Structure-activity studies on bradykinin and related peptides: agonists

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- 1 Bradykinin, kallidin, T-kinin, [Hyp³]-bradykinin and several analogues were prepared by solid-phase synthesis and purified by high performance liquid chromatography.
- 2 The various peptides were tested for their abilities to relax the dog carotid and renal arteries, or to contract the rabbit jugular vein and aorta, in order to measure their activities on BK₂ (the first three preparations) or BK₁ (the rabbit aorta) receptors. The dog renal artery without endothelium was also used as a BK₁ receptor system.
- 3 T-kinin was found to be less active than bradykinin, while the replacement of Pro³ with Hyp favoured BK₂ receptor occupation. [Hyp³,Tyr(Me)⁸]-BK was found to be a selective BK₂ receptor agonist.
- 4 Amidation or methylation of the C-terminal carboxyl decreased activity, while extension of the N-terminal with Sar or D-Arg increased affinity and selectivity for BK₁ (Sar) and affinity for BK₂ (D-Arg) receptors. Acetylation of N-terminal amide brought affinity down to 10% or less.
- 5 Replacement of the peptide bonds Phe⁸-Arg⁹ to protect from kininase I and II, decreased affinities slightly, but was incompatible with additional changes at the N-terminal or in the peptide bond Gly⁴-Phe⁵.
- 6 Substitution of C-terminal Phe in desArg⁹-BK (the BK₁ receptor stimulant) with D-Phe increased potency and selectivity for BK₁ receptors while protecting from carboxypeptidases. Sar[D-Phe⁸]desArg⁹-BK was found to be a potent and selective BK₁ receptor agonist.

Introduction

New kinins such as [Hyp⁴]-kallidin and T-kinins have recently been discovered (Bedi *et al.*, 1983; Okamoto & Greenbaum, 1983a; Mindriou *et al.*, 1988), but not yet fully characterized. Moreover, the need of selective kinins that act as pure agonists or antagonists on BK₁ or BK₂ receptors is strongly felt, especially after the reports that there may be new types of kinin receptors (Rifo *et al.*, 1987; Plevin & Owen, 1988) or that kinins may act indirectly through non-receptor mechanisms, such as the release of prostaglandins in the guinea-pig trachea (Rhaleb *et al.*, 1988) or that of histamine from rat peritoneal mast cells (Devillier *et al.*, 1985; 1988).

In the present study, some naturally occurring kinins and a few analogues of bradykinin, kallidin or desArg⁹-bradykinin were prepared and tested in various pharmacological preparations in order to identify compounds that act as pure kinin-receptor agonists and are more selective than the naturally occurring kinins or their metabolites.

Methods

Pharmacological preparations

Experiments were performed on tissues taken from rabbits (Albino New-Zealand, 1.0–1.5 kg) and mongrel dogs (9–15 kg) of either sex. The rabbits were killed by stunning and exsanguination, while the dogs were anaesthetized with sodium pentobarbitone (50 mg kg⁻¹, intraperitoneally) and killed through section of the carotid arteries. Kinins and their analogues were tested on the rabbit jugular vein, the rabbit aorta, the dog carotid artery and the dog renal artery. In previous studies (Bérubé *et al.*, 1978; Gaudreau *et al.*, 1981; Regoli *et al.*, 1977) it was shown that several rabbit veins and the rabbit aorta respond to kinins with concentration-dependent contractions. On the other hand, the dog carotid artery, while contracted with noradrenaline (2.0 × 10⁻⁸ M), responds to kinins with relaxations, provided the endothelium is intact (D'Orléans-Juste *et al.*, 1985), while the dog renal artery

responds to kinins with relaxations both when the endothelium is present or absent (Rhaleb *et al.*, 1989). The tissues were rapidly dissected away from the animals and prepared as strips, as described previously (Rhaleb *et al.*, 1989). They were then suspended in 10 ml organ baths containing warm (37°C), oxygenated (with 95% O₂ and 5% CO₂) Krebs solution of the same composition as that described by Rioux *et al.* (1973). The arterial vessels were stretched with an initial tension of 2 g and the isolated veins with 0.5 g. Changes of tension produced by the various agents were measured with Grass isometric transducers (FT 03C), calibrated as to 1 g = 30 mm. Contractions or relaxations were displayed on Grass Polygraph Model 7D. Before testing the drugs, the preparations were allowed to equilibrate for 90–120 min, during which time the tissues were repeatedly washed and tension adjusted.

Experimental protocols

Concentration-response curves were measured by cumulative (in the dog carotid and renal arteries and rabbit aorta) or consecutive (in the rabbit jugular vein) application of peptides, in order to obtain reliable estimations of the apparent affinities (which are expressed in terms of pD₂) and maximum responses. The contractile effects of kinins and analogues were measured on the rabbit jugular vein treated with captopril (1.1 × 10⁻⁶ M) in order to avoid the degradation of peptides by kininase II (Regoli *et al.*, 1986). The relaxant effects of kinins on dog carotid and renal arteries were evaluated in the presence of intact endothelium after contraction with noradrenaline (NA). Ethylenediamine-tetraacetic acid (EDTA) (5.9 × 10⁻⁵ M) and indomethacin (2.8 × 10⁻⁶ M) were added to the solution to prevent the oxidation of the catecholamines and block the synthesis of prostaglandins which may interfere with both the contractile effects of noradrenaline or the relaxing effects of the kinins. Effect of kinins on BK₁ receptors were evaluated on the rabbit aorta and the dog renal artery without endothelium contracted with NA and treated with EDTA as mentioned above. Removal of endothelium was assessed by the absence of response of the vessels to acetylcholine (2.6 × 10⁻⁶ M). The contractile effects of kinins on the rabbit aorta were measured after a period of incubation of 5 h,

in order to obtain measurable and stable responses (Regoli *et al.*, 1986).

Peptides and other agents

The following peptides were used: bradykinin (BK), kallidin (KD: Lys-bradykinin), BK amide (BK-NH₂), BK methyl ester (BK-OMe), [Tyr(Me)⁸]-BK, [Hyp³]-BK, [Hyp⁴]-KD, Ile-Ser-BK (T-kinin), [Hyp³,Tyr(Me)⁸]-BK, Sar-BK, D-Arg-BK, N-Ac-BK, [Aib⁷]-BK, Sar[Aib⁷]-BK, D-Arg[Aib⁷]-BK, [Phe⁸Ψ(CH₂-NH)Arg⁹]-BK, Sar[Phe⁸Ψ(CH₂-NH)Arg⁹]-BK, [MePhe⁵, Phe⁸Ψ(CH₂-NH)Arg⁹]-BK, [Gly⁴Ψ(CH₂-NH)-Phe⁵, Phe⁸Ψ(CH₂-NH)Arg⁹]-BK, [Phe⁵Ψ(CH₂-NH)Arg⁹]-KD, desArg⁹-BK, desArg¹⁰-KD, [D-Phe⁸]-desArg⁹-BK, Sar[D-Phe⁸]-desArg⁹-BK, Sar[Gly⁴Ψ(CH₂-NH)Phe⁵, D-Phe⁸]-desArg⁹-BK, [Hyp³]-desArg⁹-BK, [Hyp⁴]-desArg¹⁰-KD. All these peptides were prepared in our laboratory. The synthesis and purification of the peptides, except the pseudopeptide analogues (containing the isosteric bond CH₂-NH), were performed with the procedure described by Drapeau & Regoli (1988). The synthesis of the pseudo-peptides was described briefly by Drapeau *et al.* (1988).

Concentrated solutions (1–5 mg ml⁻¹) of all agents were prepared in distilled water and stored at –20°C until used.

Other drugs used were noradrenaline (Sigma), indomethacin (Sigma) EDTA (Fisher) and ascorbic acid (Baker). Solutions of noradrenaline were prepared in 5% ascorbic acid in order to prevent the oxidation of the amines and solutions of indomethacin were prepared in 0.02 M Trizma base (Sigma).

Results are presented in terms of pD₂, (the apparent affinity of agonists, calculated from at least 8 concentration-response curves) and relative activity, calculated as a percentage of that of bradykinin for the BK₂ receptor system and of that of desArg⁹-BK for the BK₁ receptor. pD₂ values evaluated in the rabbit jugular vein and the rabbit aorta were calculated from concentration-response curves in which the maximum effects were measured. pD₂ values measured on the dog carotid and renal artery are derived from concentration-response curves obtained in tissues previously contracted (to approximately 1 g) with noradrenaline and should therefore be considered as approximate.

Results

Pharmacological characterization of naturally occurring kinins and some analogues

The effects of kinins containing a hydroxyproline residue and those of T-kinin (Ile-Ser-BK), are shown in Table 1 in comparison with BK and KD. Replacement of Pro³ by Hyp in bradykinin brings about an increase of activity in the rabbit jugular vein and the dog renal artery with endothelium (two sensitive BK₂ preparations), without change of activity on the rabbit aorta (the BK₁ receptor system).

Results obtained with [Hyp⁴]-kallidin indicate that this peptide is more active than kallidin in the dog carotid and renal arteries and slightly less active than kallidin on the rabbit aorta.

T-kinin was found to be less active than BK in the three BK₂ receptor systems, as well as on the rabbit aorta (Table 1).

In an attempt to find BK₂ receptor agonists more potent and selective than BK, the Pro in position 3 of [Tyr(Me)⁸]-BK was replaced with Hyp. As shown by the data of Table 1, [Hyp³,Tyr(Me)⁸]-BK is very potent on the BK₂ receptor systems (dog carotid and renal arteries and rabbit jugular vein) and is completely inactive on the rabbit aorta. This new compound is therefore the most active and selective BK₂ receptor agonist identified until now.

Analogues of bradykinin modified at the N or C-terminals or in position 7 (Table 2)

In a second series of experiments (Table 2), it was found that amidation of the C-terminal carboxyl is accompanied by drastic decrease of activity on the BK₂ (by a factor of 142 to 1000) and on the BK₁ (by a factor of 10) receptors. Methylation of the terminal carboxyl is better tolerated on the BK₂ than on the BK₁ receptor. Activity of BK-OMe is decreased by 69% to 89% on the BK₂ and by 94% on the BK₁ receptor.

Extension of the peptide chain at the N-terminal with Sar (sarcosine) is fairly well tolerated and it is associated with a small increase of affinity on the BK₁ receptor. Extension of the chain by a D-Arg is favourable both on the BK₂ and BK₁ receptor: D-Arg-BK is more active than BK in all three BK₂ receptor systems and is twice as active as BK on the BK₁. Acetylation of the N-terminal amine function in BK brings about a marked (more than 90%) decrease of activity on the BK₂ and BK₁ receptors.

Data from the literature (Vavrek & Stewart, 1985) indicated that the replacement of Pro⁷ by aminoisobutyric acid (Aib) in BK increases affinity. This modification was made in three compounds and, as shown by the data of Table 3, it brought significant decreases of activities on the BK₂ and even more on the BK₁ receptor.

Pseudopeptide analogues of bradykinin

In order to prevent the degradation of bradykinin and kallidin at the C-terminal end, the peptide bond between Phe⁸ and Arg⁹ of BK or Phe⁹ and Arg¹⁰ of KD were replaced by the pseudopeptide bond CH₂-NH. This modification was also made in the analogue Sar-BK in order to prevent the activity of aminopeptidases. An analogue with two pseudopeptide bonds, [Gly⁴ΨPhe⁵,Phe⁸ΨArg⁹]-BK as well as [MePhe⁵, Phe⁸ΨArg⁹]-BK were also prepared and tested. The purpose of these compounds was to protect the peptide from degradation by endopeptidases that may be active on the Gly⁴-Phe⁵ bond.

The results summarized, in Table 3, indicate that the two C-terminal pseudopeptide analogues of BK and KD show

Table 1 Activities of kinins, some natural homologues and some analogues on isolated vessels containing BK₁ or BK₂ receptors

	Dog				Rabbit			
	Carotid artery		Renal artery		Jugular vein		Aorta	
	pD ₂	RA	pD ₂	RA	pD ₂	RA	pD ₂	RA
Bradykinin (BK)	8.64	100	9.85	100	8.48	100	6.22	100
Kallidin (KD)	8.44	63	9.70	71	8.63	141	7.27	1122
[Hyp ³]-BK	8.62	98	10.14	195	8.88	254	6.17	89
[Hyp ⁴]-KD	8.73	123	10.10	178	8.48	100	7.22	1000
Ile-Ser-BK	7.77	13	8.83	10	8.18	50	5.97	56
[Tyr(Me) ⁸]-BK	8.64	100	9.89	110	8.59	129	Inact	
[Hyp ³ ,Tyr(Me) ⁸]-BK	9.07	270	10.22	234	8.56	123	Inact	

pD₂: The concentration of agonists that produce 50% of the maximum effect.

RA: Relative activity as a % of that of BK. Inact: inactive.

Table 2 Activities of kinins and their analogues on four isolated vessels

	Dog				Rabbit			
	Carotid artery		Renal artery		Jugular vein		Aorta	
	pD ₂	RA	pD ₂	RA	pD ₂	RA	pD ₂	RA
BK	8.64	100	9.85	100	8.48	100	6.22	100
BK-NH ₂	6.49	0.7	6.87	0.1	5.51	0.1	5.22	10
BK-OMe	8.13	31	8.90	11	7.69	16	5.00	6
Sar-BK	8.15	32	9.78	85	8.41	85	6.39	148
D-Arg-BK	8.71	117	10.08	170	8.70	166	6.56	219
N-Ac-BK	7.68	11	8.64	6	7.31	7	5.39	1.3
[Aib ⁷]-BK	7.60	9	8.23	2	6.28	0.6		<0.1
Sar[Aib ⁷]-BK	6.96	2	7.47	0.4	6.84	2.3		<0.1
D-Arg[Aib ⁷]-BK	7.67	10.7	8.41	3.6	6.74	1.8		<0.1

pD₂ and RA same as in Table 1.

variable decrease in activities, between 35 and 70% on BK₂ receptor systems and are practically inactive on the rabbit aorta. Extension of the peptide chain at the N-terminal with a Sar appears to be unfavourable when associated with Phe⁸ΨArg⁹, since it is accompanied by a drastic decrease of activity on both BK₂ and BK₁ receptors. The same is true when the pseudopeptide bond is made simultaneously between positions 8–9 and 4–5, as well as when MePhe is used to replace Phe in bradykinin. This last compound is practically inactive on both BK₂ and BK₁ receptors.

Analogues of desArg⁹-bradykinin

Some modifications were made in the sequences of desArg⁹-BK and desArg¹⁰-KD, which represent the most active and selective BK₁ receptor stimulants. The compounds were tested in five preparations, the same three BK₂ receptor systems used in the previous experiments and two BK₁ receptor systems, namely the rabbit aorta and the dog renal artery

without endothelium. The results are summarized in Table 4. The replacement of the Pro residue in position 3 of desArg⁹-BK and position 4 of desArg¹⁰-KD with Hyp is associated with a decrease of activity on the rabbit aorta, while the activity on the dog renal artery without endothelium is increased. Indeed, this modification appears to be favourable for BK₂ as well as for the renal BK₁ receptor occupation. The other compounds analyzed in Table 4, were prepared to obtain BK₁ receptor stimulants which may be more stable than the natural compounds. Phe⁸ of desArg⁹-BK was therefore replaced by D-Phe. The analogue is a pure agonist, 3.4 times more active than desArg⁹-BK on BK₁ receptors and inactive on BK₂ receptors. Affinity for BK₁ receptors is increased further when the peptide is extended at the N-terminal by the addition of a Sar. Indeed, Sar[D-Phe⁸]desArg⁹-BK is 11 times more active than desArg⁹-BK on BK₁ and is inactive on BK₂ receptors. In order to make the compound even more stable, a pseudopeptide bond was placed between the Gly⁴ and Phe⁵, but the activity of the compound was found to be markedly diminished.

Table 3 Activities of kinin analogues, containing pseudopeptide (CH₂·NH) bonds, in four isolated vessels

	Dog				Rabbit			
	Carotid artery		Renal artery		Jugular vein		Aorta	
	pD ₂	RA	pD ₂	RA	pD ₂	RA	pD ₂	RA
BK	8.64	100	9.85	100	8.48	100	6.22	100
Ψ ⁸⁻⁹ -BK	8.11	30	8.96	13	8.29	65	Inact	
Ψ ⁹⁻¹⁰ -KD	8.29	45	9.51	46	8.23	56		<0.1
SarΨ ⁸⁻⁹ -BK	7.51	7	9.09	17	7.56	12	Inact	
[MePhe ⁵]-Ψ ⁸⁻⁹ -BK	Inact		6.30	0.03	Inact		Inact	
Ψ ⁴⁻⁵ ,Ψ ⁸⁻⁹ -BK	7.04	2.5	7.59	1	5.03	0.3		<0.1

pD₂ and RA same as in Table 1.

Ψ: pseudopeptide bond CH₂·NH.

Table 4 Activities of desArg⁹-BK and some analogues on five preparations, two of which, the dog renal artery without endothelium (– Endo) and the rabbit aorta, are sensitive BK₁ receptor systems

	Dog carotid artery		Dog renal artery		Rabbit jugular vein		Dog renal artery (– Endo)		Rabbit aorta	
	pD ₂	RA	pD ₂	RA	pD ₂	RA	pD ₂	RA	pD ₂	RA
desArg ⁹ -BK	Inact		Inact		<0.1		8.60	100	7.29	100
desArg ¹⁰ -KD	Inact		Inact		<0.1		8.60	100	8.60	2042
BK	8.64	100	9.85	100	8.48	100	8.25	45	6.22	9
[Hyp ³]desArg ⁹ -BK	5.85	0.2	<0.1		5.37	0.08	9.11	324	6.52	17
[Hyp ⁴]desArg ¹⁰ -KD	5.49	0.07	<0.1		<0.01		8.75	141	8.06	589
[D-Phe ⁸]desArg ⁹ -BK	5.74	0.01	<0.1		<0.1		8.97	234	7.82	339
Sar[D-Phe ⁸]desArg ⁹ -BK	<0.1		<0.1		<0.1		9.04	275	8.33	1096
Sar[Ψ ⁴⁻⁵ ,D-Phe ⁸]desArg ⁹ -BK	<0.1		<0.1		<0.1		8.93	216	6.71	26

pD₂ and RA same as in Table 1. Relative activity is expressed as % of that of BK in the dog carotid and renal arteries and rabbit jugular vein and as % of that of desArg⁹BK in the dog renal artery without endothelium (– Endo) and rabbit aorta.

Discussion

With a view to improving agonist selectivity and potency, various modifications were made in the sequences of bradykinin, kallidin and the corresponding C-terminal desArg-peptides. Firstly, we evaluated the effect of replacing the second Pro residue from the N-terminals (Pro³ of BK and Pro⁴ of KD) with Hyp. This modification is favourable for BK₂ receptors and rather unfavourable for the BK₁. We therefore pushed this concept further and obtained the most potent and pure BK₂ receptor stimulant, namely [Hyp³, Tyr(Me)⁸]-BK which is 2–3 times as active as BK on the dog arteries and is completely inactive on BK₁ receptor systems. T-kinin was also evaluated and found to be less active than kinins, in accordance with previous findings by Okamoto & Greenbaum (1983b) and by Sugio & Greenbaum (1988).

Secondly, we replaced the C-terminal peptide bond Phe⁸-Arg⁹ with a pseudo peptide and a selective BK₂ receptor agonist, almost as active as BK on BK₂ but inactive on BK₁ receptors was obtained (Drapeau *et al.*, 1988). The pseudo-peptide analogue of kallidin showed similar activity and no advantage with respect to the BK analogue. Extension of the chain from the N-terminal with Sar or modification of the peptide bond between Gly⁴ or Phe⁵ (either by a pseudo-peptide or by MePhe), were accompanied by a great decrease of activity. It appears that the pseudo-peptide modification cannot be easily combined with other changes, both at the N-terminal and or within the chain.

As to BK₁ receptors, the unfavourable effect of Hyp as a substitute for Pro with respect to BK₁ receptor activation was confirmed by the results obtained with [Hyp³]-desArg⁹-BK and [Hyp⁴]-desArg¹⁰-KD. The replacement of the C-terminal Phe by D-Phe, which should protect the peptide from degradation by carboxypeptidase, was found to be very good: the peptide ([D-Phe⁸]-desArg⁹-BK) showed a markedly increased affinity. Chain extension at the N-terminal by a Sar was tolerated since Sar[D-Phe⁸]-desArg⁹-BK was found to be 11 times more active than desArg⁹-BK. Although this new compound is only half as active as desArg¹⁰-KD, the presence of a Sar instead of a lysine at the N-terminal and that of D-Phe at the

C-terminal should confer resistance against both carboxy and aminopeptidases; moreover, the new compound should be much less active as histamine releaser than desArg¹⁰-KD (Devillier *et al.*, 1985). Sar[D-Phe⁸]-desArg⁹-BK appears to be a potent BK₁ selective agonist without histamine releasing activity and somewhat more stable than desArg⁹-BK.

An attempt to make the compound resistant to some endopeptidases by using a pseudo-peptide bond between positions 4 and 5 was unsuccessful, since Sar[Gly⁴ΨPhe⁵,D-Phe⁸]-desArg⁹-BK is very weak on BK₁ receptors, its relative affinity being only approximately 1/50 of that of Sar[D-Phe⁸]-BK.

In regard to the three compounds containing AiB instead of Pro, we could not confirm the results of Vavrek & Stewart (1985), who showed that [AiB⁷]BK is 4 times more active than BK on the guinea-pig ileum. In the three BK₂ receptor systems used in the present study, [AiB⁷]BK and its homologues were found to be weak agonists. A few experiments performed on guinea-pig ileum longitudinal strip treated with atropine (Rhaleb *et al.*, unpublished) indicated that the AiB containing compounds (Table 2) were found to be as active as BK. Further studies are needed to explain this discrepancy.

Naturally occurring kinins appear to be nonselective since they act on BK₂ and BK₁ receptors. Selective BK₂ receptor stimulants have been obtained either by protecting the peptide from degradation by kininase I and II or by changes in positions 3 and 8, as in [Hyp³,Tyr(Me)⁸]-BK. Affinity and selectivity for BK₁ receptors were improved by replacing the C-terminal Phe of desArg⁹-BK with a D-Phe, or by extending the peptide chain at the N-terminal with Sar. These modifications should increase the resistance of the peptides to degradation. Thus, selective agonists for BK₂ and BK₁ receptors, identified in the present experiments, should be useful for future studies on kinin receptors.

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