stitutes of Health (HE-14509, HE-14397, and RR-00396). (b) Graduate Fellow, Medical Scientist Training Program (GM 02016). (c) Research Career Development Award (HL-19586). (d) Established Investigator, American Heart Association (AHA-70-111).
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Notes

Analogs of Bradykinin with Restricted Conformational Freedom[1a]

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Three analogs of bradykinin have been synthesized which bear an α-methyl group in the place of an α proton at position 4, 5, or 8. Such analogs possess restricted conformational freedom and are of interest for three reasons. (1) They may provide information about the receptor-bound conformation of the peptide. (2) They may provide a route to antagonists of the native peptide. (3) They may be degraded slowly by proteolytic enzymes. None of the analogs described here antagonized the action of bradykinin, but one exhibited tissue specificity and decreased pulmonary inactivation in the rat.

Peptides which bear methyl groups in the place of protons at various positions on the backbone of the peptide chain may be of unusual interest for a number of reasons. Theoretical studies of the allowed dihedral angles of model peptides have shown that substitution of a methyl group for the proton on the α carbon or on the amide nitrogen of an amino acid residue results in a dramatic reduction of the conformational space available to the backbone of the peptide chain at the position where that residue occurs.[2-5] Peptide hormone analogs containing α-methyl or N-methylamino acids should therefore have a sterically rigid backbone conformation at those positions. The term "conformational analogs" has been suggested for such peptides,[6,7] since they would have a primary structure essentially identical with that of the native hormone but could assume only a more restricted set of conformations.

Should such an analog be biologically active, important constraints on the receptor-bound conformation of the parent molecule might be provided, since high biological activity would imply that the conformational restrictions imposed by the methyl substitution were compatible with the conformational requirements for binding to the receptor. Analogs whose conformations are restricted by the presence of a backbone methyl group also provide a route to a novel class of inhibitors. Schemes of analog generation that have been applied to bradykinin and other peptides nor-

mally involve varying the character or positions of amino acid side chains in the sequence. Cases could be imagined in which the receptor required all of the native peptide's side chains for binding but would bind an isofunctional analog bearing a backbone methyl group in an anomalous, nonproductive manner. Such binding could be nonproductive because due to the conformational inflexibility of the analog it might be unable to participate in any conformational transitions of the peptide–receptor complex which normally occur subsequent to binding and which are critical to the elicitation of the physiological response. There is a precedent for the production of inhibition by a peptide analog whose structure differs from that of the native peptide by only a single backbone methyl group, namely [*N*-methylphenylalanine[8]]-angiotensin II.[8]

Analogs bearing backbone methyl groups might also be expected to be metabolized slowly. The resistance to enzymatic hydrolysis of peptides containing *N*-methylamino acids (e.g., proline) is well known, but model compounds containing α-methylamino acids are also known to be resistant to chemical hydrolysis[9,10] and to enzymatic attack by both endopeptidases[11] and exopeptidases.[12,13] The synthesis of analogs containing α-methylamino acids might therefore result in the generation of long-acting agonists or antagonists.

This paper reports the application of this approach to the peptide bradykinin (BK). Although bradykinin is known to be associated with a wide variety of physiological and pathological phenomena in animal models and in man, including some prevalent and important human diseases,[14-16] its role in any of them is far from clear. This is true in part because there exists no receptor-specific bradykinin antagonist which could be used to probe the function of the kallikrein–kinin system. In addition, since the existence of bradykinin in circulating blood is so transient,[17] efforts to increase its duration of action could be of interest should the actions of bradykinin itself prove desirable in a pharmacologic agent. Finally, although some preliminary studies of the solution conformation of bradykinin have been undertaken,[18,19] very little is known about its receptor-bound conformation, and such knowledge could prove useful in the rational design of agents meant to mimic or antagonize the activity of the peptide.

Experimental Section

Materials. Bradykinin (BK) used in these studies was synthesized manually by the solid-phase protocol of Hancock et al.[20] All *tert*-butyloxycarbonylamino acids were obtained from Bachem except *tert*-butyloxycarbonyl-L-α-methylphenylalanine (Boc-α-MP), which was prepared as previously described.[6] The Boc-NO_2-L-Arg-substituted resin was synthesized by the method of Marshall and Merrifield.[21]

Analytical Methods. Thin-layer chromatography (TLC) was performed on 0.25-mm silica gel G plates (Analtech) in two systems: 1-butanol–pyridine–acetic acid–H_2O (15:10:3:12) and 2-butanol–3% NH_4OH (100:44). Mobilities in these systems will be designated R_{f1} and R_{f2}, respectively. The load of peptide was 50 μg/plate. Thin-layer plates were developed by ninhydrin and Cloroxstarch sprays.[22] Paper electrophoreses were performed in a Gilson Model D high-voltage electrophorator for 2 kV hr at pH 3.5. The electrophoretic mobility relative to arginine will be designated E_A. Peptides were hydrolyzed by the method of Westall et al.[23] for 4 hr. Amino acid analyses were performed on a Spinco 120C analyzer. To quantitate the amount of α-methylphenylalanine (α-MP) it was first necessary to remove phenylalanine from the peptide hydrolysate by the action of L-amino acid oxidase. This procedure is described elsewhere.[7]

***tert*-Butyloxycarbonylnitro-L-arginyl-L-prolyl-L-prolylglycyl-L-α-methylphenylalanyl-*O*-benzyl-L-seryl-L-prolyl-L-phenylalanylnitro-L-arginyl-resin (I).** *tert*-Butyloxycarbonylnitro-L-arginyl-resin (2 g, 0.27 mequiv/g) was placed in the reaction vessel, deprotected, neutralized, and reacted with the appropriate amino acids in the customary cycle of operations.[21] The materials used for shrinking and swelling the resin, deprotection, neutralization, and coupling were the same as those described elsewhere.[7] The only deviation from the other protocol is that Boc-α-MP was coupled to the peptidyl residue twice for 4 hr using sixfold excesses of *tert*-butyloxycarbonylamino acid, and two deprotection steps were performed before adding the next amino acid, which was also used in sixfold excess and coupled twice for 4 hr.

L-Arginyl-L-prolyl-L-prolylglycyl-L-α-methylphenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine ([α-MP[5]]-BK). Compound I was dried and placed in a polypropylene reaction vessel of an HF apparatus. Anisole (3 ml) and HF (25 ml) were added, and the suspension was stirred at 0° for 50 min. The HF was removed by a stream of nitrogen and then in vacuo. The peptide and resin were then washed with ethyl acetate and extracted with 1% acetic acid. The extract was lyophilized to give 490 mg (81%) of crude peptide which was subjected to 200 transfers in a 1-butanol–2% trifluoroacetic acid (1:1) countercurrent distribution (CCD) system. The peptide was located by the method of Lowry et al.[24] and was found to have distributed itself symmetrically in tubes 144–172 (k = 3.74). Thin-layer chromatography was performed on the contents of those tubes and the contents of tubes 148–164 were combined, concentrated under reduced pressure, diluted with H_2O, and lyophilized to give 220 mg (37%) of peptide. Amino acid analysis showed Ser 0.88, Pro 3.00, Gly 1.03, Phe 0.98, α-MP 1.10, Arg 1.84. The peptide content was 73%. One spot was seen on TLC and electrophoresis: R_{f1} 0.63, R_{f2} 0.15, E_A = 0.69. The optical activity of the peptide was $[\alpha]^{25}D$ −105.3° (*c* 0.55, methanol).

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-α-methylphenylalanyl-L-arginine ([α-MP[8]]-BK). The appropriate protected peptidyl resin was prepared in the same manner as compound I and subjected to the same cleavage–deprotection procedure, yielding 640 mg of peptide (95%). The crude material was purified as above, and 410 mg (53%) was so isolated (tubes 148–168, k = 3.74). Amino acid analysis showed Ser 0.98, Pro 2.95, Gly 1.00, Phe 1.02, α-MP 1.00, Arg 1.97. The peptide content was 75%. The material was homogenous on TLC and electrophoresis: R_{f1} 0.63, R_{f2} 0.12, E_A = 0.68. The optical activity of the peptide was $[\alpha]^{25}D$ −75.0° (*c* 0.38, methanol).

L-Arginyl-L-prolyl-L-prolyl-D-alanyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine ([D-Ala[4]]-BK). This peptide was synthesized and isolated by the same series of operations used above. The crude yield was 450 mg (82%), and 210 mg (38%) was recovered from the CCD (tubes 128–148, k = 2.24). Amino acid analysis showed Ser 0.94, Pro 3.08, Ala 1.04, Phe 2.00, Arg 2.04. The peptide content was 74%. The material was homogeneous on TLC and electrophoresis: R_{f1} 0.66, R_{f2} 0.13, E_A = 0.69. The optical activity of the peptide was $[\alpha]^{25}D$ −79.6° (*c* 0.37, methanol).

Bioassays. The oxytocic activity of the analogs was measured in rat uterus as previously described.[25] Blood pressures were measured in urethane-anesthetized rats (1.25 g/kg) by recording from the left femoral artery (P-1000A pressure transducer, physiograph). The agent was injected into the cannulated right jugular vein or left carotid artery by means of a Hamilton syringe automatic dispensing apparatus. Intraarterial (ia) and intravenous (iv) dose–response curves for BK were determined before and after the administration of each analog so that each animal served as its own control. Each analog was assayed for agonistic (ia and iv) and competitive antagonistic (ia) activity. The amount of displacement on the dosage axis of the iv dose–response curve from the ia dose–response curve was taken to reflect pulmonary degradation.

Results and Discussion

The structures of the newly synthesized analogs are presented in Figure 1. Note that the primary structure of each analog differs from that of native BK by only a single methyl group attached at some position on the backbone of the peptide chain. The agonistic activity of the analogs is summarized in Table I, the dose–response plots of the data from which the numbers in Table I are derived are presented in Figure 2. None of the analogs exhibited any antagonistic properties either in vivo or in vitro (data not shown).

[α-MP[5]]-BK is a weak agonist (1–4%) both in vivo and in vitro. Evidently the conformation imposed by the α-methyl group at position 5 is not a preferred one for maximal binding efficiency but is compatible with productive binding. The exact nature of the constraints imposed by an α-meth-

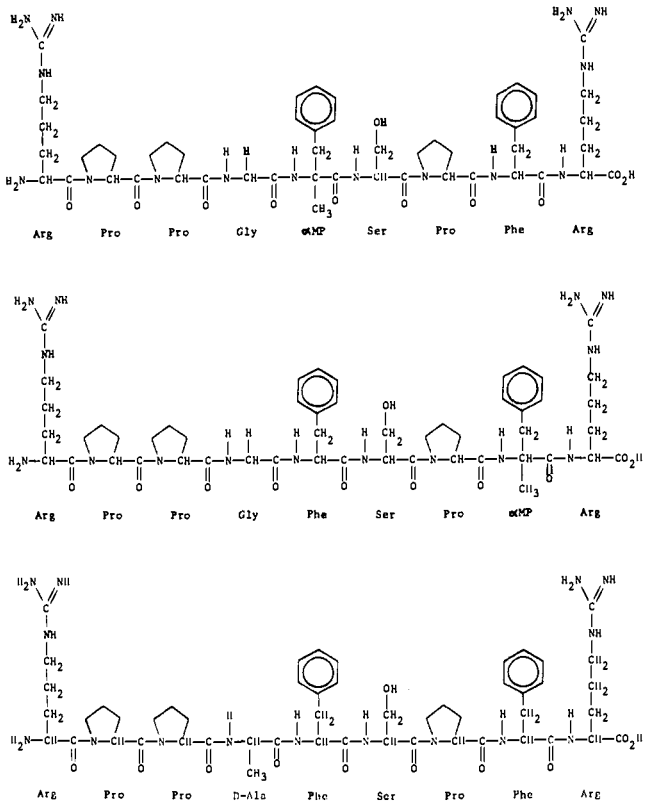

Figure 1. From top to bottom are shown the covalent structures of [α-MP^5]-BK, [α-MP^8]-BK, and [D-Ala^4]-BK.

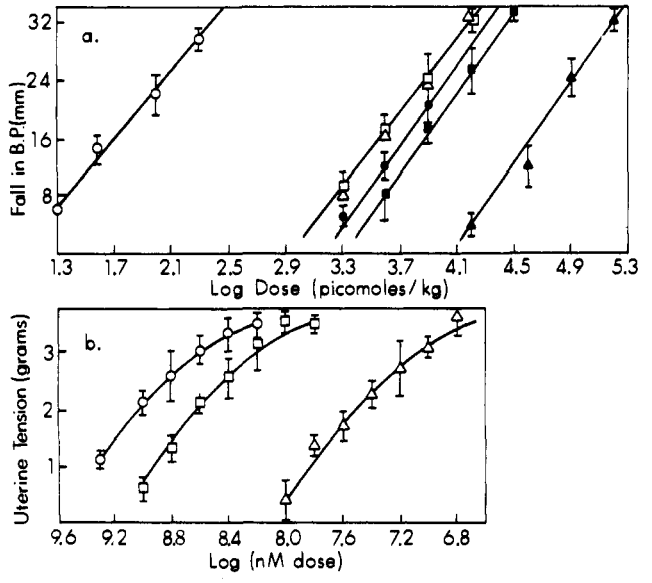

Figure 2. Dose–response plots for BK (○), [α-MP^5]-BK (△), and [α-MP^8]-BK (□) in (a) rat blood pressure assay (solid symbols for iv injection, open symbols for ia injection) and (b) rat uterus assay. Bars indicate SE (n = 4).

yl group has been fully described elsewhere,[2-5] but they can be summarized by saying that the most probable values for the torsional angles ϕ and ψ which describe the peptide's backbone conformation[26] fall primarily into two small areas centered around ($\phi = -57$, $\varphi = -47$) and ($\phi = +57$, $\psi = +47$). It is not possible to conclude that BK cannot adopt these torsional angles at position 5 in the receptor-bound conformation, however, since the low agonistic activity of [α-MP^5]-BK could also result from unfavorable steric interactions of the α-methyl group with the BK receptor. The slight decrease in the pulmonary inactivation of [α-MP^5]-BK is probably not significant. Since the analog is such a weak agonist, the intravenous (iv) levels of it required to see a depressor response probably approached saturation levels of the pulmonary kininases.

Table I. Bradykinin Analogs[a]

Analog	% activity, rat uterus	% activity, rat depressor	% pulmonary degradation
BK	100	100	99 ± 1
[α-MP^5]-BK	3.5 ± 1.6	1 ± 0.6	93 ± 3
[α-MP^8]-BK	31 ± 6	1 ± 0.4	42 ± 9
[D-Ala^4]-BK	<0.01	<0.1	–

[a] Agonistic activity is expressed as the (ratio of the dose of BK required to produce a half-maximal response to that of the analog required to produce the same response) × 100. Pulmonary inactivation is expressed as [1 − (ia ED_{30}'/iv ED_{30}')] × 100, where ia ED_{30}' and iv ED_{30}' are the intraarterial and intravenous doses of the analog required to produce a 30-mm fall in mean blood pressure. Figures shown are the mean of 16 separate determinations in four different animals. Standard deviations are indicated. Dashes indicate that the parameter in question was not determined.

[α-MP^8]-BK is a much more potent agonist in rat uterus (31%) than in the rat blood pressure assay (1%). Such a discrepancy is not unprecedented since [Lys^1]-BK is 50 times more active in the rabbit blood pressure assay than it is in the rat uterus, and [Ala^7]-BK is 200 times more active in the rat uterus than in the rabbit blood pressure assay.[27] The fact that the oxytocic activity of [α-MP^8]-BK is greater than its vasodepressor activity in the rat provides evidence that there is some intraspecies tissue specificity of BK receptors. Since the oxytocic activity of [α-MP^8]-BK is fairly high, in the receptor-bound conformation of BK in rat uterus the values for the torsional angles at position 8 probably lie close to those imposed by the 8 α-methyl group.

The fraction of [α-MP^8]-BK escaping inactivation in one passage through the lungs is increased by approximately a factor of 50 relative to BK (50 vs. 1%). This cannot be accounted for by simple saturation of the pulmonary kininases, since [α-MP^8]-BK is no less potent than [α-MP^5]-BK in the blood pressure assay. Instead, it seems likely that the 8 α-methyl group interferes with the cleavage of the 7–8 bond, which is known to be a site of attack by the pulmonary kininases.[17,28] The duration of the vasodepressor response to [α-MP^8]-BK is no longer than that to BK, however (data not shown). This suggests that either the plasma kininases[29] or the kininases in the peripheral vascular beds[30] is not affected by the presence of an α-methyl group in position 8. This may be due to the fact that kininases in the peripheral vascular beds attack other sites in the sequence.

The findings with the 4-position analogs are of some interest. Monahan et al.[31] have shown that in the case of luteinizing hormone-releasing factor (LRF), whose sequence contains a Gly in position 6, [L-Ala^6]-LRF is only 4% as active as native LRF, while [D-Ala^6]-LRF is 400% as active. On the basis of these data it was proposed that the presence of the 6 α-methyl group excluded some conformations that could normally be adopted by native LRF. In the case of the L-Ala^6 analog, a conformation preferred for binding was excluded, resulting in a loss of activity. In the case of the D-Ala^6 analog, the molecule was constrained to spend more of its time in a conformation preferred for binding. Since some of the nonproductive conformations had been excluded, an increase in activity resulted.

By analogy, since the BK sequence contains a Gly in position 4, and since [L-Ala[4]]-BK is essentially inactive,[32,33] it was thought that [D-Ala[4]]-BK might have a greater affinity for the BK receptor than BK itself and exhibit increased potency or perhaps antagonistic properties. Instead, the analog has no measurable agonistic or antagonistic activity either in vivo or in vitro. There are few regions of conformational space available to Gly that are available to neither D-Ala or L-Ala, so either [L-Ala[4]]-BK or [D-Ala[4]]-BK should be capable of assuming the correct conformation for binding at position 4. Perhaps the 4 α-methyl group interferes sterically with the peptide's approach to the receptor even if the peptide is in a conformation preferred for binding. Pointing the methyl group in another direction (as in switching L-Ala for D-Ala or vice versa) could disrupt the preferred conformation by causing unfavorable intramolecular steric interactions. In any case, the requirement for Gly in position 4 seems quite strong. Although arguments based on inactive analogs are tenuous, it is probable that a very close approximation between the receptor and the backbone of the BK chain at position 4 occurs on binding. In addition, position 4 may be centered in some tightly turned conformation of the molecule (like a β turn[34]) which minor steric influences could disrupt.

In summary, three new analogs of BK have been synthesized which bear methyl groups in the place of protons at various positions on the backbone of the peptide chain. None of the analogs antagonizes the action of BK, but one ([α-MP[8]]-BK) exhibits tissue specificity and decreased pulmonary inactivation in the rat.

References and Notes

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