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The bradykininase activities of extracts of dog lung

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Summary

1. Homogenates of dog lungs freed from blood inactivate bradykinin. The bradykininase activity is present in soluble and particulate subcellular fractions.

2. The fraction with the highest relative specific activity is that sedimenting between 8,700 *g* and 78,000 *g*. This fraction has been studied in more detail.

3. There is evidence for two bradykinin inactivating enzymes in this fraction with apparent K_m values of 7·5 μM and 120 μM.

4. The bradykininase activity is not dependent on the concentration of chloride ion.

5. The bradykininase activity is inhibited by EDTA, 2:3-dimercaptopropanol, nickel ions and some of the peptides from the venom of *Bothrops jararaca* and of *Agkistrodon halys blomhoffii* but not by 2-mercaptoethanol or N-ethylmaleimide.

6. Angiotensin II in either 15 or 170 mM chloride ion is not an inhibitor of bradykininase activity but angiotensin I at either chloride concentration is an inhibitor. It is proposed that chloride is not necessary for binding of angiotensin I to converting enzyme but is necessary to ensure the correct orientation of substrate for hydrolysis to proceed.

Introduction

Bradykinin is inactivated by a variety of tissues (Trautschold, Fritz & Werle, 1966; Iwata, Shikimi & Oka, 1969; Camargo & Graeff, 1969) and we have been particularly concerned with its inactivation in the lung which has been demonstrated *in vivo* by Ferreira & Vane (1967). There is a potent bradykininase activity in blood-free isolated lungs (Alabaster & Bakhle, 1972a) and we have studied this activity in homogenates of dog lung in greater detail. One of the aims of this study was to compare the characteristics of the bradykininase activity with those already established for angiotensin converting enzyme activity present in the cell-free extracts of dog lung (Bakhle, 1968; Huggins, Corcoran, Gordon, Henry & John, 1970), since it had been suggested by Ng & Vane (1968) that these two peptidase activities were identical. A summary of the results of some of the experiments described in detail here was presented at the 1971 meeting of the Council for High Blood Pressure Research (Alabaster & Bakhle, 1972b).

Methods

The homogenate of dog lung was prepared as described previously (Ferreira, Greene, Alabaster, Bakhle & Vane, 1970). A low speed supernatant fraction (S_1) was obtained by centrifugation at 1,000 *g* for 20 min and subsequent dialysis against

buffer (1·0 mM phosphate, pH 7·0) at 4° C overnight. This was then separated into the following fractions by differential centrifugation: P_2 the pellet after 8,700 *g* for 20 min; P_3 the pellet after 78,400 *g* for 60 min; and S_3 the supernatant after 78,400 *g* for 60 minutes. The values of relative centrifugal force are the average values obtained in the Spinco centrifuge with the No. 30 rotor. The pellets were resuspended in the phosphate buffer and all the fractions stored at −20° C. Protein was estimated by the biuret procedure. In one preparation, the resuspended P_3 fraction was dialysed against the disodium salt of ethylene diamine tetra-acetic acid (EDTA, 1 mM, pH 7·0, in the phosphate buffer) overnight and then dialysed against buffer alone.

The bradykininase activities of the various fractions were assayed in an incubation mixture consisting of: lung extract, 0·05–0·25 mg protein/ml; 5–50 μg bradykinin/ml; phosphate buffer, 40 mM, pH 7·0. The incubation was carried out in a shaking incubator at 37° C usually for 10 minutes. The zero time sample was taken immediately after adding the substrate to the other components of the incubation mixture to start the reaction. Subsequent samples were taken after the required periods of incubation. The samples were then kept in an ice bath until they were assayed. They were diluted as required with sterile isotonic saline (0·9% w/v NaCl solution) and assayed against the standard bradykinin solutions using a bracket assay method.

Bradykinin was measured on any one of four tissues; the isolated terminal ileum of the guinea-pig, the longitudinal muscle from this tissue (Rang, 1964), or strips of the cat or kitten terminal ileum (Alabaster & Bakhle, 1972a). The tissue was suspended in a 5 ml organ bath in Krebs bicarbonate solution, gassed with 95% oxygen and 5% carbon dioxide, maintained at 37° C, or superfused at a rate of 8–10 ml/min with gassed Krebs bicarbonate solution at 37° C. Hyoscine (100 ng/ml) was sometimes added to the Krebs solution to reduce spontaneous activity of the tissues. The longitudinal muscle strip from the guinea-pig ileum has much less spontaneous activity than the whole ileum, but its sensitivity to bradykinin is unchanged. When the synthetic peptides corresponding to those in *Bothrops jararaca* or *Agkistrodon halys blomhoffii* venom were used, the assay of bradykinin was carried out on strips of cat or kitten terminal ileum, as these peptides do not potentiate the action of bradykinin on the cat tissues (Alabaster & Bahkle, 1972a).

The following chemicals were used: disodium salt of ethylene diamine tetra-acetic acid (EDTA), 2:3-dimercaptopropanol (BDH); 2-mercaptoethanol (Koch-Light); N-ethylmaleimide (Sigma); cobalt sulphate, manganese sulphate, nickel nitrate, zinc sulphate and magnesium sulphate (all analytical reagent grade, BDH); angiotensin II amide (Hypertensin, Ciba); angiotensin I (Schwarz Biochemicals); bradykinin (BRS 640, Sandoz); BPF (bradykinin potentiating factor; semi-purified extract of *Bothrops jararaca* venom) was generously provided by Dr. S. H. Ferreira, the synthetic *B. jararaca* peptides by Drs. D. W. Cushman and M. Ondetti (Squibb Institute for Medical Research, USA), and the synthetic and natural *A. halys blomhoffii* peptides by Dr. H. Kato (Institute for Protein Research, Osaka).

Results

The distribution of bradykininase activity in the various subcellular fractions of the dog lung homogenate is shown in Table 1. All fractions had bradykininase

activity but the final particulate fraction (P_3) displayed the highest specific activity. Unless otherwise stated all subsequent results apply to the bradykininase activity in the P_3 fraction.

TABLE 1. *Distribution of bradykininase activity in subcellular fractions derived by differential centrifugation from homogenates of dog lung*

Fraction	Bradykininase activity	
	Specific activity	Relative specific activity
P_2 (8,700×20) *g*. min	3·1	0·8
P_3 (78,400×60) *g*. min	7·5	5·4
S_3 (78,400×60) *g*. min	2·3	0·3

The inactivation of bradykinin was measured under the standard incubation conditions: 0·1 mg protein/ml; 10 μg bradykinin/ml; buffer 40 mM; incubation at 37° C for 10 min. The units of specific activity are μg bradykinin inactivated (mg protein)$^{-1}$ min^{-1} under these conditions. Relative specific activity = % total specific activity in fraction ÷ % total protein in fraction.

The rate of inactivation of bradykinin was constant over 30 min of incubation and to about 80% of substrate (20 μg/ml) consumed. The rate was also linearly dependent on the amount of enzyme (between 0·05–0·25 mg protein/ml) in the incubation mixture. At high substrate concentrations (50–100 μg/ml), the rate decreased, suggesting that the enzyme was approaching saturation. However, when the results of these experiments were analysed by a double reciprocal plot, the points fell on a curve rather than on a straight line. This could be due to the presence of two enzymes with different Km values and when the results were plotted according to the method of Eadie & Hofstee (Mahler & Cordes, 1969), the points clearly fell on two lines of different slope (Fig. 1). The values of the apparent Km's of these

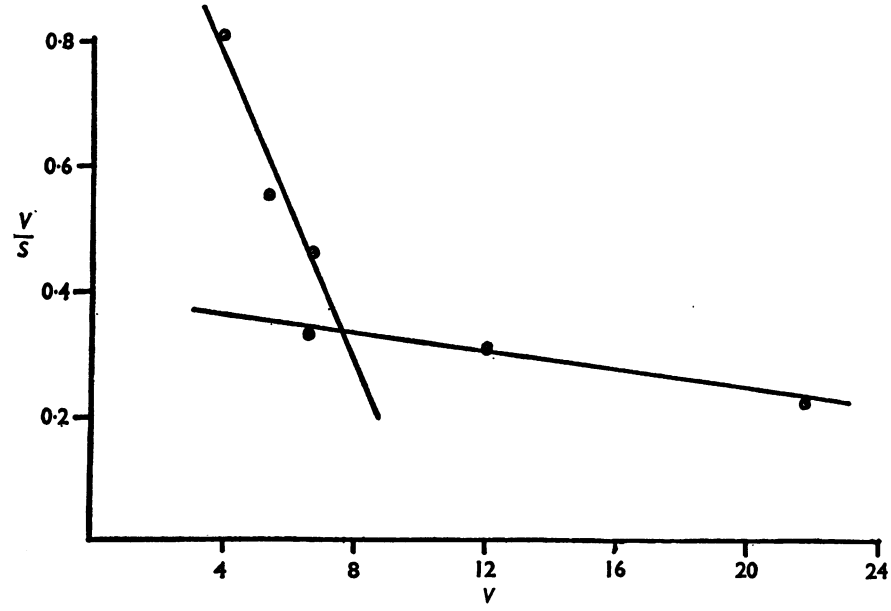

FIG. 1. Variation of the rate of inactivation of bradykinin with substrate concentration in a particulate fraction (P_3) of dog lung homogenate. The rate of reaction (V) is expressed as ng bradykinin/ml inactivated in 10 min and the substrate concentration (S) as ng bradykinin/ml. Bradykinin (5–100 μg/ml) was incubated with enzyme (0·1 mg protein/ml) for 10 minutes. The values plotted are the means of three sets of experiments.

enzymic activities are 7·3–7·5 μM (7·7–8·0 μg/ml) and 110–120 μM (126–128 μg/ml), with V_{max} values of 10 and 50 μg/mg protein/min respectively.

All subsequent experiments were carried out with substrate concentrations of 10 μg bradykinin/ml, in an attempt to work within the range of the enzyme with the lower Km and to keep clear of the transition between the activities.

Variation of the concentration of chloride ion (as NaCl) in the incubation mixture from 1·5 mM to 255 mM had no effect on the bradykininase activity. The cation requirements of this activity were studied by incubation in the presence of several metal ions. When phosphate buffer was used, only magnesium caused inhibition. In tris buffer of the same pH, nickel was strongly inhibitory with magnesium and zinc less so, and cobalt and magnesium were without effect (Table 2).

TABLE 2. *Effect of divalent cations on bradykininase activity in tris and phosphate buffers*

Cations (500 μM)	Bradykininase activity (as % of control) Tris	Phosphate
Co	112	98
Mn	100	97
Ni	10	95
Zn	64	100
Mg	69	73

Variation of effects of divalent cations on bradykininase activity in two buffers. Potassium phosphate or tris-HCl buffers (40 mM, pH 7·0) were used and the other conditions were the standard conditions given in the text. The values in the table are the means of at least three experiments.

Effects of enzyme inhibitors

2-Mercaptoethanol, an inhibitor of plasma kininases (Trautschold *et al.*, 1966) had no effect on the inactivation of bradykinin by fractions P_3, S_3 or S_1, in concentrations up to 10 mM (Table 3). A partially purified preparation of bradykinin

TABLE 3. *Effect of enzymic inhibitors on bradykininase activity*

Inhibitor	Concentration	% Inhibition
2-Mercaptoethanol	1 mM	5 (4)*
	10 mM	10 (5)
2:3-Dimercaptopropanol	1 μM	4 (1)
	10 μM	37 (3)
	100 μM	81 (3)
N-Ethylmaleimide	100 μM	0 (5)
	1 mM	3 (3)
BPF	2 μg/ml	27 (1)
	5 μg/ml	53 (2)
	20 μg/ml	67 (4)
BPF +2-Mercaptoethanol	20 μg/ml 100 μM	68 (2)

Effect of enzymic inhibitors on bradykininase activity of a particulate fraction (P_3) of dog lung homogenate. Incubations were carried out as described in the text, for 10 min with 10 μg/ml bradykinin and 0·075 mg/ml protein. BPF=bradykinin potentiating factor.
* Number of experiments.

potentiating factor (BPF) (Ferreira, 1965) inhibited the bradykininases in fractions P_3 and S_3. The enzyme activity could not be completely inhibited, a maximum of about 70% inhibition being obtained. Stewart, Ferreira & Greene (1971) described similar results with rat perfused lung but showed that by using 2-mercaptoethanol together with the BPF peptides, the pulmonary inactivation of bradykinin could be completely inhibited. However, in our experiments, addition of 2-mercapto-

ethanol to a maximally inhibitory concentration of BPF did not further inhibit bradykininase. Later the synthetic peptides corresponding to the natural components of BPF were synthesized (Greene, Stewart & Ferreira, 1970 ; Ondetti, Williams, Sabo, Pluscec, Weaver & Kocy, 1971), and some of these, as well as two of the bradykinin potentiating peptides from *A. halys blomhoffii* (Kato & Suzuki, 1971) were tested as inhibitors of the bradykininase in P_3. The results (Fig. 2) show that the peptides differ in inhibitory potency with I50 values, measured from the graph, of 0·27 μM (BJ9), 0·75 μM (BJ10), 1·7 μM (BJ5) and estimated values of about 20 μM for the peptides AH-C and E. The structures of these peptides are given in the legend to Figure 2. In all these experiments the initial bradykinin concentration was 10 μg/ml.

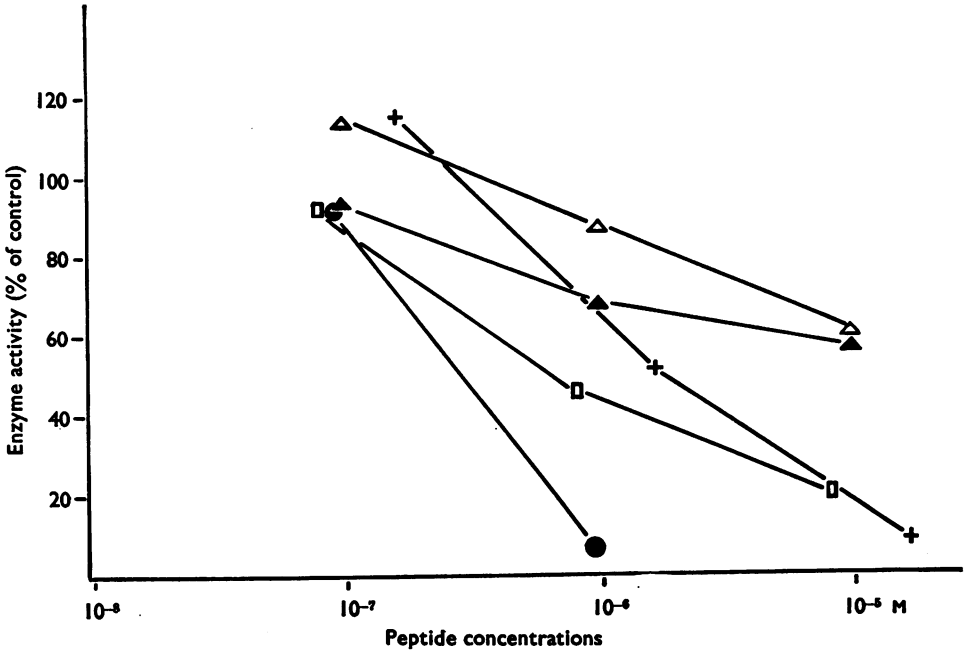

FIG. 2. Inhibition of bradykininase activity by venom peptides. The variation of enzyme activity (as % control activity) with the molar concentration of the peptide is shown. Three synthetic peptides corresponding to those found in *B. jararaca* venom (BJ5, 9, 10) and two corresponding to those in *A. halys blomhoffii* venom (AH-C and E) were added to the standard incubation mixture just before the substrate. Bioassay of bradykinin activity was carried out on strips of cat terminal ileum. At least three experiments were performed for each point, and their means are plotted.

+	BJ5	Pca-Lys-Trp-Ala-Pro
●	BJ9	Pca-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro
□	BJ10	Pca-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro
△	AH-C	Pca-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro
▲	AH-E	Pca-Lys-Trp-Asp-Pro-Pro-Pro-Val-Ser-Pro-Pro

Bradykinin is a competitive inhibitor of pulmonary angiotensin I converting enzyme (Sander, Huggins & West, 1971), and the possibility that bradykininase and converting enzyme activities may be functions of the same enzyme led us to investigate the effects of the angiotensins on our preparation of bradykininase. The results summarized in Table 4 show that angiotensin I is an inhibitor of the inactivation of bradykinin whereas angiotensin II is without effect, in either high or low concentrations of chloride. We attempted to measure the inhibition caused

by angiotensin I (20 μg/ml) over a range of bradykinin concentrations (1–10 μg/ml) in order to determine the type of inhibition caused (competitive or non-competitive). However, this amount of angiotensin I interfered seriously with the assay of the lower amounts of bradykinin and reliable data over the whole range could not be obtained.

TABLE 4. *Effect of angiotensin peptides on the inactivation of bradykinin* in vitro

	% Inactivation of substrate (mean ± S.E.M.)			
	Angiotensin I (20 μg/ml)		Angiotensin II (20 μg/ml)	
Control	15 mM Cl	170 mM Cl	15 mM Cl	170 mM Cl
62·7±2·3 (n=12)	35·4±3·8 (n=4)	37·1±3·7 (n=5)	61·8±2·1 (n=5)	63·4±1·8 (n=5)

In these experiments incubations were carried out as described in the text with 10 μg bradykinin/ml and 0·1 mg protein/ml incubated for 10 minutes. The angiotensin peptides were added to the incubation mixture just before the substrate and the bioassay carried out on strips of cat terminal ileum

The chelating agents, 2:3-dimercaptopropanol (Table 3) and EDTA (Table 5) inhibited the bradykininase activity, and this inhibition could be complete. The activity could be partially restored by the addition of divalent cations, cobalt and manganese (500 μM) being the most effective against the inhibition caused by EDTA (250 μM, Table 5). These two cations were again the most effective in restoring bradykininase activity to an enzyme preparation dialysed against EDTA (1 mM).

TABLE 5. *Inhibition of bradykininase by EDTA and reversal by divalent cations*

	Bradykininase activity (as % of control) after treatment of enzyme	
Additions	Dialysis vs. EDTA	EDTA added
None	18	7
Co	42	77
Mn	50	59
Zn	27	30
Mg	32	20

Inhibition by EDTA and its reversal by divalent cations. The enzyme preparations were either dialysed against EDTA (1 mM), or had EDTA (250 μM) added to them in the reaction mixture. The divalent cations (500 μM) were added, just before the substrate, to the incubation mixtures in which tris buffer was used. Other incubation conditions were the standard conditions given in the text. The values in the table are the means of at least 3 experiments.

The inhibition of bradykinin inactivation in perfused lungs by N-ethylmaleimide (Alabaster & Bakhle, 1972a) led us to try this compound as an inhibitor of the bradykininase activity in fraction P_3. However, despite preincubation with the enzyme for up to 30 min and in concentrations up to 1 mM, N-ethylmaleimide did not affect the inactivation of bradykinin.

Discussion

We have previously discussed the relation between the properties of the bradykininase activity, as described in detail here, and those of angiotensin I converting enzyme activity, also present in the same subcellular fraction (P_3) of dog lung homogenates (Alabaster & Bakhle, 1972b). We concluded that these two peptidase activities are closely related but may be most clearly differentiated by the effects of chloride ion. This ion is an absolute requirement for converting enzyme activity (Skeggs, Kahn, Marsh & Shumway, 1954) but not for bradykininase. A similar

result has been reported by Sander *et al.* (1971) using a particulate fraction from rabbit lung.

The multiplicity of tissue bradykininases (see **Introduction**) makes comparison of this pulmonary bradykininase with other bradykininases difficult. However, like some bradykininases (Ferreira & Rocha e Silva, 1962 ; Erdös & Yang, 1966 ; Yang & Erdös, 1967) it is inhibited by EDTA and 2:3-dimercaptopropanol. It is unaffected by 2-mercaptoethanol and manganese ions, unlike the bradykininases described by Erdös, Renfrew, Sloane & Wohler (1963), Trautschold *et al.* (1966) and Erdös & Yang (1966).

The effects of cations on the bradykininase activity provide one point worth noting ; the widely different results obtained for zinc and nickel ions in tris and phosphate buffers. This discrepancy may reflect the greater insolubility of nickel and zinc phosphates compared with that of magnesium phosphate. In earlier work (Bakhle & Reynard, 1971), tris buffers were routinely used instead of phosphate buffers in experiments involving heavy metal ions without examination of the effects of phosphate buffers. Our present results may explain why activation or inhibition by the same cation has been reported for different preparations of the same enzyme.

The relative potencies of the venom peptides as inhibitors of the bradykininase activity parallel their potencies as bradykinin potentiators on the guinea-pig ileum (Greene *et al.*, 1970 ; Kimura, Kato, Sakakibara & Suzuki, 1970) with one exception. The peptide AH-E (potentiator E) is much less effective in potentiating bradykinin on the guinea-pig ileum than peptide AH-C (potentiator C ; Kimura *et al.*, 1970), the concentrations required to double the effect of bradykinin being 46·8 μM (AH-E) and 0·53 μM (AH-C), but in our experiments AH-C and AH-E are very similar in activity as inhibitors of a lung bradykininase. This divergence between inhibition of bradykininase and potentiation of bradykinin's effects exhibited by AH-E lends support to the hypothesis advanced by Camargo & Ferreira (1971) that bradykinin potentiation is not only the result of preventing destruction of the kinin, but may also involve a direct interaction of potentiator with receptor. This type of interaction may also explain why AH-E is an ineffective potentiator of bradykinin on guinea-pig ileum but a much more effective potentiator on the rat isolated uterus (Kimura *et al.*, 1970). AH-E is also relatively more effective against bradykininase than it is against lung converting enzyme (Bakhle, 1972) which it does not inhibit at 300 μM. With this one exception the relative potencies of these venom peptides as inhibitors of bradykininase or of converting enzyme are similar though the I50 values are different for the two enzymes.

Angiotensin I inhibited the bradykininase preparation equally effectively in the presence of 15 or 170 mM chloride ion, suggesting that the binding of angiotensin I to bradykininase is unaffected by this range of chloride concentrations. Now, if bradykininase and converting enzyme are alternative functions of the same enzyme (Elisseeva, Orekhovich, Pavlikhina & Alexeenko, 1971 ; Yang, Erdös & Levin, 1971 ; Cushman & Cheung, 1972 ; Igic, Erdös, Yeh, Sorrells & Nakajima, 1972), then the binding of angiotensin I at low chloride concentrations to bradykininase suggests that angiotensin I will also bind to converting enzyme at low chloride concentrations. Thus, lack of substrate binding does not explain why converting enzyme hydrolyses angiotensin I very slowly at low chloride concentrations. Between 15 mM and 170 mM NaCl, converting enzyme activity increases 20-fold (Bakhle & Reynard, 1971 ; Alabaster & Bakhle, 1972b).

In the highly purified enzyme preparations described by Elisseeva *et al.* (1971) and Igic *et al.* (1972) both bradykinin and angiotensin I are hydrolyzed by cleavage of the C-terminal dipeptide, so the mechanism of dipeptide cleavage would seem not to be chloride-dependent. We would like to propose therefore that the effect of chloride ion on converting enzyme activity is not to increase substrate binding but to ensure the correct orientation of substrate binding. Thus at low chloride concentrations, angiotensin I may bind to the active site in an 'abortive' orientation, preventing binding of bradykinin or its own hydrolysis. In high chloride concentrations binding of angiotensin I would still occur but hydrolysis could then proceed. The inability of angiotensin II to inhibit bradykininase in either high or low chloride concentrations also suggests interaction at the active site of the enzyme since it is not a substrate for converting enzyme.

The availability of purified enzyme preparations and of synthetic analogues of the angiotensin peptides should make it possible to test critically the hypothesis proposed here.

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