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New bradykinin analogues substituted in positions 7 and 8 with sterically restricted 1-aminocyclopentane-1-carboxylic acid

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Abstract: A sterically constrained non-coded amino acid, 1-aminocyclopentane-1-carboxylic acid (Apc), was introduced in position 7 or 8 of the bradykinin (BK) B_2 receptor antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK, previously synthesized by Stewart's group. This modification is believed to reduce the flexibility of the peptides, thereby forcing the peptide backbone and side chains to adopt specific orientations. Apc substitution was combined with acylation of the *N*-terminus with 1-adamantaneacetic acid (Aaa). The activity of four new analogues was assayed in isolated rat uterus and in rat blood pressure tests. The results clearly demonstrated that the Apc residue inserted in position 7 led to a reduction of antagonistic properties in the rat uterus assay or even restored the agonism in the blood pressure test, whereas Apc at position 8 enhanced antagonistic potency in both the tests. In both cases, acylation of the *N*-terminus led to the enhancement of the antagonistic potency. On the basis of these findings, new potent and selective B_2 blockers might be designed. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin; B_2 antagonists; 1-aminocyclopentane-1-carboxylic acid; rat blood pressure assay; *in vitro* rat uterotonic test

INTRODUCTION

Kinins, such as the nonapeptide bradykinin (BK, 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, BK has been implicated in the pathogenesis of septic and endotoxic shock. BK has a short lifetime *in vivo* of the order of tens of seconds [2,3]. Passing the pulmonary circulation, more than 99% of BK is cleaved by the action of angiotensin I converting enzyme (ACE), which cleaves BK successively at the 7–8 and 5–6 bonds. Any circulating BK that escapes cleavage by ACE is cleaved by carboxypeptidase N (CPN), an enzyme that removes the *C*-terminal Arg residue. Aminopeptidase P (APP) inactivates BK by removal of the amino-terminal arginine residue. Finally, BK is cleaved at position 5 by neutral endopeptidase (NEP).

There are two types of receptors for kinins, known as B_1 and B_2. Their existence was initially defined on the basis of pharmacological criteria [4,5] and subsequently confirmed by molecular cloning techniques [5,6]. While B_2 receptors are constitutively expressed in a wide variety of cells and require the entire BK sequence for recognition, B_1 receptors have normally very limited expression and respond to [desArg^9]BK. The B_1 receptor gene is turned on following either tissue damage or inflammation. Both receptor subtypes transduce the signal via coupling primarily to $G_{\alpha q/11}$, and subsequently activate phospholipase C and PLA2 [7].

As BK is involved in many activities, it is obvious that BK antagonists are important tools that might help to investigate the role and functions of this hormone. The first report of BK analogues capable of antagonizing its action in the standard kinin assay was issued by Stewart and Vavrek [8]. A critical modification necessary to evoke B_2 antagonistic activity was replacement of Pro^7 with a D-aromatic amino acid residue. Later, the combination of the modifications that were previously used to design potent BK agonists, the D-Phe^7 substitution and addition of D-Arg

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.*, 9, 1–8 (2003). Other abbreviations: Aaa, 1-adamantaneacetic acid; Apc, 1-aminocyclopentane-1-carboxylic acid; Hyp, L-hydroxyproline; Thi, L-thienylalanine; TIS, triisopropylsilane; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PLA2, phospholipase A2.

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residue on *N*-terminus of the analogue, gave the first moderately potent B_2 antagonist [8]. Hundreds of analogues with single or multiple substitutions were later synthesized mostly in Stewart's laboratory [9]. However, these peptides have a relatively low potency, which necessitates the use of high concentrations during experiments.

Major improvements in the design of B_2 antagonists were made as soon as conformationally constrained amino acid residues were incorporated in their *C*-terminal ends [10].

In our laboratory, we discovered that acylation of the *N*-terminus of many B_2 antagonists with bulky acyl groups (e.g. 1-adamantaneacetyl, 1-adamantanecarbonyl, 4-tert-butylbenzoyl, palmitoyl, etc.) consistently improved the antagonistic potency in the blood pressure test (up to 33 times) [11]. Recently, we demonstrated that antagonistic activity at the B_2 receptor might be attributed to peptides having at position 7 a suitable achiral, non-aromatic, conformationally constrained amino acid, e.g. 1-aminocyclohexane-1-carboxylic acid (Acc). Until recently, the presence of an aromatic D-amino acid residue in position 7 was considered necessary for B_2 antagonism [12].

Having in mind the successful manipulations consisting in substitution of sterically restricted amino acids in the *C*-terminal part of BK analogues that resulted in highly potent and selective B_2 antagonists [10], we have decided to use Apc as a substituent. In comparison to the Acc residue, Apc is smaller (the cyclic side-chain ring consists of five atoms) and more restricted. Similar to the Acc substitution, it should reduce the flexibility of the peptide backbone by restricting conformational freedom. This type of modification is an example of the $C_\alpha - C_\alpha$ cyclization, whereby a dialkylated glycine residue is converted into a cyclic side chain (1-aminocycloalkane-1-carboxylic acid) [13,14]. As in our previous studies, we have used [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK, the B_2 antagonist previously synthesized in Stewart's laboratory [15], as a starting structure and substituted either position 7 or 8 with Apc. In the next two peptides, we combined this modification with acylation of the *N*-terminus with Aaa, which had previously been shown to improve the antagonistic properties. The structures of the new analogues are as follows: [D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7]BK (**I**), [Aaa^{-1}, D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7]BK (**II**), [D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK (**III**) and [Aaa^{-1}, D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK (**IV**)

EXPERIMENTAL

Instrumentation

Thin-layer chromatography (TLC) was carried out on silica plates (Merck). Spots were visualized with iodine or ninhydrin, and a solvent system butan-1-ol : acetic acid : water : ethyl acetate (1 : 1 : 1 : 1, v/v) was used throughout.

Analytical high-performance liquid chromatography (HPLC) was carried out on a Waters Breeze System equipped with a Waters 1525 binary pump, Waters 2487 dual λ absorbance detector and Waters 717*plus* autosampler. The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100Å; 150 × 4.6 mm). The following solvents were used: (A) 0.1% aqueous trifluoroacetic acid (TFA), (B) 80% acetonitrile in aqueous 0.1% TFA (v/v). A linear gradient from 10 to 70% of (B) for 40 min was applied for peptides at a flow rate of 1 ml/min. Preparative HPLC was carried out on a Waters Millennium System equipped with a Waters 600 pump with 600 controller and a Waters 2487 dual λ absorbance detector using a Kromasil C_8 column (5 μm, 100 Å, 25 × 250 mm; in a gradient running from 10 to 50% of (B) for 100 min at a flow rate of 10 ml/min, detection at 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 Bruker BIFLEX III MALDI TOF mass spectrometer (ionization 337 nm nitrogen laser).

Peptide Synthesis

The peptides were synthesized by the solid-phase method on a Symphony/Multiplex multiple peptide synthesizer (Protein Technologies Inc., USA) using the Fmoc strategy and starting from Fmoc-Arg(Pbf)-Wang resin [16] (loading 0.33 mmol/g, 50 μmol). Fmoc was removed by 20% piperidine in DMF. A fivefold excess of the respective Fmoc amino 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 tBu for Hyp and Pbf for Arg and D-Arg. All Fmoc-protected amino acids and Fmoc-Arg(Pbf)-Wang resin were purchased from NovaBiochem (Bad Soden, Germany). Aaa was coupled in the final coupling step using the same procedure as that for the Fmoc amino acids.

Cleavage of the peptides from the resin with side-chain deprotection was performed by treatment with TFA : H_2O : TIS (95 : 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 dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column eluted with aqueous acetic acid (30%) at a flow rate of 4.0 ml/h and detection at 254 nm. The eluates were fractionated, and the fractions containing the major peak were pooled and lyophilized. All peptides were purified by preparative HPLC. Physicochemical properties of the new analogues **I–IV** are presented in Table 1.

BIOLOGICAL EVALUATION

Effect of BK Analogues on Rat Blood Pressure

The antagonistic potency of the BK analogues was assessed by their ability to inhibit the vasodepressor response to exogenous BK in conscious rats. Male, intact Wistar albino rats ($N = 53$; 310–370 g) were

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Table 1 Some Physicochemical Properties of BK Analogues **I–IV**

Analogue	Formula	HPLC T_R	$[M+H]^+$	
			Calculated	Found
I	$C_{53}H_{83}N_{19}S_2O_{13}$	23.0	1258.5	1258
II	$C_{65}H_{99}N_{19}S_2O_{14}$	28.01	1434.8	1434
III	$C_{55}H_{85}N_{19}S_1O_{13}$	26.3	1252.5	1252
IV	$C_{67}H_{101}N_{19}S_1O_{14}$	28.8	1428.7	1428

HPLC was carried out on a Waters (analytical) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100 A; 150 × 4.6 mm). The following solvent systems were used: (A) 0.1% aqueous TFA, (B) acetonitrile: 0.1% aqueous TFA (80 : 20 v/v). A linear gradient from 10 to 70% of (B) for 40 min. was applied at a flow rate of 1 ml/min.

maintained on a regular chow diet and tap water at ambient temperature (22 ± 1°C).

Two days before the experiment, polyethylene tubes (PE-50, Clay-Adams, Parsippany, NJ, USA) were inserted into the right carotid and the right iliac arteries under pentobarbital (pentobarbital sodium; 40–50 mg/kg i.p.) anesthesia. A Y-type connector was mounted to the tubing from the carotid artery. Two arterial lines were led out for either injection of BK or infusion of the BK 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 the 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, USA) 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, USA; 1 mg/kg), was injected into the iliac catheter. Forty-five minutes were allowed for stabilization of blood pressure. The rats displaying MAP equal to or lower than 85 mmHg, or HR higher than 350 beats/min were excluded from the experiments ($N = 5$). Two doses of BK (Bradykinin acetate salt; Sigma, USA, dissolved in 5% D-glucose solution at a concentration of 1.25 μg/ml), 125 or 250 ng/animal, were randomly injected 2–4 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 vasodepressor response to 125 ng BK (ΔMAP) within the range of 15–30 mmHg were enrolled to this study. Furthermore, the rats that displayed a vasodepressor response to 125 ng BK higher than 82% of that evoked by 250 ng BK were also excluded from the BKA assay ($N = 3$). The BK dose of 250 ng was then employed as a standard for the BKA activity assay.

The BK analogues dissolved in the 5% D-glucose solution were infused into the other branch of the carotid catheter. A constant rate of infusion (125 μl/min) was maintained using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, Netherlands). The testing of the BK analogue potency was initiated with the 8-min infusion of the concentration 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: at 280 s and 450 s of the infusion. The concentration of the infused BK 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. If the actual BK antagonist dose–response pattern was required, additional concentrations of BK antagonists were also employed: 16, 160 and 1024 μg/ml (doses 2, 20, and 128 μg/min/rat, respectively).

Percentage inhibition of the vasodepressor response to 250 ng 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 BK 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 ± S.E.

The *in vitro* Rat Uterotonic Test

All the analogues were evaluated in the *in vitro* rat uterotonic assay using a modified Holton method [17] in Munsick solution [18] on a strip of rat uterus. Wistar rats in estrus induced by injection of estrogen 48 h before the experiments were used. BK was used as a standard over the concentration range 10 pM–10 nM. Cumulative dose–response curves were constructed both 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 maximum contraction has been obtained. The height of a single isometric contraction in response to different doses of BK was measured. The antagonist was applied to the organ bath 1 min prior to the cumulative dosing of BK. 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 BK by a factor of 2 (comparison of the linear portions of the dose–response curves). Each analogue was tested on the uteri taken

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Table 2 Pharmacological Properties of New Analogues of BK

Peptide	Uterotonic potency: % of activity of BK or pA_2	Vasodepressor potency		
		ED_{20} (mg/min)	ED_{50} (mg/min)	ED_{90} (mg/min)
[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK (Stewart's peptide)	$pA_2 = 6.88 \pm 0.08$[a]	1.73 ± 0.43[b]	—	124.17 ± 27.04[b]
I [D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7] BK	0.25	13.49 ± 4.25	166.9 ± 63.16	8302 ± 4782
II Aaa[D-Arg^0, Hyp^3, Thi^{5,8}, Apc^7] BK	0	2.83 ± 0.36	46.03 ± 12.66	2899 ± 1187
III [D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK	$pA_2 = 6.77 \pm 0.05$	0.35 ± 0.05	3.45 ± 0.37	91.04 ± 18.69
IV Aaa[D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Apc^8]BK	$pA_2 = 7.30 \pm 0.11$	0.14 ± 0.03	0.98 ± 0.06	17.57 ± 3.37
[D-Arg^0, Hyp^3, Thi^{5,8}, Acc^7] BK	0.12%[c]	3.85 ± 0.65[c]	29.14 ± 3.77[c]	453 ± 81[c]
Aaa[D-Arg^0, Hyp^3, Thi^{5,8}, Acc^7] BK	0[c]	0.98 ± 0.10[c]	9.28 ± 0.46[c]	200 ± 27[c]
D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Acc^8]BK	$pA_2 = 7.2 \pm 0.2$[c]	0.20 ± 0.03[c]	1.62 ± 0.18[c]	25.52 ± 2.15[c]
Aaa[D-Arg^0, Hyp^3, Thi^5, D-Phe^7, Acc^8]BK	$pA_2 = 7.4 \pm 0.2$[c]	0.66 ± 0.08[c]	4.89 ± 0.36[c]	79 ± 15[c]

Notes: Agonistic activity was calculated as percentage of BK activity (set to 100%); antagonistic activity was calculated as pA_2 (negative decadic logarithm of analogue concentration shifting the log dose–response curve for BK by a factor of 0.3 to the right: calculation were made from the linear portions of the curves); ED_{20}, ED_{50} and ED_{90} represent doses of BK antagonist (μg/kg/min) that inhibit the vasodepressor response to 250 ng of BK by 20, 50 and 90%, respectively.

[a] Value taken from Ref. 19.

[b] Value taken from Ref. 20.

[c] Value taken from Ref. 12.

from 3 to 5 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 BK. The activity of BK was taken as 100%.

RESULTS AND DISCUSSION

The four new BK analogues (**I–IV**) were synthesized by the Fmoc version of the solid-phase method of peptide synthesis starting from the 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 2 presents the effects of the new 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 [14], served as a reference compound for comparison of the antagonistic activities of the analogues in this study. As mentioned, this peptide served as a lead for the design of our analogues. In the *in vitro* uterus assay, compound **I** exhibited negligible agonistic properties, while its acylated counterpart **II** was inactive. Peptides **III** and **IV** showed high antagonistic activity. Their anti-BK potency (pA_2 values 6.77 and 7.3, respectively) was substantially higher than that of the model peptide.

In the BPT in rats, compound **I** and its acylated counterpart **II** exhibited weak antagonistic potency, even lower than that of Stewart's peptide. Analogue **III** turned out to be a moderately potent antagonist in BPT. At lower doses (ED_{20}) its activity was approximately 5 times higher than that of the model peptide. It was slightly more potent than the reference peptide when inhibiting the effect of BK by 90%. Acylation of peptide III with Aaa improved its potency more than twice at lower doses and 5 times at higher doses.

Recently we reported that Acc could be accepted in positions 7 and 8 of the model B_2 antagonist molecule; however the position in which it is introduced is very important for antagonistic activity [12]. We found 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. As a result of this substitution in position 7, we obtained analogues inactive in the rat uterus assay but showing antagonistic properties in BPT. However, their activity was lower than that of the model peptide. In the present work we substituted positions 7 and 8 of the model antagonist with the Apc residue. Similar to Acc modifications, Apc changed the character of the relevant fragment of the molecule from aromatic to aliphatic and reduced its conformational freedom as the residue was sterically constrained. In comparison to the Acc residue, the Apc residue is smaller and more restricted. From the results presented in Table 2 it is obvious that substitution of Apc in position 7 (similar to Acc^7 modification) is disadvantageous for B_2 antagonistic potency of the resulting analogues. Nevertheless, we could once more obtain analogues substituted in position 7 with an achiral, non-aromatic, conformationally constrained

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amino acid of antagonistic activity. Until recently, the presence of aromatic D-amino acid residue in position 7 was considered to be necessary for B_2 antagonism [9,21]. This finding is another example supporting our previous results, which have shown that the D-amino acid residue in position 7 can be replaced, together with an amino acid occupying position 8, by an appropriate sterically restricted unit [20,22,23].

From our previous data we learned that acylation of the *N*-terminus of BK antagonists with bulky groups consistently improved antagonistic potency in the BPT [11,24,25]. In view of the present data, contrary to the Acc modification [12], this appears to be valid for both pairs of analogues. However, the enhancement effect is weaker than that previously reported [11]. It is also interesting to note that peptides substituted in position 7 with Apc, which showed a moderate or a weak antagonistic potency in BPT, were completely inactive in the rat uterus assay. The same effect was observed for analogues substituted in position 7 with the Acc residue. This seems to suggest once more the possibility of the presence of different subtypes of B_2 receptors in the uterus and blood vessels, postulated by various investigators [25–28].

Summing up, we learned that the Apc^8 substitution, similar to that of Acc^8, resulted in an increase in B_2 antagonistic activity in BPT and preservation of the antagonistic properties in the rat uterus assay. Our results offer new possibilities for designing novel potent and selective B_2 blockers.

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