The kinin B₁ receptor antagonist des-Arg⁹-[Leu⁸]bradykinin: an antagonist of the angiotensin AT₁ receptor which also binds to the AT₂ receptor

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1 Agonists and antagonists of kinin B₁ and B₂ receptors were evaluated *in vitro* for their effects against angiotensin II (AII)-induced contractile responses in the rabbit aorta and for their binding properties to angiotensin AT₁ and AT₂ receptors from purified membrane of rat liver and lamb uterus respectively.

2 In aortic rings, the kinin B₁ receptor antagonist, des-Arg⁹-[Leu⁸]bradykinin (BK) (3–100 µM) caused a concentration-dependent decrease in sensitivity and a depression of the maximum response to AII. Des-Arg¹⁰-[Leu⁹]kallidin (KD), des-Arg⁹-BK, des-Arg¹⁰-KD, BK or KD at 3 µM had no effect against AII-induced contractions.

3 Des-Arg⁹-[Leu⁸]BK (3 or 100 µM) did not affect contractions of aortic rings to histamine, potassium chloride, endothelin-1, 5-hydroxytryptamine, noradrenaline and the thromboxane A₂-mimetic, U46619.

4 Des-Arg⁹-[Leu⁸]BK displaced [¹²⁵I]-Sar¹-AII binding to the AT₁ subtype in rat liver membranes with a K_i value of 1.1 ± 0.4 µM. Values of K_i for des-Arg⁹-BK and KD were 45 ± 13 µM and 25 ± 22 µM, respectively. The other kinin derivatives des-Arg¹⁰-KD, BK and des-Arg¹⁰-[Leu⁹]KD at concentrations up to 100 µM did not bind to the AT₁ receptor.

5 All the kinin derivatives except BK bound to AT₂ receptors in lamb uterus membranes. Values of K_i for des-Arg⁹-[Leu⁸]BK, des-Arg¹⁰-[Leu⁹]KD, des-Arg⁹-BK, des-Arg¹⁰-KD and KD were 0.3 ± 0.1, 0.7 ± 0.1, 1.2 ± 0.3, 1.5 ± 0.3 and 7.0 ± 1.6 µM, respectively.

6 In conclusion, des-Arg⁹-[Leu⁸]BK is an insurmountable antagonist of AII-induced contractions in the rabbit aorta and also binds with a relatively high affinity to AT₁ and AT₂ receptors in isolated membrane fractions. These additional properties of des-Arg⁹-[Leu⁸]BK should be considered when it is used as an antagonist to characterize kinin B₁ receptors.

Keywords: Kinin B₁ and B₂ receptors; angiotensin AT₁ and AT₂ receptors; rabbit aorta; vascular reactivity; binding study

Introduction

Kinin receptors have been divided into B₂ and B₁ subtypes on a pharmacological basis (Regoli & Barabé, 1980). Kallidin (KD) and bradykinin (BK) which represent the tissular and circulating forms of kinins respectively, stimulate preferentially the B₂ receptor subtype (Regoli & Barabé, 1980; Hall, 1992). In contrast, kinin derivatives lacking the C-terminal arginine such as des-Arg⁹-BK and des-Arg¹⁰-KD have been described as selective agonists at kinin B₁ receptors (Regoli & Barabé, 1980). Kinin B₁ receptors are further characterized by the ability of des-Arg⁹-[Leu⁸]BK and des-Arg¹⁰-[Leu⁹]KD to antagonize solely B₁ receptor-mediated responses (Regoli & Barabé, 1980). In this regard, des-Arg⁹-[Leu⁸]BK has been described as a specific kinin B₁ receptor antagonist (Regoli & Barabé, 1980; Regoli *et al.*, 1990) and is widely used to assess the participation of kinin B₁ receptors in experimental pathological situations (Corrêa & Calixto, 1993; Perkins *et al.*, 1993).

In the present study, we show that des-Arg⁹-[Leu⁸]BK is an antagonist of angiotensin AT₁ receptors and also binds to the AT₂ subtype. The results were obtained from functional assays on isolated rabbit aorta contracted with angiotensin II (AII) and from binding experiments conducted in membrane preparations from rat liver and lamb uterus.

Methods

Rabbit isolated aorta

Male New Zealand White rabbits weighing 2.5 to 3.5 kg were killed with an overdose of i.v. sodium pentobarbitone. The thoracic aorta was dissected out and immediately placed in a Krebs solution of the following composition (in mM): NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.17, NaHCO₃ 25, CaCl₂ 2.5, ethylenediaminetetracetic acid (EDTA) 0.026, glucose 5.5, and bubbled with 95% O₂ plus 5% CO₂. Eight rings of 4 mm in length were prepared from the aortic segment and the endothelium was rubbed off *in vitro* with a pipe cleaner which was introduced into the vessel lumen and gently moved back and forth several times. Rings were then suspended on triangular stainless-steel wires (0.3 mm in diameter) in 20 ml jacketed organ baths maintained at 37°C. One hook was suspended from a Gould-Statham UC₂ or UTC₂ transducer, and the other was fixed to a plastic support leg. Changes in isometric tension were recorded continuously on two-channel recorders (Gould BS272 or Linseis Type 7025). Rings were left unstretched for 30 min and were then stretched in a stepwise fashion to a passive force of 2 g.

After 1 h of equilibration, arterial rings were contracted with an isotonic high KCl (75 mM)-containing Krebs solution and the baths washed 3 times with normal Krebs solution. This procedure was repeated 30 min later. After return to the baseline, BK (3 µM), KD (3 µM), des-Arg⁹-BK (3 µM), des-

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Arg¹⁰-KD (3 μ M), des-Arg⁹-[Leu⁸]BK (3 to 100 μ M) or des-Arg¹⁰-[Leu⁷]KD (3 μ M) was added and 10 min was then allowed before the cumulative concentration-response curve to AII (0.0003 to 1 μ M). The effect of the kinin B₁ receptor antagonist, des-Arg⁹-[Leu⁸]BK (3 or 100 μ M) against cumulative concentration-response curves to histamine, KCl, endothelin-1, 5-hydroxytryptamine, noradrenaline or U46619 was also evaluated. Only one concentration-contraction curve was obtained in any one ring of artery.

Binding experiments

Liver membranes were purified from male Wistar rats weighing 200 to 250 g (Iffa Credo, L'Arbresle, France) according to Neville (1968) and stored frozen in liquid nitrogen. Competition binding assays were carried out in 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM MgCl₂, 1 mg ml⁻¹ bovine serum albumin (BSA) and 1 mg ml⁻¹ bacitracin, using [¹²⁵I]-Sar¹-AII as ligand, 15 μ g membrane protein per assay (final volume 90 μ l) and 30 min reaction times at 30°C.

Lamb uteri obtained from animals freshly-slaughtered at a local abattoir were ground in about 8 volumes of ice-cold grinding medium (10 mM Tris-HCl buffer at pH 7.4, 1 mM EDTA, 0.3 M KCl) using a Polytron apparatus, then a Dounce homogenizer. After 15 min of gentle stirring, the homogenate was filtered through two layers of gauze, then centrifuged for 20 min at 600 g. The supernatant was centrifuged for 20 min at 20,000 g. The resulting crude membrane pellets were washed twice with 10 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA. Aliquots of the membrane suspension kept in the same buffer were stored frozen in liquid nitrogen. Before performing AT₂ binding experiments, membranes were treated with 20 mM dithiothreitol (DTT) added to the binding medium maintained at pH 7.8, in order to inactivate AT₁ receptor sites (Whitebread *et al.*, 1989). Competition binding assays were carried out in a binding medium containing 50 mM Tris-HCl buffer at pH 7.4, 125 mM NaCl, 6.5 mM MgCl₂, 1 mM EDTA, 2 mg/ml BSA, 1 mg/ml bacitracin, with [¹²⁵I]-Sar¹-AII used as displaced ligand, 50 μ g membrane protein per assay (final volume 90 μ l) and 30 min reaction times at 30°C. Kinin derivatives were tested in 0.001–100 μ M range of concentrations. Protein measurements were performed according to Lowry *et al.* (1951).

Calculations and statistical analysis

The negative logarithm of the concentration of agonist needed to reach 50% (EC₅₀) of the maximal response to this agonist in the rabbit aorta was calculated by least-square regression analysis (Tallarida & Murray, 1981). After testing for linearity and parallelism, EC₅₀ values for contraction and maximal responses (E_{max}) were compared by Student's *t* test. Data obtained from binding experiments are given as mean $\pm$ s.d.mean. In binding experiments, values of K_i were determined as described by Ekins *et al.* (1968). *n* indicates the number of animals in vascular reactivity studies and the number of experiments in binding studies. The level of significance was *P* < 0.05.

Drugs

Bacitracin, bradykinin acetate, des-Arg⁹-bradykinin acetate, des-Arg⁹-[Leu⁸]bradykinin acetate, dithiothreitol, histamine dihydrochloride, noradrenaline hydrochloride, 5-hydroxytryptamine creatinine sulphate complex and U46619 (1,5,5-hydroxy-11,9-(epoxymethano) prosta-5z, 13E-dienoic acid) were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Neosystem (Strasbourg, France) provided des-Arg¹⁰-kallidin acetate and des-Arg¹⁰-[Leu⁷]kallidin acetate. Endothelin-1 and Sar¹-angiotensin II were from Bachem (Bubendorf, Switzerland). Sar¹-angiotensin II was radio-

iodinated (1800 Ci mmol⁻¹) as previously described (Bonafous *et al.*, 1988). All drugs were made up in distilled water or dimethylsulphoxide and kept on ice and protected from light.

Results

Vascular reactivity

The effect of des-Arg⁹-[Leu⁸]BK on the cumulative concentration-contraction curve to AII is shown in Figure 1. Des-Arg⁹-[Leu⁸]BK (3–100 μ M) produced a concentration-related, insurmountable antagonism of AII. The EC₅₀ value was 8.7 $\pm$ 0.1 in the absence and 7.8 $\pm$ 0.2, 7.3 $\pm$ 0.4, 6.8 $\pm$ 0.3, 6.3 $\pm$ 0.4 in the presence of 3, 10, 30 and 100 μ M des-Arg⁹-[Leu⁸]BK, respectively. The maximum response was reduced to 65.4 $\pm$ 7.6%, 52.1 $\pm$ 7.2%, 47.1 $\pm$ 7.5% and 53.6 $\pm$ 10.4% in the presence of 3, 10, 30 and 100 μ M des-Arg⁹-[Leu⁸]BK, respectively. Des-Arg¹⁰-BK, BK, des-Arg¹⁰-[Leu⁷]KD, des-Arg¹⁰-KD and KD at 3 μ M had no effect against the cumulative response-curve to AII (*n* = 4/group; data not shown). Des-Arg⁹-[Leu⁸]BK at 3 μ M had no effect against concentration-contraction curves to histamine, potassium chloride, endothelin-1 and the thromboxane A₂ mimetic, U46619 (Figure 2). Furthermore, des-Arg⁹-[Leu⁸]BK at 100 μ M did not affect contractile responses to noradrenaline and 5-hydroxytryptamine, two agents that similar to AII produce contractions via IP₃-mediated increases in intracellular Ca²⁺ (Figure 2).

Binding experiments

Des-Arg⁹-[Leu⁸]BK bound to the rat liver AT₁ receptor (Figure 3 and Table 1) with a K_i value of 1.1 $\pm$ 0.4 μ M (*n* = 4). KD and des-Arg⁹-BK displayed lower affinities towards AT₁ receptors with K_i values of 25 $\pm$ 22 μ M (*n* = 3) and 45 $\pm$ 13 μ M (*n* = 3) respectively whilst BK, des-Arg¹⁰-KD and des-Arg¹⁰-[Leu⁷]KD had no affinity for the AT₁ receptor (K_i > 100 μ M, Table 1).

Kinin derivatives were also tested for their ability to reverse [¹²⁵I]-Sar¹-AII binding to the AT₂ receptor in lamb uterus membranes. As for the rat liver AT₁ receptor, des-Arg⁹-[Leu⁸]BK showed the highest affinity with a K_i value of 0.3 $\pm$ 0.1 μ M (*n* = 4, Figure 3 and Table 1). BK had no binding affinity up to 100 μ M whilst KD and the other des-Arg compounds displayed K_i values in the micromolar range (Table 1).

Figure 1 Cumulative concentration-contraction curves for angiotensin II in the rabbit aorta in the absence (●) and presence of 3 (○), 10 (△), 30 (Δ) and 100 μ M (■) des-Arg⁹-[Leu⁸]BK. Values represent means $\pm$ s.e.mean of 6 experiments.

Figure 2 Concentration-response curves to endothelin-1 (ET-1), histamine, potassium chloride (KCl), U46619, noradrenaline (NA), 5-hydroxytryptamine (5-HT) obtained in the absence (●) or presence of 3 μ M (○) or 100 μ M (Δ) des-Arg⁹-[Leu⁸]BK. Values represent means $\pm$ 1 s.e. mean of 4 experiments.

Table 1 Binding of kinin derivatives to angiotensin II AT₁ and AT₂ receptor subtypes

Kinin derivatives	K _i (μ M)	K _i (μ M)
	AT ₁ (rat liver)	AT ₂ (lamb uterus)
BK	> 100	> 100
des-Arg ⁹ -BK	45 $\pm$ 13	1.2 $\pm$ 0.3
des-Arg ⁹ -[Leu ⁸]BK	1.1 $\pm$ 0.4	0.3 $\pm$ 0.1
KD	25 $\pm$ 22	7.0 $\pm$ 1.6
des-Arg ¹⁰ -KD	> 100	1.5 $\pm$ 0.3
des-Arg ¹⁰ -[Leu ⁹]KD	> 100	0.7 $\pm$ 0.1

Competition experiments were carried out as described in the Methods section, using [¹²⁵I]-Sar¹-AII (0.2 nM) as radioactive ligand and 0.001–100 μ M concentrations of the various kinin derivatives.

Values are means $\pm$ s.e. of 3–4 independent experiments. The assays within each experiment were carried out in triplicate.

Values of K_i were calculated as described by Ekins *et al.* (1968).

Discussion

The kinin B₁ receptor antagonist, des-Arg⁹-[Leu⁸]BK has been reported to have no antagonist effect against a number of other contractile agonists including AII (Regoli & Barabé,

Figure 3 Binding of des-Arg⁹-[Leu⁸]BK to the angiotensin AT₁ and AT₂ receptors. Representative typical competition experiments carried out as described in the Methods section, using [¹²⁵I]-Sar¹-AII (0.2 nM) as radioactive ligand in the presence of Sar¹-AII (○, 0.0001 to 10 μ M) or des-Arg⁹-[Leu⁸]BK (●, 0.001 to 100 μ M). Each point was obtained from a triplicate run. (a) Competition binding to AT₁ receptor from rat liver. The value of K_i for des-Arg⁹-[Leu⁸]BK in this experiment was 0.8 μ M. (b) Competition binding to AT₂ receptor from lamb uterus. The value of K_i for des-Arg⁹-[Leu⁸]BK in this experiment was 0.16 μ M.

1980; Regoli *et al.*, 1990). In the present study however, we have shown that des-Arg⁹-[Leu⁸]BK not only binds to AT₁ and AT₂ receptors but is also an insurmountable antagonist of AII-induced contractions in the rabbit aorta. The reason for the insurmountable antagonism by des-Arg⁹-[Leu⁸]BK which has been described for other peptide (Scanlon *et al.*, 1984) and nonpeptide (Timmermans *et al.*, 1991) AT₁ receptor antagonists, is unknown.

Angiotensin receptors have been divided into AT₁ and AT₂ types both of which have been cloned (Murphy *et al.*, 1991; Sasaki *et al.*, 1991; Kambayashi *et al.*, 1993; Mukoyama *et al.*, 1993). AT₁ receptors mediate most of the biological responses hitherto attributed to AII whilst the physiological role of AT₂ receptors remains uncertain (Timmermans *et al.*, 1993). Binding studies performed in rat liver membrane preparations indicate that des-Arg⁹-[Leu⁸]BK bound to the AT₁ receptor whilst BK, KD, des-Arg¹⁰-[Leu⁹]KD and des-Arg¹⁰-KD did not bind to the AT₁ receptor at concentrations up to 100 μ M. In addition, des-Arg⁹-BK, BK, des-Arg¹⁰-[Leu⁹]KD, des-Arg¹⁰-KD and KD at 3 μ M had no effect on AII-induced contractions. Thus, des-Arg⁹-[Leu⁸]BK appears to be unique amongst kinin derivatives in its additional antagonist effects at the AT₁ receptor. Furthermore, in preliminary studies des-Arg⁹-[Leu⁸]BK reversed the binding of [¹²⁵I]-Sar¹-AII or tritiated nonpeptide AT₁ antagonists to intact CHO cells transfected with cDNA encoding the AT₁ receptor (J.C. Bonnafous & A. Duvoid, personal communication).

Membranes from lamb uterus contain a mixed population

of AT₁ and AT₂ receptors. Therefore, in order to assess the binding property of des-Arg⁹-[Leu⁸]BK and other kinins to the AT₂ receptor in this tissue, membranes were first treated with DTT to selectively abolish the binding of AII to the AT₁ subtype (Whitebread *et al.*, 1989). Under these conditions, various kinin derivatives bound to the AT₂ receptor with the following potency (K_i , μM): des-Arg⁹-[Leu⁸]BK (0.3), des-Arg¹⁰-[Leu⁹]KD (0.7), des-Arg⁹-BK (1.2), des-Arg¹⁰-KD (1.5) and KD (7.0). BK was inactive at 100 μM . Although the number of kinin related compounds tested was limited, some simple structure-activity relationships appear. Compounds lacking the C-terminal arginine had a better affinity than KD and BK and a leucine residue in position 8 or 9 of des-Arg⁹-BK and des-Arg¹⁰-KD respectively, also enhanced the binding affinity.

In conclusion, we have demonstrated that the previously

accepted selective B₁ receptor antagonist des-Arg⁹-[Leu⁸]BK is an insurmountable antagonist of AII-induced contraction in rabbit aorta and binds to both AT₁ and AT₂ receptors. Thus, these additional properties of des-Arg⁹-[Leu⁸]BK should be considered in studies in which des-Arg⁹-[Leu⁸]BK is used to assess the participation of kinin B₁ receptors. Furthermore, these findings raise the question of how kinin derivatives recognize AT₁ and AT₂ receptors. Future cloning of the kinin B₁ receptor might allow delineation of the structural basis for such recognition through the comparison of binding epitopes of angiotensin II and kinin receptors.

The authors wish to thank Dr T.M. Cocks (University of Melbourne) for helpful suggestions regarding the manuscript and E. DeFrène for technical assistance.

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(Received July 15, 1994
Accepted September 15, 1994).