Endogenous aminopeptidase N decreases the potency of peptide agonists and antagonists of the kinin B₁ receptors in the rabbit aorta

Jean-Philippe Fortin, Lajos Gera, Johanne Bouthillier, John M. Stewart, Albert Adam and François Marceau*

Centre de recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Québec Qc, Canada G1V 4G2 (J.-P. F., J. B., F. M.); Faculté de Pharmacie, Université de Montréal, Montréal, Qc, Canada H3C 3J7 (A. A.); Department of Biochemistry, University of Colorado Health Sciences Center, Denver, Colorado, CO 80262, U.S.A. (L. G., J. M. S.).
Running title: Aminopeptidase N and B₁ receptor ligands

Address for correspondence, galley proofs and reprints:
François Marceau, M.D., Ph.D., Centre de Recherche en Rhumatologie et Immunologie, CHUQ, Pavillon CHUL, T1-49, 2705 Laurier Blvd., Québec (Québec), Canada G1V 4G2.
Tel. (418) 525-4444, ext. 46155; FAX: (418) 654-2765; E-mail: francois.marceau@crchul.ulaval.ca

Document statistics:
Number of text pages = 32;
of table = 1;
of figures = 7;
of references = 40
Number of words: Abstract = 249;
Introduction = 644;
Discussion = 1049.

Abbreviations:
ACE, angiotensin I converting enzyme; APN-GFP, aminopeptidase N fused to green fluorescent protein; B-9958, Lys-Lys-[Hyp³, CpG⁵, D-Tic⁷, CpG⁸]des-Arg⁹-BK; B-10350, Lys-Lys-[Hyp³, Ig¹⁵, D-Tic⁷, CpG⁸]des-Arg⁹-BK; BAEC, bovine aortic endothelial cell; Compound 11, 2-{(2R)-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; Compound A, N-[2-[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl]-2-[(2R)-1-(2-naphtylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide; des-Arg⁹-BK, des-arginine⁹-bradykinin; L-Ala-pNA, L-alanine-p-nitroanilide.
Abstract

The B₁ receptor for kinins is selectively stimulated by bradykinin-related fragments lacking the C-terminal arginine, des-arginine⁹-bradykinin (des-Arg⁹-BK) and Lys-des-Arg⁹-BK. The latter peptide is the optimal agonist at the human and rabbit receptor. The B₁ receptor is inducible as a function of inflammatory conditions in the vasculature. We studied the effect of endogenously expressed peptidases on the potency of ligands of this receptor in an established bioassay, the rabbit aorta contractility. The potency measured for agonists (EC₅₀) or antagonists (pA₂ scale) in this assay was compared to the affinity of each agent determined using [³H]Lys-des-Arg⁹-BK binding competition in cultured aortic smooth muscle cells and to the competition Kᵢ for the hydrolysis of the aminopeptidase chromogenic substrate L-Ala-ᵢ-nitroanilide by smooth muscle cell membranes. The contractile potency of the agonist Lys-des-Arg⁹-BK is decreased by in situ metabolism and aminopeptidase N mediates most of the distortion (inhibited by amastatin, but not efficiently by puromycin). At the other end of the spectrum, the fully protected agonist Sar-[D-Phe⁸]des-Arg⁹-BK is not significantly potentiated by peptidase inhibitors. A similar distortion of apparent potency was observed for some peptide antagonists used in the contractility assay, B-10350 and Lys-[Leu⁸]des-Arg⁹-BK being intensely potentiated by amastatin treatment and effective L-Ala-ᵢ-nitroanilide competitors. N-protected peptide antagonists or a nonpeptide antagonist of the B₁ receptor were not potentiated by amastatin. The co-expression of aminopeptidase N and the kinin B₁ receptor in rabbit arterial tissue is of interest for the inactivation of the high affinity agonist Lys-des-Arg⁹-BK and for the design of hydrosoluble antagonist drugs.
**Introduction**

Bradykinin-related peptides, the kinins, stimulate cellular functions following binding to two types of receptors predominantly coupled to the Gq protein, the B1 and B2 receptors (Leeb-Lundberg et al., 2005). In the vasculature, the B1 receptor is essentially an inducible gene product in most mammalian species (Marceau et al., 1998). Kinins exert antiproliferative effects on injured or cultured vascular smooth muscle cells via the B1 receptors and its expression has been shown in human atheromas (Raidoo et al., 1997; Agata et al., 2000; Dixon et al., 2002). Ischemia induces endothelial B1 receptor expression and there is mounting evidence, partly based on B1 receptor gene knockout mice, that the B1 receptor may play a detrimental role opposed to that of the B2 receptors in ischemia-reperfusion systems (Mazenot et al., 2001; Lagneux et al., 2001; Souza et al., 2004). B1 receptor mediation of ischemia-driven angiogenesis has been shown (Emanuelli et al., 2002). Of relevance to sepsis, vascular B1 receptor expression mediating hypotension also occurs following sublethal lipopolysaccharide treatments in various species (McLean et al., 1999; Schanstra et al., 2000; deBlois and Horlick, 2001; Leeb-Lundberg et al., 2005). Whether inducible B1 receptors mediate a part of the therapeutic or side effects of angiotensin converting enzyme (ACE) inhibitors is also of topical interest (Marin-Castano et al., 2002; Molinaro et al., 2002).

B1 receptors are stimulated by specific sequences derived from kininogens, namely des-arginine9-bradykinin (des-Arg9-BK) and Lys-des-Arg9-BK (des-Arg10-kallidin), that are metabolites of native kinins (bradykinin and lysyl-bradykinin or kallidin) via the action of arginine carboxypeptidases (Leeb-Lundberg et al., 2005). These peptides, like all kinins,
are short lived in vivo. Among the peptidases that hydrolyze kinins, an aminopeptidase activity inhibited by amastatin is expressed by both porcine endothelial and smooth muscle cells (more by the latter cell type; Palmieri et al., 1989). This enzyme reportedly hydrolyzes lysyl-bradykinin (kallidin) and Lys-des-Arg⁹-BK but not bradykinin or des-Arg⁹-BK (Palmieri et al., 1989; Drapeau et al., 1991). The N-terminal Lys residue is a major determinant of affinity for the human, porcine and rabbit B₁ receptors (Leeb-Lundberg et al., 2005), and the optimal agonist of these receptors, Lys-des-Arg⁹-BK produced a prolonged hypotensive response in amastatin-treated, lipopolysaccharide-pretreated rabbits (Drapeau et al., 1991b), supporting that cardiovascular responses produced by this agonist and mediated by B₁ receptors in vivo are arrested by an aminopeptidase.

The importance of peptidases in the inactivation of B₁ receptor ligands has been also shown by structural modifications. Sar-[D-Phe⁸]des-Arg⁹-BK has a decreased affinity relative to Lys-des-Arg⁹-BK at the rabbit and human B₁ receptor based on radioligand binding assays (Sabourin et al., 2002a; Leeb-Lundberg et al., 2005), but gained resistance to several peptidases present in blood plasma and kidney membranes (Drapeau et al., 1993), and is a highly persistent hypotensive agent in lipopolysaccharide-pretreated rabbits (Drapeau et al., 1991b; Audet et al., 1997). The prototype antagonist of the B₁ receptors, Lys-[Leu⁸]des-Arg⁹-BK, has also been subjected to such structural analysis (Drapeau et al., 1993). The introduction of synthetic aminoacid residues that constrain the peptide backbone has had the major impact in this field, with the production of antagonists that retain high potency (e.g., B-9958 = Lys-Lys-[Hyp³, CpG⁵, D-Tic⁷,
CpG^{8}\text{des-Arg}^{9}\text{-BK}; \text{Larrivée et al., 2000). Inflammatory pain has been their most investigated application (Leeb-Lundberg et al., 2005).}

The present study aims at evaluating the relative importance of the endogenous peptidases in the inactivation of peptide ligands of the rabbit B_{1} receptor using the contractility of the rabbit aorta. Peptidase-mediated distortions in apparent potency of the B_{1} receptor peptide agonists and antagonists have been pharmacologically addressed. A novel non-peptide antagonist (Ransom et al., 2004) was compared to peptide antagonists in this respect. An enzymatic approach was also used to measure the susceptibility of peptide ligands to an endogenously expressed aminopeptidase N (also called aminopeptidase M, CD13, EC 3.4.11.2). The latter enzyme emerges as the major inactivation pathway for both peptide agonist and antagonists of the B_{1} receptors at the vascular level.
Methods

Drugs. Compound A (N-[2-[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl]-2-[(2R)-1-
(2-naphthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide) and Compound
11 (2-[(2R)-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), non-peptide
hydrophobic B₁ receptor antagonists (Ransom et al., 2004; Morissette et al., 2004). Ac-
Lys[Leu⁸]des-Arg⁹-BK is a documented B₁ receptor antagonist (Drapeau et al., 1993) and
B-10350 (Lys-Lys-[Hyp³, Igl⁵, D-Tic⁷, CpG⁸]des-Arg⁹-BK) is a close analog of B-9958
(Larrivée et al., 2000) recently produced. Sar-[D-Phe⁸]des-Arg⁹-BK and Lys-[D-
Phe⁸]des-Arg⁹-BK are B₁ receptor agonists that integrate residue changes conferring
resistance to specific peptidases (Drapeau et al., 1991a; 1991b; 1993). The other drugs
were from Sigma-Aldrich (St. Louis, MO). The sequences of the peptide ligands are
aligned in table 1.

Contractility studies. A local ethics committee approved the procedures based on
rabbits. Rabbit aortic rings with intact endothelium (New Zealand white, 1.5-2 kg,
Charles River, St. Constant, 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 as
described (Morissette et al., 2004). Contractility studies were performed after 3 or 5.5 h
of in vitro incubation, as the response mediated by B₁ receptors is acquired in a time- and
protein synthesis-dependent manner in this preparation (Leeb-Lundberg et al., 2005). In
experiments dealing with antagonists, the procedure described by Morissette et al. (2004)
was precisely applied (construction of cumulative concentration-responses curves for des-
Arg9-BK at times 3.5 and 5.5 h from the beginning of the incubation of the preparation; antagonists or their vehicle introduced 30 min before the construction of the second curve; pA2 value calculation as described). The curves constructed at 3.5 h are internal controls, and only the second ones are shown. In experiments dealing with agonists, cumulative concentration-effects curves (5.5 h) were determined for des-Arg9-BK, Lys-des-Arg9-BK or Sar-[D-Phe8]des-Arg9-BK in separate tissues for the determination of apparent contractile potencies. Additional concentration-effect determinations were performed in the presence of a cocktail of peptidase inhibitors (3 µM amastatin, 1 µM captopril, 1 µM phosphoramidon) or of separate peptidase inhibitors introduced 45 min before the construction of the second concentration-effect curve. These concentrations of peptidase inhibitors are similar to those used in metabolic studies dealing with the metabolism of bradykinin-related peptides (Palmieri et al., 1985; Orawski et al., 1989; Drapeau et al., 1993). Contractility results were expressed as a percent of the maximal response recorded in each tissue (all the antagonists used were surmountable as judged by the conservation of the E_{max} from the first concentration-effect curve).

**Binding assay.** The binding of 1 nM [3H]Lys-des-Arg9-BK (PerkinElmer Life Sciences, Boston, MA; 80 Ci/mmol) to adherent intact rabbit aortic smooth muscle cells was evaluated as described in cells maintained in the culture medium supplemented with 10% fetal bovine serum and interleukin-1β (5 ng/ml, for the last 4 h) (Sabourin et al., 2002b). The assay was applied to evaluate binding competition by unlabelled drugs. These drugs were present during the 60 min period allowed for radioligand binding equilibration in the binding buffer (consisting of Medium 199 supplemented with 0.1% bovine serum
albumin, 3 µM amastatin, 1 µM captopril, 1 µM phosphoramidon and sodium azide 0.02% w/v). Kᵢ values for unlabelled competitors were derived from the relationship:

\[ Kᵢ = \frac{IC₅₀}{1 + S/K_D} \]  
(equation 1)

where K_D is the dissociation constant of the radioligand, S the radioligand concentration and IC₅₀, the unlabelled drug concentration that displaces half of the specific radioligand binding (graphic determination).

**Membrane and cytosol preparations, enzymatic assay.** Several primary lines of rabbit aortic smooth muscle cells were initiated and cultured as described (Sabourin et al., 2002b). Bovine aortic endothelial cells (BAECs, gift from Dr. Darren Richard, CHUQ, Quebec City), maintained in Dulbecco’s Minimum Essential Medium supplemented with 10% fetal bovine serum, were selected for their low background expression of membrane aminopeptidase activity. They were transfected as described (Sabourin et al., 2002a) with the vector coding for human aminopeptidase N fused to green fluorescent protein (APN-GFP), a variant based on the Clontech pEGFP-N1 vector of a reported fluorescent fusion protein (Kehlen et al., 2003; gift from Dr. Astrid Kehlen, Halle, Germany). Membranes from either cell type or from freshly desendothelialized rabbit aortic rings (representing smooth muscle) or a cytosolic fraction from smooth muscle cells were prepared as sources of enzyme. Briefly, cells or tissues were homogenized (up to 24 75-cm² flasks per day or ~50 mg of fresh tissue) in 0.5 ml sucrose buffer (250 mM sucrose, 20 mM tricine buffer, 1 mM PMSF, 10 mg/ml leupeptin, 2 mg/ml pepstatin, and 10 mg/ml soybean trypsin inhibitor, pH 7.5). In the sequential centrifugation steps applied, the first
(600 g, 5 minutes) and second pellets (15 000 g, 5 minutes) were discarded; the third (150 000 g, 3 hours) pellets was resuspended in the same buffer as a source of membrane enzyme (normalized as to the basis of protein concentration, BCA Protein Assay, Pierce). In some experiments, the last supernatant from smooth muscle cells was also used as a cytosolic extract (also normalized as protein content).

Enzymatic assays, based on the chromogenic substrate L-alanine-p-nitroanilide (L-Ala-pNA, Sigma-Aldrich; 0.08-5 mM) and 30 µg of membrane or cytosol proteins co-incubated at 37°C for 1-2 h in 200 µl of PBS, pH 7.4, were performed precisely as described (Lendeckel et al., 1996). Colorless solutions of peptide ligands of the B1 receptors (agonists or antagonists, 10 µM) were used as competitors of L-Ala-pNA hydrolysis in some experiments. Michaelis-Menten enzyme kinetics and competitive inhibition $K_i$ values were evaluated using a computer program (Tallarida and Murray, 1987).
Results

Pharmacological study of the agonists. The concentration-effect relationship for the B₁ receptor agonists in the rabbit aorta indicated the following order of potency when the assay was performed in control tissues: Lys-[D-Phe⁸]des-Arg⁹-BK > Sar-[D-Phe⁸]des-Arg⁹-BK ≈ Lys-des-Arg⁹-BK > des-Arg⁹-BK (fig. 1A). In the presence of a peptidase inhibitor cocktail consisting of amastatin, captopril and phosphoramidon, this order of potency changed to Lys-des-Arg⁹-BK ≈ Lys-[D-Phe⁸]des-Arg⁹-BK > Sar-[D-Phe⁸]des-Arg⁹-BK > des-Arg⁹-BK (fig. 1B). Using Lys-des-Arg⁹-BK, the agonist for which the cocktail produces the most important change in apparent potency (13.8-fold increase), the effect of the separate components of the peptidase inhibitor mixture were tested (fig. 1C). Amastatin is the most important component of the cocktail, producing alone a 12.6-fold potency shift, while captopril or phosphoramidon effects (1.58- and 1.33-fold increases, respectively) were barely recordable. Puromycin, a selective inhibitor of a cytosolic aminopeptidase (Solhonne et al., 1987; Minnasch et al., 2003), was ineffective to potentiate Lys-des-Arg⁹-BK (fig. 1D).

A competition assay for the binding of [³H]Lys-des-Arg⁹-BK to rabbit cultured aortic smooth muscle cells showed that the agonist order of potency is Lys-des-Arg⁹-BK > Lys-[D-Phe⁸]des-Arg⁹-BK > Sar-[D-Phe⁸]des-Arg⁹-BK > des-Arg⁹-BK (raw data in fig. 2, Kᵢ values for unlabeled peptides in table 1 and fig. 3 derived from the Kᵥ value of 0.14 nM for this radioligand and these cells; Sabourin et al., 2002b). The binding assay was applied to monolayers of smooth muscle cells (minimal impairment of distribution) and
in the presence of the same peptidase inhibitor cocktail as the one used in experiments reported in fig. 1B. Running the binding competition assay for unlabelled Lys-des-Arg⁹-BK without the peptidase inhibitor cocktail did not significantly change the estimated potency (data not shown).

In the contractility assay, a significant gain of potency in the presence of peptidase inhibitors was limited to Lys-des-Arg⁹-BK and, almost as importantly, to its [D-Phe⁸] isomer, as shown in fig. 3. This representation shows that the contractility EC₅₀ values moved closer to the binding Kᵢ values for the two peptides in the presence of the peptidase inhibitor cocktail.

**Pharmacological studies of the antagonists.** The binding competition assay was also applied to several B₁ receptor antagonists (fig. 2, Kᵢ values in table 1). This evaluation of potency was compared to pA₂ scale values determined using the dextral shift of the contractile concentration-effect curve for the agonist des-Arg⁹-BK, itself not importantly affected by the peptidase inhibitor cocktail (fig. 4; Schild plots from fig. 4 data in fig. 5). The nonpeptide Compound A was a very potent and apparently surmountable antagonist, with a pA₂ value of 10.01 ± 0.20 (s.e.m.) (fig. 4A). The historical antagonist prototype [Leu⁸]des-Arg⁹-BK and the novel peptide antagonist B-10350 were of comparable potencies (pA₂ value of 6.7 and 7.4, respectively; fig. 4I, C). Ac-Lys-[Leu⁸]des-Arg⁹-BK is a peptide in which the N-terminus was protected at the expense of affinity, being less potent than the nonacetylated form (pA₂ 7.37 ± 0.08; fig. 4E, relative to 7.88 ± 0.27 for Lys-[Leu⁸]des-Arg⁹-BK; fig. 4G). It can be seen that the tested antagonists, all
surmountable, are dispersed over more than 3 log units on the pA2 affinity scale. The analysis presented in fig. 6 shows the relationship between the pA2 values in the contractility assay and the binding Ki value and includes the pA2 value recently obtained by our laboratory with identical procedures for an additional B1 receptor antagonist, the nonpeptide Compound 11 (Morissette et al., 2004). In this representation, points corresponding to antagonists that are not aminopeptidase substrates, like the nonpeptide Compounds A and 11 and [Leu8]des-Arg9-BK, lay close to an identity line Ki = 10^{pA2}. The coordinates of other peptide antagonists, namely B-10350 and Lys-[Leu8]des-Arg9-BK, were located further away from this line. The suggestion that the experimental pA2 values are distorted by the action of aminopeptidase N for at least a subset of antagonists is supported by the higher apparent potency of B-10350 and of Lys-[Leu8]des-Arg9-BK in the presence of amastatin (gain of 0.9 and 1.0 log unit, respectively; fig. 4, 5). In the graph of Ki values as a function of pA2 values (fig. 6), the addition of amastatin moved B-10350 and Lys-[Leu8]des-Arg9-BK coordinates closer to a line where those of most other antagonists lay. The gain of affinity for the peptide antagonist Ac-Lys-[Leu8]des-Arg9-BK in the presence of amastatin was much smaller (0.1 log unit; fig. 4F, 6) and null for [Leu8]des-Arg9-BK (fig. 4J, 6). The potency of Compound A was essentially unaffected by the presence of amastatin (fig. 4B, 6), consistent with its nonpeptide chemistry. Thus, some peptide antagonists were also subjected to potency estimate distortion due to the presence of aminopeptidase N in the rabbit aorta.

**Enzyme activity.** L-Ala-pNA is a usual substrate for aminopeptidase N (Riemann et al., 1999); at a standard substrate concentration of 2.5 mM (Lendeckel et al., 1996),
membranes (30 µg/reaction) from both fresh deendothelialized rabbit aorta and cultured aortic smooth muscle cells contained measurable aminopeptidase activity (fig. 7A). Pre-incubating aortic rings for 6 h in sterile Krebs medium, a procedure that sharply upregulates B₁ receptor expression (Sabourin et al., 2002), modestly increased the aminopeptidase activity. All these activities were massively inhibited by amastatin (3 µM), but much less efficiently by puromycin (5 µM). Experiments performed using large lots of cultured smooth muscle cell membranes showed that the enzyme that cleaves L-Ala-pNA exhibits a Kₘ of 0.63 mM (V_max 4.11 pkat; fig. 7B). Puromycin is a useful inhibitor to differentiate aminopeptidase N (relatively insensitive to it) from an ubiquitous and abundant intracellular puromycin-sensitive form of aminopeptidase (EC 3.4.11.14) that is also inhibited by amastatin but confined to the cytosolic fraction of cells (Solhonne et al., 1987; Minnasch et al., 2003). This was confirmed in the present experiments by the demonstration of a hydrolytic activity for L-Ala-pNA in the smooth muscle cell cytosol extract (30 µg protein; Kₘ 0.79 mM, high V_max, fig. 7B) that was effectively inhibited by both amastatin and puromycin (fig. 7A). Therefore, the relative efficacy of inhibitors is consistent with the presence of authentic aminopeptidase N, an ectoenzyme, in smooth muscle cell membranes. Recombinant human APN-GFP expressed in BAECs exhibited a Kₘ of 0.73 mM and was more sensitive to amastatin than to puromycin inhibition (fig. 7A, B), like the smooth muscle cell membrane activity. A significant background aminopeptidase activity was present in the membrane fraction of BAECs (one third of that of transfected cells, fig. 7B).
The B₁ receptor ligands, added at the fixed concentration of 10 µM to the reaction mixtures, were tested for apparent enzyme competition (source of enzyme, membranes of cultured smooth muscle cells). The data were found to approximate competitive inhibition better than noncompetitive kinetics. The hydrolysis of L-Ala-pNA is inhibited by the agonists Lys-des-Arg⁹-BK and Lys-[D-Phe⁸]des-Arg⁹-BK (estimated Kᵢ values reported in table 1), but only marginally by des-Arg⁹-BK and not by Sar-[D-Phe⁸]des-Arg⁹-BK (fig. 7C; double reciprocal plot representation). Lys-des-Arg⁹-BK is a mediocre competitor of the smooth muscle cell cytosolic aminopeptidase (calculated Kᵢ 129 µM, data not shown). The same type of analysis performed on the set of peptide antagonists showed that Lys-[Leu⁸]des-Arg⁹-BK competes with L-Ala-pNA for the smooth muscle cell membrane aminopeptidase (Kᵢ 26.2 µM), but that both [Leu⁸]des-Arg⁹-BK and Ac-Lys-[Leu⁸]des-Arg⁹-BK competed with low affinity (table 1; fig. 7D). B-10350 was the most potent tested peptide competitor of the chromogenic substrate (Kᵢ 1.89 µM; fig. 7C).
Discussion

The potentiation of peptide agonists of the kinin B₁ receptor by peptidase inhibitors suggests that the breakdown of some peptides decreased agonist drug concentration in the tissue extracellular fluid at the vicinity of receptors in a manner that is not completely compensated by diffusion from the bathing fluid. This is a situation of drug removal from the receptor compartment as theoretically developed by Kenakin (1987). The compact structure of the contractile tissue (smooth muscle cells, positive for α-actin expression) is shown in an inset of fig. 3.

The inhibitor cocktail used in experiments reported in fig. 1B and 2 covered at least ACE (captopril), aminopeptidases (amastatin) and neutral endopeptidase (phosphoramidon). Sar-[D-Phe⁸]des-Arg⁹-BK is reportedly stable in the presence of any of these peptidases (Drapeau et al., 1993), consistent with the fact that the peptidase inhibitor cocktail exerted a minimal effect on its apparent potency in the contractility assay. Lys-des-Arg⁹-BK is metabolized by aminopeptidase N, which cleaves the N-terminal Lys residue and yields the much less potent des-Arg⁹-BK, a partial inactivation reaction. The isomerization of Phe⁸ into D-Phe⁸ in Lys-[D-Phe⁸]des-Arg⁹-BK or Sar-[D-Phe⁸]des-Arg⁹-BK confers a complete protection against purified ACE and neutral endopeptidase (Drapeau et al., 1991a; 1993). The EC₅₀ shift recorded for Lys-[D-Phe⁸]des-Arg⁹-BK in the presence of the peptidase inhibitor cocktail probably isolated the relative role of aminopeptidase N, which remained important. Des-Arg⁹-BK is not as much susceptible to aminopeptidase N degradation, because the peptide bond preceding a proline is resistant to this enzyme (Riemann et al., 1999). Further, inactivation of des-Arg⁹-kinins by ACE is
a low affinity reaction relative to the one that hydrolyzes bradykinin (Drapeau et al., 1991a), consistent with the small effect of an ACE inhibitor the the concentration-effect relationship of Lys-des-Arg^9^-BK (fig. 1C). It should be noted that ACE is functionally detectable in such rabbit aortic rings with intact endothelium (loss of apparent potency for angiotensin I in the presence of an ACE inhibitor; Fortin et al, 2003). The K_i values derived from the competition of L-Ala-pNA hydrolysis by peptide agonists essentially confirmed the inferences made from the pharmacological analyses of contractility. Thus, aminopeptidase N mediates the major inactivation pathway for the optimal B_1 receptor agonist, Lys-des-Arg^9^-BK, in the rabbit aorta. The recent report by Pelorosso et al. (2005) also stresses that a peptidase sensitive to amastatin is the dominant inactivation pathway for Lys-des-Arg^9^-BK on the basis of potentiation of the contractile effect mediated by B_1 receptors in the human isolated umbilical artery. Kokkanen et al. (1999) have analyzed the metabolism of bradykinin and lysyl-bradykinin in cardiac tissue. While the latter peptide was converted into bradykinin by a tissue aminopeptidase, both native kinins were metabolized efficiently by neutral endopeptidase and ACE, showing that these preferential agonists of the B_2 receptors appear to be inactivated differently from the B_1 receptor agonist. ACE2 is a recently discovered homologue of ACE that does not metabolize bradykinin, but reportedly inactivates both des-Arg^9^-BK and Lys-des-Arg^9^-BK, among other vasoactive peptides, by hydrolyzing the C-terminal Phe residue (Oudit et al., 2003). However, this enzyme, insensitive to conventional ACE inhibitors, has a limited tissue distribution (heart, kidney, testis) and is not likely to participate to the inactivation of B_1 receptor ligands in smooth muscle cells.
The pA\textsubscript{2} determination requires that drug equilibrium can be reached at the vicinity of receptors (Kenakin, 1987), which is not the case for B-10350 and Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK. The latter peptides gained 0.9-1.0 log unit of potency in the presence of amastatin, whereas Ac-Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK and [Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK were practically unaffected. These pharmacological data, along with the fitting K\textsubscript{i} values derived from the L-Ala-pNA hydrolysis competition assay, confirmed a dominant role of aminopeptidase N in the degradation of peptide B\textsubscript{1} receptor antagonists. The effect of acetylation of the N-terminus is clear, as it affords nearly complete protection against aminopeptidase hydrolysis. B-10350 structure unexpectedly determined the highest apparent affinity for aminopeptidase N (perhaps because the primary reaction product is also a likely substrate of the same enzyme). The free amino terminus and N-terminal Lys residue certainly contribute to affinity for the rabbit B\textsubscript{1} receptor for both agonist and antagonist peptides (10-fold lower binding K\textsubscript{i} for Lys-[D-Phe\textsuperscript{8}]des-Arg\textsuperscript{9}-BK than for Sar-[D-Phe\textsuperscript{8}]des-Arg\textsuperscript{9}-BK, 14.4-fold lower binding K\textsubscript{i} for Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK relative to Ac-Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK). However, these chemical features introduce a susceptibility to aminopeptidase N. This knowledge can be applied for the future development of novel peptide antagonists of high potency and in vivo stability.

Aminopeptidase N assumes widely divergent functions in the organism such as the digestion of protides at the surface of the intestinal mucosa and the degradation of some cytokines, like interleukin-8, by leukocytes (Riemann et al., 1999). The peptidase is upregulated in several tumor cell lines and tumor stromal elements (Riemann et al., 1999; Curnis et al., 2002; Kehlen et al., 2003). Interestingly, aminopeptidase N is progressively
expressed in several organs during the fetal development of the rat, including the aortic wall (Jardinaud et al., 2004).

The vascular expression of aminopeptidase N, an ectoenzyme bound to membranes and relatively resistant to puromycin, is of interest for the in vivo inactivation of Lys-des-Arg⁹-BK, the likely physiological agonist for this receptor in the human, rabbit and pig (Leeb-Lundberg et al., 2005). A very recent study of an alternate model, the human umbilical artery, has led to substantially similar conclusions, although limited to the B₁ receptor agonists and based only on the pharmacological analysis of contractility (Pelorosso et al., 2005). In the present study, the comparative determination of absolute receptor affinity using a radioligand competition assay and of the capacity of each peptide to compete for the hydrolysis of a synthetic aminopeptidase N substrate allowed us to show that the observed contractile potency of the B₁ receptor agonists in the isolated rabbit aorta is a function of both parameters. Further, the present study shows unequivocally that susceptibility to aminopeptidase N is critical for the design of hydrosoluble peptide antagonists, as illustrated with B-10350. Hydrosoluble antagonists may be well adapted to intravenous administration in intensive care units for future applications that are still under investigation, such as sepsis, wasting states and pain (Leeb-Lundberg et al., 2005). Alternatively, peptide antagonists may be suitable for topical administration with minimal systemic distribution. For instance, allergic inflammation of the human nasal mucosa determines a local B₁ receptor upregulation (Christiansen et al., 2002), and the possible benefits of receptor blockade remain to be determined in this condition.
Acknowledgements

We thank Dr. Guy Drapeau for the gift of some peptides and for reading the manuscript and Dr. Douglas J. Pettibone, Merck Research Laboratories (West Point, PA) for the gift of nonpeptide antagonists.
References


Audet R, Rioux F, Drapeau G, and Marceau F (1997) Cardiovascular effects of Sar-[D-Phe\textsuperscript{8}]des-Arg\textsuperscript{9}-bradykinin, a metabolically protected agonist of B\textsubscript{1} receptor for kinins, in the anesthetized rabbit pretreated with a sublethal dose of bacterial lipopolysaccharide. *J Pharmacol Exp Ther** **280**:6-15.


Footnotes to title page: Supported by the Canadian Institutes of Health Research (CIHR, grant MOP-14077) and the Fonds de la recherche en Santé du Québec (Studentship award to J.-P. F.).

*Author for correspondence; e-mail: francois.marceau@crchul.ulaval.ca
**Figure legends**

Fig. 1. Effect of agonists of B₁ receptors on rabbit aortic contractility. A, B.

Concentration-effect relationship in the absence (A) or presence (B) of a peptidase inhibitor cocktail (3 µM amastatin, 1 µM captopril, 1 µM phosphoramidon). C. Effect of separate peptidase inhibitors on Lys-des-Arg⁹-BK-induced contraction in the rabbit aorta. D. Effect of puromycin on Lys-des-Arg⁹-BK-induced contraction in the rabbit aorta. All the results shown are derived from the concentration-response curves constructed at 5.5 h with each tissue. Values are means ± s.e.m. of the number of determinations indicated by n.

Fig. 2. Competition of radioligand binding to B₁ receptors by unlabelled drugs. The total binding of 1 nM [³H]Lys-des-Arg⁹-BK to rabbit aortic vascular smooth muscle cells expressing the natural B₁ receptor is presented; unlabelled drugs were co-incubated at the indicated concentrations with the radioligand. Values are the mean of two experiments composed of duplicate determinations and expressed as a percent of the total binding recorded in the absence of antagonist. The non-specific binding was not subtracted. The agonist and the antagonist competitors are presented separately.

Fig. 3. Relationship between the Kᵢ values obtained using cultured smooth muscle cells and the contractility EC₅₀ values of B₁ receptor agonists as a function of the presence of the peptidase inhibitor cocktail in the contractility assay. Insets: immunohistochemistry for α-actin (black) in rabbit aortic smooth muscle cells, either cultured (bottom, to show...
the well spread out morphology) or in situ in a freshly isolated aorta (top, compact organization, prominent non-muscle structures; intimal surface towards the bottom).

Fig. 4. Effects of antagonists on des-Arg⁹-BK-induced contraction of the rabbit isolated aorta. The concentration-effect curves recorded at time 5.5 h are shown and were constructed in the presence of an antagonist or its vehicle (DMSO ≤ 0.1% v/v when indicated). Values are means ± s.e.m. of the number of determinations indicated by n. A, B. Compound A without or with amastatin (3 µM), respectively. C, D. B-10350 without or with amastatin. E, F. Ac-Lys-[Leu⁸]des-Arg⁹-BK without or with amastatin. G, H. Lys-[Leu⁸]des-Arg⁹-BK without or with amastatin. I, J. [Leu⁸]des-Arg⁹-BK without or with amastatin.

Fig. 5. Schild plot analyses derived from publication fig. 4 data and based on the agonist EC₅₀ values from the averaged concentration-effect curves (DR, dose ratio = EC₅₀ in the presence of the antagonists divided by the control EC₅₀). Calculated pA₂ values (Tallarida and Murray, 1987) are reported in text.

Fig. 6. Relationship between the Kᵢ derived from radioligand binding competition studies and the pA₂ derived from the contractility of the isolated rabbit aorta for a series of B₁ receptor antagonists. The dotted line is the theoretical identity (Kᵢ = 10⁻pA₂). The potency of five of the drugs (Compound A, B-10350, Ac-Lys-[Leu⁸]des-Arg⁹-BK, Lys-[Leu⁸]des-Arg⁹-BK, [Leu⁸]des-Arg⁹-BK) was also assessed in the presence of amastatin (3 µM; circles).
Fig. 7. Use of the L-Ala-pNA hydrolysis assay to evaluate the affinity (competition $K_i$) of B$_1$ receptor ligands for endogenously expressed aminopeptidase. A. Hydrolysis of L-Ala-pNA (2.5 mM) in the presence of 30 $\mu$g of membrane proteins from freshly isolated rabbit aorta (further incubated or not for 6 h in sterile Krebs solution), from cultured aortic smooth muscle cells (SMC, membranes or cytosol) or BAECs expressing recombinant APN-GFP (membranes; fluorescence was checked as an additional proof of expression). Relevant aminopeptidase inhibitors (amastatin, puromycin) were used to characterize the activity. B. Effect of the substrate concentration on the reaction velocity (4 sources of enzyme; see text for analysis). C, D. Double reciprocal plot representation of the effect of various receptor ligands (10 $\mu$M) on the hydrolysis of L-Ala-pNA (0.3-5 mM) by the smooth muscle cell membranes (C, agonists; D, peptide antagonists).
Table 1. Aligned structure of the B1 receptor peptide ligands used in the present study and their affinities in two assays (competition of 
[^3]H)Lys-des-Arg⁹-BK to B1 receptors expressed by smooth muscle cells, competition for the hydrolysis of L-Ala-pNA  by the 
aminopeptidase present in smooth muscle cell membranes).

<table>
<thead>
<tr>
<th>Agonists</th>
<th>B1 receptor binding Ki (nM)</th>
<th>Enzyme Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>des-Arg⁹-BK</td>
<td>24.8</td>
<td>86.7</td>
</tr>
<tr>
<td>Lys-des-Arg⁹-BK</td>
<td>0.14</td>
<td>14.3</td>
</tr>
<tr>
<td>Lys-[D-Phe⁸]des-Arg⁹-BK</td>
<td>0.28</td>
<td>16.8</td>
</tr>
<tr>
<td>Sar-[D-Phe⁸]des-Arg⁹-BK</td>
<td>2.80</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonists²</th>
<th>B1 receptor binding Ki (nM)</th>
<th>Enzyme Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Leu⁸]des-Arg⁹-BK</td>
<td>54.3</td>
<td>124</td>
</tr>
<tr>
<td>Lys[Leu⁸]des-Arg⁹-BK</td>
<td>0.52</td>
<td>26.2</td>
</tr>
<tr>
<td>Ac-Lys[Leu⁸]des-Arg⁹-BK</td>
<td>7.53</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>B-10350</td>
<td>0.74</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Abbreviations: Ac : N-acetyl ; CpG, α-cyclopentylglycine; Hyp, trans-4-hydroxyproline; Igl, α-(2-indanyl)glycine; Sar, sarcosine (N- methylglycine) ; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

². The B1 receptor binding Ki values for the nonpeptide Compounds 11 and A are 0.013 and 0.032 nM, respectively.
Fig. 1

A. $B_1$ receptor agonists (n = 4-6)

B. Agonists + peptidase inhibitors (n = 4)

C. Separate peptidase inhibitors (n = 4)

D. Effect of puromycin (n = 4)
fig. 2

**Agonists**
- des-Arg⁹-BK
- Lys-des-Arg⁹-BK
- Lys-[D-Phe⁸]des-Arg⁹-BK
- Sar-[D-Phe⁸]des-Arg⁹-BK

**Antagonists**
- [Leu⁸]des-Arg⁹-BK
- Lys-[Leu⁸]des-Arg⁹-BK
- Ac-Lys-[Leu⁸]des-Arg⁹-BK
- B-10350
- Compound 11
- Compound A

![Graph showing residual total binding for agonists and antagonists](attachment:image.png)
**Fig. 3**

The graph illustrates the EC$_{50}$ (nM) values plotted against $K_i$ (nM) for variousBK analogues. The X markers represent the control, while the o markers indicate the concentration with a cocktail. The dashed line indicates $K_i = EC_{50}$.

- **Lys-des-Arg$^9$-BK**
- **Sar-[D-Phe$^8$]des-Arg$^9$-BK**
- **Lys-[D-Phe$^8$]des-Arg$^9$-BK**

The graph shows the following concentrations:

- **Lys-des-Arg$^9$-BK**: 100 nM
- **Sar-[D-Phe$^8$]des-Arg$^9$-BK**: 10 nM
- **Lys-[D-Phe$^8$]des-Arg$^9$-BK**: 0.1 nM

This article has not been copyedited and formatted. The final version may differ from this version.
A. **Compound A (n = 4)**

B. **Compound A + amastatin (n = 4)**

C. **B-10350 (n = 3-4)**

D. **B-10350 + amastatin (n = 4)**

E. **Ac-Lys-[Leu⁸]des-Arg⁹-BK (n = 4)**

F. **Ac-Lys-[Leu⁸]des-Arg⁹-BK + amastatin (n = 4)**

G. **Lys-[Leu⁸]des-Arg⁹-BK (n=5)**

H. **Lys-[Leu⁸]des-Arg⁹-BK + amastatin (n=4)**

I. **[Leu⁸]des-Arg⁹-BK (n = 4)**

J. **[Leu⁸]des-Arg⁹-BK + amastatin (n = 5)**

**Contraction (% maximum)** vs. **[des-Arg⁹-BK] (nM)**
A. Compound A

B. B-10350

C. Ac-Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK

D. Lys-[Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK

E. [Leu\textsuperscript{8}]des-Arg\textsuperscript{9}-BK

---

-\log[Antagonist]

-\log(DR-1)
Ki (nM)

- Control
- With amastatin

Ki = 10^{-pA_2}

- Compound 11
- Compound A
- Lys-[Leu^8]des-Arg^9-BK
- Ac-Lys-[Leu^8]des-Arg^9-BK
- B-10350
- [Leu^8]des-Arg^9-BK
**A. L-Ala-pNA 2.5 mmol/L**

- control
- amastatin 3 µM
- puromycin 5 µM

**B.**

- SMC membranes (n=8)
- SMC cytosol (n=3)
- BAEC membranes (n=1-2)
- BAEC / APN-GFP (n=2)

**C. Agonists (n=2)**

- control
- + des-Arg⁹-BK
- + Lys-des-Arg⁹-BK
- + Lys-[D-Phe⁸]des-Arg⁹-BK
- + Sar-[D-Phe⁸]des-Arg⁹-BK

**D. Peptide antagonists (n=3-4)**

- control
- + [Leu⁸]des-Arg⁹-BK
- + Lys-[Leu⁸]des-Arg⁹-BK
- + Ac-Lys-[Leu⁸]des-Arg⁹-BK
- + B-10350

This article has not been copyedited and formatted. The final version may differ from this version.