INFLUENCE OF *C*-TERMINAL MODIFICATIONS OF BRADYKININ ANTAGONISTS ON THEIR ACTIVITY

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In the present study we have described the synthesis and some pharmacological properties of four new analogues of bradykinin (BK). Two peptides were designed by substitution of positions 7 and 8 of known B_2 antagonists with *N*-methyl-L-phenylalanine [Phe(Me)]. The next two analogues were obtained by replacement of D-Phe residue in position 7 of known B_2 antagonist with 1-naphthyl-D-alanine or 2-naphthyl-D-alanine. The antagonistic potency of peptides was assessed by their ability to inhibit vasodepressor response to exogenous bradykinin in conscious rats. Although our studies demonstrated disadvantegous influence of $Phe(Me)^{7,8}$ modification for B_2 antagonism, we showed that D-amino acid residue in position 7 of BK antagonists may be replaced by suitable L-amino acid residue. As regards $(D\text{-}Nal)^7$ substitution, we found strikingly different antagonistic potencies of analogues which differ only in the presence of D-1-Nal and D-2-Nal. We assume that it is due to different conformations of these peptides, proving the importance of the shape of the *C*-terminal part of B_2 antagonists for their activity.

Key words: Peptides; Bradykinin; B_2 antagonists; Blood pressure.

Modifications currently used for structure–activity relationships study of peptides are of various types: addition, deletion, substitution of one or more amino acid residues, cyclization, or use of peptide bond isosters. In this field, *N*-methylation is considered as one of the local and subtle modes of conformational constraint. There are many information outlining structural perturbations induced by *N*-methylation: steric constraints, suppression of a proton donating N–H group capable of hydrogen bonding, reduction of the predominance of the *trans vs cis* peptide bond and increased basicity of the carbonyl group. Detailed conformational studies indicate that the influence of *N*-methylation on a conformation depends to a large extent on the chirality of the residues surrounding the modified peptide bond[1]. The biological significance of this modification still remains a question, there are suggestions that it either provides enhanced resistance against biodegradation or increases hydrophobicity.

Having all this in mind we decided to check how replacement of amino acid residues in positions 7 and 8 of the Stewart's antagonist $[D\text{-}Arg^0,Hyp^3,Thi^{5,8},D\text{-}Phe^7]BK$

with *N*-methyl-L-phenylalanine [Phe(Me)]* will influence pharmacological properties of the resulting analogue **1**. We also found it interesting to apply the same modification for Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK, one of our previously synthesized potent B_2 antagonists[2] (analogue **2**). This analogue, in fact, may be considered as a derivative of **1** with the 1-adamantaneacetyl group (Aaa) on its *N*-terminus.

It is believed that the critical change conferring bradykinin antagonist activity upon analogues is the replacement of Pro^7 with aromatic D-amino acids[3]. D-Phe has been most widely used and appears to be acceptable in analogues with a wide variety of additional modifications. Later it was shown that only a narrow range of D-amino acid residues is acceptable for production of antagonistic activity. Naphthylalanine (Nal) is one of the amino acids which lead to less active compounds[3]. On the other hand, our results obtained for modification of position 3 of arginine vasopressin (AVP) or its analogues with L-2-Nal and L-1-Nal have proved there to be great differences in activities of compounds, which are distinguished only by the presence of these two residues. These amino acids differ only because the naphthalene ring is connected by its position 1 and 2 to the backbone of the molecule, respectively. The hindering effect caused by bulky naphthalene ring near the peptide bond is in the case of L-1-Nal much greater than for L-2-Nal. In our opinion this may have a significant impact on conformation of an analogue and can thus influence its interaction with receptors[4].

This finding prompted us to investigate how substitution of position 7 of our previously synthesized antagonist, which we already used as a model to design analogue **2**, with D-1-Nal and D-2-Nal will affect pharmacological properties of resulting compounds **3** and **4**.

All synthesized analogues have the following structure:

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

Analogue	X	Y	Z
1	H	Phe(Me)	Phe(Me)
2	Aaa	Phe(Me)	Phe(Me)
3	Aaa	D-1-Nal	Thi
4	Aaa	D-2-Nal	Thi

* Abbreviations: The symbols of the amino acids and peptides are in accordance with 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (European J. Biochem. *138*, 9 (1984)). Other abbreviations: Aaa, 1-adamantaneacetic acid; DCM, dichloromethane; DMF, dimethylformamide; HOBt, *N*-hydroxybenzotriazole; D-1-Nal, 1-naphthyl-D-alanine; D-2-Nal, 2-naphthyl-D-alanine; TBTU, benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluoronium tetrafluoroborate; Thi, α-(2-thienyl)alanine.

EXPERIMENTAL

The optical rotations were measured by means of a Perkin–Elmer Model 141 polarimeter. For amino acid analysis, the peptides (0.5 mg) were hydrolyzed with constantly boiling hydrochloric acid (400 μl), containing phenol (20 μl), in evacuated sealed ampoules for 18 h at 110 °C. The analyses were performed on a Microtechna type AAA881 analyzer. The elemental analyses were determined on a Carlo–Erba Model 1106 analyzer. TLC was carried out on silica plates (Merck), and the spots were visualized by iodine or ninhydrine. The following solvent systems were used: (A) butan-1-ol–acetic acid–water, 4 : 1 : 5 (v/v), upper phase; (B) ethyl acetate–pyridine–acetic acid–water, 5 : 5 : 1 : 3 (v/v); (C) butan-1-ol–pyridine–acetic acid–water, 52 : 12 : 12 : 25 (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 × 150 mm). Solvent system: (1) 0.1% trifluoroacetic acid (TFA), (2) acetonitrile–0.1% TFA, 80 : 20 (v/v), linear gradient from 30 to 90% of (2) for 20 min, λ = 226 nm, flow rate 1 ml/min. Each analogue gave a single peak. The purity of all peptides was between 95 and 97% as determined from the integrated areas recorded at 226 nm.

N-tert-Butoxycarbonyl-*N*-methylphenylalanine was obtained in 86% yield from *N-tert*-butoxycarbonylphenylalanine (7.95 g, 0.05 mol), methyl iodide (15 ml, 0.24 mol) and sodium hydride dispersion (3.96 g, 0.09 mol) according to Cheung[5] as an oil, M^+ = 279 (*m/z*); dicyclohexylammonium salt: $[\alpha]_D^{20}$ = –24.1° (*c* 1, methanol). For $C_{27}H_{44}N_2O_4$ (480.6) calculated: 70.4% C, 9.6% H, 6.1% N; found: 70.2% C, 9.7% H, 6.1% N.

Peptide Synthesis

All peptides were prepared by the solid phase synthesis method by stepwise coupling of Boc-amino acids to the growing peptide chain on a Merrifield resin[6]. Boc-Arg(Tos)-resin (Sigma; 0.35 mmol of amino acid per gram; 1.0 g) was converted to the protected decapeptidyl resins (analogue **1**) or acyldecapeptidyl resins (analogues **2**, **3**, **4**) in the nine and ten cycles of standard solid phase synthesis, respectively[6]. Boc-Arg(Tos)-OH, Boc-D-Arg(Tos)-OH, D-1-Nal-OH and D-2-Nal-OH were dissolved prior to coupling in a mixture DMF–DCM (3 : 1). For coupling of Boc-Phe(Me)-OH, TBTU/HOBt in a mixture DMF–DCM (2 : 1) was used. Boc-Hyp-OH was coupled without protection of the OH-group. *N*-1-Adamantaneacetic acid was used in the final coupling steps for analogues **2**, **3** and **4**. The completion of all coupling reactions was monitored by the Kaiser test[7]. After synthesis was completed, 1 g of the protected acylpeptidyl resin was treated with 10 ml of liquid hydrogen fluoride containing 1 ml of anisole at –70 °C and stirred for 50 min at 0 °C. After the removal of the HF and anisole *in vacuo* the mixture was washed with anhydrous diethyl ether (3 × 30 ml) and then with 30% acetic acid (5 × 40 ml). The acetic acid extracts were combined and lyophilized to yield the crude product. The material was desalted by gel chromatography on a Sephadex G-15 column (120 × 2.9 cm) with 50% aqueous acetic acid at a flow rate of 5 ml/h. Fractions comprising the major peak were pooled and lyophilized, and the residue was further subjected to gel chromatography on a Sephadex LH-20 column (120 × 1.4 cm) with 30% aqueous acetic acid at a flow rate of 2 ml/h. The peptide was eluted as a single peak. Lyophilization of the pertinent fractions gave the bradykinin analogue. Physico-chemical properties of the new analogues (**1**–**4**) are presented in Table I.

Effect of 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[2,8], as follows. Male, intact Wistar albino rats (300–350 g) were maintained on a regular chow diet, as well as tap water in a room at constant

temperature (23 ± 1 °C). One day before the experiment, the right carotid and the iliac artery were catheterized with polyethylene tubing (PE50) under light ether anaesthesia. A "Y" type connection was attached to the carotid artery for injection of bradykinin and for infusion of the bradykinin analogues. All catheters were exeriorized subcutaneously at the back of the neck. On the day of the experiment, the rats were conscious and unrestrained in plastic cages. Mean arterial pressure (MAP) and heart rate (HR) were monitored through a Gould–Statham P23-ID pressure transducer (Gould, Cleveland, OH, U.S.A.) connected to the iliac catheter and recorded on a paper chart recorder. A 30 min stabilization period was allowed before initiation of the experiment. Angiotensin-converting enzyme inhibitor, Enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, U.S.A., 1 mg/kg) was injected through the iliac catheter. Thirty to sixty minutes later, when a stable blood pressure was obtained, bradykinin acetate salt (Sigma) (62.5, 125, 250 ng) dissolved in 5% glucose to 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 two or three times until the vasodepressor responses to exogenous bradykinin were stable. The vasodepressor response to BK was plotted against the logarithm of the bradykinin dose. The average values of the responses to 125 and 250 ng were calculated from the regression line obtained from the log dose–effect plot. Both vasodepressor responses to 125 and 250 ng were taken, respectively, as the control responses. The BK analogue dissolved in 5% dextrose solution was infused to a branch other than the BK of the carotid catheter. A constant rate of infusion, 125 μl/min, was provided using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, The Netherlands). The bradykinin analogue administration was initiated with an 8-min infusion at a concentration of 0.5 μg/ml (this gave a dose of 62.5 ng/min). By the end of the 3rd and the 7th minute of this infusion 250 ng of BK was injected into the carotid artery. In some experiments, where the vasodepressive responses to BK were different from each other by more than 3 mm Hg, the infusion was prolonged by 150 s and the third dose of BK was injected by the end of this infusion. The mean value of the vasodepressive responses to BK were taken for further data analysis. The dose of bradykinin antagonists was then increased (0.5, 2, 8, 32, 120 and, if necessary, 400 and 1 000 μg/ml) and the some procedure was repeated until the vasodepressor response to exogenous bradykinin decreased to less than 10% of the control response.

The antagonistic potency of the bradykinin analogues was quantitatively expressed as the effective doses; ED_{20}, ED_{50} and ED_{90}. The effective doses: ED_{20}, ED_{50} and ED_{90} represented doses of bradykinin antagonist (μg/min) that inhibit 20%, 50% and 90%, respectively, the vasodepressor response

TABLE I
Physico-chemical characteristics of bradykinin analogues

Peptide	M^+ *m/z*	R_F			$[\alpha]_D^{20}$ (c = 1, 1M AcOH)	Yield[a], % (crude)	Yield[b], % (purified)
		A	B	C			
1	1 316	–	0.68	0.44	–85.0	78	21
2	1 492	–	0.66	0.27	–105.5	62	12
3	1 520	0.11	0.77	–	–72.1	75	27
4	1 520	0.13	0.75	–	–69.7	67	22

[a] Yields were calculated on the basis of the arginine content of the starting resin. [b] All peptides gave expected amino acid analysis ratios after hydrolysis (±0.05). The purity of analogues determined on HPLC was between 95 and 97%.

to its agonist (250 ng of bradykinin). For evaluation of the ED values linear regression analysis was used. For each experiment, the relationship between the dose of the tested bradykinin analogue and the inhibition of the vasodepressor response to bradykinin was determined by fitting a least-square regression line. In all cases significant linear regression was found between the dose and the effect of bradykinin antagonist (correlation coefficient r was never lower than 0.96 and P was never higher than 0.05). The ED values were finally assessed from the regression line. In some experiments, the ED_{90} (bradykinin analogues **2** and **3**) exceeded the applied bradykinin analogue dose range and the values of those ED were evaluated from the regression lines obtained by extrapolation.

Results are reported as mean values of ±S.E. Comparison of the two analogues was accomplished by Student's non-paired t-test[9]. Differences were considered to be significant for $P < 0.05$.

RESULTS AND DISCUSSION

Four new, *C*-terminal-related analogues of bradykinin were synthesized using the solid-phase method on chloromethylated Merrifield resin. The antagonistic potency of peptides was evaluated by their ability to inhibit vasodepressor response to exogeneous bradykinin in conscious rats[2,8]. Some pharmacological properties of new analogues **1**–**4** in comparison with those of some previously synthesized antagonists, which we tested under the conditions of our assay, are presented in Table II. We assessed relatively high values of S.E. in our assay. Since bradykinin and also its analogues are known to exhibit potent sensory effects (*e.g.*, itching, pain) the vasomotoric responses evoked are not usualy uniform in conscious animals and this is probably the reason for the diversity obtained within the experimental subpopulations. On the other hand, bradykinin is highly involved in the afferent part of the cardiovascular reflexes controlling blood

TABLE II
Pharmacological data on bradykinin analogues

Analogue		n^a	Antagonistic potency, μg/min		
			ED_{20}	ED_{50}	ED_{90}
[D-Arg^0,Hyp^3,Thi^{5,8},(NMe)Phe^{7,8}]BK	(**1**)	3		weak agonist	
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},(NMe)Phe^{7,8}]BK	(**2**)	4	4.58 ± 2.75	49.18 ± 41.17	1 676.36 ± 1 088.78
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-1-Nal^7]BK	(**3**)	8	1.54 ± 0.99	27.72 ± 18.11	1 148.34 ± 867.32
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-2-Nal^7]BK	(**4**)	6	0.37 ± 0.09	1.99 ± 0.42	18.57 ± 3.27
[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK[b]	(**5**)	7	1.49 ± 0.29	10.52[c]	142.5 ± 26.4
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK[b]	(**6**)	7	0.84 ± 0.09	2.80[c]	13.94 ± 1.69

[a] Number of rats tested. [b] Data from ref.[10]. This peptide was previously designed by Stewart's group[3]. As we used a different assay for the evaluation of antagonistic properties of our peptides we tested this analogue in our system as a reference. [c] The ED_{50} value was evaluated by extrapolation from the ED_{20} and ED_{90} values.

pressure. Anaesthesia fades these reflexes considerably and alters cardiovascular responses to bradykinin.

In our assay analogue **4** is a very potent antagonist whereas peptides **2** and **3** exhibit rather low potency. Analogue **1** is a weak agonist. In lower doses (ED_{20}) our most active peptide **4** is about 4 and 2 times more potent, respectively, than antagonists synthesized previously in Stewart's (compound **5**) and our (compound **6**) laboratory. At higher doses (ED_{90}), peptide **4** is approximately 9 times more potent than compound **5** and equipotent with compound **6**. Activities of new antagonists **2** and **3** are of similar range as those of **5** and **6** only for ED_{20}. In higher doses both new analogues are much less active than **5** and **6**.

From the results presented it is clear that (NMe)Phe^{7,8} modification results in dramatic decrease of antagonistic activity (analogue **2**) or even conversion it into weak agonist (analogue **1**). On the other hand, analogue **2** is the first example of B_2 antagonist having L-amino acid residue in position 7. The presence of D-amino acid residue, until now, was considered to be necessary for B_2 antagonism. The present data seem also to support our previously raised thesis concerning interaction between analogues acylated with bulky substituent and receptor. It is evident that peptides **1** and **2**, which differ only by the presence of 1-adamantaneacetyl group on the *N*-terminus of **2**, showing opposite types of activities, *e.g.* agonism and antagonism, are good examples of the influence of bulky acyl substituent on antagonistic potencies of analogues.

Passing on to compounds **3** and **4**, regardless of the previous knowledge about disadvantageous effect of D-Nal^7 substitution[3], we decided to substitute D-Phe^7 in our potent B_2 antagonist, Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK, with D-1-Nal and D-2-Nal. As mentioned in the introduction, the hindering effect caused by the bulky naphthalene ring near the peptide bond is in the case of L-1-Nal much greater than for L-2-Nal. Indeed, analogues **3** and **4** having strikingly different antagonistic potencies proved the importance of conformation of the *C*-terminal part of the peptide for its antagonistic properties. It should be emphasized, that such great difference of activities is due to minor change in the structure of analogues, which differ only because the naphthalene ring is connected by its position 1 or 2 to the backbone of the molecule. We can assume that this factor, *e.g.* hindering effect of naphthalene ring, has a significant impact on the bioactive conformations of molecules which contain these amino acids and thus can influence their interaction with B_2 receptors.

Summing up, although our studies demonstrated that the Phe(Me)^{7,8} modification is disadvantageous for B_2 antagonism, we showed that D-amino acid residue in position 7 of BK antagonists which, until now, was considered to be necessary for B_2 antagonism, may be replaced by suitable L-amino acid residue. As regards D-Nal^7 substitution, the great difference of activities due to minor change in the structure caused by the presence in position 7 of analogues D-1-Nal or D-2-Nal support the known thesis about importance of conformation of *C*-terminal part of B_2 antagonists for their activity. Our

study besides providing new information about structure–activity relationship of bradykinin antagonists, resulted in the highly active analogue **4** which may be useful in clarifying the role of BK in various physiological effects.

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