



Bradykinin Receptors: Agonists, Antagonists, Expression, Signaling, and Adaptation to Sustained Stimulation

François Marceau, MD, PhD¹; Johanne Bouthillier¹; Steeve Houle, PhD¹; Thierry Sabourin, MS, MD¹; Jean-Philippe Fortin, PhD¹; Guillaume Morissette, PhD¹; Robert Lodge, PhD²; Sébastien Fortin, PhD³; René C.-Gaudreault, BPharm, LPharm, PhD⁴; Marie-Thérèse Bawolak, PhD¹; Gérémy A. Koumbadinga, PhD¹; Caroline Roy, MS¹; Xavier Charest-Morin, BS¹; and Lajos Gera, PhD⁵

¹Centre de Recherche en Rhumatologie et Immunologie, CHU de Québec, Québec, QC, Canada; ²Laboratory of Human Retrovirology, Institut de Recherches Cliniques de Montréal, Montreal, QC, Canada; ³Département de Chimie-Biologie, Université du Québec à Trois-Rivières, Trois-Rivières, QC, Canada; ⁴Unité des Biotechnologies et de Bioingénierie, CHU de Québec, Québec, QC, Canada; and ⁵Department of Biochemistry, University of Colorado Denver, Aurora, CO, USA

ABSTRACT

Bradykinin-related peptides, the kinins, are blood-derived peptides that stimulate 2 G protein-coupled receptors, the B₁ and B₂ receptors (B₁R, B₂R). The pharmacologic and molecular identities of these 2 receptor subtypes will be succinctly reviewed, with emphasis on drug development, receptor expression, signaling, and adaptation to persistent stimulation. Peptide and nonpeptide antagonists and fluorescent ligands have been produced for each receptor. The B₂R is widely and constitutively expressed in mammalian tissues, whereas the B₁R is mostly inducible under the effect of cytokines during infection and immunopathology. Both receptor subtypes mediate the vascular aspects of inflammation (vasodilation, edema formation). On this basis, icatibant, a peptide antagonist of the B₂R, is approved in the management of hereditary angioedema attacks. Other clinical applications are still elusive despite the maturity of the medicinal chemistry efforts applied to kinin receptors. While both receptor subtypes are mainly coupled to the G_q protein and related second messengers, the B₂R is temporarily desensitized by a cycle of phosphorylation/endocytosis followed by recycling, whereas the nonphosphorylatable B₁R is relatively resistant to desensitization and translocated to caveolae on activation.

INTRODUCTION

The pharmacology of bradykinin (BK)-related peptides, the kinins, has come a long way in recent decades, with the pharmacologic and then the molecular definition of 2 G protein-coupled receptors (GPCRs) that mediate their cellular actions, the B₁ and B₂ receptors (B₁R, B₂R).¹ Additional modern research tools include mouse strains in which one or both of the genes coding for kinin receptors have been deleted ("gene knockout" models).² The formation of kinins and their degradation by interesting peptidases, such as angiotensin-converting enzyme, will not be covered in the present text. The arduous nature of the analytic biochemistry of BK-related peptides is outlined elsewhere.³ We rather propose an excursion into the pharmacology of kinins, the first author having been a spectator and an actor in the field for the past 35 years. There will be a strong autobiographic bias in the selection of the illustration material. We hope this partiality will be forgiven in light of the generous invitation he has received to summarize the field during an oral presentation at the 8th C1-Inhibitor Deficiency Workshop.

BRADYKININ RECEPTOR LIGANDS: AGONISTS AND ANTAGONISTS

The receptors for bradykinin were initially defined in the late 1970s and 1980s using pharmacologic criteria. The B₁R was historically the first defined using both a typical potency order of agonists and a class of specific antagonists. This somewhat atypical receptor subtype is optimally responsive to fragments of the native kinins (BK and Lys-BK) in which the Arg⁹ residue has been removed (des-Arg⁹-BK, Lys-des-Arg⁹-BK, respectively; Fig. 1).⁴ The early peptide antagonists were simply des-Arg⁹ sequences in which Phe⁸ was replaced with a residue possessing an aliphatic side chain, such as Leu. In retrospect, it is now clear that the native kinins produced by the kallikreins, either BK or Lys-BK (kallidin), are selective agonists of the physiologically prominent B₂R subtype that has been consolidated with specific peptide antagonists in the 1980s by Professor John M. Stewart and colleagues. The B₂R antagonists typically possess a constrained peptide backbone due to the inclusion of bulky nonnatural amino acids that also confer resistance



to inactivation by peptidases (Fig. 1). Icatibant (Firazyr® Shire Human Genetic Therapies, Inc, Lexington, Massachusetts, Hoe 140)⁵ is an excellent prototype of the peptide B₂R antagonists exploited in hundreds of basic science studies; it is presently approved for the management of hereditary angioedema attacks.⁶ Icatibant is apparently a competitive (surmountable) antagonist at the human form of the B₂R, but the peptide may be insurmountable and/or a partial agonist in other mammalian species.^{7,8} The des-Arg⁹ form of icatibant is predominantly a B₁R antagonist,¹ showing that the constrained peptide design is also viable at this receptor subtype; other highly specific peptide antagonists of this type include B-9958 (Fig. 1).

Peptidase-resistant agonists selective for either receptor type are interesting laboratory tools, and one of the selective B₂R agonists of this category, B-9972, has distinctive effects on B₂R cycling (see below). Another selective B₂R agonist resistant to carboxypeptidases, labradimil (Cereport®, formerly manufactured by Alkermes, Inc. Cambridge, MA, RMP-7; Fig. 1), has reached clinical development as an adjuvant to chemotherapy for brain tumors. In this case, the proinflammatory effect of BK was deliberately exploited to temporarily open the blood-brain barrier. However, a trial of labradimil as an adjuvant of carboplatin was inconclusive in children with brain tumors.⁹ Current preclinical research indicates that a B₁R agonist resistant to peptidases, Sar-Lys[D-Phe⁸]desArg⁹-BK, has a superior potential to open the blood-brain barrier at the level of gliomas, the B₁R being selectively expressed at lesion sites.¹⁰

The pharmaceutical industry has developed a large number of nonpeptide antagonists for both BK receptor subtypes¹¹; let us mention only one for each. The B₂R antagonist anatibant (LF 16-0687; Fig. 1) was used in a clinical trial for the prevention of brain edema after head injury (an inconclusive trial).¹² The clinical development of the brain penetrant B₁R antagonist MK-0686¹³ as an analgesic was interrupted after at least 2 trials for inflammatory pain, apparently because of lack of efficacy.¹¹ The anti-inflammatory effect of kinin receptor antagonists, well established in numerous preclinical studies, has not been evaluated in humans, but is of considerable potential interest. For instance, in inflammatory bowel disease, diarrheal symptoms may be driven by both BK receptor subtypes that mediate chloride and water secretion at the level of the intestinal epithelium.¹⁴

In the course of structure-activity studies of nonpeptide B₂R antagonists, Fujisawa scientists have discovered several nonpeptide partial agonists of this receptor. Compound 47a, illustrated in Figure 1, is one of them; it has limited structural commonalities with anatibant and is an intriguing laboratory tool.¹⁵

Docking models for peptide and some nonpeptide ligands have been proposed for both the B₁ and B₂Rs.¹ They

notably explain why the B₁R excludes peptides possessing the Arg⁹ residue and also some species discrepancies, for example, the fact that the presence of Lys at the position “zero” is critical for good affinity at the human form of the B₁R. The models predict that the C-terminus of the ligands plunges into the central receptor cavity, whereas the N-terminus remains closer to the extracellular fluid. Based on these premises, Professor Lajos Gera has developed the full set of fluorescent agonists and antagonists for both the B₁R and B₂R by prolonging their structure at the N-terminus (Fig. 1).¹⁶

BRADYKININ RECEPTORS: EXPRESSION AND SIGNALING

In mammalian genomes, the 2 genes encoding the BK B₂R and B₁R, termed *BDKRB2* and *BDKRB1*, respectively, are located next to each other, in tandem and in this order (Fig. 2, marker 1). In the human chromosome 14q32 region schematically represented (about 70 kb, from position 94.66 to 94.73 Mb), the 3 major exons of each gene are shown (not precisely to scale).¹ B₂R expression is constitutive in a large number of tissues. The B₁R is an exceptional GPCR that is inducible, notably under the influence of tissue injury, cytokines, and the signaling systems mentioned in Figure 2.^{17,18} The injection of bacterial lipopolysaccharide in laboratory animals, including a primate species, is a historic model to sensitize the whole cardiovascular system to B₁R agonists, with such responses as hypotension, vasodilation, and increased vascular permeability (Fig. 2, marker 2).^{19,20} This can be modeled at the cellular level: for instance, the B₁R is synergistically upregulated in human umbilical vein endothelial cells (HUVECs) by cotreatment with tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (mRNA measurements or radioligand binding assay, Fig. 2, marker 3, graph on right).¹⁸ Thus, B₁R induction is clearly regulated at the transcriptional level, with very small concentrations in healthy tissues, and extends the organism's response to a broader spectrum of kinin metabolites as a function of time and physiological state. Although the cellular concentration of the mRNA coding for B₂R often varies in parallel with that of B₁R in immunopathology or in response to cytokines (as in HUVECs),¹⁸ the former receptor subtype does not seem much regulated at the level of transcription, possibly because the B₂R protein is constitutive, recycled, and long-lived, as we will see below. Baseline populations of B₁Rs are also observed in cultured cells that are assumed to be resting, as in radioligand binding studies applied to human vascular cells,^{17,18} for example, in HUVECs maintained in commercial endothelial cell growth medium (Fig. 2, marker 3). However, it seems to be an artifact linked to cell stimulation with serum, growth factors, and culture conditions. Stimulation with various cytokines (TNF- α , IFN- γ , interleukin-1, epidermal growth factor etc.) clearly upregulates the B₁R protein and function in these models.



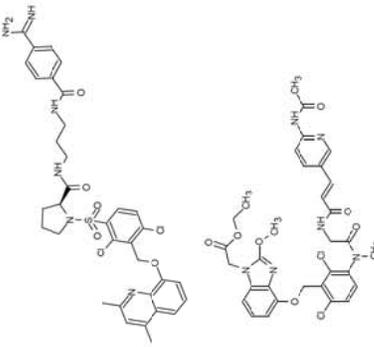
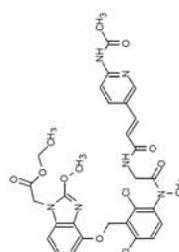
Ligands	B ₂ R		B ₁ R	
	Developed Structure	Ligands	Fluorescent Version	Ligands
Natural peptide agonists	Met-Lys-BK-Ser-Ser	BK* Lys-BK PR3 kinin ³⁷	CF-ε-ACA-BK ²⁹ B-10378 ³⁵	Lys-des-Arg ⁹ -BK* des-Arg ⁹ -BK
Peptidase-resistant agonists	[Hyp ³ , Thi ⁵ , 4-Me-Tyr ⁶ ψ(CH ₂ -NH)-Arg ⁷]-BK D-Arg-[Hyp ³ , Igl ⁵ , Oic ⁷ , Igl ⁸]-BK	labradimil ³⁸ B-9972 ³¹	FTC-B-9972 ¹⁶	Sar-[D-Phe ⁷] ^{des} -Arg ⁹ -BK ³⁷ Sar-Lys[D-Phe ⁸] ^{des} -Arg ⁹ -BK ¹⁰
Peptide antagonists	D-Arg-[Hyp ³ , Thi ⁵ , D-Tic ⁷ , Oic ⁸]-BK D-Arg-[Hyp ³ , Igl ⁵ , D-Igl ⁷ , Oic ⁸]-BK	icatibant ⁵ B-9430	B-10380 ⁴¹ FTC-B-9430 ¹⁶	Lys-Lys-[Hyp ³ , CpG ⁵ , D-Tic ⁷ , CpG ⁸] ^{des} -Arg ⁹ -BK
Nonpeptide antagonists		anantibant ⁴²		MK-0686 ¹³
Nonpeptide partial agonist		compound 47a ¹⁵		

Figure 1. Selected ligands of the bradykinin receptors. For peptides, the reference structure is the nonapeptide bradykinin (BK; H-Arg¹-Pro²-Pro³-Gly⁴-Phe⁵-Ser⁶-Pro⁷-Phe⁸-Arg⁹-OH). Des-Arg⁹ indicates a peptide with the Arg⁹ residue removed. Nonnatural amino acids: CF, 5(6)-carboxyfluorescein; CpG, α-cyclopentylglycine; ε-ACA, ε-aminocaproic acid; FTC, fluorescein-5-thiocarbamoyl; Hyp, trans-4-hydroxyproline; Igl, α-(2-indanyl)glycine; Oic: (3as,7as)-octahydroindole-2-carboxylic acid; Sar, sarcosine; Thi, β-2-thienylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. *Commercially available as a tritiated radioligand.



Both B₁ and B₂Rs are mainly coupled to protein G_q, itself activating a phosphoinositide phospholipase C-β that hydrolyzes phosphatidylinositol 4,5-bisphosphate present in the plasma membrane into inositol 1,4,5-trisphosphate (IP₃) + diacylglycerol (Fig. 2, marker 4). The latter lipid stays in the membrane and recruits various isoforms of protein kinase C that may sequentially determine the activation of the MEK/ERK mitogen-activated protein kinases and c-Fos expression and phosphorylation.^{15,21} The phosphosugar IP₃ is the activator of the IP₃ receptor of the endoplasmic reticulum (ER) that is a high conductance ligand-gated calcium channel. Thus, stimulation of either BK receptor subtype rapidly releases ER Ca²⁺ stores to raise cytosolic Ca²⁺ concentrations. This was assessed by the variation of FURA-2 fluorescence read at 510 nm under excitation at 340 nm in HUVECs cultured as described^{18,22}; cells were stimulated with BK with or without a submaximal concentration of the B₂R antagonist anantibant (unpublished results from our laboratory, Fig. 2, marker 3, left graph). Other mechanisms may contribute to late Ca²⁺ entry from the extracellular fluid in activated vascular cells.²³

Ca²⁺ activates relevant enzymatic systems in endothelial cells, either directly (cytosolic phospholipase A₂) or via its binding to calmodulin (endothelial nitric oxide synthase; myosin light chain kinase, MLCK). Messenger molecules produced by the endothelium, of which NO and prostaglandin I₂ are prime examples, relax the arteriolar smooth muscle cells (Fig. 2, marker 5), supporting the vasodilation that is the basis of specific cardinal signs of inflammation (*rubor, calor*). The MLCK-mediated activation of the contraction of actin-myosin cytoskeleton leads to the retraction of endothelial cells, making them leaky, particularly at the level of postcapillary venules, and to the exocytosis of Weibel-Palade bodies containing P-selectin.²⁴ This leads to the extravasation of fluid, proteins and, eventually, inflammatory cells that supports *tumor*, tissue edema. As kinins stimulate the abundant afferent nerves ending present in tissues (eg, in airway mucosae), neurogenic inflammation driven by the antidromic release of proinflammatory neuropeptides and tissue leukocytes responsive to them can amplify edema.²⁵

The proinflammatory effects of BK-related peptides are largely asymmetric, favoring the vascular manifestations of the process vs leukocyte recruitment; there are few credible effects of kinins on blood leukocytes. The B₁R gene knockout mouse exhibits a minor deficit of phagocyte migration at inflammatory sites; this effect may be primarily vascular as des-Arg⁹-BK increases chemokine production in the endothelium, the likely explanation for the B₁R-mediated facilitation of neutrophil extravasation.²⁶

BRADYKININ RECEPTOR ADAPTATION

The adaptation function of the agonist-stimulated B₂R is a

typical feature of GPCRs in general in the sense that it follows a sequence of events well documented for a number of other receptors¹: a Ser/Thr rich domain of the intracellular C-terminal tail of the B₂R sequence is phosphorylated by several GPCR kinases; the 2 nonvisual arrestins (β-arrestin₁ and ₂) can associate with the phosphorylated receptor and compete with the G protein, thus desensitizing the receptor. Adaptor and structural proteins, which probably include AP-2 and clathrin in the case of the B₂R,²⁷ direct the receptor to a pit that can leave the plasma membrane and acquire the properties of the early endosome (Fig. 3, bottom, schematic representation). These events can be modeled using fluorescent receptors, arrestins, and ligands in HEK 293 (a) cells (Fig. 3, top). The rabbit B₂R fused to the green fluorescent protein (GFP) is translocated from the cell surface to multiples and polymorphic endosomes on BK stimulation; various approaches (microscopy, binding assays, immunoblots) show the complete recycling of the receptor in 1 to 3 hours.²⁸ The β-arrestins have a smooth cytosolic distribution in resting cells but, in cells expressing a nonfluorescent B₂R that are further stimulated with BK, the fluorescence associated with the fusion protein β-arrestin₂-GFP condenses in endocytic structures (Fig. 3).¹⁵

Other cells that express nonfluorescent B₂Rs can be labeled with the fluorescent antagonist B-10380 or agonist CF-εACA-BK (peptide structures given in Fig. 1, confocal imaging of HEK 293a cells in Fig. 3), but the subcellular distribution of the fluorescence is very different. While the antagonist labels the resting B₂R essentially at the cell surface, the fluorescent agonist labels endosomal structures, and evidence of endosomal breakdown of the agonist is provided by the fact that some of the carboxyfluorescein label of the peptide is transferred as a function of time in the cytosol (Fig. 3).²⁹ Thus, the B₂R transports the agonist ligands into cells and this leads to their degradation. Microscopic studies in short-term experiments (30-min incubation periods with the agonist) fully support the colocalization of the agonist-B₂R-arrestin triad in early endosomes; Rab5 is also colocalized at this stage. Then, protein phosphatases dephosphorylate the B₂Rs, which are ready to be recycled to the cell surface (Fig. 3, bottom).¹

Recent evidence based on cytoskeletal-disrupting drugs and dominant negative (GDP-locked) Rab GTPases points out that the progression of the ligand-B₂R-arrestin cargo from the cell surface to the perinuclear space is dependent on Rab5-mediated displacement of early endosomes along tubulin fibers and that the recycling process is entirely different, dependent on Rab4 and Rab11 and the actin cytoskeleton.³⁰ The intraendosomal inactivation of BK is also critical for the time course of B₂R recycling as shown by an inactivation-resistant peptide agonist, B-9972, or by a nonpeptide partial agonist,

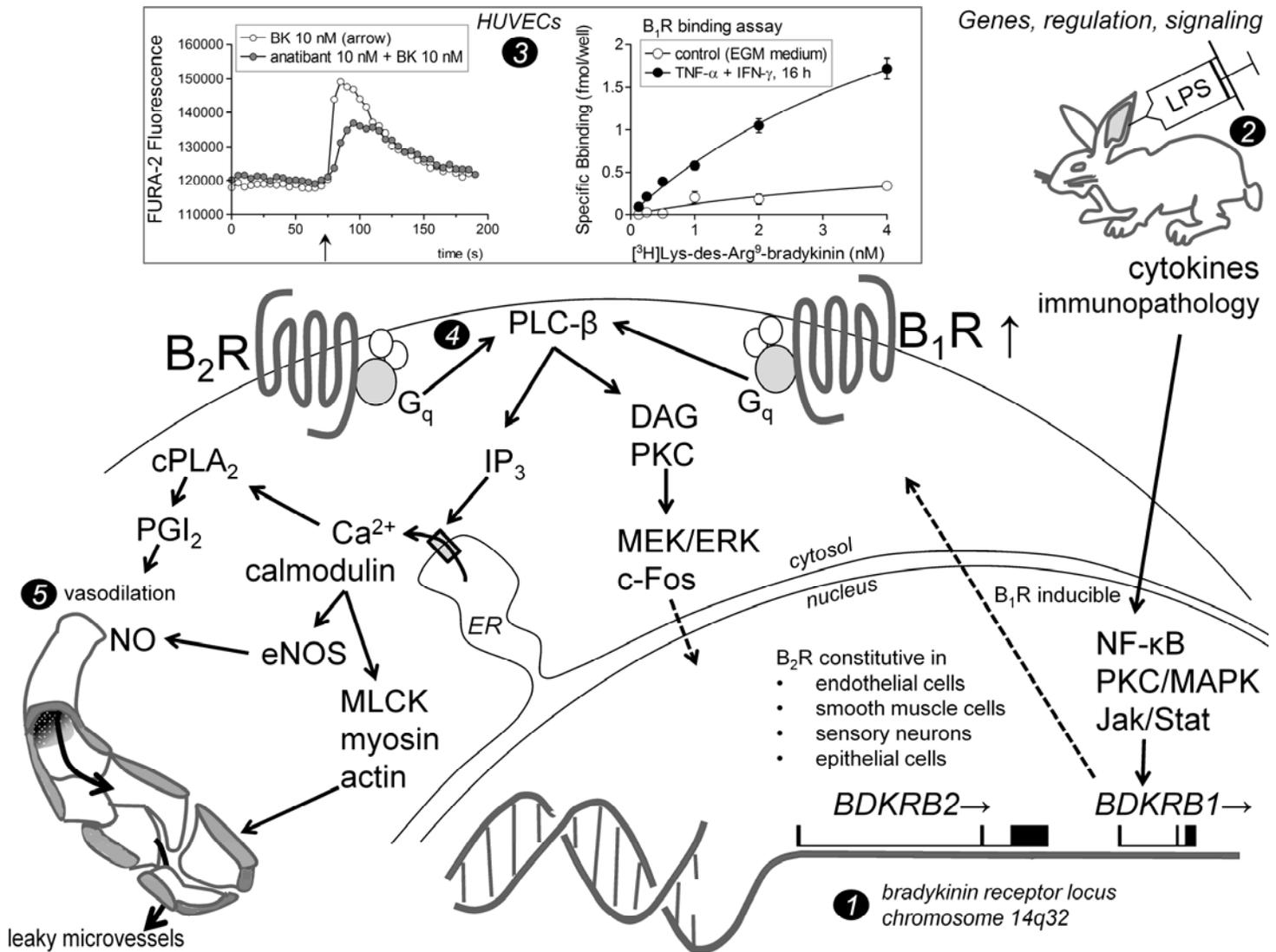


Figure 2. Bradykinin (BK) receptor genes, regulation, and signaling. The schematic representations and experimental data are described in the main text using markers 1-5. The graph to the right of marker 3 is reproduced from Koumbadinga et al¹⁸ with the permission of Elsevier. B₁R, B₁ receptor; B₂R, B₂ receptor; cPLA₂, cytosolic phospholipase A₂; DAG, diacylglycerol; EGM, endothelial growth medium; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; G, glycoprotein; HUVECs, human umbilical vein endothelial cells; IFN- γ , interferon- γ ; IP₃, inositol 1,4,5-trisphosphate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; MLCK, myosin light chain kinase; NF- κ B, nuclear factor κ B; PG, prostaglandin; PKC, protein kinase C; PLC- β , phospholipase C- β ; TNF- α , tumor necrosis factor- α ;



compound 47a (structures in Fig. 1).^{15,31} An amphibian BK homologue termed maximakinin also acts as an inactivation-resistant agonist.³² In all these cases, no or little B₂R recycling is observed in the 12-hour incubation period following cell stimulation, and a sizeable proportion of the receptor is destroyed (immunoblot evidence), presumably after progression into the late endosome-lysosome continuum.

The B₁R has an intracellular C-terminal tail that is not well conserved in sequence between mammalian species and that is very short in some mammalian species.¹ Accordingly, the B₁R is not phosphorylated on agonist stimulation³³ and does not promote condensation of the cytosolic β-arrestins at endosomal or plasma membrane levels (Fig. 3 top, unpublished results). Thus B₁Rs lack a basic desensitization mechanism of the B₂R and may signal for prolonged periods in some systems.¹ However, an unexpected discovery based on a fusion protein of the

rabbit B₁R with the yellow fluorescent protein (YFP) was the lateral agonist-induced translocation of the B₁R into structures that remain close to the plasma membrane plane (confocal microscopy, Fig. 3, top).³⁴ These structures are disrupted by cholesterol extraction from the cells and are likely to be caveolae, the lipid rafts that can be resolved in optical microscopy. This view is supported by the labeling of nonfluorescent rabbit or human B₁R by fluorescent ligands (Fig. 3, top; the human B₁R is illustrated using confocal microscopy). While the antagonist B-10376 essentially labels resting B₁R in the plasma membrane in a continuous manner, the agonist B-10378 has no significant endosomal distribution, but a rather spotty plasma membrane location (Fig. 3, top; peptide structures in Fig. 1).³⁵

Microscopic studies support that caveolin-1 is colocalized with B-10378³³ and with agonist-stimulated B₁R-YFP.³⁴ In addition, cell fractionation studies that aimed to recover

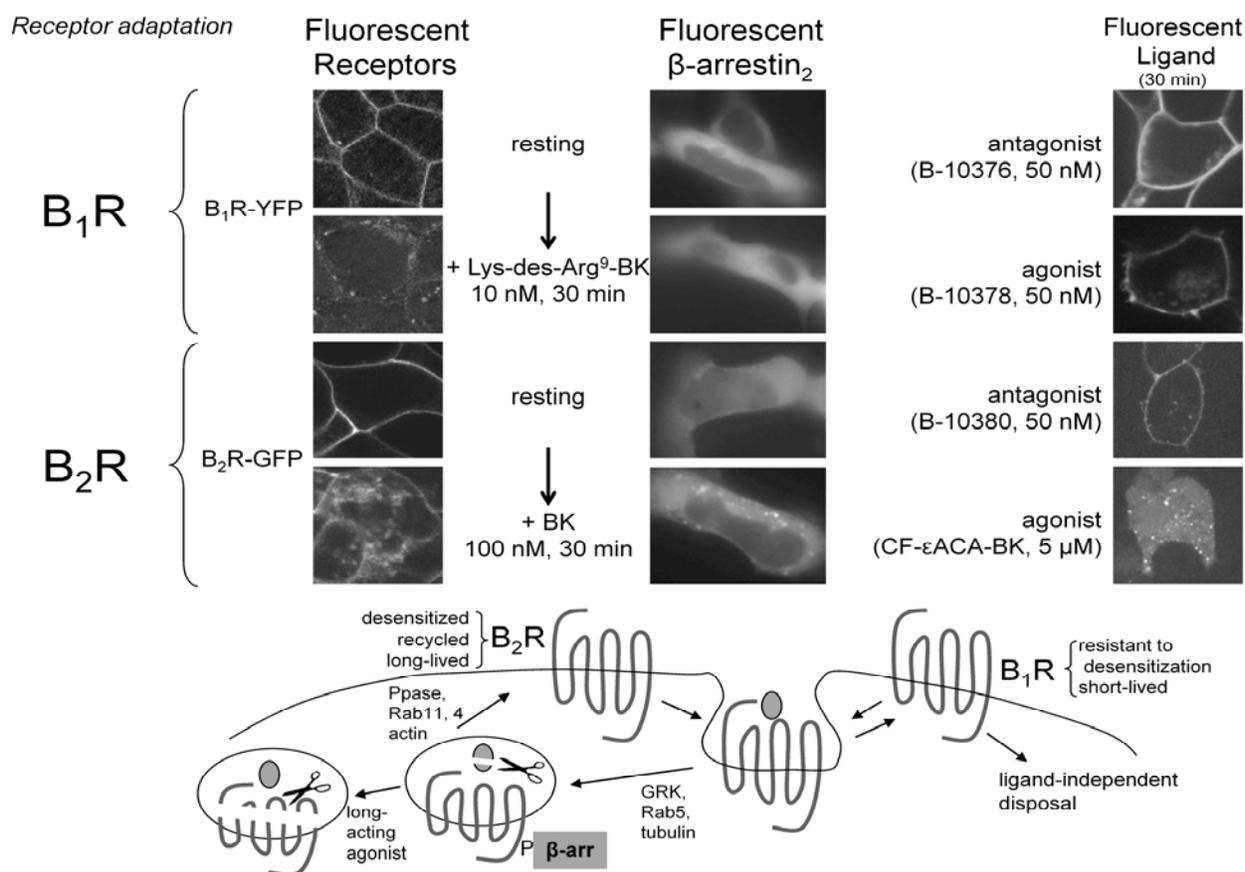


Figure 3. Bradykinin (BK) receptor adaptation. Top: Subcellular location of receptors, β-arrestin₂, and ligands in HEK 293 (left-most column) or HEK 293a cells (2 right-most columns) expressing recombinant bradykinin receptors. The fluorescent receptors or the distribution of fluorescent ligands were examined using confocal microscopy, as described elsewhere.^{16,28,34} Imaging of B₁R-YFP reproduced from Sabourin et al³⁴ with the permission of ASPET. Bottom: schematic representation of BK receptor kinin adaptation. B₁R, B₁ receptor; B₂R, B₂ receptor; β-arr, β-arrestin; CF, carboxyfluorescein; ε-ACA, ε-aminocaproic acid; GFP, green fluorescent protein; GRK, G protein-coupled receptor kinase; Ppase, protein phosphatase; YFP, yellow fluorescent protein.



buoyant caveolae-related lipid rafts from cells prelabeled with either an agonist or an antagonist radioligand showed that the agonist version is highly enriched in such rafts, as opposed to the antagonist. This applied both to HEK 293 cells overexpressing B₁R-YFP³³ or to cultured human vascular smooth muscle cells expressing endogenous B₁Rs (see supplemental material). Caveolae, particularly prominent in vascular endothelial cells, are signaling platforms of interest where the protein G_{αq}, a signaling partner of the B₁R, is enriched.

Another noticeable difference of interest between the 2 BK receptor subtypes is their apparent half-life as mature proteins at the cell surface. The discussion above about the B₂R cycling makes clear that it is economically managed and it is also long-lived.³⁶ Like other gene products induced during inflammatory conditions (eg, inducible nitric oxide synthase), the B₁R is short-lived (t_{1/2} 2-4 h), being eliminated from the cell surface in a ligand-independent manner (schematic representation, Fig. 3, bottom). This difference of half-life was established for both recombinant and naturally expressed receptors.

Some potentially important molecular issues have not been discussed in this text, such as the possible homo- and heterodimerization of receptors or development of biased agonists, because of the uncertain nature of the limited reported findings applied to the B₁Rs and B₂Rs. Suffice it to say that B-9972 behaves as an apparent biased agonist of the B₂R simply because it changes the kinetics of receptor cycling.³⁶

CONCLUSIONS

Kinins have emerged as inflammatory mediators particularly implicated in the development of the cardinal signs of inflammation that are dependent on vascular responses (vasodilation, increased microvascular permeability). Despite the maturity of the medicinal chemistry efforts aimed at antagonizing the BK receptors, clinical applications remain limited, hereditary angioedema presently being the showcase of the kallikrein-kinin system in human therapeutics. Indeed, the B₂R antagonist icatibant and the plasma kallikrein inhibitor ecallantide (Kalbitor™, Dyax Corp, Burlington, Massachusetts; DX-88) are now clinically used for this indication. Despite the disappointing analgesic effect of BK receptor antagonists in humans, other therapeutic avenues are worth pursuing (eg, anti-inflammatory effects). Potential salutary effects of endogenous kinins in the circulation are also of great interest, especially during stressful situations (ischemia, renal injury, etc). Whether accidental and catastrophic side effects of BK antagonists may arise in individuals for whom these mechanisms are compensatory is of interest for the clinical development of such drugs.

ACKNOWLEDGMENTS

Studies from Dr. Marceau's laboratory were supported by

the Canadian Institutes of Health Research (MOP-93773). The authors thank multiple trainees, employees, and collaborators in this field of research.

FINANCIAL DISCLOSURE

The authors have no financial interests in the materials or the subject dealt with in this article.

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CORRESPONDING AUTHOR:

François Marceau, MD, PhD, Centre de Recherche en Rhumatologie et Immunologie, Room T1-49, CHU de Québec, 2705 Laurier Blvd., Québec, QC, Canada G1V 4G2. Tel: 418-525-4444, ext. 46155. Fax: 418-654-2765. E-mail: francois.marceau@crchul.ulaval.ca