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 vivo. 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.

Kininogenase Activity

Kininogenase activity was determined by incubation of kallikrein or urine with kininogen and measurement of the liberated kinins 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 μg 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 3 μg 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 μg 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 antisera. If any sample consumed more than 15% 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.

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††The abbreviation used is RIA, radioimmunoassay.
Effect of Cations on the Activity of Human Urinary Kallikrein

The amidase activity of untreated urine (0.04 ml) was determined with and without the addition of 2 ng of purified kallikrein, and the activity of purified kallikrein was determined in the absence of added urine. The percent recovery of added kallikrein activity in urine was determined as follows:

\[
\text{Activity of urine and purified kallikrein} = \frac{\text{activity of urine} - \text{activity of purified kallikrein}}{\text{activity of purified kallikrein}} \times 100
\]

Esterase Assay

Esterase activity was determined using a standard radiochemical technique (5) with N\(^{-}\)\(\text{N}^{\prime}\)(p-tosyl)-L-arginine \([\text{H}]\)methyl ester as substrate. The incubation mixture contained 0.025 ml of tosylarginine \([\text{H}]\)methyl ester, 0.04 ml of 0.1 M Tri-HCl buffer, pH 8.5, 0.01 ml of pure human urinary kallikrein (3.5 \(\times\) 10\(^{-3}\) esterase units) diluted in buffer, and 0.025 ml of water (for 100% activity) or appropriate concentrations of salt solutions.

The inhibitory effect of urine on esterase activity was determined by incubating 0.025 ml of untreated urine, with the same proportions of buffer and substrate, with and without the addition of purified kallikrein. The recovery of activity of the added kallikrein was calculated for each urine sample as described above.

Immunoreactive (Total) Kallikrein

The radioimmunoassay procedure for human urinary kallikrein has been previously described in detail (9). This assay measures total (active + inactive) kallikrein.

Urine Samples

Eight urine samples were obtained from normal laboratory personnel and were stored frozen at -70 °C. Five samples were obtained in random fashion while three were obtained after the subjects had ingested approximately 200 ml of water in order to obtain dilute specimens.

Desalting of Urine

Urines 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 urines 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 (I\(_50\)) was determined by interpolation from linear regressions obtained by relating per cent inhibition (20 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 Cations 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 0.3 ± 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
Effect of Cations on the Activity of Human Urinary Kallikrein

The effect of ions on the amidase activity of human urinary kallikrein is markedly inhibited by the presence of cations in the assay system. The effect is unrelated to ionic strength since magnesium chloride results in greater activation than sodium chloride (p < 0.01) and sodium chloride is more potent than sodium sulfate (p < 0.01) at equivalent ionic strength. Also, the nature of the anion appears to be unimportant since sodium is equally effective as an activator with either sulfate or chloride as the accompanying anion (Fig. 1).

The Effect of Cations on the Amidase Activity of Kallikrein—
In contrast to the effect of cations on kininogenase activity, the amidase activity of human urinary kallikrein is markedly inhibited by the presence of cations in the assay system. In Table I, the molar concentration (mM) required for 50% inhibition ($I_50$) of amidase activity of purified kallikrein is given for a number of inorganic and organic cations. Sodium, potassium, ammonium, magnesium, and calcium represent the major cations found normally in human urine. All these cations markedly inhibit the amidase activity of kallikrein at concentrations in which they are found in urine. Divalent cations (calcium and magnesium) are approximately 20-fold and 10-fold more potent as inhibitors than the univalent cations. The inhibitory effect of the organic cations choline ($M_i = 105$), tetraethylammonium ($M_i = 130$), and tetrapropylammonium ($M_i = 187$) appears to be directly related to the size of the cation. Thus, both charge and size determines the degree to which cations inhibit amidase activity.

The inhibitory effect of ions is clearly unrelated to an ionic strength effect. Sodium chloride, calcium chloride, and tetrapropylammonium chloride result in 50% inhibition of amidase activity at ionic strengths of 52, 6.6, and 0.2, respectively. Also, as shown in Table I, the nature of the anion does not influence the inhibitory effect of sodium or calcium.

The effect of urea on amidase activity was also tested since urea is present in urine in concentrations of up to 500 to 600 mM. Urea did not inhibit amidase activity as concentrations in the assay system of less than 100 mM. 150 mM urea inhibited amidase activity 16 ± 2% and 250 mM urea 23 ± 5%. Therefore, urea may contribute to some extent to the inhibitory effect of urine on amidase activity.

Effect of Ions on the Esterase Activity of Kallikrein—
The effect of ions on the esterase activity of kallikrein is shown in Table II. 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>$I_50$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Chloride</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td>51</td>
</tr>
<tr>
<td>Potassium</td>
<td>Chloride</td>
<td>47</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>Chloride</td>
<td>2.2</td>
</tr>
<tr>
<td>Choline</td>
<td>Chloride</td>
<td>6.6</td>
</tr>
<tr>
<td>Tetraethylammonium</td>
<td>Chloride</td>
<td>2.4</td>
</tr>
<tr>
<td>Tetrapropylammonium</td>
<td>Chloride</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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

**Table II**

<table>
<thead>
<tr>
<th>Concentration in assay system</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride 25</td>
<td>9 ± 09</td>
</tr>
<tr>
<td>Potassium chloride 25</td>
<td>33 ± 19</td>
</tr>
<tr>
<td>Magnesium sulfate 25</td>
<td>27 ± 22</td>
</tr>
<tr>
<td>Calcium chloride 30</td>
<td>29 ± 40</td>
</tr>
<tr>
<td>Tetraethylammonium 25</td>
<td>47 ± 60</td>
</tr>
</tbody>
</table>

**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.

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*The text continues with further discussion and tables.*
Effect of Desalting with Sephadex G-25 on Kininogenase Activity and Immunoreactive Kallikrein in Normal Human Urine—Kininogenase activity and immunoreactive (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 immunoreactive 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 a diuresis, anti-diuresis, 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 vivo 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 amide and esterase activity. 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 amidease 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 200-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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