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New Analogues of Bradykinin Substituted in the C-Terminal Part of the Molecule with Naphthylalanine*

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This paper describes the synthesis and some pharmacological properties of eight new analogues of a previously synthesized bradykinin antagonist, D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg. Two peptides were designed by substitution of Thi[8] with L-1- and L-2-naphthylalanine. In two further analogues this modification was combined with placement in position 7 of D-2-naphthylalanine residue. Finally, we obtained four analogues by acylation of N-terminus of the peptides mentioned above with 1-adamantaneacetic acid. The activity of analogues was assessed by their ability to inhibit vasodepressor response to exogenous bradykinin (rat blood pressure test). Our results indicate that the modification proposed decreased the B_2 antagonistic activity, however, the range of this effect was different. We also observed that even minor changes in the structure of the C-terminal part of the B_2 antagonists may significantly influence their activity.

Key words: rat uterus assay, bradykinin, B_2 antagonists

Bradykinin (BK) is a naturally occurring nonapeptide that is split from the serum alpha$_2$ – globulin precursor by the enzyme called "kalikrein". To date, two major classes of receptors of this hormone, B_1 and B_2, have been defined. The data published so far indicate that most of the clinically relevant effects of BK are functions of B_2 receptors. For this reason, most attempts to synthesize effective BK antagonists have been directed towards analogues acting as B_2 blockers [1]. In the short period after Stewart and Vavrek reported, in 1985, the first bradykinin analogues with antagonistic

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* Abbreviations: The symbols of the amino acids and peptides are in accordance with 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.*, **138**, 9 (1984)]. Other abbreviations: Aaa, 1-adamantaneacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Oic, octahydroindole-2-carboxylic acid; Igl, α-(2-indanyl) glycine; L-1-Nal, L-3-(1-naphthyl)-alanine; L-2-Nal, L-3-(2-naphthyl)-alanine; DIEA, N,N-diisopropylethylamine; DCM, dichloromethane; DMF, dimethylformamide; Hyp, hydroxyproline; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; TBTU, O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Thi, β-thienyl-L-alanine; TIS, triisopropylsilane.

activity at the B_2 receptors, hundreds of antagonists have been designed, synthesized and pharmacologically evaluated. These compounds have been extensivelly used in studies of BK receptor subtypes and the role of this hormone in blood-pressure regulation, inflammatory diseases, endocrinological regulation, pain and airway diseases. However, the breakthrough in this field took place in the early 1990's, when the second generation of blockers was introduced [2]. This and the following generations of antagonists consists of conformationally constrained peptides having at the C-terminal end of their molecules amino acids such as 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), octahydroindole-2-carboxylic acid (Oic), α-(2-indanyl) glycine (Igl) and pentafluorophenyl-alanine [3]. In our laboratory, we also explored structural factors that may influence the antagonistic properties of BK analogues. We reported that acylation of the N-terminus of several known B_2 antagonists with various kinds of bulky acyl groups has consistently improved their antagonistic potency in rat blood pressure assay [4–6]. However, although more than four decades have passed since the discovery of BK stable, potent and specific antagonists of this hormone, which may be used as therapeutic agents in the treatment of BK mediated disorders continue to be unavailable. For this reason, future structure-activity studies, which will lead to improvements in potency, selectivity and oral activity of bradykinin antagonists, are needed. Moreover, the prospect of uncovering other BK receptor subtypes offers scientists the opportunity to attain atractive design and synthetic goals.

In 1997 we reported that two analogues synthesized in our laboratory, formed by replacement of D-Phe in position 7 of one of potent B_2 antagonists, Aaa (D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7) BK with D-1- or D-2-Nal, showed strikingly different potency in rat blood pressure assay: peptide substituted with D-2-Nal exhibited a much greater antagonistic activity than its counterpart having D-1-Nal [7]. These two peptides differ only in that the naphthalene ring is connected to the backbone of the molecule through position 2 or 1. The hindering effect, due to the bulky naphthalene fragment near the peptide bond in the case of D-3-(1-naphthyl)-alanine, is much greater than for D-3-(2-naphthyl)-alanine. We assumed that this factor has a significant impact on the bioactive conformations of molecules that contain these amino acids, and can thus influence their interaction with receptors.

Bearing all this in mind, we decided to check how replacement of Thi^8 in B_2 antagonist previously synthesized by Stewart and Vavrek [8], namely (D- Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7)BK (model peptide) with L-3-(1-naphthyl)-alanine (L-1-Nal) and L-3-(2-naphthyl)-alanine (L-2-Nal) will influence the pharmacological properties of resulting analogues I and III. In peptides II and IV we combined the above-mentioned modifications with acylation of the N-terminus with 1-adamantaneacetic acid (Aaa). Next, two analogues V and VII we designed by substitution in the model peptide D-Phe^7 with 2-D-Nal combined with introduction in position 8 of either L-1-Nal or L-2-Nal residues. In peptides VI and VIII we additionally acylated the N-terminal ends of both compounds (V and VII) with 1-adamantaneacetic acid.

The structures of eight new analogues are:

X-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Y-O-Arg
where:

X = H	Y = D-Phe	O = L-1-Nal	(I)
X = Aaa	Y = D-Phe	O = L-1-Nal	(II)
X = H	Y = D-Phe	O = L-2-Nal	(III)
X = Aaa	Y = D-Phe	O = L-2-Nal	(IV)
X = H	Y = D-2-Nal	O = L-1-Nal	(V)
X = Aaa	Y = D-2-Nal	O = L-1-Nal	(VI)
X = H	Y = D-2-Nal	O = L-2-Nal	(VII)
X = Aaa	Y = D-2-Nal	O = L-2-Nal	(VIII)

EXPERIMENTAL

Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Microtechna type AAA881 analyzer. For analysis, the peptides (0.5 mg) were hydrolyzed by azeotropic hydrochloric acid (400 μl), containing phenol (20 μl), in evacuated sealed ampoules at 110°C for 18 h. TLC was carried out on silica plates (Merck), and the spots were visualized using iodine or ninhydrin. The following solvent systems were used: A: butan-1-ol – acetic acid – water 4:1:5 (v/v), upper phase; B: butan-1-ol – acetic acid – water – ethyl acetate 1:1:1:1 (v/v/v/v).

The purity of the peptides was also ascertained by HPLC. Analyses of the analogues were performed on a Gold System Beckman chromatograph with an Ultrasphere ODS column (5 μm, 4.6×200 mm) and with an ODS precolumn ((5 μm, 4.6×50 mm). Solvent system: (A) 0.1% trifluoroacetic acid (TFA), (B) acetonitrile – 0.1% TFA (80:20 v/v), linear gradient 10–70% of (B) for 25 min, λ = 226 nm, flow rate 1 ml/min. Mass spectra were determined on Finningan Mat 95 spectrometer with FAB ionization technique.

Peptide synthesis: Peptides I–IV were synthesized manually, peptides V–VIII were synthesized by the solid-phase method on a Symphony/Multiplex$_{TM}$ Multiple Peptide Synthesizer (Protein Technologies Inc., USA) using the Fmoc-strategy and starting from Fmoc-Arg(Pbf)-Wang resin (10) (loading 0.33 mmol/g, 50 μmol). Fmoc was removed by 20% piperidine in DMF. A five-fold excess of the respective Fmoc-amino acids was activated *in situ* using TBTU (1 eq.) / HOBt (1 eq.) in DMF and coupling reactions were base catalyzed with DIPEA (4 equivalents). 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 commercially available (NovaBiochem, Bad Soden, Germany). 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 seconds. 2. Equilibrating the resin with 2.5 ml of a 20% solution of piperidine (Pip) in DMF, twice for 5 minutes each time. 3. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) six times for 30 seconds. 4. Equilibration with a solution of five-fold excess of Fmoc-amino acid followed by the addition of five-fold excess of a solution of: TBTU (1 eq.) / HOBt (1 eq.) / DIPEA (4 equivalents) in DMF and mixing for 45 minutes. Coupling reactions were performed twice without monitoring. 5. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) three times for 30 seconds.

Cleavage of the peptides from the resin with side-chain deprotection was performed by treatment with TFA:H_2O:TIS (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 crude material was desalted by gel filtration on a Sephadex G-15 column (10×1.5 cm) eluted with aqueous acetic acid (50%) at a flow rate of 4 ml/h. Fractions comprising the major peak were pooled and lyophilized, and the residue was subjected

further to reversed-phase HPLC on an Ultrasphere ODS column (5 μm, 10×200 mm). Purification was performed on a Gold System Beckman chromatograph. Solvent system: (A) 0.1% trifluoroacetic acid (TFA), (B) acetonitrile – 0.1% TFA (80:20 v/v); isocratic system – 40% of (B), λ = 226 nm, flow rate 2 ml/min. Lyophilization of the pertinent fractions gave the bradykinin analogues.

Bioassay methods. ***Effect of bradykinin analogues on rat blood pressure:*** The antagonistic potency of the analogues was assessed by their ability to inhibit the vasodepressor response to exogenous bradykinin in conscious rats. Male, intact Wistar albino rats (350–400 g) were maintained on a regular chow diet and tap water at a constant room temperature of (23 ± l°C).

One day before the experiment polyethylene tubing (PE5O) was inserted into the right carotid and the iliac artery under pentobarbital (pentobarbital sodium – 20 mg/kg i.p.) anaesthesia. We provided 24 hours 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 bradykinin, which might interfere with our assay.

A Y-type connection was attached to the carotid artery for injection of bradykinin and infusion of the bradykinin analogues. All catheters were exteriorized subcutaneously at the back of the neck. On the day of the experiment, the rats were put into 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). Thirty minutes prior to the experiment, pentobarbital (pentobarbital sodium – 10 mg/kg) was injected i.p. to sedate the rats. The next dose of pentobarbital (5 mg/kg) was repeated 60–70 min later. Following the injection of the first dose, a 30-min stabilization period was allowed prior to the start of the experiment. Since we observed in our previous studies performed on conscious rats, that some analogues of bradykinin evoked agitation of animals related to direct sensory effect, we prevented rats from anxiety in the present assay with the use of a moderate dose of an anesthetic, pentobarbital sodium. The measures implied resulted in a relatively small deviation of the mean.

An angiotensin-converting enzyme inhibitor, enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, USA; l mg/kg) was injected into the iliac catheter. Thirty to sixty minutes later, after stabilization of the blood pressure, bradykinin acetate salt (Sigma) (62.5, 125, 250 ng) dissolved in 5% D-glucose solution at a concentration of 2.5 μg/ml, was injected every 4 to 5 min. into one branch of the carotid catheter. Each dose was repeated twice or three times until the vasodepressor responses to exogenous bradykinin were stable. *(Prior to the administration of the doses of BK, MAP averaged 102 ± 2 mm Hg and HR was 342 ± 6 beats/min. BK evoked significant dose-dependent vasodepressive response (–23.7 ± 1.1, –31.7 ± 1.3 and –39.4 ± 1.7 mm Hg following 62.5, 125 and 250 ng BK, respectively), accompanied by a transient increase in HR (23 ± 5; 45 ± 7 and 56 ± 8 beats/min, following 62,5, 125 and 250 ng BK, respectively)).*

The vasodepressor response to BK was plotted against the logarithm of the bradykinin dose. The average values of the responses to 125 ng and 250 ng were calculated from the regression line obtained from the log dose-effect plot. Both vasodepressor responses to 125 ng and 250 ng were taken as the control responses. The BK analogue dissolved in 5% D-glucose solution was infused to a branch of the carotid catheter other than the BK. A constant rate of infusion – 125 μl/min was provided using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, Netherlands). The BK analogue administration was initiated with its 8-min infusion at a concentration of 0.4 μg/ml (giving a dose of 50 ng/min), during the infusion, 250 ng BK was injected into the carotid artery. This procedure was repeated twice or three times until the vasodepressor responses were stable. The dose of bradykinin antagonist infused was afterwards increased (1, 4, 16, 64, 150 or 256 and, if necessary, 500 and 1000 μg/ml) and the same procedure was repeated until the vasodepressor response to 250 ng of exogenous bradykinin decreased to less than 10% of the control response.

The inhibition of the vasodepressor response to 250 ng BK by each BK-antagonist tested was plotted against the logarithm of its dose. This dose-effect plot was used for quantitative estimation of the antagonistic potencies of the tested compounds. As indices of these potencies we used the effective doses: ED_{20}, ED_{50} and ED_{90}, representing the respective doses of bradykinin antagonist (μg/kg/min) that inhibit the

vasodepressor response to its agonist (250 ng of BK) by 20, 50 and 90%. We also calculated pA_2 from the dose-effect plot as a supplementary index. In our assay pA_2 represents a negative logarithm of the molar concentration of an antagonist (concentration injected divided by the estimated volume of distribution – 67 ml/kg), which reduces the response to the double dose of agonist (250 ng BK) to a value obtained as a response to its single dose (125 ng BK) [11]. The results are reported as mean values of ± S.E. A comparison of the two analogues was accomplished by Student`s non-paired t-test [12]. Differences were considered to be significant for $P < 0.05$.

RESULTS AND DISCUSSION

The antagonistic potencies of 8 new analogues of bradykinin compared with that of antagonist synthesized by Stewart's group [9] ([D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK) and our previously obtained peptides (IX,X) [4,7] are presented in Table 1. The antagonistic potency of analogues was assessed by their ability to inhibit vasodepressor response to exogenous BK in conscious rats [4,5]. In this assay, peptides II and V are moderately potent antagonists. In lower doses (ED_{20}) our most active analogue V is equipotent with Stewart's peptide, however, in higher doses (ED_{50} and ED_{90}), its activity is slightly lower. On the other hand, in lower doses peptide V is about 50% less active than IX, whereas in higher doses its activity is 5 times lower. Passing to the next analogues we observed diminished antagonistic activity of peptides I, III and IV and only negligible potency of peptides IV, VII and VIII.

As mentioned in the introduction, analogues of bradykinin designed by substitution of D-Phe^7 in one of the potent B_2 antagonists (Aaa [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK) with D-1- or D-2-Nal showed a strikingly different potency in rat blood pressure assay [7], *i.e* peptide formed by replacement with D-2-Nal exhibited a much higher activity than its counterpart having D-1-Nal in position 7. This observation and earlier data, which had suggested that the presence of two suitable, bulky amino acid residues in positions 7 and 8 may result in potent and selective B_2 antagonists [2,3], prompted us to design, synthesize and pharmacologically evaluate two analogues, in which we combined the introduction of D-2-Nal into position 7 with replacement of Thi^8 with L-1-Nal or L-2-Nal (V, VII). The next two peptides described (VI, VIII) were additionally acylated on their N-terminus with 1-adamantaneacetic acid in order to check whether our earlier finding that such operation improved activity of many B_2 antagonists is valid also in this case. We also found it interesting to evaluate the pharmacological properties of two compounds (I, III), in which we kept D-Phe in position 7, but introduced L-1- or L-2-Nal into position 8. For the same reason we also synthesized two analogues of peptides I and III acylated with 1-adamantaneacetic acid (II, IV).

It is clear from the results presented, that the modifications proposed led to a drop in antagonistic activities of the new analogues, especially when compared with peptides IX and X. Our present studies demonstrated that the effect of substitution of Thi^8 with L-1-Nal or L-2-Nal is either diverse or not so dramatic as it was in the case described earlier for placement of their D-enantiomers in position 7. Our data showed that D-2-Nal^7 substitution combined with L-1-Nal in position 8 (V) gives a more

potent antagonist, whereas when position 7 is occupied by D-Phe, the presence of either L-1-Nal or L-2-Nal in position 8 does not seem to differentiate pharmacological properties of the resulting analogues (I and III).

Our earlier data had suggested that acylation of the N-terminus of many BK antagonists with bulky groups consistently improved the antagonistic potency [4–6]. However, in view of the present results, this regularity appears to be valid only for two pairs of analogues: I, II and VII, VIII. As regards peptides III, IV and V, VI the acylated compounds are less potent than the nonacylated ones.

Moreover, it is worth pointing out the significant difference of activites of pairs of peptides: II, IV; V, VII and VI, VIII, which differ only in that the naphthalene ring is connected to the backbone of the molecule through positions 2 or 1. One can only assume that the different hindering effect for L-1-Nal and L-2-Nal has a significant impact on the bioactive conformations of molecules and can thus influence their interaction with receptors. This observation proves that even minor changes in the structure of the C-terminal part of B_2 antagonists may have a considerable impact on their activity.

Judging from the above results, our studies have demonstrated that the proposed modification decreased B_2 antagonistic potency, however, the range of this effect was different. Our results, though not impressive from the point of view of activity, may be of certain value in the design of new B_2 antagonists.

Table 1. Pharmacological properties of bradykinin analogues.

Peptide		N	Antagonistic potency			
			ED_{20} [a]	ED_{50} [a]	ED_{90} [a]	pA_2 [b]
[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,1-Nal^8]BK	I	8	3.36±0.44	33.96±3.76	749.7±105.8	7.25±0.08
Aaa[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,1-Nal^8]BK	II	7	1.80±0.12	10.43±0.6	111.29±10.99	7.36±0.16
[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,2-Nal^8]BK	III	9	2.04±0.24	25.59±2.43	608.1±56.55	7.34±0.08
Aaa[D-Arg^0,Hyp^3,Thi^5,D-Phe^7,2-Nal^8]BK	IV	5	8.24±0.75	125.18±13.56	>1000	6.72±0.07
[D-Arg^0,Hyp^3,Thi^5,D-2-Nal^7,1-Nal^8]BK	V	9	0.59±0.06	5.24±0.45	98.27±8.96	7.96±0.04
Aaa[D-Arg^0,Hyp^3,Thi^5,D-2-Nal^7,1-Nal^8]BK	VI	4	3.02±0.35	25.29±3.43	495±136.1	7.35±0.05
[D-Arg^0,Hyp^3,Thi^5,D-2-Nal^7,2-Nal^8]BK	VII	7	68.96±8.28	650±113.4	>1000	5.78±0.07
Aaa[D-Arg^0,Hyp^3,Thi^5,D-2-Nal^7,2-Nal^8]BK	VIII	4	11.11±1.24	133.32±16.56	>1000	6.53±0.13
[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK[c] (Stewart's peptide)		9	0.68±0.04	5.04±0.28	75.90±7.51	7.98±0.05
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-2-Nal^7]BK[d]	IX		0.37±0.09	–	18.57±3.27	–
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK[e]	X		0.84±0.09	–	13.94±1.69	–

N – Number of rats tested.

[a] ED_{20}, ED_{50} and ED_{90} represent the respective doses of bradykinin antagonist (μg/kg/min) that inhibit the vasodepressor response to its agonist (250 ng of BK) by 20, 50 and 90%.

[b] pA_2 represents a negative logarithm of the molar concentration of an antagonist (concentration injected divided by the estimated volume of distribution – 67 ml/kg, [11]) which reduces the response to the double dose of agonist (250 ng BK) to a value obtained as a response to its single dose (125 ng BK).

[c] This peptide was previously designed by Stewart's group [9]. As we used a different assay for evaluation of our peptides, we tested this analogue in our system as a reference.

[d] Data from [7].

[e] Data from [4].

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