Renal Inactivation of Substance P in the Rat

By PATRICK E. WARD and ALICE R. JOHNSON

Department of Pharmacology, University of Texas Health Science Center at Dallas,
5323 Harry Hines Boulevard, Dallas, TX 75235, U.S.A.

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The activity and distribution of substance P-catabolizing enzyme(s) were studied in the rat kidney. Kidney homogenates inactivate substance P 5–20 times as fast as do homogenates of intestine, liver, lung, heart or brain. The catabolizing activity was highest in the cortex and decreased progressively down the papilla. Cortex of rat kidney was homogenized and fractions enriched in microsomal membrane, final supernatant, plasma membrane, endoplasmic reticulum, brush border and intact glomeruli were prepared. The identity and homogeneity of the preparations were determined by assaying marker enzymes and by morphological examination. Substance P was catabolized most rapidly by the microsomal and plasma-membrane-enriched fractions, and least rapidly by endoplasmic reticulum or final supernatant fractions. Purified brush border of proximal tubules inactivated substance P more than 10 times as fast as isolated glomeruli. Our experiments show that substance P is catabolized at a rate that is similar to the rates of inactivation of bradykinin and angiotensin II. Further, the distribution of substance P-catabolizing activity in various kidney fractions is similar to the distribution of kininase and angiotensinase activities previously reported.

Substance P is a pharmacologically active peptide that was discovered in extracts of brain and small intestine nearly half a century ago (von Euler & Gaddum, 1931). Although this undecapeptide lowers blood pressure, dilates blood vessels and stimulates contraction of smooth muscle (Pernow, 1953; Lembeck & Zetler, 1972) and secretion from salivary glands (Vogler *et al.*, 1963), the physiological role of substance P is still unknown.

The possibility that substance P might be a neurotransmitter has stimulated intense investigations (Lembeck, 1953; Hökfelt *et al.*, 1975; Barker, 1976). In addition, substance P may also play an important role in renal function. Mills *et al.* (1974) found that substance P was among the most potent natriuretic substances so far described. Concentrations as low as 0.1 ng/ml infused into the renal artery caused diuresis and natriuresis in the dog. Gullner *et al.* (1977) confirmed these findings and established that substance P also suppressed renin release. Although Arendshorst *et al.* (1976) used micropuncture to show that substance P significantly decreased the reabsorption of salt and water from the proximal tubule of rat kidney, the mechanisms by which it exerts its effects on renal function are not clear. Bradykinin is also natriuretic, but substance P is approx. 100 times as potent (Mills *et al.*, 1974; Gullner *et al.*, 1977). Either the high potency of substance P relative to bradykinin may be due to the high intrinsic activity of the undecapeptide, or substance P may act indirectly by releasing another natriuretic agent (Mills *et al.*, 1974). Another explan-

ation for the difference in potency may be that bradykinin is more readily inactivated than substance P, since the kidney is a very rich source of tissue kininases. We have investigated the last suggestion by comparing the distribution of substance P-inactivating enzyme(s) in rat kidney with the distribution of kininase and angiotensinase (Ward *et al.*, 1976, 1977b). Since substance P and several derivatives have been synthesized (Tregear *et al.*, 1971), we used the synthetic undecapeptide and its biologically active C-terminal heptapeptide derivative as substrates for the study of substance P-inactivating enzymes in the kidney. Part of this work has appeared in abstract form (Ward *et al.*, 1977a).

Methods

Animals

Unanaesthetized male Sprague–Dawley rats (250–350g) were killed by decapitation or cervical dislocation. The aorta was clamped above and below the renal arteries, the vena cava was opened, and the kidneys were perfused with 0.9% NaCl through the aorta until free of blood. All subsequent procedures were carried out at 4°C. For each experiment, about 10g of renal cortex was dissected from three to six rats, pooled and minced with fine scissors.

Subfractionation

Renal cortex, outer medulla and papilla were sectioned with curved scissors. Dissection was per-

formed on 1–2 mm-thick transverse sections of kidney. Renal homogenates were prepared in 50 mM-Tris/HCl buffer (pH 7.4) with a hand-held glass/Teflon homogenizer (12 strokes). Microsomal, final supernatant, plasma membrane and endoplasmic-reticulum-enriched fractions were prepared as described by Jakobsson (1974). Brush border of proximal tubules was prepared by the method of Wilfong & Neville (1970). Glomeruli were isolated with a magnet after infusion with iron oxide or by gravity after infusion with tantalum powder (Meezan *et al.*, 1973). Our adaptations of these techniques are described in detail in previous papers (Ward *et al.*, 1976, 1977b).

Enzyme assays

Inactivation of substance P and the heptapeptide derivative was measured by bioassay on atropine-treated strips of guinea-pig ileum. Substance P stimulates a rapid contraction of the ileum that is proportional to the amount of peptide added to the muscle bath (Pernow, 1953). A concentration–effect relationship was established with standards of substance P and heptapeptide before testing the kidney samples for enzymic activity. Inactivation of the synthetic peptides was measured by following the decrease in spasmogenic activity with time in a reaction mixture that contained the peptide substrate (10 μ M) and tissue extract in a buffer of 200 mM-Tris/HCl and 200 mM-NaCl (pH 7.4). Incubation of the peptides with buffer alone did not result in loss of activity over a 60 min period. The peptide substrate was added to a chilled mixture of enzyme and buffer and the reaction was initiated by rapidly mixing the contents of the tube on a vortex mixer and placing it in a shaking water bath at 37°C. A zero-time sample of the chilled mixture was rapidly withdrawn after the mixing but before warming. The tubes were then incubated at 37°C and portions were withdrawn every 2.5 min for 15 min. The amount of substance P (or heptapeptide) remaining in the sample at each time point was determined by comparison of the height of contraction with contractions produced by standard concentrations of the peptides. Where inhibitors were tested, they were added to the enzyme and incubated for 15 min before addition of the peptide substrate. Renal kininase and angiotensinase were assayed at 37°C in a shaking water bath as previously described (Ward *et al.*, 1976). Membrane fractions were characterized by several marker enzymes. Alkaline phosphatase (EC 3.1.3.1) was assayed with *p*-nitrophenyl phosphate substrate (Linhardt & Walter, 1965). Glucose 6-phosphatase (EC 3.1.3.9) was assayed in the presence of 4 mM-EDTA and 2 mM-KF (Harper, 1965; Hübscher & West, 1965). The phosphate released from the substrate was determined by the method of Fiske & SubbaRow (1925). Protein was measured by the

method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Materials

Ficoll (mol.wt. approx. 400 000) was purchased from Pharmacia (Piscataway, NJ, U.S.A.) and tantalum powder (particle size 1.0 μ m) was from Fansteel (Chicago, IL, U.S.A.). The sieves used in the isolation of the glomeruli were prepared from Nitex Nylon monofilament bolting cloth in the designated mesh sizes from Tetko (Houston, TX, U.S.A.). Substance P (H-Arg¹-Pro²-Lys³-Pro⁴-Gln⁵-Gln⁶-Phe⁷-Phe⁸-Gly⁹-Leu¹⁰-Met¹¹-NH₂) and substance P-(5–11)-heptapeptide were purchased from Beckman (Palo Alto, CA, U.S.A.) and from Peninsula Laboratories (San Carlos, CA, U.S.A.). Bradykinin and sucrose (Ultrapure) were purchased from Schwartz/Mann (Orangeburg, NY, U.S.A.). Soya-bean and lima-bean trypsin inhibitors were obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Trasylol (1200 units/mg as defined by Webster, 1970) was generously supplied by Dr. G. L. Haberland (Bayer A.G., Wuppertal-Elberfeld, West Germany).

Results

Distribution

The inactivation of substance P by crude homogenates of rat kidney, small intestine, liver, lung, heart or whole brain was determined by bioassay. Kidney homogenates were 5–20 times as active as the other whole-tissue preparations when expressed per g wet wt. of tissue (Table 1).

After dissection of the kidney, we found that within renal tissues substance P was inactivated fastest by the cortex. The mean $\pm$ S.E.M. specific activities of cortex, outer medulla and papilla were 0.65 ± 0.18 , 0.24 ± 0.04 and 0.08 ± 0.03 μ mol/h per mg respectively.

Microsomal membranes

Microsomal and final supernatant fractions were prepared from 12 rats in four separate experiments. The microsomal fraction is composed predominantly of plasma membranes and endoplasmic reticulum (Ward *et al.*, 1976) and it inactivated substance P at a rate of 4.3 μ mol/h per mg of protein, which is nearly 6 times as fast as crude homogenate. This increase in the rate of inactivation corresponded with a 5.6-fold enrichment in glucose 6-phosphatase, a microsomal marker enzyme (Table 2). Substance P-inactivating enzyme(s) were less concentrated in the final supernatant; total soluble activity accounted for only approx. 7% of the activity in whole kidney homogenates.

In three separate experiments microsomal membranes from 12 kidneys were subfractionated into plasma membrane and endoplasmic-reticulum-enriched fractions by rate differential centrifugation in a linear continuous sucrose/Ficoll gradient. Fig. 1 shows the relative distribution of the endoplasmic reticulum and the plasma-membrane marker enzymes, glucose 6-phosphatase and alkaline phosphatase. The distribution of these marker enzymes demonstrates the separation of endoplasmic reticulum from plasma membranes that contain brush border. The different morphology of the membranes collected at the top and bottom of the gradient was established previously by transmission electron microscopy (Ward *et al.*, 1976). The top fractions contained vesicles of smooth and rough endoplasmic reticulum. The bottom of the gradient was enriched in plasma membrane and fragments of brush-border membrane. Although other plasma-membrane marker enzymes were not determined in the present study, Jakobsson (1974) found that the distribution of all plasma-membrane-bound enzymes parallels that of alkaline phosphatase.

Renal inactivation of substance P was most pronounced in fractions that contained plasma membrane. The distribution of this enzyme (or enzymes) was nearly identical with that of the membrane marker enzyme alkaline phosphatase.

Table 1. Inactivation of substance P in rat tissues
Homogenates were prepared and assayed as described in the Methods section. All values are given as means±S.E.M. for four experiments.

Organ	Activity (μmol/h per g of tissue)
Kidney	44.9 ± 14.5
Small intestine	8.2 ± 2.0
Liver	6.2 ± 0.4
Lung	3.6 ± 1.5
Heart	2.2 ± 0.6
Brain	1.9 ± 0.6

Brush border

Brush border of the proximal tubules makes up the major fraction of plasma membranes of renal cortex. We purified intact brush borders from the proximal tubule of 12 rats in three experiments. The identity of this preparation with brush border *in situ* has been established previously (Ward *et al.*, 1976, 1977b). In the present study, alkaline phosphatase, the marker enzyme of renal brush border, was enriched over 12-fold (Table 2). The low activity of glucose 6-phosphatase indicated that there was minimal contamination by endoplasmic reticulum. The inactivation of substance P increased 5–6-fold in the purified brush-border preparations. The rates of inactivation of substance P and heptapeptide derivative by four separate brush-border preparations were similar, 3.2±1.0 and 3.5±0.8 μmol/h per mg of protein respectively.

Fig. 1. Distribution of glucose 6-phosphatase (○), alkaline phosphatase (●) and substance P-inactivating enzyme(s) (Δ) after rate-differential centrifugation of the microsomal fraction of homogenized cortex of the rat kidney in a sucrose/Ficoll gradient by the method of Jakobsson (1974). Relative specific activity is (specific activity of fraction)/(specific activity of microsomal fraction).

Table 2. Distribution of enzymes in homogenized rat kidney
The subfractionation and assay of the renal homogenate was carried out as described in the Methods section. Values are means±S.E.M. for four experiments, except for brush border where numbers shown are those obtained from a typical preparation. Relative specific activity is (mean specific activity in the fraction)/(mean specific activity in the homogenate). Percentage of total activity is given in parentheses.

	Substance P inactivation		Glucose 6-phosphatase		Alkaline phosphatase	
	Specific activity (μmol/h per mg)	Relative specific activity	Specific activity (μmol/h per mg)	Relative specific activity	Specific activity (μmol/h per mg)	Relative specific activity
Homogenate	0.73 ± 0.21	1.0	3.0 ± 0.06	1.0	—	—
Microsomal fraction	4.30 ± 1.11 (25)	5.9	16.9 ± 1.8 (22)	5.6	—	—
Final supernatant fraction	0.36 ± 0.12 (7)	0.5	—	—	—	—
Brush border	4.8 (7)	5.6	2.4 (0.9)	0.8	58.2 (14)	12.2

Glomeruli

Intact glomeruli were isolated with an estimated purity of greater than 98%. At physiological perfusion pressures (≤ 150 mmHg), approx. 35% of the glomeruli were recovered in the final purified preparation.

Enzymes that inactivate substance P, bradykinin and angiotensin II could be detected in every preparation of glomeruli assayed. However, their specific activities were less than those of the crude homogenate and less than one-tenth of those found in the purified brush border (Table 3).

Inhibition of inactivation

In an attempt to identify the renal enzyme or enzymes that inactivate substance P, samples of purified brush border were treated with various inhibitors before combination with the peptide substrate. Addition of 1 mM-1,10-phenanthroline to the reaction mixture inhibited the inactivation of substance P by more than 80% in five separate experiments. EDTA (1 mM) also significantly decreased the rate of inactivation. Other enzyme inhibitors, including di-isopropyl phosphorofluoridate, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor and Trasylol were inactive. Similar results were obtained when the heptapeptide was used as substrate. These results are given in Table 4.

Table 3. *Inactivation of peptides by isolated glomeruli*
Glomeruli were prepared as described by Ward *et al.* (1977b) by using iron oxide or tantalum powder. All values are means $\pm$ S.E.M. for five experiments. Relative specific activity is (mean specific activity in the preparation)/(mean specific activity in the homogenate).

Inactivated peptide	Specific activity (μ mol/h per mg)	Relative specific activity
Substance P	0.31 ± 0.2	0.5 ± 0.2
Bradykinin	0.12 ± 0.1	0.4 ± 0.3
Angiotensin II	0.04 ± 0.02	0.6 ± 0.2

Discussion

Homogenates of rat kidney inactivated synthetic substance P faster than did homogenates of other tissues, including intestine, liver, lung, heart and brain. This finding suggests that the kidney is a major site for inactivation of this peptide *in vivo*. In this regard, our study confirms the previous observations by Eber & Lembeck (1956) that kidney homogenates are more active than other tissue homogenates in inactivation of substance P.

Substance P was inactivated fastest by microsomal fractions of homogenized kidney. Microsomal fractions were 12 times as active as the final supernatant (soluble) fractions and approximately 6 times as active as the original homogenates. When microsomal fractions were further separated into plasma-membrane and endoplasmic-reticulum-enriched fractions, plasma membranes contained most of the activity. Since the enzyme activity was most concentrated in the brush border, which is part of the proximal tubules, the primary site of hydrolysis of filtered substance P by the kidney is probably the proximal tubules. Furthermore, brush-border preparations inactivated the heptapeptide derivative of substance P, which indicates that not only the native undecapeptide but also shorter active peptide fragments can be metabolized by brush border. However, since substance P inactivation was not as enriched in the brush-border preparation as the marker enzyme alkaline phosphatase, this may indicate that the brush border is not the only site for renal substance P inactivation.

Although isolated purified glomeruli inactivated substance P, the rate of inactivation was less than one-tenth of that of the renal brush border. This would suggest that the peptide is filtered intact and is subsequently degraded by contact with the surface of the brush border in the proximal tubules. It is unlikely that the low amounts of substance P-catabolizing activity in the isolated glomeruli are due to loss of activity during purification. As reported previously (Ward *et al.*, 1977b), scanning and transmission electron microscopy of isolated purified glomeruli showed that they were intact and morphologically

Table 4. *Effect of enzyme inhibitors on inactivation of substance P and the C-terminal heptapeptide*
Data are percentages of control (uninhibited) inactivation rates. Numbers of experiments are given in parentheses. Assays and preincubations of inhibitors were carried out as described in the Methods section.

Inhibitor	Concn.	Substrate	
		Substance P	Heptapeptide
Trasylol	2000 μ g/ml	139 ± 19 (3)	143 ± 3 (4)
Lima-bean trypsin inhibitor	100 μ g/ml	148 ± 28 (4)	126 ± 6 (4)
Soya-bean trypsin inhibitor	100 μ g/ml	116, 103 (2)	—
Di-isopropyl phosphorofluoridate	1 mM	110 ± 9 (3)	$85, 124$ (2)
1,10-Phenanthroline	1 mM	23 ± 10 (5)	6 ± 2 (3)
EDTA	1 mM	59 ± 10 (5)	62 ± 9 (3)

similar to glomeruli *in situ*. Attached Bowman's capsules were the major contaminant, and there were no tubular structures in the preparations. In addition, the isolated glomeruli had marker-enzyme activities similar to those of glomeruli studied by others (Taylor *et al.*, 1971; Brendel & Meezan, 1973).

Purified preparations of brush border were incubated with various enzyme inhibitors in an attempt to identify the enzymes that inactivate substance P and its heptapeptide derivative. Since the only inhibitors that effectively blocked hydrolysis of either peptide were 1,10-phenanthroline and EDTA, the major inactivating enzyme must require a metal cofactor. The ineffectiveness of di-isopropyl phosphorofluoridate, Trasylol and trypsin inhibitors in blocking inactivation of the peptides shows that the enzyme is not a serine proteinase.

Enzymes that metabolize substance P and its C-terminal octapeptide were found in cultured human endothelial cells (Johnson & Erdös, 1977). One of them was partially inhibited by 1,10-phenanthroline. The enzyme in the brush border may be similar to a renal microsomal enzyme described by Erdös & Yang (1967), who found that a microsomal fraction from pig kidney inactivated eldoisin and that the inactivation was blocked by 1mM-EDTA. Substance P is structurally closely related to eldoisin (Chang & Leeman, 1971).

Three peptidases that could be expected to hydrolyse substance P, i.e. neutral endopeptidase (Kerr & Kenny, 1974*a,b*), dipeptidyl peptidase IV (Kenny *et al.*, 1976) and aminopeptidase M (George & Kenny, 1973), have been purified from brush border and may account for a large percentage of the substance P-catabolizing activity.

The present experiments show that substance P is metabolized at a rate that is similar to the rate of inactivation of bradykinin and angiotensin II. Further, the inactivation of substance P by various kidney fractions follows the pattern of inactivation of kinins and angiotensins by the same fractions (P. E. Ward & E. G. Erdös, unpublished work). Because of the differences in the structures of substance P, angiotensin II and bradykinin, it is highly unlikely that the same peptidase would inactivate all of them. Thus the high potency of substance P in increasing renal blood flow, natriuresis and diuresis (Mills *et al.*, 1974; Arendshorst *et al.*, 1976; Gullner *et al.*, 1977), compared with those of bradykinin and angiotensin, is not related to a slower renal inactivation of substance P.

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