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Bradykinin potentiating peptides isolated from α-casein tryptic hydrolysate

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On the basis of a previous observation made by Henriques *et al.* [1], showing that a peptone preparation, obtained by partial tryptic hydrolysis of casein, potentiates bradykinin activity toward isolated guinea-pig ileum, we decided to investigate which of the casein chains are responsible for that potentiation. "Sigma" casein was used for the separation of the α, β and γ chains [2]. Those fractions were separately hydrolyzed by trypsin-L-1-tosylamide-2-phenylethyl chloromethyl ketone (trypsin-TPCK). Each fraction was separately dissolved in a minimum volume of 20 mM ammonium bicarbonate, pH 8; to this mixture 10 mg of trypsin-TPCK was added for each gram of casein fraction, and the mixture was incubated at 37° for 15 min. The hydrolysis was interrupted by heating the mixtures for 5 min in a boiling water bath. Comparing the bradykinin potentiating activity of each hydrolyzed casein fraction on the isolated guinea-pig ileum, we observed that the α-casein hydrolysate was the most active among them. Therefore, we chose this hydrolysate as a source for the isolation of the bradykinin-potentiating peptides. The hydrolysate of α-casein was chromatographed on a Sephadex G-25 M column, as shown in Fig. 1. The potentiating activity of the fractions was measured on the isolated guinea-pig ileum. One potentiating unit was defined as the amount of protein able to double the activity of bradykinin toward that structure [3].

To determine the potentiation by a preparation, a log-dose–response curve for the effect of bradykinin on an isolated ileum preparation was made first. Then the same structure was tested by measuring the effect of a fixed amount of bradykinin plus increasing amounts of the preparation being assayed, in order to find the dose of potentiator beyond which no further increase of the ileum response to the chosen bradykinin dose would occur. The height of contraction observed was converted into nanograms of bradykinin with the aid of a log-dose–response bradykinin curve; the maximal potentiation activity shown in Table 1 is the ratio between that figure and the fixed dose of bradykinin.

The active fractions (Fig. 1) eluted from the G-25 M column were pooled (110–165), lyophilized and submitted to filtration chromatography on a Sephadex G-25 F column (Fig. 2). From this column, fractions that potentiated the effect of bradykinin on the guinea-pig ileum 11 to 14 times (fractions 64–69) were pooled and lyophilized. The whole procedure, up to this last phase, was repeated twice, starting with the casein fractionation and chromatography on the same Sephadex columns, with similar results. The average activity found for the three preparations from the G-25 M column was 2.93 ± 0.2 and after G-25 F, 13.3 ± 0.3.

The specificity of the active fractions for bradykinin potentiation is indicated by the results obtained in a set of three experiments which showed that those fractions did not change significantly (Fig. 3) the effects of acetycholine (highest contraction: without potentiator, 22.3 ± 1.9 mm; with potentiator, 21.3 ± 2.4 mm); histamine (highest contraction: without potentiator, 19.3 ± 0.67 mm; with potentiator, 19.7 ± 1.8 mm); angiotensin II (highest contraction: without potentiator, 23.0 ± 5.8 mm; with potentiator, 22.7 ± 6.7 mm); and $BaCl_2$ (highest contraction: without potentiator, 29.0 ± 0.58 mm; with potentiator, 30.7 ± 0.88 mm; see Fig. 4).

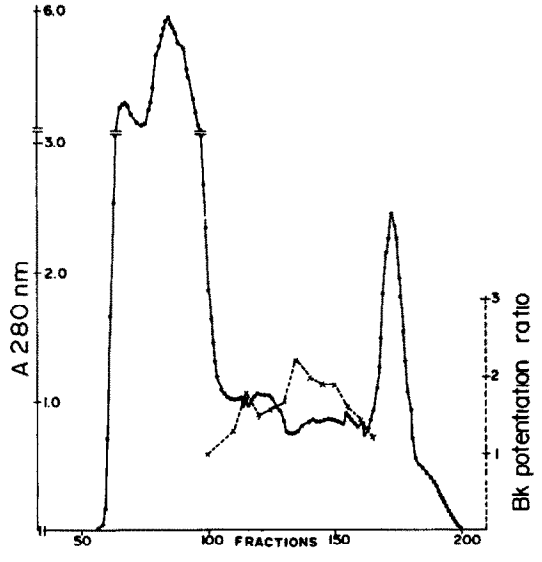

Fig. 1. Gel filtration of α-casein tryptic hydrolysate on a Sephadex G-25 M column (5 × 150 cm). Buffer: 20 mM ammonium bicarbonate. Fraction volume: 16 ml. Bradykinin potentiation was measured toward guinea-pig ileum; bradykinin was added to the ileum bath 1 min after addition of the eluate containing the crude peptides.

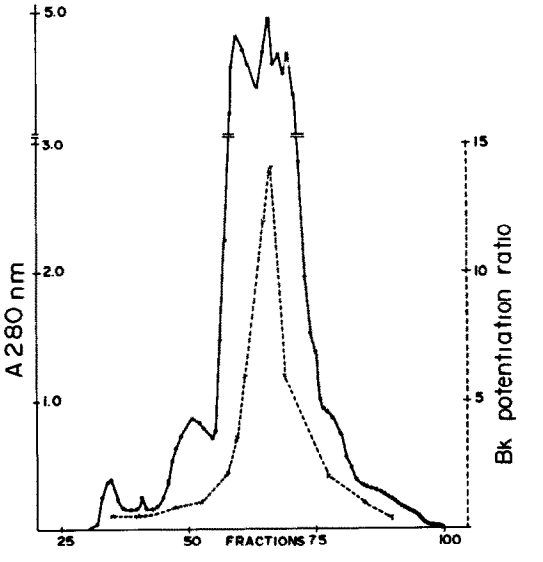

Fig. 2. Gel filtration on a Sephadex G-25 F column (2 × 180 cm) of the bradykinin potentiating fractions obtained after filtration on a Sephadex G-25 M column (5 × 150 cm). Buffer: 20 mM ammonium bicarbonate. Fraction volume: 3.5 ml. Bradykinin potentiation was measured as described in Fig. 1.

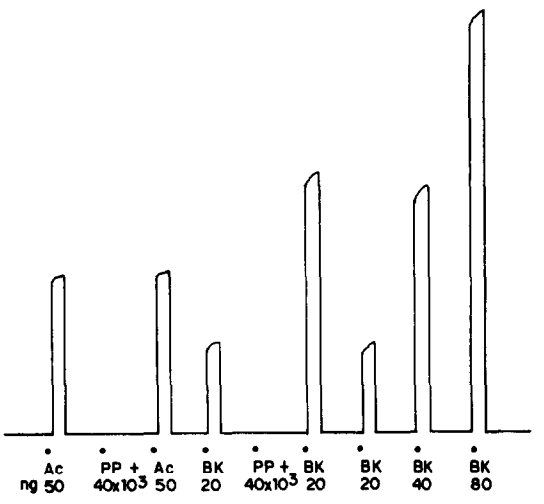

Fig. 3. Specificity of the bradykinin (BK) potentiating activity of the partially purified α-casein fractions (PP) on the guinea pig ileum. The presence of the potentiator did not alter the ileum response to acetylcholine.

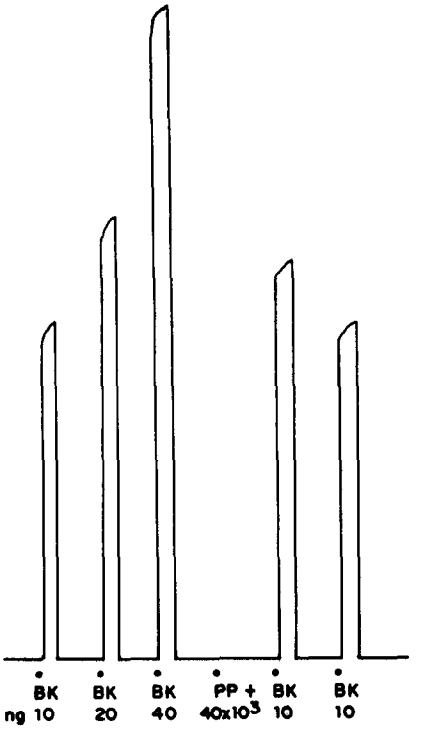

Fig. 5. Potentiation of the effect of bradykinin (BK) by the partially purified α-casein fraction (PP) in conditions where the endogenous angiotensin converting enzyme of the ileum was inhibited by Captopril (1 μg/ml Tyrode).

The peptides obtained from α-casein were able to potentiate the effect of bradykinin even though the endogenous angiotensin-converting enzyme of the ileum was inhibited by Captopril (Fig. 5). This observation suggests that the potentiating activity of the peptides obtained from α-casein, apart from being due to the inhibition of the endogenous enzymes, as described in the experiment presented in this paper, was also dependent on a direct effect of the peptides.

We have also observed that the fractions described above potentiate bradykinin when rat uterus preparation is used, a structure known to be almost free of kininase II [4].

To evaluate the activity of the peptides obtained by hydrolysis of α-casein as inhibitors of the plasma angiotensin-converting enzyme (ACE), the I_{50} for the hydrolysis of Hip-His-Leu by this enzyme was determined by fluorometry [5]. The activities of the inhibitors derived from the α-casein hydrolysate, as well as those from the partially purified peptides, were compared to those of Captopril (SQ 14225) and MK-422, both considered to be specific inhibitors of ACE.

The data presented in Table 1 show that the tryptic hydrolysate of α-casein contained peptides that acted as ACE inhibitors with an I_{50} in the micromolar range (molecular weight–2000 daltons). The results also show that the mixture of partially purified peptides potentiated, *ca.* 14 times, bradykinin activity toward the guinea-pig ileum, a potentiating activity that was ±2 times higher than that of Captopril and ±3 times than that of MK-422.

The high bradykinin potentiating activities of the partially purified peptides from α-casein hydrolysates, when compared with the activities of Captopril and MK-422, suggest that α-casein hydrolysate contains peptides that have potentiating activities which seem to be partially independent of ACE inhibitors. This property could be useful in studies of the participation of bradykinin in physiological or physiopathological situations.

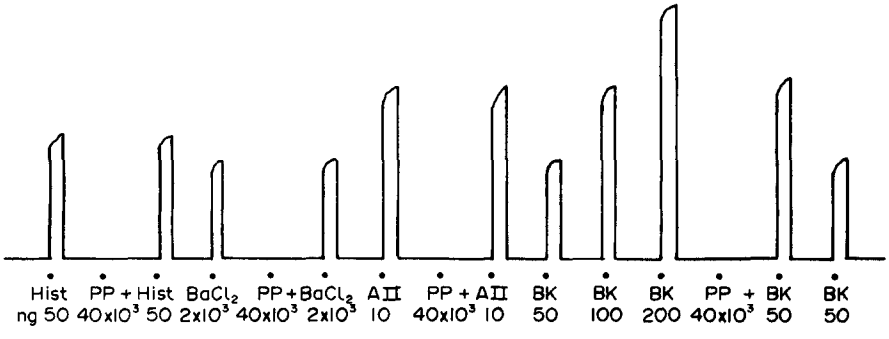

Fig. 4. Specificity of the bradykinin (BK) potentiating activity of α-casein fractions (PP) on the guinea pig ileum. There was no change of histamine (Hist), $BaCl_2$ or angiotensin II (A II) activities.

Table 1. Bradykinin (BK) potentiating action on guinea-pig ileum and inhibition of human plasma ACE by captopril, MK-422 and α-casein fractions

Substance	PU* (μg/ml)	I_{50} ACE† (μg/ml)	Maximal potentiation BK‡
Captopril	$(8.1 \pm 3.6) \times 10^{-4}$§	$(6.0 \pm 0.12) \times 10^{-3}$	7.2 ± 0.6
MK-422	$(2.3 \pm 0.4) \times 10^{-3}$	$(4.3 \pm 0.15) \times 10^{-3}$	5.2 ± 0.12
Tryptic hydrolysate	95.0 ± 4.0	81.0 ± 1.05	2.0 ± 0.18
Sephadex G-25 F active fractions	2.0 ± 0.09	23.0 ± 1.15	13.3 ± 0.3

* PU—potentiating unit: amount of peptide per milliliter able to double the action of a single dose of BK on the isolated guinea-pig ileum.
† I_{50} ACE—50% inhibition of angiotensin converting enzyme.
‡ For definition, see text.
§ Standard errors with two degrees of freedom.

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Evidence for the microsomal metabolism of glycolonitrile

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Saturated aliphatic nitriles are commercially important chemicals used in the manufacture of plastics, pharmaceuticals, pesticides and synthetic fibers [1]. The acute toxicity of the saturated nitriles has been attributed to a metabolite, CN^-* [2].

The mechanisms of metabolism of saturated nitriles to CN^- have not been well characterized. Ohkawa *et al.* [3] hypothesized that the initial step in nitrile metabolism is a mixed-function oxidase-dependent oxidation at the alpha-carbon, resulting in the formation of a cyanohydrin, which may be unstable and breakdown non-enzymatically to HCN and an aldehyde. Based upon data which indicated that 4-methylpyrazole and ethanol delayed the time to death in mice administered succinonitrile, Doherty *et al.* [4] suggested that alcohol dehydrogenase may catalyze the liberation of CN^- from the cyanohydrin of succinonitrile and, further, that the metabolism of other nitriles may also involve two enzymatic steps. Ethanol and pyrazole-compounds are also inhibitors of certain hepatic mixed-function oxidase systems [5, 6], however, and these chemicals may delay toxicity by inhibiting the initial oxidation of the parent nitrile. In our research on the metabolism of MeCN [7], we observed and report here that HCHO was not a metabolite of this nitrile. The present studies were thus initiated to study the liberation of CN^- from GCN, the cyanohydrin of MeCN. The data presented here indicate that a microsomal enzyme, probably cytochrome P-450, may catalyze the metabolism of this cyanohydrin to CN^- via a reaction which does not involve the release of HCHO.

* Abbreviations: MeCN, acetonitrile; CN^-, cyanide; HCHO, formaldehyde; and GCN, glycolonitrile.

Materials and methods

Chemicals. MeCN (99+%) and GCN (70%) were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and Alfa Products (Danvers, MA) respectively. Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and NADP were obtained from the Sigma Chemical Co. (St. Louis, MO). Cobaltic protoporphyrin 9-chloride (cobalt heme) was purchased from Porphyrin Products (Logan, UT). All other chemicals were of the highest grade commercially available. All buffers and water were passed through Chelex 100 (Bio-Rad Laboratories, Richmond, CA) prior to use to reduce the concentrations of contaminating trace metals.

Microsomal preparations. Female Sprague–Dawley rats, obtained from the Charles River Breeding Laboratories (Lakeview Facility, NJ) and weighing 170–260 g, were used in these studies. The animals were dosed with either a 25% (v/v) aqueous solution of acetone (1960 mg/kg, p.o.) or water (10.0 ml/kg, p.o.). The rats were killed (+24 hr), and the livers were collected, pooled by dosage group, and homogenized in 0.1 M sodium phosphate buffer containing 0.15 M KCl, pH 7.4. Microsomes were prepared by differential centrifugation, washed with 0.15 M KCl, resus-