Isolation of Membrane-Bound Renal Kallikrein and Kininase

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Fractions highly enriched in plasma membrane, endoplasmic reticulum or brush border were prepared from homogenized rat kidney cortex. Kallikrein was concentrated in the plasma-membrane fraction, but not in the brush border of the proximal tubules. Kininase II or angiotensin I-converting enzyme was localized in the brush-border membrane. It is suggested that kallikrein in the urine may originate from the plasma membrane of the distal tubules and the conversion of angiotensin I and the inactivation of bradykinin may occur on the lumen membrane of the proximal tubular cells.

Kinins are active polypeptides released by kallikrein from a plasma α_2 -globulin. Kallikrein in the urine is thought to originate from the kidney (Nustad & Vaaje, 1975). Although isoenzymes of the kallikrein in the urine have been isolated in highly purified form (Nustad & Pierce, 1974), less is known about kallikrein in renal tissue. Carvalho & Diniz (1966) suggested that kallikrein is in the lysosomal fraction of homogenized kidney, but Nustad (1970) found it in the microsomal fraction.

The kidney is rich in kininases that inactivate kinins (Erdős & Yang, 1966) and most of this activity sediments with the microsomal fraction. Erdős & Yang (1966) described three of these kininases. One of these, kininase I (EC 3.4.12.7), is similar to carboxypeptidase N of plasma, and the other (kininase II; peptidyl-dipeptide hydrolase; EC 3.4.15.1) is identical with the angiotensin I-converting enzyme (Yang *et al.*, 1970, 1971; Oshima *et al.*, 1974).

The presence of kallikrein and kininases in kidney cortex and the excretion of kallikrein and kinins in urine (Erdős, 1970) suggest that kinins may affect renal function. Changes in kallikrein excretion can coincide with changes in sodium excretion and occur in certain hypertensive conditions (Marin-Grez *et al.*, 1972; Croxatto *et al.*, 1973; Margolius *et al.*, 1974; Geller *et al.*, 1975; Mills & Ward, 1975). Kinins are natriuretic, diuretic (Webster & Gilmore, 1964; Nasjletti *et al.*, 1975) and hypotensive, and these properties suggest that they have a physiological significance.

For these reasons we studied the cellular localizations of kallikrein and kininases in the kidney. Fractions highly enriched in plasma membrane or in endoplasmic reticulum were prepared and their kallikrein and kininase activities were determined. Kininase II was localized in the brush-border membrane of the proximal tubules, but kallikrein was found in a different plasma-membrane fraction.

Methods

Animals. Unanaesthetized male Sprague-Dawley

rats (250–350 g) were killed by decapitation. The kidneys were perfused blood-free with a solution of 0.25 M-sucrose and 5 mM-NaHCO₃, pH 7.5. Renal cortical tissue from three to six rats (≈ 10 g) was pooled, minced, weighed, and kept at 4°C.

Plasma-membrane preparation. Plasma-membrane preparations were obtained by a modification (Birckbichler *et al.*, 1973) of the method of Wallach & Kamat (1966) by using 15% (w/v) Dextran 110 instead of Ficoll for the separation of microsomal membranes into plasma-membrane (PM)-enriched fraction and endoplasmic-reticulum (ER)-enriched fractions.

Minced tissue was gently homogenized in 0.25 M-sucrose, 10 mM-Tris (pH 7.4) and 0.2 mM-MgSO₄ in a Dounce homogenizer with a loose pestle. The tissue suspension was equilibrated for 20 min at 800 lb/in² (5.6 MPa) N₂, and the cell membranes were fragmented into small vesicles by a sudden release of pressure (Dowben *et al.*, 1968). The microsomal fraction containing the membrane vesicles was sedimented from the supernatant by centrifugation at 100 000g for 60 min after initial centrifugations at 10 000g for 15 min and 30 000g for 15 min. The microsomal membranes were washed in low-ionic-strength media to remove trapped and intravesicular soluble protein (Wallach & Kamat, 1966), dialysed against 1 mM-Tris (pH 8.6) and 1 mM-MgSO₄ for 2 h, and 7 ml of the suspension microsomal membranes was layered on a discontinuous gradient of 10 ml of 15% (w/v) Dextran 110 in 1 mM-Tris (pH 8.6) and 1 mM-MgSO₄. The gradients were centrifuged for 15 h in a SW 27 rotor (27 000 rev./min; r_{av} , 11.5 cm) in a Sorvall OTD-2 ultracentrifuge. Approx. 2 ml of the PM-enriched fraction was collected at the gradient interface and the ER-enriched fraction collected as the pellet.

Brush-border preparation. Large fragments of brush-border membranes from the proximal convoluted tubular cells were prepared by the method of Wilfong & Neville (1970). The membranes were produced by gentle homogenization in a hypo-

osmotic medium and recovered from the low-speed precipitate (500g, 20min).

Enzyme assays. Renal kallikrein was assayed by incubating samples of renal tissue (50–100 μ l) with kininogen substrate (100 μ l) in 0.1M-Tris-HCl, pH 8.5 (500 μ l), in the presence of the kininase inhibitors nonapeptide SQ 20881 (100 μ l, 1 mM), EDTA (100 μ l, 50 mM) and *o*-phenanthroline (100 μ l, 10 mM) at 37°C. Kininogen was prepared by heating dog plasma. The amount of kinin generated was determined on an isolated rat uterus (1 unit of kallikrein activity is the amount of enzyme that forms the equivalent of 1 ng of bradykinin in 1 min). By using inhibitor, purified enzyme and differential bioassays, we confirmed that all the material which contracted the rat uterus was released from the kininogen substrate by kallikrein.

The esterolytic activity of kallikrein was assayed with α -N-tosyl-L-arginine [^{3} H]methyl ester substrate (Beaven *et al.*, 1971).

Renal kininase was assayed by incubating samples of renal tissues (20 μ l), bradykinin (200 ng in 200 μ l) and 0.1M-Tris, pH 7.4, 0.2M-NaCl (200 μ l) at 37°C and following the inactivation of the peptide on the isolated rat uterus (1 unit of kininase activity is the amount of enzyme that inactivates the equivalent of 1 ng of bradykinin in 1 min).

Renal angiotensin I-converting enzyme (kininase II) hydrolyses the synthetic substrate hippurylglycylglycine and is inhibited by the nonapeptide SQ 20881 (Yang *et al.*, 1971). Converting enzyme was assayed by incubating samples of renal tissues (50 μ l) with 1 mM-hippurylglycylglycine in 0.2M-Tris, pH 7.4, containing 0.2M-NaCl at 37°C. Converting-enzyme activity was calculated as the amount of hippurylglycylglycine hydrolysed that could be inhibited by SQ 20881 at 0.1 mM. The amount of diglycine released was assayed in a Beckman 121C amino acid analyser.

The activities of various marker enzymes were also determined. Alkaline phosphatase was assayed with *p*-nitrophenyl phosphate substrate (Linhardt & Walter, 1965). Glucose 6-phosphatase was assayed in the presence of 4 mM-EDTA and 2 mM-KF (Harper, 1965; Hübscher & West, 1965). Membrane ATPase* was assayed by the method of Post & Sen (1967). Inorganic phosphate was determined by the method of Fiske & SubbaRow (1925). Protein was determined by the method of Lowry *et al.* (1951).

Dextran 110 was purchased from Pharmacia (Piscataway, N.J., U.S.A.) and α -N-tosyl-L-arginine [^{3} H]methyl ester from Biochemical and Nuclear Corp. (Burbank, Calif., U.S.A.) Bradykinin, hippurylglycylglycine and sucrose (ultrapure) were from Schwarz-Mann, Orangeburg, N.Y., U.S.A. All other reagents used were of analytical grade.

* Abbreviation: ATPase, adenosine triphosphatase.

Results

The renal microsomal fraction is predominantly composed of fragments of plasma membrane and endoplasmic reticulum. The method of Wallach & Kamat (1966) separates these membranes into fractions predominantly rich in plasma membrane or in endoplasmic reticulum.

Membranes were isolated from 21 rats in six experiments. The PM-enriched fraction collected from the gradient interface has a low glucose 6-phosphatase activity and high alkaline phosphatase and membrane ATPase activities. The ER-enriched fraction has a high glucose 6-phosphatase activity and low alkaline phosphatase and membrane ATPase activities (Table 1). The specific activity of glucose 6-phosphatase in the ER-enriched fraction was over seven times that of the PM-enriched fraction, whereas the specific activity of alkaline phosphatase was only half of that in the PM-enriched fraction. The presence of alkaline phosphatase indicated that PM-enriched fraction contained brush border.

The PM-enriched fraction had approx. 10% of the total kallikrein activity of the crude homogenate and a specific activity five times that of the ER-enriched fraction. The relative specific activity was 28 times higher than in the crude homogenate. Preliminary experiments with renal homogenates showed that total α -N-tosyl-L-arginine [^{3} H]methyl esterase activity in the homogenate was not due entirely to kallikrein. However, like renal kallikrein, the specific esterase activity in the PM-enriched fraction was approximately three times that of the washed microsomal fraction and six times that of the ER-enriched fraction.

The specific kininase activity in the PM-enriched fraction was not significantly higher than that in the ER-enriched fraction. However, the kininase inhibitor EDTA was used in the preparation of these membranes. Transient exposure to this inhibitor may affect renal kininase distribution and activity.

Brush borders from renal tissue are derived from plasma-membrane fragments of the proximal convoluted tubular cells (Dalton & Haguenau, 1967). Because, as described above, kallikrein was bound to a renal plasma membrane, we wished to determine whether renal kallikrein was associated with the brush-border portion of plasma membrane.

Brush borders were prepared from 15 rats in five experiments. The relative specific activity of the marker enzyme of renal brush border, alkaline phosphatase, was nearly identical with that previously reported (Wilfong & Neville, 1970; Table 2). Although endoplasmic-reticulum contamination was present, the relative specific activity of glucose 6-phosphatase remained near unity. Electron and phase-contrast microscopy confirmed that our preparation consisted mainly of renal brush border.

Table 1. *Distribution of enzyme activity in membrane fractions isolated from rat kidney cortex*

The preparation of membrane fractions and the assay of enzymes were as described under 'Methods'. All values are means $\pm$ s.e.m. Relative specific activity is (mean specific activity in the fraction)/(mean specific activity in the homogenate).

	α -N-Tosyl-L-arginine [³ H]methyl ester hydrolysis				Kininase activity				Glucose 6-phosphatase activity				Alkaline phosphatase				Membrane ATPase			
	Kallikrein		Sp. activity [amount of the compound (as c.p.m.) min per mg of protein]		Sp. activity (μ g of brady- kinin hydro- lysed/min per mg of protein)		Relative sp. activity		Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)		Relative sp. activity		Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)		Relative sp. activity		Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)		Relative sp. activity	
	Recovery (%)	Sp. activity (ng of brady- kinin equiv. released/min per mg of protein)	Relative sp. activity	Sp. activity (μ g of brady- kinin hydro- lysed/min per mg of protein)	Relative sp. activity	Recovery (%)	Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)	Relative sp. activity	Recovery (%)	Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)	Relative sp. activity	Recovery (%)	Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)	Relative sp. activity	Recovery (%)	Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)	Relative sp. activity	Recovery (%)	Sp. activity (μ mol of sub- strate hydro- lysed/min per mg of protein)	Relative sp. activity
Homogenate	100	1.0 $\pm$ 0.2	1	0.34 $\pm$ 0.11	1	100	0.05 $\pm$ 0.001	1	100	0.10 $\pm$ 0.004	1	100	0.39 $\pm$ 0.05	1	100	0.39 $\pm$ 0.05	1	100	0.39 $\pm$ 0.05	1
Washed microsomes	36	9.5 $\pm$ 2.1	9.5	1290 $\pm$ 210	1	24	0.33 $\pm$ 0.03	6.5	18	0.52 $\pm$ 0.11	5.3	10	0.84 $\pm$ 0.18	2.1	10	0.84 $\pm$ 0.18	2.1	10	0.84 $\pm$ 0.18	2.1
Plasma-membrane fraction	10	28 $\pm$ 5.1	28	3100 $\pm$ 350	2.4	0.3	0.06 $\pm$ 0.01	1.1	3	0.64 $\pm$ 0.13	6.5	1	1.40 $\pm$ 0.42	3.5	1	1.40 $\pm$ 0.42	3.5	1	1.40 $\pm$ 0.42	3.5
Endoplasmic-reticulum fraction	8	5.6 $\pm$ 1.1	5.6	520 $\pm$ 130	0.4	10	0.43 $\pm$ 0.09	8.5	8	0.35 $\pm$ 0.09	3.6	2	0.60 $\pm$ 0.09	1.5	2	0.60 $\pm$ 0.09	1.5	2	0.60 $\pm$ 0.09	1.5
No. of expts.	4			2		5		3	3			3			3					
Total activity	825 $\pm$ 164					52 $\pm$ 3						84 $\pm$ 14								

Table 2. *Enzyme activity in the isolated brush-border preparation of rat kidney cortex*

Brush-border membranes were prepared as described by Wilfong & Neville (1970). All values given as means $\pm$ s.e.m. Relative specific activity is (mean specific activity in the brush-border fraction)/(mean specific activity in the homogenate). Specific activities are as defined in Table 1.

	Kallikrein				Kininase activity				Glucose 6-phosphatase				Alkaline phosphatase				Kininase II			
	Relative sp. activity		Sp. activity		Relative sp. activity		Sp. activity		Relative sp. activity		Sp. activity		Relative sp. activity		Sp. activity		Relative sp. activity		Sp. activity	
	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity	Sp. activity	Relative sp. activity
Homogenate	1.6 $\pm$ 0.5	1	0.27 $\pm$ 0.08	1	0.05 $\pm$ 0.001	1	0.08 $\pm$ 0.01	1	0.08 $\pm$ 0.01	1	0.05 $\pm$ 0.001	1	0.08 $\pm$ 0.01	1	0.08 $\pm$ 0.01	1	0.12 $\pm$ 0.02	1	0.12 $\pm$ 0.02	1
Brush-border fraction	0.2 $\pm$ 0.1	0.1	2.35 $\pm$ 0.45	8.7	0.05 $\pm$ 0.011	1	1.31 $\pm$ 0.21	16.2	1.31 $\pm$ 0.21	16.2	0.05 $\pm$ 0.011	1	1.31 $\pm$ 0.21	16.2	1.31 $\pm$ 0.21	16.2	1.20 $\pm$ 0.15	10	1.20 $\pm$ 0.15	10
No. of expts.	3		3		3		3		3		3		3		3		3		3	

The relative specific activity of kallikrein in the final brush-border preparation was very low (0.14; Table 2). Thus, unlike the marker enzyme, little or no kallikrein activity was associated with the brush border. The lack of kallikrein activity on the brush border cannot be attributed to loss of enzymic activity since the total recovery of kallikrein from all fractions was essentially 100% (not shown in Table 2). In contrast with kallikrein, the activities of kininase and angiotensin I-converting enzyme were concentrated in the brush border. The relative specific total-kininase and converting-enzyme activities in the brush border were 8.7 and 10.0. These values were similar to that of the marker enzyme alkaline phosphatase.

Discussion

Renal kallikrein is located predominantly in the PM-enriched fraction. Purified brush-border membranes have little or no renal kallikrein activity. Although the absence of kallikrein in the brush border does not exclude its existence in proximal tubular cells, it does suggest that urinary kallikrein is not secreted into the proximal tubule. Thus possibly the plasma membrane that contains kallikrein came from the distal tubules. This view is supported by the recent stop-flow experiments of Scicli *et al.* (1975), which indicated that kallikrein is not excreted from the proximal but rather from the distal tubule.

It has previously been reported that kallikrein in the crude renal homogenate could be activated by incubation in hypo-osmotic medium or acid pH (Carvalho & Diniz, 1966; Ward *et al.*, 1975). Our preliminary experiments now indicate that it is the membrane-bound kallikrein that is activated by such treatment.

In contrast with kallikrein, renal kininase II or the angiotensin I-converting enzyme is present in relatively high concentration in brush-border membranes. The concentration of kininase on the brush border could account for the rapid destruction of infused kinin. In support of this concept, Carone *et al.* (1975) have shown in micro-perfusion studies that there is a rapid removal of bradykinin by enzymic cleavage at the lumen surface of the proximal tubular cells.

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