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AN ENZYME IN HUMAN BLOOD PLASMA THAT INACTIVATES BRADYKININ AND KALLIDINS*

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Abstract—Human plasma fraction IV-1 contains an enzyme that rapidly inactivates bradykinin and kallidin II. The activity of the enzyme was characterized with biological and chemical techniques; it inactivates bradykinin by breaking its $Phe_8.Arg_9$ bond. Zn^{2+} or Co^{2+} can reactivate the enzyme, which is inhibited by chelating agents, but Co^{2+} is the only accelerator found. Numerous other compounds inhibit only. The enzyme in human red blood cells that destroys bradykinin is different from the plasma enzyme. The suggestion was made to name the enzyme in human plasma fraction IV-1 "carboxypeptidase N".

BRADYKININ† was discovered by Rocha e Silva *et al.*[5] who noticed that proteolytic enzymes release a hypotensive polypeptide, from plasma globulins, which stimulates smooth muscle. The activity of the peptide rapidly disappears upon standing in plasma. This inactivation was attributed to an enzyme, which was named after the substrate "kininase".[6] Fractions of human blood plasma that contain the precursors of bradykinin are usually contaminated with kininase.[6]

Kallidin has a longer history, research on the releasing enzyme, kallikrein, having begun in the 1920's.[7] Werle and colleagues[7] were the first to report that kallikreins liberate an active factor from plasma globulins. This factor also is destroyed by plasma or serum; the inactivator responsible for the destruction was called "kallidinase".[8]

Arg.	Pro.	Pro.	Gly.	Phe.	Ser.	Pro.	Phe.	Arg
1	2	3	4	5	6	7	8	9

FIG. 1. Structure of bradykinin.

Lys.	Arg.	Pro.	Pro.	Gly.	Phe.	Ser.	Pro.	Phe.	Arg
1	2	3	4	5	6	7	8	9	10

FIG. 2. Structure of kallidin II.

The structure of bradykinin recently has been established[9] and the peptide synthesized;[10] it is one of the strongest biologically active agents.[11] The structure of kallidin also was disclosed recently.[12] Two peptides were found in the preparations; according to Pierce and Webster[12] kallidin I is identical with bradykinin (Fig. 1), and kallidin II is a decapeptide (Fig. 2).

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† Abbreviations: N- and C-terminal, Reference 3; amino acids in the figures: Arg., arginine; Pro., proline; Gly., glycine; Phe., phenylalanine; Ser., serine; Lys., lysine; a period after the abbreviation indicates a peptide bond. For structures of side chains on amino acids found in proteins see Reference 4. Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetic acid.

The enzyme(s) in plasma that inactivates these peptides, may influence the duration of their action *in vivo*; this consideration led us to undertake the following project.

MATERIALS AND METHODS

Bradykinin (Fig. 1) and kallidin II (Fig. 2) were synthesized by Nicolaides and De Wald,[13] (Parke, Davis and Co.) who generously supplied us with samples. Kallidin II contained some impurities and probably was partly racemized.[14]

The biological activity of bradykinin and kallidin II was determined on the isolated rat uterus in estrus and twice on rabbit blood pressure. Rats were pretreated with a single subcutaneous injection of 0·1 mg of diethylstilbestrol. The isolated uterus was suspended in an oxygenated DeJalon's solution at 30 °C. The isotonic contractions were registered with the help of a new device built in this institute. The instrument is basically a converted density balance. The uterus in the Magnus bath was attached to the balance arm; the movements of the arm were converted by differential transformers and by the necessary circuits to signals, which were recorded automatically on a recorder. The details of this technique were published elsewhere.[15]

Because various sources and inhibitors of enzymes can interfere with the biological assay of bradykinin, the incubation mixture of substrate (bradykinin) and enzyme was diluted to the point at which only bradykinin remained active. The concentration of bradykinin incubated with the enzyme at 30 °C in a 0·1 M tris buffer of pH 7·4 was 3·4 μg/ml (3×10^{-6} M). In the majority of experiments the source of enzyme was human plasma fraction IV-1 (Cutter, Pentex, Protein Foundation, Inc.) in the concentration of 3 mg/ml. Samples were withdrawn at regular intervals, diluted immediately in saline, and injected into the muscle bath. Depending upon the sensitivity of the muscle, bradykinin was usually diluted 5000 to 40,000 fold or, in other words, its final concentration was in the order of 0·1 mμg/ml or less. The enzymic hydrolysis of kallidin II was similarly determined. Kallidin II, 5 μg/ml, was incubated with the enzyme and subsequently diluted in saline. The final concentration in the muscle bath was in the order of 1 mμg/ml. The activator or various inhibitors were usually preincubated with the enzyme for 60 min. Sequestering agents were added at zero time, the reactivating metals at 30 min, and the substrate at 60 min.

The blood pressure of the rabbit in pentobarbital narcosis was recorded in the carotid artery with the help of a Grass polygraph; injections were made into the femoral vein.

For paper chromatography and electrophoresis the "finger-printing" technique of Katz *et al.*[16] was employed. Descending chromatography was carried out for 17 hr on a Whatman 3MM paper. The solvent was a mixture of butanol:H_2O:acetic acid (4:5:1). The chromatography was followed in the second dimension by a 60-min electrophoresis (43 v/cm) in a pyridine:acetic acid:H_2O (1:10:289) buffer, pH 3·5. The high-voltage electrophoresis apparatus contained Varsol, and was built here according to the instructions of Katz *et al.*[16]

For "finger-printing", 0·5 mg of fraction IV-1 was incubated with 0·5 mg of bradykinin in an 0·1 M tris buffer of pH 7·4 in the presence of $1 \cdot 10^{-3}$ M $CoCl_2$ for 18 hr at 30° C. (When necessary, the concentration of enzyme and substrate was increased in the 1:1 ratio.) The mixture was applied to the chromatography paper without further treatment. In the experiments with carboxypeptidase A, 2·5 mg of the diisopropylfluorophosphate (DFP)-treated enzyme (Worthington) was incubated with

the substrate in an 0·05 M tris buffer, pH 7·7, for 4 hr at 31 °C. After the incubation the enzyme was precipitated with an equal volume of 6% dichloroacetic acid. The precipitate was removed in the centrifuge; the supernatant was dried in vacuum, diluted again, and analyzed. The spots were eluted from the unstained papers with 50% methanol. Generally the papers were stained on one side with a combination of Sakaguchi's technique, according to Irrevere's modification,[17] and with ninhydrin on the other. In all of these studies an adequate number of control experiments was done.

After the reaction with fraction IV-1 or carboxypeptidase A was completed, the presence of phenylalanine or arginine in the eluates was also shown with the help of a Spinco automatic amino acid analyzer, model 120. The hydrolysis of hippuryl-L-arginine was ascertained with the finger-printing technique; 4 μmoles hippuryl-L-arginine were incubated with 1 mg of fraction IV-1 in 0·2 ml tris and 10^{-3} M $CoCl_2$ at 31 °C for 18 hr. In the attempts to show hydrolysis of hippuryl-L-arginine[18] with the spectrophotometer technique, a Cary model 11 automaticrecording spectrophotometer was used.

Pooled plasma was collected in heparinized tubes from healthy male donors; 0·05 ml was diluted to 1 ml with tris buffer and used as a source of enzyme. Human red blood cells were washed in saline three times, then hemolyzed with equal amounts of distilled water.

RESULTS

Biological studies on the enzyme

When 3·4 μg bradykinin/ml was incubated with human plasma fraction IV-1, the biological activity of the peptide rapidly disappeared, as shown with the rat uterus technique (Fig. 3) and twice with the rabbit blood pressure. Only relatively small

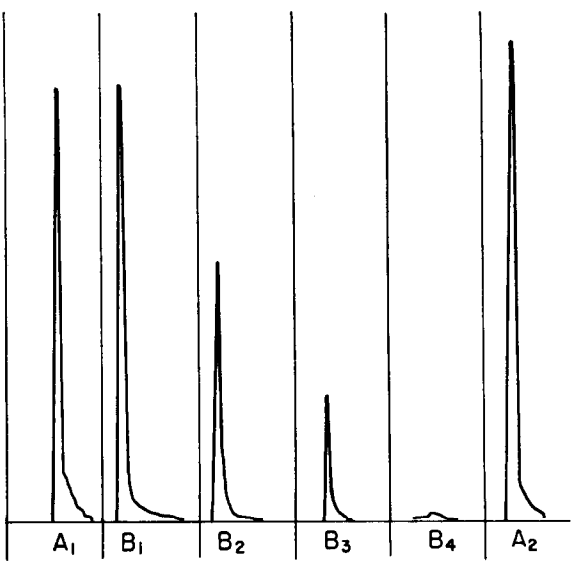

FIG. 3. Inactivation of bradykinin by plasma fraction IV-1. Rat uterus in estrus was used. A_1 and A_2 = 0·17 mμg bradykinin/ml. B_1, B_2, B_3, and B_4 have the same amount of bradykinin, but were incubated with the enzyme, and injected at 0, 15, 30, and 45 min (see Methods).

changes in the concentration of bradykinin could be determined with the first method. For kinetic studies the enzyme bradykinin mixture was diluted (see Methods) 1:20,000 v/v; after the activity disappeared at this concentration, the dilution was decreased to 1:5000 and later correspondingly lowered; thus the destruction of bradykinin was followed beyond 90 per cent completion of the reaction. Plotting the log of mean decrease in activity in three experiments against time, and from equation $t_{1/2} =$

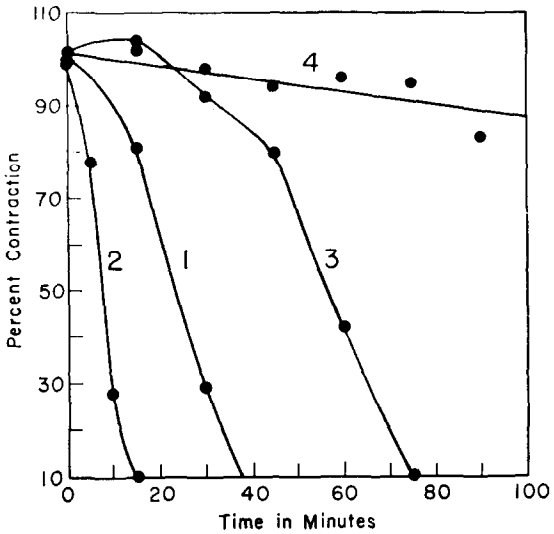

FIG. 4. Destruction of the activity of synthetic bradykinin ($3 \cdot 10^{-6}$ M) by human plasma fraction IV-1 (3 mg/ml). Ordinate: per cent of contraction of isolated rat uterus. Abscissa: time in minutes. 1, Control; 2, 1×10^{-3} M $CoCl_2$; 3, 3×10^{-3} M cysteine; 4, 3×10^{-4} M $CdSO_4$.

$0{\cdot}693/k$, a first-order rate constant of $k = 0{\cdot}024$ min^{-1} was calculated. In a few experiments human plasma fractions IV-4 and IV-6-3 also destroyed bradykinin.

The properties of the enzyme in question were further probed with the help of various agents. In the experiments shown in Fig. 4 $CoCl_2$ accelerated the enzyme, while cysteine was a moderate, and $CdSO_4$ a strong, inhibitor. The inhibition studies were summarized in Table 1. Here the most effective inhibitors are arbitrarily marked $++$, the less potent ones $+$, the inactive compounds θ. Some heavy metals (Mn^{2+}, Cd^{2+}, Hg^{2+}) are good inhibitors, but the —SH agent *p*-chloromercuriphenylsulfonic acid (CMPS) was inactive. $ZnCl_2$ in the concentration of $3 \cdot 10^{-4}$ M did not influence the reaction; $1 \cdot 10^{-3}$ M $ZnCl_2$, however, stopped it completely. (This was possibly owing to the precipitation of plasma proteins by the metal ions.) Chelating agents, such as 1,10-phenanthroline or EDTA and its calcium and magnesium complexes, block the enzyme, but Co-EDTA is inactive. The addition of $CoCl_2$ in equimolar quantities to the enzyme-EDTA or enzyme-phenanthroline mixture, or the addition of $ZnCl_2$ to the phenanthroline mixture, overcomes the inhibition and restores the activity of the enzyme. (Contrary to the findings of others[19] $CoCl_2$ itself has no effect on the stability of bradykinin solutions.) Arginine, ϵ-amino-*n*-caproic acid and δ-amino-*n*-valeric acid, inhibitors of carboxypeptidase B,[20] were also effective here.

Other inhibitors of proteolytic enzymes, like DFP and trypsin or kallikrein inhibitors (see Table 1), had little or no effect. Since fraction IV-1 contains also powerful enzymes that destroy organophosphorus compounds,[21, 22] the results with DFP are not conclusive. No inhibition was observed, however, even when the length of preincubation with DFP was varied; 6 M urea completely destroyed the activity of the enzyme at pH 7·7 and room temperature within 1 hr; 4% butanol had no significant effect.

TABLE 1. INHIBITORS OF THE ENZYMATIC HYDROLYSIS OF BRADYKININ

Inhibitor	Concentration (M)	Relative degree of inhibition*
$MnCl_2$	1×10^{-3}	++
$CdSO_4$	3×10^{-4}	++
$HgCl_2$	1×10^{-4}	++
CMPS	1×10^{-3}	θ
$NiSO_4$	1×10^{-3}	θ
$ZnCl_2$	3×10^{-4}	θ
1,10-Phenanthroline	3×10^{-4}	++
EDTA	3×10^{-3}	++
Ca-EDTA	3×10^{-3}	++
Mg-EDTA	3×10^{-3}	++
Co-EDTA	3×10^{-3}	θ
Cysteine	3×10^{-3}	+
Arginine	3×10^{-3}	++
ε-amino-*n*-caproic acid	3×10^{-3}	++
δ-amino-*n*-valeric acid	1×10^{-2}	++
DFP	1×10^{-4}	θ
Soybean trypsin inhibitor	0·6 mg/ml	θ
Ovomucoid trypsin inhibitor	0·6 mg/ml	θ/+
Trasylol kallikrein inhibitor	150 U/ml	θ/+

* Most effective inhibitors, ++; less potent, +; inactive compounds, θ.

Chemical studies on the enzyme

The biological experiments indicated that "kininase" in fraction IV-1 is a metallopeptidase, thus possibly an exopeptidase. Because bradykinin contains arginine at both ends, the closer characterization of the enzyme had to be done with chemical techniques. When bradykinin was incubated with fraction IV-1 in the presence of $CoCl_2$, the combined paper chromatography and electrophoresis showed two Sakaguchi and ninhydrin-positive main spots (Fig. 5). One spot behaved like free arginine [Fig. 5 (3).] The presence of arginine was confirmed by elution of the active material

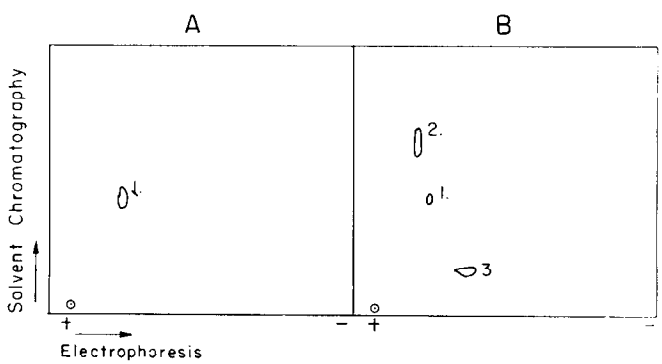

FIG. 5. "Finger-printing" of the products of enzymatic hydrolysis of bradykinin. A, 0 time reaction; B, after 18 hours of incubation. 1, Bradykinin; 2 and 3, split products (see Results).

from the paper and by analysis on a Spinco automatic amino acid analyser. The second spot probably represented the octapeptide [Fig. 5, (2)] remainder of bradykinin. In order to determine whether the liberated arginine was C- or N-terminal, the octapeptide spot also was eluted from the paper and treated with purified carboxypeptidase A. The amino acid analyzer showed the presence of phenylalanine after the incubation in the enzyme. The phenylalanine, liberated by carboxypeptidase A, very likely corresponds to Phe_8. in the structure of bradykinin. This, taken with the purple color of the ninhydrin reaction, indicates that the plasma enzyme inactivates bradykinin by splitting off its C-terminal arginine, or, in other words, it behaves like a carboxypeptidase (Fig. 6).

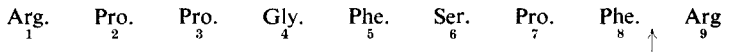

FIG. 6. Enzymic inactivation of bradykinin.

In the ultraviolet spectrophotometer no appreciable hydrolysis of hippuryl-L-arginine by fraction IV-1 was detected, but after an overnight incubation the fingerprinting showed the hydrolysis of this substrate.

Inactivation of kallidin II

In studies paralleling those with bradykinin, fraction IV-1 destroyed the biological effect of kallidin II. The enzymatic activity was accelerated by $CoCl_2$, and inhibited by $CdSO_4$, EDTA, or 1,10-phenanthroline. The inhibition by EDTA was prevented by adding $CoCl_2$ to the mixture of enzyme and complexing agent. CMPS or $NiSO_4$ did not delay the inactivation. Since kallidin II has the same C-terminal end as bradykinin,[12] we can assume from these results that the two peptides are inactivated by the same enzyme in plasma.

Human blood plasma and red cells

Diluted human plasma inactivates bradykinin rapidly. The inhibition of the enzyme in plasma by chelating agents resembles that of fraction IV-1. Four-hr digestion of bradykinin by fresh plasma gave spots comparable with those obtained with fraction IV-1 in the combined paper chromatography-electrophoresis. Preliminary studies with inhibitors showed a definite difference, however, between the enzyme in erythrocytes that destroys bradykinin on one hand and the peptidase in plasma on the other.

DISCUSSION

In addition to bradykinin and kallidin, numerous other hypotensive peptides that stimulate smooth muscle have been found. These are released from plasma proteins by colostrum, plasmin, contact with glass, dilution[23] or are present in human urine,[24, 25] in wasp venom,[26] etc. It was suggested that these substances be called "kinins".[6] The name indicates a similarity of action, but not necessarily an identity of structure; the term is not yet generally accepted.[27] In accord with this terminology the enzymes in the circulation and in the tissues, which destroy kinins, were named "kininases".[6]

Little was known about these enzymes beyond a few isolated observations. For example, released kallidin and bradykinin were preserved in plasma by the addition of cysteine[6, 8] and Armstrong *et al.* noticed that the activity of a factor in plasma liberated by glass was retained in the presence of EDTA.[28]

Our experiments indicated that in plasma fraction IV-1, the "kininase" that destroys bradykinin and kallidin II is a carboxypeptidase-type enzyme. Very likely, the same enzyme in plasma is responsible for the destruction of both agents.

The C-terminal $Phe_8.Arg_9$ end of bradykinin has been described as susceptible to attack by two purified proteolytic enzymes. Chymotrypsin[9, 10] and carboxypeptidase[29] (probably a mixture of A- and B-type enzymes) can liberate arginine from the peptide. The enzyme characterized in this article can easily be distinguished from chymotrypsin. The activity of chymotrypsin is accelerated by calcium only,[30] but calcium is not a cofactor of the enzyme,[30] and chymotrypsin is not included among the metallopeptidases.[31] It is not inhibited by EDTA[32] but by DFP,[33] by trypsin inhibitors,[33] and by human plasma.[34]

Carboxypeptidase A does not split off basic C-terminal amino acids;[35] the bonds of arginine, lysine, and ornithine are broken by carboxypeptidase B.[35] The two carboxypeptidases have numerous properties in common. Both contain zinc,[18, 35] both are accelerated by cobalt,[36–38] and inhibited by phenanthroline,[35] cadmium, etc.[36] Carboxypeptidase B has been found only in the pancreas of domestic animals as a zymogen.[39] It is not inhibited by EDTA, or at least not with hippuryl-L-arginine substrate.[18] It stays active in 6 M urea, and *n*-butanol enhances the activity.[40]

The source of our enzyme, on the other hand, was a fraction of human plasma. Besides inhibitors of carboxypeptidase B, urea and EDTA blocked the hydrolysis of bradykinin; *n*-butanol had no effect. Hippuryl-L-arginine, the specific substrate of carboxypeptidase B, was only very slowly hydrolyzed by fraction IV-1; possibly, in blood, the level of the enzyme is just not high enough for spectroscopic assay with this substrate. The inhibition by chelating agents could be reversed with cobalt chloride; the inhibition by phenanthroline also could be reversed with zinc chloride. At the present degree of purity we cannot state which metal is required in the first place.

Thus human plasma contains a carboxypeptidase B-type enzyme which, like the carboxypeptidase B of the pancreas, can release the C-terminal arginine. The apparent differences between the two enzymes were summarized above. For the present we suggest "carboxypeptidase N" as the name of the enzyme in plasma fraction IV-1 that inactivates bradykinin and probably kallidin II. The enzymes that can terminate the action of peptides have often been named after one substrate (e.g., angiotensinase, vasopressinase, bradykininase, kallidinase, etc.[1]). However, we would like to conform with the terminology of the biochemist by naming the enzyme that inactivates bradykinin and kallidin II after the bond it breaks, and not after the whole peptide chain.

A future communication will deal with the effect of purified proteolytic enzymes on kallidin and bradykinin.

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