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The effect of calcium and magnesium ions on urinary urokinase and sialidase activity

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Summary. The effect of a promoter (calcium) and an inhibitor (magnesium) of urolithiasis was spectrophotometrically studied on urokinase (0.45 IU) and sialidase (5 mM). Although these mineral did not affect the sialidase activity, total inhibition of urokinase activity was observed with either 0.05 M calcium chloride or 0.1 M magnesium chloride. This observation might explain why calcium and magnesium respectively function as a promoter and an inhibitor of stone formation.

Key words: Calcium – Kidney stones – Magnesium – Plasmin – Sialidase – Urokinase

The process of kidney stone formation is not clear. At present, three theories exist to explain this process, namely the theory on lack of inhibitors, the precipitation theory and the matrix theory. According to the matrix theory both the urinary protein and calcium concentrations would be of utmost importance in stone formation, because it is reckoned that a protein, such as uromucoid, possibly activates the initial crystallisation process by binding calcium [4]. Analogous to this theory, increases in uromucoid [7, 12, 13] and calcium [5, 15] excretion have been observed with stone subjects.

In contrast to the action of calcium, magnesium which is also a divalent cation, forms soluble complexes with calcium and oxalate and consequently reduces the ionic activity product [9, 10]. Calcium and magnesium are therefore classified respectively as promoters and inhibitors of stone formation [5].

It has recently been demonstrated that the urinary enzymes, urokinase and sialidase, may be important factors in urolithiasis. This conclusion was based on the fact that renal stone patients have lower urinary urokinase [1, 2] and higher sialidase activities [3]. Little is known about this phenomenon. The lower urinary urokinase activity may be attributed to increased urate [1] and trypsin [16] inhibition of urokinase or to a lower urinary testosterone concentration [2]. However, the question has arisen as the whether calcium and magnesium would affect the activity of urokinase and sialidase. The present study was therefore undertaken to investigate the in vitro effects of both the promoter formation and the inhibitor (magnesium) of renal stones (calcium) on urokinase and sialidase activity.

Materials and methods

Reagents and chemicals

All reagents were "Analar" grade. Merck (Darmstadt, FRG) and BDH (Poole, UK) supplied the reagents sodium phosphate, sodium azide. Triton X-100, calcium chloride, magnesium chloride, tris(hydroxymethyl)aminomethane (TRIS), dimethyl sulphoxide (DMSO) and polyethyleneglycol. The substrates plasminogen (human plasma), D-valyl-L-leucyl-L-lysine *p*-nitroanilide, benzoyl-L-valyl-glycyl-L-arginine *p*-nitroanilide (Chromozym, UK), L-pyroglutamyl-glycyl-L-arginine *p*-nitroanilide (S-2444), *N*-Cbz-glycyl-glycyl-arginine 7-amido-4-methylcoumarin (Cbz-Gly-Gly-Arg-AMC) as well as plasmin (human plasma), 7-amino-4-methylcoumarin (AMC), low molecular weight human kidney urokinase (EC 3.4.21.31), antipain, aprotinin and *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) were obtained from Sigma (St. Louis, USA). Boehringer (Mannheim, FRG) supplied nicotinamide adenine dinucleotide, reduced (NADH), rabbit muscle lactate dehydrogenase (EC 1.1.1.27) in ammonium sulphate solution. *N*-acetylneuraminic acid aldolase (EC 4.1.3.3) from *Escherichia coli* neuraminidase (sialidase, EC 3.2.1.18) from *Clostridium perfringens*, bovine colostrum *N*-acetylneuraminosyl-D-lactose (sialyllactose) and *N*-acetylneuraminic acid. Bovine fibrinogen, enriched with plasminogen, was obtained from Organon Teknika b.v., Boxtel, The Netherlands. The bovine thrombin (5000 NIHU) was a Parke-Davis (Morris Plains, N.J., USA) product.

Determination of urokinase/plasmin activity

Two methods were used to determine the urokinase/plasmin activity, namely the spectrophotometric and the fibrin plate method.

Spectrophotometric method. A standard activity curve of the coupled urokinase/plasmin system in the presence and absence of ethylenediaminetetraacetic acid (EDTA) was obtained by incubating different amounts of urokinase in a stirred waterbath for 90 min at 37°C [1].

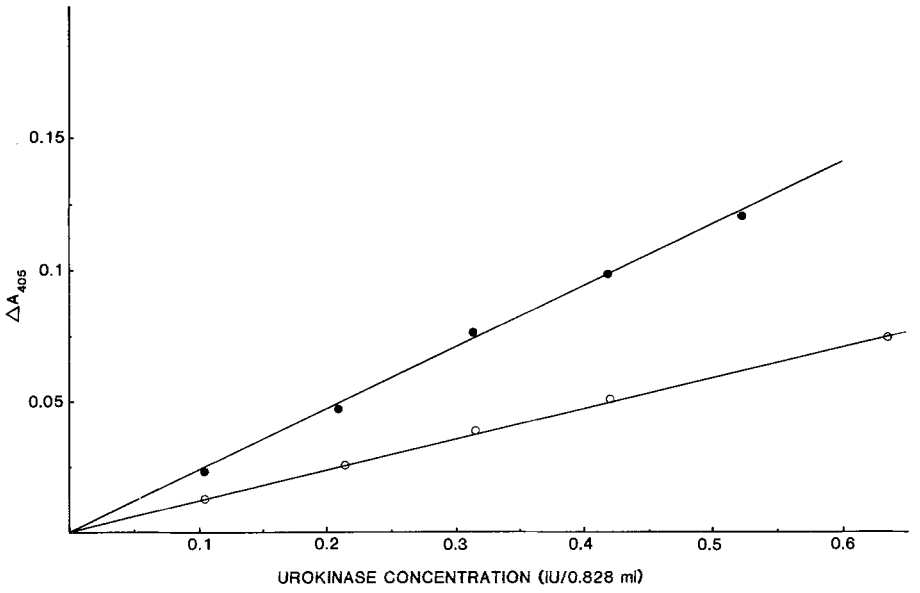

Fig. 1. Standard curve of urokinase activity in the presence (●) and absence (○) of EDTA. The incubation time was 90 min and was carried out in a stirred waterbath at 37°C. Each point represents the mean of two determinations. ΔA_{405}, difference in absorption at 405 nm between the blank and controls

This assay was done according to a modified method of Wiman et al. [18]. Activator reagent (400 µl) was added to 323 µl of 0.1 M sodium phosphate buffer, pH 7.3, containing 0.1 g/l sodium azide and 0.1 g/l Triton X-100. The activator reagent consisted of 1 µM plasminogen and 0.6 mM D-valyl-L-leucyl-L-lysine *p*-nitroanalide, which was dissolved in 0.1 M sodium phosphate buffer. The blank consisted of the activator reagent and buffer. The total volume for each was brought up to 828 µl with distilled water. After the desired incubation time, the reaction was stopped instantly by inserting the glass tubes in ice and adding 0.1 ml of 50% acetic acid solution to all the tubes [11]. Enzyme activity was estimated from the product concentration recorded at 405 nm, on a Hitachi (Tokyo, Japan) 150-20 spectrophotometer connected to a data processor. The molar absorption coefficient for *p*-nitrophenol was taken as 9620 mol l^{-1} cm^{-1} [17]. The urokinase activity was expressed in international units (IU) where 1 IU equals 0.5 nmol [6].

Since our studies were aimed at determining the effect of calcium and magnesium on urokinase, different concentrations of these minerals in 100-µl aliquots were added to different glass tubes and assayed as described for the standard curve of the urokinase/plasmin system. The same amount of urokinase was added to all the tubes (average, 0.45 IU). The blank rate value was substracted from the rate value obtained in the presence of the enzyme to obtain the real value. This experiment was repeated at least three times in duplicate

Fibrin plate methods. The fibrinolytic activity of 0.4 IU urokinase was determined using plasminogen-rich bovine fibrinogen (0.1%, m/v) dissolved in 0.05 M TES, pH 7.5/5% (v/v) DMSO [19]. This was coagulated with bovine thrombin in Nunc (Roskilde, Denmark) tissue culture dishes (60 × 15 mm) containing different concentrations of either calcium or magnesium. After incubation for 17 h at 37°C in a moist zone, the product of two diameters at right angles was used to determine the lysed circular areas. All determinations were performed in triplicate.

Determination of plasmin activity

The plasmin activity determinations was done according to the spectrophotometric assay method described by Cs-Szabo et al. [8], in TRIS buffer, pH 7.3, at 37°C, in the Hitachi 150-20 spectrophotometer. Once again D-valyl-L-leucyl-L-lysine *p*-nitrophenol (130.5 µM) was used as substrate. Plastic cuvettes were used for the measurement of the production of *p*-nitrophenol, which was repeated at least twice.

Urokinase activity with synthetic substrates

Inhibition studies on urokinase were carried out with two synthetic tripeptidyl *p*-nitroanalide substrates. As described under "Determination of urokinase/plasmin activity", 100 µl of 1.0 mM Chromozym UK or S-2444 substrate was added to 628 µl phosphate buffer (0.1 M). This buffer also contained 0.1% (w/v) polyethylene glycol [14]. The blanks were composed of buffer and substrate, while 0.342 and 0.56 IU were added to the Chromozym UK and S-2444 controls, respectively. The samples contained different concentrations of calcium or magnesium. The total volume was 828 µl. Enzyme activity was estimated from the product concentration recorded at 405 nm and at 37°C in a Hitachi 150-20 spectrophotometer connected to a data processor. The rate assay of urokinase was also conducted with the synthetic fluorogenic peptide substrate. Cbz-Gly-Gly-Arg-AMC [19]. Briefly, the control reaction medium (900 µl) consisted of about 0.002 IU urokinase in the presence of 0.05 mM substrate in 0.05 M TES, pH 7.5/5% (v/v) DMSO. The urokinase powder was dissolved in TES buffer. The blank consisted of buffer with substrate, while the samples contained different concentrations of the minerals (100 µl), which were dissolved in distilled water. After the desired incubation time, namely 90 min at 37°C, the enzyme activity was measured on a Hitachi 650-10S fluorescence spectrophotometer at activation and emission wavelengths of 383 nm and 455 nm, respectively. The instrument was standardised with AMC. The effect of calcium and magnesium on the activity of urokinase was then expressed as a percentage of the control.

Determination of sialidase activity

The working suggestion for the determination of sialidase activity was supplied by Boehringer Mannheim [3]. This assay consists in principle of three enzymatic reactions, namely sialidase (5×10^{-3} U), *N*-acetylneuraminic acid aldolase (± 0.4 U) and lactate dehydrogenase (0.5 mg). In determining the action mechanism, this assay system was divided into two complexes. Complex 1 consisted of all the enzymes, while complex 2 consisted of only *N*-acetylneuraminic acid aldolase and lactate dehydrogenase. Sialyllactose (0.15 mM) and sialic acid (0.15 mM) served as the substrates in complex 1 and 2, respectively. Activity assays were repeated three times.

Table 1. Effect of minerals on lysis of fibrin by 0.4 IU urotinase

Concentration of calcium or magnesium (M)	Lysis of fibrin (% control)	
	Calcium (%)	Magnesium (%)
0	100	100
0.001	96 (± 5)	Liquefied
0.01	90 (± 7)	Liquefied
0.1	78 (± 7)	70 (± 17)

Data are means of three values (± SD)

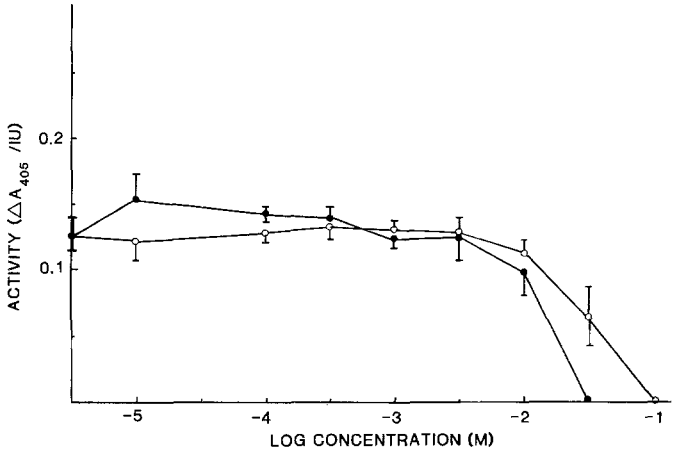

Fig. 2. The effect of calcium (●) or magnesium (○) on the urokinase/plasmin system in the absence of EDTA. Each *point* represents the mean of three experiments carried out in duplicate. About 0.45 IU of urokinase was used

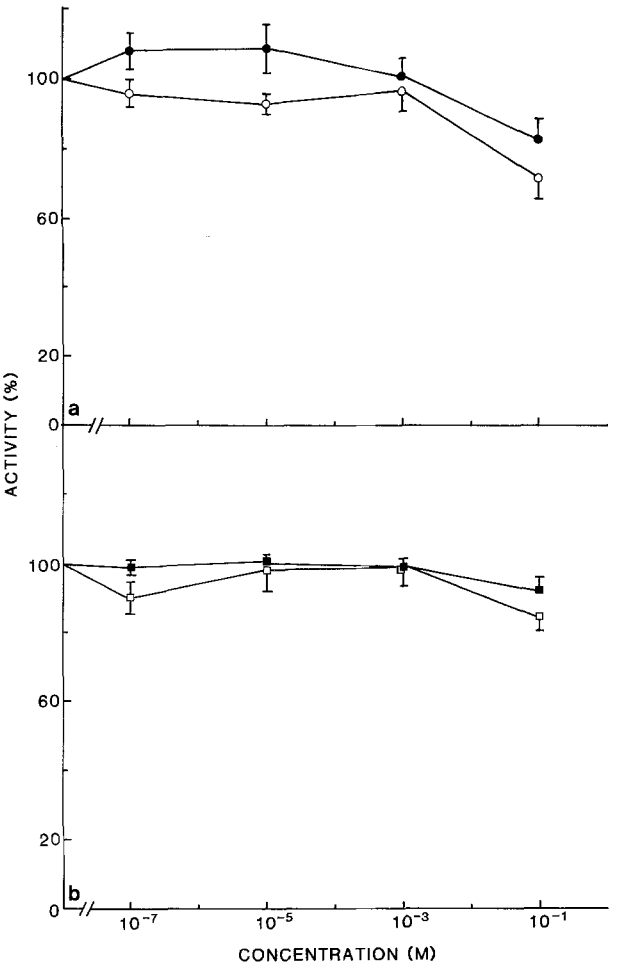

Fig. 3a, b. The effect of calcium and magnesium on the sialidase activity. **a** Calcium: complex 1 (●), complex 2 (○); **b** Magnesium: complex 1 (■), complex 2 (□). The complexes are described in Materials and methods

Results

To determine the linearity of the coupled urokinase system in the presence and absence of EDTA, it was necessary to establish its kinetic patterns. Different urokinase concentrations were used (Fig. 1). Inhibition of this system had already been established using 1 μM antipain, which reflected its specificity for urokinase.

The effects of the different minerals on urokinase were studied with the fibrin plate (Table 1) and spectrophotometric assays (Fig. 2). Although the fibrin plate method did not work as desired, significant results were obtained with the spectrophotometric study. Total inhibition of the urokinase/plasmin system was obtained with 0.05 M calcium. Since it was then necessary to determine which of the coupled enzymes was specifically involved in the inhibition, two consecutive inhibition studies were designed using plasmin and urokinase separately. Firstly, the influence of the minerals on plasmin was studied. No modification of the plasmin activity occurred with either 0.1 M calcium or magnesium. Contrastingly, the inhibitor aprotinin (0.064 TIU) completely inhibited the activity of the enzyme.

Due to the above-mentioned result, we examined the inhibition of urokinase. Three different synthetic substrates were used, namely Chromozym UK, S-2444 and Cbz-Gly-Gly-Arg-AMC. Unfortunatelly spontaneous hydrolysis of these substrates occurred in the presence of the minerals.

The activity of sialidase was studied next using different mineral concentrations. Both calcium (Fig. 3a) and magnesium (Fig. 3b) had little effect on either complex 1 or 2. The inhibition of complex 1 at 0.1 M calcium chloride could possibly be attributed to the inhibition of complex 2.

Discussion

This study was conducted to investigate the in vitro effects of a promoter (calcium) and an inhibitor (magnesium) of stone formation on urokinase and sialidase activity. Although sialidase activity was not affected by these minerals, a significant effect was observed with the urokinase/plasmin system. This inhibition could only be

attributed to the urokinase activity, because no effect was exercised by the minerals on the plasmin.

These results could contribute to a better understanding of urolithiasis if urokinase controls the uromucoid concentration which is referred to in the matrix theory. The difference between the inhibition of the urokinase activities at 0.05 M of the promoter (calcium) and the inhibitor (magnesium) was more than 50% (Fig. 2). This could possibly lead to an increased uromucoid concentration in urine containing calcium rather than magnesium. Considering this, together with the fact that magnesium reduces the ionic activity product by complex formation, it is expected that the chance for stone formation would be higher in urine with increased calcium concentrations. This agrees with published data, namely that the urine of stone formers contains increased uromucoid [7, 12, 13] and calcium [5, 15] concentrations, and decreased urokinase activity [1, 2]. It may therefore be understandable that calcium and magnesium are respectively referred to as promoter and inhibitor of renal stone formation.

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