Journal of Peptide Science
J. Peptide Sci. **4**: 92–100 (1998)

Bradykinin Antagonists with Dehydrophenylalanine Analogues at Postion 5

GEORG GREINER[1], UTZ DORNBERGER[1], INGE PAEGELOW[2], BERNWARD A. SCHÖLKENS[3], CLAUS LIEBMANN[1] and SIEGMUND REISSMANN[1]

[1]Friedrich-Schiller-University of Jena, Institute of Biochemistry and Biophysics, Jena, Germany
[2]University of Rostock, Institute of Pharmacology and Toxicology, Rostock, Germany
[3]Hoechst AG, SBU Cardiovascular Agents, Frankfurt (Main), Germany

Received 12 February 1997
Accepted 30 May 1997

Abstract: Continuing the studies on structural requirements of bradykinin antagonists, it has been found that analogues with dehydrophenylalanine (ΔPhe) or its ring-substituted analogues (ΔPhe(X)) at position 5 act as antagonists on guinea pig pulmonary artery, and on guinea pig ileum. Because both organs are considered to be bradykinin B_2 receptor tissues, the analogues with ΔPhe or ΔPhe(X) at position 5, but without any replacement at position 7, seem to represent a new structural type of B_2 receptor antagonist. All the analogues investigated act as partial antagonists; they inhibit the bradykinin-induced contraction at low concentrations and act as agonists at higher concentrations. Ring substitutions by methyl groups or iodine reduce both the agonistic and antagonistic activity. Only substitution by fluorine gives a high potency. Incorporation of ΔPhe into different representative antagonists with key modifications at position 7 does not enhance the antagonist activity of the basic structures, with one exception. Only the combination of ΔPhe at position 5 with DPhe at position 7 increases the antagonistic potency on guinea pig ileum by about one order of magnitude. Radioligand binding studies indicate the importance of position 5 for the discrimination of B_2 receptor subtypes. The binding affinity to the low-affinity binding site (K_L) was not significantly changed by replacement of Phe by ΔPhe. In contrast, ring-methylation of ΔPhe results in clearly reduced binding to K_L. The affinity to the high-affinity binding site (K_H) was almost unchanged by the replacement of Phe in position 5 by ΔPhe, whereas the analogue with 2-methyl-dehydrophenylalanine completely failed to detect the K_H-site. The peptides were synthesized on the Wang-resin according to the Fmoc/Bu^t strategy using Mtr protection for the side chain of Arg. The dehydrophenylalanine analogues were prepared by a strategy involving PyBop couplings of the dipeptide unit Fmoc-Gly-ΔPhe(X)-OH to resin-bound fragments. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin; antagonists; dehydrophenylalanine; smooth muscle contraction; radio-ligand binding studies

Abbreviations: DIEA, diisopropylethylamine; DTE, dithioerithritol; D-Tic, D-tetrahydroisoquinoline-3-carboxylic acid; GPI, guinea pig ileum; HOE-140, DArg[Hyp^3,Thi^5,$DTic^7$,Oic^8]-BK; Hyp, hydroxyproline; NMePhe, *N*-methyl phenylalanine; Mtr, 4-methoxy-2,3,6-trimethyl-benzene-sulphonyl; Oic, octahydroindole-2-carboxylic acid; PA, guinea pig pulmonary artery; ΔPhe, (*Z*)-α,β-dehydro phenylalanine; ΔPhe(4-F), (*Z*)-α,β-dehydro 4-fluorophenyl alanine; ΔPhe(4-I), (*Z*)-α,β-dehydro 4-iodophenyl alanine; ΔPhe(2-Me), (*Z*)-α,β-dehydro 2-methylphenyl alanine; ΔPhe(2,5-Me), (*Z*)-α,β-dehydro 2,5-dimethylphenyl alanine; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBrop, bromo-tris-pyrrolidino phosphonium hexafluorophosphate; RUT, rat uterus; Thi, β-2-thienylalanine; TES, *N*-Tris(hydroxy-methyl)methyl-2-aminoethane sulphonic acid; WANG-resin, *p*-benzyloxycarbonyl alcohol-resin.

CCC 1075-2617/98/020092-09

Address for correspondence: Prof. Dr. S. Reißmann, Friedrich-Schiller-University of Jena, Institute of Biochemistry and Biophysics, Philosophenweg 12, D-07743 Jena, Germany.

INTRODUCTION

Antagonists of the nonapeptide bradykinin are of great therapeutical interest, because of their potential use in treatment of rhinitis, bronchitis, sepsis, asthma, pain and other allergic diseases or kinds of inflammation. Since the development of the first antagonist in 1984 by Vavrek and Stewart [1], new generations of more active analogues have been developed (for a review see [2]) and some of these are undergoing clinical trials. More recently some groups have engaged in the development of non-peptide antagonists [3,4].

All the recently designed bradykinin antagonists are based on the same key modification, the replacement of proline in position 7 by aromatic or hydrophobic D-amino acids. In comparison with D-Phe many of these replacements enhance antagonistic potency. But, until now, it has been impossible to understand why these compounds act as antagonists. There have been some attempt to determine the receptor-bound conformations of agonists and antagonists [5–11]. But the findings on the conformational differences of agonists and antagonists are to some degree contradictory, e.g. in regard to β-turns in the N-terminal or C-terminal sequence.

In preceding work we have studied, more or less systematically, the structural requirements of bradykinin antagonists. Thus, we replaced proline in position 7 by series of C^{α}- and C^{β}-substituted amino acids or ring-substituted phenylalanines ([12], and manuscript in preparation). The substitution of proline in position 2 by *N*-methylphenylalanine provides an antagonist on the guinea pig lung strip without any other amino acid replacements in the sequence [13–15]. From these studies we draw the conclusion that there is available more than one key modification to convert bradykinin agonists into antagonists.

A systematic search on the influence of position 5 on biological activity led us to another structure of antagonists. The replacement of phenylalanine in position 5 by the *Z*-configuration of dehydrophenylalanine or its ring-substituted analogues provides partial antagonists, with antagonist activity at very low or low concentrations and agonist activities at higher concentrations. To study the influence of ΔPhe on the biological activity we have incorporated it into different representative bradykinin agonists and antagonists. The systematic search for new structures of bradykinin antagonists helps to understand the structural and conformational requirements for antagonists and helps furthermore to develop more selective peptide and non-peptide therapeutics for treatment of different kinds of inflammation processes.

MATERIALS AND METHODS

Syntheses of the Peptides

Fmoc-amino acids, D,L-Phe(4-F) and D,L-Phe(4-I) were purchased from Bachem, Switzerland, and Novabiochem, Switzerland. Fmoc-D-Tic and Fmoc-Oic were a gift from Hoechst AG. D,L-Phe(2-Me) and D,L-Phe(2,5-Me) were synthesized by the acetamidomalonate method [16].

General Procedure for the Synthesis of Fmoc-Gly-ΔPhe(X)-OH. The ring-substituted racemic phenylalanine analogues (10 mmol) were converted to the corresponding analogues of glycyl-(*Z*)-α-β-dehydro phenylalanine according to a procedure described by Phelps and Gaeta [17] with minor modifications. By this procedure each phenylalanine analogue was lyophilized and suspended with dichloroacetic anhydride (4.80 g, 20 mmol) in 50 ml ethyl acetate. After refluxing for 2 h, the mixture was cooled to room temperature, 10 ml 1N HCl were added, and the mixture was stirred overnight.

After addition of 100 ml ethyl acetate the organic layer was washed with saturated sodium chloride solution. The organic solution was dried with anhydrous sodium sulphate and evaporated to dryness giving a solid residue, which was treated with hexane and dried *in vacuo* over potassium hydroxide. The crude chloroacetylated analogue of phenylanaline was dissolved in a solution of 75 ml methanol and 75 ml saturated aqueous ammonia. After stirring for 48 h at room temperature in a closed vessel the solvent was evaporated to dryness, the residue was washed with acetone, and finally recrystallized from water to remove traces of ammonium chloride.

Some 3 mmol of each dipeptide was dissolved in 50% aqueous dioxane containing sodium hydrogen carbonate (0.50 g, 6 mmol). Fmoc-OSu (1.01 g, 3 mmol) was added. After stirring overnight, the solvent was evaporated and the residue was treated with a mixture of ethyl acetate and 5% potassium hydrogen sulphate. The organic phase was extracted three times with 5% potassium hydrogen sulphate and saturated sodium chloride solution. After drying with anhydrous sodium sulphate, the organic solu-

Table 1 Chemical Characterization of Fmoc-Gly-ΔPhe(X)-OH Building Units

No.	Compound	Formula	MW	FAB-MS MH	m.p. (°C)	TLC [R_F][c] I	TLC [R_F][c] II	HPLC[a] K′	Elementary analysis [%][b] C	H	N
18	Fmoc-Gly-ΔPhe-OH	$C_{26}H_{22}N_2O_5$	442.2	443.4	161–165	0.45	0.15	20.0	69.80	4.95	6.03
									(70.58)	(5.01)	(6.33)
19	Fmoc-Gly-ΔPhe(2-Me)-OH	$C_{27}H_{24}N_2O_5$	456.2	457.1	207–210	0.50	0.20	21.3	70.58	5.19	6.15
									(71.04)	(5.30)	(6.14)
20	Fmoc-Gly-ΔPhe(2,5-Me)-OH	$C_{28}H_{26}N_2O_5$	470.2	471.4	203–205	0.60	0.25	23.5	70.86	5.55	5.90
									(71.48)	(5.57)	(5.95)
21	Fmoc-Gly-ΔPhe(4-F)-OH	$C_{26}H_{21}N_2O_5F_1$	460.1	461.2	177–181	0.50	0.15	20.6	67.20	4.52	6.01
									(67.82)	(4.60)	(6.08)
22	Fmoc-Gly-ΔPhe(4-I)-OH	$C_{26}H_{21}N_2O_5I_1$	568.1	569.3	138–142	0.45	0.15	23.8	55.47	3.63	4.89
									(54.94)	(3.72)	(4.93)

[a]HD-SIL-C18-5-80, 4 × 250 mm; gradient, 20–80% A/60 min; A, acetonitrile/0.1%TFA; B, water/0.1%TFA, 1 ml/min.
[b]( ) C,H,N calculated.
[c]solvent I, $CHCl_3$/MeOH/acetic acid (9 : 1 : 0.1); solvent II, benzene/acetone/acetic acid (27 : 10 : 0.5).

tion was evaporated to dryness. The substituted Fmoc-Gly-ΔPhe(X)-OH dipeptide was recrystallized from ethyl acetate/hexane (for analytical characterization see Table 1).

Solid-phase Synthesis, Purification and Analytical Characterization of the Bradykinin Analogues. The peptides were synthesized on a solid support according to the Fmoc/Bu^t-strategy starting with Fmoc-Arg(Mtr)-Wang-resin (Bachem, 0.55 mmol/g) on a 0.05 mmol scale. Common couplings were performed twice with TBTU/DIEA in DMF using a 4-fold excess of carboxy component. Fmoc-NMePhe and Fmoc-Gly-ΔPhe(X)-OH dipeptides were coupled with PyBop/DIEA. Couplings to NMePhe were performed with PyBrop. The nonapeptides were cleaved from the resin using a cocktail of trimethylbromosilane/TFA/thioanisole (11%:83%:6%) for 3 h at 4 °C.

The analogues were purified by RP-HPLC (Shimadzu LC-8A, SPD-6A) using a VYDAC C8 reversed-phase column (25 × 2.2 cm) with a linear gradient from 20 to 50% CH_3CN in 0.1% aqueous trifluoracetic acid at a flow rate of 8 ml/min with UV detection at 233 nm. The purity of the free peptides was investigated by analytical HPLC with gradient elution (see details in Table 2). Amino acid analyses and molecular weights were in good agreement with the calculated values (Table 2).

Pharmacological Assays and Receptor Binding Assay

Rat Uterus (in Estrus). Female Wistar rats weighing 180–200 g were injected with diethylstilbestrol (100 μg/kg s.c.) 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 500 mg. BK (1 or/and 2×10^{-9} M) was added to the organ bath at 4 min intervals and left in the organ bath for 45 s. The activity of the peptides was estimated by means of the four-point method described by Schild [18]. To investigate an inhibitory effect, peptides were added to the organ bath 5 min before cumulative dose–response curves for BK were made. Antagonistic activities are expressed and calculated as pA_2-value (−log of the concentration of the antagonist that reduces the effect of a double dose of an agonist to that of a single dose) [19].

Bradykinin Agonism (%) and Antagonism (pA_2) on the Guinea Pig Ileum. Guinea pigs of either sex (200–250 g body weight) were killed by a blow on the neck and exsanguinated. The ileum was excised, washed and prepared to about 20 cm length. From this strip segments of 1.5 cm length (unstretched) were cut for use in experiments. The segments were suspended in an organ bath containing Tyrode solution at 37 °C.

Isotonic contractions were recorded under a resting tension of 500 g. BK (5 and 10×10^{-8} M) was added to the organ bath at 4 min intervals and left in the organ bath for 30 s. The agonistic activity of the analogues was estimated by means of the four-point method described by Schild [18]. Antagonistic activities were expressed and calculated as pA_2-values [19] after recording cumulative dose–response curves of BK. The analogues were added

Table 2 Analytical Data of Bradykinin Analogues

No.	Peptide	Amino acid analysis[a]								Formula	MW	FAB-MS MH	HPLC K'[b]
		Pro	Phe	Gly	Arg	Ser	Oic	Tic	Hyp				
2	[ΔPhe^5]-BK	3.02	1.03	1.00	1.98	0.97	–	–	–	$C_{50}H_{71}N_{15}O_{11}$	1057.6	1058.6	5.41
3	[ΔPhe(2-Me)^5]-BK	3.04	1.07	1.00	1.95	0.95	–	–	–	$C_{51}H_{73}N_{15}O_{11}$	1071.6	1072.7	6.00
4	[ΔPhe(2,5-Me)^5]-BK	2.99	1.05	1.00	1.69	0.87	–	–	–	$C_{52}H_{75}N_{15}O_{11}$	1085.6	1086.7	6.56
5	[ΔPhe(4-I)^5]-BK	3.28	1.05	1.00	1.97	0.91	–	–	–	$C_{50}H_{70}N_{15}O_{11}I_1$	1183.4	1184.6	6.94
6	[ΔPhe(4-F)^5]-BK	3.12	1.06	1.00	1.97	0.94	–	–	–	$C_{50}H_{70}N_{15}O_{11}F_1$	1075.5	1076.9	5.73
9	[ΔPhe^5, DPhe^7]-BK	1.99	1.95	1.00	1.94	0.88	–	–	–	$C_{54}H_{73}N_{15}O_{11}$	1107.6	1108.5	7.93
10	[ΔPhe^5, DTic^7, Oic^8]-BK	1.83	–	1.00	1.94	0.86	1.01	0.99	–	$C_{55}H_{76}N_{15}O_{11}$	1122.6	1123.3	7.18
11	[Hyp^3, ΔPhe^5, DTic^7, Oic^8]-BK	0.92	–	1.00	1.90	0.88	0.98	1.06	0.99	$C_{55}H_{76}N_{15}O_{12}$	1138.6	1140.5	6.90
12	[DArg^0, Hyp^3 ΔPhe^5, DTic^7, Oic^8]-BK	0.94	–	1.00	2.90	0.87	0.98	1.24	0.98	$C_{61}H_{87}N_{19}O_{13}$	1293.7	1295.7	6.91
15	[ΔPhe^5, DNMePhe^7]-BK	1.97	0.99	1.00	1.98	0.92	–	–	–	$C_{55}H_{75}N_{15}O_{11}$	1121.6	1122.5	6.93
16	[NMePhe^2, ΔPhe^5]-BK	2.01	1.02	1.00	1.95	0.96	–	–	–	$C_{55}H_{75}N_{15}O_{11}$	1121.6	1122.7	7.38
17	[NMePhe^2, ΔPhe^5, DNMePhe^7]-BK	0.98	1.05	1.00	1.97	0.89	–	–	–	$C_{56}H_{77}N_{15}O_{11}$	1135.6	1136.8	8.50

[a]reference amino acid: Gly, ΔPhe decompose during hydrolysis with 6N HCl to NH_3 and α-keto acid.
[b]Vydac C18, 4 × 250 mm; gradient, 10–50% A/40 min; A, acetonitrile/0.1%TFA; B, water/0.1%TFA, 1 ml/min.

5 min to the bath before the cumulative does–response curves for BK were made.

To investigate an inhibitory effect in low concentrations, contraction of the ileum segments was recorded *isometrically* with a load of 1 g on the tissues. After an equilibration period of about 30 min a stable baseline tone was reached. Then the segments were contracted with BK (4×10^{-8} M). At the end of the contraction the isolated tissue was washed for 3 min and then allowed to rest for 20 min. At that time, the segments had relaxed to baseline levels. Two or three control contractions were carried out in the absence of the test compounds, and only segments producing reproducible recordings (± 15%) were used subsequently. The last response was taken as 100% and subsequent results were expressed at a percentage of this. The test substances were incubated with the segments for 10 min repeating the contraction. IC_{50} values were estimated according to the earlier described procedure [20].

Guinea Pig Pulmonary Artery The pulmonary artery was quickly removed from stunned guinea pigs (450–500 g) and immersed in Ringer solution at room temperature. Spirally cut strips (45°, 25 × 4 mm) were suspended with a preload of 0.5 g in a 10 ml bath with Ringer solution at 37 °C at pH 7.4 and oxygenated with 95% O_2 and 5% CO_2. Isotonic recordings were made with a lever displacement measuring system (Hugo Sachs Elekronik KG, Freiburg, Germany). The composition of the Ringer solution was as follows (mM): NaCl 154, KCl 5.6, $CaCl_2$ 1.9, $NaHCO_3$ 2.4, glucose 5.0. After an equilibration period of 1 h, each strip was contracted by BK at a final bath concentration of 2×10^{-7} M. After a washout, the pulmonary arteries were preincubated in concentrations of 2×10^{-7} to 2×10^{-5} M for 10 min. Using this approach a bradykinin-like effect could be demonstrated. Additional administration also enabled an antagonistic effect or a potentiation to be demonstrated.

Radioligand Binding Studies

Preparation of rat myometrial as well as guinea pig ileum membranes and binding assays were carried out as described in detail [21] with minor modifications. Briefly, membranes (between 0.3 to 0.5 mg 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 μM DTE, bacitracin (140 μg/l), bovine serum albumin

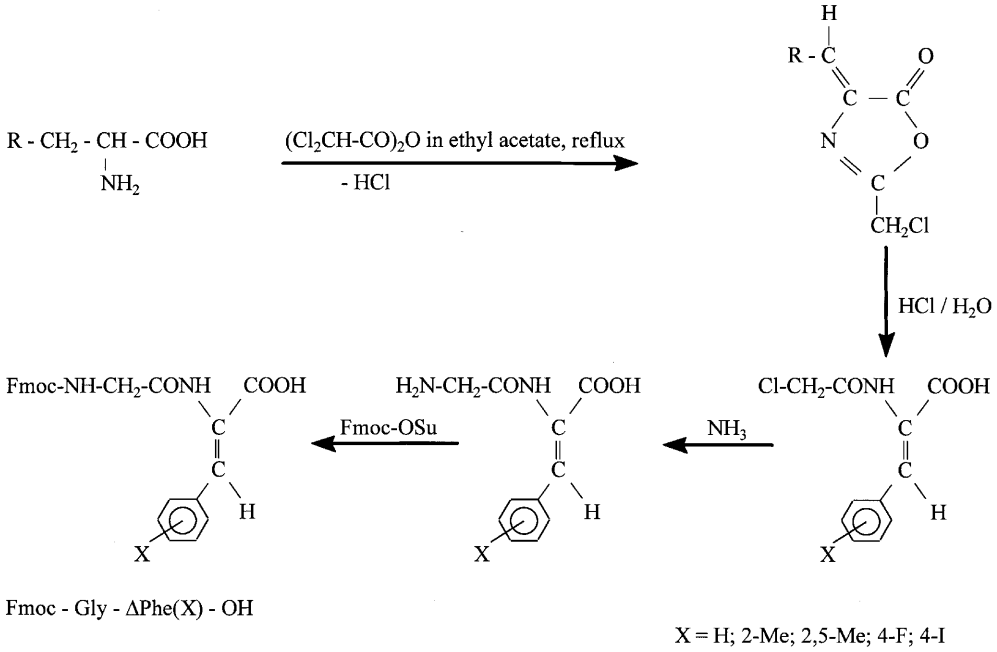

Figure 1 Route of synthesis for Fmoc-Gly-⊿Phe(X)-OH.

(1 g/l), [3H] BK (0.03–0.05 nM, obtained from NEN with a specific activity of 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 fibre filters pretreated with 0.1% (w/v) aqueous polyethylenimine solution using a Brandel harvester. The filters were washed three times with 5 ml TES buffer (10 mM, pH 6.8), transferred into scintillation vials, dried and counted for radioactivity in 6 ml of a toluene-based scintillator. Non-specific binding was determined in the presence of 1 μM BK. The IC_{50}-values were calculated using the programme of Tobler and Engel [22].

RESULTS

The protected dipeptides Fmoc-Gly-⊿Phe(R)-OH were synthesized by the route shown in Figure 1. The syntheses were performed according to the procedure described by Phelps and Gaeta [17]. Yields and chemical characterization of the ⊿Phe derivatives are given in Table 1. This procedure gives a dehydrophenylalanine residue in the *Z*-configuration [17]. Peptide syntheses were performed on the solid support using Wang-resin. No complications resulting from the dehydrophenylalanine were observed. The incorporation of ⊿Phe into peptides together with *N*-methylphenylalanine (sterically hindered) requires strong activation. The synthetic route for one of those analogues is shown in Figure 2. Some of the peptides were purified by gel chromatography and additionally by semi-preparative HPLC.

The ⊿Phe-containing bradykinin analogues, published first by Fisher *et al.* [23], show a high agonistic activity. That amino acid was incorporated into potent agonists and antagonists, to check its influence on the activity. Furthermore ring-substituted dehydrophenylalanine analogues were used to estimate the influence of the ring-substitution on the activity. From preceding work with bradykinin agonists and antagonists the necessity of estimating the bioactivity at different organs [9, 13, 14, 15, 24]

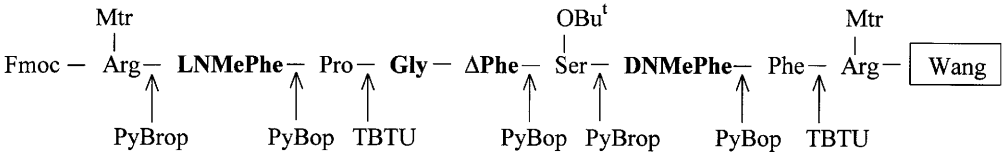

Figure 2 Synthesis of bradykin analogues with ⊿Phe and NMePhe.

Table 3 Biological Activities on Different Smooth Muscle Organs

		Agonistic activity (%)		Antagonistic activity (GPI)	
No.	Peptide	RUT	GPI	IC_{50}	pA_2
1	BK	100	100	–	–
	1. Series: ΔPhe and ΔPhe analogue at position 5				
2	[ΔPhe^5]-BK	170 ± 48	150 ± 40	8.8×10^{-9}	–
3	[ΔPhe(2-Me)^5]-BK	28 ± 7	3.7 ± 0.7	1.2×10^{-6}	–
4	[ΔPhe(2,5-Me)^5]-BK	0.30 ± 0.02	0.07 ± 0.01	1.0×10^{-6}	–
5	[ΔPhe(4-I)^5]-BK	16.5 ± 6.5	14.5 ± 6.5	3.0×10^{-8}	–
6	[ΔPhe(4-F)^5]-BK	113 ± 48	121 ± 33	7.6×10^{-8}	–
	2. Series: Incorporation of ΔPhe into antagonists with key modification at position 7				
7	[DPhe^7]-BK	1.70 ± 0.39	0	$> 10^{-5}$	4.90 ± 0.32
8	HOE-140	0	0	1.1×10^{-8}	8.41 ± 0.48
9	[ΔPhe^5, DPhe^7]-BK	0	0	3.1×10^{-7}	5.76 ± 0.31
10	[ΔPhe^5, DTic^7, Oic^8]-BK	0	0	2.7×10^{-8}	7.33 ± 0.50
11	[Hyp^3, ΔPhe^5, DTic^7, Oic^8]-BK	0	0	1.1×10^{-7}	6.98 ± 0.25
12	[DArg^0, Hyp^3, ΔPhe^5, DTic^7, Oic^8]-BK	0	0	1.1×10^{-8}	7.71 ± 0.64
	3. Series: combination of ΔPhe with NMePhe at positions 2 and 7				
13	[DNMePhe^7]-BK	136 ± 23	0.04 ± 0.01	–	–
14	[NMePhe^2]-BK	35 ± 4	4.0 ± 0.8	–	–
15	[ΔPhe^5, DNMePhe^7]-BK	7.5 ± 1.5	0.30 ± 0.10	–	–
16	[NMePhe^2, ΔPhe^5]-BK	183 ± 129	31.6 ± 10.2	–	–
17	[NMePhe^2, ΔPhe^5, DNMePhe^7]-BK	3.08 ± 2.00	0.05 ± 0.03	–	–

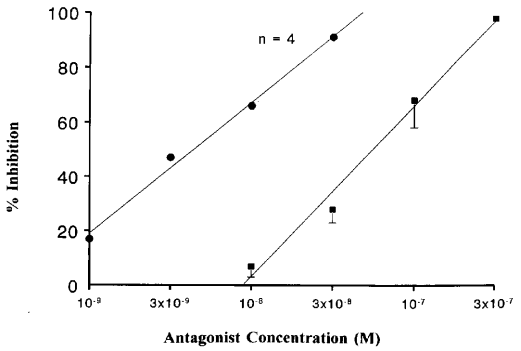

Figure 3 Inhibition of BK-induced contraction in the isolated pulmonary artery of the guinea pig by HOE-140 (●) and [ΔPhe^5]-BK (■).

had been established. Thus, the smooth muscle organs rat uterus (RUT), guinea pig ileum (GPI) and guinea pig pulmonary artery (PA) were used. Very surprisingly, bradykinin analogues with ΔPhe or ΔPhe(X) showed inhibition of bradykinin-induced contractions on GPI and PA in low concentrations, lower than necessary for agonistic action (Table 3). The analogues **2, 3, 4, 5** act as partial agonists; they inhibit the bradykinin action and act at higher concentrations as agonists. Compound **2** shows a parallel shift of the dose–response curve on the pulmonary artery: this means a pure competitive antagonism to BK (Figure 3).

In radio-ligand binding studies BK was found to recognize two binding sites, one with pM (K_H-site) and one with nM (K_L-site) affinity in both RUT [25] and GPI [26] membranes. Compared with BK showing binding affinities (IC_{50}-values) of 13 pM and 0.6 nM at RUT membranes and 5 pM and 0.3 nM at GPI, very similar IC_{50}-values were obtained for compound **2** at GPI (4 pM and 0.4 nM), whereas at RUT membranes the affinity towards the K_H-site was slightly enhanced (3 pM versus 0.5 nM). In contrast, compound **3** displayed a clearly reduced affinity to the K_L-site only with IC_{50}-values of 13 pM (RUT) and 9 nM (GPI), respectively (Figure 4).

The investigations of the effect of combining dehydrophenylalanine in position 5 with such other amino acid replacements as yield activity in other antagonist types were disappointing. These analogues are in most cases less active than with one modification alone. Only the combination with D-Phe in position 7 (Table 2, compound **7**) provides an enhanced antagonistic activity (pA_2-value) on GPI, enhanced for about one order of magnitude.

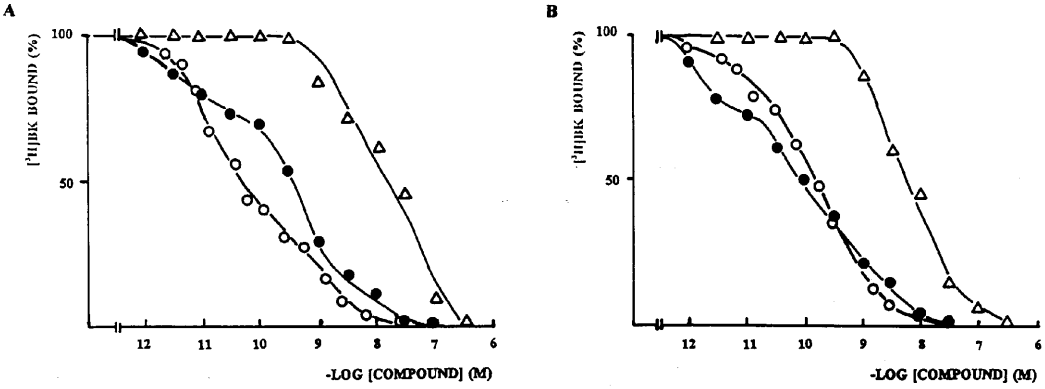

Figure 4 Inhibition of specific [^{3}H]BK (0.03–0.05 nM) binding to membranes from rat uterus (A) and guinea pig ileum (B) by BK (○), [Δ^{5}Phe]-BK (●) and [Δ^{5}Phe(2-Me)]-BK (△). Each point is the mean of two separate experiments with duplicate determinations. Interassay determinations differed by $< 5\%$.

DISCUSSION

The biological activities of the new bradykinin analogues containing Δ Phe or its ring-substituted analogues in position 5 emphasize the important role of that sequence position. In good agreement with findings from Fisher *et al.* [23] the agonist activity of bradykinin analogues without modification in position 7 was enhanced by Δ Phe and its analogues.

The binding curves underline the role of modifications in position 5 for the recognition of the high- and low-affinity binding site. In both, RUT and GPI, the BK-induced contraction appears to be mediated via the K_L-site by stimulation of inositoltriphosphate formation [27] and, subsequently, increase in cytosolic Ca concentration. The biological function of the K_H site which inhibitorily coupled to adenylate cyclase in both tissues [28, 29] is not yet understood. Neither the binding affinity nor the agonist activity were significantly changed by replacement of Phe by Δ Phe in position 5. In contrast, ring-methylation of Δ Phe resulted in a clearly reduced affinity to K_L as well as in a drastically diminished biological activity in both tissues.

Surprisingly, all the Δ Phe-containing analogues investigated act as partial antagonists on GPI and PA, having both agonist and antagonist activity. They inhibit at low concentrations the bradykinin-induced contraction and act at higher concentrations as agonists. Because GPI and PA are organs with B_2 receptor, this finding encouraged us to consider these compounds as a new structural type of bradykinin B_2 receptor antagonists. This new type does not require any amino acid replacement in position 7. This result is in agreement with very early findings of Stewart and Woolley [30] who estimated antibradykinin activity (partial antagonism) in analogues with modifications in positions 5 and 8, without modification in position 7.

The conformational constraints resulting from Δ Phe could help to define the conformational requirements. The flexibility of both the dehydropeptide backbone as well as the specific side chain of the dehydro residue is expected to be restricted on account of the double bond between C^α and C^β atoms [31, 32]. Many authors have postulated turns in the bradykinin molecule, as well as in the N-terminal part and in the C-terminal pentapeptide. In contrast to these assumptions the analogues with Δ Phe seem to give evidence for a turn in the middle of the nonapeptide.

Ring-substitution of Δ Phe influences the agonistic and antagonistic activities differently. Methylation of the ring reduces both the antagonist and agonist activity. That fact could be caused by the positive I-effect of the methyl groups together with their steric hindrance by the receptor binding. Despite the negative I-effect of iodine the *para* substitution of Δ Phe reduces the agonistic and antagonistic activity (compound **5**). Only the strongly electronegative and very small fluorine atom gives an analogue (compound **6**) with nearly the same agonistic and antagonistic activity as the lead compound **2**. The result is in good agreement with the high agonistic activity of a BK-analogue with *p*-fluorophenylalanine in position 5, developed by Fisher *et al.* [33].

Incorporation of Δ Phe into antagonists with amino acid replacements in position 7 (compounds **9, 10, 11, 12,**) leads to compounds with antagonistic potencies in the same range as for the basic

structures. There is only an enhancement of the antagonistic pA_2-value by combination of ΔPhe in position 5 with D-Phe in position 7 (compound **9**). But, the IC_{50}-value is higher for (ΔPhe^5)-BK alone.

In the highly active agonist **13** the incorporation of ΔPhe in position 5 (compound **15**) reduces the activity strongly, indicating different conformational requirements for agonists and antagonists.

The new analogues indicate that there exist more than one possibility for converting bradykinin agonists into antagonists. Analogues with ΔPhe in position 5 seem to represent a new structural type of B_2-receptor antagonistic. Because of their agonistic activity these analogues are not of therapeutic interest, but they could help to define the different structural and conformational requirements for agonists and antagonists. Their novelty lies in their structure, not in their activity. Also the first generation of bradykinin antagonists with D-phenylalanine at position 7 showed agonistic and antagonistic activity depending on the smooth muscle organ. With the increasing antagonistic potency their partial antagonism disappeared by developing the next generations of this antagonist type.

To find new structural types of antagonists is of great importance from a theoretical point of view. We conclude from these findings that attemps in the literature to find an optimum antagonist with an optimum structure and conformation are misconceived. We think that there exist different types of antagonist that disturb the interaction of bradykinin with its receptor in different ways.

Acknowledgements

We thank B. Schilling, H. Rohde and I. Schumann for skilful technical assistance. We gratefully acknowledge the assistance of G. Eberhardt in the preparation of this manuscript. D-Tic- and Oic-derivatives used were a gift from the Hoechst AG. The work was performed with financial support of the Bundesministerium fü;r Forschung und Technologie (BEO 21/19992A) and of the Hoechst AG.

REFERENCES

1. R. J. Vavrek and J. M. Stewart (1985). Competitive antagonists of bradykinin. *Peptides 6*, 161–164.
2. J. M. Stewart (1995). Bradykinin antagonists: Development and applications. *Biopolym. (Peptide Sci.) 37*, 143–155.
3. J. M. Salvino, P. R. Seoane, B. D. Douty, M. A. Awad, D. Hoyer, T. M. Ross, R. E. Dolle, W. T. Houck, D. M. Faunce and D. G. Sawutz (1995). Structure activity relationships of non-peptide bradykinin B_2 receptor antagonists. *Bioorg. Med. Chem. Lett. 5*, 357–362.
4. D. G. Sawutz, J. M. Salvino, R. E. Dolle, F. Casiano, S. J. Ward, W. T. Houck, D. M. Faunce, B. D. Douty, E. Baizman, M. M. A. Awad, F. Marceau and P. R. Seoane (1994). The nonpeptide WIN 64338 is a bradykinin B_2 receptor antagonist. *Pharmacology 91*, 4693–4697.
5. S. Chakravarty, D. Wilkins and D. J. Kyle (1993). Design of potent, cyclic peptide bradykinin receptor antagonists from conformationally constrained linear peptides. *J. Med. Chem. 36*, 2569–2571.
6. W. Guba, R. Haeßner, J. Knolle and H. Kessler (1995). A novel combined approach of NMR and molecular dynamics within a biphasic membran mimetic-conformation and orientation of the bradykinin antagonist Hoe 140. *J. Am. Chem. Soc. 116*, 7532–7540.
7. D. J. Kyle, S. Chakravarty, J. A. Sinsko and T. M. Stormann (1994). A proposed model of bradykinin bound to the rat B_2 receptor and its utility for drug design. *J. Med. Chem. 37*, 1347–1354.
8. A. Otter, P. Bigler, J. M. Stewart and G. Kotovych (1993). A proton magnetic resonance study of two synthetic agonist–antagonist pairs of bradykinin analogues. *Biopolymers 33*, 769–780.
9. L. Seyfarth, L. F. Pineda De Castro, I. Liepina, I. Paegelow, C. Liebmann and S. Reissman (1995). New cyclic bradykinin antagonists containing dsulfide and lactam bridges at the N-terminal sequence. *Int. J. Peptide Protein Res. 46*, 155–165.
10. J. K. Young and R. P. Hicks (1994). NMR and molecular modeling investigations of the neuropeptide bradykinin in three different solvent systems: DMSO, 9 : 1 dioxane/water, and in the presence of 7.4 mM lysophosphatidylcholine micelles. *Biopolymers 34*, 611–623.
11. M. Pellegrini and D. F. Mierke (1997). Threonine^6-bradykinin: Molecular dynamics simulations in a biphasic membrane mimetic. *J. Med. Chem. 40*, 99–104.
12. S. Reissmann, G. Greiner, C. Schwuchow, L. F. Pineda De Castro and C. Liebmann (1993). In: *Chemistry of Peptides and Proteins*, D. Brandenburg, V. Ivanov and W. Voelter, Eds., p. 377–388, DWI Reports, Aachen.
13. S. Reissmann, C. Schwuchow, L. Seyfarth, L. F. Pineda De Castro, C. Liebmann, I. Paegelow, H. Werner and J. M. Stewart (1996). Highly selective bradykinin agonists and antagonists with replacement of proline residues by D- and L-*N*-methylphenylalanine. *J. Med. Chem. 39*, 929–936.
14. S. Reissmann, G. Greiner, L. Seyfarth, I. Paegelow, H. Werner, G. Vietinghoff, S. Boeckmann, E. Schultz, U. Wartner and L. Gera (1996). A new type of bradykinin antagonists: Analogues with *N*-alkyl amino acids in position 2. *Immunopharmacology 33*, 73–80.
15. I. Paegelow, H. Werner and S. Reissmann (1995). Effects of bradykinin and bradykinin analogues on spleen cells of mice. *Eur. J. Pharmacol. 279*, 211–216.

16. J. Porter, J. Dykert and J. Rivier (1987). Synthesis, resolution and characterization of ring substituted phenylalanines and tryptophans. *Int. J. Peptide Protein Res. 30*, 13–21.
17. D. J. Phelps and F. C. A. Gaeta (1982). A convenient synthesis of glycyl-(β-aryl)-dehydroalanines. *Synthesis 1982*, 234–235.
18. H. O. Schild (1942). A method of conducting a biological assay on a preparation giving repeated graded responses illustrated by the estimation of histamine. *J. Physiol. 101*, 115.
19. J. M. van Rossum, J. A. Th. M. van Hurkmans and C. J. J. Wolters (1963). Cumulative dose–response curves. II. Technique for the making of dose–response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. 143*, 299–330.
20. F. J. Hock, K. Wirth, U. Albus, W. Linz, H. J. Gerhards, G. Wiemer, St. Henke, G. Breipohl, W. König, J. Knolle and B. A. Schölkens (1991). Hoe 140 a new potent and long acting bradykinin antagonist: *In vitro* studies. *Br. J. Pharmacol. 102*, 769–773.
21. C. Liebmann, St. Nawrath, B. Ludwig and I. Paegelow (1993). Pharmacological and molecular actions of the bradykinin B_2 receptor antagonist, Hoe 140, in the rat uterus. *Eur. J. Pharmacol. 235*, 183–188.
22. H. J. Tobler and G. Engel (1983). Affinity spectra: A novel way for the evaluation of equilibrium binding experiments. *Naunyn-Schmiedeberg's Arch. Pharmacol. 322*, 183–192.
23. G. H. Fisher, P. Berryer, J. W. Ryan, V. Chauhan and C. H. Stammer (1981). Dehydrophenylalanyl analogues of bradykinin: Synthesis and biological activities. *Arch. Biochem. Biophys. 211*, 269–275.
24. G. Vietinghoff, I. Paegelow and S. Reissmann (1996). Induction of histamine release from rat mast cells by bradykinin analogues. *Peptides 17*, 1467–1470.
25. C. Liebmann, S. Offermanns, K. Spicher, K.-D. Hinsch, M. Schnittler, J. L. Morgat, S. Reissmann, G. Schultz and W. Rosenthal (1990). A high-affinity bradykinin receptor in membranes from rat myometrium is coupled to pertussis toxin-sensitive G-proteins of the G_i-family. *Biochem. Biophys. Res. Commun. 167*, 910–917.
26. C. Liebmann, R. Bossé and E. Escher (1994). Discrimination between putative bradykinin B_2 receptor subtypes in guinea pig ileum smooth muscle membranes with a selective, iodinatable, bradykinin analogue. *Mol. Pharmacol. 46*, 949–956.
27. R. W. Ransom, C. B. Goodman and G. S. Young (1992). Bradykinin stimulation of phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle. *Br. J. Pharmacol. 105*, 919–924.
28. C. Liebmann, K. Mammery and A. Graness (1994). Bradykinin inhibits adenylate cyclase activity in guinea pig ileum membranes via a separate high-affinity bradykinin B_2 receptor. *Eur. J. Pharmacol. 288*, 35–43.
29. C. Liebmann and S. Reissmann (1990). Bradykinin inhibits rat myocetrial adenylate cyclase activity via a high-affinity receptor. *Biomed. Biochim. Acta. 49*, 1231–1234.
30. J. M. Stewart and D. W. Woolley (1966). In: *Hypotensive Peptides.*, E. G. Erdös, N. Back and F. Sicuteri, Eds., p. 23–31, Springer, Berlin.
31. R. Jain and V. S. Chauhan (1996). Conformational characteristics of peptides containing α,β-dehydroamino acid residues. *Biopolym. (Peptide Sci.) 40*, 105–119.
32. G. Pietrzyński, B. Rzeszotarska, E. Cizak and M. Lisowski (1994). Turn tendency of model α,β-dehydropeptides. *Polish J. Chem. 68*, 1015–1021.
33. G. H. Fisher, J. W. Ryan and P. Berryer (1977). Biologically active bradykinin analogues containing *p*-fluorophenylalanine. *Cardiovas. Med. 2*, 1179–1181.

