Rabbit Tissue Peptidases that Hydrolyse the Peptide Hormone Bradykinin

DIFFERENCES IN ENZYMIC PROPERTIES AND CONCENTRATION IN RABBIT TISSUES

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The distribution and properties of neutral peptidases acting on the peptide hormone bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) were determined in several rabbit tissues. The supernatant and particulate fractions prepared from tissue homogenates (25000g for 60min) were studied. Bradykinin inactivation (kininase activity) was measured by bioassay with the isolated guinea-pig ileum. The sites of peptide-bond cleavage were determined in the amino acid analyser, which permits detection and measurement of amino acids and peptides derived from bradykinin. The results indicate that kininases are present in a wide range of concentrations in different tissues, kidney and lung having the most activity. Kininases present in different tissues were distinguished on the basis of sensitivity to the effects of EDTA, dithiothreitol and ZnCl₂ and by the site of peptide-bond hydrolysis in bradykinin.

The study of the number, distribution and properties of tissue peptidases active at physiological pH represents a necessary approach to the determination of the enzymes involved in protein turnover and activation and inactivation of enzymes and peptide hormones (Schimke & Bradley, 1975). We have attempted to determine whether several tissues from rabbit contain the same or different classes of peptidases capable of hydrolysing bradykinin (Arg¹-Pro²-Pro³-Gly⁴-Phe⁵-Ser⁶-Pro⁷-Phe⁸-Arg⁹). Similar studies have been performed by Rapley *et al.* (1971) using di-, tri- or tetra-peptides as substrates for enzymes from human tissues. They concluded that the same peptidases exist in the tissues studied, but that they are present in a wide range of concentrations.

The combination of the bioassay method, which measures the loss of biological activity when any peptide bond of bradykinin is hydrolysed, and an analytical chromatographic procedure for the identification of bradykinin peptide fragments was used to detect kininase activity and to identify the sites of peptide-bond hydrolysis. The results reported here indicate that the enzymes which are responsible for the inactivation of bradykinin in homogenates of several tissues exhibit different properties. These properties include the activation or inhibition by several compounds and the site of peptide-bond hydrolysis in bradykinin.

Experimental

Materials

Aminex A-5 and A-6 resins were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Bradykinin and the other synthetic peptides used in this study were synthesized by Dr. A. C. M. Paiva (Escola Paulista de Medicina, São Paulo, Brazil). The peptides had integral molar ratios of constituent amino acids after acid hydrolysis and were homogeneous by high-voltage electrophoresis at pH3.5.

Methods

Enzyme preparation. Mature rabbits were anaesthetized with pentobarbital (40mg/kg body wt.) and both carotid arteries were cannulated. After the jugular veins were excised, cold 0.9% NaCl solution was infused to eliminate blood. The tissues (liver, brain, spleen, kidney, lung, heart, skeletal muscle and ileum) were rapidly removed from the animal and transferred to cold 0.9% NaCl solution. The smooth muscle was obtained from the ileum. The tissues were cut into small pieces, washed several times with cold 0.9% NaCl solution and homogenized with a Potter-Elvehjem homogenizer in 7 vol. (v/w) of 0.25 M-sucrose/0.01 M-Tris/HCl, pH 7.5. The homogenates were centrifuged at 25000g for 1 h at 4°C. The precipitate was suspended in 5 vol. of 0.25 M-sucrose/0.01 M-Tris/HCl, pH 7.5. Blood was collected into heparin from an anaesthetized animal by cardiac puncture and centrifuged at 750g for 1 h at 4°C. Samples (2ml each) of particulate material (p) (i.e. pellet after centrifugation), supernatant (s) and also plasma were dialysed against 700 vol. of 0.1 M-sodium phosphate buffer, pH 7.5 at 4°C. The buffer was replaced twice at 4h intervals. These preparations, which constitute the source of tissue

kininases, were kept at -20°C and used within 20 days.

Determination of bradykinin inactivation: kininase assay. A bioassay with the isolated guinea-pig ileum was used to measure the concentration of bradykinin. The response, the extent of contraction of the smooth muscle, was standardized with known amounts of the hormone. The kininase assay consists of the determinations of residual bradykinin activity by matching the response of the test solution with that of the control (Camargo *et al.*, 1972). The isolated guinea-pig ileum was bathed at 37°C in 10 ml of Tyrode buffer (0.138 M-NaCl/18 μM -CaCl₂/2.68 mM-KCl/0.49 mM-MgCl₂/11 mM-NaHCO₃/0.36 mM-NaH₂PO₄) containing 0.35 M-atropine, 1.7 M-di-phenylhydramine and 2.8 mM-glucose.

Bradykinin (5–80 nmol) was incubated at 37°C with enzyme in 0.05 M-sodium phosphate buffer, pH 7.5, containing 0.1 M-NaCl. The concentration of enzyme was selected so as to give 40–60% inactivation within 18 min. Residual bradykinin was determined after 6, 12 and 18 min of incubation. The rate of reaction was essentially linear with time in the range studied. All determinations were carried out in duplicate.

Determination of the effect of various compounds on kininase activity. EDTA, dithiothreitol, peptide BPP_{9a} (<Glu-Trp-Pro-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and ZnCl₂ were incubated with the dialysed tissue fractions for 10 min at 0°C at the concentration given in the legend to Table 1, and the kininase activity was determined by bioassay. A separate control tube containing bradykinin and compound, but no enzyme, was used as reference to correct for the effect of the compound on the response of the guinea-pig ileum to bradykinin. The only compound for which this correction was necessary was peptide BPP_{9a}, which potentiates the activity of bradykinin on the isolated guinea-pig ileum (Greene *et al.*, 1972).

Determination of peptides and amino acids derived from bradykinin. An automatic amino acid analyser (Alonzo & Hirs, 1968) was operated as previously described (Camargo *et al.*, 1973). Samples were analysed by the two-column system of Spackman *et al.* (1958) with the buffer systems described by Oliveira *et al.* (1976). The short-column (Aminex A-5 resin) system permits the detection and determination of the following peptides: Arg-Pro-Pro-Gly, Arg-Pro-Pro-Gly-Phe, Arg-Pro-Pro-Gly-Phe-Ser, Arg-Pro-Pro-Gly-Phe-Ser-Pro, Phe-Ser-Pro-Phe-Arg, Ser-Pro-Phe-Arg and Phe-Arg. Des-Arg¹-Pro²-bradykinin and Pro-Phe-Arg were the only basic peptides that could not be detected with this system. Arg-Pro-Pro was not completely separated from Arg-Pro-Pro-Gly-Phe-Ser-Pro, and the peaks of Ser-Pro-Phe-Arg and des-Arg⁹-bradykinin overlapped. When both peptides were present, determination was not possible. However, it was possible to detect the presence

of one peptide mixed with the other by the A_{570}/A_{440} ratio. The ratios were: Arg-Pro-Pro, 1.5; Arg-Pro-Pro-Gly-Phe-Ser-Pro, 3.6; Ser-Pro-Phe-Arg, 4.9; des-Arg⁹-bradykinin, 1.8. The long column (Aminex A-6 resin) was used to detect acidic or neutral free amino acids and Ser-Pro. The relative colour yields (area/concentration) and the chromatographic behaviour of the synthetic peptides are described in Oliveira *et al.* (1976).

Protein determination. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Results

Kininase activity in tissue homogenates

Kininase activity was determined for the two fractions prepared from the homogenate of each tissue by a single centrifugation procedure where most of the sedimentable particles (p) were separated from the soluble fraction (s). The activity of each fraction was measured at two substrate concentrations. The rates of bradykinin inactivation obtained with low substrate concentration (5 μM) were not less than one-half those obtained with a substrate concentration 16 times higher for all fractions studied, except liver supernatant. The results in Table 1 indicate that the specific activities (per mg of protein or per g wet wt.) for kidney and lung (both s and p fractions) were about 160 and 1200 times greater respectively than that of plasma, which had the lowest specific activity. The activity present in lung and kidney was several times greater than that found in the other tissues studied. The amount of kininase activity was equally distributed in the s and p fractions, or was predominantly in the s fraction, in all tissues except for kidney, where most of the activity was recovered in the p fraction.

Susceptibility of tissue kininases to various compounds

The kininase response to the effect of various inhibitors or activators was compared by using compounds selected on the basis of their effect on kininases described in the literature (Erdös & Yang, 1970; Camargo *et al.*, 1973). Table 2 demonstrates that the effects of inhibitors and activators are different on kininases from the different tissues. Two groups of tissue kininases can be distinguished on the basis of the EDTA effect and then further classified with respect to sensitivity to dithiothreitol and ZnCl₂.

Group I: EDTA-sensitive kininases. Kininases from lung (s and p), liver (s and p), plasma, skeletal muscle (p) and smooth muscle (p) were completely inhibited by 1 mM-EDTA, whereas kininases from heart and spleen (s and p) and from smooth muscle (s) and kidney (p) were only partially inhibited by 1 mM-EDTA. The kininases present in both the super-

Table 1. *Kinase activity in rabbit tissues*

Bradykinin (5 or 80 nmol) was incubated at 37°C with dialysed supernatant or particulate fraction in 1 ml of 0.05 M-Tris/HCl, pH 7.5, containing 0.1 M-NaCl. The rate of bradykinin inactivation was measured by bioassay on the isolated guinea-pig ileum as described under 'Methods'. The values for kinase activity are the average obtained for two tissue preparations, each determined in duplicate.

Tissue	Supernatant fraction			Particulate fraction		
	Protein (mg/g wet wt.)	Kininase activity (nmol/min per mg of protein)		Protein (mg/g wet wt.)	Kininase activity (nmol/min per mg of protein)	
		5 nmol/ml	80 nmol/ml		5 nmol/ml	80 nmol/ml
Bradykinin ...						
Kidney	32.4	11.10	11.3	46.8	53.00	48.00
Lung	25.8	3.30	6.72	25.9	6.60	6.06
Heart	18.9	1.93	2.32	60.1	0.26	0.52
Spleen	44.6	1.35	1.60	49.3	1.66	2.20
Brain	17.6	1.61	1.80	81.2	0.31	0.35
Liver	47.2	1.00	0.72	80.8	0.31	0.30
Skeletal muscle	28.8	0.75	0.80	47.0	0.06	0.19
Smooth muscle	9.1	0.42	0.48	57.1	0.14	0.08
Plasma	47.7	0.04	0.064	—	—	—

Table 2. *Effect of various compounds on the rate of bradykinin inactivation by rabbit tissue peptidases*

Results are expressed as the percentage change from the activity without added compound. Tissue extracts were preincubated with and without the compounds in 0.05 M-sodium phosphate buffer (pH 7.5)/0.1 M-NaCl for 10 min at 0°C. The kininase assay conditions were: supernatant or particulate fraction (from 0.04 to 13.7 mg/ml of protein) was incubated at 37°C for 18 min with bradykinin (5 nmol/ml) in the same buffer. Bradykinin activity was measured with the isolated guinea-pig ileum. —, Inhibition; +, activation, 0, no effect.

Compound	Supernatant fraction						Particulate fraction					
	EDTA		Dithiothreitol		ZnCl ₂	Peptide BPP _{9a}	EDTA		Dithiothreitol		ZnCl ₂	Peptide BPP _{9a}
	0.5 mM	1 mM	0.5 mM	1 mM	1 mM	25 µM	0.5 mM	1 mM	0.5 mM	1 mM	1 mM	25 µM
Kidney	0	0	0	0	-10	-100	-30	-30	-40	-50	-40	-70
Lung	-100	-100	0	0	+100	-100	-100	-100	0	0	+15	-60
Heart	-5	-10	-50	-50	-25	-100	-40	-40	-40	-50	-30	-40
Spleen	-25	-30	-70	-80	-20	-70	-10	-60	-50	-100	-100	-50
Brain	0	0	0	0	-100	-40	0	0	+25	+50	-80	-20
Liver	-50	-100	-70	-75	-70	-100	-75	-100	-70	-70	-100	-100
Skeletal muscle	0	0	+50	+50	0	-50	-80	-100	0	-40	0	-50
Smooth muscle	-30	-50	-20	-30	0	-75	-80	-100	-30	-60	0	-50
Plasma	-100	-100	-50	-75	0	-80	—	—	—	—	—	—

nant and particulate fraction of lung differ from all other group-I enzymes in that they are activated by 1 mM-ZnCl₂ and not inhibited by dithiothreitol.

Group II: EDTA-insensitive kininases. Kininase activity from kidney (s), brain (s and p) and skeletal muscle (s) is not affected by 1 mM-EDTA. Enzymes from heart (s) are little affected by this chelating agent. However, the response of this group of enzymes to dithiothreitol and to ZnCl₂ shows that other differences in properties exist. Kidney kininases (s) are not sensitive to the thiol reagent and are only

slightly inhibited by 1 mM-ZnCl₂. However, skeletal muscle (s) and brain (p) kininases are activated by dithiothreitol, but only those from brain (s and p) are strongly inhibited by 1 mM-ZnCl₂.

The peptide BPP_{9a} (25 µM) inhibited the activity of both supernatant and particulate fractions of all tissues studied. The inhibition was slightly more pronounced for the supernatant fractions at this concentration. However, more detailed studies are required to obtain a more effective discrimination among these tissues.

Fig. 1. Hydrolysis of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin) by supernatant fractions derived from rabbit tissue homogenates

The values indicated by the width of the bars are nmol of the peptide or free amino acids recovered after approx. 50% hydrolysis of bradykinin. The enzymic hydrolysates were prepared as follows: bradykinin (80nmol) was incubated in 1.0ml of 0.05M-sodium phosphate buffer (pH7.5)/0.1M-NaCl at 35°C with tissue-supernatant fractions. The extent of hydrolysis of bradykinin was determined by bioassay. After 50% of the bradykinin had been hydrolysed, the reaction was stopped by the addition of 1.2ml of a 30% (v/v) solution of poly(ethylene glycol) in 0.32M-sodium citrate buffer containing 0.05M-HCl. The hydrolysis products were determined by chromatographic analysis (see under 'Methods').

Sites of peptide-bond hydrolysis in bradykinin by tissue peptidases

Figs. 1 and 2 show the pattern of products obtained from bradykinin when 50% of the hormone had been inactivated by enzymes from tissue supernatant and particulate fractions respectively. The products of bradykinin degradation recovered in these experiments account for 60–80% of the peptide hydrolysed. Arginine and phenylalanine in approximately equal concentrations were the major products. The amount of glycine, proline and serine recovered (not shown in Figs. 1 and 2) was equivalent to 10–20% of the arginine in most of the incubation mixtures. There are, however, large differences in the peptide products of bradykinin. The complementary peptides Arg-Pro-Pro-Gly-Phe-Ser-Pro and Phe⁸-Arg⁹ were released in higher quantities by kidney, lung and smooth muscle than by the other tissues. On the other hand, two other complementary peptides, Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg, were recovered in higher amounts in hydrolysate containing enzymes from brain (s and p), heart (s and p), liver (p) and spleen (p).

The detailed interpretation of these patterns of products requires knowledge of the specificity and properties of the enzymes. This is possible for the kininase activity of plasma (Fig. 1), which can be interpreted in terms of kinase I and II, which have been characterized by Erdős and collaborators (Erdős & Sloane, 1962; Yang *et al.*, 1971). Kininase I (EC 3.4.12.8) is carboxypeptidase N, which hydrolyses the Phe⁸-Arg⁹ peptide bond. Kininase II, peptidyl dipeptidase (EC 3.4.15.1) hydrolyses the Pro⁷-Phe⁸ bond of bradykinin. All of the expected products, Arg-Pro-Pro-Gly-Phe-Ser-Pro, Phe⁸-Arg⁹, free arginine and Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe, were detected, and all but the octapeptide were determined quantitatively. The presence of free arginine and free phenylalanine is probably due to hydrolysis of the Phe-Arg dipeptide, and the small excess of arginine relative to phenylalanine most likely reflects the activity of kininase I (carboxypeptidase N). Thus it is possible to rationalize the products that were recovered in terms of characterized kininases of plasma. It should be emphasized that no other peptide fragments were in fact detected.

Fig. 2. Hydrolysis of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin) by particulate fractions derived from rabbit tissue homogenates
The conditions are the same as given in the legend to Fig. 1.

Although detailed interpretation of the patterns obtained with other tissues is more complicated, it is clear that the patterns of products obtained are different for each tissue (see the Discussion section).

Discussion

The bioassay method used to measure kinase activity is based on the loss of smooth-muscle-contracting activity of bradykinin. Since the hydrolysis of any peptide bond in bradykinin abolishes biological activity (Suzuki *et al.*, 1969) at the concentrations used in our assay, it is reasonable to expect that a variety of tissue peptidases could inactivate bradykinin. Some fragments of bradykinin at concentrations orders of magnitude higher than used in the assay do have some smooth-muscle-contracting activity. The ability of several tissues to inactivate bradykinin at pH 7.5 and the properties of this class of neutral peptidases were studied to determine if similarities and/or differences exist among rabbit tissues. As a first approach to this problem, we studied the total kinase activity present in the tissues instead of using purified preparations, to avoid losses of enzymes during the purification procedure. Although the study of the mixture of enzymes present in the tissue homogenate does not

permit conclusions on the number and characteristics of the individual enzymes, it does allow the comparison of properties of the kinetically predominant kinases present in the tissues.

The relative capacity of tissue homogenates to inactivate bradykinin (calculated as specific activity relative to weight of tissue or protein content) varied over a 1200-fold range with kidney having the highest and plasma the lowest values. Differences of 2-fold in the initial rate of hydrolysis, which was linear with time up to 50% inactivation of bradykinin, were observed when substrate was 5 or 80 μM. The rate of hydrolysis was higher at the higher substrate concentration in 15 out of 17 fractions studied. The factor of 2 in the initial rate is very small compared with the large differences observed between some tissues. Differences in content and specific activity have been demonstrated for angiotensin-converting enzyme among the tissues of rat (Cushman & Cheung, 1971) and also in several common laboratory animals (Cushman & Cheung, 1972). These differences for angiotensin-converting enzyme in the rat were smaller than we have observed for kinase in the rabbit, and the ranking of the tissues in terms of specific activity does not correspond.

The data demonstrate that the most active kinases present in each tissue can be readily distinguished on the basis of their sensitivity to EDTA, ZnCl₂ and

dithiothreitol. For some tissues, these differences are quite striking. For example, 1 mM-EDTA completely inhibits kinase activity in lung (s and p), liver (s and p) and plasma, but it does not affect kinase activity in brain (s and p). Large differences in sensitivity to EDTA were also detected between the supernatant and particulate fractions prepared from kidney, heart and skeletal muscle.

The differences in the pattern of peptide products obtained for kinase activity, though less direct than the enzyme-inhibitor studies, provides a second type of evidence, which indicates the presence of different peptidases in the tissues studied. The analysis of the products generated by the plasma enzymes is consistent with the presence of kinases I and II, described in the literature (Erdős & Sloane, 1962; Yang *et al.*, 1971) (see the Results section). Similarly, the products released by brain enzymes are consistent with the specificity of kinases A and B, which hydrolyse the Phe⁵-Ser⁶ and Pro⁷-Phe⁸ bonds respectively (Oliveira *et al.*, 1976). However, additional dipeptidases must be invoked to account for the free amino acids detected (Smith, 1960; Campbell *et al.*, 1966; Hayman & Patterson, 1971). The recovery of the complementary peptides Arg-Pro-Pro-Gly-Phe and Ser-Pro-Phe-Arg, as well as Arg-Pro-Pro-Gly-Phe-Ser-Pro and Phe⁸-Arg⁹, suggest that enzymes with specificity similar to brain kinase A and B exist also in other tissues such as heart (s and p) and skeletal muscle (s). However, hydrolysis of the same peptide bond does not necessarily indicate that the enzymes are identical. For example, both brain kinase B and angiotensin-converting enzyme (peptidyl dipeptidase, EC 3.4.15.1) hydrolyse bradykinin only at the Pro⁷-Phe⁸ peptide bond. Kinase B inactivates both angiotensin I and II (C. Spadaro, unpublished work), whereas the converting enzyme transforms angiotensin I into angiotensin II and does not act on angiotensin II. Experiments with double-labelled peptides or more detailed kinetic studies would be required to demonstrate that the complementary fragments are derived from the same molecule.

The kinases may be part of the intracellular machinery for protein breakdown, or may participate in the metabolism of peptide hormones, or both. It is difficult to interpret the large differences in activity among the tissues or to relate the multiplicity of kinases to a physiological function. The isoenzymes of lactate dehydrogenase (Wilkinson, 1970), hexokinase (Katzen, 1967) and esterase (Coates *et al.*, 1975) illustrate one type of selective distribution of enzymes that act on the same substrate. It has been possible to establish a relationship between the metabolic role of the tissue and the isoenzyme tissue distribution in terms of specificity for alternative substrates and the effect of hormones and inhibitors on enzyme activity (see Wilkinson, 1970). If the

kinases are not isoenzymes, then the multiplicity would seem to indicate that the tissues do not share a common proteolytic system to degrade the nonapeptide bradykinin unless the localization of an enzyme makes its contribution the most significant. This may be true for circulating kinins, which are hydrolysed by the dipeptidyl carboxypeptidase located on the membrane of the endothelial cell of the vascular bed (Ryan *et al.*, 1975; Caldwell *et al.*, 1976).

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