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A Novel Nonpeptide Antagonist of the Kinin B_1 Receptor: Effects at the Rabbit Receptor

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ABSTRACT

The kinin B_1 receptor (B_1R) 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-1*H*-imidazol-2-yl)phenyl]ethyl}acetamide) is a high-affinity nonpeptide antagonist for the human B_1R, but it is potent at the rabbit B_1R as well: its K_i value for the inhibition of [^{3}H]Lys-des-Arg^9-BK (bradykinin) binding to a novel myc-labeled rabbit B_1R 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-Arg^9-BK- or Lys-des-Arg^9-BK-induced contraction of the rabbit isolated aorta (pA_2 values of 10.6 $\pm$ 0.14 and 10.4 $\pm$ 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-Arg^9-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-Arg^9-BK in serum-starved rabbit aortic smooth muscle cells and the agonist-induced translocation of the fusion protein B_1R-yellow fluorescent protein expressed in human embryonic kidney (HEK) 293 cells. Compound 11 does not importantly modify the expression of myc-B_1R 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 B_1R in models of inflammation, pain, and sepsis based on the rabbit.

The kinin B_1 receptor (B_1R) is a G protein-coupled receptor selectively stimulated by sequences related to bradykinin (BK) but not by BK itself. Instead, des-Arg^9-BK, Lys-BK (kallidin) and Lys-des-Arg^9-BK (des-Arg^{10}-kallidin) stimulate the human and rabbit recombinant B_1Rs, the last peptide with the highest affinity (Menke et al., 1994; MacNeil et al., 1995). The B_1R is also strongly regulated by inflammatory conditions, to the point of being largely inducible in several experimental systems (Marceau et al., 1998). The kinin B_1R, expressed by vascular and epithelial cells, fibroblasts, some leukocytes, and sensory and other neurons (Marceau et al., 1998; Ricupero et al., 2000; Christiansen et al., 2002; Shughrue et al., 2003), has attracted the interest of medicinal chemists as a therapeutic target because this molecule is involved in sustained inflammation and inflammatory pain perception (Perkins et al., 1993; Fox et al., 2003; Su et al., 2003).

The regulation of the B_1R at the cell level has revealed a number of findings of interest. Experimental results support a role of inflammatory cytokines and nuclear factor-κB in B_1R induction (Ni et al., 1998; Schanstra et al., 1998; Sabourin et al., 2002b; Passos et al., 2004). In systems where the B_1R expression is inducible over a ~2- to 6-h period by cytokine treatment or tissue injury, protein synthesis inhibitors prevent its surface expression and function (Marceau et al., 1998; Fortin et al., 2003b). Radioligand binding and phosphate labeling have shown that the B_1R neither is subjected

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ABBREVIATIONS: B_1R, B_1 receptor; B_2R, B_2 receptor; BK, bradykinin; YFP, yellow fluorescent protein; Ang II, angiotensin II; AT_1R, AT_1 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-1*H*-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-trifluoromethyl pyrazole; SSR240612, (2*R*)-2-[((3*R*)-3-(1,3-benzodioxol-5-yl)-3-{[(6-methoxy-2-naphtyl)sulfonyl]amino}propanoyl)amino]-3-(4-{[2*R*,6*S*)-2,6-dimethylpiperidinyl]methyl}phenyl)-*N*-isopropyl-*N*-methylpropanamide hydrochloride.

to important agonist-induced endocytosis nor phosphorylated, unlike the related BK B_2 receptor (B_2R) (Faussner et al., 1998; Blaukat et al., 1999). Agonist-induced cellular redistribution has been studied using a fusion protein composed of the rabbit B_1R fused with the yellow fluorescent protein (YFP). Lys-des-Arg^9-BK induces a condensation of cell surface B_1R-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 B_1R 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 B_1R signaling as the inflammatory condition is being resolved.

Several nonpeptide antagonists of the parent BK B_2R 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 B_1R, compound 11 (Fig. 1A), is potent at the rabbit B_1R as well: its K_i value for the inhibition of [^{3}H]Lys-des-Arg^9-BK binding to the rabbit recombinant B_1R expressed in Chinese hamster ovary cells was reportedly 50 pM (Su et al., 2003). SSR240612 is another example of nonpeptide B_1R 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 B_1R. Furthermore, we have studied the possibility that this nonpeptide kinin receptor antagonist alters the surface expression of the B_1R. Previous results based on the vasopressin V_2 and the

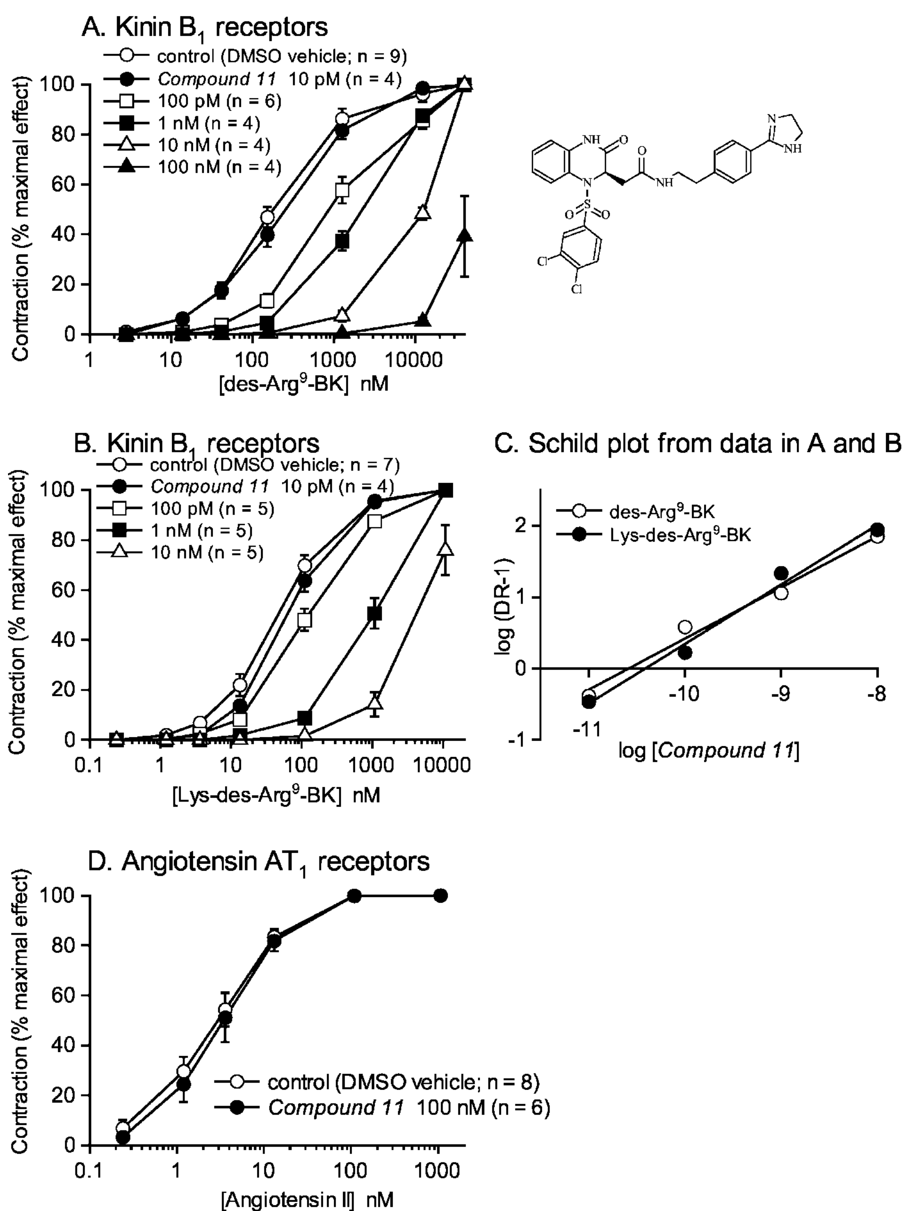


Fig. 1. Effect of compound 11 on contractility assays in the rabbit isolated aorta. A, effect of compound 11 on des-Arg^9-BK-induced contraction mediated by B_1Rs and recorded at time 5.5 h post-tissue mounting. B, effect of compound 11 on Lys-des-Arg^9-BK-induced contraction mediated by B_1Rs. In A 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 AT_1 receptors (recorded at time 7.5 h postmounting). Presentation as in A. See text for analysis.

δ-opiod 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; Petäjä-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-[Hyp^3, Igl^5, D-Igl^7, Oic^8]des-Arg^9-BK), Hoe 140 (icatibant, D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK), and LF 16-0687 (1-[[2,4-dichloro-3-[[(2,4-dimethylquinolin-8-yl)oxy] methyl] phenyl]sulfonyl]-*N*-[3-[[4-(aminoimethyl) phenyl] carbonylamino] propyl]-2(*S*)-pyrrolidinecarboxamide) were gifts from Laboratoires Fournier (Daix, France). These compounds are, respectively, a peptide B_1R antagonist (Larrivée et al., 2000), a peptide, and a nonpeptide B_2R antagonist (Houle et al., 2000). The nonpeptide B_2R antagonist bradyzide (Burgess et al., 2000; Dziadulewicz et al., 2000) is a gift from Dr. E. Dziadulewizc (Novartis, London). Losartan, a nonpeptide antagonist of the angiotensin II (Ang II) AT_1 receptor (AT_1R), and compound 11 (2-{(2*R*)-1-[(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl}-*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}acetamide) were gifts from Merck Research Labs (West Point, PA). The synthesis and some pharmacological properties of compound 11 are reported by Su et al. (2003). Sar-[D-Phe^8]des-Arg^9-BK, a peptidase-resistant B_1R agonist (Marceau et al., 1998), was a gift from Prof. D. Regoli (Faculty of Medicine, University of Sherbrooke, Sherbrooke, Canada). BK and Lys-des-Arg^9-BK (des-Arg^{10}-kallidin) was purchased from Bachem Biosciences (King of Prussia, PA), and purified human α-thrombin was from Calbiochem (La Jolla, CA). The prostacyclin analog iloprost was a gift from Berlex (Lachine, QC, Canada). The other drugs were purchased from Sigma-Aldrich (St. Louis, MO).

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% O_2, 5% CO_2) and warmed (37°C) Krebs' solution as described previously (Larrivée et al., 2000). Some experiments were also performed on rabbit jugular veins, cut into 2-cm strips, and suspended under a tension of 1 g in 5-ml tissue baths containing oxygenated and warmed Krebs' solution to which 1 μM captopril was added (Larrivée et al., 2000) and rings of mesenteric arteries (suspended under a tension of 1 g; Petitclerc et al., 1994).

Contractility studies in the aortic preparation were based upon the construction of cumulative concentration-responses curves for des-Arg^9-BK (a B_1R agonist on this tissue) and an additional one for Ang II (an AT_1R agonist in the aorta; Fortin et al., 2003a). These studies aimed to investigate the potency, surmountability, and specificity of the B_1 receptor antagonist in the vascular smooth muscle preparation. In each tissue, two cumulative concentration-response curves for des-Arg^9-BK were constructed at times 3.5 and 5.5 h from the beginning of the incubation of the preparation. Compound 11 or its DMSO vehicle in control tissues was introduced at time 5 h and maintained in the bathing fluid during the construction of the second curve (effect of the antagonist at 5.5 h). An additional cumulative concentration-response curve for Ang II was constructed in some tissues at time 7.5 h in tissues re-exposed to compound 11 or the DMSO vehicle at time 7 h. In different sets of tissues, two concentration-response curves were constructed at times 3.5 and 5.5 h for des-Arg^9-BK and Lys-des-Arg^9-BK, respectively, to calculate the potency of compound 11 (applied at time 5 h) against the latter high-affinity B_1R agonist. Contractility results were expressed as a percentage of the maximal response recorded in each tissue when constructing the 5.5- or 7.5-h curves.

The contractility experiments in the rabbit jugular vein, which responds to kinins via B_2Rs and histamine via H_1 receptors, were performed to investigate the specificity of the recently produced B_1R antagonist (as in Larrivée 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-Arg^9-BK mostly by a prostaglandin (PG)-mediated relaxation, and this response is acquired in vitro as a function of time (Churchill and Ward, 1986; Deblois 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-Arg^9-BK.

Construction of the Rabbit myc-B_1 Receptor Conjugate (myc-B_1R). To assess the abundance of kinin receptors at the cell surface in a radioligand-independent manner, we produced and characterized a rabbit B_1R labeled with the N-terminal (extracellular) myc epitope. Using the pCDNA3-based vector for the wild-type rabbit B_1R (Larrivée et al., 2000) as a template, the coding region of the B_1R gene was amplified by polymerase chain reaction (PCR). 5′-GAACGAATTCGATGGCCTCACAGGGCCCCCTGGAG-3′ and 5′-TGATTCTAGATTAATTCCGCCAGAAACCCCAGAGCATTTC-3′ were used as sense and anti-sense PCR primers, respectively (primers derived from the rabbit B_1R sequence; MacNeil et al., 1995). The pair of primers contained additional *Eco*R1 and *Xba*I sites, respectively (underlined), needed for the directional cloning of the rabbit B_1R coding region in the eukaryotic expression vector pCI-neo-myc (Promega, Madison, WI). The PCR fragment and the pCI-neo-myc vector were digested with *Eco*R1 and *Xba*I (Invitrogen, Carlsbad, CA) and ligated at room temperature for 2 h. The resultant vector (myc-B_1R) 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).

Binding Assays. The binding of [^{3}H]Lys-des-Arg^9-BK (PerkinElmer Life and Analytical Sciences, Boston, MA; 80 Ci/mmol) to adherent intact COS-1 cells transiently expressing the myc-B_1R 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 (pCI-neo-myc vector as a control). The assays were applied to generate saturation curves and evaluate binding competition by unlabeled drugs. These drugs were present during the 60-min period allowed for radioligand binding equilibration in the binding buffer specific for each assay. Competition of [^{3}H]Ang II binding to HEK 293 cells expressing the fusion protein AT_1R-YFP by unlabeled drug was performed as described previously (Fortin et al., 2003a).

Microscopy and Cytofluorometry. HEK 293 cells stably expressing AT_1R-YFP (Fortin et al., 2003a) or transiently expressing B_1R-YFP (Sabourin et al., 2002a) 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-B_1R coding or control vectors were fixed 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.

Immunoblot-Based Assays. We tested the effect of the novel B_1R 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

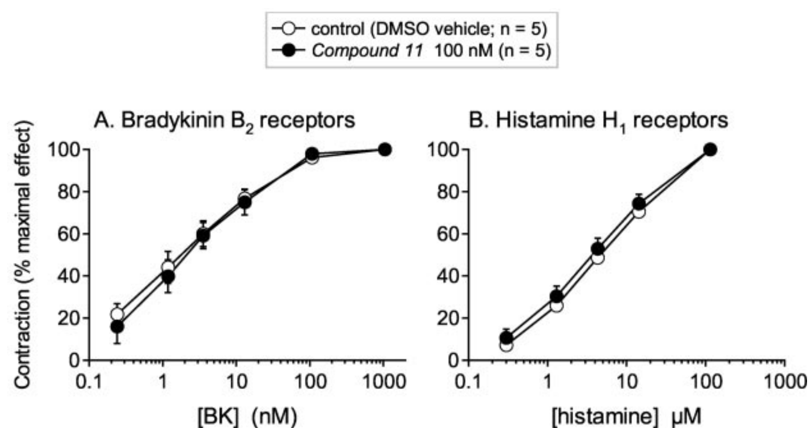


Fig. 2. Lack of effect of compound 11 (100 nM) on contractility assays based on the rabbit isolated jugular vein. A, BK-induced contraction mediated by B_2 receptors. B, histamine-induced contractions mediated by H_1 receptors. Values are the means ± S.E.M. of the number determinations indicated by n. Presentation as in Fig. 1A.

B_1R (Sabourin et al., 2002b). The assay was performed as described using 75-cm^2 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-B_1R (dilution 1:1000–1:500) using the same general methods. Membranes were prepared from 75-cm^2 flasks of HEK 293 cells transiently expressing myc-B_1R 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.

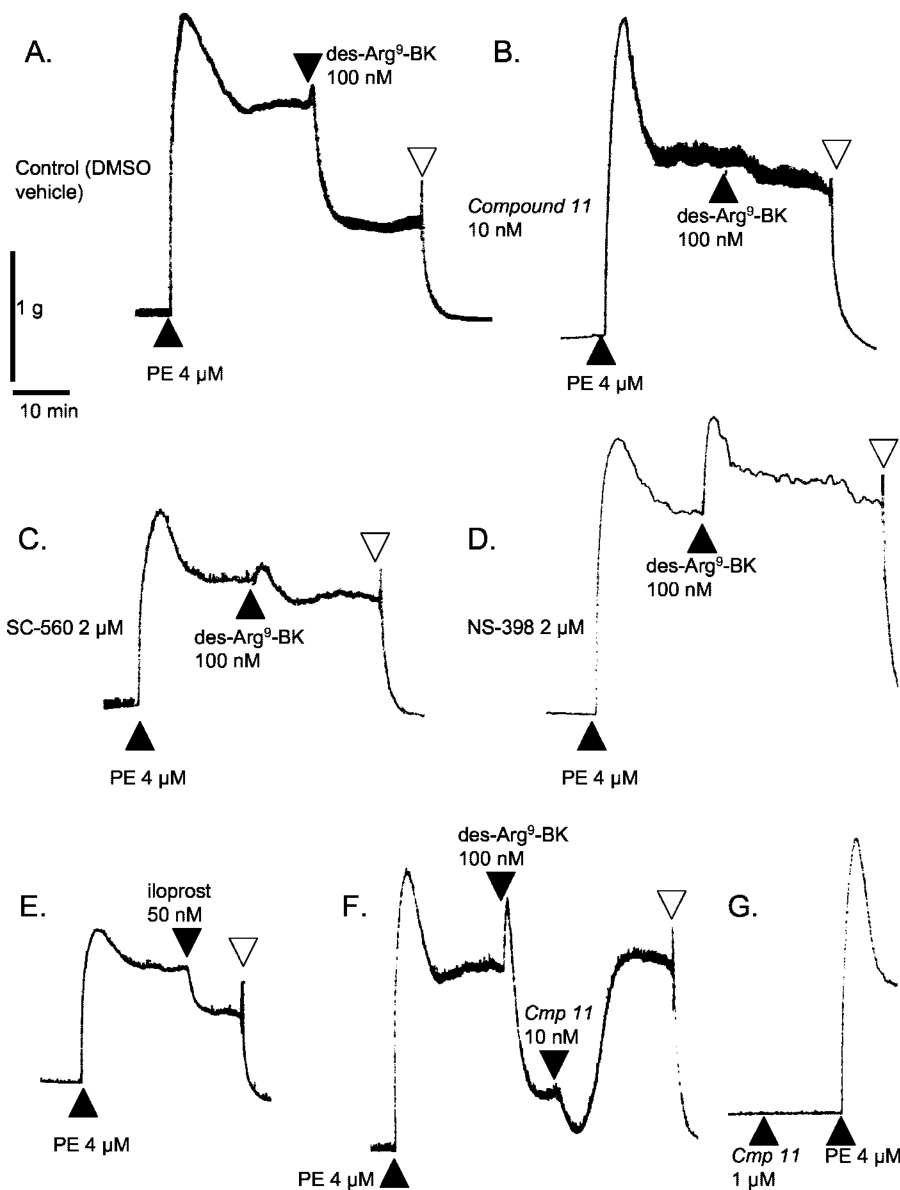


Fig. 3. A to D, representative tracings of the tension developed by rings of rabbit mesenteric artery sequentially treated with an inhibitory drug or their DMSO vehicle, phenylepherine (PE; 4 μM) and the B_1R agonist des-Arg^9-BK (100 nM, administered on a stable PE-induced contraction plateau). E, relaxing effect of iloprost on the precontracted mesenteric artery. F, effect of compound 11 on a tissue fully relaxed with the B_1R 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 PGI_2 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

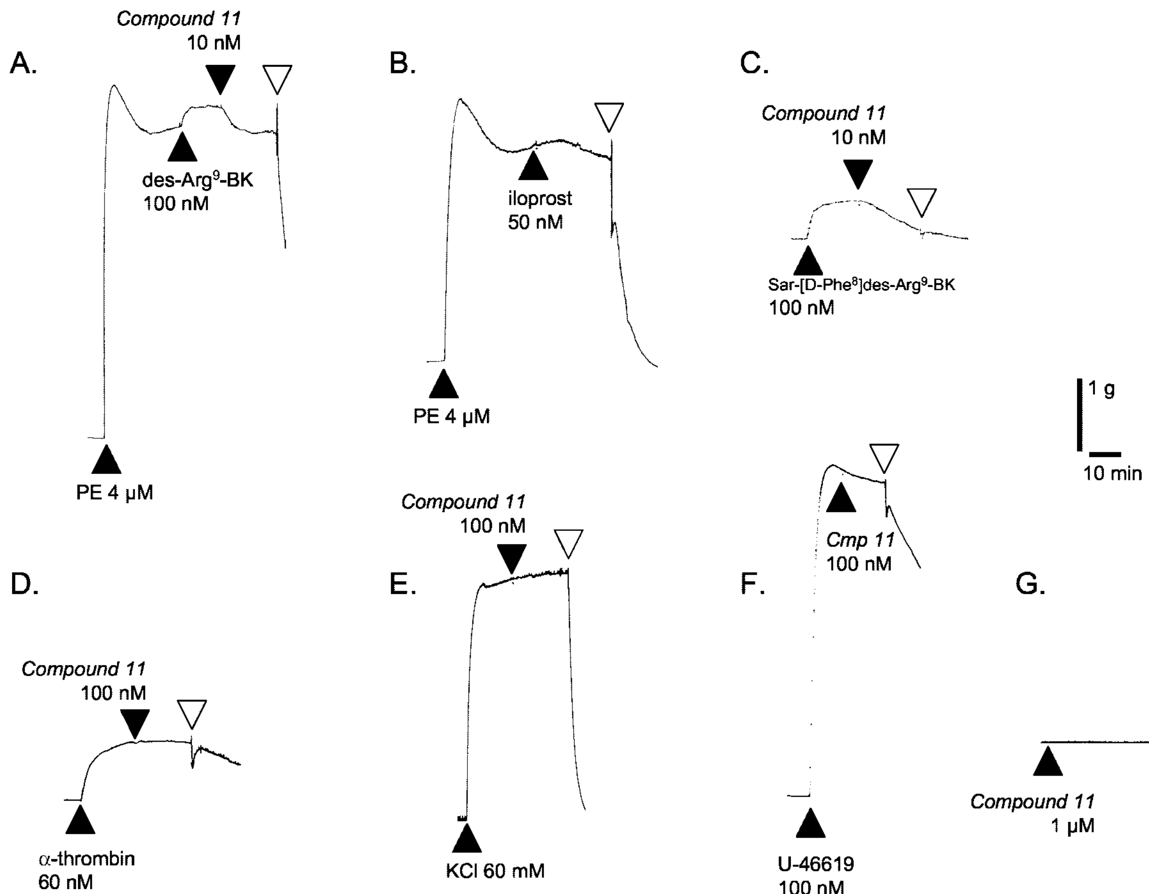


Fig. 4. Representative tracings of the tension developed by rabbit aortic rings in response to various drugs. Presentation as in Fig. 3.

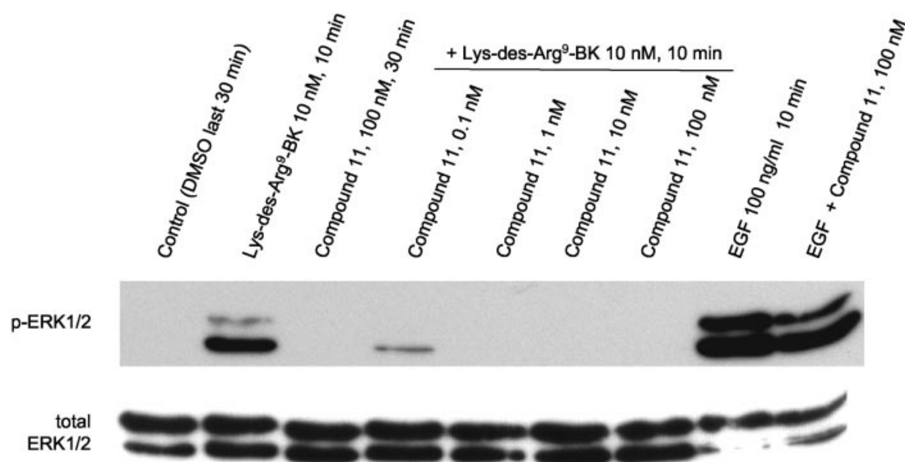


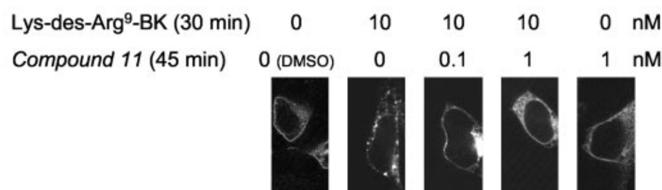
Fig. 5. Effect of compound 11 on ERK1/2 phosphorylation. Serum-starved (24 h) smooth muscle cells derived from the rabbit aorta were treated with Lys-des-Arg^9-BK (10 nM; 10 min), recombinant human EGF (100 ng/ml; 10 min), Compound 11 or its DMSO vehicle (last 30 min of incubation), or a combination of the antagonist with the tested stimulants. The same total cell extracts were immunoblotted for phospho-ERK1/2 and total ERK1/2 (to assess comparable loading of tracks; bands of 42 and 44 kDa). Representative results of two experiments.

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-Arg^9-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 B_1R agonist Sar-[D-Phe^8]des-Arg^9-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 $F_{2\alpha}$) (Fig. 4).

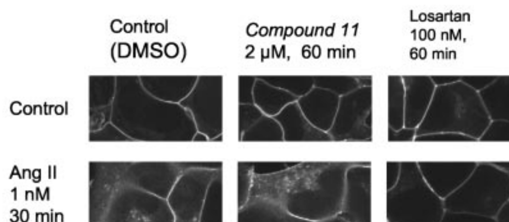
Effect of Compound 11 on Cell-Based Assays. Rabbit aortic smooth muscle cells express the B_1R in a regulated manner and those are coupled to the phosphorylation of the ERK1/2 MAP kinases (Fortin et al., 2003c). ERK1/2 was phosphorylated in response to Lys-des-Arg^9-BK in smooth muscle cells derived 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 ($\geq$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 B_1R-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 B_1R-YFP, whereas Lys-des-Arg^9-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 ($\geq$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 AT_1R-YFP. Compound 11 failed to inhibit Ang II-induced endocytosis of AT_1R-YFP in HEK 293 cells (Fig. 6B); losartan

A. HEK 293 cells transiently expressing B_1R-YFP



B. HEK 293 cells stably expressing AT_1R-YFP



C. Competition of [^{3}H]Ang II (1 nM) binding to AT_1R-YFP

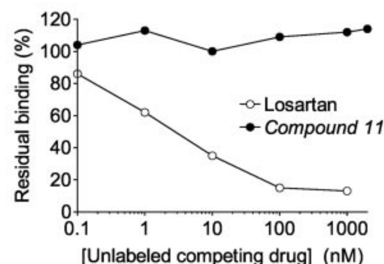


Fig. 6. A, subcellular localization of the B_1R-YFP fusion protein in transiently transfected HEK-293 cells maintained in the complete culture medium and treated with cycloheximide (71 μM) and the indicated concentration of the agonist Lys-des-Arg^9-BK or compound 11 for 30 to 45 min. The large side of rectangular microscopic fields is 40 μm in length. The selected confocal planes are halfway to the thickness of most cells. Representative results of several microscopic fields in two separate days of experiments. B, effect of compound 11 (2 μM) or losartan (100 nM) on Ang II-induced endocytosis of receptors in HEK 293 cells stably expressing AT_1R-YFP. Presentation as in A. C, competition of [^{3}H]Ang II binding to HEK 293 cells stably expressing AT_1R-YFP by unlabeled antagonists. Values are the mean of duplicate determinations.

is active in this respect in the same system (Fig. 6B; Fortin et al., 2003a). Accordingly, unlabeled losartan, but not compound 11, displaced [^{3}H]Ang II binding from AT_1R-YFP-expressing cells (Fig. 6C).

A novel myc-B_1R construction, based on the rabbit B_1R, was pharmacologically characterized (Fig. 7). COS-1 cells transiently transfected with the pCI-neo-myc vector (sham transfection) bound little [^{3}H]Lys-des-Arg^9-BK, whereas cells that expressed myc-B_1R 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 (Larrivée et al., 2000). The B_{max} estimate was 6.45 ± 0.81 fmol/well (Fig. 7, A and B). The pharmacological profile of the myc-B_1R construction transiently expressed in COS-1 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 drugs used at 1 μM (Fig. 7C). The agonists Lys-des-Arg^9-BK, Lys-BK and des-Arg^9-BK could displace significantly the radioligand, the first one being optimal, and the peptide antagonist of the B_1R, B-9858, was also a very effective competitor at 1 μM. However, the selective B_2R agonist BK and its antagonists Hoe 140, LF 16-0687, and bradyzide (a peptide and two nonpeptides, respectively; Dziadulewicz et al., 2000; Houle et al., 2000) failed to compete for the radioligand binding to myc-B_1R (Fig. 7C). All these findings were compatible with the pharmacological profile of the rabbit B_1R (MacNeil et al., 1995). Compound 11 is a powerful competitor of [^{3}H]Lys-des-Arg^9-BK binding to myc-B_1R (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_1R. 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_1R 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_1R 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_1R 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_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (1 μM), compound 11, or bradyzide did not

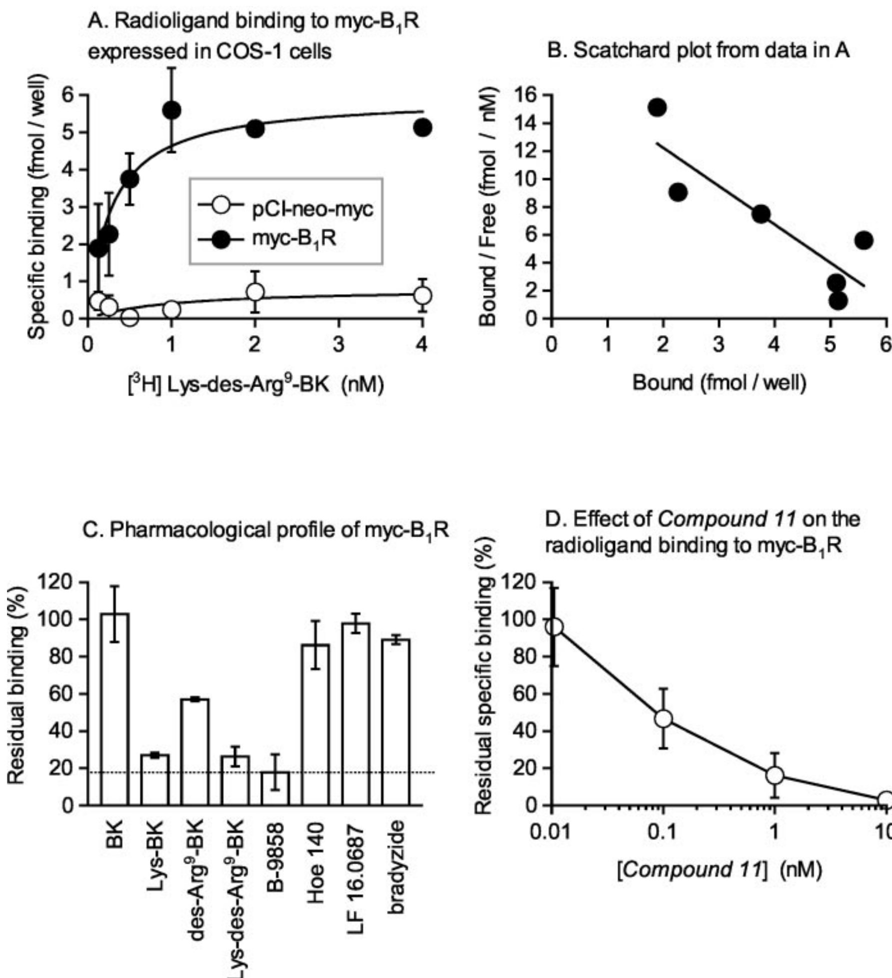
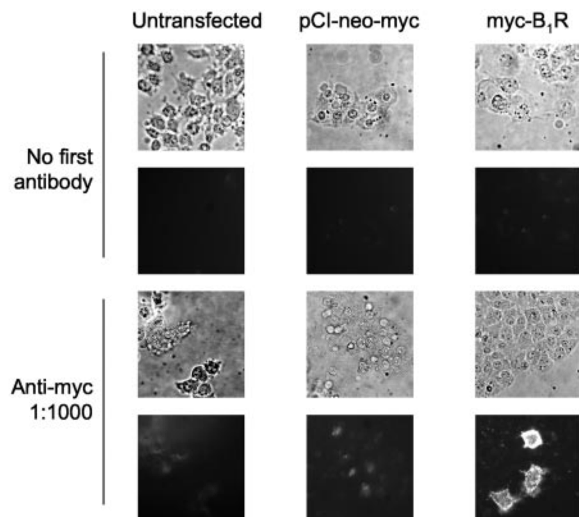


Fig. 7. Pharmacological characterization of myc-B_1R. A, saturation curves in COS-1 cells transfected with the indicated vector. Values are the mean ± S.E.M. of two experiments made of duplicate determinations. B, Scatchard plot derived from binding data in A for myc-B_1R. C, competition of the binding of [^{3}H]Lys-des-Arg^9-BK (1 nM) to COS-1 cells transiently expressing myc-B_1R by a panel of unlabeled drugs (1 μM of each; average ± S.E.M. of two experiments composed of duplicate determinations). The total residual binding is presented (the dotted line represents the nonspecific binding). D, effect of compound 11 on the binding of [^{3}H]Lys-des-Arg^9-BK (1 nM) to COS-1 cells transiently expressing myc-B_1R. Values are the means of four experiments made of duplicate determinations. See text for analysis.



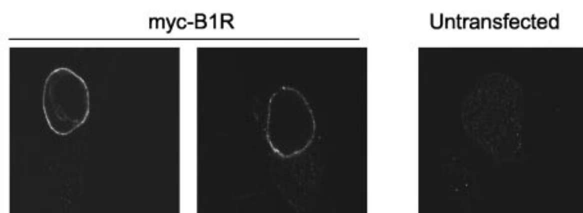


Fig. 8. Immunofluorescence of the myc epitope in transiently transfected cells (48 h). Cells were fixed but not permeabilized. A, COS-1 cells were studied untransfected or transfected with vectors for myc-B_1R or the control vector pCI-neo-myc. Pairs of matched phase contrast and epifluorescence fields are shown one above the other. B, HEK 293 cells were transfected with the vector coding for myc-B_1R or remained untransfected. Confocal microscopy presentation as in Fig. 6.

importantly change the distribution of the specific fluorescence (Fig. 9C). Immunoblots of myc-B_1R 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 B_1R antagonist at a concentrations as low as 100 pM (contractility, ERK1/2 phosphorylation, B_1R-YFP translocation assays). This affinity surpasses that of the alternate nonpeptide B_1R antagonist SSR240612 by about 1 log unit in the rabbit aorta contractility assay (pA_2 of 9.4; Gougat et al., 2004); on the other hand, the latter drug is more potent at rodent B_1Rs 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-Arg^9-BK or Lys-des-Arg^9-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 B_1R 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 B_1R antagonists in this preparation (Larrivée et al., 2000). Nevertheless, the pA_2 estimate of 10.4 to 10.6 for compound 11 is congruent with the K_i values of 22 to 50 pM against cloned rabbit B_1Rs (Su et al., 2003; present results based on myc-B_1R).

Compound 11 also inhibited the biphasic effect of des-Arg^9-BK on the precontacted mesenteric artery (Fig. 3). It was known that the COX inhibitor indomethacin reversed the relaxation into a superimposed contraction (Churchill and Ward, 1986; Deblois 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 PGE_2 and PGI_2, but that the latter tissue is exquisitely sensitive to both (Förstermann et al., 1984). We confirmed this difference by showing that the PGI_2 mimetic iloprost relaxes only the mesenteric artery. The B_1R agonist des-Arg^9-BK releases profuse amounts of PGE_2 and of the PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ in the bathing fluid of aortic rings and in a manner sensitive to the peptide antagonist Lys-[Leu^8]des-Arg^9-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 B_1R 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 B_1R; thus, the inducible COX-2 molecule can work in concert with the inducible B_1R in a simulated inflammatory condition. The transient relaxing effect of compound 11 on mesenteric arteries already relaxed with des-Arg^9-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 B_1R-initiated PG-mediated relaxation and consistent with the fact that a small contraction precedes the relaxation in most tissues stimulated with des-Arg^9-BK. Addition of compound 11 to mesenteric arteries eventually reverses all components of the kinin effect in about 15 min. The inhibition of B_1R-mediated PG release from peripheral, inflamed tissues is a plausible mechanism of the analgesic effect of B_1R 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 AT_1Rs, that of BK mediated by B_2Rs and the effect of histamine mediated by H_1 receptors. Furthermore, the nonpeptide

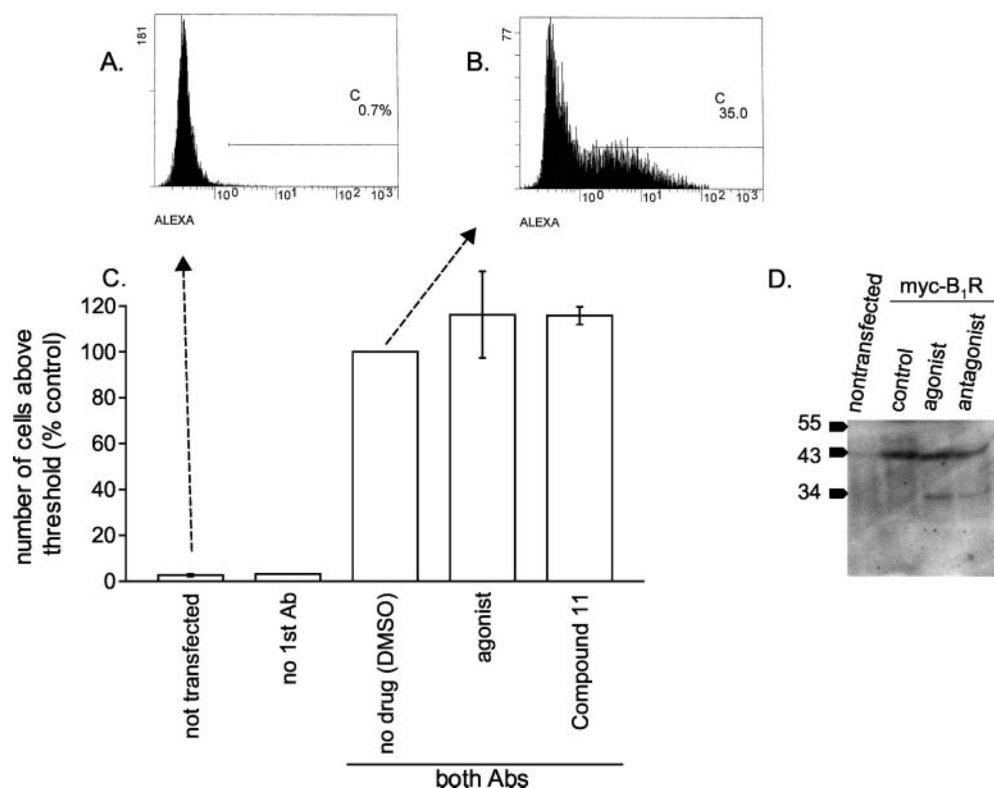


Fig. 9. Cytofluorometry of HEK 293 cells transiently transfected (48 h) with the myc-B_1R vector (indirect immunofluorescence). Sample fluorescence distributions of untransfected cells (A) or transfected, control cells (B) are shown. C, percentage of cells above a set threshold as a function of the drug treatment applied to the cell culture before suspension and assay. Drug concentrations and treatment duration: agonist Sar-[D-Phe^8]-des-Arg^9-BK, 1 μM, 24 h and reapplied 1 h before cell harvesting; compound 11, 1 μM, 24 h; DMSO vehicle of the antagonist, 0.1% (v/v), 24 h. Results are the mean $\pm$ 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-B_1R coding vector (48 h) and optionally treated as in panel C for the last 24 h of culture.

drug did not affect assays based on AT_1R-YFP (binding, agonist-induced endocytosis). The B_2R and AT_1R are the closest relatives of the B_1R based on amino acid sequence homology (Menke et al., 1994). Such a high degree of selectivity for compound 11 effect on kinin receptor subtypes is a practical advance over some recently developed peptide B_1R antagonists (e.g., B-9858), which also antagonize the B_2R with an ~200-fold selectivity for the B_1R (Larrivée et al., 2000). Su et al. (2003) noted that compound 11 exhibits a >5000-fold selectivity for the B_1R in a panel of assays representing hundreds of enzymes, receptors and transporters, significantly better than the reported ~750-fold selectivity for the human B_1R over the human B_2R for the other nonpeptide SSR240612 (Gougat et al., 2004). Compound 11 failed to antagonize EGF-induced ERK1/2 phosphorylation in rabbit aortic smooth muscle cells, implying that the drug is not an antagonist of EGF, an inhibitor of the EGF receptor tyrosine kinase activity, or of any further enzyme that links the receptors to the MAP kinase activation. The mechanisms of B_1R 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-Arg^9-BK, not in tissue from healthy volunteers (Christiansen et al., 2002). In that report, the B_1R-ERK1/2 coupling was speculatively linked to gene expression via the activation of the transcription factor AP-1.

The myc-labeled B_1R 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-B_1R 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 D_2 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 B_1R expression (the "autoregulation hypothesis"; Schanstra et al., 1998; Phagoo et al., 1999) is controversial and may not be widely applicable (Sabourin et al., 2001, 2002b).

In summary, compound 11 is a powerful and selective antagonist suitable for further physiopathological investigations of the role of the kinin B_1R in models of inflammation, pain, and sepsis based on rabbit models. This nonpeptide kinin receptor antagonist does not act as pharmacological chaperone.

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References

Blaukat A, Herzer K, Schroeder C, Bachmann M, Nash N, and Müller-Esterl W (1999) Overexpression and functional characterization of kinin receptors reveal subtype-specific phosphorylation. *Biochemistry* **38:**1300–1309.

Burgess GM, Perkins MN, Rang HP, Campbell EA, Brown MC, McIntyre P, Urban L, Dziadulewicz EK, Ritchie TJ, Hallett A, et al. (2000) Bradyzide, a potent non-peptide B_2 bradykinin receptor antagonist with long-lasting oral activity in animal models of inflammatory hyperalgesia. *Br J Pharmacol* **129:**77–86.

Christiansen SC, Eddleston J, Woessner KM, Chambers SS, Ye R, Pan ZK, and

Zuraw BL (2002) Up-regulation of functional kinin B1 receptors in allergic airway inflammation. *J Immunol* **169:**2054–2060.
Churchill L and Ward PE (1986) Relaxation of isolated mesenteric arteries by des-Arg[9]-bradykinin stimulation of B_1 receptors. *Eur J Pharmacol* **130:**11–18.
Deblois D and Marceau F (1987) The ability of des-Arg[9]-bradykinin to relax rabbit isolated mesenteric arteries is acquired during in vitro incubation. *Eur J Pharmacol* **142:**141–144.
Dziadulewicz EK, Ritchie TJ, Hallett A, Snell CR, Davies JW, Wrigglesworth R, Dunstan AR, Bloomfield GC, Drake GS, McIntyre P, et al. (2002) Nonpeptide bradykinin B_2 receptor antagonists: conversion of rodent-selective bradyzide analogues into potent, orally-active human bradykinin B_2 receptor antagonists. *J Med Chem* **45:**2160–2172.
Dziadulewicz EK, Ritchie TJ, Hallett A, Snell CR, Ko SY, Wrigglesworth R, Hughes GA, Dunstan AR, Bloomfield GC, Drake GS, et al. (2000) 1-(2-Nitrophenyl)thiosemicarbazides: a novel class of potent, orally active non-peptide antagonist for the bradykinin B_2 receptor. *J Med Chem* **43:**769–771.
Faussner A, Proud D, Towns M, and Bathon JM (1998) Influence of the cytosolic carboxyl termini of human B1 and B2 kinin receptors on receptor sequestration, ligand internalization and signal transduction. *J Biol Chem* **273:**2617–2623.
Förstermann U, Hertting G, and Neufang B (1984) The importance of endogenous prostaglandins other than prostacyclin, for the modulation of contractility of some rabbit blood vessels. *Br J Pharmacol* **81:**623–630.
Fortin JP, Bouthillier J, Bastien L, Bachvarov DR, and Marceau F (2003a) Characterization of a fluorescent conjugate of the rabbit angiotensin AT_1 receptor. *Br J Pharmacol* **138:**1495–1504.
Fortin JP, Bouthillier J, and Marceau F (2003b) High agonist-independent clearance of rabbit kinin B_1 receptors in cultured cells. *Am J Physiol* **284:**H1647–H1654.
Fortin JP, Gobeil F Jr, Adam A, Regoli D, and Marceau F (2003c) Do angiotensin-converting enzyme inhibitors directly stimulate the kinin B_1 receptor? *Am J Physiol* **285:**H277–H282.
Fox A, Wotherspoon G, McNair K, Hudson L, Patel S, Gentry C, and Winter J (2003) Regulation and function of spinal and peripheral neuronal B_1 bradykinin receptors in inflammatory mechanical hyperalgesia. *Pain* **104:**683–691.
Gougat J, Ferrari B, Sarran L, Planchenault C, Poncelet M, Maruani J, Alonso R, Cudennec A, Croci T, Guagnini F, et al. (2004) SSR240612, a new non-peptide antagonist of the bradykinin B_1 receptor. Biochemical and pharmacological characterization. *J Pharmacol Exp Ther* **309:**661–669.
Houle S, Larrivée JF, Bachvarova M, Bouthillier J, Bachvarov DR, and Marceau F (2000) Antagonist-induced intracellular sequestration of rabbit bradykinin B_2 receptor. *Hypertension* **35:**1319–1325.
Houle S and Marceau F (2003) Wortmannin alters the intracellular trafficking of the bradykinin B_2 receptor: role of phosphoinositide 3-kinase and Rab5. *Biochem J* **375:**151–158.
Kaplanski J, Pruneau D, Asa I, Artru AA, Azez A, Ivashkova Y, Rudich Z, and Shapira Y (2002) LF 16-0687 Ms, a bradykinin B_2 receptor antagonist, reduces brain edema and improves long-term neurological function recovery after closed head trauma in rats. *J Neurotrauma* **19:**953–964.
Larrivée JF, Gera L, Houle S, Bouthillier J, Bachvarov DR, Stewart J, and Marceau F (2000) Non-competitive pharmacological antagonism at the rabbit B_1 receptor. *Br J Pharmacol* **131:**885–892.
Levesque L, Drapeau G, Grose JH, Rioux F, and Marceau F (1993) Vascular mode of action of kinin B_1 receptors and development of a cellular model for the investigation of these receptors. *Br J Pharmacol* **109:**1254–1262.
MacNeil T, Bierilo KK, Menke JG, and Hess JF (1995) Cloning and pharmacological characterization of a rabbit bradykinin B_1 receptor. *Biochim Biophys Acta* **1264:**223–228.
Marceau F, Hess JF, and Bachvarov DB (1998) The B_1 receptors for kinins. *Pharmacol Rev* **50:**357–386.
Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck KA, Ransom RW, Strader CD, Linemeyer DL, and Hess FJ (1994) Expression cloning of a human B_1 bradykinin receptor. *J Biol Chem* **269:**21583–21586.
Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, et al. (2000) Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J Clin Investig* **105:**887–895.
Ni A, Chao L, and Chao J (1998) Transcription factor nuclear factor κB regulates the inducible expression of the human B_1 receptor gene in inflammation. *J Biol Chem* **273:**2784–2791.
Passos GF, Fernandez ES, Campos MM, Araujo JGVC, Pesquero JL, Souza GEP, Avellar MCW, Teixeira MM, and Calixto JB (2004) Kinin B_1 receptor up-regulation after lipopolysaccharide administration: role of proinflammatory cytokines and neutrophil influx. *J Immunol* **172:**1839–1847.
Perkins MN, Campbell E, and Dray A (1993) Antinociceptive activity of the bradykinin B_1 and B_2 receptor antagonists, des-Arg[9], [Leu[8]]-BK and HOE 140, in two models of persistent hyperalgesia in the rat. *Pain* **53:**191–197.
Petäjä-Repo UE, Hogue M, Bhalla S, Laperrière A, Morello JP, and Bouvier M (2002) Ligands act as pharmacological chaperones and increase the efficiency of δ opioid receptor maturation. *EMBO (Eur Mol Biol Organ) J* **21:**1628–1637.
Petitclerc E, Poubelle PE, and Marceau F (1994) Rapid protein synthesis and turnover is involved in interleukin-1-induced relaxation of the rabbit isolated mesenteric artery. Analysis of the arachidonate cascade. *J Pharmacol Exp Ther* **268:**1419–1425.
Phagoo SB, Poole S, and Leeb-Lundberg LM (1999) Autoregulation of bradykinin receptors: agonists in the presence of interleukin-1beta shift the repertoire of receptor subtypes from B2 to B1 in human lung fibroblasts. *Mol Pharmacol* **56:**325–333.
Pruneau D, Paquet JL, Luccarini JM, Defrène E, Fouchet C, Franck RM, Loillier B, Robert C, Bélichard P, Duclos H, et al. (1999) Pharmacological profile of LF 16-0687, a new potent nonpeptide bradykinin B_2 receptor antagonist. *Immunopharmacology* **43:**187–194.
Ricupero DA, Romero JR, Rishikof DC, and Goldstein RH (2000) Des-Arg[10]-kallidin engagement of the B1 receptor stimulates type I collagen synthesis via stabilization of connective tissue growth factor mRNA. *J Biol Chem* **275:**12475–12480.
Sabourin T, Bastien L, Bachvarov DR, and Marceau F (2002a) Agonist-induced translocation of the kinin B_1 receptor to caveolae-related rafts. *Mol Pharmacol* **61:**546–553.
Sabourin T, Guay K, Houle S, Bouthillier J, Bachvarov DR, Adam A, and Marceau F (2001) Absence of ligand-induced regulation of kinin receptor expression in the rabbit. *Br J Pharmacol* **133:**1154–1162.
Sabourin T, Morissette G, Bouthillier J, Levesque L, and Marceau F (2002b) Expression of kinin B_1 receptor in fresh or cultured rabbit aortic smooth muscle: role of NF-κB. *Am J Physiol* **283:**H227–H237.
Schanstra JP, Bataille E, Marin Castano ME, Barascud Y, Hirtz C, Pesquero JB, Pêcher C, Gauthier F, Girolami JP, and Bascands JL (1998) The B_1-agonist [des-Arg[10]]-kallidin activates transcription factor NF-κB and induces homologous upregulation of the bradykinin B_1-receptor in cultured human lung fibroblasts. *J Clin Investig* **101:**2080–2091.
Shughrue PJ, Ky B, and Austin CP (2003) Localization of B1 bradykinin receptor mRNA in the primate brain and spinal cord: an in situ hybridization study. *J Comp Neurol* **465:**372–384.
Silvestri S, Seeman MV, Negrete JC, Houle S, Shammi CM, Remington GJ, Kapur S, Zipursky RB, Wilson AA, Christensen BK, et al. (2000) Increased dopamine D2 receptor binding after long-term treatment with antipsychotics in humans: a clinical PET study. *Psychopharmacology* **152:**174–180.
Su DS, Markowitz MK, DiPardo RM, Murphy KL, Harrell CM, O'Malley SS, Ransom RW, Chang RS, Ha S, Hess FJ, et al. (2003) Discovery of a potent, non-peptide bradykinin B_1 receptor antagonist. *J Am Chem Soc* **125:**7516–7517.
Tallarida RJ and Murray RB (1987) *Manual of Pharmacologic Calculations with Computer Programs*. Springer, New York.
Zausinger S, Lumenta DB, Pruneau D, Schmid-Elsaesser R, Plesnila N, and Baethmann A (2002) Effects of LF 16-0687 Ms, a bradykinin B_2 receptor antagonist, on brain edema formation and tissue damage in a rat model of temporary focal cerebral ischemia. *Brain Res* **950:**268–278.

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