Synthesis and Properties of New Bradykinin Potentiating Peptides†

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In a study of the relationship between structure and activity of bradykinin potentiating peptides (BPP), six analogs and homologs of peptides occurring in the venoms of *Bothrops jararaca* and *Agkistrodon halys blomhoffii* were synthesized and assayed in the isolated guinea pig ileum and rat uterus. None of the peptides had bradykinin-like activity and their bradykinin potentiating activity was much greater in the guinea pig ileum than in the uterus. The following observations were made with the guinea pig ileum. The introduction of Gln as the eighth residue in potentiator B (pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro) and potentiator C (pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro) produced a small increase in their bradykinin potentiating activity. Removal of the two N-terminal residues of [Gln^8]-potentiator B and [Gln^8]-potentiator C led to alterations in activity that paralleled those described earlier for the parent compounds (potentiators B and C). The peptide with the sequence pGlu-Trp-Pro-Arg-Pro-Lys-Trp-Ala-Pro was seven times as active as BPP5a, while the most potent natural BPP, a nonapeptide from *B. jararaca* venom, is reported to be only four times as active as BPP5a. An analog of the above-mentioned nonapeptide containing Pro^8 instead of Ala^8 was only as active as BPP5a. For all of the peptides, as well as for potentiators B and C and BPP5a, the concentration *vs.* potentiating activity curves had similar shapes, with a plateau at twofold potentiation and a maximum potentiation of 10- to 11-fold. A direct action on the bradykinin receptors may be responsible for the effects observed at lower BPP concentration while the effects at higher concentrations may be due to kininase inhibition. The potentiating activities of potentiator B and its Gln^8 analog persisted after the peptides were removed from the medium. This "sensitizing activity" was not observed with any of the other peptides.

Several bradykinin potentiating peptides (BPP) have been isolated from the venoms of *Bothrops jararaca*[2,3] and *Agkistrodon halys blomhoffii*[4] (Table I). These peptides, when administered with bradykinin, increase the effects produced by the latter, both *in vivo* and upon isolated smooth muscle preparations.

Among the most active of these peptides, the pentapeptide pGlu-Lys-Trp-Ala-Pro (BPP5a) from *B. jararaca* differs from the others by its size and by the C-terminal sequence. The other highly active peptides isolated from the venoms of the two snakes contain 9–13 amino acid residues and the sequence Ile-Pro-Pro at the C-terminal end.

The mechanism of BPP action has not been completely clarified, although it is certain that they act as inhibitors of peptidases that destroy bradykinin (kininases). There are several such kininases in the circulating blood and other tissues by Stewart, *et al.*,[9] showed that BPP5a appears to selectively inhibit a lung enzyme that cleaves bradykinin at its Ser^6-Pro^7 bond. However, multiple mechanisms for potentiation of bradykinin response are probably involved, and no simple criteria for determining potentiating activity have evolved from studies of structure–activity relationships.[8]

We have attempted to obtain more information about this problem by studying the effect, upon biological activity, of the length of the peptide chain and of the nature of residue X in the C-terminal sequence -X-Ile-Pro-Pro. In the most active longer peptides X is Gln (Table I), while in potentiators B and C this position is occupied by Lys and Pro, respectively. In order to find whether replacement of these residues by Gln would increase the bradykinin potentiating activity of these peptides we have synthesized [Gln^8]-potentiator B and [Gln^8]-potentiator C (Table II, **15** and **17**, respectively). Since the synthetic potentiator C (3-11) nonapeptide homolog was shown to be more active than the natural undecapeptide parent compound,[6] we have also synthesized the (3-11) nonapeptide analogs of potentiators B and C containing Gln in place of Lys (**16**) and Pro (**18**), respectively.

We have also synthesized a hybrid nonapeptide (**19**) containing the C-terminal tetrapeptide sequence of BPP5a and the N-terminal five residue sequence of peptide **3**, which is the BPP with greatest potentiating activity in the isolated guinea pig ileum. To further test the importance of the C-terminal Pro-Pro sequence, we have also prepared an analog of **19** in which Ala in position 8 is replaced by Pro (**20**).

The six new peptides listed in Table II were assayed for their bradykinin potentiating activity in the isolated guinea pig ileum and rat uterus.

Results

None of the peptides studied showed detectable bradykinin-like activity upon the isolated guinea pig ileum or rat uterus. All of them had potentiating activity when added to the muscle bath simultaneously with bradykinin. The potentiating effect of some of the peptides persisted after several washings of the preparation with fresh medium. Because of this "sensitizing activity,"[4] the muscle segment was discarded and replaced by a new one after each addition of these peptides, in the study of their potentiating activity.

The effect of BPP concentration on the response of the guinea pig ileum to bradykinin was very similar for BPP5a, potentiators B and C, and the six new peptides. An example of this concentration–effect relationship is presented in Figure 1, which shows the results obtained with [Gln^8]-potentiator C. The maximum potentiation produced by each of the peptides studied was 10- to 11-

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Table I. Bradykinin Potentiating Peptides Isolated from the Venoms of *Bothrops jararaca* and *Agkistrodon halys blomhoffii*

Compd	Name	Ref	Amino acid sequence	Biological act.[a]
1	BPP5a	3	pGlu-Lys-Trp-Ala-Pro	100
2		3	pGlu-Ser-Trp-Pro-Gly-Pro[b]	9
3		3,5	pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	410
4		3,5	pGlu-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	47
5		5	pGlu-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro[b]	100
6		3,5	pGlu-Ser-Trp-Pro-Gly-Pro-Asn-Ile-Pro-Pro	80
7	Potentiator A	6,7	pGlu-Gly-Arg-Pro-Pro-Gly-Pro-Pro-Ile-Pro	0.1[c]
8		3,5	pGlu-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro	34
9	Potentiator B	4	pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	20[c]
10	Potentiator C	4	pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	11[c]
11	Potentiator E	4	pGlu-Lys-Trp-Asp-Pro-Pro-Pro-Val-Ser-Pro-Pro	0.2[c]
12		3	pGlu-Gln-Trp-Ala-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro[b]	380
13		3,5	pGlu-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro	200
14		3	pGlu-Gly-Gly-Leu-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro[b]	90

[a]As percentage of the bradykinin potentiating activity of 1, on the guinea pig ileum, on a molar basis; these data were taken from ref 8. [b]Sequences of these peptides were not determined; sequences shown were deduced from physical properties and by comparison with related peptides. [c]No direct comparison of these peptides with the others has been made and the relative biological activities given here were estimated from the concentrations reported to produce twofold potentiation of bradykinin.

Table II. New BPP Analog and Homolog Peptides

Compd	Name	Amino acid sequence
15	[Gln[8]]-potentiator B	pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Gln-Ile-Pro-Pro
16	[Gln[8]]-potentiator B (3–11)	Leu-Pro-Pro-Arg-Pro-Gln-Ile-Pro-Pro
17	[Gln[8]]-potentiator C	pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Gln-Ile-Pro-Pro
18	[Gln[8]]-potentiator C (3–11)	Leu-Pro-Pro-Gly-Pro-Gln-Ile-Pro-Pro
19	Hybrid nonapeptide	pGlu-Trp-Pro-Arg-Pro-Lys-Trp-Ala-Pro
20	[Pro[8]]-hybrid nonapeptide	pGlu-Trp-Pro-Arg-Pro-Lys-Trp-Pro-Pro

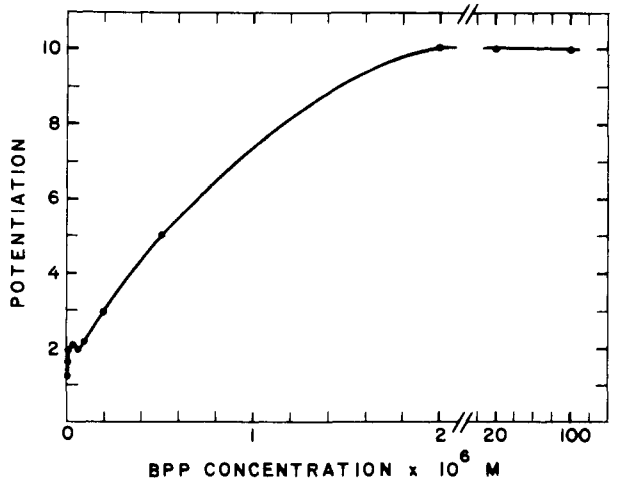

Figure 1. Potentiation of the isolated guinea pig ileum's response to bradykinin by different concentrations of potentiator C.

fold. However, at concentrations around 10^{-7} *M* the concentration–effect curves presented a plateau at about twofold potentiation. This is illustrated in Figure 2 for the case of compounds **15**, **16**, and **17**. Since the relationship was approximately linear at the lowest BPP concentrations, we obtained the least-squares straight line through these points in order to determine the minimum concentration that produced twofold potentiation. Using this method we estimated the potentiating activity of BPP5a, potentiator B and C, and the six new peptides (Table III).

The bradykinin potentiating activities of the peptides were also assayed in the isolated rat uterus, and the results are also shown on Table III.

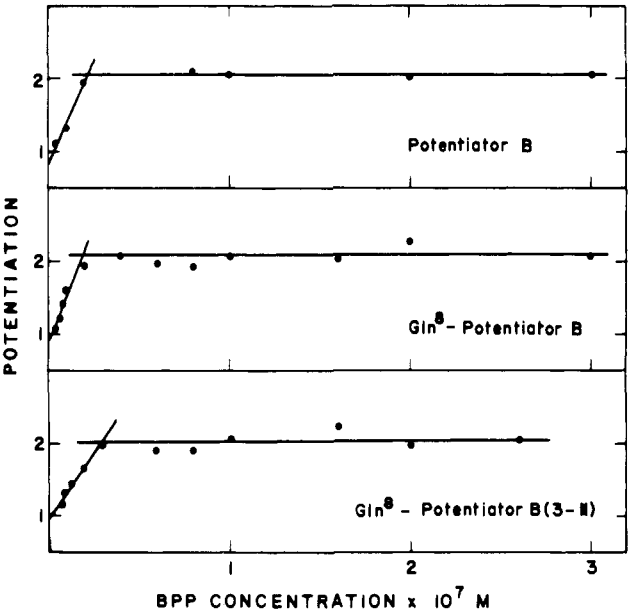

Figure 2. Effect of low concentrations of potentiator B, [Gln[8]]-potentiator B, and [Gln[8]]-potentiator B (3-11) on the isolated guinea pig ileum's response to bradykinin.

Discussion

The peptides assayed in this study had bradykinin potentiating activity in the guinea pig ileum and in the rat uterus. However, the concentrations needed to produce similar effects were three to four orders of magnitude greater in the uterus than in the ileum. This resulted in a

Table III. Bradykinin Potentiating Activity of Analogs and Homologs of Potentiators B and C and BPP5a

Compd	Isolated guinea pig ileum			Isolated rat uterus		
	Sensitizing act.[a]	Concn for twofold potentiation (mM)	Rel act.[b]	Sensitizing act.[a]	Concn for twofold potentiation (mM)	Rel act.[b]
9	+	0.021	2.8	+	20	1.0
15	+	0.018	3.2	+	10	2.0
16	0	0.029	2.0	0	10	2.0
10	0	0.29	0.20	0	40	0.5
17	0	0.21	0.28	0	16	1.2
18	0	0.10	0.58	0	20	1.0
1	0	0.058	1.0	0	40	0.5
19	0	0.0081	7.2	0	10	2.0
20	0	0.053	1.1	0	20	1.0

[a] A + sign indicates that the potentiating activity remained after washing with fresh medium. [b] Relative to the activity of BPP5a taken as 1.0.

much smaller precision of the data obtained with the uterus. These data are included in Table III, but our discussion will be based only on the results obtained with the guinea pig ileum with which a more detailed study was made. The shape of the concentration-effect curves for the nine bradykinin potentiating peptides in the guinea pig ileum was strikingly similar. The two plateaus (Figures 1 and 2) indicate that two different mechanisms are involved in the phenomenon, as first proposed by Ferreira.[10] A direct action on the bradykinin receptors may be responsible for the effects observed with the lower BPP concentrations, while the effects at higher concentrations may be due to inhibition of bradykinin inactivation by peptidases.

The plateau at twofold potentiation is very interesting because BPP activities have usually been reported on the basis of the concentrations needed to produce a doubling in the response to bradykinin. Therefore, it is necessary to make a careful determination of the minimum concentration that produces such an effect. Figure 2 shows that this could be done for the peptides studied because of the sharp break between the two straight lines drawn through the data points.

The bradykinin potentiating activities estimated with this method (Table III) were in good agreement with those previously reported for BPP5a[3,9] and potentiator C.[4] However, we have found potentiator B to be 15 times as active as previously reported.[4] This emphasizes the difficulty in comparing values for absolute specific activities obtained in different laboratories because they depend on small, usually unreported, experimental details of the smooth muscle preparations. This should be taken into account when considering the biological activities listed on Table I, which is a compilation of data from different laboratories.

The introduction of Gln as the eighth residue in potentiators B and C produced a small increase in their bradykinin potentiating activity. The nonapeptide homolog of [Gln[8]]-potentiator B (**16**) was about 62% as active as the undecapeptide 15 while a doubling of the activity was observed when the two N-terminal residues were removed from [Gln[8]]-potentiator C (compare 17 and 18, Table III). These relationships closely parallel the findings with the parent peptides, containing Lys (**9**) or Pro (**10**) in position 8; in the case of potentiators B and C, the nonapeptides had 50 and 230%, respectively, of the undecapeptides' activity.[6] Thus, the contribution of the pGlu-Gly N terminus to the potentiating activity of potentiators B and C is clearly dependent on whether Arg or Gly is present in position 6 of the peptide chain and does not depend on the residue in position 8.

The hybrid nonapeptide (**19**) was the most potent of the bradykinin potentiating peptides studied (Table III), being seven times as active as BPP5a. The most active BPP previously described was the nonapeptide **3**, reported to be four times as active as BPP5a on the guinea pig ileum (Table III). Thus, the addition of the N-terminal 5 residues of **3** to the C-terminal 4 residues of BPP5a yielded a nonapeptide that was more active than either of the naturally occurring parent compounds. The Ala-Pro C-terminal sequence proved to be important for the high activity of this hybrid nonapeptide, since replacement of Ala by Pro (**20**) led to a reduction of the activity to the level of that of BPP5a.

We have confirmed the observation that potentiator B's action on the guinea pig ileum persists even after several washings of the organ with fresh medium. This "sensitizing activity" was also observed with [Gln[8]]-potentiator B, but not with any of the other peptides studied (Table III). This property of **9** and **15** may be caused by a slow dissociation of the peptide, either from the bradykinin receptors or from kininases. The relationship between structure and "sensitizing activity" merits further investigation because persistence of action is a desirable attribute of compounds with BPP activity.

Experimental Section

Materials. Potentiators B and C were gifts from Professor S. Sakakibara. *tert*-Butyloxycarbonylamino acids were purchased from Schwarz-Mann. Bradykinin was a synthetic product from this laboratory.

Peptide Syntheses. The peptides were synthesized by the solid-phase method.[11,12] Boc-Pro was attached to 2% cross-linked chloromethyl polymer, and chain elongation was performed on an automatic peptide synthesizer.[13] $CHCl_3$ was used as solvent for all reagents and *tert*-butyloxycarbonylamino acids. Arginine was introduced as Boc-Arg (NO_2), which was dissolved in $CHCl_3$–DMF (2:1). The Boc groups were removed with 25% (v/v) CF_3COOH in $CHCl_3$ for 30 min. The couplings were done with 2.5 equiv of *tert*-butyloxycarbonylamino acid and DCI and monitored with the ninhydrin reaction.[14] For the coupling of the third residue from the C terminus, the usual order of treatment with *tert*-butyloxycarbonylamino acid before DCI was reversed to minimize diketopiperazine formation.[15] After coupling of the last residue, the peptide-resin was treated with anhydrous HF for 45 min at 0°. After removal of HF by vacuum distillation of washing with

Table IV. Physical Properties of Compounds 15-20

Compd	Yield,[a] %	Mp, °C	$[\alpha]^{20}D$,[b] deg	K[c]	R_f[d] A	R_f[d] B	R_f[d] C	Electrophoretic migration[e] pH 2.8 (R_{His})	pH 5.0 (R_{Arg})	pH 9.9 (R_{Picr})
15	21	220-225	+192.4	0.68	0.71	0.50	0.45	0.41	0.20	−0.23
16	26	183-185	+210.0	0.20	0.53	0.40	0.37	0.68	0.53	−0.11
17	21	178-183	+207.2	1.78	0.80	0.51	0.50	0.17	−0.14	0.16
18	25	167-168	+204.4	0.59	0.76	0.48	0.42	0.44	0.23	0.31
19	16	220-225	+105.5	0.53	0.64	0.56	0.52	0.44	0.37	−0.22
20	20	227-235	+116.0	0.83	0.71	0.56	0.52	0.51	0.36	−0.28

[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] See Experimental Section.

EtOAc, the peptides were extracted with 0.1 *M* HOAc and freeze-dried.

No attempt was made to detect diketopiperazine formation during coupling of the third amino acid residue. However, there is reason to believe that, if diketopiperazine was formed, it was in less than 10% of the peptide chains; no contaminating peptides lacking the two C-terminal residues were detected in the crude products which were obtained after HF cleavage in yields greater than 85% in every case.

The lyophilized crude peptides were submitted to 200 transfers of countercurrent distribution in *n*-BuOH–HOAc–H_2O (4:1:5) and the distribution coefficient (K) found for each one is given in Table IV. The pooling of the fractions from countercurrent distribution was done with emphasis on purity rather than on yield. After vacuum evaporation of the solvent, the peptides were dissolved in water and lyophilized. All the peptides were found to be homogeneous by chromatography with *n*-BuOH–HOAc–H_2O (4:1:1) on Whatman No. 1 paper (A) and by tlc on 0.1-mm silica gel plates (Eastman "Chromagram") with the following solvent systems: (B) *n*-BuOH–EtOAc–HOAc–H_2O (1:1:1:1); (C) *n*-BuOH–pyridine–HOAc–H_2O (30:20:6:24). The location of the peptides was done with the chlorine spray[12] and, in the case of **16** and **18**, also with ninhydrin.

All the peptides were also homogeneous to paper electrophoresis at 1000 V for 60 min, with the following buffer systems: 1 *M* HOAc (pH 2.8), 0.1 *M* pyridine acetate (pH 4.9), and 0.2 *M* sodium carbonate–bicarbonate (pH 9.9). The electrophoretic behavior of the peptides is reported on Table IV by the ratios of their migration to that of a simultaneously run sample of either histidine (R_{His}) at pH 2.8, or arginine (R_{Arg}) at pH 4.9, or picric acid (R_{Picr}) at pH 9.9. The migrations were given a negative sign when peptide and standard had opposite charges.

Amino acid analyses were made on the Beckman 120 C amino acid analyzer, after hydrolysis with 2 ml of 6 *N* HCl containing 0.1 ml of 10% mercaptoethanol and 0.1 ml of 5% phenol, under nitrogen, for 72 hr at 120°. **20** and **21** were also hydrolyzed with *p*-toluenesulfonic acid, containing 1% indole, for 48 hr at 105°, for the determination of tryptophan content. The amino acid molar ratios found were the following: **15**, Glu (1.90), Gly (1.00), Leu (0.89), Pro (4.80), Arg (1.00), Ile (0.91); **16**, Leu (0.96), Pro (5.06), Arg (0.95), Glu (1.00), Ile (1.03); **17**, Glu (2.13), Gly (1.86), Leu (1.03), Pro (4.80), Ile (1.00); **18**, Leu (1.04), Pro (5.28), Gly (0.99), Glu (1.05), Ile (1.00); **19**, Glu (0.95), Trp (1.72), Pro (2.81), Arg (0.95), Lys (0.95), Ala (1.00); **20**, Glu (1.00), Trp (1.77), Pro (3.85), Arg (1.02), Lys (0.94).

Bioassays. The isolated guinea pig ileum[16] and rat uterus[17] were prepared as described earlier. The bath volume was 5 ml, the media were aerated with a stream of air, and the isotonic contractions were recorded under a 1-g load with sixfold magnification. After obtaining a dose-response curve, two bradykinin concentrations were selected and alternatively administered as controls and in the presence of different BPP concentrations. The peptides to be assayed were added to the medium 1 min before the addition of bradykinin and the interval between additions was 5 min. After each contraction reached a maximum the preparation was washed 2-3 times with fresh medium. In some cases, the potentiating action persisted after the washing and subsequent additions of up to ten bradykinin controls. For the assay of peptides with this "sensitizing activity," a new organ segment was mounted in the muscle bath after each treatment with the peptide.

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