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Bradykinin Potentiating and Sensitizing Activities of New Synthetic Analogues of Snake Venom Peptides

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The structural requirements for prolonged residual ("sensitizing") activity in bradykinin-potentiating peptides (BPP's) were investigated through a study of seven synthetic BPP's including three not previously described: [Lys^6]-BPP9a, [Gly^6]potentiator B, and [Lys^6,Gln^8]potentiator B. The quantitation of the sensitizing activities in the isolated guinea pig ileum indicated that the structural requirements for bradykinin potentiation and for sensitization were not the same. The most potent sensitizers were potentiator B and [Lys^6]-BPP9a.

Several peptides isolated from the venoms of *Bothrops jararaca*[1,2] and *Agkystrodon halys blomhoffii*,[3] when administered with bradykinin, potentiate the effects of this hormone, both in vivo and in vitro. These bradykinin-potentiating peptides are potent inhibitors of bradykininase activity,[4] but their potentiation of bradykinin effects may be partly due to a direct action on the receptors to this peptide.[5,6] Most of the natural BPP's are short-acting, and their effect on isolated smooth muscles disappears upon washing with fresh medium. One exception is the undecapeptide potentiator B (Table I), which continues to potentiate bradykinin in the isolated guinea pig ileum and rat uterus after it is removed from the medium.[3] This residual potentiation of bradykinin, termed sensitizing action, was also observed with the synthetic analogue [Gln^8]potentiator B but not with any of the other BPP's that were previously tested.[6]

The relationship between BPP structure and sensitizing activity merits further investigation because persistence of action is a desirable attribute of these compounds. In particular, it would be interesting to know whether BPP9a[7] also has sensitizing activity. This nonapeptide is one of the longer acting inhibitors of angiotensin I conversion in vivo,[8] and this activity is related to bradykinin potentiation because of the identity between angiotensin converting enzyme and bradykininase (kininase II).[8b,9]

In this paper we present the results of a determination of BPP9a's sensitizing activity and the study of three new synthetic analogues (Table I): [Gly^6]potentiator B (**3**), [Lys^6,Gln^8]potentiator B (**4**), and [Lys^6]-BPP9a (**7**). Their bradykinin-potentiating activities on the isolated guinea pig ileum preparation[10] were evaluated by the minimum concentration required for twofold potentiation, as described in detail elsewhere.[6]

In order to compare the sensitization produced by the different peptides, it was necessary to quantitate this activity, previously described only qualitatively.[3,6] The typical results obtained with [Lys^6]-BPP9a illustrate how this quantitation was achieved. Figure 1 shows that, in the presence of this peptide, bradykinin was markedly potentiated and that some potentiation remained in the five responses following removal of the BPP from the medium. No significant differences between these five responses were found for any of the BPP's tested. Although the sensitization persisted for several hours, a quantitation of this longer permanence was impaired by a slow spontaneous increase in sensitivity to bradykinin, observed in control organs left without treatment for 2 h or more. However, during 20 min taken for the five test doses, control organs showed no significant alteration of their responses to bradykinin. Accordingly, the sensitizing activity of each peptide could be estimated by the average potentiation measured in the five responses subsequent to its removal from the medium.

The effect of BPP concentration on sensitizing activity is also shown in Figure 1 for the typical case of [Lys^6]-BPP9a. Potentiating (before washout) and sensitizing (after washout) effects were concentration dependent, but the sensitizing activity reached a maximum at 0.4 μM peptide concentration. Similar observations were made with the other peptides, which differed on the maximum degree of sensitization produced. Although the minimum concentration needed for maximum sensitization also differed, this maximum could be obtained with 0.4 μM concentration of all the peptides that were studied. The bradykinin potentiating as well as the sensitizing activities of these peptides are shown on Table I. No relationship between potentiating and sensitizing activities was apparent, indicating that these two properties have different structural requirements.

The most active sensitizer was potentiator B. Replacement of either Arg^6 or Lys^8 by a neutral residue caused large losses of sensitizing potency, but the resulting analogues **2**–**4** still retained a significant activity (Table I). However, replacement of both basic amino acids at positions 6 and 8 by proline or glutamine residues, in potentiator C (Table I) or [Gln^8]potentiator C,[6] resulted in total loss of sensitizing activity.

We have found that BPP9a has a sensitizing activity slightly smaller than those of **2**–**4**. Since this nonapeptide

Table I. Bradykinin Potentiating and Sensitizing Effects of 0.4 μM BPP on the Isolated Guinea Pig Ileum

Compd	Name	Amino acid sequence	Rel potentiating act.[a]	Sensitization[b] Av	SE	n
1	Potentiator B	pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	2.8[c]	2.40	0.05	17
2	[Gln^8]potentiator B	pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Gln-Ile-Pro-Pro	3.2[c]	1.29	0.15	7
3	[Gly^6]potentiator B	pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Lys-Ile-Pro-Pro	0.6	1.28	0.07	21
4	[Lys^6,Gln^8]potentiator B	pGlu-Gly-Leu-Pro-Pro-Lys-Pro-Gln-Ile-Pro-Pro	0.8	1.48	0.13	16
5	Potentiator C	pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	0.2[c]	1.00	0.02	7
6	BPP9a	pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	9.6	1.20	0.02	10
7	[Lys^6]-BPP9a	pGlu-Trp-Pro-Arg-Pro-Lys-Ile-Pro-Pro	5.8	2.18	0.09	20

[a] Relative to the activity of BPP5a, based on the minimum BPP concentration needed for twofold potentiation of bradykinin (see ref 6). [b] Average residual potentiation measured in five responses to bradykinin subsequent to BPP washout; value of unity indicates absence of sensitization. [c] From ref 6.

Table II. Physical Properties of New Bradykinin-Potentiating Peptides

Compd	Yield,[a] %	Mp, °C	$[\alpha]^{20}_D$[b]	K[c]	R_f[d] A	B	C	Electrophoretic migration[e] pH 2.8 (R_{His})	pH 4.9 (R_{Arg})	pH 9.9 (R_{Picr})
3	24	210-215	+215.2	0.41	0.17	0.20	0.38	0.32	0.15	-0.03
4	25	200-208	+248.8	0.56	0.06	0.21	0.27	0.32	0.18	0.08
7	15	210-220	+164.0	0.43	0.05	0.31	0.32	0.57	0.43	-0.31

[a] Based on the initial amount of Boc-Pro polymer. [b] c 0.5, H_2O. [c] Partition coefficient in n-BuOH-HOAc-H_2O (4:1:5). [d] A, B, and C refer to the chromatographic systems described in the Experimental Section. [e] R_{His}, R_{Arg}, and R_{Picr} are the migrations relative to histidine, arginine, and picric acid, respectively.

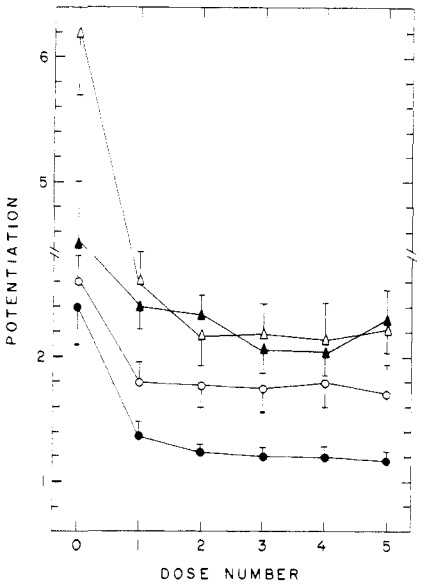

Figure 1. Potentiation of the effect of 0.04 μM bradykinin on the isolated guinea pig ileum in the presence of [Lys^6]-BPP9a (dose number 0) and in five administrations (doses number 1–5) subsequent to the washout of the BPP from the medium. The interval between doses was 5 min, and bradykinin was left in contact with the organ for 90 s. [Lys^6]-BPP9a concentration: (●) 0.02 μM; (○) 0.06 μM; (▲) 0.4 μM; (△) 2.0 μM.

has only one basic residue in position 4 (corresponding to position 6 in the undecapeptide, see Table I), one would expect a greater sensitizing potency in the analogue [Lys^6]-BPP9a, whose C-terminal 7-residue sequence is identical with that of potentiator B. Table I shows, indeed, that [Lys^6]-BPP9a had a high sensitizing activity, which approached that of potentiator B.

[Lys^6]-BPP9a is a very interesting analogue because it combines high potentiating activity (compared to that of potentiator B) with high sensitizing action (compared to that of BPP9a). It holds promise as a more efficient kininase inhibitor for in vivo studies, in which extensive use is presently made of BPP9a since this peptide was found to be the longest acting of the BPP's.[7]

Experimental Section

Potentiators B and C were gifts from Professor S. Sakakibara. The synthesis of [Gln^8]potentiator B was previously described.[6] BPP9a and bradykinin were also synthesized in this laboratory. *tert*-Butyloxycarbonylamino acids were brought from Bachem, Inc.

Peptide Syntheses. The peptides were synthesized by the solid-phase method,[11] following the usual procedures,[12] with the aid of an automatic peptide synthesizer.[13] The *tert*-butyloxycarbonylamino acids with reactive side chains were used as the following protected derivatives: Lys(ε-Z); Arg(N^g-Tos); Trp(N^i-formyl).[14] CH_2Cl_2 was used as solvent for all reagents, except that Boc-Arg(N^g-Tos) was dissolved in CH_2Cl_2–DMF (2:1), and TFA (30%, v/v) was dissolved in $CHCl_3$. The couplings were done with 2.5 equiv of *tert*-butyloxycarbonylamino acid and DCI for 2 h, except in the case of *tert*-butyloxycarbonylglutamine, which was coupled by the active ester method, with 4 equiv of the *p*-nitrophenyl ester in DMF for 6 h. When the couplings were not completed after the normal time, as evidenced by the ninhydrin reaction,[15] a new coupling cycle was carried out and a negative ninhydrin test was obtained within 2–6 h. To minimize diketopiperazine formation in the coupling of the third residue from the C-terminus, the usual order of treatment with *tert*-butyloxycarbonylamino acid and DCI was reversed,[16] and the neutralization step was done with 5% (v/v) *N*-methylmorpholine.[17] The peptide was cleaved from the resin by treatment with anhydrous HF, containing 5% anisole (v/v), for 45 min at 0 °C. After removal of HF and anisole, by vacuum distillation and washing with EtOAc, the peptide was extracted with 1 M HOAc and freeze-dried.

Peptides 4 and 7 were submitted to a first purification by countercurrent distribution in n-BuOH–HOAc–H_2O (4:1:5), and their detection was made by the ninhydrin reaction. Crude **3** was first purified by chromatography on a silica gel 60 column using n-BuOH–HOAc–H_2O (4:1:1) as eluent, and the ninhydrin reaction (after alkaline hydrolysis[18]) was used for peptide location in the eluate fractions. This was followed by removal of the formyl group from the tryptophan side chain, by treatment with 1 M ammonium bicarbonate buffer (pH 9) for 24 h. All the peptides were further purified by gel filtration on Sephadex G-15 equilibrated and eluted with 0.1 M HOAc, monitored by the absorbance at 206 nm.

The peptides were homogeneous by the following criteria. Only one component was detected upon TLC of 0.1 μmol on silica gel plates (Merck, 0.2 mm) with the following solvent systems: (A) n-BuOH–HOAc–H_2O (4:1:1); (B) n-BuOH–EtOAc–HOAc–H_2O (1:1:1:1); (C) n-BuOH–pyridine–HOAc–H_2O (15:10:3:12). Only

one component was detected upon paper electrophoresis (Whatman 3MM, 1000 V, 60 min) with the following buffer systems: pH 2.8 (1 M HOAc); pH 4.9 (0.1 M pyridine acetate); pH 9.9 (0.2 M sodium carbonate–bicarbonate). The peptides were located with a hypochlorite spray and, in the case of 4 and 7, also with ninhydrin. The properties of the three new peptides are summarized on Table II.

Amino acid analyses were made on a Beckman 120C instrument, after hydrolysis with 2 mL of 6 N HCl, containing 0.5% (v/v) mercaptoethanol and 0.2% (v/v) phenol, in a nitrogen atmosphere for 72 h at 110 °C. For estimation of tryptophan content the hydrolysis was also done with *p*-toluenesulfonic acid, containing 1% indole, for 48 h at 110 °C. The following amino acid ratios were found: **3**, Lys (1.03), Glu (1.01), Pro (5.0), Gly (1.99), Ile (1.02), Leu (0.93); **4**, Lys (1.01), Glu (1.92), Pro (5.13), Gly (0.95), Ile (0.99), Leu (1.02); **7**, Lys (0.98), Arg (1.04), Trp (0.98), Glu (1.04), Pro (4.12), Ile (1.04).

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(+)- and (−)-3-Methoxycyproheptadine. A Comparative Evaluation of the Antiserotonin, Antihistaminic, Anticholinergic, and Orexigenic Properties with Cyproheptadine

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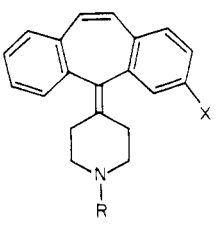
The synthesis and resolution of (±)-3-methoxycyproheptadine [(±)-4] are described. As a peripheral serotonin antagonist, (±)-4 was found to be one-half as potent as cyproheptadine (**1b**). The peripheral anticholinergic and antihistaminic activities as well as the orexigenic property of (±)-4 are less than those of **1b**. A further comparison of the enantiomers (+)-4 and (−)-4 shows that all of the anticholinergic activity of (±)-4 resides solely in the dextrorotatory enantiomer, (+)-4, while the antiserotonin activity, which is similar to that of **1b**, resides in the levorotatory enantiomer, (−)-4. Antihistaminic and orexigenic activity also resides in (−)-4 but these properties are reduced compared to those of **1b**.

A previous report[1] from this laboratory described the stereoselective antipsychotic and central anticholinergic activities that were found to reside in the levo- and dextrorotatory enantiomers of the cyproheptadine analogue, 1-cyclopropylmethyl-4-(3-trifluoromethylthio-5*H*-dibenzo[*a*,*d*]cycloheptene-5-ylidene)piperidine (**1a**), respectively. During an investigation into the stereostructure–activity relationships of other enantiomeric 3-substituted cyproheptadine analogues and derivatives, (±)-3-methoxycyproheptadine [(±)-4] was prepared and resolved. In a preliminary pharmacological evaluation, the racemate (±)-4 was found to have a biological profile similar to that observed with cyproheptadine (**1b**), a clinically useful serotonin and histamine antagonist with anticholinergic and orexigenic properties.[2,3] This finding prompted a comparative evaluation of the enantiomers (+)- and (−)-4 with **1b** to determine whether enantiomeric differences in biological activities existed.



1a, X = SCF_3; R = CH_2-c-C_3H_5
b, X = H; R = CH_3
c, X = CN; R = CH_3

Chemistry. (±)-3-Methoxycyproheptadine [(±)-4] was prepared by the addition of 1-methyl-4-piperidyl-