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New acylated bradykinin analogues: effect on rat blood pressure and rat uterus

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Abstract: It was previously reported that acylation of the *N*-terminus of several known B_2 antagonists with various types of bulky acyl groups consistently improved their antagonistic potency in the rat blood pressure assay. On the other hand, earlier results seem to suggest that the effects of acylation on the contractility of isolated rat uterus depend substantially on the chemical character of the acyl group, as it was observed that this modification may either change the range of antagonism or even transform it into agonism.

Bearing all this in mind, three new analogues of bradykinin were designed by modifying the moderately potent B_2 antagonist, previously synthesized by Stewart's group, D-Arg-Arg-Pro-Hyp-Gly-Thr-Ser-D-Phe-Thi-Arg. New analogues were obtained by acylation of the *N*-terminus of the above peptide with succinic acid, 12-aminododecanoic acid and 4-aminobenzoic acid in order to confirm whether either the positive or the negative charge on the *N*-terminal end of the peptide is responsible for the transformation of activity. The activity of analogues was assessed on blood pressure and in uterotonic *in vitro* tests. The modifications proposed either preserved or increased the antagonistic potency in the rat blood pressure test. On the other hand, the three substituents, depending on their chemical character, differently influenced the interaction with the rat uterine receptors. The results may be of value in the design of new B_2 agonists and antagonists. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin; B_2 antagonists; rat blood pressure assay; rat uterotonic test *in vitro*

INTRODUCTION

Almost all cells in the majority of species express kinin receptors which mediate the various physiological and pathophysiological activities of bradykinin (BK). These receptors belong to a G-protein-coupled family and their activation stimulates smooth muscle cells [1,2], sensory nerve endings [3,4], causes vasodilation and microvascular leakage [1,2] and modulates the response of immunocompetent cells [2,5]. There is also considerable evidence that BK contributes to the inflammatory responses. Moreover, after injection to the skin, BK produces all the basic signs of inflammation [6,7]. Due to its ability to lower blood pressure, BK has been implicated in the pathogenesis of several shock syndromes, particularly septic or endotoxic shock. Two receptors, B_1 and B_2, mediate the biological activities of BK. While B_2 receptors require the entire BK sequence for recognition, B_1 receptors recognize and bind des-Arg^9-BK only.

The aforementioned listing of different types of bradykinin activities illustrates why the search for antagonists of this hormone started immediately after the elucidation of its structure in 1960. However, the first report on bradykinin analogues able to antagonize the effects of BK in standard kinin assays, such as rat uterus, guinea-pig ileum or rat blood pressure, came 25 years later, with the description of the [D-Phe^7]- and [$Thi^{5,8}$, D-Phe^7]- bradykinins [8]. Subsequently, in many laboratories the investigation of structural features, which were thought to produce potent and selective blockers of B_2 receptors, commonly called B_2-bradykinin antagonists, resulted in the synthesis and pharmacological evaluation of thousands of compounds exhibiting various types and ranges of activities. In the course of these studies the role of amino acid residues in all positions of BK, as well as the influence of various combinations of substitutions on pharmacological activity of the resulting compounds were carefully investigated [9]. However, the breakthrough in this field took place in the early 1990s, when potent B_2 blockers, carrying conformationally constrained amino acid residues in their *C*-terminal ends were synthesized [10,11].

Another approach was adopted, consisting of acylation of the *N*-terminus of the already known

Abbreviations: Aaa, 1-adamantaneacetic acid; Aba, 4-aminobenzoic acid; Aca, 1-adamantanecarboxylic acid; Adc, 12-aminododecanoic acid; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Sua, succinic acid; TIS, triisopropylsilane.

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B_2-antagonists with various bulky groups in order to improve antagonistic potency and selectivity. It was found that such modifications consistently improved the antagonistic potency in the rat blood pressure assay [12]. Our results also suggest that even minor changes in the structure of acylated bradykinin analogues might be crucial for designing more potent B_2-blockers. This was indicated by a substantial difference in B_2 antagonistic potency between analogues obtained by acylation of known antagonists with 1-adamantaneacetic or 1-adamantanecarboxylic acids. It was demonstrated that the latter peptide, differing from the former only in the lack of an CH_2 group in the *N*-acyl residue, was three times as potent in the rat blood pressure assay [13]. All these findings prompted us to continue investigations into the influence of *N*-terminal acylation on pharmacological activity of B_2 antagonists. It was decided to synthesize three new peptides, I–III, by acylation of a B_2-antagonist reported by Stewart and Vavrek [14], D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg, with 12-aminododecanoic acid, 4-aminobenzoic acid or succinic acid.

EXPERIMENTAL PROCEDURES

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

Peptide Synthesis

The peptides were synthesized by the solid-phase method using the Fmoc-strategy and starting from Fmoc-Arg(Pbf)-Wang resin [15] (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 catalysed with DIPEA (4 eq). The amino acid side chain protecting groups were tBu for Hyp and Pbf for Arg and D-Arg. All the Fmoc-protected amino acids and Fmoc-Arg(Pbf)-Wang resin were commercially available (NovaBiochem, Bad Soden, Germany).

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 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 (50%) at a flow rate of 4.0 ml/h, $\lambda = 254$ nm. The eluates were fractionated, and the fractions containing the major peak were pooled and lyophilized. All peptides were purified by preparative HPLC.

The physicochemical properties of peptides I–III are presented in Table 1.

Biological Evaluation

Effect of bradykinin analogues on rat blood pressure. The antagonistic potency of the bradykinin analogues was assessed by their ability to inhibit the vasodepressor response to exogenous BK in conscious rats. Male, intact Wistar albino rats ($n = 31$; 250–320 g) were maintained on a regular chow diet and tap water at ambient temperature (22° ± *l* °C).

Two days before the experiment, polyethylene tubes (PE-5O, Clay-Adams, USA) were inserted into the right carotid and the iliac artery under pentobarbital (pentobarbital sodium −40 mg/kg i.p.) anaesthesia. A Y-type connector was mounted to the tubing from the carotid artery. Two arterial lines were led out for injection of either bradykinin, or infusion of the bradykinin analogues. All catheters were exteriorized subcutaneously at the back of the neck. Some 40 to 44 h was allowed 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).

Table 1 Physicochemical Properties of BK Analogues I–III

Analogue	20%–80% of [B] for 30 min		[M + H]+	
	HPLC tr	Purity[%]	Calculated	Found
Adc [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK (I)	11.95	98.23	1491.83	1492.1
Aba [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK (II)	10.87	97.35	1413.36	1413.3
Sua [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK (III)	10.92	98.87	1394.58	1394.8

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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 was provided for stabilization of blood pressure. The rats displaying MAP equal to or lower than 85 mmHg were excluded from further procedures. BK acetate salt; (Sigma, USA): 250 ng per animal; dissolved in 5% D-glucose solution at a concentration of 2.5 μg/ml; was injected three to five times, every 4 to 5 min, into one branch of the carotid catheter, until the evoked vasodepressor responses were stable. Prior to the administration of BK, MAP averaged 121.42 ± 1.45 mmHg (ranging from 110 to 137 mmHg; $n = 31$, mean ± SE) and HR was 297.5 ± 3.3 beats/min (ranging from 266 to 352 beats/min). BK evoked a significant vasodepressive response (30.84 ± 0.94 mmHg). To provide homogeneity of the assay, only the rats which exhibited a vasodepressive response to 250 ng BK (Δ MAP) within the range of 20 to 50 mmHg were exclusively enrolled and used in this study.

The BK analogues dissolved in 5% D-glucose were infused to a branch of the carotid catheter the other than that for BK. A constant rate of infusion of 125 μl/ min was maintained using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, Netherlands). The BK analogue infusion was initiated with the 8 min infusion in a concentration of 0.5 μg/ml (this gave a dose of 62.5 ng/min). During this infusion, a single dose of BK was injected into the carotid artery two or three times (150 s, 300 s, or if necessary 480 s of BK analogue) until the vasodepressor responses were stable. The concentration of infused bradykinin antagonist was then increased (1, 8, 16, 32, 64 or 128, and, if necessary, up to 512 μg/ml) to give doses of 0.125, 1, 2 or 4, 8 or 16 and 64 μg/ min /rat, respectively. As soon as the vasodepressor response to BK fell below 10% of the standard response, the procedure was completed.

Inhibition of the vasodepressor response to 250 ng BK by the tested BK antagonist was plotted against the logarithm of its dose. This dose-effect plot was used to quantify the antagonistic potencies of the tested compounds. As indices of these potencies, effective doses: ED_{20}, ED_{50} and ED_{90} were used representing respective doses of the bradykinin antagonist (μg/ min) that inhibit the vasodepressor response to its agonist (250 ng of BK) by 20%, 50% and 90%. The results are reported as mean values ±SE.

The in vitro rat uterotonic test. All analogues were evaluated in the *in vitro* rat uterotonic assay using a modified Holton method [16] in Munsick solution [17] on a strip of rat uterus. Wistar rats with oestrus induced by injection of oestrogen 48 h before the experiments were used. As a standard, bradykinin was used over the concentration range 10 pM to 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 maximal contraction has been obtained. The height of the single isometric contraction in response to the different doses of bradykinin was measured. The antagonist was applied to the organ bath 1 min prior to the cumulative dosing of bradykinin. The antagonistic activity was expressed as pA_2, i.e. the negative decadic logarithm of the concentration of the analogue, which shifted the dose response curve of bradykinin by a factor of two. Each analogue was tested on uteri taken from 4–5 rats.

RESULTS AND DISCUSSION

The three new BK analogues (I–III) were synthesized by stepwise coupling of Fmoc amino acids to the growing peptide chain starting from Fmoc-Arg(Pbf)-Wang resin. Cleavage of the analogues from the resin with side-chain deprotection was performed by treatment with TFA–H_2O–TIS. The crude products were purified on Sephadex G-15 and by RP-HPLC.

The antagonistic potencies of analogues I–III, compared with that of the antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] BK, synthesized by Stewart's group [14] and which may be considered as their parent compound, are summarized in Table 2. The potencies of the analogues were assessed by their ability to inhibit the vasodepressor response of exogenous BK in conscious rats and by their ability to inhibit the contractions of isolated rat uterus evoked by BK. In the first assay, all the compounds exhibited moderate antagonistic potency. The analogues were approximately equipotent to Stewart's peptide when inhibiting the effect of BK by 20%. However, analogues I and II inhibited 90% of the effect of BK at lower concentrations (ED_{90}) thus exhibiting twice higher activity, while III was equipotent to the parent peptide. In the rat uterus test, peptide I showed higher potency than the parent peptide, analogue II was equipotent, whereas analogue III was practically inactive.

It should be pointed out that all the analogues in both tests did not show any agonistic activity.

The present study continued efforts to explore the structural requirements which, in the case of BK analogues, result in high B_2 antagonistic activity. As mentioned in the introduction, previously it was reported that the presence of a bulky acyl substituent (1-adamantaneacetic acid, 4-tert-butylbenzoic acid, 1-adamantanecarboxylic acid, etc) at the *N*-terminus of B_2 antagonists significantly influenced the interaction of the peptide with B_2 receptors thus increasing potency [18]. The results also suggested that even minor changes in the structure of analogues may be of importance in designing more potent B_2 antagonists. On the other hand, the effects of acylations might vary substantially with the chemical character of the acyl group [19,20]. It seemed that either the positive or negative charge on the *N*-terminal acyl group influenced the activity of analogues, as suppressed antagonistic potency was observed due to these modifications. However, all acyl substituents studied so far were aromatic. In the present work three new groups were placed in the *N*-terminus of the standard antagonist (Stewart's peptide or parent peptide): 12- aminododecanoic acid, 4-aminobenzoic acid and succinic acid. Two of these, Ada and Aba, are basic, differing in their base strength. The last-named acyl group, Sua, is acidic. As seen in Table 2, it is clear that the modifications either preserved or increased

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Table 2 Pharmacological Properties of BK Analogues

Peptide	Antiuterotonic potency (pA_2)	Vasodepressor potency: Infusion concentration giving 20%, 50% or 90% decrease of the response to standard dose of BK		
		ED_{20} (μg/ min)	ED_{50} (μg/ min)	ED_{90} (μg/ min)
[D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7] BK	6.88 ± 0.08^a	1.73 ± 0.43^b	—	124.17 ± 27.04^b
Adc [D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7] BK (I)	7.55 ± 0.15	1.94 ± 0.25	7.65 ± 0.94	50.69 ± 9.46
Aba [D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7] BK (II)	7.04 ± 0.21	1.10 ± 0.14	5.99 ± 0.47	64.52 ± 13.28
Sua [D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7] BK (III)	<6.0	1.17 ± 0.20	8.30 ± 0.56	112.89 ± 11.16

[a] Value taken from ref. [20].
[b] Value taken from ref. [19].

the antagonistic potency in the rat blood pressure test, which did not confirm our previous findings [20]. Moreover the range of antagonism did not seem to depend on the magnitude of basicity.

On the other hand, the three substituents differently influenced the interaction with the rat uterine receptors. In this case higher basicity resulted in a more active B_2 blocker (approximately seven times higher activity compared with Stewart's peptide in the case of analogue I). Moreover, very interesting is the lack of antagonistic properties of analogue III, which has the acidic group on its *N*-terminus. However, it should be noticed that the size of this group is relatively small in comparison with the Aba or Ada groups. This is a very promising observation as it may be of importance for designing selective B_2 antagonists, as this modification did not suppress the antagonistic potency of the peptide in the rat blood pressure assay. It is obvious that further studies are needed to confirm these extremely interesting findings.

In summary, this study provides new information on the structure–activity relationship of bradykinin analogues and may have an impact on the design of selectively acting antagonists of bradykinin.

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