Research Article

Journal of PeptideScience

Received: 15 September 2010 Revised: 30 October 2010 Accepted: 12 December 2010 Published online in Wiley Online Library: 15 March 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1351

Novel analogues of bradykinin conformationally restricted in the C-terminal part of the molecule‡

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In the present work, achiral non-coded amino acids, N-(Bzl)-Gly, X_1 or X_2, were substituted at position 7 of the model B_2 receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK. The N-terminal amino group of the analogues was either free or acylated with 1-Aca or Aaa. Biological activity of the compounds was assessed in the *in vitro* rat uterus test and the *in vivo* rat blood pressure test. The X_1^7 substitution resulted in a decrease in antagonistic potency of the new peptide in both assays. The X_2^7 and N-(Bzl)-Gly^7 substituted analogues showed weak agonistic properties in the rat uterus test. Interestingly, the latter compound exhibited dual activity in the pressor test, i.e. intrinsic vasodepressor action and at the same time a weak antagonistic effect. Acylation of the N-terminus enhanced antagonistic properties of the resulting peptides in the rat blood pressure test in the case of compounds containing X_1 or X_2 modification. Our studies provide new information about structure–activity relationship of the BK antagonists which may be helpful for designing more potent B_2 receptor blockers. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin analogues; B_2 receptor antagonists; sterically restricted residue; *in vivo* rat blood pressure test; *in vitro* rat uterus test

Introduction

BK and related kinins are autacoid peptides produced by catalytic action of kallikrein enzymes on plasma and tissue precursors called kininogens. Kinins play an integral role in pathophysiological processes that accompany acute and chronic pain and inflammation. Their biological actions are mediated by at least two major G-protein-coupled BK receptors termed B_1 and B_2. The B_2 receptor is constitutively expressed on most cell types, whereas the B_1 receptors are not present in tissues under normal conditions but their expression is induced during inflammatory insults [1,2]. The putative role of kinins, and specifically that of BK, in the management of pain and inflammation has provided the impetus for developing potent and selective BK antagonists. In recent years, this effort has been heightened with the expectation that useful therapeutic agents endowed with analgesic and anti-inflammatory properties would be discovered.

The disclosure of the first antagonists for B_2 receptor in 1984 revitalized the quiescent kinin field [3]. A critical structural change in the BK sequence to induce B_2 antagonist activity consisted in substitution of the Pro at position 7 with a D-aromatic amino acid residue [D-Phe was first used (A), see Table 1]. Perhaps the most useful analogue of the first generation was a peptide known as NPC-349 (Stewart's peptide) [4,5]. This compound had additionally two Phe at position 5 and position 8 substituted with β-thienylalanine residue (Thi), which greatly enhanced receptor affinity. A D-Arg residue was added to the N-terminus to block aminopeptidase action, and a 4-hydroxyproline residue (Hyp) was used to replace Pro at position 3. Although this kind of antagonists had relatively low affinity for receptors and short *in vivo* lifetimes in rats (a few minutes only), they were used to demonstrate, for the first time, clear involvement of BK in several processes [6]. The second generation of BK antagonists started with a Hoechst peptide exemplified by HOE-140 (icatibant), which contained two new unnatural amino acids in the structure of the first generation antagonist, D-Tic at position 7 and Oic at position 8.

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‡ *Special issue devoted to contributions presented at the 12th Naples Worshop on Bioactive Peptides and 2nd Italy-Korea Symposium on Antimicrobial Peptides, 4–7 June 2010, Naples, Italy.*

Abbreviations used: *Aaa, 1-adamantaneacetic acid; Aca, 1-adamantanecarboxylic acid; Acc, 1-aminocyclohexane-1-carboxylic acid; D-1-Nal, 1-naphthyl-D-alanine; D-2-Nal, 2-naphthyl-D-alanine; D-f5f, D-pentafluorophenylalanine; D-Hpe, D-cis-4-propoxyproline; D-Tic, D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; HR, heart rate; Igl, α-2-indanylglycine; L-1-Nal, 1-naphthyl-L-alanine; MAP, mean arterial pressure; N-(Bzl)-Gly, N-benzylglycine; NMP, 1-methyl-2-pyrrolidone; Oic, octahydroindole-2-carboxylic acid; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group; TIS, triisopropylsilane; X_1, 2-aminomethylphenylacetic acid; X_2, 3-aminophenylacetic acid.*

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Table 1. Ligands for kinin B_2 receptors

BK	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
A	Arg-Pro-Pro-Gly-Phe-Ser-D-Phe-Phe-Arg
NPC-349 (Stewart's antagonist)	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg
HOE-140	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg
NPC-17731	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Hpe-Oic-Arg
CP-0127	Y(D-Arg-Arg-Pro-Hyp-Gly-Phe-Cys-D-Phe-Leu-Arg)$_2$
B-9430	D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg
B-9858	Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic
B-10056	D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-f5f-Igl-Arg

Y, bis-maleimidohexane joins the Cys side chains.

These modifications blocked degradation by carboxypeptidase N and greatly enhanced the *in vivo* lifetime [7]. Investigators at Nova introduced an aliphatic residue, D-Hpe, at position 7 that afforded a potent and long-acting antagonist, NPC-17731, but little work has been reported on the use of this peptide [8]. A different approach was taken by Cortech researchers, who showed that dimerization of the Cys-containing first generation antagonist by a bis-maleimidohexane crosslinker gave a superior B_2 receptor blocker (CP-0127) [9,10]. The third generation of BK antagonists was developed again in the Stewart laboratory – they introduced a new amino acid residue, Igl, at positions 5 (L-isomer) and 7 (D-isomer) of the HOE-140 structure [11,12]. The Igl residue at position 5 blocked the action of the neutral endopeptidase. B-9430 and similar compounds are not degraded by the lung or kidney homogenates, have *in vivo* lifetimes of hours and show moderate oral bioavailability in rats.

Perhaps the most remarkable feature of the biological activity of B-9430 is the fact that this peptide is an effective antagonist at both B_2 and B_1 receptors, although it is a full-chain analogue, containing a C-terminal Arg residue [13]. Des-Arg^9 peptides related to B-9430, for example B-9858, are extremely potent, selective antagonists of B_1 receptors [14]. The next important advancement in the case of peptide BK antagonists came with introduction of the D-f5f at position 7 [14,15]. Some BK antagonists with this modification, for instance B-10056, showed in some assays a higher potency than that of any previously reported compounds. Moreover, they also exhibited a combined B_2/B_1 antagonist activity, similar to that of the third generation antagonists.

As mentioned above, the key to design a BK antagonist was to substitute Pro in position 7 with a D-aromatic residue. This modification is still dominant in essentially all B_2 receptor blockers. The aromatic residue used in the first generation antagonists was D-Phe, but in successive generations this was replaced by unnatural amino acids having special steric properties.

In our laboratory, during almost 15 years of investigations, we have synthesized plenty of analogues with conformationally constrained amino acid residues incorporated into their C-terminal ends. Some of them exhibited potent antagonism in the rat uterus test, comparable or even higher than that of the Stewart's peptide [16,17] and some were antagonists in the rat blood pressure test [18]. A couple of compounds, for example [D-Arg^0,Hyp^3,Thi^5, D-2-Nal^7,L-1-Nal^8]-BK or [D-Arg^0,Hyp^3,Thi^5,D-Phe^7,Acc^8]-BK, turned out to be potent B_2 receptor blockers in both assays [16,19]. We also reported that acylation of the N-terminus of BK antagonists with various bulky groups (e.g. 1-adamantaneacetyl, 1-adamantanecarbonyl, 4-tert-butylbenzoyl, palmitoyl, etc) is regularly improving their antagonistic potency in the rat blood pressure test (up to 33 times) [20]. However, this relationship does not appear to be so obvious in analogues that are conformationally restricted in their C-terminal part, where the introduction of an N-terminal acyl substituent led to suppression of B_2 antagonistic activity of the resulting counterparts [16,19,21–23].

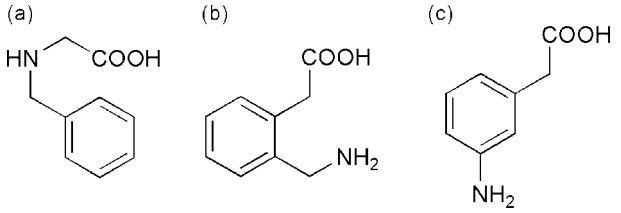

Figure 1. Structures of non-coded amino acids introduced into position 7 used in the study: (a) N-(Bzl)-Gly, (b) X_1 and (c) X_2.

In the present study, D-Phe at position 7 of the peptide [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-BK (NPC-349) [4] was replaced with N-(Bzl)-Gly residue, a structural isomer of Phe, in which the side chain is displaced from C_α to the nitrogen atom (Figure 1A). Insertion of such a peptoid unit into a peptide chain may be considered as an alkylation of the peptide bond, which is attractive as a local and subtle mode of conformational constraint. Recently we demonstrated that the N-(Bzl)-Gly^8 substitution resulted in an impressive increase in B_2 antagonistic potency in the rat blood pressure test [24]. This finding prompted us to investigate how the replacement of the amino acid residue at position 7 of the model peptide with this peptoid unit would influence its pharmacological properties (analogue **I**). We also obtained two other compounds designed by substitution of position 7 of the Stewart's antagonist with achiral non-coded amino acids, X_1 (analogue **III**) and X_2 (analogue **V**). Like the N-(Bzl)-Gly residue, these non-coded amino acids also contain the aromatic ring, but it is located between the carboxyl and amino groups and consequently is a part of the peptide scaffold (Figure 1b and c). The structure of the X_1 and X_2 residues differs only slightly; the distance between the carboxyl group and amino group in both cases is bridged by four carbon atoms. However, while in the case of X_1 it includes two carbons of the aromatic ring, in the case of X_2 it includes three carbon atoms of the ring. The conformation of the latter residue may thus be considered as being more restricted, i.e. more rigid. The aforementioned three analogues (**I, III** and **V**) were also synthesized in the N-acylated form using either Aca (analogue **II**) or Aaa (analogues **IV** and **VI**). The structures of the new compounds are as follows:

Y-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Z-Thi-Arg

Y = H	Z = N-(Bzl)-Gly	(**I**)
Y = Aca	Z = N-(Bzl)-Gly	(**II**)
Y = H	Z = X_1	(**III**)
Y = Aaa	Z = X_1	(**IV**)
Y = H	Z = X_2	(**V**)
Y = Aaa	Z = X_2	(**VI**)

Materials and Methods

General

TLC was carried out on silica plates (Merck) and the spots were visualized with iodine. The solvent system used was butan-1-ol/acetic acid/water/ethyl acetate (1 : 1 : 1 : 1, v/v). HPLC was carried

out on a Waters (analytical and preparative) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The following solvent system was used: [A] 0.1% aqueous TFA and [B] acetonitrile in 0.1% aqueous TFA (80 : 20, v/v). Semi-preparative HPLC was carried out using a Waters C_{18} column (15 μm, 100 Å; 7.8 mm × 300 mm) in a linear gradient from 15 to 45% of [B] for 60 min (analogues **I** and **II**), and from 15 to 25% of [B] for 60 min (analogues **III** and **V**), and from 25 to 35% of [B] for 60 min (analogues **IV** and **VI**), all at a flow rate of 2.5 ml/min. The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer (Biflex III Bruker, $\lambda = 337$ nm). The purity of the peptides was determined on a Hypersil C_{18} column (5 μm, 100 Å, 4.6 mm × 250 mm). Linear gradients from 22 to 45% of [B] for 40 min (analogues **I** and **II**) or from 1 to 80% of [B] for 60 min (analogues **III–VI**) were applied at a flow rate of 1 ml/min.

All the amino acid derivatives were purchased from NovaBiochem, except for Fmoc-X_1, Fmoc-X_2 and Aaa, Aca which were provided by PolyPeptide Laboratories, Inc. and Sigma-Aldrich, Co. respectively. Fmoc-N-(Bzl)-Gly was prepared according to the procedures described previously [25,26].

Peptide synthesis

All the peptides were obtained manually by the solid-phase method, i.e. by stepwise coupling of Fmoc-amino acids to the growing peptide chain on a polystyrene resin (preloaded Wang resin, capacity 0.4 mmol/g). The amino acid side chain protecting groups were the Bu^t for Hyp and Ser and the Pbf for Arg and D-Arg. Fully protected peptide resins were synthesized according to standard procedures [27] involving (i) deprotection steps using a 20% solution of piperidine in DMF, 5 and 10 min and (ii) couplings in a mixture of DMF/NMP (1 : 1, v/v) using TBTU, HOBt in the presence of NMM. In most cases, the amino acids were coupled at a threefold excess. The couplings after Fmoc-N-(Bzl)-Gly, Fmoc-X_1 and Fmoc-X_2 were mediated by HATU, HOAt in the presence of NMM in the mixture of DMF/NMP (1 : 1, v/v). The completeness of each coupling reaction was monitored by the Kaiser [28] or chloranil test [29]. Recoupling was performed when the test was positive. Aca and Aaa were coupled in the final coupling step (analogues **II** and **IV, VI**, respectively) using the same procedure as that for Fmoc-amino acids. After completion of the synthesis, the protected peptidyl resins were treated with TFA : H_2O : TIS (95.5 : 2.5 : 2.5, v/v/v) and stirred for 4 h. Solutions of the cleaved peptides were filtered and evaporated *in vacuo* to a volume of about 1 ml. Then the peptides were precipitated with diethyl ether to afford crude products. The solvents were evaporated under reduced pressure and the resulting materials were dissolved in water, frozen, and lyophilized to give the final products. The crude peptides were desalted on a Sephadex G-15 column, and eluted with aqueous acetic acid (30%) at a flow rate of 3 ml/h. After freeze-drying, the fractions comprising the major peak were purified by RP-HPLC as described above. The purity of the peptides was checked using analytical HPLC. MALDI TOF mass spectrometry (molecular ion) was used to confirm the identity of the pure products.

Biological evaluation

The in vivo rat blood pressure test

The ability of the BK analogues to inhibit the vasodepressor response to exogenous BK in conscious rats was assessed. Male Wistar rats were maintained on a regular chow diet and tap water at ambient temperature (22±1 °C). The assay was based on the previously published procedure [18,19,30] with minor modifications. Handling of the experimental animals was done under supervision of the local Ethics Committee of the Medical University of Gdańsk.

The rats had two arteries catheterized. A Micro-Renathane® polyurethane-based elastomer tubing (MRE-040, Braintree Scientific, USA) was inserted into the left carotid artery, while a RenaPulse® High Fidelity Pressure Tubing (RPT 040, Braintree Scientific, USA) with a tip made of Micro-Renathane® MRE-040 tubing was indwelled into the right iliac artery. Both catheters were exteriorized subcutaneously at the back of the neck. A Y-type connector was mounted to the carotid artery line. Following a 40–44 h recovery, MAP and HR were monitored through a Gould-Statham P23-ID pressure transducer (Gould, Cleveland, OH, USA) connected to the iliac catheter. Angiotensin converting enzyme (kininase II) inhibitor, enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, USA; 1 mg/kg), was injected into the iliac artery to stabilize the blood pressure. To provide homogeneity of further assay, rats with MAP within the range of 85–125 mmHg and HR 270–360 beats/min were arbitrarily enrolled to the study. Two doses of BK (BK acetate salt; Sigma, USA), 125 and 250 ng per animal, at a concentration of 1.25 μg/ml in a 5% D-glucose, were randomly injected 4–5 times, every 4–5 min, into one branch of the carotid catheter. The rats which exhibited vasodepressor response to 250 ng BK (ΔMAP) within the range of 20–45 mmHg were enrolled to the study. In accordance with the previous study [30], those rats which displayed the vasodepressor response to 125 ng BK higher than 82% of that evoked by 250 ng BK were excluded from testing. The BK dose of 250 ng was used as a standard in further assay.

The BK analogues dissolved in the 5% D-glucose were infused (constant infusion rate 80 μl/min) to the other branch of the carotid catheter. The testing was initiated with the 10-min infusion at a concentration of 1 μg/ml (this gave a dose of 0.08 μg/min/rat). During this infusion, the 250 ng dose of BK was injected twice: at 270 and at 570 s. The concentration of the infused BK antagonist was then increased to 4, 16, 64, 256 and 1024 μg/ml and, if necessary, up to 2048 or 3580 μg/ml, to give the doses of 0.32, 1.28, 5.12, 20.48, 81.92, 163.84 and 286.40 μg/min/rat, respectively. The BK analogue infusion at concentrations of 1024 μg/ml and higher was limited to 5 min and BK was injected only once by the end of the infusions. As soon as the vasodepressor response to BK was lower than 10% of the standard response, the procedure was completed.

The percent inhibition of the vasodepressor response to 250 ng BK by the tested BK antagonist 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 the BK antagonist (μg/min) 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.M.

The in vitro rat uterus test

All the analogues were evaluated in the uterotonic assay using a modified Holton's method [31] in the Munsick solution [32] on a strip of the rat uterus [33]. Wistar rats in estrus induced by injection of estrogen 48 h before the experiments were used. Handling of the experimental animals was done under supervision of the Ethics Committee of the Academy of Sciences according to §23 of the law of the Czech Republic no. 246/1992. BK was used as a standard over a concentration range of 10 pM–10 nM. Cumulative dose–response curves were constructed both in the absence and presence of various concentrations of the analogues, i.e. doses of BK were added successively to the organ bath in doubled concentrations and at 1-min intervals, without the fluid

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being changed, until the maximum contraction of the uterine tissue was 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 common logarithm of the analogue concentration which shifted the dose–response curve of BK by a factor of two (comparison of the linear portions of the dose–response curves). Each analogue was tested on uteri taken 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%.

Table 2. Physicochemical properties of analogues **I–VI**

Analogue	Formula	HPLC T_R (min)	MW [M+H$^+$] Calculated	MW [M+H$^+$] Found
I	$C_{56}H_{83}N_{19}O_{13}S_2$	24.9[a]	1294.9	1294.7
II	$C_{67}H_{97}N_{19}O_{14}S_2$	38.3[a]	1457.2	1456.8
III	$C_{56}H_{82}N_{19}O_{13}S_2$	30.73[b]	1295.4	1294.2
IV	$C_{68}H_{98}N_{19}O_{14}S_2$	38.93[c]	1470.4	1470.9
V	$C_{55}H_{80}N_{19}O_{13}S_2$	29.58[b]	1281.3	1280.9
VI	$C_{67}H_{96}N_{19}O_{14}S_2$	37.50[c]	1456.3	1456.7

[a] Linear gradient from 15 to 45% of [B] for 60 min.
[b] Linear gradient from 15 to 25% of [B] for 60 min.
[c] Linear gradient from 25 to 35% of [B] for 60 min.
The following solvent system was used: [A] 0.1% aqueous TFA and [B] acetonitrile in 0.1% aqueous TFA (80 : 20, v/v).

Results

The six new analogues of BK (**I–VI**) were obtained as crude products in satisfactory yields. After HPLC purification, their purity was higher than 98% as determined by analytical HPLC. The MALDI TOF mass spectrometry confirmed that the purified peptides were the desired products. Physicochemical properties of the new compounds are presented in Table 2.

Pharmacological data of analogues **I–VI** together with those of Stewart's antagonist (used as a positive control) and some related peptides are summarized in Table 3. The potency was assayed in the *in vivo* rat blood pressure test using conscious rats and in the *in vitro* rat uterus test. (see Section on Materials and Methods).

Basal characteristics of the rats assayed in the rat blood pressure test are typical of unrestrained rats at daytime (Table 4). The whole group of rats was homogeneous, since no significant differences among selected rat subsets were found. Relatively low HR recorded during experiments has confirmed good recovery of the rats from surgical procedure and also that rats were well accustomed to experimental conditions. No signs of discomfort were noticed during the test.

In the rat blood pressure test, data for analogue **I** (Table 3), which was designed by replacement of D-Phe at position 7 of the model peptide by the N-(Bzl)-Gly residue, showed that this modification resulted in a moderate antagonist of BK. Its acylated (with Aca residue) form (analogue **II**) was characterized by a comparable degree of antagonism. Compound **III**, which had X_1 residue at position 7, exhibited weak antagonistic properties. However, its acylation with Aaa residue (analogue **IV**) strongly potentiated the activity, almost up to the level of the potency of the Stewart's antagonist. Peptide **V**, which had X_2 residue at position 7, showed a moderate antagonism at low doses only. Acylation of this peptide with Aaa residue significantly increased the antagonistic properties up to 6 times (analogue **VI**). However, the response was very irregular.

Table 3. Pharmacological properties of the new BK analogues

Analogue		Vasodepressor potency[a] ED_{20} (μg/min)	ED_{50} (μg/min)	ED_{90} (μg/min)	Uterotonic potency % of activity of BK or pA_2[c]
I	[D-Arg^0,Hyp^3,Thi^{5,8},N-(Bzl)-Gly^7]-BK	2.17 ± 0.60[b]	13.64 ± 3.46	146.39 ± 29.30	15.5%
II	Aca-[D-Arg^0,Hyp^3,Thi^{5,8}, N-(Bzl)-Gly^7]-BK	2.14 ± 0.55[b]	12.93 ± 2.74	198.23 ± 64.69	0.03%
III	[D-Arg^0,Hyp^3,Thi^{5,8},X$_1$ ^{7}]-BK	4.57 ± 1.45	215.15 ± 169.22	62 358 ± 65 644[b]	$pA_2 \sim 5.6$
IV	Aaa-[D-Arg^0,Hyp^3,Thi^{5,8},X$_1$ ^{7}]-BK	0.82 ± 0.20	5.24 ± 2.61	74.58 ± 59.69	$pA_2 \sim 5.6$
V	[D-Arg^0,Hyp^3,Thi^{5,8},X$_2$ ^{7}]-BK	1.83 ± 0.35	28.85 ± 14.63	2797 ± 2876[b]	0.4%
VI	Aaa-[D-Arg^0,Hyp^3,Thi^{5,8},X$_2$ ^{7}]-BK	1.23 ± 0.36	10.02 ± 5.78	416.99 ± 232.93	$pA_2 = 5.60 \pm 0.30$
Stewart's antagonist[d]	[D-Arg^0-Hyp^3,Thi^{5,8},D-Phe^7]-BK	0.43 ± 0.03	3.19 ± 0.33	52.60 ± 10.59	$pA_2 = 7.70 \pm 0.13$
Peptide 1[e]	[D-Arg^0,Hyp^3,Thi^5, D-D-Phe^7, N-(Bzl)-Gly^8]-BK	0.18 ± 0.02	1.06 ± 0.13	12.30 ± 2.37	–
Peptide 2[e]	Aca-[D-Arg^0,Hyp^3,Thi^5,D-Phe^7, N-(Bzl)-Gly^8]-BK	0.08 ± 0.01	0.51 ± 0.06	6.22 ± 0.36	–

[a] ED_{20}, ED_{50} and ED_{90} represent doses of BK antagonist (μg/min) that inhibit the vasodepressor response to 250 ng of BK by 20, 50 and 90%, respectively.
[b] Values ED_{20} or ED_{90} extrapolated from the dose–response curve.
[c] Agonistic activity was calculated as percentage of the BK activity (set to 100%); antagonistic activity was calculated as pA_2 (negative common logarithm of analogue concentration shifting the log dose–response curve for BK by a factor of 0.3 to the right: the calculations were made from the linear portions of the curves).
[d] This peptide was previously designed by Stewart's group [4]. As we used a different assay for evaluation of biological activities, we tested this analogue in our system as a reference.
[e] Data from Ref. 24.

Table 4. Basal characteristics of the tested rats and blood pressure responses to BK analogues

Analogue	*n*	Body weight	Basal MAP (mmHg)	Basal HR (beats/min)	MAP response (mmHg)
I	8	420.0 ± 8.0	111.00 ± 3.83	321.12 ± 4.98	−12.55 ± 4.57[a]
II	8	419.1 ± 7.5	114.12 ± 4.35	312.50 ± 6.34	−5.03 ± 1.65[a]
III	4	324.0 ± 14.5	96.75 ± 5.58	330.50 ± 7.91	+3.62 ± 0.86
IV	4	321.2 ± 12.6	98.25 ± 6.40	313.75 ± 7.58	–
V	4	320.5 ± 6.6	102.37 ± 5.47	326.50 ± 9.35	−5.62 ± 1.32[a]
VI	4	343.8 ± 7.3	96.75 ± 4.20	317.75 ± 8.12	+3.00 ± 1.31 ns[b]
Stewart's antagonist	7	402.0 ± 6.2	116.57 ± 6.29	307.86 ± 8.46	ns[b]

n, number of rats tested; ns, non-significant response.
[a] Regular or occasional vasodepressor response to the BK analogue infusion corresponding to the BK agonistic properties.
[b] Irregular MAP response up to +5.5 mmHg.

Two out of the six tested analogues, **I** and **II**, also elicited direct vasodepressor response over the dose range of 0.25–8 and 0.25–1 μg/min respectively. Although endogenous BK is not the major vasodilatory contributor that takes part in the regulation of arterial pressure, the evoked decrease in MAP is likely to suggest some BK agonistic potency. On the other hand, all of the aforementioned peptides substantially inhibited the vasodepressor responses to exogenous BK that is a proof and quantitative measure of their antagonistic potency. The reason for the appearing discrepancies is not clear. The point is that direct agonist-like effect on blood pressure of the BK analogues may not necessarily reflect their interaction with BK receptors responsible for vasodilation caused by injection of exogenous BK. The standard decrease in MAP noticed following BK injection is strongly related to vasodilation of small arteries of the resistance type. This is not the case in the direct vasodepressor response observed during infusion of some BK analogues. Such response may involve either relaxation of smooth muscle in larger arteries or may result from central blood pressure deregulation [34–36]. It is well known that BK receptors, including those of B_2 type, are not homogenous in the vasculature and in the regulatory circuits of the central nervous system [35,37–39]. It is possible that some BK analogues may bind to one, rather narrow pool of BK receptors, to activate the cellular response and may simultaneously block the other pool of BK receptors easily accessible for exogenous BK in the bloodstream [34,36,39].

In the rat uterus test, compounds **III, IV** and **VI** exhibited very low, almost negligible antagonistic effect, about two orders of magnitude lower than that of the Stewart's peptide. Analogues **I, II** and **V** displayed agonistic potency which was, however, much weaker than that of BK. The dose–response curves of these peptides were parallel to that of BK and it was possible to reach the same maximal contraction.

Discussion

The present study is a continuation of our previous efforts aimed at finding structural requirements in effective BK antagonists of B_2 receptors. When designing the new analogues we focused once again on position 7 of the Stewart's antagonist NPC-349 (Table 1). Three conformationally constrained amino acids were introduced into this position. The results show clearly that the D-Phe[7] substitution with the N-(Bzl)-Gly, X_1 or X_2 residue led to suppression of antagonistic activity of the new peptides. However, these non-coded achiral amino acids are still acceptable at that position. This is consistent with our earlier data suggesting that the presence of the D-amino acid residue at position 7 is not necessary for B_2 antagonism [18,19,21,40,41].

Recently we have found that N-(Bzl)-Gly at position 8 of the Stewart's antagonist caused an increase in B_2 antagonistic potency in the pressure test [24]. However, as can be seen from Table 3, introduction of this modification into position 7 (analogue **I**) resulted in a substantial reduction of the antagonistic activity in this test. As the antagonistic properties were preserved, this achiral non-coded amino acid in this position is still acceptable by the receptors. On the other hand, this analogue displayed an agonistic effect at low doses. A substantial agonistic potency was exhibited by this compound in the uterus test. This points out to differences in the interaction of peptide **I** with B_2 pressor receptors and B_2 receptors in the uterus.

Substitution of D-Phe at position 7 by the other two achiral non-coded amino acids, X_1 and X_2 (Figure 1), which differ only slightly in their structures, led to even less potent analogues in the pressor test. In the uterus test, one of the compounds showed low antagonistic activity (peptide **III**) and the other the low degree of agonism (peptide **V**). As the X_1 residue may be considered as conformationally less restricted and less extended than the X_2 residue, it can be concluded that the enhanced restriction and extension of this part of the peptide chain is likely to result in antagonism. These findings seem to support our previous data regarding BK analogues with the D-1-Nal and D-2-Nal residues at position 7 [40].

As far as the acylation is concerned, its effect was different in the case of both the pressor test and the uterus test. While in the pressor test the N-terminal introduction of the acyl group enhanced the antagonism of compounds **III** and **V** (analogues **IV** and **VI**, respectively in Table 3) and preserved antagonistic properties of compound **I** (analogue **II**), in the uterus test the agonistic potency of peptide **I** was strongly reduced by acylation. Passing on to compound **III**, its negligible antagonistic activity was not affected, while with peptide **V** its low agonistic potency was converted into antagonism. These findings again reveal nonidentity of the analogue–receptor interaction in both tests. The different consequences of acylation in the case of analogue **II** on the one hand and analogues **IV** and **VI** on the other hand, may be due to difference between the acyl agents (Aca vs Aaa).

The pharmacological discrepancies observed with BK and its antagonists still remain difficult to explain. Up to the present, there is no clear evidence for the existence of subtypes of BK receptor within a single species. Furthermore, the results of the studies excluded the possibility of the appearance of more than one gene for B_2 receptor within the same species and the B_2 receptor gene knockout mouse has lost all responses to exogenous BK [42]. This

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notwithstanding, an alternative splicing of a single gene cannot be ruled out. The recent discovery of a novel human orphan G-protein-coupled receptor, which is present in locations where kinins act (such as heart, skeletal muscle, some endocrine glands, brain areas) may contribute to elucidation of this enigmatic issue in the BK receptor field [43].

Acknowledgements

This work was supported by the State Committee for Scientific Research (Poland), grant No. PB 0380/H03/2006/31 and by research project No. Z40550506 of the Academy of Sciences of the Czech Republic. Partial funding for this work was also provided by the Gdańsk University grant DS/8453-4-0169-0.

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