was supported by the Canadian Institutes of Health Research (Grant MOP-14077) and the Fonds de la recherche en Santé du Québec (Studentship award to J.-P.F.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.104.071266.

ABBREVIATIONS: B₁R, B₁ receptor; B₂R, B₂ receptor; BK, bradykinin; YFP, yellow fluorescent protein; Ang II, angiotensin II; AT₁R, AT₁ receptor; DMSO, dimethyl sulfoxide; PG, prostaglandin; compound 11, 2-{(2(R)-1-[[3,4-dichlorophenyl]sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]-N-[2-(4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl}acetamide; COX, cyclooxygenase; PCR, polymerase chain reaction; HEK, human embryonic kidney; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; NS-398, N-[2-cyclohexyloxy-4-nitrophenyl]-methanesulfonamide; SC-560, 5-[4-chlorophenyl]-1-(4-methoxyphenyl)-3-fluorouethyl pyrazole; SSR240612, 2(R)-2-[[[3R]-3-(1,3-benzodioxol-5-yl)-3-[[6-methoxy-2-naphthyl]sulfonyl]amine]propanoyl]amino]-3-(4-[[2R,6S]-2,8-dimethylpiperidiny]methyl)phenyl|N-isopropyl|N-methylpropanamide hydrochloride.
to important agonist-induced endocytosis nor phosphorylated, unlike the related BK B₂ receptor (B₂R) (Faussner et al., 1998; Blaukat et al., 1999). Agonist-induced cellular redistribution has been studied using a fusion protein composed of the rabbit B₁R fused with the yellow fluorescent protein (YFP). Lys-des-Arg⁹-BK induces a condensation of cell surface B₁R-YFP into aggregates that remain associated with the plasma membrane and that were identified as cholesterol rich rafts; this redistribution was slowly reversible on washing at 37°C and distinct from endocytosis (Sabourin et al., 2002a). Finally, it has been proposed that the rapidly induced B₁R is subjected to an accelerated cellular degradation when its synthesis subsides in cellular models (Fortin et al., 2003b). This mechanism, based on a form of agonist-independent endocytosis, could contribute to terminate B₁R signaling as the inflammatory condition is being resolved.

Several nonpeptide antagonists of the parent BK B₂R have been discovered over the last decade (Pruneau et al., 1999; Dziadulewicz et al., 2000, 2002) with proposed applications in inflammatory pain and the prevention of vasogenic edema of the brain (Burgess et al., 2000; Kaplanski et al., 2002; Zausinger et al., 2002). One of the first reported nonpeptide antagonist of the human B₁R, compound 11 (Fig. 1A), is potent at the rabbit B₁R as well: its $K_i$ value for the inhibition of $[^{3}H]$Lys-des-Arg⁹-BK binding to the rabbit recombinant B₁R expressed in Chinese hamster ovary cells was reportedly 50 pM (Su et al., 2003). SSR240612 is another example of nonpeptide B₁R agonist with significant structural similarity to compound 11 (Gougat et al., 2004). The characterization of low molecular weight and cell-permeant nonpeptide antagonists for both types of kinin receptors opens new experimental possibilities, by comparison with the previously available peptide antagonists.

We have verified the potency and specificity of compound 11 for several effects mediated by the rabbit B₁R. Furthermore, we have studied the possibility that this nonpeptide kinin receptor antagonist alters the surface expression of the B₁R. Previous results based on the vasopressin V₂ and the

![Fig. 1. Effect of compound 11 on contractility assays in the rabbit isolated aorta. A, effect of compound 11 on des-Arg⁹-BK-induced contraction mediated by B₁Rs and recorded at time 5.5 h post-tissue mounting. B, effect of compound 11 on Lys-des-Arg⁹-BK-induced contraction mediated by B₁Rs. In A and B, each tissue was subjected to the construction of two full cumulative concentration-effect curves, in the absence of antagonist (3.5 h; not shown) and in the presence of an antagonist or its DMSO vehicle applied 30 min earlier (5.5 h). Values are the means ± S.E.M. of the number determinations indicated by n. The structure of compound 11 is also shown on the right. C, Schild plot analysis based on the averaged data in A and B. D, effect of compound 11 (100 nM) on angiotensin II-induced contraction of the rabbit aorta mediated by AT₁ receptors (recorded at time 7.5 h postmounting). Presentation as in A. See text for analysis.](image-url)
δ-opioid receptors have led to the suggestion that cell-permeant antagonists of G protein-coupled receptors may facilitate the surface expression and transit along the secretory pathways by binding to newly formed, presumably misfolded receptors (Morello et al., 2000; Petajä-Repo et al., 2002). The drugs capable of stabilizing a favorable receptor conformation have been termed "pharmacological chaperones".

Materials and Methods

Drugs. B-9858 (Lys-Lys-Hyp3,4Ig1, d-Ig1, Oic4I-des-Arg6-BK), Hoe 140 (ictibant, d-Arg5-Hyp3,4Ig1, d-Ig1, Oic4I-des-Arg6-BK), and LF 16-0687 (1-[2,4-dichloro-3-[[2,4-dimethylquinolin-8-yl]oxy] methyl]phenyl)sulfonyl]-N-[3-[4-(aminomethyl) phenyl] carbonylamino] propyl]-2(S)-pyrrolidinecarboxamide) were gifts from Laboratoires Sanofi-Aventis (Flamborough, ON, Canada) as directed by the manufacturer (pCI-neo-myc-B1R coding or control vectors were fixed and submitted to immunofluorescence staining). The Elite ESP cytofluorometry apparatus was used (Beckman Coulter, Inc., Fullerton, CA) with the excitation set at 495 nm and the emission recorded above 520 nm. The Elite ESP cytofluorometry apparatus was used (Beckman Coulter, Inc., Fullerton, CA) with the excitation set at 495 nm and the emission recorded above 520 nm. The excitation set at 495 nm and the emission recorded above 520 nm.

Contractility Studies. A local ethics committee approved the procedures based on rabbits. Rabbit aortic rings (New Zealand White, 1.5–2 kg; Charles River Canada, St. Constant, QC, Canada) were suspended under a tension of 2 g in 5-ml tissue baths containing oxygenated (95% O2, 5% CO2) and warmed (37°C) Krebs’ solution maintaining oxygenation (95% O2, 5% CO2) and warmed (37°C) Krebs’ solution. The contractility experiments in the rabbit jugular vein, which respond to kinins via B1R and histamine via H1 receptors, were performed to investigate the specificity of the recently produced B1R antagonist (as in Larriviére et al., 2000). Two cumulative concentration-response curves for BK (2 h) and histamine (3.5 h) were constructed; Compound 11 or its DMSO vehicle was introduced 30 min before the agonist stimulation in the bathing fluid. Precontracted rabbit mesenteric artery rings respond to des-Arg9-BK mostly by a prostaglandin (PG)-mediated relaxation, and this response is acquired in vitro as a function of time (Churchill and Ward, 1986; Debois and Marceau, 1987). Compound 11 or one of isoform-specific cyclooxygenase (COX) inhibitors were introduced 30 min before the phenylephrine stimulation used to induce tissue precontraction to analyze the mechanism of action of des-Arg9-BK.

Construction of the Rabbit myc-B1, Receptor Conjugate (myc-B1R). To assess the abundance of kinin receptors at the cell surface in a radioligand-independent manner, we produced and characterized a rabbit B1R labeled with the N-terminal (extracellular) myc epitope. Using the pCDNA3-based vector for the wild-type rabbit B1R (Larriviére et al., 2000) as a template, the coding region of the B1R gene was amplified by polymerase chain reaction (PCR). 5'-GAACGAAATTCCATGCGCTACAGGCGCCCTCAGGAG-3' and 5'-TGATCTTAGATATCTCCGCCAGAACACCCAGACATTCC-3' were used as sense and anti-sense PCR primers, respectively (primers derived from the rabbit B1R sequence; MacNeil et al., 1995). The pair of primers contained additional 5'-3'-extension sequence for cell-permeability of myc epitope peptide (MEQKLISEEDLNS) fused in frame with the corresponding rabbit receptor coding sequence at its carboxy terminus. The sequence of the construction was verified (RSVS core laboratory, Pavillon Marchand, Laval University, Ste. Foy, QC, Canada).

Binding Assays. The binding of [3H]Lys-des-Arg5-BK (PerkinElmer Life and Analytical Sciences, Boston, MA; 80 Ci/mmol) to adherent intact COS-1 cells transiently expressing the myc-B1R was evaluated as described previously (Sabourin et al., 2002a). The cells had been transfected 48 h before the binding assay with the above described expression vector using the Ex-Gen 500 transfection reagent (MBI Fermentas, Flamborough, ON, Canada) as directed by the manufacturer (pCDNA3-based vector for the wild-type rabbit B1R coding region in the eukaryotic expression vector pC1-neo-myc (Promega, Madison, WI). The PCR fragment and the pC1-neo-myc vector were digested with EcoRI and XbaI (Invitrogen, Carlsbad, CA) and ligated at room temperature for 2 h. The resultant vector (myc-B1R) contains the myc epitope peptide (MEQKLISEEDLNS) fused in frame with the corresponding rabbit receptor coding sequence at its carboxy terminus. The sequence of the construction was verified (RSVS core laboratory, Pavillon Marchand, Laval University, Ste. Foy, QC, Canada).

Microscopy and Cytofluorometry. HEK 293 cells stably expressing AT1R-B1YFP (Fortin et al., 2003a) were used in confocal microscopy (35-mm petri dishes), precisely as described previously (Sabourin et al., 2002a). COS-1 or HEK 293 cells transiently transfected with myc-B1R coding or control vectors were introduced and submitted to indirect immunofluorescence using an anti-myc monoclonal antibody (clone 9e10, dilution 1:1000; Convance, Richmond, CA) revealed using an Alexa-Fluor 488-labeled anti-mouse goat antibody (dilution 1:1000; Molecular Probes, Eugene, OR), the cells were further observed in epifluorescence microscopy, confocal microscopy, or cytofluorometry (cells used for the latter technique were suspended using 0.5 mM EDTA before immunofluorescence staining). The Elite ESP cytofluorometry apparatus was used (Beckman Coulter, Inc., Fullerton, CA) with the excitation set at 495 nm and the emission recorded above 519 nm; results were analyzed using the Expo software version 2.0.

Immunohot-Based Assays. We tested the effect of the novel B1R antagonist on agonist-induced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) in primary cultures of smooth muscle cells derived from the rabbit aorta and known to express the
B1R (Sabourin et al., 2002b). The assay was performed as described using 75-cm² flasks of serum-starved (24 h) cells (Fortin et al., 2003c). The 9e10 monoclonal antibodies or the alternate anti-myc monoclonal 4A6 (Upstate, Charlottesville, VA) were applied to the immunoblot of myc-B1R (dilution 1:1000–1:500) using the same general methods. Membranes were prepared from 75-cm² flasks of HEK 293 cells transiently expressing myc-B1R or of untransfected cells as described previously (Houle et al., 2000) and submitted to electrophoresis (50 μg of protein per track) followed by transfer and immunoblotting.

Fig. 2. Lack of effect of compound 11 (100 nM) on contractility assays based on the rabbit isolated jugular vein. A, BK-induced contraction mediated by B2 receptors. B, histamine-induced contractions mediated by H1 receptors. Values are the means ± S.E.M. of the number determinations indicated by n. Presentation as in Fig. 1A.

Fig. 3. A to D, representative tracings of the tension developed by rings of rabbit mesenteric artery sequentially treated with an inhibitory drug or their DMSO vehicle, phenylephrine (PE; 4 μM) and the B1R agonist des-Arg9-BK (100 nM, administered on a stable PE-induced contraction plateau). E, relaxing effect of iloprost on the precontracted mesenteric artery. F, effect of compound 11 on a tissue fully relaxed with the B1R agonist. G, lack of direct effect of compound 11 in a resting tissue. Abscissa scale, time; ordinate, isometric contraction (grams). The closed symbols refer to the application of agents and the open symbols, to washout of stimulants. Each experiment was performed at least twice using tissues from different animals.
Data Analysis. When applicable, numerical values are means ± S.E.M. The parameters of the Scatchard (binding saturation data) and Shild (antagonist potency) plots were calculated using a computer program (Tallarida and Murray, 1987).

Results

Effect of Compound 11 on Vascular Contractility in Rabbit Isolated Blood Vessels. Des-Arg^9^-BK-induced contraction in the rabbit aorta incubated for 5.5 h exhibited an average EC_{50} of 180 nM (Fig. 1A) and Lys-des-Arg^9^-BK with an EC_{50} of 47 nM (Fig. 1B). We have elected to calculate antagonist potency based only on the 5.5-h concentration-effect curves, with different control (DMSO vehicle-treated) and drug-treated tissues sampled from the same aortas. The 3.5-h concentration-effect curves constructed in all tissues allowed judgment of the surmountability of the antagonist drugs.

Compound 11 (10 pM–100 nM) antagonized des-Arg^9^-BK-induced contraction in an apparently surmountable and concentration-dependent manner (Fig. 1A). The calculated pA_2 value for compound 11 was 10.6 ± 0.14 (Schild regression; Fig. 1C). The corresponding slope of the regression (0.72 ± 0.06) differed from unity. Similarly, the pA_2 of compound 11 against the alternate high-affinity B_1R agonist Lys-des-Arg^9^-BK was 10.4 ± 1.12 (slope, 0.83 ± 0.07; Fig. 1, B and C). Compound 11 at 100 nM did not significantly influence Ang II-induced contraction of the rabbit aorta (Fig. 1D).

The rabbit isolated jugular vein is an established bioassay for the BK B_2R and the histamine H_1 receptor; compound 11 at 100 nM failed to influence significantly the contractions induced by either BK or histamine in this tissue (Fig. 2). Compound 11 did not exert direct contractile effects on either type of isolated blood vessel (up to 1 μM, Figs. 3G and 4G).

The B_1R agonist des-Arg^9^-BK (100 nM) exerted biphasic effects on the precontracted rabbit mesenteric arteries (Fig. 3A), the relaxing phase amounting to 69.1 ± 5.3% of the contraction plateau (n = 9). This effect was largely prevented by treatment with compound 11 (10 nM; residual relaxing effect 6.3 ± 6.3%, n = 2, P < 0.01 by Dunnett’s test), without influencing the action of the agent used to induce the precontraction (the α-adrenoceptor agonist phenylephrine; Fig. 3B). The relaxing effect of the kinin is mediated by PGs in this system (Churchill and Ward, 1986), but the relevant COX isoform coupled to B_1R mediation has not been determined. The COX-1 and -2 isoform-selective inhibitors SC-560 and NS-398 both attenuated the relaxing effect of des-Arg^9^-BK (residual relaxing effect of 5.82 ± 5.8 and 0.1 ± 8.3, respectively, n = 3, P < 0.01 for both values by Dunnett’s test) (Fig. 3, C and D). The precontracted mesenteric artery rings were relaxed by the PGI2 mimetic drug iloprost (Fig. 3E). Some precontracted tissues were exposed to compound 11 when the full relaxing effect of des-Arg^9^-BK was obtained (Fig. 3F): before reversing the relaxation and regaining the plateau tension, tissues were transiently further relaxed in the minutes after the application of the B_1R antagonist (observed in all replicates; n = 4).

The major mechanical response to B_1R stimulation in the rabbit isolated aorta, contraction, is opposed to the relaxation...
observed in the mesenteric artery. To ascertain that the different experimental procedures do not determine these conclusions, aortic rings precontracted with phenylephrine were stimulated with des-Arg⁹-BK at the contraction plateau (Fig. 4A). Only an additional contractile response to the kinin superimposed to the plateau was observed; adding compound 11 to the tissue baths at the end of the recording relaxed the preparation only to the level of the phenylephrine-induced plateau (Fig. 4A). Other precontracted aortic rings were used to show that the preparation is not responsive to iloprost (Fig. 4B). The rabbit aorta preparation develops sustained contraction plateaus in response to persistent stimulation with several contractile agonists. Compound 11 was applied at the contraction plateau induced by various agents to record any antagonism of the persistent contractile signaling (Fig. 4, C–E). The drug (10 nM) reversed a large fraction of the contraction established using the B₁R agonist Sar-[D-Phe⁸]des-Arg⁹-BK, but at 100 nM failed to significantly relax tissues contracted with α-thrombin, KCl, or the thromboxane mimetic U-46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂α) (Fig. 4).

Effect of Compound 11 on Cell-Based Assays. Rabbit aortic smooth muscle cells express the B₁R in a regulated manner and those are coupled to the phosphorylation of the ERK1/2 MAP kinases (Fortin et al., 2003c). ERK1/2 was phosphorylated in response to Lys-des-Arg⁹-BK in smooth muscle cells derived from the rabbit aorta (Fig. 5). This response was strongly inhibited by the antagonist compound 11 at 100 pM and abrogated at higher concentrations of the drug (≥1 nM), which itself was devoid of direct effect (Fig. 5). Furthermore, human recombinant epidermal growth factor (EGF) has a strong effect on the assay via endogenous receptors, and this effect was not significantly inhibited by compound 11 (100 nM; Fig. 5).

Confocal fluorescence microscopy reveals that B₁R-YFP labels plasma membrane of HEK 293 cells as a continuous line (Fig. 6A; Sabourin et al., 2002a). Compound 11 (1 nM) failed to modify the cellular distribution of B₁R-YFP, whereas Lys-des-Arg⁹-BK (10 nM; 30 min) concentrated fluorescent receptors in structures that remained close to the membrane surface, which is not continuously labeled anymore (the aggregates were previously proposed to be cholesterol rich rafts; Sabourin et al., 2002a). Compound 11 (≥0.1 nM) was effective to prevent the effect of the agonist on cells treated with both agents. An alternate receptor construction based on the same fluorescent protein and same promoter is AT₃R-YFP. Compound 11 failed to inhibit Ang II-induced endocytosis of AT₃R-YFP in HEK 293 cells (Fig. 6B); losartan...
is active in this respect in the same system (Fig. 6B; Fortin et al., 2003a). Accordingly, unlabeled losartan, but not compound 11, displaced \[^3H\]Ang II binding from AT\(_1\)R-YFP-expressing cells (Fig. 6C).

A novel myc-B\(_1\)R construction, based on the rabbit B\(_1\)R, was pharmacologically characterized (Fig. 7). COS-1 cells transiently transfected with the pCI-neo-myc vector (sham transfection) bound little \[^3H\]Lys-des-Arg\(^\alpha\)-BK, whereas cells that expressed myc-B\(_1\)R exhibited specific and saturable binding (Fig. 7A). The affinity estimate derived from Scatchard plot analysis (Fig. 7B) was \(K_D = 0.36\) nM; this value is close to the previously reported estimate in COS-1 cells for the wild-type receptor (Larriveé et al., 2000). The \(B_{\text{max}}\) estimate was 6.45 ± 0.81 fmol/well (Fig. 7, A and B). The pharmacological profile of the myc-B\(_1\)R construction transiently expressed in COS-1 cells was investigated by the competition of \[^3H\]Lys-des-Arg\(^\alpha\)-BK (1 nM) binding to cells by a panel of cold BK-related drugs used at 1 \(\mu\)M (Fig. 7C). The agonists Lys-des-Arg\(^\alpha\)-BK, Lys-BK and des-Arg\(^\alpha\)-BK could displace significantly the radioligand, the first one being optimal, and the peptide antagonist of the B\(_1\)R, B-9858, was also a very effective competitor at 1 \(\mu\)M. However, the selective B\(_2\)R agonist BK and its antagonists Hoe 140, LF 16-0687, and bradyzide (a peptide and two nonpeptides, respectively; Dzidealiewicz et al., 2000; Houle et al., 2000) failed to compete for the radioligand binding to myc-B\(_1\)R (Fig. 7C). All these findings were compatible with the pharmacological profile of the rabbit B\(_2\)R (MacNeil et al., 1995). Compound 11 is a powerful competitor of \[^3H\]Lys-des-Arg\(^\alpha\)-BK binding to myc-B\(_1\)R (IC\(_{50}\) for the specific component of the binding = 83 pM; Fig. 7D; estimated \(K_i = 22\) pM). This value is similar to the \(K_i\) value of 50 pM estimated by Su et al. (2003) for the binding of this antagonist to the wild-type rabbit B\(_1\)R. By comparison, the peptide antagonist B-9858 exhibited an IC\(_{50}\) value of 3 nM (\(K_i\) of 0.8 nM; data not shown).

Immunofluorescence with an effective anti-myc antibody is instrumental in assessing the abundance of the surface myc-B\(_1\)R in a manner independent from ligands. The feasibility of this approach is shown in fixed, but nonpermeabilized, transiently transfected cells (Fig. 8). Cells expressing myc-B\(_1\)R exhibit a specific surface immunofluorescence (COS-1 and HEK 293 cells; Fig. 8, A and B, respectively). COS-1 cells transfected with pCI-neo-myc may theoretically express a 2.6-kDa peptide (MEQKLISEEDLNSRVPLESTRAAASL) that includes the myc epitope and the translation of the multiple cloning site (up to the first stop codon), but this peptide should be restricted to the cytosol; accordingly, the surface labeling of myc is small in these cells (Fig. 8A).

Figure 9 shows the cytofluorometric evaluation of HEK 293 expressing myc-B\(_1\)R as a function of drug treatments (24 h). Untransfected cells exhibited a background fluorescence similar to that of cells stained without the anti-myc antibodies. Treatment with the peptidase-resistant B\(_1\)R agonist Sar-[Phe\(^\beta\)]des-Arg\(^\alpha\)-BK (1 \(\mu\)M), compound 11, or bradyzide did not
A. COS-1 cells

![Image of Confocal microscopy presentation as in Fig. 6.](Image)

**Fig. 8.** Immunofluorescence of the myc epitope in transiently transfected cells (48 h). Cells were fixed but not permeabilized. A, COS-1 cells were studied untransfected or transfected with vectors for myc-B1R or the control vector pCI-neo-myc. Pairs of matched phase contrast and epifluorescence fields are shown one above the other. B, HEK 293 cells were transfected with the vector coding for myc-B1R or remained untransfected. Confocal microscopy presentation as in Fig. 6.

importantly change the distribution of the specific fluorescence (Fig. 9C). Immunoblots of myc-B1R based on the same antibody as those used in cytofluorometry (Fig. 9D) or on an alternate monoclonal (4A6; not shown) revealed a ~42-kDa major protein whose concentration in the membrane extract was not importantly changed by treatments with the agonist or antagonist ligands (Fig. 9D).

**Discussion**

The present experiments provide evidence that compound 11 is an exceptionally potent antagonist, with strong effects as a B1R antagonist at a concentrations as low as 100 pM (contractility, ERK1/2 phosphorylation, B1R-YFP translocation assays). This affinity surpasses that of the alternate nonpeptide B1R antagonist SSR240612 by about 1 log unit in the rabbit aorta contractility assay (pA2 of 9.4; Gougat et al., 2004); on the other hand, the latter drug is more potent at rodent B1Rs than compound 11 (Su et al., 2003), illustrating again the species-specific pharmacological divergences observed for a number of kinin receptor antagonists (Marceau et al., 1998; Burgess et al., 2000). Compound 11 is active as an apparently surmountable antagonist of des-Arg9-BK or Lys-des-Arg9-BK in the rabbit aorta, but the Schild regression slope is significantly inferior to 1, a finding usually interpreted as lack of competitive behavior. However, a progressive increase of B1R agonist maximal effect has been observed in this preparation (Larrivée et al., 2000), a phenomenon attributed to the postisolation formation of these receptors (Sabourin et al., 2002b). This influx of novel surface receptors has been shown to distort the evaluation of both the potency and the competitive behavior of peptide B1R antagonists in this preparation (Larrivée et al., 2000). Nevertheless, the pA2 estimate of 10.4 to 10.6 for compound 11 is congruent with the Ki values of 22 to 50 pM against cloned rabbit B1Rs (Su et al., 2003; present results based on myc-B1R).

Compound 11 also inhibited the biphasic effect of des-Arg9-BK on the precontracted mesenteric artery (Fig. 3). It was known that the COX inhibitor indomethacin reversed the relaxation into a superimposed contraction (Churchill and Ward, 1986; Deblouis and Marceau, 1987) in this system, and that the difference between the rabbit isolated aorta and mesenteric artery is that the first tissue does not respond to exogenous vasorelaxant PGs such as PGE2 and PGI2, but that the latter tissue is exquisitely sensitive to both (Förstermann et al., 1984). We confirmed this difference by showing that the PGL2 mimetic iloprost relaxes only the mesenteric artery. The B1R agonist des-Arg9-BK releases profuse amounts of PGE2 and of the PGI2 metabolite 6-keto-PGF1α, in the bathing fluid of aortic rings and in a manner sensitive to the peptide antagonist Lys-[Leu6]des-Arg9-BK (radioimmunoassay; Levesque et al., 1993). However, indomethacin does not influence the contractile effect of the kinin in this system, consistent with the ideas that the aorta is refractory to relaxant PGs, but that the B1R is efficiently coupled to PG synthesis. The pharmacological approach applied in present experiments shows that both COX-1 and -2 isoforms may participate to PG production controlled by the B1R; thus, the inducible COX-2 molecule can work in concert with the inducible B1R in a simulated inflammatory condition. The transient relaxing effect of compound 11 on mesenteric arteries already relaxed with des-Arg9-BK (Fig. 3F) is not interpreted as a partial agonist effect of the drug but is probably the result of the fact that the contractile component of the tissue response to the kinins is coupled more immediately to receptor stimulation than the B1R-initiated PG-mediated relaxation and consistent with the fact that a small contraction precedes the relaxation in most tissues stimulated with des-Arg9-BK. Addition of compound 11 to mesenteric arteries eventually reverses all components of the kinin effect in about 15 min. The inhibition of B1R-mediated PG release from peripheral, inflamed tissues is a plausible mechanism of the analgesic effect of B1R antagonists in animal models (Perkins et al., 1993), especially for peptide antagonists with little central nervous system penetration. It remains to be determined whether a more lipophilic drug will influence pain perception in a different manner by both a central and a peripheral effect.

Tested as an antagonist of other receptors expressed in rabbit vascular smooth muscle, compound 11 (100 nM) failed to influence the contractile effects of Ang II mediated by AT1Rs, that of BK mediated by B2Rs and the effect of histamine mediated by H1 receptors. Furthermore, the nonpeptide
but it did not importantly influence myc-B1R abundance at type receptor. Compound 11 is presumably cell-permeant, macological properties (affinity, profile), relative to the wild-type receptor. Compound 11 failed to antagonist EGFr-induced ERK1/2 phosphorylation in rabbit aortic smooth muscle cells, implying that the drug is not an antagonist of EGFr, an inhibitor of the EGFr receptor tyrosine kinase activity, or of any further enzyme that links the receptors to the MAP kinase activation. The mechanisms of B1R coupling to the ERK1/2 MAP kinase pathway has not been fully elucidated, but it was observed in inflamed nasal mucosa tissue removed from human allergic patients challenged with Lys-des-Arg9-BK, not in tissue from healthy volunteers (Christiansen et al., 2002). In that report, the B1R-ERK1/2 coupling was speculatively linked to gene expression via the activation of the transcription factor AP-1.

The myc-labeled B1R was documented to conserve its pharmacological properties (affinity, profile), relative to the wild-type receptor. Compound 11 is presumably cell-permeant, but it did not importantly influence myc-B1R abundance at the cell surface; thus, such high-affinity antagonists may not always act as a pharmacological chaperone. This property may apply to some, but not all antagonists, for peptide hormone receptors (Morello et al., 2000; Petäjä-Repo et al., 2002). These considerations may be important for the safety of drugs, as a form of antagonist-induced receptor up-regulation followed by metabolic drug disposition could theoretically exacerbate a pathology associated with this receptor. Chaperone-induced supersensitivity would represent an alternative to the situation where agonist-induced down-regulation is significant and chronic deprivation of receptor stimulation by antagonists leads to supersensitivity and receptor up-regulation (e.g., as for the dopamine D2 receptor blockers; Silvestri et al., 2000). Agonist-induced down-regulation is difficult to observe with recombinant kinin receptors (Houle and Marceau, 2003). A positive feedback mechanism where kinin receptor stimulation determines further B1R expression via the activation of the transcription factor AP-1.

The myc-B1R coding vector (48 h) and option-

A. Number of cells above threshold (% control)

B. Flow cytometry of HEK 293 cells transiently transfected (48 h) and option-

C. Flow cytometry of HEK 293 cells transiently transfected (48 h) with the myc-B1R vector (indirect immunofluorescence). Sample fluorescence distributions of untransfected cells (A) or transfected, control cells (B) are shown. C, percentage of cells above a set threshold as a function of the drug treatment applied to the cell culture before suspension and assay. Drug concentrations and treatment duration: agonist Sar-[d-Phe8]-des-Arg9-BK, 1 μM, 60 min; compound 11, 1 μM, 24 h; DMSO vehicle of the antagonist, 0.1% (v/v), 24 h. Results are the mean ± S.E.M. of two experiments and are normalized as percentage of control. D, the same anti-myc antibodies were applied to immunoblot of membrane extracts from HEK 293 cells transfected or not with the myc-B1R coding vector (48 h) and optionally treated as in panel C for the last 24 h of culture.

Fig. 9. Flow cytometry of HEK 293 cells transiently transfected (48 h) with the myc-B1R vector (indirect immunofluorescence). Sample fluorescence distributions of untransfected cells (A) or transfected, control cells (B) are shown. C, percentage of cells above a set threshold as a function of the drug treatment applied to the cell culture before suspension and assay. Drug concentrations and treatment duration: agonist Sar-[d-Phe8]-des-Arg9-BK, 1 μM, 60 min; compound 11, 1 μM, 24 h; DMSO vehicle of the antagonist, 0.1% (v/v), 24 h. Results are the mean ± S.E.M. of two experiments and are normalized as percentage of control. D, the same anti-myc antibodies were applied to immunoblot of membrane extracts from HEK 293 cells transfected or not with the myc-B1R coding vector (48 h) and optionally treated as in panel C for the last 24 h of culture.

**Acknowledgments**

We thank Nancy Roberge for help with the cytotoxicometric technique and Dr. D. J. Pettibone (Merrick Research Labs) for the gift of compound 11.

**References**


Christiansen SC, Eddleston J, Woessner KM, Chambers SS, Ye R, Pan ZK, and...
Morissette et al.

Fortin JP, Gobeil F Jr, Adam A, Regoli D, and Marceau F (2003c) Do angiotensin-


Dziadulewicz EK, Ritchie TJ, Hallett A, Snell CR, Davies JW, Wrigglesworth R, 

Levesque L, Drapeau G, Grose JH, Rioux F, and Marceau F (1993) Vascular mode of

Larrivee JF, Gera L, Houle S, Bouthillier J, Bachvarov DR, Stewart J, and Marceau 

Fortin JP, Bouthillier J, Bastien L, Bachvarov DR, and Marceau F (2003a) Charac-


Churchill L and Ward PE (1986) Relaxation of isolated mesenteric arteries by

Morello JP, Salahpour A, Laperriere A, Bernier V, Arthur MF, Lonergan M, Petaja-

Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck KA, Ransom 


Shanmugan K, Bataille E, Marin Castano ME, Barascud Y, Hirtz C, Pesquero JB, 


Address correspondence to: Dr. François Marceau, Centre Hospitalier Universitaire de Québec, Centre de recherche, Pavillon L’Hôtel-Dieu de Québec, 11 Côte-du-Palais, Québec, QC, Canada G1R 2J6. E-mail: françois.marceau@crhdq.ulaval.ca