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COMPONENTS OF THE KALLIKREIN–KININ SYSTEM IN RAT URINE*

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Abstract—Using a direct radioimmunoassay and a kininogenase assay, we determined that 68% of rat urinary kallikrein was enzymatically active while 32% was in an inactive form which was activated by trypsin. Inorganic cations, at concentrations found in rat urine, were inhibitory in an amidase assay but appeared to potentiate kininogenase activity of pure rat urinary kallikrein. In random urines, kinin concentration was 4.2 ± 0.7 ng/ml. Trypsinization of the urines generated 52.9 ± 25.8 ng kinin/ml, indicating that kininogen was present. The rate of kinin formation *in vivo* may depend on the availability of kininogen and the concentration of inorganic cations in urine, as well as on other well-recognized factors, such as the kallikrein activity of the urine.

The renal kallikrein–kinin system may be involved in the regulation of salt and water excretion and in the pathophysiology of hypertension [1, 2]. The activity of the system usually is monitored by assays for urinary kallikrein (EC 3.4.21.8), which has been assumed to be the rate-limiting component [3, 4]. However, in some circumstances [5] the urinary level of kallikrein does not correlate well with urinary kinin excretion. Information about the presence in urine of other components of the system should help to determine the significance of such discrepancies.

In human urine, the components of the kallikrein system are now well defined in terms of the amounts and characteristics of kallikrein, kinin and kininogen [6–9]. However, such information is not available for rat urine despite frequent use of this animal in studies of experimental hypertension and renal hemodynamics. The purpose of this investigation was to determine the levels and certain relevant biochemical characteristics of several components of the kallikrein–kinin system in rat urine.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: chloramine-T iodination beads (Pierce Chemical Co., Rockford, IL); prolyl-phenylalanyl-arginyl-[3H]-benzylamide (Ventrex Laboratories, Inc., Portland, ME); ^{125}I-Tyr^{8}-bradykinin (New England Nuclear Corp., Boston, MA); tosyl phenylalanine chloromethyl ketone (TPCK)-treated trypsin and trypsin-Sepharose (Worthington Biochemical Corp., Freehold, NJ); and goat anti-rabbit IgG and normal rabbit IgG (Miles Laboratories, Inc., Elkhart, IN).

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Characterization of rabbit anti-rat urinary kallikrein serum (anti-kallikrein). The anti-kallikrein serum used in the present study was that described in previous publications [10–12]. The protocol for the direct radioimmunoassay (RIA) of kallikrein was identical to that already described [11, 12], with the exception that radiolabeling of kallikrein was performed using insoluble chloramine T.

Determination of kininogenase activity by kinin RIA. Bovine kininogen was partially purified from the plasma by ammonium sulfate and zinc acetate precipitations, as described by Kato *et al.* [13]. In selected instances, pure low molecular weight (LMW) kininogen, supplied by Dr. H. Kato, was employed. Generation and determination of kinin by kinin RIA were performed as previously reported [14].

Determination of amidase activity. Amidase activity of rat urine was determined by a radiochemical technique using a tripeptide substrate, prolyl-phenylalanyl-arginyl[^{3}H]-benzylamide, as previously described [14].

Determination of catalytic efficiency of rat urinary kallikrein in the presence of inorganic ions. Kininogenase activity of pure rat urinary kallikrein was determined in a low molarity (5 mM) sodium phosphate buffer, while the amidase activity was determined in 0.1 M 2-amino-2-methyl-2,3-propanediol (AMPD) buffer. Kininogenase and amidase activity of pure rat urinary kallikrein were measured in aliquots of the respective buffers to which selected concentrations of sodium chloride and magnesium chloride had been added.

Determination of active and inactive kallikrein. A 24-hr sample of urine was collected at 25° from Charles River rats, housed separately in metabolic cages. The urine sample was divided into three aliquots. The first aliquot was analyzed without any treatment. The second aliquot was activated with insoluble trypsin as previously described [6]. The third aliquot was adsorbed with insoluble Trasylol

[6] to remove active enzyme. A portion of the last aliquot was trypsinized. All of these samples were analyzed by the direct assay and by the kininogenase assay.

Determination of endogenous kininogen in rat urine. Seven rats were housed in individual metabolic cages, and urine was collected at 25° over 18–20 hr into 1 ml of 2 N HCl and 10 μg pepstatin. An aliquot was heated at 60° for 2 hr and then concentrated approximately 2- to 8-fold by filtration through an Amicon UM 10 filter. (Preliminary studies confirmed that no loss of kininogen occurred during the filtration step. Total kinins generated after trypsinization of urokininogen were also found to be the same whether the heating step was done at 60° or in a boiling water bath.) Concentrated urine was taken up in 0.1 M phosphate buffer, pH 8.5, containing 3 mM phenanthroline and 30 mM sodium EDTA. The volume was brought to 0.4 ml, and 100 μl (0.5 mg) of trypsin (pretreated with TPCK) was added to consume the kininogen. TPCK-treated trypsin was used to inhibit the traces of α-chymotrypsin contaminant known to occur in trypsin preparations. The reaction was incubated at 37° for 20 min and then terminated by immersing the tubes in a boiling water bath for 5 min. The generated kinins were analyzed by kinin RIA. Control reaction consisted of trypsin without urine. An aliquot of untreated urine was also analyzed to determine the level of preformed kinins.

RESULTS

Activity of rat kallikrein. We studied the catalytic efficiency of kallikrein in the presence of inorganic cations. In three experiments, the amidase activity of rat urinary kallikrein was inhibited markedly by addition of cations to the standard electrolyte-free reaction mixture, as shown in Table 1. Both univalent and divalent cations inhibited kallikrein activity. The amidase activity of the enzyme was inhibited 50% by either 30 mM NaCl or 1 mM $MgCl_2$. In a separate single experiment, the rate of kinin generation by kallikrein from purified LMW kininogen was approximately doubled when 10 mM NaCl or 2 mM $MgCl_2$ was added to the low molarity assay system (5 mM sodium phosphate, pH 8.5, containing 3.0 mM 1,10-phenanthroline and 1 mM Captopril). Activity (ng kinin per 20 min per μg kallikrein) increased from 200 to 500 when 10 mM NaCl was added, and to 450 when 2 mM $MgCl_2$ was added.

RIA for rat kallikrein. Using insoluble chloramine T, the radiolabeled iodine was incorporated at 20–30 μCi/μg in rat urinary kallikrein. A high titer antiserum was employed in the direct RIA. In a 1:100,000 dilution, the antiserum bound approximately 30% of tracer antigen. A linear plot with an $r = 0.95$ was obtained by Scatchard analysis. The calculated K_{eq} from ten separate experiments was $1.1 \times 10^{12} M^{-1}$ and the K_d was 9.1×10^{-13} M, indicating a high affinity of the antiserum.

In Fig. 1, the standard RIA curve from ten different experiments is shown. The variability of the assay (6.1% intra; 9.5% inter) and the recovery (104 ± 8%) of added enzyme were similar to those already described [11]. Also shown in Fig. 1 are actual displacement readings of three different rat urines and three samples of urine from which active kallikrein had been removed by adsorption with insoluble Trasylol. The parallelism of these curves from untreated urine and urine free of active kallikrein suggests that the antibody recognized active as well as inactive kallikrein in RIA. The minor cross-reactivity of rabbit and human urine was nonparallel.

Immunoreactive kallikrein and kininogenase activity in seven rat urines before and after trypsinization are shown in Fig. 2. As illustrated in the upper half of the figure, immunoreactive kallikrein both in untreated urine and in urine pretreated to remove active kallikrein was unchanged after trypsinization. In marked contrast, the kininogenase activity (lower half of the figure) was increased 1.5 times after

Table 1. Effects of ions on the amidase activity of rat urinary kallikrein*

	Final concn in assay system (mM)	% Inhibition (± S.E.M.)
Sodium chloride	5	13.8 ± 3.4
	10	26.1 ± 3.9
	20	41.8 ± 1.1
	40	59.8 ± 2.3
	80	78.4 ± 1.1
	120	81.4 ± 0.6
Magnesium chloride	0.1	8.4 ± 3.4
	0.2	13.3 ± 5.4
	0.5	38.4 ± 7.2
	1.0	46.9 ± 4.4
	2.0	68.8 ± 4.4
	4.0	76.7 ± 7.0

* Amidase activity of pure rat urinary kallikrein was determined (N = 3 experiments) in 0.1 m AMPD buffer, pH 9.5, in the absence and presence of stated concentrations of inorganic ions. The reaction was initiated by addition of enzyme, incubated at 37° for 15 min, and then terminated by addition of 1 ml of 0.1 M NaOH.

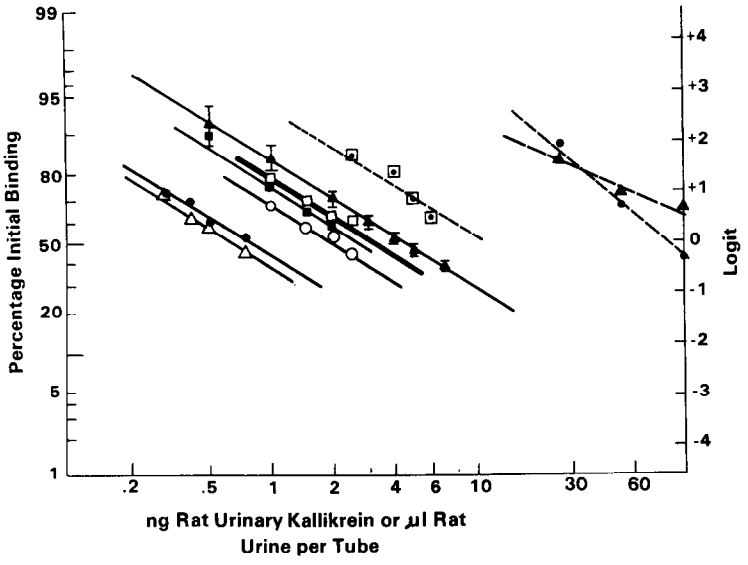

Fig. 1. Log-logit transformations: ten standard curves of rat urinary kallikrein (mean ± S.D. ▲—▲); rat urine samples 1 (△—△), 2 (●—●) and 3 (■—■); rat urines devoid of active kallikrein, 1 (⊡---⊡), 2 (□—□) and 3 (○—○); and rabbit (●---●) and human (▲---▲) urine samples.

trypsinization of urines and increased 3.8 times after trypsinization of urines which had been pretreated to remove active enzyme. From the effect of trypsin on kininogenase activity, active kallikrein is calculated to be 66% of total urinary kallikrein (355/540 × 100). From the determination of immunoreactive kallikrein, total and inactive (after adsorption on Trasylol–Sepharose), active kallikrein is calculated to be 70% of the total (4.13–1.24/4.13 × 100). Thus, approximately two-thirds of rat urinary kallikrein is in an active form. The remaining one-third is in inactive form which can be activated with trypsin.

Kininogen levels in seven random rat urines are presented in Table 2. The preformed kinin level in these urines was 4.2 ± 0.7 ng/ml. When urines were incubated with TPCK-trypsin, significant kinin was generated in every sample. After trypsinization, kinins increased to 52.9 ±25.8 ng/ml.

DISCUSSION

The levels of active and inactive kallikrein in rat urine were determined using a direct radioimmunoassay for kallikrein and a kininogenase assay for kallikrein activity* in which kinins generated were measured by RIA. The concentration of kallikrein by direct RIA, about 4 µg/ml, is in the same range as found by others [4, 11]. By analyzing immunoreactive kallikrein before and after trypsin treatment, we have shown that our rabbit anti-rat urinary kallikrein recognizes both active and inactive enzyme. This observation was supported by the following facts: (1) untreated urine as well as urines treated with Trasylol to remove active enzyme gave displacements of [^{125}I]-kallikrein–anti-kallikrein conjugate which were parallel to the standard curve (Fig.1); (2) the dose–response curves of untreated urines depleted of active enzyme were linear; and (3) after urines were trypsinized, the immunoreactive level was not affected (left side of Fig.2), while the kininogenase activity increased. We also have demonstrated (Fig. 2) that approximately two-thirds of kallikrein in rat urine is in the active form, while one-third is inactive. In human urine, Pisano *et al.* [9] and our own group [6] have found that about half of urinary kallikrein is in the inactive form. The nature of inactive urinary

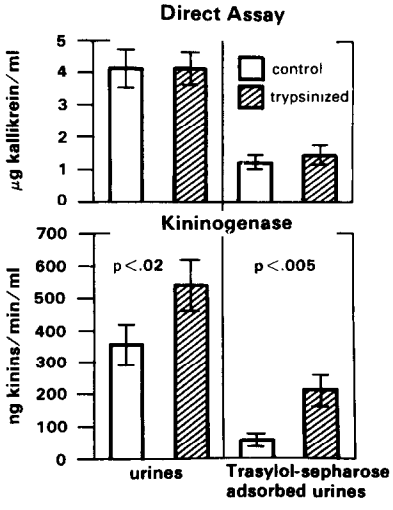

Fig. 2. Determination of active and inactive kallikrein in rat urines. Values are mean ± S.E.M. for urines from seven rats. P values were calculated by Student's *t*-test for paired samples before and after trypsinization.

* Esterase A_2 in rat urine has significant kininogenase activity with dog kininogen but only trivial activity with bovine substrate [15]. Since bovine kininogen was used in our kininogenase assay, it should be an accurate index of kallikrein activity in rat urine.

Table 2. Kinin generation from urokininogen

Urine sample	Kinin (ng/ml urine)	
	Before trypsin	After trypsin
1	7.3	195
2	5.8	37
3	3.5	84
4	3.4	4
5	1.3	7
6	3.5	20
7	4.8	22
Mean ± S.E.M.	4.2 ± 0.7	52.9 ± 25.8

kallikrein is not known. Recent evidence suggests that it is a precursor enzyme [16], although the possibility that some or all of the inactive form is an inhibitor–enzyme complex has not been ruled out [17].

In a previous study from this laboratory [14], the catalytic efficiency of human urinary kallikrein was found to be greatly influenced by inorganic cations. The activity on a synthetic substrate was markedly inhibited, while kininogenase activity was potentiated in the presence of ions such as sodium and magnesium. We now report similar effects of ions on the rat enzyme. The amidase activity of rat urinary kallikrein was inhibited 50% with either 30 mM sodium chloride or with 1 mM $MgCl_2$. In contrast, in a separate experiment, kininogenase activity, which was very low in a low molarity buffer, increased about 2-fold in the presence of 10 mM NaCl or 2 mM $MgCl_2$.

Pisano *et al.* [9], using the substrate depletion technique, found low molecular weight kininogen in human urine. The technique is reproducible provided the kininogen is denatured to facilitate access of trypsin to key sites in the substrate [18]. A possible problem with this method is that trypsin digestion may produce factors which potentiate the biological action of bradykinin [19]. In the present work we used an immunological, not a biological, assay for kinins to avoid this problem.

We have found that rat urine contains kallikrein, kinin and kininogen. There was approximately 4 μg/ml immunoreactive kallikrein in rat urine, of which 68% or 2.7 μg was in the active form (Fig. 2). The level of kininogen in the rat urines can be estimated as follows. After trypsin treatment, 52.9 ± 25.8 ng/ml kinin was generated in the urines (Table 2). If 1 mg low molecular weight kininogen is assumed to generate 20 μg kinin equivalence [20], the kininogen content of these urines was about 2.7 μg/ml. This calculation is admittedly very approximate; it includes as kininogen any metabolized fragments capable of releasing kinins after trypsinization. Nevertheless, it suggests that the molar concentration of kininogen is in the range of 39 pmoles/ml (mol. wt 70,000; Ref. 21). Active kallikrein appears to be about 60 pmoles/ml (mol. wt 45,000; Ref. 10). Thus, substrate (kininogen) and enzyme (active kallikrein) are present in rat urine at roughly comparable molar concentrations. This is contrary to the recent suggestion [22] that kininogen may be present only at very low concentrations relative to kallikrein in rat urine.

Our data do not explain why there is so much intact kininogen despite the presence of active kallikrein in urine. The rate of kinin formation may be limited by multiple factors, such as enzyme concentration, substrate concentration, affinity constant (K_m) for enzyme–substrate complex formation, and by the pH and cation concentration at the site of kinin formation *in vivo*. It is not known whether product and/or substrate inhibition significantly alters the *in vivo* rate of kinin formation.

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