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New Bradykinin Analogues Modified in the C-Terminal Part with Sterically Restricted 1-Aminocyclohexane-1-carboxylic Acid

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In the present work, a sterically constrained noncoded amino acid, 1-aminocyclohexane-1-carboxylic acid (Acc), was substituted in position 8 of the peptide chain of bradykinin (BK) and position 6, 7, or 8 of its B_2 receptor antagonist [D-Arg^0,Hyp^3,Thi,^{5,8}D-Phe^7]BK, previously synthesized by Stewart's group, to reduce the flexibility of the peptides, thus forcing the peptide backbone and side chains to adopt specific orientations. Knowing that acylation of the N-terminus of several known B_2 blockers with a variety of bulky groups has consistently improved their antagonistic potency in the rat blood pressure assay, the Acc substituted analogues were also synthesized in the N-acylated form with 1-adamantaneacetic acid (Aaa). The activity of eight new analogues was assayed in isolated rat uterus and in rat blood pressure tests. The results clearly demonstrated the importance of the position in the peptide chain into which the sterically restricted Acc residue was inserted. Meanwhile, Acc at positions 6 and 7 led to reduction of antagonistic qualities or even restored the agonism, respectively. Acc at position 8 enhanced antagonistic qualities in both tests. The Acc at position 8 of BK strongly reduced the agonistic potency. In most cases acylation of the N-terminus led either to enhancement of antagonistic potencies or to further decrease of agonistic potency. Our findings offer new possibilities for designing new potent and selective B_2 blockers.

Introduction

Kinins, such as the nonapeptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), are important mediators of various physiological and pathophysiological responses including inflammatory disease, asthma, rhinitis, cell division, pain, vascular permeability, and allergic reactions.[1] Owing to its ability to lower blood pressure, bradykinin (BK) has been implicated in the pathogenesis of septic and endotoxic shock. The activity of BK is terminated by several enzymes: kininase I, which liberates active metabolite desArg^9-BK, kininase II, and endopeptidases, which produce inactive metabolites. This is the reason BK has a very short lifetime in the circulation and acts in the vicinity of the site of production on a wide variety of peripheral and central cell types.[2]

Two types of receptors, B_1 and B_2, mediate the biological effects of BK. They were initially defined on the basis of pharmacological criteria[3,4] and subsequently confirmed by molecular cloning techniques.[4,5] While B_2 receptors exist on cell membranes and require the entire bradykinin sequence for recognition, B_1 receptors are induced following either tissue damage or inflammation and have higher affinity to desArg^9-BK. Both receptor subtypes transduce the signal via coupling primarily to $G_{\alpha q}$/11 and subsequent activation of phospholipase C and PLA2.[6]

The aforementioned listing of different types of bradykinin activities illustrates the importance of tools that might help to investigate the role and functions of this hormone. The search for bradykinin antagonists began in many laboratories shortly after the announcement of the structure of the hormone. The first antagonist was developed by Vavrek and Stewart; their key modification consisted of replacement of Pro^7 with either D-Phe or other aromatic amino acids. Subsequently they introduced other modifications, e.g., addition of D-Arg to the amino end and substitution of hydroxyproline in position 3 and thienylalanine in positions 5 and 8 that resulted in first generation of antagonists.[7]

Since that time, many laboratories have attempted to develop more potent and selective antagonists. In the course of these studies, the role of amino acid residues in all positions of BK and the influence of various combinations of substitutions on pharmacological activity were carefully investigated.[7] However, massive progress in this field took place after 1991, when potent B_2 blockers carrying conformationally constrained amino acid residues in their C-terminal ends have been synthesized.[8]

In our laboratory we also explored several structural factors that might affect the antagonistic properties of BK analogues. For instance, we reported that acylation of the N-terminus of several known B_2 antagonists with various bulky acyl groups has consistently improved their antagonistic potency in rat blood pressure assay.[9]

Bearing in mind successful manipulations consisting of substitution of sterically restricted amino acids in the C-terminal part of BK analogues that resulted in highly

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Table 1. Pharmacological Properties of New Analogues of BK

analogue	rat uterus in vitro[a]	rat pressor test		
		ED_{20} (mg/min)[b]	ED_{50} (mg/min)[b]	ED_{90} (mg/min)[b]
[D-Arg^0,Hyp^3,Thi^5,Phe,^{7}THI^8]BK	$pA_2 = 6.9 \pm 0.1$[c]	1.73 ± 0.43[d]	-	124 ± 27[d]
[D-Arg^0,Hyp^3,Thi^{5,8},Acc,^{6}D-Phe^7]BK (**I**)	$pA_2 \approx 5.5$	agonist	agonist	agonist
[Aaa[D-Arg^0,Hyp^3,Thi^{5,8},Acc,^{6}D-Phe^7]BK (**II**)	$pA_2 = 6.3 \pm 0.2$	8.94 ± 1.89	86.05 ± 11.35	2392 ± 726
[D-Arg^0,Hyp^3,Thi^{5,8},Acc^7]BK (**III**)	0.12%	3.85 ± 0.65	29.14 ± 3.77	453 ± 81
[Aaa[D-Arg^0,Hyp^3,Thi^{5,8},Acc^7]BK (**IV**)	0	0.98 ± 0.10	9.28 ± 0.46	200 ± 27
[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,Acc^8]BK (**V**)	$pA_2 = 7.2 \pm 0.2$	0.20 ± 0.03	1.62 ± 0.18	25.52 ± 2.15
[Aaa[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,Acc^8]BK (**VI**)	$pA_2 = 7.4 \pm 0.2$	0.66 ± 0.08	4.89 ± 0.36	79 ± 15
[Acc^8]BK (**VII**)	0.03%	agonist	agonist	agonist
[Aaa^0,Acc^8]BK (**VIII**)	0	14.94 ± 3.67	784 ± 161	156500 ± 35000

[a] Agonistic activity calculated as percentage activity of bradykinin (put to 100%), antagonistic activity calculated as pA_2 (negative decadic logarithm of analogue concentration shifting the log dose response curve for bradykinin by a factor of 0.3 to the right; calculation made from the linear portions of the curves). [b] ED_{20}, ED_{50}, and ED_{90} represent doses of bradykinin antagonist ((μg/kg)/min) that inhibit the vasodepressor response to 250 ng of BK by 20%, 50%, and 90%, respectively. [c] Value taken from ref 27. [d] Value taken from ref 16.

potent and selective B_2 antagonists, we have now decided to use 1-aminocyclohexane-1-carboxylic acid (Acc) as a substituent. The Acc should reduce the flexibility of the peptide backbones by restricting conformational freedom. This modification is an example of the $C_\alpha - C_\alpha$ cyclization whereby the dialkylated glycine residue is converted into a cyclic side chain (1-aminocycloalkane-1-carboxylic acid).[10,11] In this case, the ring consists of six atoms. We decided to use [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK, the B_2 antagonist previously synthesized in Stewart's laboratory,[12] as a starting structure and substitute its position 6, 7, or 8 with Acc (analogues **I**, **III**, and **V**, respectively). In the next three peptides we combined these changes with acylation of the N-terminus with 1-adamantaneacetic acid (Aaa), which previously was demonstrated to improve antagonistic properties (analogues **II**, **IV**, **VI**). We have also substituted Acc in position 8 of BK (analogue **VII**) and its acylated analogue (analogue **VIII**).

Information about the new analogues are in Table 1.

Results

The eight new BK analogues (**I**−**VIII**) were synthesized by the Fmoc version of the solid-phase method of peptide synthesis starting from Fmoc-Arg(Pbf)-Wang-resin. Cleavage of the analogues from the resin with simultaneous side chain deprotection was performed by treatment with TFA-H_2O-TIS. The crude products were purified by gel filtration on Sephadex G-15 and by RP-HPLC.

Table 1 presents the structure and effects of the new BK analogues on the contractility of isolated rat uterus and their potency to inhibit vasodepressor response to exogenous BK in conscious rats. BK was used as a standard agonist in both tests, whereas [D-Arg^0, Hyp^3, Thi^{5,8},D-Phe^7]BK, the B_2 antagonist previously synthesized in Stewart's laboratory,[12] served as a reference compound for comparison of the antagonistic activities of our analogues. As mentioned above, in most cases this peptide served as a starting structure when designing our analogues.

Basal hemodynamic characteristics of the tested rats are typical for unrestrained rats at daytime. The whole population appeared homogeneous, since no significant differences among selected rat subsets were found. Prior to the administration of BK, mean arterial pressure (MAP) averaged 104.58 ± 1.34 mmHg (ranging from 90 to 124 mmHg; $N = 53$, mean $\pm$ SE) and heart rate (HR) was 308.6 ± 2.6 beats/min (ranging from 278 to 340 beats/min). BK (125 ng) evoked a significant vasodepressive response (decrease from 104.58 ± 1.34 to 23.60 ± 0.55 and 33.25 ± 1.01 mmHg; 125 and 250 ng of BK, respectively). Relatively low HR recorded during experiments has confirmed good recovery from surgical procedures and also that rats were well accustomed to the experimental conditions.

It is noteworthy that analogue **I**, and to a lesser extent analogue **II**, exhibited toxicity at high concentrations that manifested itself in anxiety, sniffing, bradycardia, bradyarrhythmia, and in some cases fatal cardiac arrest usually preceded by seizures. High concentrations of BK analogues **I**, **II**, **VII**, and **VIII** (160−1024 μg/mL; i.e., doses 20−128 μg/min) usually evoked a pronounced short-lasting (1−3 min) increase in MAP (up to 40 mmHg) followed by its decrease. In the light of the accompanying behavioral changes, anxiety, and sniffing, the observed vasopressor effect is not necessarily due to any BK antagonistic potency, but it might have resulted from some sensory effects undefined in this study. On the other hand, BK acting through B_2 and atypical receptors is involved in central mechanisms of cardiovascular regulation, and its cardioinhibitory effect may be attributed to deregulation of the autonomic control of the heart rhythm.[13,14]

In the in vitro uterus assay, compounds **III** and **VII** exhibited negligible agonistic properties, while their acylated counterparts **IV** and **VIII** were inactive. Peptides **I**, **II**, **V**, and **VI** showed varying degrees of antagonistic activity ranging from very low antagonism ($pA_2 \approx 5.5$) to rather potent antagonism ($pA_2 = 7.4$). From the results presented, it is clear that only analogues substituted with Acc at position 8 exhibited higher antagonistic potency than the model reference peptide.

In the rat blood pressure test (BPT), compounds **I** and **VII** were weak B_2 agonists, while their acylated derivatives **II** and **VIII** possessed weak antagonistic properties. Peptide **III**, obtained by substituting D-Phe at position 7 of the model peptide with Acc, and its acylated counterpart **IV** exhibited moderate antagonistic potencies. Both were, however, less potent than the model reference peptide. Results for analogue **V**, which was designed by replacement of Thi at position 8 of the Stewart's peptide by the Acc residue, showed that this modification resulted in a potent antagonist in BPT. At lower doses (ED_{20}), its activity was approximately 8 times higher than that of the model peptide and the analogue was 5 times more potent when inhibiting the

effect of BK by 90%. Acylation of peptide V with 1-adamantaneacetic acid did not improve its potency. On the contrary, this acylated peptide was about 3 times less potent than the parent one.

Discussion

To make contributions to studies concerning structural requirements of BK analogues that are responsible for B_2 antagonistic activity, in the present work we decided to substitute consecutively positions 6−8 of the model antagonist peptide with Acc. We also introduced Acc into position 8 of the BK molecule. In the case of modifications of positions 7 and 8, the Acc changed the character of the relevant fragment of molecule from aromatic to aliphatic and reduced its conformational freedom as the residue is sterically constrained. It is obvious that replacement by Acc in position 6 is disadvantageous for B_2 antagonistic potency of the resulting analogues. A similar, though less distinct, effect was also observed following Acc^7 substitution. Nevertheless, we should bear in mind that not a long time ago, the presence of an aromatic D-amino acid at position 7 was considered to be a prerequisite for B_2 antagonism.[7,15] However, our previous results have shown that such a residue can be replaced, together with an amino acid occupying position 8, by a suitable sterically restricted ethylene bridged dipeptide unit (-Phe-Phe).[16,17] In the present work we have gone a step further by demonstrating that antagonistic activity at the B_2 receptor might be an attribute of peptides having suitable achiral, nonaromatic, conformationally constrained amino acid at position 7.

Our previous data had suggested that acylation of the N-terminus of BK antagonists with bulky groups consistently improved antagonistic potency in the BPT.[9,18,19] In view of the present data, this regularity appears to be valid for three pairs of analogues: **I**−**II**, **III**−**IV**, and **VII**−**VIII**. However, the enhancing effect is much weaker than that previously reported.[9] On the other hand, it is worthwhile to note that acylation of [Acc^8]-BK converted its activity from agonistic to antagonistic. It is also interesting that peptides **IV** and **VIII**, which showed a moderate or a weak antagonistic potency in BPT, do not interact with B_2 receptors in the uterus. This seems to support the possibility of the presence of different subtypes of B_2 receptors in the uterus and blood vessels. The existence of multiple B_2 receptor subtypes has been postulated by various investigators.[20−22] However, one should consider the option that because of the presence of nonproteinogenic amino acid residues in the molecules of new analogues, metabolism also may account for the differences in the activities just discussed.

Considering that our peptides, because of the reduction of flexibility caused by substitution with Acc, seem to interact in different modes with the B_2 receptor, it is interesting to check if they also interact with the B_1 receptor.

Conclusion

Finally, we learned that the Acc^8 substitution resulted in an increase in B_2 antagonistic activity in BPT and preservation of the antagonistic properties in the rat uterus assay. It is also interesting to note that acylation diminished the B_2 antagonistic potency of the resulting analogue in BPT. We have already described such examples with respect to antagonists conformationally restricted in their C-terminal part. One can only assume that the analogue−receptor interaction of numerous B_2 antagonists acylated with bulky substituents is changed owing to the more rigid structure of the C-terminal part of the molecule,[16,17,23] which results in an interference with the effective signal transduction.

In summary, our studies have shown the importance of the position of the antagonist peptide chain into which a sterically restricted amino acid is incorporated. We also demonstrated, contrary to previous knowledge, that position 7 of B_2 antagonists can be occupied by a nonaromatic, achiral, sterically restricted amino acid. Moreover, we confirmed our finding that acylation of the N-terminus of the B_2 antagonists with a bulky group (Aaa) does not always increase antagonistic potencies of the resulting peptides as we thought, until recently.[16,17,23] Two of the new analogues are potent B_2 antagonists in both assays. Our findings offer new possibilities for designing new potent and selective B_2 blockers.

Experimental Section

Thin-layer chromatography (TLC) was carried out on silica plates (Merck). The spots were visualized with iodine or ninhydrin, and the solvent system butan-1-ol/acetic acid/water/ethyl acetate (1:1:1:1, v/v) [a] was used throughout. High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector. The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100A; 150 mm × 3.9 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA); [B] acetonitrile/aqueous 0.1% TFA (80:20 v/v). A linear gradient from 10% to 70% of solvent system B for 30 min was applied for peptides. Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 mm × 250 mm) in a gradient running from 10% to 50% of solvent system B for 100 min at a flow rate of 10 mL/min (λ = 226 nm). FAB/MS of peptides were recorded on a TRIO-3 mass spectrometer at 7 keV with argon as the bombarding gas and on a MALDI TOF mass spectrometer.

Peptide Synthesis. Peptides were synthesized by the solid-phase method on a Symphony/Multiplex multiple peptide synthesizer (Protein Technologies Inc.) using the Fmoc strategy and starting from Fmoc-Arg(Pbf)-Wang0resin[24] (loading 0.33 mmol/g, 50 μmol). Fmoc was removed by 20% piperidine in DMF. A 5-fold excess of the respective Fmocamino acids was activated in situ using TBTU (1 equiv)/HOBt (1 equiv) in DMF, and coupling reactions were base-catalyzed with DIEA (4 equiv). The amino acid side chain protecting groups were *t*Bu for Hyp and Pbf for Arg and D-Arg. All Fmoc-protected amino acids and Fmoc-Arg(Pbf)-Wang-resin were commercially available (NovaBiochem, Bad Soden, Germany). 1-Adamantaneacetic acid (Aaa) was coupled in the final coupling step using the same procedure as for Fmoc-amino acids.

Each cycle of solid-phase synthesis consisted of the following steps: (1) washing the resin with a 2.5 mL portion of dimethylformamide (DMF) three times for 30 s; (2) equilibrating the resin with 2.5 mL of a 20% solution of piperidine (Pip) in DMF, twice for 5 min each time; (3) washing the resin with a 2.5 mL portion of dimethylformamide (DMF) six times for 30 s; (4) equilibration with a solution of a 5-fold excess of Fmoc-amino acid followed by the addition of a 5-fold excess of a solution of TBTU/HOBt/DIEA (1:1:4, equiv) in DMF and mixing for 45 min, with coupling reactions being performed twice without monitoring; (5) washing the resin with a 2.5 mL portion of DMF three times for 30 s.

Cleavage of the peptides from the resin with side chain deprotection was performed by treatment with TFA/H_2O/TIS

Table 2. Calculated Molecular Weights and Molecular Weights from MS and HPLC Experiments[a] of the New BK Analogues **I**−**VIII**

		[M + H$^+$]		20−80% of solvent system B for 20 min		10−70% of solvent system B for 30 min	
analogue	formula	calcd	found	HPLC t_r (min)	purity (%)	HPLC t_r (min)	purity (%)
[D-Arg^0,Hyp^3,Thi^{5,8},Acc,^{6}D-Phe^7]BK (**I**)	$C_{60}H_{89}N_{19}S_2O_{12}$	1332.8	1332.8	9.55	98.3	17.27	96.2
[Aaa[D-Arg^0,Hyp^3,Thi^{5,8} Acc,^{6}D-Phe^7]BK (**II**)	$C_{72}H_{105}N_{19}S_2O_{13}$	1508.9	1508.8	12.26	93.0	21.34	92.9
[D-Arg^0,Hyp^3,Thi^{5,8} Acc^7]BK (**III**)	$C_{54}H_{86}N_{19}S_2O_{13}$	1272.5	1272.6	7.01	97.7	13.44	98.7
[Aaa[D-Arg^0,Hyp^3,Thi^{5,8} Acc^7]BK (**IV**)	$C_{66}H_{87}N_{19}S_2O_{14}$	1448.6	1448.9	10.09	97.5	18.09	95.4
[D-Arg^0,Hyp^3,Thi^5 D-Phe^7,Acc^8]BK (**V**)	$C_{56}H_{87}N_{19}S_1O_{13}$	1266.5	1266.9	7.54	99.6	14.17	98.2
[Aaa[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,Acc^8]BK (**VI**)	$C_{68}H_{103}N_{19}S_1O_{14}$	1442.6	1443.4	10.42	99.0	18.61	96.2
[Acc^8]BK (**VII**)	$C_{48}H_{75}N_{15}O_{11}$	1038.2	1039	6.52	98.8	12.02	95.7
[Aaa^0,Acc^8]BK (**VIII**)	$C_{60}H_{91}N_{15}O_{12}$	1214.3	1215	11.18	98.8	19.56	96.9

[a] High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical) chromatograph equipped with a UV detector (λ = 226 nm). The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100A; 150 mm × 3.9 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA); [B] acetonitrile/0.1% aqueous TFA (80:20 v/v). A linear gradient from 10% to 70% of solvent system B for 30 min and from 20% to 80% of solvent system B for 20 min was applied at a flow rate of 1 mL/min.

(95.5:2.5:2.5 v/v/v) for 4 h. The total volume of the TFA filtrate was reduced to about 1 mL, and the peptides were precipitated with cold diethyl ether. The solvents were evaporated under reduced pressure, and the resulting materials were dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column, eluting with aqueous acetic acid (30%) at a flow rate of 4.0 mL/h, λ = 254 nm. The eluates were fractionated, and the fractions containing the major peak were pooled and lyophilized. All peptides were purified by preparative HPLC. Calculated molecular weights and molecular weights from MS and HPLC experiments are presented in Table 2 (for details see the footnote).

Biological Evaluation. Effect of Bradykinin Analogues on Rat Blood Pressure. The antagonistic potency of the bradykinin analogues was assessed by their ability to inhibit the vasodepressor response to exogenous BK in conscious rats. Intact male Wistar albino rats (N = 53; 310−370 g) were maintained on a regular chow diet and tap water at ambient temperature (22 ± l °C).

Two days before the experiment, polyethylene tubes (PE-50, Clay-Adams, Parsippany, NJ) were inserted into the right carotid and the right iliac artery under pentobarbital (sodium pentobarbital, 40−50 mg/kg ip) anesthesia. A Y-type connector was mounted to the tubing from the carotid artery. Two arterial lines were led out for either injection of bradykinin or infusion of the bradykinin analogues. All catheters were exteriorized subcutaneously at the back of the neck. We allowed 40−44 h for recovery from the surgical catheterization to reduce the effect of neurohumoral activation related to trauma. In particular, we tried to minimize incoherent reactions related to an increase in endogenous catecholamines and BK, which might interfere with our assay.

On the day of the experiment, the rats were placed in plastic cages. Mean arterial pressure (MAP) and heart rate (HR) were monitored through a Gould-Statham P23-ID pressure transducer (Gould, Cleveland, OH) connected to the iliac catheter and recorded on a paper chart recorder (TZ 4200, Laboratorni Pristroje, Prague, Czech Republic).

The ACE inhibitor enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ; l mg/kg) was injected into the iliac catheter. Forty-five minutes were provided for stabilization of blood pressure. Rats displaying MAP equal to or lower than 85 mmHg or displaying HR higher than 350 beats/min were arbitrarily excluded from further procedures (N = 5). Two doses of BK (bradykinin acetate salt; Sigma), 125 or 250 ng per animal dissolved in a 5% D-glucose solution at 1.25 μg/mL, were randomly injected two to four times every 4−5 min into one branch of the carotid catheter until the evoked vasodepressor responses were stable. To provide homogeneity of the assay, only the rats that exhibited vasodepressive response to 125 ng of BK (ΔMAP) within the range 15−30 mmHg were enrolled in this study. Furthermore, rats that displayed a vasodepressive response to 125 ng of BK higher than 82% of that evoked by 250 ng of BK were excluded from the BPT (N = 3). The BK dose, 250 ng, was then employed as a standard in the assay.

The BK analogues dissolved in the 5% D-glucose solution were infused into another branch of the carotid catheter, other than that used for BK. A constant rate of infusion (125 μL/min) was maintained using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, The Netherlands). The testing of the BK analogue was initiated with an 8-min infusion of 1 μg/mL (this gave a dose of 125 ng/min). During this infusion, a single 250 ng dose of BK was injected twice, in 280 and 450 s of the infusion. The concentration of the infused bradykinin antagonist was then increased to 2, 8, and 64 (if necessary, up to 512) μg/mL to give the doses of 0.25, 1, 8 (64) (μg/min)/rat, respectively. As soon as the vasodepressor response to BK fell below 10% of the standard response, the procedure was completed. For the actual BK antagonist dose response pattern, additional concentrations of BK antagonists were also employed: 16, 160, and 1024 μg/mL (doses 2, 20, and 128 (μg/min)/rat, respectively).

Percent inhibition of the vasodepressor response to 250 ng of BK by the tested BK antagonists was plotted against the logarithm of the dose. From these dose response curves, effective doses ED_{20}, ED_{50}, and ED_{90} were determined representing doses of bradykinin antagonist (μg/mL) that inhibit vasodepressor response to the agonist (250 ng of BK) by 20%, 50%, and 90%, respectively. The results are reported as mean values ± SE.

In Vitro Rat Uterotonic Test. All analogues were evaluated in the in vitro rat uterotonic assay using a modified Holton method[25] in Munsick solution[26] on a strip of rat uterus. Wistar rats in estrus induced by injection of estrogen 48 h before the experiments were used. Bradykinin was used as a standard over a concentration range from 10 pM to 10 nM. Cumulative dose response curves were constructed in the absence and presence of various doses of the analogues; i.e., standard doses of BK were added successively to the organ bath in doubled concentrations and at 1 min intervals without the fluid being changed until the maximal contraction had been obtained. The height of a single isometric contraction in response to different doses of bradykinin was measured. The antagonist was applied to the organ bath 1 min prior to the cumulative dosing of bradykinin. The antagonistic activity was expressed as pA_2, i.e., the negative decadic logarithm of the analogue concentration, which shifted the dose response curve of bradykinin by a factor of 2 (comparison of the linear portions of the dose response curves). Each analogue was tested on uteri taken from four to five rats. As far as the agonistic activity of the analogues is concerned, it was calculated by comparing the threshold doses of cumulative dose response curves of the analogue and that of standard bradykinin. The activity of bradykinin was taken as 100%.

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Appendix

Abbreviations. The symbols of the amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature and "A Revised Guide to Abbreviations in Peptide Science" published in *J. Pept. Sci.* **2003**, *9*, 1−8. Other abbreviations are as follows: Aaa, 1-adamantaneacetic acid; Acc, 1-aminocyclohexane-1-carboxylic acid; TIS, triisopropylsilane; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PLA2, phospholipase A2.

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