Synthesis and Biological Evaluation of Bradykinin B_1/B_2 and Selective B_1 Receptor Antagonists

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We recently described a potent bradykinin B_2 receptor agonist (JMV1116) obtained by replacing the D-Tic-Oic dipeptide moiety of HOE140 by a (3*S*)-amino-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety. This compound inhibited the specific binding of [^{3}H]BK on membranes of CHO cells expressing the human cloned B_2 receptor with nanomolar affinity and contracted both isolated rat uterus and human umbilical vein. These data demonstrated that D-BT could be a good mimic of the Pro-Phe dipeptide. In the present study we characterized B_1 receptor antagonists containing the D-BT moiety. We prepared an analogue of compound JMV1116 deleting the C-terminal arginine residue. The resulting compound (**1**) had an affinity of 83 nM for the human cloned B_1 receptor. The most remarkable property of **1** is its ability to bind also the B_2 receptor with an affinity of 4.4 nM despite the absence of the C-terminal arginine residue. Modifications at the N-terminal part of **1** associated with the substitution of the thienylalanine residue by α-(2-indanyl)glycine resulted in analogues selectively binding to the B_1 receptor with an affinity in the picomolar range.

Introduction

Both bradykinin (BK, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and kallidin (Lys-BK) are involved in numerous physiological and pathophysiological processes including smooth muscle contraction, vasodilation, pain, hypotension, and acute inflammation such as trauma, burns, shock, and allergy.[1−5] Effects of kinins are mediated through B_1 and B_2 receptors that were initially identified from classical bioassay experiments. Corresponding human B_1 and B_2 receptors were subsequently cloned in 1994 and 1992.[6,7] B_2 receptors which are constitutively expressed in a variety of tissues are activated by BK or Lys-BK peptide and mediate most of the biological actions of kinins.[8] In contrast, B_1 receptors which are induced after inflammation are activated by carboxypeptidase degradation products of BK and Lys-BK which are [des-Arg^9]-BK and [des-Arg^{10}]-kallidin, respectively.[9] The pronounced up-regulation of the B_1 receptor in a variety of chronic inflammatory conditions supports a role for this receptor in the maintenance of associated pain.[9−11] The growing evidence that the B_1 receptor, in addition to the B_2 receptor, plays an important role in various pathophysiological processes stimulated research for B_1 receptor antagonists.

The first B_1 receptor antagonists described by Regoli were [Leu^8,des-Arg^9]-BK and [Leu^9,des-Arg^{10}]-KD (CP 0298),[12] but these compounds suffered from rapid in vivo degradation.[8] Deletion of the C-terminal arginine residue of the potent B_2 antagonist HOE140[13] produced [des-Arg^{10}]-HOE140 which has been described as a potent and stable B_1 antagonist.[14] However, Stewart et al.[15] have found this compound 10-fold weaker on the human B_1 receptor than Lys-[Leu^8,desArg^9]-BK. Cheronis et al.[16] designed heterodimer peptides with combined B_1 and B_2 antagonist activity. More recently, Stewart and co-workers[15] have described a series of bradykinin antagonists containing the amino acid indanylglycine (Igl) at positions 5 and 7 of the bradykinin. Among these compounds, B-9430 (H-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg-OH) exhibited potent antagonist activity at the human B_2 receptor both in vitro and in vivo. Moreover, this compound exhibited high affinity for the B_1 receptor despite the presence of the C-terminal arginine residue. Subsequent deletion of the C-terminal arginine residue led to a series of potent B_1 antagonists. The most potent compound (B9858: H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH) showed subnanomolar affinity on the human cell line IMR-90. However, this compound also bound to the human B_2 receptor with high affinity (pIC_{50} on the human cloned B_2 receptor of 7.7).[17]

Recently,[18,19] we have reported the substitution of the dipeptide D-Tic-Oic in HOE140 by various constrained dipeptide mimetics and found that replacement by a (3*S*)-amino-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety yielded a potent B_2 receptor agonist (JMV1116, H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-Arg-OH). Therefore, we proposed that D-BT could be a good mimic of Pro-Phe (or D-Tic-Oic) dipeptide. Since dual B_1 and B_2 receptor antagonists or selective B_1 antagonists could be attractive targets, we were interested in the design of compounds containing constrained dipeptide mimics in their sequence. For this purpose we investigated the synthesis of des-Arg analogues of our potent B_2 agonist JMV1116.

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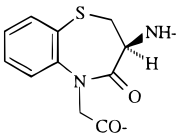
Table 1. Binding Affinities of Bradykinin and HOE140 Analogues to B_2 and B_1 Human Receptors

D-BT = 

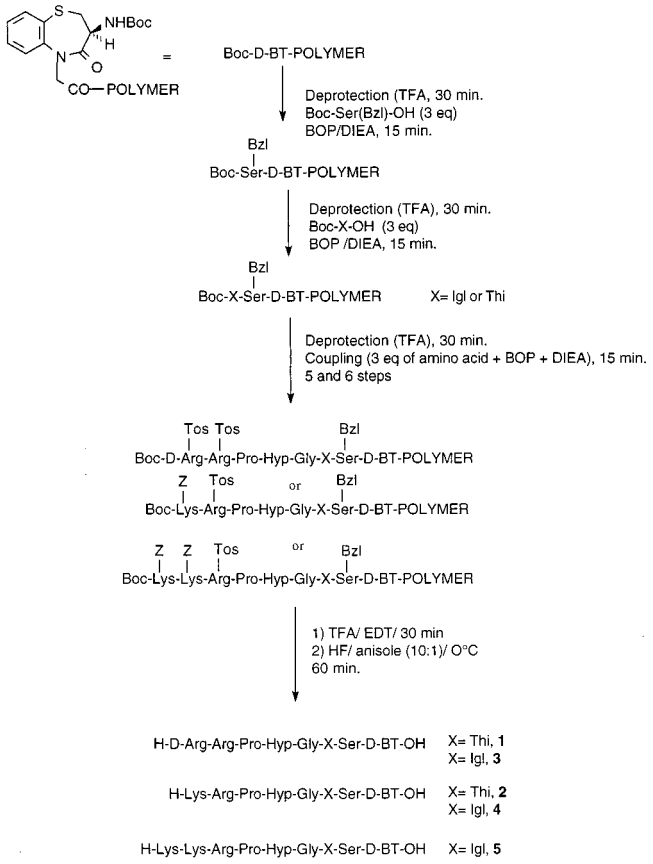
compd	sequence	K_i (nM), human receptor		pA_2 or pD_2, HUV	
		B_2[a]	B_1[b]	B_2[c]	B_1[d]
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	0.11(22)	>10³(23)	7.9 (pD_2)	nd
HOE140	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH	0.08(22)	>10³(23)	8.2	<5
[des-Arg⁹,Leu⁸]-BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH	>10³(22)	382(23)	<5	6.4
[des-Arg¹⁰,Leu⁹]-KD	H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH	>10³(22)	0.9(23)	<5	7.9
[des-Arg¹⁰]-HOE140	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-OH	nd	24	<5	7.5
1, JMV1431	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-OH	4.4	83	nd	nd
2, JMV1669	H-Lys-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-OH	0.40	0.22	pK_p[e] 4.8	7.4
3, JMV1638	H-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	325	5.60	<5	nd
4, JMV1645	H-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	9.2	0.023	<5	8
5, JMV1639	H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	62	0.035	5.5	8.5

[a] Competition binding experiments with [³H]bradykinin on human cloned B_2 receptors. [b] Competition binding experiments with [³H][des-Arg¹⁰,Leu⁹]-kallidin on human cloned B_1 receptors. [c] Inhibition of the contraction of the human umbilical vein (HUV) induced by different concentrations of BK. [d] Inhibition of the concentration response curve to [des-Arg¹⁰]-kallidin in HUV incubated for 16 h in Eagles Medium containing 1% FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. Results are means of at least 3 separate experiments; nd, not determined. [e] pK_p represents the agonist affinity of a partial agonist (Kenakin et al. In *Pharmacology of Drug−Receptor Interaction*; Kenakin, T., Ed.; Ravin Press: New York; pp 175−181). It is estimated by comparing equiactive concentrations of a full agonist in the absence and presence of a partial agonist according to the formula log(1/slope − 1)=log [P] − log K_p.

Chemistry

The constrained dipeptide mimetic Boc-D-BT-OH was prepared as previously described.[18] All compounds were synthesized on a chloromethylated resin by the solid-phase method with Boc-D-BT-OH bound to the resin via its cesium salt.[20] N^{α}-*tert*-Butyloxycarbonyl (Boc) protection was used as temporary protection of the N-terminal amino groups, and tosyl, benzyloxycarbonyl (Z), and benzyl groups were used for side-chain protections. Couplings of protected amino acids were carried out with BOP reagent.[21] After deprotection of the Boc group by TFA in the presence of ethanedithiol as scavenger, BK analogues were deprotected and cleaved from the resin using standard liquid HF procedure in the presence of anisole, and compounds **1**−**5** were purified by preparative reverse-phase HPLC on a C_{18} column and characterized by mass spectrometry. Their purity was checked by analytical HPLC and capillary electrophoresis (Table 2). The synthetic routes used for their preparation are described in Scheme 1.

Scheme 1. Synthesis of Compounds **1**−**5**



Results and Discussion

Bradykinin analogues were evaluated for their ability to bind the human cloned B_2 receptor expressed in CHO cells[22] as well as the human cloned B_1 receptor expressed in 293 cells.[23] The most potent analogues were tested for their ability to interact with BK or [des-Arg¹⁰,Leu⁹]-KD-induced contraction of the human umbilical vein. All compounds **1**−**5** inhibited in a concentration-dependent manner the binding of [³H]BK to the human cloned B_2 receptor and [³H][des-Arg¹⁰,Leu⁹]-kallidin to the human cloned B_1 receptor with affinity in the picomolar to micromolar range (Table 1).

Deletion of the C-terminal arginine residue of JMV1116 yielded compound **1** (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-OH) which bound to the B_1 receptor with high affinity (83 nM). Although removal of the C-terminal arginine residue is critical to obtain a selective B_1 receptor antagonist, compound **1** also displayed high affinity for the B_2 receptor (4.4 nM).

Substitution of the N-terminal D-arginine residue by a lysine yielded compound **2** which had 380 and 10 times increased affinity for B_1 and B_2 receptors, respectively. Compound **2** bound with almost the same potency to human cloned B_2 and B_1 receptors (0.40 and 0.22 nM, respectively). This compound acted as a weak B_2 partial agonist at high concentrations on the human umbilical vein (pK_p[24] = 4.8) and as a potent B_1 antagonist (pA_2 = 7.4) with no residual agonist effect.

Table 2. Analytical Data of B_2 and B_1 Bradykinin Analogues

compd	sequence	retention time (min)[d] HPLC[a]	retention time (min)[d] CE[b]	MS (M + H)[c]
1, JMV1431	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-OH	27.2	6.27	1072
2, JMV1669	H-Lys-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-OH	25.5	6.38	1044
3, JMV1638	H-DArg-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	30.6	4.14	1092
4, JMV1645	H-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	26.9	4.51	1064
5, JMV1639	H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-BT-OH	29.2	4.67	1192

[a] HPLC was performed on a Delta-Pak RP C_{18} column (5 μm, 100 Å, 3.9 × 150 mm) with a mixture of A (H_2O, 1% TFA) and B (CH_3CN, 1% TFA) as eluent; used gradient: 0→50′→50% B. [b] CE was performed with uncoated fused silica capillaries (75 μm × 50 cm × 800 μm aperture), pressure injection and the following run conditions: 15 min, 20 °C, 30 kV, borate buffer (boric acid 0.4%; Borax 0.3%, water) at pH 8.6. [c] FAB mass spectrometry was run on a JEOL JMS-DX-300-SX102 instrument. [d] Purity > 98% according to both methods.

Our binding results confirmed that one of the requirements for high affinity at the B_1 receptor is the presence of a lysine residue at the N-terminal of BK analogues.

Stewart et al.[15] developped a series of B_2/B_1 receptor antagonists by introducing an indanylglycine (Igl) at positions 5 and 7 in the HOE140 sequence. Their antagonists exhibited high affinity at the B_1 receptor despite the presence of the C-terminal arginine. This antagonist property could be attributed to the presence of Igl residues. To increase the potency and the selectivity of our compounds for the B_1 receptor, we substituted the thienylalanine by an indanylglycine residue in compounds **1** and **2**. As expected, the resulting analogues **3** and **4** were approximately 15 and 10 times more potent at the B_1 receptor than their thienylalanine analogues. While compound **3** showed a dramatic decrease in affinity for the B_2 receptor (K_i = 325 nM) as compared to compound **1** (K_i = 4.4 nM), it retained high affinity for the B_1 receptor (K_i = 5.6 nM) which was increased by a factor of approximately 15 as compared to compound **1**. Compound **4** having a N-terminal lysine instead of D-arginine showed high affinity for the B_2 receptor (K_i = 9.2 nM). However, it had both an impressive high affinity for the B_1 receptor (0.023 nM) and a resulting 400-fold selectivity for this receptor.

It has been observed[17] that the N-terminal extension of BK and analogues with lysine brought a significant gain of affinity for the B_1 receptor. To check the significance of an extra N-terminal lysine in our analogues, we have synthesized compound **5** which is an N-terminal lysine-extended analogue of **4.** This modification resulted in a compound which showed a 7-fold decrease in affinity for the B_2 receptor (K_i = 62 nM) while its affinity for the B_1 receptor remained high (K_i = 0.035 nM). The most potent compounds in binding competition studies on B_1 and B_2 receptors were tested on the contraction of the isolated human umbilical vein induced by [des-Arg[10]]-kallidin and BK. None of the compounds but **2,** which exhibited a partial agonist activity, were able to contract human umbilical vein even at high concentration (1 μM). Compound **5** was able to slightly inhibit BK-induced contraction (pA_2 of 5.5). We have recently shown that replacing the dipeptide D-Tic-Oic in the potent BK antagonist HOE140 unexpectedly resulted in potent agonists.[18] The results of the present study suggest that the absence of the C-terminal arginine residue is not important for binding to the B_2 receptor while it is a crucial determinant for exhibiting biological activity at this receptor. In fact compounds **4** and **5** having significant affinity at the B_2 receptor were poor antagonists, while they exhibited high affinity at the B_1 receptor (K_i = 0.023 and 0.035 nM). They exhibited high antagonist activity at the B_1 receptor (pA_2 = 8.0 and 8.5, respectively).

We have shown in this study that suppression of the C-terminal arginine residue in D-BT-containing bradykinin analogues resulted in B_1 ligands still exhibiting affinity for the B_2 receptor. However, replacement of thienylalanine by indanylglycine in these analogues conferred high selectivity for the B_1 receptor. Although D-BT-containing bradykinin analogues were agonists at the B_2 receptor,[18] their des-Arg derivatives behaved as B_1 receptor antagonists.

Experimental Section

Compounds were prepared by solid-phase synthesis using Boc strategy. The starting chloromethylated Merrifield resin was purchased from Pierce; Boc-amino acids were obtained from Bachem except for the (3*S*)-[*tert*-butyloxycarbonylamino]-5-carboxymethyl-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (Boc-D-BT-OH). The following protected amino acids were used: Boc-Lys(Z)-OH, Boc-D-Arg(Tos)-OH, Boc-Arg(Tos)-OH, Boc-Ser(Bzl)-OH. Boc-D-BT-OH was coupled to the chloromethylated resin according to the Gisin method.[25] Final compounds were cleaved from the resin by HF in the presence of anisole and crude products were purified by reverse-phase HPLC on a Waters Delta-Prep 4000 chromatograph equipped with a Waters 486 UV detector with detection at 214 nm, using a Delta-Pak C_{18} column (40 × 100 mm, 15 μm, 100 Å) at a flow rate of 50 mL/min of a binary eluent system of A/B (A: H_2O, TFA 0.1%; B: CH_3CN, TFA 0.1%).

Boc-D-BT-O-Merrifield Resin. Boc-D-BT-OH (2 g; 5.7 mmol) was solubilized in a mixture of ethanol 95%/H_2O (80/20). Cesium carbonate (936 mg; 2.87 mmol) was added under stirring. After 15 min stirring, the solvent was removed in vacuo and the resulting oil was lyophilized. The obtained cesium salt was then solubilized in DMF, the Merrifield resin was added (2.87 g; 0.5 to 1 mmol substitution) and the mixture was slowly stirred at 60 °C for 48 h. The resin was filtred, largely washed with DMF, DCM, MeOH, H_2O and DCM, and dried in vacuo to yield 3.7 g. The obtained fonctionalization was evaluated at 0.98 mmol/g (by rise of weight).

General Procedure for the Preparation of Analogues 1−5. Boc-D-BT-O-Merrifield (255 mg; 0.25 mmol) was used and the following amino acids were coupled in DCM in the presence of BOP as coupling reagent and DIEA to the growing peptide chain in stepwise fashion: 3 equiv of Boc-Ser(Bzl)-OH (or 3 equiv of Boc-Thr(Bzl)-OH), 3 equiv of Boc-Thi-OH (or 3 equiv of Boc-Igl-OH), 3 equiv of Boc-Gly-OH, 3 equiv of Boc-Hyp-OH, 6 equiv of Boc-Pro-OH, 6 equiv of Boc-Arg(Tos)-OH, 3 equiv of Boc-Lys(Z)-OH (or 3 equiv of Boc-D-Arg(Tos)-OH), and 3 equiv of Boc-Lys(Z)-OH. Reaction time for complete couplings were 20 min for Boc-Ser(Bzl)-OH (or Boc-Thr(Bzl)-OH), Boc-Thi-OH (or Boc-Igl-OH), Boc-Gly-OH, Boc-Hyp-OH and Boc-D-Arg(Tos)-OH (or Boc-Lys(Z)-OH), 1 h for Boc-Pro-OH and Boc-Arg(Tos)-OH. Completion of the reaction was checked by the Kaiser test. Removal of the Boc protecting group was achieved with a mixture of TFA/DCM/ethanedithiol (40/60/2). Washings of the substituted resin were performed with isopropyl alcohol and DCM. After coupling the last amino

acid Boc-D-Arg(Tos)-OH (or Boc-Lys(Z)-OH), the Boc protecting group was removed by TFA/DCM/ethanedithiol (40/60/2) before complete deprotection.

HF Cleavage. The total deprotection and the cleavage of the compounds from the resin was performed with HF. The peptidyl resin was placed in a Teflon reactor containing anisole (1 mL/g of resin). After distillation of HF (10 mL/g of resin) into the reactor, the mixture was stirred 1 h at 0 °C. HF was removed by distillation. The expected compound was precipitated by addition of ether, washed with ether and finally purified by reverse-phase HPLC with a mixture of acetonitrile/water/TFA (50/50/0.1) and lyophilized to yield a white fluffy solid.

Pharmacological Studies. Materials. HOE140 (D-Arg^0-[Hyp^3,Thi,^{5}D-Tic^7,Oic^8]bradykinin), [Leu^8,des-Arg^9]-BK, [Leu^9,des-Arg^{10}]-KD and [des-Arg^{10}]-HOE140 were synthesized in our laboratory. MERGETPA (DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid) was obtained from Calbiochem (La Jolla, CA). All molecular biology and cell culture reagents were purchased from Life Technologies (Cergy-Pontoise, France). Other chemicals were from Sigma Chemical Co. (St. Louis, MO). The cDNA of the rat B_2 receptor subcloned in pRC/CMV was kindly provided by Prof. J. Navarro (University of Texas Medical Branch, Galveston, TX). [^{3}H]Bradykinin and [^{3}H][des-Arg^{10},Leu^9]-kallidin (80−120 Ci/mmol; 1 Ci = 37 GBq) were purchased from New England Nuclear.

Cloning of Human B_2 and B_1 Receptors. CHO cells expressing the human B_2 receptor or the rat B_2 receptor have been previously characterized.[22] HER293 cells expressing the human B_1 receptor have been described elsewhere.[23]

Cell Culture and Transfection. CHO cells were maintained in HAM F12 containing 10% fetal calf serum, 4.5 g/L glucose, 100 mg/L streptomycin and 10^5 units/L penicillin. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum, 4.5 g/L glucose, 1% Glutamax (v/v), 1% nonessential amino acids (v/v), 1 mM sodium pyruvate, 100 mg/mL penicillin and 100 μg/mL streptomycin. Cells were transfected with the different cDNA containing vectors (10 μg/plate of 150 mm in diameter) using the calcium phosphate precipitation method. After 48−72 h of recovery, the selection of transfectants was performed using 500 μg/mL Geneticin. Cell clones were isolated by dilution plating, screened for receptor expression and then propagated.

Binding Studies. Stably transfected CHO cells were scrapped from dishes in 5 mL of binding buffer containing 20 mM TES (pH 6.8), 1 mM 1,10-phenantroline, 140 μg/mL bacitracine and 0.1% bovine serum albumin; 293 cells stably transfected with the B_1 receptor were treated as described above except that TES was used at 25 mM and pH 7.4. Cell membranes were obtained by centrifugation (40000*g* for 15 min). Competition binding experiments were carried out by incubating membranes with the competitor ligands and 400 pM [^{3}H]bradykinin for the B_2 receptor (0.5 mL final volume for 90 min) or 1 nM [^{3}H][des-Arg^{10},Leu^9]-kallidin for the B_1 receptor (0.5 mL final volume for 60 min). Nonspecific binding was determined in the presence of 10 μM bradykinin or [des-Arg^{10},Leu^9]-kallidin. Reactions were terminated by filtration with a brandel cell harvester through Whatman GF/B filters presoaked for 2 h in poly(ethylenimide) 0.1% (w/v). Filters were washed three times with ice-cold 50 mM TES or Tris and the radioactivity retained onto the filters was counted with a Beckman liquid scintillation counter. Protein concentration was measured by the method of Bradford.[26]

Human Umbilical Vein (HUV) Contraction. Human umbilical cords were collected post-delivery and immediately placed in Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.5, and EDTA 0.026. Umbilical vein rings (3−4 mm in length) were incubated overnight for B_1 induction at 37 °C in Krebs solution containing 100 U/mL penicillin and 100 μg/mL streptomycin (to reduce bacterial growth) bubbled with 95% O_2 and 5% CO_2. Tissues were set up in 8-mL jacketed organ baths containing Krebs solution maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2. Strips were left unstretched for 1 h, during which the bath fluid was changed every 15 min with fresh solution. Strips were then stretched to 1 g. All rings were contracted twice by KPSS (Krebs solution in which NaCl was replaced by KCl) in order to obtain the maximal contraction. After the rings were washed twice with normal Krebs solution and returned to the baseline, captopril (10 μM), DL-thiorphan (1 μM), mepyramine (1 μM), atropine (1 μM), indomethacin (3 μM), NG-nitro-L-arginine (30 μM) and nifedipine (0.1 μM) were added into the organ bath. Thirty minutes later concentration−response curves to BK (for activity at the B_2 receptor) or [des-Arg^{10}]-kallidin ([des-Arg^{10}]-KD) (for activity at the B_1 receptor) were obtained in the presence of vehicle or antagonist added 15 min before. Each ring was used for a single concentration−response curve. The contractile responses to agonists are expressed as percent (%) of the maximal contraction obtained by adding the thromboxane A_2 mimetic, U46619 (1 μM).

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