ACYLATION OF THE N-TERMINUS OF B_2 BRADYKININ ANTAGONISTS WITH BULKY SUBSTITUENTS DRAMATICALLY INCREASES POTENCY

Bernard LAMMEK[a], Yasushi ITO[b], Irene GAVRAS[b] and Haralambos GAVRAS[b]

[a]*Department of Chemistry, University of Gdansk, Sobieskiego 18, 80-952 Gdansk, Poland*
[b]*Hypertension Section and Department of Medicine, Thorndike Memorial Laboratory, Boston City Hospital and the Department of Medicine, University Hospital, Boston University School of Medicine, U.S.A.*

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While studying structural factors which can influence B_2 antagonistic activity we designed and synthesized five new bradykinin (BK) antagonists. Two of them, namely Aaa[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK and Aca[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK are among the most potent B_2-antagonists of exogeneous BK described to date. The remaining three analogs are also potent B_2-antagonists. None of the new compounds contain Thi at position 5 and 8, a change that markedly reduces their cost. The new antagonists have obvious potential as valuable pharmacological tools in the investigation of the role of endogenous BK in cardiovascular regulation.

The possible role of bradykinin in cardiovascular regulation has intrigued scientists for many years. A member of the kinin system discovered in the late 1920's, bradykinin (BK) is a nonapeptide whose existence was first recognized by Rocha a Silva[1] from its effects on intestinal smooth muscle. Since then, several other actions of BK were described, including vascular concentration and relaxation, participation in the process of inflammatory reactions, interaction with central and peripheral neural structures, stimulation of synthesis and release of various vasoactive substances, etc.

All of the clinically relevant effects of bradykinin appear to be mediated via the classical B_2 receptors and, wherever tested, were found to be reversible by B_2 receptor antagonists. B_2-antagonists are very important pharmacological tools in the research of mechanisms of hypertension, myocardial ischemia, postmyocardial infarction (MI), arrhythmia and congestive heart failure. They may also have a therapeutic potential in treating pain from burns, anginal pain and myocardial reperfusion arrhythmias, bronchial asthma and allergic rhinitis, toxic shock and circulatory collapse, orthostatic hypotension and possibly other B_2 mediated effects of bradykinin that are still ill-defined, such as pain, bowel hypermobility and diarrhea of chronic inflammatory intestinal disease, painful arthritis, neuralgia, etc.

The key modification that confers B_2-antagonistic properties to an analog is replacement of the proline residue at position 7 of bradykinin with D-phenylalanine residue[2].

Other substitutions such as replacement of proline by hydroxyproline (Hyp) in positions 2 or 3; substitutions of the phenylalanine residues at positions 5 and 8 with β-2-thienyl-L-alanine (Thi); or addition of one or two amino acid residues to the N-terminus, can potentiate the antagonistic properties of analogs[2]. Various bradykinin derivatives appear to exhibit different tissue specificity. One of the commonly used in the literature and most potent antagonists of bradykinin (designated henceforth as peptide *I*) has the sequence: D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg[2,3]. Using it as the parent peptide we reported that acylation of its N-terminus with 1-adamantaneacetic acid results in an analog (designated here as peptide *II*) with more than ten times enhanced potency[4].

Subsequently we designed more analogs in order to investigate the role of various acyl groups at the N-terminus of peptide *I*. We found that introduction of additional D-Arg or acylation with propionic acid of peptide *I* results in analogs (designated here as peptides *III* and *IV*) with similar or weaker antagonistic potency as compared to peptide *I*, respectively[5]. As a conclusion from these studies it appeared that the presence of a bulky acyl substituent at the N-terminus of B_2-antagonists significantly influences the interaction of the peptide with the B_2 receptor and therefore increases the potency. Strong support for such hypothesis came recently from our finding that acylation of peptide *I* with 1-adamantanecarboxylic acid results in an analog (designated here as peptide *V*) with more than 33 times enhanced potency over the parent peptide[6]. It should be noted that acetylation of B_2-antagonists was found by Regoli et al.[7] to eliminate in some cases the agonist effect of these compounds. However their results clearly indicate that such modification not only does not increase but even diminishes antagonistic potencies of analogs. Their findings are in good agreement with those of Stewart's group, who found that acetylated antagonists have potencies not much different from the same antagonists lacking an acetyl group[2].

As a continuation of our effort to develop more potent B_2-antagonists we wished to explore how acylation with 1-adamantaneacetic acid and 1-adamantanecarboxylic acid of other B_2-antagonists, some of them recently developed[8], will influence their pharmacological properties. Primary structure of peptides *I* – *V* and five new analogs are shown in Table I.

During the preparation of this manuscript we learned that the substitutions of positions 7 and 8 with D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and (*S*,*S*,*S*)-2-azabicyclo[3,3,0]octane-3-carboxylic acid or L-octahydroindole-2-carboxylic acid, respectively, combined with changes which potentiate antagonistic potency, resulted in very potent B_2-antagonists of bradykinin[9 - 11]. However, due to a different bioassay than ours applied by the authors to determine the antagonistic potency of the analogs, comparison with our compounds is not possible.

EXPERIMENTAL

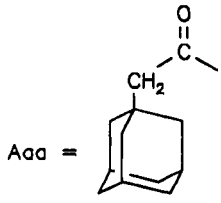
Peptide Synthesis

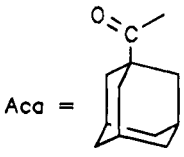
All peptides were synthesized by the solid phase peptide synthesis method by stepwise coupling of BOC-amino acids to the growing peptide chain on a Merrifield resin[12]. 1-Adamantaneacetic acid (Aldrich) and 1-adamantanecarboxylic acid (Aldrich) were used in the final coupling steps. The completion of all coupling reactions was monitored by the Kaiser test[13]. After synthesis was complete, 1 g of the protected

TABLE I
Structures of bradykinin analogs *I* – *X*

Compound[a]	Structure
I	D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-OH [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK
II	Aaa-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-OH Aaa[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK
III	D-Arg-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-OH
IV	Prop-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-OH
V	Aca-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-OH Aca[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK
VI	Aaa-D-Arg-Arg-Pro-Hyp-Gly-Phe-Ser-D-Phe-Leu-Arg-OH Aaa[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK
VII	Aca-D-Arg-Arg-Pro-Hyp-Gly-Phe-Ser-D-Phe-Leu-Arg-OH Aca[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK
VIII	Aaa-D-Arg-Arg-Pro-Hyp-Gly-Leu-Gly-D-Phe-Leu-Arg-OH Aaa[D-Arg^0, Hyp^3, Leu^{5,8}, Gly^6, D-Phe^7]BK
IX	Aca-D-Arg-Arg-Pro-Hyp-Gly-Leu-Gly-D-Phe-Leu-Arg-OH Aca[D-Arg^0, Hyp^3, Leu^{5,8}, Gly^6, D-Phe^7]BK
X	Aaa-D-Arg-Arg-Pro-Pro-Gly-Phe-Ser-D-Phe-Phe-Arg-OH Aaa[D-Arg^0, D-Phe^7]BK

[a] BK bradykinin,





Prop = CH_3-CH_2-CO- .

acylpeptidylresin 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 removal of the HF and anisole in vacuo, the mixture was washed with anhydrous diethyl ether (3 times, 30 ml portions) and then five times with 40 ml portions of 15% acetic acid. The acetic acid extracts were combined and lyophilized to yield crude analog. This material was desalted by gel filtration on Sephadex G-15 (50% acetic acid) and purified twice on Sephadex LH-20 (30% acetic acid). The purity and identity of the peptides were ascertained by thin-layer chromatography in two different solvent systems (1-butanol–acetic acid–water, 4 : 1 : 5, upper phase, and ethyl acetate–pyridine–acetic acid–water, 5 : 5 : 1 : 3), HPLC (reverse phase column Aquapore RP-300, 7 μm, 250 × 4.5 mm, linear gradient of 10 – 60% acetonitrile in 0.1% trifluoroacetic acid, flow rate 2 ml/min) and by amino acid analysis.

Bioassay Method

The bioassay for assessment of antagonistic potency of peptides in vivo has been published in detail elsewhere[4,6]. In short, chronically catheterized Wistar rats receive bolus bradykinin injections of 250 mg (Sigma, St. Louis, MO, U.S.A.) before, during and after infusion of various doses of the antagonist, under concurrent ACE inhibition with enalapril *IV*. The reduction of the blood pressure lowering effect of BK by the antagonists is assessed at each point.

The antagonistic potency of the bradykinin analogs is quantitatively expressed as the effective dose (ED), ED_{20} and ED_{90}. The ED is defined as the dose (in μg/min) of antagonist that reduces the response to two units of agonist (250 ng of bradykinin) to equal the response obtained previously by one unit of agonist (125 ng of bradykinin) in the absence of antagonist.

ED_{20} and ED_{90} represent the doses of bradykinin antagonist (μg/min) that inhibit by 20% and 90%, respectively, the vasodepressor response to its agonist (250 ng of bradykinin). These indices were estimated by linear regression analysis using the linear part of each dose-response curve on a logarithmic dose scale.

Results are reported as mean values ± standard error of the mean (SE). Comparison of mean values was accomplished by one way analysis of variance. Differences at the 5% level ($p < 0.05$) were considered to be statistically significant.

RESULTS

The antagonistic potencies of the new bradykinin analogs *VI* – *X* in comparison to those of the parent peptide *I* and the previously synthesized by us BK antagonists (BKAs) *II* – *V* are presented in Table II. Peptide *I* until recently, was considered to be one of the most potent BKAs tested both in vivo and in vitro[3,14]. We synthesized and assayed it to obtain reference data for comparison with our analogs.

All new analogs *VI* – *X* are antagonists of the vasodepressor response to exogenous BK. In low doses (ED_{20}) analogs *VI* and *VII* are 15 times more potent than peptide *I*. At higher doses (ED_{90}), which most investigators choose during experiments, they are 33 times more potent than peptide *I*. When analogs *VIII* and *IX* are compared to peptide *I*, their potency at low doses is 5 times higher and at higher doses more than 10 times higher than that of peptide *I*. Compounds *VI* and *VII* are equipotent to peptide *V*, which is considered as one of the most potent B_2-antagonists of exogenous BK described to date. The ED_{20} of analog *X* is about 2.5 times higher than that of peptide *I*. We did not

determine ED_{90} for this analog since doses higher than 100 μg/min caused a significant drop in blood pressure.

DISCUSSION

The new analogs were designed to find out how acylation with the bulky substituents 1-adamantaneacetic acid (Aaa) or 1-adamantanecarboxylic acid (Aca), which were previously shown to confer certain advantages to BK analogs[4,6] may change the pharmacological properties of some other B_2-antagonists. The peptides *VI* – *IX* were designed following the report by Steranka et al.[5] and by Rhaleb et al.[8] that thienylalanine substitution at positions 5,8 may not be as essential as it was previously considered to be for the enhancement of B_2 antagonistic properties of analogs. In an attempt to obtain antagonists by the elimination of aromatic residues (Phe) they used Leu in position 5 or 8 or both, to replace Phe. We decided to choose two peptides with the above-mentioned modifications, which were some of the most active in the described series, to check how acylation with Aaa or Aca will influence the B_2 antagonistic potency of the resulting acylated compounds. Specifically, we modified by acylation the structure of [D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK (*XI*) and [D-Arg^0, Hyp^3, Gly^6, D-Phe^{7}Leu^{5,8}]BK (*XII*).

TABLE II
Pharmacological data on bradykinin analogs *I* – *X*

No.	Compound[a]	*N*[b]	Antagonistic potency[c]	
			ED_{20}	ED_{90}
I	[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK[d]	7	1.49 ± 0.29	142.5 ± 26.44
II	Aaa[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK	7	0.84 ± 0.09	13.94 ± 1.69
III	D-Arg[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK	5	10.07 ± 1.68	314.31 ± 109.49
IV	Prop[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK	5	3.59 ± 1.44	209.10 ± 36.35
V	Aca[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK	9	0.094 ± 0.02	4.23 ± 0.46
VI	Aaa[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK	5	0.11 ± 0.02	4.38 ± 0.43
VII	Aca[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]BK	5	0.09 ± 0.02	4.28 ± 0.44
VIII	Aaa[D-Arg^0, Hyp^3, Gly^6, Leu^{5,8}, D-Phe^7]BK	5	0.27 ± 0.06	12.19 ± 1.63
IX	Aca[D-Arg^0, Hyp^3, Gly^6, Leu^{5,8}, D-Phe^7]BK	5	0.29 ± 0.06	13.56 ± 1.41
X	Aaa[D-Arg^0, D-Phe^7]BK	5	3.9 ± 0.8	*e*

[a] For structure of analogs see Table I. [b] Number of rats tested. [c] ED_{20} and ED_{90} represent the doses of bradykinin antagonist (μg/min) that inhibit by 20% and 90% the vasodepressor response to its agonist (250 ng of BK). [d] This analog was previously designed by Stewart[2,3]. We assayed it to obtain reference data for comparison with our analogs. [e] We did not determine ED_{90} for the peptide *X*. The 100 μg/min dose inhibits by 77.1% ± 4.4 the vasodepressor response to its agonist (250 ng of BK). Higher doses of analog *X* significantly decreased the blood pressure of the animals.

These peptides have been described[8] to have essentially similar potency to peptide *I*. Their acetyl derivatives were shown to have reduced residual B_2-agonistic activity with unchanged or slightly decreased antagonistic properties as compared to the parent peptides. The data from our studies demonstrate that the acylation with Aaa or Aca of peptides *XI* and *XII* results in compounds with greatly improved B_2 antagonistic properties. This substantial enhancement of antagonistic potency appears to be due as in previous cases (peptides *II* and *V*) to the presence of a bulky substituent at the N-terminus of the molecule. Our results also confirm that Phe^5 and Phe^8 need not be replaced with thienylalanine in order to achieve the high antagonistic potency of analogs. This suggestion was previously made for antagonism in vitro[8], but on the basis of our present results it appears to be valid for antagonism in vivo as well.

From our previous studies it appeared that acylation of peptide *I* with Aca is advantageous in terms of potency when compared to the Aaa modification[4,6]. However, comparison of the B_2-antagonistic properties of analogs *VI* – *IX* clearly shows that the above-mentioned modification did not lead to different potencies of these particular compounds.

With respect to the properties of peptide *X*, the presence of Aaa residue at the N-terminus of the analog results in a peptide with B_2-antagonistic potency lower than that of peptide *I*. However, it is worth pointing out that the parent peptide for analog *X*, i.e. [D-Arg^0,D-Phe^7]BK shows either weak antagonistic properties on guinea-pig ileum (GPI) and blood pressure assays or insignificant agonistic activity on rat uterus (RUT)[2]. Nevertheless, the relatively low potency of analog *X* demonstrates that acylation of the N-terminus of an analog must be combined with more than D-Arg^0 and D-Phe^7 substitutions in order to achieve high B_2-antagonistic potency.

In summary, we have reported the synthesis and some pharmacological properties of five new B_2-antagonists of bradykinin. Peptides *VI* and *VII* are equipotent to one of the most active B_2-antagonists described to date. Analogs *VIII* and *IX* with ED_{90} values close to 10 μg/min are also among the potent BKAs. Analogs *VI* – *IX* could thus serve as valuable pharmacological tools in the investigation of the role of endogenous BK in the regulation of systemic and regional hemodynamics, the development of various types of hypertension or cardiac disease, and in the antihypertensive effects of angiotensin-converting enzyme inhibition. From our present and previous[4,6] efforts to modify B_2-antagonists, it appears that acylation with 1-adamantaneacetic acid or 1-adamantanecarboxylic acid has consistently improved antagonistic activity. Thus, we believe that this type of modification may be valuable in the design of more potent B_2-antagonists.

Linear analogs of AVP substituted in position 1 with Aaa have been shown in Manning's laboratory to exhibit high V_2/V_1 antagonistic activity. Consequently it may be interesting to check how this type of modification might change the pharmacological properties of B_1-antagonists of bradykinin as well as antagonists of other hormones such as somatostatin, substance P, etc.

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