Highly Selective Bradykinin Agonists and Antagonists with Replacement of Proline Residues by *N*-Methyl-D- and L-phenylalanine†

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For further studies on the structural and conformational requirements of positions 2, 3, and 7 in the bradykinin sequence, we replaced the proline residues by the more hydrophobic and conformationally restricted *N*-methyl-L- and D-phenylalanine (NMF). The biological activities of the new analogs were evaluated on rat uterus, guinea pig ileum, and guinea pig lung strip. Receptor binding of the analogs was studied in membranes from rat uterus and guinea pig ileum. Influence of bradykinin analogs on the release of cytokines from mouse spleen cell cultures was also measured. Bradykinin analogs were synthesized by the solid phase method, using Boc strategy on PAM or Merrifield resins. The best results in the formation of the *N*-methylamide bond were obtained with the coupling reagent PyBrop. In position 7 the substitution of D-Phe by D-NMF, retaining the configuration of the amino acid, converts bradykinin antagonists into agonists. The bradykinin analogs with D-NMF at position 7 gave the highest known tissue selectivity for rat uterus among agonists. [L-NMF^2]bradykinin has moderate agonist activity on rat uterus but antagonist activity on guinea pig lung strip. It represents a new antagonist for B_2 receptors without any replacement at position 7. The same analog completely inhibits bradykinin-evoked cytokine expression by mononuclear cells.

Introduction[1]

Bradykinin (BK), an endogenous linear nonapeptide hormone with the amino acid sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, is involved in a variety of physiological and pathophysiological processes. It stimulates smooth muscle cells[2,3] and sensory nerve endings,[4,5] modulates the response of immunocompetent cells,[3,6] and causes vasodilatation and microvascular leakage.[2,3] Bradykinin contributes to the inflammatory response; it produces pain, swelling, redness, and heat.[3,4,7]

Because of the pathophysiological role of bradykinin, its antagonists are of great interest. Vavrek and Stewart[8] developed the first antagonists in 1984; their key sequence alteration with respect to bradykinin was the replacement of 7-proline with D-phenylalanine or other D-aromatic amino acids. Following this key substitution, they introduced other changes (particularly addition of D-arginine to the amino end and introduction of hydroxyproline at position 3 and thienylalanine at positions 5 and 8) that gave the widely-used "first generation" antagonists, especially D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]BK, known as NPC-349.[9] Since then, many laboratories have attempted to develop more potent antagonists. In particular, the antagonist D-Arg-[Hyp^3,D-Phe^7,Leu^8]BK[10] was converted to the 6-cysteine-containing analog, then cross-linked via bis(maleimido)hexane, forming a dimeric peptide (known as CP-0127).[11] Knolle et al.[12] replaced the amino acids in positions 7 and 8 of NPC-349 by D-tetrahydroisoquinoline-3-carboxylic acid (Tic) and octahydroindole-2-carboxylic acid (Oic) to give the antagonist HOE-140. A third series of potent antagonists was synthesized by Kyle et al.[13] They replaced proline in position 7 by ethers of hydroxyproline, and phenylalanine in position 8 by Oic. One of the most active compounds, NPC-17761, contains *trans*-4-(phenylthio)-D-proline at position seven. Both HOE-140 and NPC-17761 are more potent than the earlier antagonists containing 7-D-phenylalanine.

In recent years various laboratories have attempted to estimate the conformation of bradykinin agonists and antagonists, especially the bioactive conformation. One of the most interesting questions is a possible difference in the conformation of agonists and antagonists. Because of the lack of strongly conformationally restricted cyclic agonists and antagonists with high biological activity and specificity, the proposed models give only preliminary information about the bioactive conformation. From NMR measurements and calculations of the dihedral angles ϕ, ψ, and χ, Kyle et al.[14] estimated two turns in bradykinin and bradykinin antagonists, one turn in the N-terminal part and a β-turn in the C-terminal part. Cyclization of the N-terminal part of their antagonists[14d] led to large losses in potency. In contrast to this model, NMR studies of agonist−antagonist pairs by Liu et al. and Otter et al.[15] showed the C-terminal β-turn only in agonists while antagonists had only one β-turn in the N-terminal part.

As shown for various di- and oligopeptides[16−19] the allowed dihedral angles in *N*-methyl amino acids are very restricted. Generally, N-methylation is considered to be one of the local and subtle modes of conformational constraint. It reduces the energy barrier to rotation around the N-methylated peptide bond and thus may

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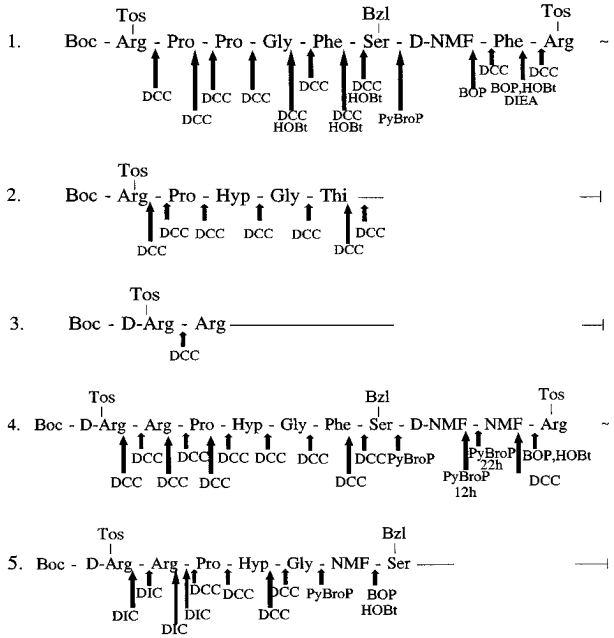
reduce the predominance of the *trans* vs *cis* peptide bond. With *N*-methylphenylalanine (NMF) the rate constant for *cis*−*trans* interconversion is in the same range as in proline.[17,19] In this respect *N*-methyl amino acids are able to simulate the situation of proline in peptide bonds. On the one hand, the *cis*−*trans* interconversion enhances the flexibility, while on the other hand, significant conformational restriction is imposed by the *N*-methyl group. Furthermore, N-methylation of a peptide bond eliminates its ability to donate a hydrogen bond. With respect to the biological activity, in some cases N-methylation has given compounds with improved pharmacological properties, such as enhanced potency and selectivity.[17,20,21]

To study the influence of N-methylation on biological activity we replaced the phenylalanine residue at several positions in bradykinin antagonists by D- and L-NMF. In order to help define conformational requirements about the critical position 7, we synthesized analogs having D- or L-NMF at that position. Since potency and tissue selectivity depend not only upon replacement in position 7 but on the whole sequence, we put D- and L-NMF into various positions in highly potent and selective compounds from the [D-Phe[7]]BK series. Continuing studies of one of the authors[22] on bradykinin analogs with L-NMF and *N*-methy-L-arginine, we also investigated replacement of both the phenylalanine residues at positions 5 and 8. Because of the analogy of proline to *N*-methyl amino acids with respect to the *cis*−*trans* conversion, we also replaced the proline residues at positions 2 and 3 with L- and D-NMF. Because it may be possible to find new types of bradykinin antagonists having their key modifications at different positions in the sequence, one goal of these studies was to find new classes of antagonists for different pharmacological assay systems.

The chemistry of bradykinin synthesis is very well established. Starting from Boissonnas and Guttmannn's first synthesis[23] in solution and Merrifield's synthesis[24] on a solid phase, many synthetic variants have been tested in the last three decades. We synthesized the bradykinin analogs by different solid phase strategies, using the Boc strategy on PAM or Merrifield resin. One of the anticipated problems was coupling of the sterically hindered *N*-methyl amino acid and the formation of the *N*-methylamide bond at the following step in the synthesis.

Kinin receptors have been divided into three classes: B_1, B_2, and B_3.[25,26] B_1 receptors recognize primarily the octapeptide bradykinin(1−8), and may be important in chronic inflammation, while most responses to bradykinin are mediated by B_2 receptors, which recognize bradykinin and larger homologs and respond only very weakly to bradykinin(1−8). Many laboratories have attempted to subdivide B_2 receptors on the basis of relative potencies of bradykinin analogs in different tissues.[25] Thus Regoli[27] suggested from differences in agonist and antagonist activities three different subtypes: B_2A, B_2B, and B_2H. We have therefore estimated the biological activities of the new series for contraction of rat uterus (RUT), guinea pig ileum (GPI), and guinea pig lung strip (LS) and for inhibition of bradykinin-evoked cytokine release from mononuclear lymphocytes. RUT, GPI, and LS are classified as tissues with B_2 receptor subtypes.[2,25,26] The goal of this structure−activity study is not only to subdivide the B_2 receptors but also to develop highly potent and highly selective agonists and antagonists. Enzymatic degradation of the agonists in the pulmonary circulation was evaluated from the difference in the hypotensive response following intravenous and intraarterial administration. In addition to these classical tests, we estimated the influence of the bradykinin agonists and antagonists on interleukin release from mononuclear cells.[28] Binding studies using displacement of ^{3}H-labeled bradykinin from membrane fractions of RUT and GPI were performed to estimate the correlation between receptor affinities and pharmacological activities. Binding parameters of the analogs were calculated by use of the Tobler program.[29] The radioligand binding experiments combined with a sophisticated calculation of parameters give additional and more detailed information about receptor interactions than the pharmacological assays alone.

Scheme 1. Synthesis of Bradykinin Analogs with L- and D-NMF at Positions 5, 7, and 8, Using Boc Strategy on Merrifield Resin[a]



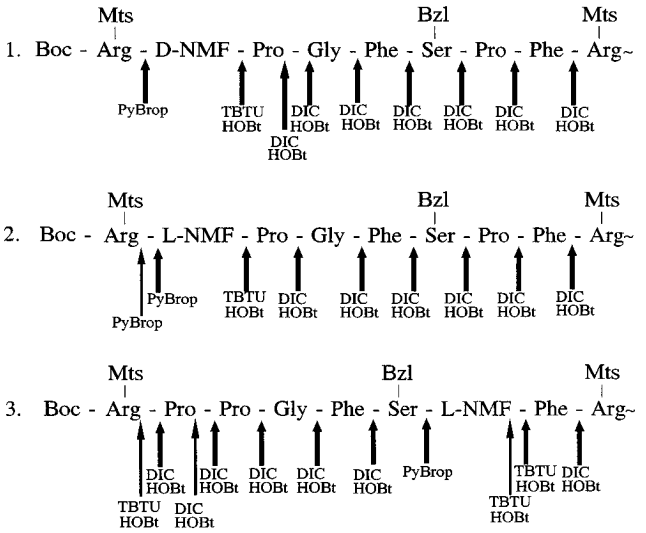
[a] N^G-Tos protection, PyBrop as coupling reagent for formation of methylamide bonds, BOP for sterically hindered amino acids, and DCC for normal coupling steps.

Results and Discussion

Synthesis. Coupling of the hindered Boc-NMF requires strong activation; BOP/HOBt was used. For acylation of NMF residues, best results in formation of the CO−NCH_3 bond were obtained with the coupling agent PyBrop. The synthetic routes used for Boc and Fmoc syntheses of peptides containing NMF are shown in Schemes 1 and 2.

The combination of two or three NMF residues in one peptide caused the most difficulty. Thus, peptide **7**, containing three NMF residues, including the sequence [NMF[7],NMF[8]], was obtained only in low yield and with a considerable amount of byproducts. It could be purified only by a combination of CCD and preparative HPLC. Unexpected difficulties were encountered in the syntheses of [L-NMF[3]]- and [D-NMF[3]]BK, resulting in impure products and low yields. In both cases the coupling of proline at position 2 required two recoupling steps with a total time of 72 h. Even a change of solvent

Scheme 2. Synthesis of Bradykinin Analogs with L-and D-NMF at Positions 2 and 7 Using Boc Strategy on PAM Resin[a]



[a] N^G-Mts protection, PyBrop as coupling reagent for formation of methylamide bonds, TBTU for sterically hindered amino acids, and DIC for normal coupling steps.

and addition of $NaClO_4$[30] did not reduce the coupling time. Standard HF cleavage and deprotection of the peptides on Merrifield resin (Scheme 1) yielded a considerable amount of incompletely deprotected peptides. In contrast to this result, the use of the TFMSA cocktail described for the syntheses on PAM resin (Scheme 2) delivered peptides with better purity and in higher yield.

Conversion of Antagonists into Agonists by Replacement at Position 7. Table 1 shows that all analogs having D-NMF at position 7 are agonists on RUT and GPI. Thus, the simple replacement of D-Phe at position 7 by D-NMF converts the anticipated antagonists into agonists in all analogs tested. Analog **8** with L-NMF in position 7 is an agonist as well. In good agreement with the studies of Mazur et al.[31] on the biological activity of bradykinin analogs containing L-NMF, the replacement of Phe at positions 5 and 8 in our compounds **6** and **7** reduced the agonist potency and stabilized the peptide against enzymatic degradation in the circulation. The strongly conformationally restricted compounds **6** and **7** are interesting objects for conformational studies.

Given the state of our present knowledge, it is impossible to explain the conversion of antagonists with D-amino acids at position 7 into agonists with D-NMF. We compared the calculated conformational energy maps for *N*-methyl amino acids, especially D- and L-NMF, with those for BK and the amino acid combinations D-Phe-Oic, D-Tic-Oic, and *trans*-D-Hyp-Oic in antagonists. The conformational energy maps for *N*-methyl amino acids with the peptide bond in the *cis* or *trans* configuration were taken from the literature[16−18] and estimated for di- and oligopeptides. The dihedral angles for the dipeptides in the C-terminal part were estimated by Kyle and co-workers.[14] Neither D-NMF nor L-NMF is able to have the same allowed areas for dihedral angles as has been estimated for position 7 in antagonists, but the conformational ϕ, ψ energy maps also do not correspond to that of proline. Nevertheless, the analogs with D-NMF at position 7 act as agonists.

The functional activities of compounds **3** to **7** (Table 1) show great differences in potency between the rat uterus and the guinea pig ileum. The decrease in potency between the key analog **3** and the other compounds (**4**, **5**, **6**, and **7**) in this series suggests that there exist different structural requirements for agonists and antagonists. We may draw this conclusion from the finding that in contrast to results with antagonists the replacements of phenylalanine at position 5 by thienylalanine and of proline at position 3 by hydroxyproline in these agonist peptides do not increase, as expected, but rather reduce their potency. The replacement of phenylalanine residues by NMF in the peptides enhances the stability against enzymatic inactivation. The differences in the potencies for blood pressure reduction between intraarterial and intravenous administration of these agonists in comparison to BK (**3**, ia 60%, iv 250%; **4**, ia 40%, iv 160%; **5**, ia 14%, iv 760% of BK potency) demonstrate enhanced resistance to degradation in the pulmonary circulation. This degradation is principally due to angiotensin I converting enzyme (ACE).[8] Furthermore, the peptide with D-NMF in position 7 (**3**) is unable to interact with isolated ACE (inhibition of ACE activity: 10^{-5} M, 0%; 10^{-4} M, 20%, $IC_{50} \gg 10^{-4}$ M; W.-E. Siems, personal communication). This finding is in good agreement with the stability *in vivo* of [D-Phe[7]]BK analogs.[32]

Replacement of Proline Residues at Positions 2 and 3 by D- and L-NMF. Replacement of the imino acid proline at positions 2 and 3 by the more hydrophobic and conformationally restricted imino acid *N*-methylphenylalanine decreases the biological activity strongly in most cases (Table 1, compounds **11**−**13**). Only [L-NMF[2]]BK (**10**) shows moderate activity on RUT. This fact emphasizes the different structural requirements for positions 2 and 3 and the tissue differences in GPI and RUT.

Summarizing these results from the viewpoint of amino acid configuration, we can conclude that on the one hand at position 7 NMF in both configurations gives agonists, whereas on the other hand at position 2, only the L-configuration provides moderate agonist potency.

A New Type of Antagonist for the B_2 Receptor. Both the ileum and lung of guinea pigs have B_2 receptors with similar structural requirements for BK and analogs, although the lung may be more complex.[33] A significant difference between these tissues is indicated by peptide **10**, which has L-NMF at position 2; this analog is an antagonist on the lung strip. This analog is one of very few antagonists[26] for B_2 receptors without any change at position 7.

On the basis of differences in potencies of agonists and antagonists, Farmer et al.[33] proposed that guinea pig lungs contain two receptor subtypes: B_2 and B_3. With our analogs we found only small differences between the agonist activities on the GPI and LS. The only exception is compound **10**. Thus, we would suggest that [L-NMF[2]]BK is a new antagonist for the B_2 receptor type. This compound is a weak antagonist on LS but the strongest known antagonist on mononuclear cells, as shown below. This new antagonist and the B_2A selective antagonists DArg-[Hyp[3],Leu[8]]BK and DArg-[Hyp[3],Gly[6],Leu[8]]BK developed by Regoli et al.[27] indicate different structural requirements for different types of

Table 1. Biological Activities of Bradykinin Analogs Containing *N*-Methylphenylalanine[a]

no.	peptide structure	activity (% of bradykinin) RUT	GPI	LS
1	BK	100	100	100
2	[D-Phe^7]BK	1.7 ± 0.03	pA_2 5.56	0.9 ± 0.7
3	[D-NMF^7]BK	136 ± 23	0.04 ± 0.01	1.4 ± 0.03
4	[Hyp^3,Thi^5,D-NMF^7]BK	89 ± 4.7	0.02*	0.04 ± 0.007
5	D-Arg-[Hyp^3,Thi^5,D-NMF^7]BK	86 ± 14.4	0.006*	0.04 ± 0.01
6	D-Arg-[Hyp^3,D-NMF^7,L-NMF^8]BK	40 ± 2.5	0.01*	0.45 ± 0.04
7	D-Arg-[Hyp^3,D-NMF^7,L-NMF^{5,8}]BK	15 ± 5.1	0.005*	0.5 ± 0.05
8	[L-NMF^7]BK	0.2 ± 0.04	0.03 ± 0.004	0.1 ± 0.02
9	D-Arg-[L-NMF^7]BK	0.5 ± 0.04	0.07 ± 0.01	0.08 ± 0.02
10	[L-NMF^2]BK	35 ± 4	4 ± 0.8	1.4 ± 0.01 pA_2 5.59 ± 0.47
11	[D-NMF^2]BK	0.15 ± 0.02	0.15 ± 0.09	0.05 ± 0.01
12	[L-NMF^3]BK	0.03 ± 0.009	0.05 ± 0.005	0.03 ± 0.002
13	[D-NMF^3]BK	0.02 ± 0.008	0.03 ± 0.004	0.03*

[a] Assays were performed as described in the Experimental Section: RUT, rat uterus; GPI, guinea pig ileum; LS, guinea pig lung strip. Antagonist activities of peptides **2** and **10** are given as the pA_2. The pD_2 of BK is 7.9 in RUT and 7.4 in GPI. The results are means ± SEM of three to seven separate determinations. Data marked with an asterisk (*) are the means of duplicate determinations that varied by less than 10%.

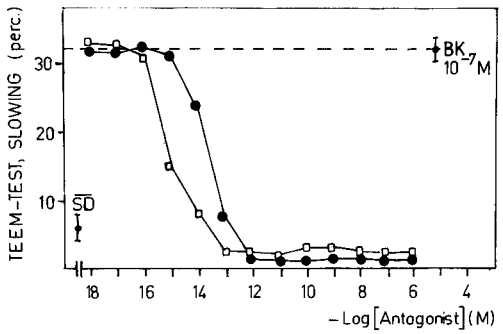


Figure 1. Effect of HOE-140 and [L-NMF^2]BK on the BK-induced secretion of charge-changing cytokines in mouse spleen cell cultures. Spleen cells (10^6/mL) were incubated for 4 h, 37 °C with BK alone (10^{-7} M) (- - -) or with this BK dose after preincubation (0.5 h, 37 °C) with graded concentrations of HOE-140 (●) or [L-NMF^2]BK (□). Cell-free supernatants were incubated with target cells (sulfosalicylic acid stabilized, tanned sheep erythrocytes) for 1 h, 23 °C, and the migration time of the targets was estimated in a cytopherometer. The slowing effect was calculated using targets and targets plus peptides (to exclude side effects) as controls. Values are mean ±SD, taken from three experiments in duplicate cultures. The mean of all SD was calculated as residual variance.

antagonists and show that the various analogs with replacements of proline at position 7 represent only one type.

In other words, from these and other recent findings we might assume that there exists more than one position in the sequence where alterations may give antagonists, but nothing is known about the conformational requirements at these positions. Cyclization and subsequent conformational analysis of the new analogs by different physical and computational methods will be required.

Cytokine Release from Mononuclear Cells. The TEEM (tanned erythrocyte electrophoretic mobility) test was used to characterize the cytokines released from mononuclear cells by the peptides.[28] Bradykinin releases cytokines from mouse spleen cells, mainly interleukins 1, 2, and 6. Figure 1 shows inhibition of cytokine release by the bradykinin antagonists HOE-140 and [L-NMF^2]BK. The latter compound completely inhibited cytokine expression at a concentration of 10^{-13} M. Other antagonists are less potent, e.g. [DPhe^7]BK with an inhibitory concentration of 10^{-10} M and HOE-140 with 10^{-12} M. Structure−activity relationships in this assay and receptor binding of several series of bradykinin analogs will be published separately.

Tissue Selectivity. One of our goals is the development of highly selective bradykinin agonists and antagonists. Table 2 shows the tissue selectivity of bradykinin agonists for RUT and GPI. The quotient RUT/GPI indicates the selectivity of various bradykinin analogs for the rat uterus. In comparison with the most selective agonists from the literature,[9,34,35] some of our analogs with D-NMF at position 7 considerably exceed their selectivity quotients and are the most selective agonists known for rat uterus.

Receptor Binding Parameters. For both rat uterus and guinea pig ileum there are contradictory results postulating a single binding site[36] or two binding sites, one with picomolar and one with nanomolar affinity.[37] The biphasic displacement curves (Figure 2) obtained for most, but not all, analogs tested here indicate an interaction with both of the previously described bradykinin binding sites. There is no obvious correlation between the recognition of one or two bindings sites and the functional activities. However, the functional potencies of most analogs relative to bradykinin give a closer correlation with the low-affinity site (Table 3), thus confirming our assumption, based on previous evidence, that the smooth muscle contracting effect of bradykinin seems to be mediated by the site with nanomolar affinity[38] that is linked to phospholipase C.[39] It has recently been shown that the high-affinity (picomolar) binding site on guinea pig ileum correlates with inhibition of adenylate cyclase in that tissue.[40] It should be noted that [L-NMF^2]BK showed a higher biological activity but lower binding affinity on the rat uterus compared with that on the guinea pig ileum. It may be assumed, therefore, that there exists a higher signal amplification in the rat uterus, or that there are different signal transduction pathways in the two tissues.

While most of the bradykinin analogs reported here bind to both the high-affinity and low-affinity sites on ileal membranes, several did not (Table 3); this group of peptides showed only one affinity. This has previously been observed for some other agonist analogs.[41] Another puzzle is the very large difference between binding affinity and functional potency of certain of the analogs, especially in ileum (Table 1). This difference is apparent for bradykinin itself, since the pD_2 (the

Table 2. Tissue Selectivity for Smooth Muscle Contraction of Bradykinin Agonists[a]

no.	compound	RUT	GPI	RUT/GPI
	bradykinin (BK)	100%	100%	1
	Lys-Lys-[ΔPhe^7]BK[b]	12	0	
	[*p*-GuanidoPhe^{1,9}]BK[c]	0.001	2	0.001
	[Lys^9]BK[c]	0.2	20	0.01
	[Aib^7]BK[b]	55	418	0.13
	[D-Phe^6,Aib^7]BK[b]	124	426	0.291
	[D-Phe(Cl)^6,Aib^7]BK[b]	97	321	0.30
	[D-Trp^6,Aib^7]BK[b]	100	226	0.442
	[Thi^{5,8}]BK[b]	1000	200	5.0
	[Thi^8]BK[b]	500	40	13
	D-Ile-Ser-BK[b]	271	11	25
	D-Arg-[Hyp^3,Thi^{5,8}]BK[b]	237	7	30
	[Gly^6,D-Phe^7]BK[b]	31	0.3	100
	[Thi^{5,8},ΔPhe^7]BK[b]	15	0.1	200
3	[D-NMF^7]BK	136	0.4	300
4	[Hyp^3,Thi^5,D-NMF^7]BK	89	0.02	4000
5	D-Arg-[Hyp^3,Thi^5,D-NMF^7]BK	86	0.006	10000
6	D-Arg-[Hyp^3,D-NMF^7,NMF^8]BK	40	0.01	4000
7	D-Arg-[Hyp^3,NMF^{5,8},D-NMF^7]BK	15	0.005	3000

[a] Activities are given as percent of BK activity. [b] Reference 35. [c] Reference 34. The ratio RUT/GPI indicates the selectivity of the analogs for rat uterus.

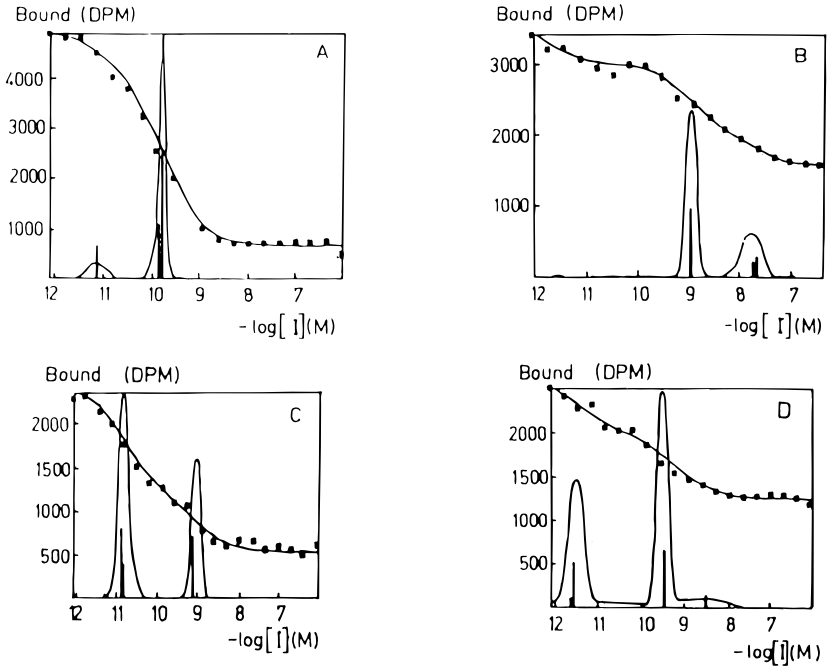


Figure 2. Inhibition of specific [^{3}H]BK (30 pM) binding to membranes of guinea pig ileum by BK (A) and [D-NMF^7]BI (B) and membranes of rat myometrium by BK (C) and [D-NMF^7]BK (D). Plots from the Tobler program[29] are shown. Calculations were from untransformed data (total binding values). Nonspecific binding is reflected by the curve shape at BK concentrations higher than 1 μM and can differ between several experiments. Locations of the vertical bars indicate the IC_{50} values of the binding sites. Their height represents the relative concentrations of the binding sites. The affinity spectra (continuous curves) allow for visual differentiation between statistically valid and spurious peaks. Data points are means from duplicate determinations in a representative experiment.

negative logarithm of the ED_{50}) for bradykinin is 7.9 on rat uterus and 7.4 on guinea pig ileum, indicating an EC_{50} between 10 and 100 nM, whereas the binding IC_{50} is about 1 nM for the low-affinity site in both tissues. With the peptides reported here, the dissociation between binding and functional potency is particularly impressive on ileum. Lower potencies on ileum are often attributed to the known greater activity of various kininase enzymes in ileum. Binding studies are done in low ionic strength medium, at low temperature, and in the presence of enzyme inhibitors, whereas the functional assays are carried out at physiological temperature and ionic strength. Physiological ionic strength media have been reported to inhibit bradykinin receptor binding.[39] Internalization of agonist−receptor complexes, which may contribute in an undefined way to the lower functional potencies of agonists in various tissues, is inhibited in binding studies by use of low temperature.

At this time only one bradykinin B_2 receptor amino acid sequence has been identified for any given species, although from very early in bradykinin antagonist research peptides were obtained which discriminated between tissues within a species as well as between species.[9] As mentioned above, several subclasses of B_2 receptors have been proposed on the basis of pharmacological responses to different antagonists.[27] The molecular basis of these differences remains an open question, although different post-translational processing of receptors in different tissues is a likely cause. A recent study of the rat B_2 receptor gene identified differential processing leading to two different mRNAs

Table 3. Binding Affinities of Bradykinin Analogs to Membranes from Rat Uterus and Guinea Pig Ileum[a]

		IC_{50}(nM)			
		RUT		GPI	
no.	peptide structure	HA	LA	HA	LA
1	BK	0.01	1.00	0.01	1.75
2	[D-Phe^7]BK	6.6	3.5	14	
3	[D-NMF^7]BK	0.11	4.80	1.0	132
4	[Hyp^3,Thi^5,D-NMF^7]BK	0.49	65	29	
5	D-Arg-[Hyp^3,Thi^5,D-NMF^7]BK	0.30	21	38	
6	D-Arg-[Hyp^3,D-NMF^7,L-NMF^8]BK	0.33	18	33	
8	[L-NMF^7]BK	500		700	
9	D-Arg-[L-NMF^7]BK	33		1.0	
10	[L-NMF^2]BK	3.5	232	1.0	25
11	[D-NMF^2]BK	100		57	3780
12	[L-NMF^3]BK	0.2	375	2.6	8000
13	[D-NMF^3]BK	0.3	57	1400	

[a] Conditions for assays and analysis of results are given in the Experimental Section. IC_{50} values were calculated using the program of Tobler and Engel.[29] Data are means of duplicate determinations, which did not differ by more than 5%. Most analogs interact with both high- (HA) and low-affinity (LA) bindings sites. Some analogs cannot discriminate between the HA and LA sites; values for these are given in the middle column for each tissue: RUT, rat uterus; GPI, guinea pig ileum.

for the receptor, both of which contained the same coding sequence.[42] These messages did contain alternate translation start codons, which could lead to alternate protein sequences. Definitive statements about receptor heterogeneity must await actual receptor protein sequence information.

Experimental Section

Peptide Synthesis. All of the analogs were synthesized by solid phase methods, using Boc strategy. Boc-Arg(Tos)-Merrified resin (0.234 mequiv/g, Bachem, CA) and Boc-Arg-(Mts)-PAM resin (0.5 mequiv/g, Bachem, Switzerland), were purchased. Boc-amino acids were obtained from Orpegen, Bachem, CA and Bachem, Switzerland. Boc-D-NMF was synthesized by the procedure of Olsen.[43]

The first strategy (Scheme 1) with Boc-Arg(Tos) resin was carried out on the Beckman 990 synthesizer, and the synthetic route with Boc-Arg(Mtr) resin (Scheme 2) on the PSS-80 synthesizer. With the PSS-80, coupling reactions were monitored by the internal automatic monitoring system with dimethoxytrityl chloride. With the Beckman Synthesizer and as an additional control on the PSS-80 we used the Kaiser test.

Peptides were purified by CCD, by gel filtration on Biogel P2 in 2% acetic acid and by RP-HPLC (Shimadzu LC-8A, SPD-6A) on a Nucleosil 100, C18, 5 μm reversed phase columm (25 cm $\times$ 1.6 cm) and 30% CH_3CN in 0.1% aqueous trifluoracetic acid at a flow rate of 6 mL/min with UV detection at 233 nm. Purity of the free peptides was evaluated by TLC in two solvent systems on silica gel, by paper electrophoresis at pH 2.3, and by analytical HPLC (see details in Table 4). Amino acid analyses were performed on a Beckman 6300 amino acid analyzer and gave acceptable results for all peptides (Table 5). The $(M + H)^+$ molecular ions and fragmentation patterns were obtained by FAB$-$MS and were in good agreement with the calculated molecular weights for each peptide (Table 5).

Example A: Synthesis of [D-NMF^7]Bradykinin on Merrifield Resin (Scheme I). Boc-Arg(Tos)-Resin (0.85 g, 0.2 mmol) was used, and the following protected amino acids were coupled to the growing peptide chain in stepwise fashion: Boc-Phe, Boc-D-NMF, Boc-Ser(Bzl), Boc-Phe, Boc-Pro, Boc-Pro, and Boc-Arg(Tos). All amino acids except Boc-D-NMF and Boc-Ser(Bzl) were coupled with DCC. Boc-D-NMF was coupled with BOP/HOBt and Boc-Ser(Bzl) with PyBrop. Reaction times for complete couplings were 2 h with DCC and PyBrop and 12 h with BOP/HOBt. The Boc group was removed with 25% TFA in DCM with indole as scavenger in two steps: 1.5 min prewash and 30 min deprotection. After coupling of the last amino acid, the Boc protecting group was removed, and the resin was washed with DCM and ethanol and dried.

Table 4. Analytical Characteristics of Bradykinin Analogs

	TLC[a] R_f values		electrophoresis[b]	HPLC[c]	route of
no.	A	B	R_{Arg}	K'	synthesis[d]
3	0.53	0.35	0.95	14.20	A,B
4	0.5	0.30	0.95	13.50	A
5	0.45	0.13	1.0	13.10	A
6	0.5	0.15	1.0	14.30	A
7	0.5	0.15	1.0	14.60	A
8	0.53	0.35	0.95	13.60	B
9	0.5	0.15	1.0	13.50	B
10	0.60	0.38	0.95	13.30	B
11	0.60	0.38	0.95	13.30	B
12	0.60	0.35	0.95	13.10	B
13	0.60	0.35	0.95	13.20	B

[a] Silica gel F254, 250 μm (Merck 5715) glass plates were used. Solvent systems: (A) pyridine/ethyl acetate/acetic acid/H_2O: 5/5/1/3 (v/v/v/v); (B) 1-butanol/acetic acid/water: 48/18/24 (v/v/v). [b] Paper electrophoresis in 6% aqueous acetic acid. R_f values are relative to Arg. [c] Capacity factor for Bioselect 300$-$5 C18 reversed phase columm (250 $\times$ 4 mm), elution A, 0.1% aqueous TFA; B, 0.1% TFA in acetonitrile; gradient 0$-$50% B in 50 min, flow rate 1 mL/min, monitored at 220 nm. [d] A: synthesis on Merrifield resin according to Scheme 1 and example A. B: synthesis on PAM resin according to Scheme 2 and example B.

Cleavage and deprotection of the peptide-resin was done with HF:anisole (10:1) at 0 °C, 40 min. HF was removed by vacuum at 0 °C, the product was washed with ethyl ether, and the peptide was extracted with 16 mL of glacial acetic acid and lyophilized. The crude product was dissolved in 8 mL of a mixture of 1-butanol and 1% aqueous TFA (1:1) and purified by 100-step CCD in the same solvent system. The distribution was monitored by the quantitative Sakaguchi test. Fractions were pooled, evaporated, and evaluated by TLC and analytical HPLC. Analytical data are presented in Tables 4 and 5. Yield: 75 mg of white lyophilized powder; mp 145$-$160 °C; $[\alpha]^{D}_{20} = -34.8 \pm 2$ ($c = 1$, H_2O).

Example B: Synthesis of [D-NMF^7]Bradykinin on PAM Resin (Scheme 2). Boc-Arg(Mts)-PAM-resin (0.57 g, 0.285 mmol) was used with DIC/HOBt as coupling agent, except that the couplings of Boc-Phe at position 8 and Boc-Arg(Mts) at position 1 were done with TBTU/HOBt and Boc-NMF was coupled with PyBrop. The internal monitoring of the PSS-80 synthesizer showed that under these conditions couplings of more than 99.5% were attained. Only [Boc-Pro^2] required recoupling to reach this value. Cleavage from the resin and deprotection was carried out with a cocktail containing 1.5 mL of TFA, 0.15 mL of TFMSA, 0.11 mL of thioanisole, and 0.055 mL of EDT for 200 mg of dry resin. The reaction mixture was stirred for 10 min at 0 °C and for 60 min at room temperature, filtered, and poured into ethyl ether. The precipitate was isolated by centrifugation. The crude peptide was dissolved in 20 mL of *tert*-butyl alcohol and lyophilized (yield 350 mg). The product was dissolved in 2% aqueous acetic acid (1 mg/5 mL), passed through an ion exchange resin (Amberlite IRA 410; 12 mL of resin/mequiv of peptide), and relyophilized. The crude product was purified by HPLC as described above. The fractions were collected, evaporated, lyophilized, and evaluated by TLC and analytical HPLC (for analytical data see Tables 4 and 5).

Radioligand Binding. Binding assays on rat myometrial and guinea pig ileum membranes were carried out as described previously[44] with minor modifications. Briefly, membranes (0.3$-$0.5 mg of protein per assay, always freshly prepared) were incubated in a total volume of 1 mL containing 25 mM TES buffer, pH 6.8, 1 μM captopril, 1 mM DTE, bacitracin (140 mg/L), bovine serum albumin (1 g/L), [^{3}H]BK (0.05 nM, obtained from New England Nuclear, specific activity 102 Ci/mmol), and the various peptides in increasing concentrations. After incubation (4 °C, 30 min), the samples were filtered through Whatman GF/C glass fiber filters pretreated with 0.1% (w/v) aqueous polyethylenimine solution, using a Brandel harvester. The filters were washed three times with 5 mL of TES buffer (10 mM, pH 6.8), transferred into scintillation vials, dried, and counted for radioactivity in 6 mL of a toluene-based scintillator. Nonspecific binding was determined in the presence of 1 μM BK. The IC_{50} values were calculated using the

Table 5. Amino Acid Compositions and Molecular Weights of the Peptides

									FAB-MS (M + H)	
no.	Arg	Pro	Hyp	Gly	Phe	Thi	Ser	NMF	calcd	found
3	1.9 (2)	2.0 (2)		1.0 (1)	2.1 (2)		0.85 (1)	1.3 (1)	1124.2	1124.5
4	2.0 (2)	1.0 (1)	0.95 (1)	1.0 (1)	0.97 (1)	1.0 (1)	0.83 (1)	1.15 (1)	1146.0	1146.5
5	3.0 (2)	1.0 (1)	0.95 (1)	1.0 (1)	0.97 (1)	1.0 (1)	0.87 (1)	1.15 (1)	1302.2	1302.6
6	3.0 (2)	0.85 (1)	0.75 (1)	1.2 (1)	1.0 (1)		1.2 (1)	2.3 (2)	1310.2	1310.7
7	3.0 (2)	0.75 (1)	0.75 (1)	0.95 (1)			0.8 (1)	3.2 (3)	1324.1	1324.7
8	2.0 (2)	1.9 (2)		1.0 (1)	2.1 (2)		0.9 (1)	1.1 (1)	1124.2	1124.4
9	2.9 (3)	2.0 (2)		0.95 (1)	2.0 (2)		0.85 (1)	1.15 (1)	1280.3	1280.7
10	1.7 (2)	1.7 (2)		1.1 (1)	2.0 (2)		0.85 (1)	1.0 (1)	1124.2	1124.5
11	1.7 (2)	1.7 (2)		1.0 (1)	2.0 (2)		0.85 (1)	0.9 (1)	1124.2	1124.8
12	1.9 (2)	1.8 (2)		1.0 (1)	2.0 (2)		0.9 (1)	0.9 (1)	1124.2	1124.5
13	1.9 (2)	1.9 (2)		0.95 (1)	2.0 (2)		0.87 (1)	1.1 (1)	1124.2	1124.9

program of Tobler and Engel.[29] The Tobler program was supplemented with a special data input program and combined with subsequent nonlinear regression analysis as recently described by Schnittler et al.[45] The calculation program of Tobler and Engel was not altered.

Estrus Rat Uterus Assay. Female Wistar rats weighing 180−200 g were injected with diethylstilbestrol (100 μg/kg sc) 16 h before the uterus was used. The distal parts of the uterine horns were suspended at 28 °C in de Jalon solution. Isotonic contractions were recorded under a resting tension of 0.5 g. BK (half-maximal dose) was added to the organ bath at 4 min intervals and left in the organ bath for 45 s. To investigate inhibition, analogs were added to the organ bath 1 min before the BK dose. Peptides without antagonist action were tested for agonist activity by means of the 4-point method of Schild.[46] In some cases cumulative dose−response curves for bradykinin were made to estimate the antagonist activities, which are expressed as pA_2 values (negative log of the concentration of the antagonist that reduces the effect of a double dose of BK to that of a single dose).[47]

Guinea Pig Ileum Assay. Food was withheld from animals for 16 h before use. Terminal ileum (1.5 cm) was suspended in Tyrode solution at 37 °C. Isotonic contractions were recorded under a resting tension of 0.5 g. BK was applied in a cumulative way, reaching a maximum contraction at a BK concentration of 0.3 μM. Antagonist analogs were added to the bath 5 min before the cumulative dose−response curve for BK was made. The pA_2 value of antagonists was estimated as described above. BK analogs without inhibitory action were tested for agonist action by the 4-point method of Schild[46] using BK doses of 1 and 2 nM.

Guinea Pig Lung Strip Assay. Male or female guinea pigs weighing 400−600 g were used. The lungs were dissected out and placed in a modified Krebs solution. Sections of lung tissue about 2−3 mm wide were cut quickly from the lower lobes parallel to the margin. The isolated strips, loaded with 1 g tension, were placed in an organ bath containing Krebs−Henseleit solution at 37 °C. Subsequent changes in the tension were measured isometrically. The tissues were allowed to equilibrate for 1 h with solution changes at 15 min intervals. Cumulative dose−response curves for BK were made in the concentration range 0.01−10 μM BK. To examine antagonist activity the peptides were incubated with the lung strip 5 min before addition of an ED_{50} dose of BK. The pA_2 values were calculated by the method of van Rossum et al.[47]

Interleukin Release Assay. Tanned Erythrocyte Electrophoretic Mobility (TEEM) Test. Mouse spleen cells were incubated with the different BK analogs in a serum-free short-time (5 h, 37 °C) culture to evoke interleukin release.[28] The supernatants were prepared by centrifugation and partially purified by gel filtration (AcA54, column 0.9 × 60 cm; 25 mL/min; fractions of 1 mL).

Interleukin standards (IL-1α, IL-1β, IL-3, IL6, TNFα) were all from Genzyme, Cambridge, MA. Culture supernatants or fractions with or without monoclonal antibodies (hamster anti-mouse IL-1α, rat anti-mouse IL-2, rat anti-mouse IL-3, rat anti-mouse IL-6, hamster anti-mouse TNFα/β, all from Genzyme; anti-IL-2 receptor, mouse, Boehringer Mannheim, Germany) were added to the targets (sulfosalicylic acid-stabilized tanned sheep red blood cells) and incubated for 1 h at 23 °C. The migration time of the target cells was estimated in an Opton cytopherometer. The slowing effect (percent) was calculated using targets or targets plus peptides (to exclude side effects) as controls. In order to test antagonist activities of selected BK analogs, an inhibition variant of the TEEM test was used: spleen cells of mice were preincubated (0.5 h, 37 °C) with the potential antagonists followed by incubation with the optimal agonist dose of BK (10^{-7} M) for 4 h at 37 °C. Interleukin activity in the cell-free supernatant was determined as described above.

Inhibition of Isolated Angiotensin Converting Enzyme. Inhibitory activities of some peptides were determined with an enzyme preparation from pig lung, using benzoyl-Gly-His-Leu as substrate. The method was described elsewhere.[48]

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(1) Symbols and abbrevations are in accord with the recommendations of the IUPAC−IUB Commission on Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Other abbreviations: ACE, angiotensin I converting enzyme; Aib, α-aminoisobutyric acid; BK, bradykinin; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; CCD, countercurrent distribution; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DIC, diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DTE, dithioerithritol; EDT, ethanedithiol; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HOE-140, Darg-[Hyp^3,Thi^5,DTic^7,Oic^8]BK; ia, intraarterial; iv, intravenous; LS, lung strip; Mts, mesitylene-2-sulfonyl; NMF, *N*-methyl-Phe; Oic, octahydroindole-2-carboxylic acid; DPhe(Cl), *p*-chloro-D-Phe; ΔPhe, 2,3-dehydro-Phe; PyBrop, bromotrispyrrolidinophosphonium hexafluorophosphate; RUT, rat uterus; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEEM, tanned erythrocyte electrophoretic mobility; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; Tic, tetrahydroisoquinoline-3-carboxylic acid; Thi, β-2-thienylalanine; Tos, *p*-toluenesulfonyl.

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