The Effect of Cations on the Activity of Human Urinary Kallikrein*

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We studied the effect of ions on the ability of purified human urinary kallikrein to cleave its natural substrate (kininogen) as well as two synthetic substrates, tosylarginine [3H]methyl ester and Pro-Phe-Arg-[3H] benzylamide.

The kininogenase activity of kallikrein is markedly dependent upon the concentration of cations in vitro. Kininogenase activity is very low when measured in a low electrolyte buffer. The addition of cations to the reaction mixture increases activity by up to 27-fold. Maximum activity is achieved with 100 mM sodium, 100 mM potassium, or 20 mM magnesium. The activity is stable at higher concentrations of cation. Renal kallikrein is believed to act within distal tubular fluid in vitro. The concentration of cations in this fluid varies widely in response to alterations in salt and water metabolism. Thus, the relationship of kininogenase activity to the concentration of cations demonstrated in vitro may be relevant to the activity of kallikrein at its presumed site of action in the kidney.

In separate experiments, we evaluated the effect of ions on the amidase and esterase activities of kallikrein which are the basis of several assays in routine use for physiological studies. In contrast to their stimulatory effect on kininogenase activity, cations inhibit amidase and to a lesser extent esterase activity. Additional studies indicate that urinary cations probably account entirely for the well known ability of normal urine to inhibit the amidase and esterase activities of kallikrein.

URINARY KALLIKREIN—Urinary kallikrein (EC 3.4.21.8) is a serine protease synthesized by the kidney and excreted in the urine (1) which liberates kallidin from its natural substrate kininogen. Evidence has accumulated recently which suggests that the renal kallikrein–kinin system may play a role in the regulation of renal function and in certain diseases such as hypertension (2).

Renal kallikrein is believed to release kinines in the distal nephron (3). The ionic composition of distal tubular fluid varies markedly in response to alterations in salt and water metabolism. Prado et al. (4) have demonstrated that the kininogenase activity of horse urinary kallikrein is dependent upon the presence of cations in the assay system. We have studied the effect of ions on the kininogenase activity of human urinary kallikrein and report that the activity is strikingly dependent upon the concentration of cations in vitro.

In addition, we have studied the effect of ions on the ability of human urinary kallikrein to cleave synthetic substrates. Most studies of urinary kallikrein excretion have used a synthetic substrate to measure kallikrein by means of its esterase or amidase activity. In contrast to their enhancement of kininogenase activity, cations inhibit the amidase activity of kallikrein markedly and esterase activity to a lesser extent. It is known that normal human urine inhibits the esterase and amidase activities of urinary kallikrein (5, 6). We have found that this inhibition can be attributed entirely to the effect of the cations present in urine. We have also found that desalting urine samples by gel filtration results in consistent loss of kininogenase activity and of immunoreactive kallikrein.

EXPERIMENTAL PROCEDURES

Materials

Ultrace grade sodium chloride and polyethylene glycol (Baker), tosylarginine [3H]methyl ester (Amersham), Captopril (SQ14,225, Squibb), Sephadex G-25 (Pharmacia), lactoperoxidase iodination kit and 3'-5'-lodo-Tyr-bradykinin (New England Nuclear), and goat anti-rabbit IgG and bovine y-globulin serum (Miles) were obtained from commercial sources indicated.

Kininogenase Activity

Kininogenase activity was determined by incubation of kallikrein or urine with kininogen and measurement of the liberated kinines by radioimmunoassay.

Generation of Kinins

Effect of Ions on Kininogenase Activity—In order to study the effect of ions on kininogenase activity, homogenous human urinary kallikrein and bovine low molecular weight kininogen were reacted in a low molarity sodium phosphate buffer to which increasing concentrations of ions were added. The kallikrein was isolated to apparent homogeneity according to the method of Oza and Ryan (7). Purified bovine low molecular weight kininogen was generously supplied by Dr. H. Kato, Kyushu University, Fukuoka, Japan. The substrate generated approximately 20 ng of kinin equivalents per mg of protein when incubated with an excess of trypsin.

The reaction was carried out in 5 mM sodium phosphate buffer, pH 8.5, containing 3.0 mM 1,10-phenanthroline and 1 mM Captopril. The reaction mixture contained 0.01 to 0.06 ml of various concentrations of salt solutions dissolved in buffer and 30 ng of kininogen (approximately 60 ng of kinin equivalents) in 0.02 ml of phosphate buffer and was brought to a final volume of 0.5 ml with the phosphate buffer.

The reaction was initiated by addition of 1 to 4 ng of pure kallikrein in 0.01 ml of phosphate buffer. Samples were incubated at 37 °C for 20 min and the reaction was terminated by heating in a boiling water bath for 15 min.

The substrate displayed no cross-reactivity with the antiserum. If any sample consumed more than 10% of the substrate, the incubation was repeated with a smaller amount of kallikrein. The recovery of added bradykinin following both incubation and RIA steps was 106 ± 6.2%. Activity is expressed as nanograms of bradykinin equivalents generated/ng of kallikrein/20 min of incubation.

Kininogenase Activity in Urine—This activity was determined by a modification of the above reaction conditions. Bovine kininogen, in this case, was partially purified by ammonium sulfate and zinc acetate.

† The abbreviation used is RIA, radioimmunoassay.

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Kinin Radioimmunoassay

The generated kinins were measured by RIA using rabbit anti-bradykinin serum generously supplied by Dr. Colin Johnstone (Melbourne, Australia). The RIA was performed in 0.1 M Tris-Cl buffer, pH 7.4, containing 10 mM Na₂ ethylenediaminetetraacetic acid, 1.0 mM 1,10-phenanthroline, 0.2% gelatin, and 0.1% neomycin. The reaction mixture contained 0.025% of [125I]-bradykinin (approximately 4,000 cpm), 0.1% of normal serum (for nonspecific binding) or anti-bradykinin serum, and 0.025:1,000 pg of unlabeled bradykinin or 0.01 to 0.05 μl of sample, and the RIA buffer to yield a total volume of 0.5 ml. Samples were incubated for 2 h at 4°C. Free and antibody-bound [125I]-bradykinin were separated by precipitation with bovine γ-globulin (1 g/100 ml dissolved in RIA buffer) was added to each tube. 0.6 ml of polyethylene glycol (25% in 0.1 M Tris-HCl, pH 7.4, at 4°C) was then added and each tube was agitated and then centrifuged at 3000 rpm for 30 min. The supernatant was decanted and the precipitate containing the antibody-bound [125I]-bradykinin was counted for radioactivity. A standard curve was obtained by plotting the per cent of initial binding (B/B₀) against the bradykinin standard. Nonspecific binding, determined in each assay by substituting nonimmune serum for the anti-bradykinin serum, ranged from 0.2 to 0.3% (S.D.) of the kinins generated by incubation of the same urine samples with kininogen. Thus, no correction for preformed kinins in the urine samples was necessary since they represented a negligible fraction of the generated kinins.

Amidase Assay

Amidase activity of urinary kallikrein was determined by a previously described radiochemical technique (8) using a tripeptide substrate (Pro-Phe-Arg-[125I]benzylationdine) The reaction mixture contained 0.05 ml of substrate buffer with 0.2 μg of human urinary kallikrein (3.9 X 10⁻⁴ esterase units) diluted in 0.05 ml of buffer to yield 1 ml of activity of purified kallikrein was determined in the absence of added salt (i.e., in 0.1 M 2-amino-2-methyl-2,3-propanediol as initial (100%) activity.

The Effect of Urine on the Activity of Kallikrein

Urine was desalted with Sephadex G-25 (fine) using a modification of a previously described technique (5). One ml of each urine sample was desalted by centrifuging it through 4 ml of hydrated gel placed in a 5-ml plastic syringe barrel. The desalted urine samples were stored frozen at -70°C. Electrolyte Measurements

Sodium and potassium were measured by a flame photometer (Instrumentation Laboratories Inc., Boston, MA). Calcium and magnesium were measured by atomic absorption (Instrumentation Laboratories). Urea was measured by a standard technique (10).

Statistics

All data are expressed as the mean ± S.E. All values represent the mean of 3 to 6 analyses. The concentration of cation required for 50% inhibition of amidase activity (I₅₀) was determined by interpolation from linear regression equations obtained by relating per cent inhibition (0 to 80%) to the log of the concentration of cation in the assay system. Covariance analysis and Scheffe's multiple comparison test were used to compare the relationship between ionic strength and the kininogenase activity for the various salts tested. RESULTS

Effect of Ions on the Kininogenase Activity of Kallikrein—Human urinary kallikrein has very little kininogenase activity in a low molarity phosphate buffer (5 mM sodium phosphate). One ng of kallikrein released 0.5 ± 0.03 ng of kinins/20 min of incubation. This represents only 0.5% consumption of substrate. The activity increased markedly with the addition of cations to the assay system (Fig. 1) to a maximum of 8.3 ± 0.8 ng of kinins/ng of kallikrein/20 min of incubation. Maximum
The esterase activity of kallikrein is inhibited both by univalent and divalent cations. However, at comparable concentrations of cations, the esterase activity of kallikrein is inhibited to a lesser extent than amidase activity.

Inhibition of Kallikrein Activity by Normal Urine and Matched Electrolyte Solutions (Fig. 2) — Normal human urine is known to inhibit the amidase and esterase activities of added purified human urinary kallikrein (5, 6). In order to determine the extent to which cations in urine account for this inhibition, the effect of eight urine samples and eight matched electrolyte solutions on the activity of added kallikrein was determined.

Eight samples of normal human urine (ranging in osmolality from 97 to 1943 mosm/kg) inhibited amidase activity by 41 ± 7% and esterase activity by 16 ± 5%. Eight electrolyte solutions were prepared which matched the eight urine samples in urea, sodium, potassium, calcium, and magnesium content. The accompanying anion was chloride. These solutions produced the same degree of inhibition of amidase (40 ± 7%) and esterase activity (18 ± 4%) of added kallikrein as the urine samples (Fig. 2).

**TABLE I**

<table>
<thead>
<tr>
<th>Cation</th>
<th>Anion</th>
<th>Inhibition (%)</th>
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</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Chloride</td>
<td>52</td>
</tr>
<tr>
<td>Potassium</td>
<td>Chloride</td>
<td>50</td>
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<tr>
<td>Ammonium</td>
<td>Chloride</td>
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<tr>
<td>Calcium</td>
<td>Chloride</td>
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<tr>
<td>Magnesium</td>
<td>Chloride</td>
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<tr>
<td>Phosphate</td>
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<td>Acetate</td>
<td>Chloride</td>
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<td>Chloride</td>
<td>Chloride</td>
<td>12</td>
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<tr>
<td>Tetrapropylammonium</td>
<td>Chloride</td>
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<tr>
<td>Tetraethylammonium</td>
<td>Chloride</td>
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</table>

* Concentration of cation (mM) in assay system that inhibits activity of kallikrein by 50%.

**TABLE II**

<table>
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<tr>
<th>Cation</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Sodium chloride</td>
<td>25</td>
<td>10 ± 0.9</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>25</td>
<td>10 ± 0.9</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
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<td>10 ± 0.9</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>30</td>
<td>10 ± 0.9</td>
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**AMIDASE ACTIVITY**

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<th>Matched Electrolyte Solutions</th>
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**ESTERASE ACTIVITY**

<table>
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<th>Urines</th>
<th>Matched Electrolyte Solutions</th>
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**FIG. 1**. Kininogenase activity of human urinary kallikrein as a function of the log of increasing concentrations of cation added to the assay system (5 mM sodium phosphate buffer, pH 8.5). ○ sodium (as sodium chloride); □ sodium (as sodium sulfate); □ potassium (as potassium chloride); △ magnesium (as magnesium chloride).

FIG. 2. Comparison of the inhibitory effect of eight normal human urine samples with that of eight electrolyte solutions of equivalent cationic composition on the amidase and esterase activities of human urinary kallikrein.
Effect of Desalting with Sephadex G-25 on Kininogenase Activity and Immunoactive Kallikrein in Normal Human Urine—Kininogenase activity and immunoactive (total) kallikrein was measured in eight urine samples before and after desalting with Sephadex G-25. (In the kininogenase assay, the volume of urine is so small (see “Experimental Procedures”) that variations in urinary electrolyte concentration do not alter the ionic composition of the reaction mixture significantly. Therefore, the loss of ions from the urine samples during the desalting procedure per se does not affect kininogenase activity.) In all urine samples, desalting results in loss of both active and total kallikrein. There was a 22 ± 4% loss of kininogenase activity and a 31 ± 3% loss of immunoactive kallikrein. The per cent loss of kallikrein varied widely among individual urine specimens, ranging from 15 to 40%.

Discussion

We have demonstrated, using homogeneous enzyme and substrate, that the kininogenase activity of human urinary kallikrein is critically dependent upon the presence of cations in the incubation mixture (Fig. 1). Renal kallikrein has been localized to the tubular cells of the distal nephron by immunofluorescent studies (11). Furthermore, stop-flow studies suggest that kallikrein is secreted into tubular fluid along the distal nephron and that kinins are produced within distal tubular fluid (3). The ionic composition of distal tubular fluid varies widely depending on whether the kidney is undergoing diuresis, antidiuresis, natriuresis, or antinatriuresis. Thus, the dependence of kininogenase activity upon the concentration of ions that we have demonstrated in vitro may have considerable physiologic relevance to the activity of kallikrein within distal tubular fluid in vivo.

Recent studies have reported a lack of correlation between urinary kallikrein and kinin excretion (12). Discrepancies between the availability of enzyme and the amount of product (kinins) formed might in certain circumstances be due to alterations in the ionic environment of distal tubular fluid. For example, during dietary sodium deprivation, urinary kallikrein excretion increases substantially but urinary kinin excretion does not change (12). This observation could be explained by the fall in sodium concentration that occurs in distal tubular fluid during sodium restriction. Our data would predict that the fall in sodium concentration would inhibit kinin generation. Thus, kininogenase activity of urine measured under standard conditions in vitro may not reflect the activity of kallikrein at its presumed site of action within the distal nephron. However, further in vitro studies are necessary in order to determine whether ion concentration in distal tubular fluid in fact influences the activity of kallikrein in various physiologic states.

In contrast to their ability to activate kininogenase activity of kallikrein, univalent and divalent cations inhibit the amidase and esterase activity of the enzyme. In physiological and clinical studies, the enzymatic activity of kallikrein in urine has been most often measured by such methods. Information about the effect of univalent ions and divalent cations such as calcium and magnesium on glandular kallikrein is contradictory. Worthington and Cuschieri (13) found that sodium chloride decreased esterase activity of pig pancreatic kallikrein, while Takami (14) and Fiedler and Werle (15) could find no inhibitory effect of univalent cations on esterase activity of pig pancreatic kallikrein. Our studies, the first using human urinary kallikrein, indicate that the cations found normally in urine inhibit amidase and esterase activity.

Previous investigations have provided evidence that human urine can inhibit both the esterase and amidase activity of urinary kallikrein (5, 6). The factors responsible for this inhibition have not been determined. Zschiedrich et al. (6) have postulated that this inhibitory effect may be due to a specific inhibitor similar to that described in rat renal tubules by Geiger and Mann (16). However, our data indicate that a specific inhibitor need not be invoked to account for the inhibitory effect of urine on kallikrein activity measured with synthetic substrates. This effect appears to be due entirely to the inhibitory action of urinary cation and urea (Fig. 2).

In most studies in which kallikrein activity of urine has been measured using artificial substrates, the inhibitory effect of urine has been eliminated by desalting urine with Sephadex G-25 prior to assay (5, 12). We have found that this maneuver results in a substantial and variable loss of both active and total kallikrein. The interference with the measurement of kallikrein activity by urinary salts is best overcome by using a highly sensitive assay in which the urine sample can be diluted so greatly in the assay buffer that the ionic composition of the reaction mixture is not materially altered by any conceivable variation in urinary composition. For this reason, we measure urinary kininogenase activity by diluting untreated urine 100- to 300-fold in the reaction mixture.

In summary, the ability of urinary kallikrein to cleave the artificial substrates tested is markedly inhibited by cations. This effect of cations appears to account for the well described inhibitory effect of human urine on the esterase and amidase activity of kallikrein. In contrast, the kininogenase activity of human urinary kallikrein is critically dependent upon the presence of cations in vitro. This finding may have important physiologic implications, since kallikrein is thought to act within distal tubular fluid where ionic composition varies widely.

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References

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