Endogenous Aminopeptidase N Decreases the Potency of Peptide Agonists and Antagonists of the Kinin B₁ Receptors in the Rabbit Aorta

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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 (p⁰A₂ scale) in this assay was compared with the affinity of each agent determined using [³H]Lys-des-Arg⁹-BK binding competition in cultured aortic smooth muscle cells and with the competition Kᵢ for the hydrolysis of the aminopeptidase chromogenic substrate l-Ala-p-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 (Lys-Lys-[Hyp³, Igl⁵, D-Tic⁷, CpG⁸]des-Arg⁹-BK) and Lys-[Leu⁶]des-Arg⁹-BK being intensely potentiated by amastatin treatment and effective l-Ala-p-nitroanilide competitors. N-Protected peptide antagonists or a nonpeptide antagonist of the B₁ receptor were not potentiated by amastatin. The coexpression 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.

Bradykinin-related peptides, the kinins, stimulate cellular functions following binding to two types of receptors predominately coupled to the Gₛ 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 receptors mediate a part of the therapeutic or inducible B₁ receptors. 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 antihypertensive effect of aminopeptidase N fusion protein needs to be confirmed in vivo. Endogenous aminopeptidase N decreases the potency of peptide agonists and antagonists of the kinin B₁ receptors in the rabbit aorta.
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-arginine-bradykinin (des-Arg9-BK) and Lys-des-Arg9-BK (des-Arg9-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 the kinins, are short-lived in vivo. Among the peptidases that hydrolyze kinins, porcine endothelial and smooth muscle cells express aminopeptidase activity inhibited by amastatin (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., 1991a). 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 the idea 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 also been 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 it 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-[Leu]des-Arg9-BK, has also been subjected to such structural analysis (Drapeau et al., 1993). The introduction of synthetic amino acid residues that constrain the peptide backbone has had a major impact in this field, with the production of antagonists that retain high potency (e.g., B-9958; Larrièrè et al., 2000). Contractility studies were performed after 3 or 5.5 h of in vitro incubation because 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-response 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 were introduced 30 min before the construction of the second curve; pA4 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-effect 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 mixture of pepti-
dase inhibitors (3 μM amastatin, 1 μM captopril, and 1 μM phosphoramidon) or of separate peptide inhibitors introduced 45 min before the construction of the second concentration-effect curve. These concentrations of peptide inhibitors are similar to those used in metabolic studies dealing with the metabolism of bradykinin-related peptides (Palmeri et al., 1985; Oravski et al., 1989; Drapeau et al., 1993). Contractility results were expressed as a percentage of the maximal response recorded in each tissue (all the antagonists used were surmountable as judged by the conservation of the $E_{\text{max}}$ from the first concentration-effect curve).

**Binding Assay.** The binding of 1 nM [3H]Lys-des-Arg9-BK (80 Ci/mmol) (PerkinElmer Life and Analytical Sciences, Boston, MA) 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 unlabeled 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 0.02% sodium azide w/w). $K_s$ values for unlabeled competitors were derived from the relationship $K_s = IC_{50}/(1 + S/K_p)$, where $K_p$ is the dissociation constant of the radioligand, $S$ is the radioligand concentration, and $IC_{50}$ is the unlabeled 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 previously described (Sabourin et al., 2002b). Bovine aortic endothelial cells (BAECs; gift from Dr. Darren Richard, Centre Hospitalier Universitaire de Québec, Quebec City, QC, Canada), 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 previously 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 BD Biosciences Clontech (Palo Alto, CA) pEGFP-N1 vector of a reported fluorescent fusion protein (Kehlen et al., 2003; gift from Dr. Astrid Kehlen, Institute of Medical Immunology, University of Halle-Wittenberg, Halle, Germany). Membranes from either cell type or from freshly de-endothelialized 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 of sucrose buffer (250 mM sucrose, 20 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine buffer, 1 mM phenylmethylsulfonyl fluoride, 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 min) and second pellets (15,000 g, 5 min) were discarded; the third (150,000g, 3 h) pellets were resuspended in the same buffer as a source of membrane enzyme (normalized as to the basis of protein concentration; BCA Protein Assay; Pierce, Rockford, IL). 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 to 2 h in 200 μl of phosphate-buffered saline, pH 7.4, were performed precisely as previously described (Lendeckel et al., 1996). Colorless solutions of peptide ligands of the B1 receptor (agonists or antagonists, 10 μM) were used as competitors of L-Ala-pNA hydrolysis in some experiments. Michaelis-Menten enzyme kinetics and competitive inhibition $K_s$ 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-[p-Phe]des-Arg9-BK > Sar-[p-Phe]des-Arg9-BK ∼ Lys-des-Arg9-BK > des-Arg9-BK (Fig. 1A). In the presence of a peptide inhibitor mixture consisting of amastatin, captopril, and phosphoramidon, this order of potency changed to Lys-des-Arg9-BK > Sar-[p-Phe]des-Arg9-BK > des-Arg9-BK > des-Arg9-BK (Fig. 1B). Using Lys-des-Arg9-BK, the agonist for which the mixture produces the most important change in apparent potency (13.8-fold increase), the effect of the separate components of the peptide inhibitor mixture was tested (Fig. 1C). Amastatin is the most important component of the mixture, producing alone a 12.6-fold potency shift, whereas 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-[p-Phe]des-Arg9-BK > Sar-[p-Phe]des-Arg9-BK > des-Arg9-BK (raw data in Fig. 2; $K_s$ values for unlabeled peptides in Table 1 derived from the $K_p$ 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 inhibi-
In the contractility assay. Insets, immunohistochemistry for α-actin (black) in rabbit aortic smooth muscle cells, either cultured (bottom, well spread-out morphology) or in situ in a freshly isolated aorta (top, compact organization and prominent nonmuscle structures; intimal surface to 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; Figs. 4 and 5). In the graph of K_i 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; Figs. 4F and 6) and null for [Leu8]des-Arg9-BK (Figs. 4J and 6). The potency of compound A was essentially unaffected by the presence of amastatin (Figs. 4B and 6), consistent with its nonpeptide chemistry. Thus, some peptide antagonists were also subjected to potency estimate distortion because of 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 fresh de-endothelialized rabbit
aorta and cultured aortic smooth muscle cells contained measurable aminopeptidase activity (Fig. 7A). Preincubating aortic rings for 6 h in sterile Krebs' medium, a procedure that sharply up-regulates 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 $K_M$ of 0.63 mM ($V_{max}$, 4.11 picokatals; 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 of protein; $K_M$, 0.79 mM, high $V_{max}$; Fig. 7B) that was effectively inhibited by 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 $K_M$ of 0.73 mM and was more sensitive to amastatin than to puromycin inhibition (Fig. 7, A and B), like the smooth muscle cell membrane activity. A significant background aminopeptidase activity was present in the membrane fraction of BAECs (1/3 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 $K_i$ values reported in Table 1) but only marginally by des-Arg9-BK and not by Sar-[D-Phe8]des-Arg9-BK (Fig. 7C;
of the chromogenic substrate (7D). B-10350 was the most potent tested peptide competitor in the presence of drugs (compound A, B-10350, Ac-Lys-[Leu8]des-Arg9-BK, Lys-[Leu8]des-Arg9-BK). A relationship between the agonist EC50 values from the averaged concentration-effect curves (DR, dose ratio = EC50/EC50) in the presence of the antagonists divided by the control EC50. Calculated pA2 values (Tallarida and Murray, 1987) are reported in text.

**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 mixture used in experiments reported in Figs. 1B and 2 covered at least ACE (captopril), aminopeptidases (aminopeptidase N, 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 mixture 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-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-des-Arg9-BK in the presence of the peptidase inhibitor mixture 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). Furthermore, 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 on the concentration-effect relationship of Lys-des-Arg9-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 Kd 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 B1 receptor agonist, Lys-des-Arg9-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-Arg9-BK on the basis of potentiation of the contractile effect mediated by B1 receptors in the human isolated umbilical artery. Kokkonen et al. (1999) have analyzed the metabolism of bradykinin and lysyl-bradykinin in cardiac tissue. Whereas 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 B2 receptors seem to be inactivated differently from the B1 receptor agonist. ACE2 is a recently discovered homolog of ACE that does not metabolize bradykinin but reportedly inactivates des-Arg9-BK and Lys-des-Arg9-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, and testis) and is not likely to participate in the inactivation of B1 receptor ligands in smooth muscle cells.

The pA2 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-[Leu8]des-Arg9-BK. The double reciprocal plot representation). Lys-des-Arg9-BK is a mediocre competitor of the smooth muscle cell cytosolic aminopeptidase (calculated Kd 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 (Kd 26.2 μM) but that [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 (Kd 1.89 μM; Fig. 7C).
latter peptides gained 0.9 to 1.0 log unit of potency in the presence of amastatin, whereas Ac-Lys-[Leu8]des-Arg9-BK and [Leu8]des-Arg9-BK were practically unaffected. These pharmacological data, along with the fitting $K_i$ values derived from the L-Ala-pNA hydrolysis competition assay, confirmed a dominant role of aminopeptidase N in the degradation of peptide B1 receptor antagonists. The effect of acetylation of the N terminus is clear because 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 B1 receptor for agonist and antagonist peptides (10-fold lower binding $K_i$ for Lys-[D-Phe8]des-Arg9-BK than for Sar-[D-Phe8]des-Arg9-BK; 14.4-fold lower binding $K_i$ for Lys-[Leu8]des-Arg9-BK relative to Ac-Lys-[Leu8]des-Arg9-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 up-regulated 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 (Peloro 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. Furthermore, the present study shows unequivocally that susceptibility to aminopeptidase N is critical for the design of hydrosoluble peptide antagonists, as illus-
trated with B-10350. Hydrosoluble antagonists may be well adapted to i.v. 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 example, allergic inflammation of the human nasal mucosa determines a local B2 receptor up-regulation (Christiansen et al., 2002), and the possible benefits of receptor blockade remain to be determined in this condition.

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