Kidney International, Vol. 31 (1987), pp. 744–751

Angiotensin I converting enzyme and kinin–hydrolyzing enzymes along the rabbit nephron

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Angiotensin I converting enzyme and kinin–hydrolyzing enzymes along the rabbit nephron. Angiotensin I converting enzyme (ACE) and kininase activities were measured in various segments of the rabbit nephron. ACE was determined with tritiated hippuryl-glycylglycine as substrate. Lysyl-bradykinin (LBK) hydrolysis (kininase activity) was measured by radioimmunoassay. ACE was only found in the glomerulus and in the two parts of proximal tubule: the convoluted proximal tubule and the pars recta (PR). It was distributed along a concentration gradient which increased from the glomerulus to PR. Kininase activity was found in both proximal and distal parts of the nephron. Besides intense LBK-hydrolyzing activity in the proximal tubule, a kininase activity was also found in the medullary collecting tubule (MCT). Kininase activity in the glomerulus and the proximal tubule was completely inhibited by chelating agents. Captopril inhibited this activity only in the PR and at high concentrations (above 10^{-7} M). These results indicate that several types of enzymes other than ACE hydrolyze kinins in the glomerulus and in the proximal tubule. The contribution of ACE to kinin hydrolysis appears only minimal. The kininase activity found in MCT was different from ACE and other proximal tubule kininases because it was not inhibited by chelating agents. This kininase may play a physiological role in inactivating the kinins formed by kallikrein at or beyond the connecting tubule.

Rapid kinin hydrolysis by the kidney is now well established [1]. This hydrolysis is the consequence of the activity of several types of peptidases, collectively called kininases. Although most reports indicate that kinin hydrolysis mainly occurs in proximal tubules [2], the results of stop–flow experiments suggest the presence of kininase activity in the terminal part of the nephron [3]. Among the kininases that may play a part in kinin inactivation is kininase II. The latter enzyme has been intensively studied, because, in addition to hydrolyzing kinins, it converts angiotensin I to angiotensin II [4]. Kininase II, generally referred as angiotensin I converting enzyme (ACE), is the dipeptidyl-carboxypeptidase EC 3-4-15-1. It is also able to hydrolyze in vitro several short–peptide substrates. In the kidney, it is mainly localized in the brush–borders of the proximal tubule cells [5]. However, even if ACE is able to hydrolyze kinins, its contribution to the renal inactivation process of kinins is not clearly defined. Furthermore, since urinary kinins are produced by kallikrein in the distal tubule [3], it may be physiologically important to establish if kininase activity is present in the late nephron segments. The present study was therefore designed to investigate and quantify kinin–hydrolyzing activity in the different portions of the nephron and to explore the possible contribution of ACE to this activity. For this purpose, we began by determining the distribution of ACE along the microdissected rabbit nephron. This was done by measuring hydrolysis of a tritiated ACE substrate. The specificity of this measurement was established by studying its inhibition by low doses of captopril. We also searched for other kininase activities along the nephron by quantifying the hydrolysis of lysyl–bradykinin. We then attempted to establish whether or not the tubular kininase activity measured could be inhibited by compounds capable of inactivating ACE, such as chelating agents and captopril.

The results confirm the presence of both ACE and other kininases in the proximal tubule, and demonstrate the occurrence of a concentration gradient for converting enzyme along this segment. Kininase activity was also found in the terminal part of the nephron, particularly in the medullary collecting tubule. However, this activity is enterly due to an enzyme other than ACE.

Methods

Biochemical materials

Collagenase (CLS, 146 U/mg) from Clostridium histolycum was purchased from Worthington Biochemical Corp. (Freehold, New Jersey, USA). Deoxycholic acid, HEPES 4-(2-hydroxyethyl)1-piperazine-ethanesulfonic acid and lysozyme were from Sigma Chemical Co. (St. Louis, Missouri, USA); ethylenediamine tetracetic acid disodium salt (EDTA), o-phenanthroline hydrochloride and sodium sulfate came from Merck (Darmstadt, FRG). Lysyl–bradykinin and hippuryl–glycylglycine were from Bachem AG, Bubendorf, Switzerland. CHAPS 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate, was a product of Serva. Captopril was a gift from Squibb (Paris, France). [^{3}H] hippuryl-glycylglycine (about 20 Ci/mmole) was synthetized and tritiated as previously described [6].

Solutions

The purfusion solution was a modified Hank's solution [7] and contained in mM, 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 10 Tris-HCl and 1 $CaCl_2$, pH = 7.4.

Received for publication April 29, 1986
and in revised form August 13, 1986

The microdissection medium was identical to the perfusion medium except for $CaCl_2$, whose concentration was 0.25 instead of 1 mM.

Buffer I (0.1 M sodium phosphate, pH = 7.0, containing 0.1% lysozyme) was used for radioimmunoassay of bradykinin (BK).

Buffer II consisted of 0.3 M Hepes, pH = 8.0, containing 0.3 M NaCl and 1.8 M Na_2SO_4 and was used to measure converting enzyme activity.

Microdissection of tubules

Single nephron segments were isolated according to the method described previously in detail [8]. Briefly, male New Zealand rabbits weighing 1 to 1.5 kg were anesthetized and bled from the carotid artery. The left kidney was immediately removed and infused with 20 ml of perfusion solution containing 0.1% collagenase and bovine serum albumin. Thin pyramids were cut along the corticopapillary axis and incubated at 35°C for 30 minutes in the presence of collagenase. After incubation, the pyramids were rinsed with perfusion medium and kept on ice.

Microdissection was performed by hand, in ice-cold solution under stereomicroscopic observation. The glomeruli and different nephron segments were isolated and identified according to the usual criteria [9]. These different segments were: the convoluted part of proximal tubule (PCT) and its straight part called pars recta (PR), the thin descending limb of Henle's loop (TDL), the medullary and cortical segments of the thick ascending limb (MAL and CAL), the bright (DCT_b) and granular (DCT_g) portions of the distal convoluted tubule, the cortical medullary segments of the collecting tubule (CCT_1 and MCT).

Each piece of tubule was transferred in microdissection solution into a concave bacteriological glass slide, covered with a second slide, and photographed to permit subsequent measurement of its length.

Permeabilization and solubilization of tubules

Before measuring enzyme activities, the different nephron segments were either permeabilized by an osmotic shock followed by freezing and thawing, or solubilized by a detergent.

The osmotic shock was done by sucking out the excess of microdissection medium and replacing it with 0.3 μl of a hypo-osmotic solution containing 10^{-3} M Tris-HCl, pH 8. This step was performed under stereomicroscopic observation. Addition of the hypo-osmotic solution resulted in rapid swelling of the cells, especially in the proximal tubule. The samples were then frozen to −20°C and kept at this temperature overnight. On the following day, the samples were thawed and refrozen a second time before measurement. This procedure was adopted after checking that the enzyme activities measured under these conditions, that is after 18 hours storage, were not different from those determined on the day of microdissection.

However, in case this permeabilization procedure was not sufficient to allow full access of the substrate to all active enzyme sites, additional measurements were carried out in some experiments after solubilization of the tubule. This was done by preincubating the samples for 30 minutes at 30°C with either 0.5% deoxycholic acid or 3 mM CHAPS.

Micromethod for measurement of angiotensin I converting enzyme activity in the tubule by hydrolysis of hippuryl–glycylglycine

The micromethod used for measuring the activity of angiotensin I converting enzyme (ACE) was a miniaturized version of Ryan's method [10]. This procedure measures the rate of hydrolysis of the protected tritiated tripeptide [^{3}H]-hippuryl–glycylglycine (^{3}H-HGG) into glycylglycine and [^{3}H]-l-abeled hippurate. The hydrolysis is done at pH 8, in the presence of unlabeled hippuryl–glycylglycine (HGG) and of sulfate. In our experiments, the reaction proceeded in a final volume of 3 μl on the glass slide containing the tubule, which had been permeabilized or solubilized as described above. Each sample was adjusted to 1 μl with 10^{-3} M Tris-HCl, pH = 8. The ACE reaction was started by adding 2 μl of buffer II containing 30 nmoles HGG and 150,000 cpm ^{3}H-HGG. The final concentration of the substrate was 10 mM (apparent ACE km = 5 mM) [10] and its final specific activity was 5 μC/μmol. After one or two hours of incubation, the reaction was stopped by diluting the medium with 50 μl of 0.1 M HCl. The sample was then transferred into a small glass tube and the slide was rinsed twice with 50 μl HCl. The volume in the tube was adjusted to 200 μl. The hippurate was extracted with 200 μl of ethyl acetate. The aqueous and ethyl acetate phases were mixed by shaking for 30 seconds, and the layers were separated by centrifugation (1500 g for 10 min). Forty μl of the ethyl acetate was transferred into a small scintillation vial containing 3 ml of scintillation fluid and counted for 10 minutes. To measure the specific radioactivity, 2 μl of buffered substrate was directly added to five scintillation vials.

In each experiment, five blank samples containing no tubule were simultaneously treated with the experimental samples. Under these conditions, less than 5% of substrate was partitioned into ethyl acetate.

We checked that the reaction was inhibited by low concentrations of captopril (a potent inhibitor of ACE) [11] and by chelating agents (since ACE is a metalloenzyme), in order to insure that [^{3}H]-HGG hydrolysis specifically reflected ACE activity. The chelating agents were 1 mM EDTA and 1 mM o-phenanthroline used together. Dose–response curves for captopril were established on PCT and PR using concentrations from 0.7. 10^{-9} M to 10^{-8} M. In each routine experiment, it was systematically verified that the hydrolysis of HGG by each type of segment studied was totally inhibited by 10^{-7} M captopril.

Converting enzyme activity was expressed as pmoles HGG hydrolyzed per minute.

Micromethod for the measurement of kininase activity in the tubules by hydrolysis of lysyl–bradykinin (LBK)

In preliminary experiments we checked that the urine kallikrein of rabbit releases LBK from kininogen (and not BK as in the rat) [12]. LBK was therefore used to determine the kininase activity in the different nephron segments. For the assay, 8.4 pmoles of LBK (in 0.7 μl) were added to the glass slides containing the permeabilized or deoxycholate–treated tubule. Hydrolysis proceeded at 37°C in a final volume of 1.6 μl of 0.15 M Tris-HCl at pH 8 (LBK concentration = 5. 10^{-6} M). The reaction was stopped after 15 or 30 minutes of incubation by adding 50 μl of buffer I containing 10^{-3} M EDTA and o-

phenanthroline, and the sample was transferred into a Minisorp tube. The slide was then rinsed twice with 50 μl of buffer I, which were added to the Minisorp tube. Each sample was adjusted to one ml and kept frozen until the LBK was measured. This was done by radioimmunoassay using an antibody which recognized BK and LBK but not fragments of BK [12].

In each experiment, five control samples containing LBK but no tubule were run to check LBK stability during incubation. The LBK recovery measured under these conditions was 86.2 ± 1.2 (N = 21).

To check the accuracy and reproducibility of the small volume of LBK added in the enzymatic assay (0.7 μl), three additional control samples were also run in each experiment. They were prepared by directly adding LBK to Minisorp tubes. The quantity of LBK measured in these control samples was similar to the expected amount (8.64 ± 0.23 pmoles, N = 21 experiments, vs. 8.40 pmoles). The interassay variability coefficient calculated according to RODBARD [13], from the SEM/mean × 100 ratio was therefore 2.6%.

To calculate kininase activity, the amount of LBK remaining after incubation in the presence of tubule was subtracted from the amount measured after incubation in the absence of tubule. Kininase activity was expressed in picomoles of LBK hydrolyzed per minute and per millimeter of tubule.

Effect of chelating agents and captopril on kininase activity

The inhibition test was performed as follows: either 0.3 μl of the EDTA plus o-phenanthroline mixture or 0.3 μl of captopril was added to the permeabilized or solubilized tubule. The samples were then preincubated at 37°C for 10 minutes before the addition of LBK. Separate controls containing the substrate and chelators or captopril but not tubules were also run, to check that these inhibitors did not interfere with LBK measurements.

Checking of collagenase batches

As noted in the Microdissection of tubules section, the isolation of nephron segments required collagenase treatment of the kidney, which created a potential problem because the commercial collagenase batches used were not pure. It was therefore necessary to check that the impurities did not impair the activity of ACE or of kininases. Since these enzymes are known to be present in the brush border of proximal tubule cells and in the glomerulus [14], the effect of collagenase was studied on their activities. For this purpose, both kidneys of the same rabbit were prepared: one, as usual, was infused with the perfusion medium containing collagenase and serum albumin, and the other was infused with Hank's solution only. The ACE and kininase activities of microdissected glomeruli and portions of proximal tubule from both kidneys were then measured.

We also checked that the perfusion medium was not able to hydrolyze LBK by incubating 0.2 μl of perfusion medium with LBK under the usual conditions but in the absence of tubule. After 30 minutes of incubation, LBK was measured.

Calculations

Except where otherwise indicated, results are expressed as means ± SEM, and statistical analysis was performed using the non-paired Student's t-test. The criterion for statistical significance was taken as $P < 0.05$.

Results

Angiotensin I converting enzyme activity along the rabbit nephron

Effect of collagenase on HGG hydrolysis. When tested in the pars recta (PR), angiotensin I converting enzyme activity was not affected by collagenase treatment (53.5 ± 3.2 pmoles · min^{-1} · mm^{-1}, N = 7 samples from the collagenase treated kidney vs. 52.7 ± 1.8 pmoles · min^{-1} · mm^{-1}, N = 7 samples from the untreated kidney). When tested in the glomerulus, ACE activity was barely detectable after such treatment, but it became highly significant in the absence of collagenase (0.65 ± 0.06 pmoles · min^{-1} per glomerulus, N = 8, vs. 2.16 ± 0.21 pmoles · min^{-1} per glomerulus, N = 8). Consequently, the measurements were always performed on glomeruli from kidneys not treated with collagenase.

Distribution of angiotensin I converting enzyme activity. The hydrolysis of [^{3}H]hippuryl–glycylglycine (^{3}H-HGG) by nephron segments was considered to reflect ACE activity specifically because it was completely inhibited by low concentrations of captopril.

Only the glomerulus and the proximal tubule (PCT and PR) were found to contain angiotensin I converting enzyme (ACE). Figure 1 shows the ACE activities obtained when the samples were permeabilized by osmotic shock and subsequent freezing and thawing. ACE activity in the PR was four times higher than that measured in the first mm of PCT (PCT_a). The activity in PCT_b (that is, the fragment located 2.5 to 3 mm away from PCT_a) and in PCT_c (the fragment located 1.5 to 2 mm away from PCT_b) doubled and tripled, respectively, as compared to that in PCT_a. ACE activity in the glomerulus was low compared to that in the PR. Thus, if the amount of protein is assumed to be 75 ng per glomerulus and 200 ng per mm of PR (15-8), it can be calculated that the corresponding enzyme activities were 29 pmoles · min^{-1} and 265 pmoles · min^{-1} per μg of protein in the glomerulus and the PR, respectively.

The right part of Figure 1 shows the ACE activity in successive portions isolated from seven PCT_s dissected over 7 mm or more. This figure clearly indicates the existence of a converting enzyme concentration gradient along proximal tubules.

No statistically significant level of ACE activity was measured in the other nephron segments (TDL: 0.57 ± 0.55, CAL: 0.45 ± 0.13, DCT_b: 0.31 ± 0.20, DCT_g: 0 ± 0, CCT_1: 0.30 ± 0.20, and MCT: 0.41 ± 0.20 pmoles · mm^{-1} · min^{-1}).

ACE activity in the proximal tubule (PCT and PR) was also measured after solubilization of these segments with either 0.5% deoxycholate or 3 mM CHAPS. After deoxycholate, the ACE activity was three times less than in permeabilized tubules. In spite of this reduction, an activity gradient was again observed along all the proximal tubules (Table 1). In PR treated with CHAPS, ACE activity was similar to that found in the permeabilized PR (41.2 ± 1.8, N = 8, vs. 42.6 ± 2.5 pmoles · min^{-1} · mm^{-1}, N = 7).

The effects of chelating agents and captopril on the activities measured in the proximal tubule and in the glomerulus correspond to the known properties of ACE: thus full inhibition was induced by 10^{-3} M EDTA and o-phenanthroline and by 10^{-7} M captopril.

As shown by the dose–response curves of Figure 2, the

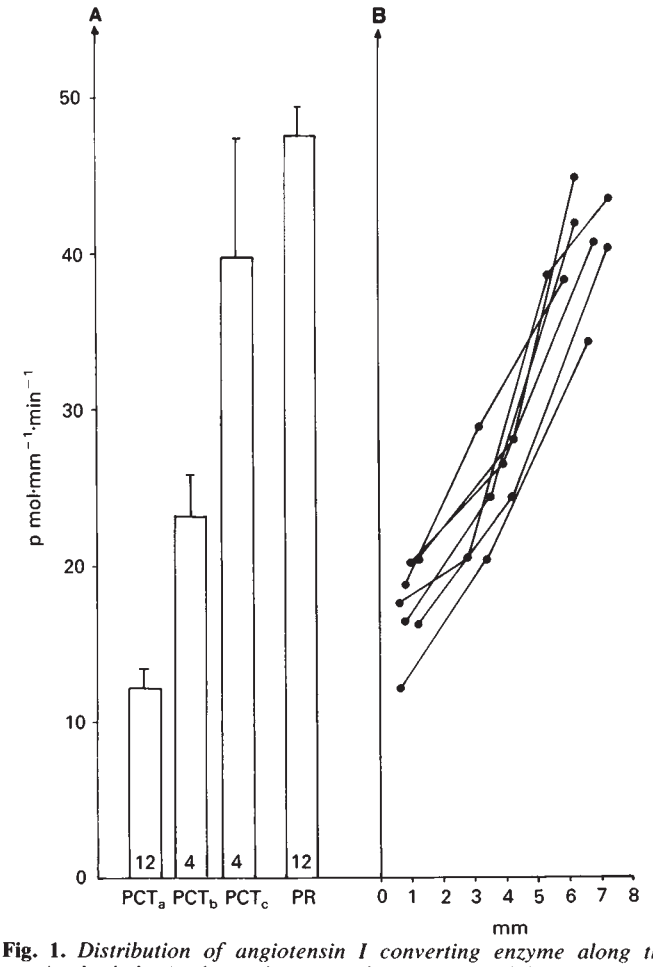

Fig. 1. *Distribution of angiotensin I converting enzyme along the proximal tubule.* **A.** shows the converting enzyme activity measured in the early, middle and late parts of the PCT, and in the PR. PCT_a denotes the first millimeter following the glomerulus. PCT_b denotes the fragments located 2.5 to 3 mm from PCT_a. PCT_c denotes the fragments located 1.5 to 2 mm from PCT_b. For each fragment, 6–10 nephrons from the same rabbit were tested. The number of rabbits studied is indicated in the bars. **B.** shows the converting enzyme activity measured in successive portions isolated from seven PCT, measuring over 7 mm each. Each PCT was divided into 3 fragments (except for one, which was divided into 4). The activity of each portion (ordinate) is expressed in pmoles mm^{-1} min^{-1} and plotted at the abscissa, which corresponds to the distance between the glomerulus and the mid-point of the portion. The values obtained for the same PCT are joined by lines.

activity measured in PCT and PR was highly sensitive to captopril, since $1.7.10^{-9}$ M caused 50% inhibition.

LBK hydrolyzing activity along the rabbit nephron

Effect of collagenase on LBK hydrolysis. The LBK-hydrolyzing activity measured in glomeruli, PCT and PR from untreated and collagenase–treated kidneys of the same animal showed that collagenase treatment did not significantly modify that activity in the PCT or PR, but reduced it to some extent in the glomeruli (Table 2).

In addition, when 8.4 pmoles of LBK was incubated in the presence of 0.2 μl of perfusion medium containing collagenase, the amount LBK recovery after 30 minutes of incubation (mean ± SD) was similar to that measured in control samples incubated without collagenase (6.67 ± 0.40 pmoles, $N = 5$ vs 6.99 ± 0.29, $N = 5$).

Table 1. Effect of deoxycholate on angiotensin I converting enzyme activity in the proximal tubule

Segment	Osmotic shock	Deoxycholate
PCT_a	17.1 ± 1.2 (8)	4.7 ± 0.5 (9)
PCT_b	30.6 ± 1.4 (8)	10.3 ± 1.1 (8)
PCT_c	52.3 ± 3.2 (6)	14.0 ± 1.7 (6)
PR	59.5 ± 9.1 (6)	18.1 ± 1.6 (6)

The fragments PCT_a, PCT_b, PCT_c are described in the legend to Fig. 1. Numbers in parenthesis denote the number of tubules.

Distribution of kininase activity along the rabbit nephron. After an osmotic shock followed by two freezings and thawings, glomeruli, early PCT and late PR hydrolyzed LBK at a fast rate: after 15 minutes of incubation, 40% of LBK was hydrolyzed per single glomerulus, and 47% per 0.5 mm of PCT or PR. Under the same conditions, MCT hydrolyzed LBK only at low rate which was not statistically significant. Therefore, the experiments were repeated by incubating the inactive or poorly active segments for 30 minutes with LBK. After that, a statistically significant kininase activity was always measured in the MCT (Fig. 3A). One millimeter of MCT hydrolyzed 2.8 pmoles of LBK per 30 minutes, that is, 33% of the initial amount. When the rate of LBK hydrolysis was correlated with the length of tubule in the MCT samples incubated (Fig. 4), a linear relationship was found ($y = 2.58x - 0.17$ and $r = 0.843$).

Kininase activity was also detected in the bright and granular portions of the distal convoluted tubule (DCT_b and DCT_g) and in the cortical collecting tubule (CCT_l). However, it was not statistically significant in any experiment. The mean percentage of LBK hydrolyzed per mm after 30 minutes was 15% for DCT_b, 21% for DCT_g and 20% CCT_l.

Solubilization with deoxycholic acid did not significantly alter LBK hydrolysis by the glomerulus, PCT or PR. In contrast, it greatly reduced hydrolysis in the other segments, including MCT (Fig. 3B).

Inhibition of LBK-hydrolyzing activities in the glomerulus, PCT, PR and MCT by chelating agents and captopril

The LBK hydrolysis produced by the glomerulus, PCT and PR was almost completely inhibited in the presence of 1 mM of EDTA and o-phenanthroline. The inhibition occurred regardless of whether the tubules were treated by osmotic shock or deoxycholic acid. In contrast, the hydrolysis of LBK was not affected by these chelating agents in MCT, suggesting that kininase activity was not due to the presence of a metalloenzyme in this nephron segment (Table 3).

As regards the captopril, tested at 5.10^{-6} M, it did not change LBK hydrolysis in the glomerulus or PCT, and only partially inhibited that of the PR (Fig. 5). When the tubules were submitted to osmotic shock, this inhibition was 32.0 ± 3.6%. It was more marked when tubules were solubilized with deoxycholic acid (48.3 ± 5.1 $P < 0.01$). Figure 5 shows that LBK hydrolyzing activity also decreased in MCT, but the inhibition which averaged 29% was rather variable. Even when tested at 5.10^{-5} M, captopril remained ineffective in reducing

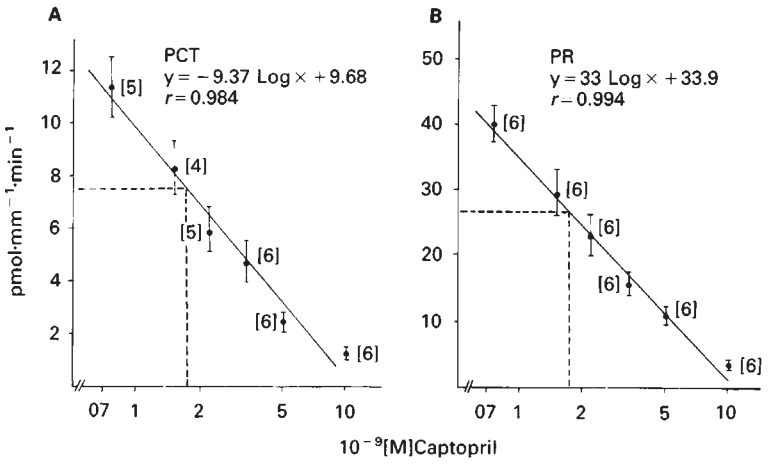

Fig. 2. *Plot for the inhibition of angiotensin I converting enzyme activity in the PCT (A) and PR (B) by captopril concentrations of 10^{-9} M to 10^{-8} M.* 0.7 μl of captopril was added to the glass slides containing the permeabilized tubule. The number of samples measured for each concentration is indicated in the figures.

Table 2. Effect of collagenase on the kininase activity of glomeruli, PCT and PR[a]

Nephron segments	Without collagenase	With collagenase
Glomeruli	2.77 ± 0.27 (6)	1.84 ± 0.35 (7)
PCT	8.66 ± 0.91 (5)	7.59 ± 1.02 (7)
PR	8.49 ± 0.98 (5)	7.86 ± 1.18 (7)
PR	7.04 ± 1.91 (8)[b]	6.77 ± 1.89 (8)[b]

[a] Kininase activity is expressed as picomoles of LBK hydrolyzed per millimeter of tubule during 15 minutes of incubation. Values represent mean ± SD. Numbers in parenthesis denote the number of tubules.

[b] The values without symbols represent the activity of glomeruli, PCT and PR from the same rabbit. The values marked ([b]) were obtained with PR from another rabbit during an additional experiment.

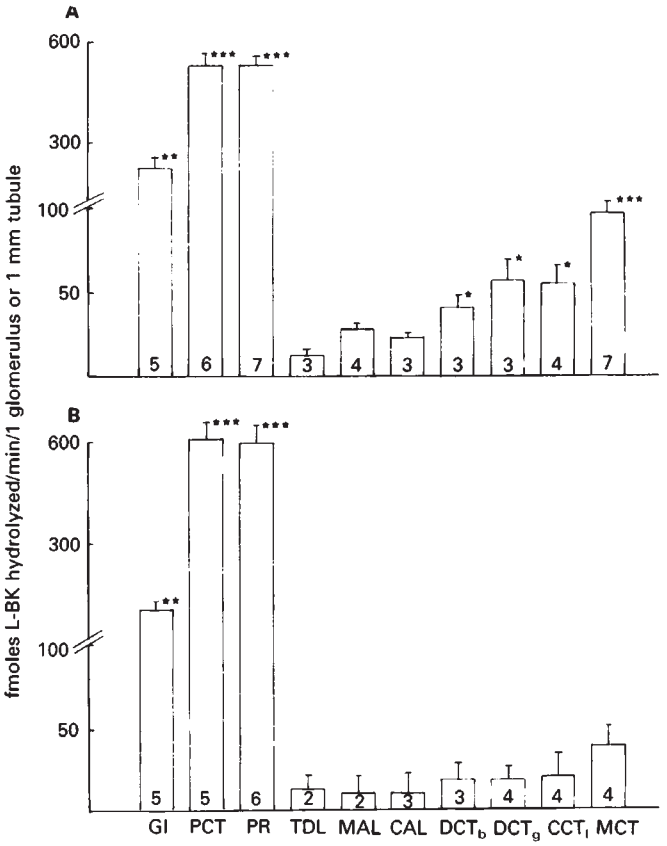

Fig. 3. *Distribution of kininase activity along the rabbit nephron.* Before incubation, nephron segments were submitted either to an osmotic shock followed by 2 freezings and thawings (**A**), or solubilized by 0.5% deoxycholate (**B**). For each rabbit, 6–10 replicate samples per nephron segment were tested. The number of rabbits studied is indicated in the bars. All nephron segments could not be obtained from the same experiment, but PCT or PR was studied in every case. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

the kininase activity of glomeruli and PCT (data not shown). Finally, the dose–response curves established for the inhibition by captopril of LBK hydrolysis in PR and MCT clearly indicate it was more sensitive to captopril in PR than in MCT (Fig. 6).

Discussion

The present study shows that in the rabbit nephron, ACE and other kininases are mainly present in the proximal tubule. Whereas total kininase activity was similar along the entire tubule, the ACE concentration gradually increases from glomerulus to pars recta. A kinin–hydrolyzing activity was also found in the terminal parts of nephron, particularly in the medullary collecting tubule.

Collagenase treatment of the kidney is a procedure commonly used to permit microdissection of different nephron segments. Without such treatment, it is very difficult to isolate certain nephron segments. However, as collagenase contains impurities which are liable to impair enzyme activity, we began by testing segments, such as glomeruli or short fragments of early PCT and PR, which could be dissected without it, and compared the results to those obtained with collagenase–treated segments. We found that in the PCT and PR fragments, collagenase treatment affected neither ACE nor kinin hydrolyzing activity. However, in the glomerulus, it reduced both these activities, particularly that of ACE, which became barely detectable. This drastic reduction might be explained by the fact that at least part of the activity measured in the glomerulus originated from the endothelial cells of the vascular pole formed by afferent and efferent arterioles, and that after collagenase treatment, less of this vascular tissue was simultaneously dissected with the glomerulus.

We noticed that the permeabilization of tubules by osmotic shock plus freezing and thawing was the best procedure for

Table 3. Effect of chelating agents on LBK hydrolysis in the glomerulus, PCT, PR and MCT

		Glomerulus	PCT	PR	MCT
Osmotic shock	Control	2.65 ± 0.12 (7)	6.35 ± 0.36 (6)	10.10 ± 0.69 (7)	2.63 ± 0.09 (7)
	EDTA + o-phenanthroline 10^{-3}M	0.12 ± 0.10 (7)	0.15 ± 0.11 (7)	0.21 ± 0.15 (7)	2.98 ± 0.20 (7)
Deoxycholate	Control	2.86 ± 0.22 (6)	9.37 ± 1.30 (6)	10.97 ± 0.70 (6)	—
	EDTA + o-phenanthroline 10^{-3}M	0.39 ± 0.20 (6)	0 ± 0 (7)	0.10 ± 0.10 (6)	—

0.3 μl of a mixture of EDTA and o-phenanthroline prepared in 0.2 M Tris (pH = 8) was added to concave glass slides containing either a permeabilized or solubilized tubule. LBK was added and the final concentration of EDTA and o-phenanthroline was 10^{-3}M. The glomerulus, PCT and PR were incubated for 15 minutes, and the MCT, for 30 minutes. Mean ± SEM, pmoles · mm^{-1}

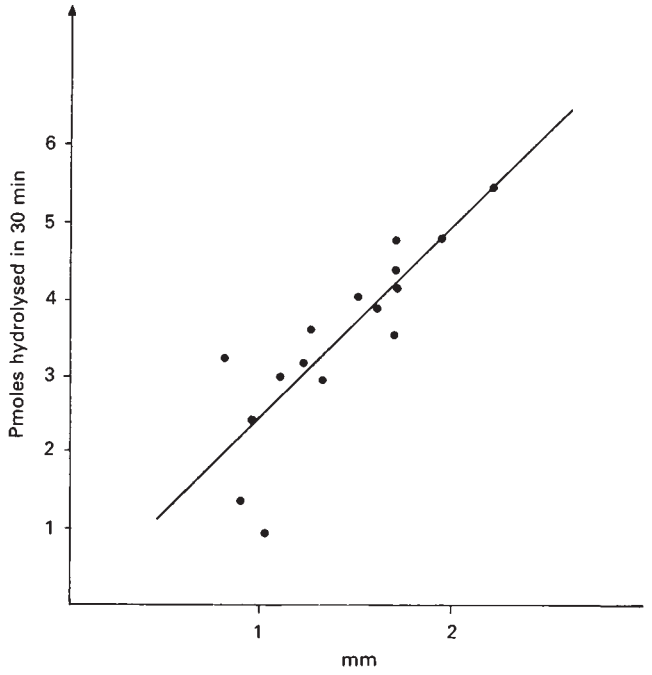

Fig. 4. *MCT length dependency of LBK hydrolysis.* MCT of different lengths were incubated for 30 min. at 37°C with 8.4 pmoles of LBK. Abscissa, mm of tubule; ordinate, pmoles of LBK hydrolyzed in 30 min. $r = 0.843$; $y = 2.58x - 0.17$.

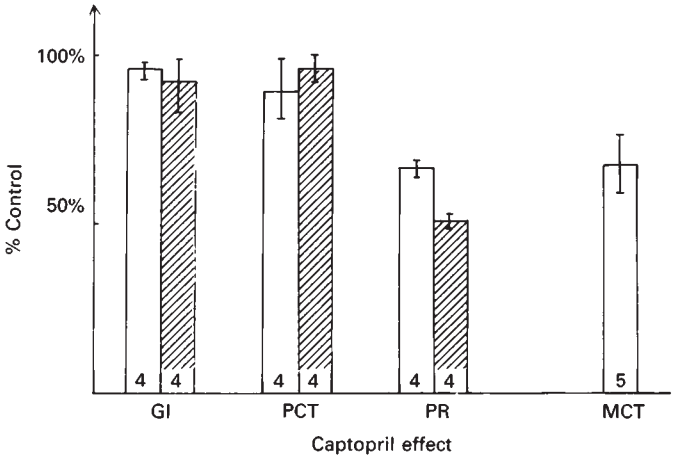

Fig. 5. *Effect of 5.10^{-6} M captopril on the LBK hydrolysis produced by the glomerulus, PCT, PR and MCT.* 0.3 μl of captopril in 0.2 M TRIS pH 8 was added to the samples containing the permeabilized or solubilized tubules. Slides were then preincubated for 10 min before addition of LBK. The final concentration of captopril was 5.10^{-6} M. The glomerulus, PCT and PR samples were incubated for 15 min, whereas the MCT samples were incubated for 30 min. For each rabbit and each experimental condition, 6–8 samples of the same segment were tested. Number of rabbits is indicated in bars, (□) osmolic shock, (▨) deoxycholate. Results are expressed as the percentage of the mean control kininase activity measured in the same experiment, in the absence of captopril.

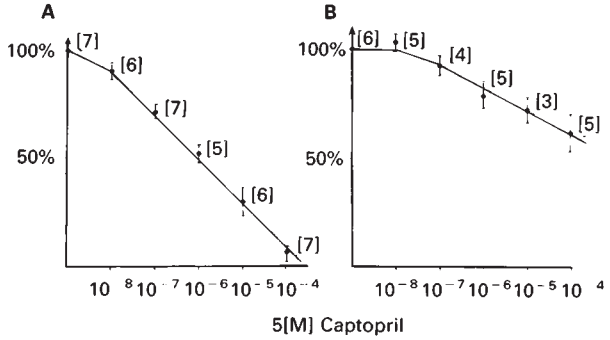

Fig. 6. *Plot of percent inhibition of kininase activity in PR (A) and MCT (B) by captopril concentrations ranging from 10^{-8} M to 10^{-4} M.* The experiment was performed as described in the legend to Fig. 5, by adding 0.3 μl of increasing concentrations of captopril. PR were previously solubilized by deoxycholate and MCT were permeabilized by osmotic shock and freezing and thawing. The number of samples studied for each concentration is indicated on the curves.

revealing the enzyme activities studied. However, to make sure that this method enabled all the activity to be measured, we compared the results to those obtained using detergents, since ACE is known to be present in the brush-borders of proximal tubule cells [15], a site which might be poorly accessible to the substrate in isolated non-perfused tubules. However, solubilization of nephron segments by deoxycholate did not reveal more kininase activity in the glomerulus, PCT or PR than osmotic shock plus freezing and thawing, and even inhibited the kinin-hydrolyzing activity of MCT (Fig. 3). Deoxycholate also inhibited ACE activity (Table 1). Inhibition of enzyme activity by deoxycholate has already been reported, and the use of a less drastic detergent such as the zwitterion CHAPS has been suggested [16]. In our experiments with CHAPS, the level of ACE in the proximal tubule was neither decreased, nor increased compared to that measured after permeabilization. This latter observation indicates that the permeabilization procedure we used allowed the ACE and LBK hydrolyzing activity in the tubular cells to be fully revealed.

We showed that ACE is distributed in the proximal tubule along a gradient which increased from the glomerulus to the pars recta. The highest level of activity was found in the PR. This observation agrees with the results of Sudo for the rat nephron [15]. Like Ward et al [14], we also found low ACE

activity in the glomerulus. None at all was measured in the descending and ascending limbs, the two portions of distal convoluted tubule or in the collecting tubule, although immunofluorescence test suggested that it may also be present in the distal tubule of species other than the rabbit [17].

The ACE activity found here in the glomerulus, PCT and PR was completely inhibited by chelating agents, and was inhibited by captopril to a similar degree in the PCT and PR (Fig. 2) which demonstrate that the same enzyme activity was measured in the PCT and PR. In addition, the characteristics of captopril inhibition for ACE in PCT and PR were very similar to those reported for ACE in intestinal and vascular surface membrane [18] and to those which we found with a purified ACE preparation from human kidney (data not shown).

As previously observed by micropunctures of rat nephron [1], kinins are very quickly hydrolyzed by the proximal tubule. A similar rate was found here for LBK hydrolysis at the beginning and end of the proximal tubule (PCT and PR). This hydrolysis was completely inhibited by EDTA and o-phenanthroline in both segments. Captopril, however, affected each one differently: it inhibited kinin–hydrolyzing activity in PR only, but it did not affect the activity of the early PCT, even at a concentration of 5.10^{-5} M. Captopril also failed to inhibit kininase activity in the glomerulus. Consequently, kinin hydrolysis in the glomerulus, PCT and PR must involve different enzymes. The fact that captopril had no inhibitory effect in the glomerulus or PCT indicates that ACE, although present, does not contribute to LBK hydrolysis in these parts. In the pars recta, the inhibition of kinin–hydrolyzing activity was total when the captopril concentration was high (5.10^{-4} M, Fig. 4). This concentration was higher than that required to inhibit the LBK-hydrolyzing activity of a purified ACE preparation from human kidney, measured under our experimental conditions, since half the activity of this preparation was inhibited by a captopril concentration of only 10^{-7} M, and complete inhibition was obtained with 10^{-6} M (results not shown). These concentrations were respectively 50 and 500 times lower than those required to inhibit by 50% and 100% the LBK-hydrolyzing activity of the pars recta (Fig. 6). It is, therefore, very likely that at the high concentrations used here to inhibit this activity in the pars recta, captopril simultaneously inhibited ACE and the other kininases present. Although, our results do not allow quantification of the contribution of ACE to kinin hydrolysis in pars recta, it cannot be very high despite the fact that this segment contained a large amount of ACE.

The physiological significance of the ACE concentration gradient we found here in the proximal tubule remains unexplained, and the role of ACE in the kidney has not in fact been well defined as yet in either the renin angiotensin or kallikrein kinin systems. Its localization in the proximal tubule does not allow its involvement in the inactivation of kinins generated by the kidney. The characteristics of the intrarenal kallikrein–kinin system (localization of kallikrein in the connecting tubule and of kininogen in the collecting duct system) [19–23] rather involve the terminal parts of nephron. However, ACE is presumably involved in the intrarenal formation of angiotensin II, which might control sodium reabsorption in the proximal tubule [24] by acting not only on the peritubular side but also on the luminal side [25–27].

One original observation of the present study is the presence of kininase activity in the MCT. This activity was not due to ACE, since it was not inhibited by EDTA and o-phenanthroline, and was little affected by captopril, even at high concentrations such as 5.10^{-4} M (Table 3 and Figs. 5, 6). The absence of inhibition by chelating agents also indicates that kininase activity in the MCT may not be due to the presence of enkephalinase A (NEP), an enzyme which, however, highly contributes to the degradation of bradykinin in the urine [28]. The nature of the kininase present in MCT remains unknown, but its activity might resemble the type of kinin–hydrolyzing activity which is insensitive to EDTA and was observed by Cicclini et al [29] in the soluble fraction of rabbit kidney homogenate. In the literature, divergences subsist as regards the presence of kininase activity in the terminal parts of the nephron. On the one hand, Carone et al [1] did not find any inactivation of bradykinin after its injection into the distal part of the rat nephron. On the other, Scicli, Gandolfi and Carretero [3] used the stop flow technique to determine the site of formation of kinins and found evidence for some kininase activity in the terminal segments. The differences between these results might be due to the low level of kininase activity observed.

The presence of kininase in the MCT might be physiologically important because this segment is a target site for the action of kinin, as demonstrated by the existence of bradykinin receptors along the CCT and MCT [30]. At the present time, we can only speculate on the physiological function of this enzyme in MCT because it is not known now if it is localized on the cell membrane and/or intracellularly. However, because this kininase activity is located in the terminal parts of the nephron, it may be involved in the hydrolysis of the kinins formed by the kidney, at and/or beyond its production site in the connecting tubule [19–23]. Partial inactivation of kinins by the terminal part of the nephron might explain, at least in part, the difficulty of correlating urinary kallikrein activity and kinin excretion [31]. Studies designed to determine the nature and function of this kininase activity are now in progress.

Acknowledgments

The authors are indebted to Professor F. Morel for helpful suggestions and critical advice. We are also grateful to Dr. J.P. Morgat for his gift of tritiated hippuryl–glycylglycine, Dr. F. Bastide for help in drafting the manuscript, to M. Dreyfus for English language revision and S. Bayon for secretarial assistance. This work was presented in part at the International Kinin Congress, Savannah, October 1984.

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