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The Effect of Cations on the Amidase Activity of Human Tissue Kallikrein: 1-Linear Competitive Inhibition by Sodium, Potassium, Calcium and Magnesium. 2-Linear Mixed Inhibition by Aluminium*

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Hydrolysis of D-valyl-L-leucyl-L-arginine *p*-nitroanilide by human tissue kallikrein (hK1) was studied in the absence and in the presence of increasing concentrations of the following chloride salts: sodium, potassium, calcium, magnesium and aluminium. The data indicate that the inhibition of hK1 by sodium, potassium, calcium and magnesium is linear competitive and that divalent cations are more potent inhibitors of hK1 than univalent cations. However the inhibition of hK1 by aluminium cation is linear mixed, with the cation being able to bind to both the free enzyme and the ES complex. This cation was the best hK1 inhibitor. Aluminium is not a physiological cation, but is a known neurotoxicant for animals and humans. The neurotoxic actions of aluminium may relate to neuro-degenerative diseases.

Keywords: Human tissue kallikrein cation inhibition; Tissue kallikrein inhibition; Enzyme inhibition; Enzyme kinetics; Human kallikrein

INTRODUCTION

Serine proteases are proteolytic enzymes with an active serine residue in their catalytic site. The kallikreins (EC 3.4.21.8) are a subgroup of the serine protease family which is known to have diverse physiological functions.[1] The kallikreins are found in glandular cells, neutrophils and biological fluids. The kallikreins are divided into two main groups: plasma (EC 3.4.21.34) and tissue (EC 3.4.21.35) kallikreins. The two groups differ significantly in their molecular weight, isoelectric point, substrate specificity, immunological characteristics, gene structure and the type of kinin released.[2,3] A single gene (KLKB1) encodes for the plasma kallikrein or Fletcher factor, which is located on human chromosome 4q35.[3] Plasma kallikrein participates in surface-dependent activation of blood clotting, fibrinolysis, regulation of vascular tone, inflammation and also releases bradykinin from high molecular weight (HMW) kininogen.[3,4] Tissue kallikreins belong to a large multigene family that demonstrate considerable similarities at the gene and protein level as well as in the structure.[3] The human kallikrein gene family was thought to have only three members: the tissue (pancreatic-renal) kallikrein (KLK1, encoding for hK1), the human glandular kallikrein (KLK2, encoding for hK2), and prostatic specific antigen (KLK3, encoding for PSA or hK3). Recently, new kallikrein and kallikrein-like genes have been discovered.[4] The human kallikrein gene family has

*This paper is dedicated to the memory of Prof. Carlos Ribeiro Diniz and Prof. Marcos Luiz dos Mares–Guia, who died respectively on July 8 and August 23 2002.

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now been fully characterized and includes 15 members located in tandem on chromosome 19q13.4.[5] Recent data suggest that at least a few of these kallikrein genes are connected to malignancy.[6] Tissue kallikreins are expressed in many tissues including kidney, pancreas, salivary glands, prostate, breast, testis, uterus, and in the central nervous system.[2,3,7] Most tissue kallikreins are predicted to have trypsin-like enzymatic activity except three which are probably chymotrypsin-like.[5]

Human tissue kallikrein (hK1)[7] is related in many regards to trypsin, although with a higher specificity for the polypeptide substrate cleavage site.[8] Its principal known biological function is the highly selective cleavage of the plasma protein low molecular weight kininogen at two different peptide bonds (Met^{379}-Lys^{380} and Arg^{389}-Ser^{390}, using the prekininogen numbering) to release stoichiometrically the vasoactive and spasmogenic decapeptide kallidin (Lys-bradykinin).[8,9] Human tissue kallikrein (hK1), also hydrolyzes various synthetic substrates such as Nα-substituted arginine and lysine derivatives as esters, amides and fluorogenic peptides.[10] Like other serine proteases, hK1 is inhibited by diisopropylfluorophosphate, chloromethyl ketones of arginine and lysine, and bovine pancreatic trypsin inhibitor (BPTI) (also known as aprotinin, Trasylol® or Kunitz pancreatic trypsin inhibitor). On the other hand, soybean trypsin inhibitor, a strong inhibitor of trypsin, plasma kallikrein and other serine proteinases, does not inhibit hK1.[11]

Renal kallikrein is believed to release kinins in the distal nephron.[12] Evidence has accumulated 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.[13]

The interactions between kallikreins and monovalent and divalent cations are not clear. Thus, the monovalent cations of Tyrode's solution (Na^+ and K^+) were required for expression of the kininogenase activity of horse urinary kallikrein, but not for that of porcine pancreatic kallikrein (pK1).[14] Fiedler and Werle[15] showed that hydrolysis of *N*α-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) by pK1 was slightly stimulated by up to 0.05 M NaCl, although Takami[16] observed no such effect. On the other hand, Worthington and Cushieri[17] reported that 0.1 M NaCl decreased the Bz-Arg-OEt hydrolysis by pK1. Lieberthal *et al.*[13] studied the effects of cations on the amidase, esterase and kininogenase activities of hK1. The authors observed that the amidase and esterase activities on synthetic substrates (Pro-Phe-Arg-[^{3}H]benzylamide and *N*α-*p*-tosyl-L-arginine-[^{3}H]methyl ester) were reduced by monovalent (Na^+, K^+ and NH_4^+) and divalent (Ca^{2+} and Mg^{2+}) cations although the kininogenase activity, on bovine kininogen, was stimulated. Chao *et al.*[18] found that the enzymatic activities (*N*α-*p*-tosyl-L-arginine methyl ester esterase activity and kininogenase activity) and the binding to specific antibodies (immunoreactivities) of rat and human urinary kallikreins were inhibited by monovalent cations (NaCl, KCl, LiCl, choline chloride, NaH_2PO_4 and KH_2PO_4). The degree of inhibition, which exceeded 80% with physiological concentrations of cations, was dependent on temperature and pre-incubation time. The type of the inhibition and its kinetic constants were not described by these authors.

One of the purposes of the present study is to examine in depth the kinetics of the inhibition of hK1 amidase activity by the cations Na^+, K^+, Ca^{2+} and Mg^{2+} in order to identify the precise mechanism of inhibition and to determine their accurate inhibition constants (K_i).

Aluminium is not a physiological cation, but a cation known to be neurotoxic to animals and to man.[19] According to Zatta *et al.*[20] the inhibitory effects of Al(III) on the activity of many enzymatic systems have been well documented even if the mechanism(s) are yet to be fully understood. The authors investigated the interference of Al(III) with Tos-Arg-OMe esterase activity of trypsin and with Bz-Arg-OEt esterase activity of α-chymotrypsin, respectively. The authors reported that their results demonstrate that Al(III) inhibits *in vitro* the protease activity of trypsin and α-chymotrypsin with different kinetic mechanism. On the other hand, Dörnyei *et al.*[21] reported that their results concerning the effects of Al(III) on the Bz-Arg-OEt esterase activity of trypsin strongly suggest that there is no direct involvement of Al(III) in the enzymatic reactions of the enzyme. Similar results were found by Krejpcio *et al.*[22] who found that Al(III) did not significantly affect the proteolytic activity of trypsin. Thus, according to Dörnyei *et al.*[21] the results published so far on the influence of Al(III) on trypsin activity are contradictory.

On the other hand, since it is already well established that the brain is capable of expressing hK1 and other tissue kallikreins,[3,23] another purpose of the present study is to check the effects of Al^{3+} on the amidase activity of the serine protease hK1 and also identify the mechanism of inhibition and to determine its accurate inhibition constant (K_i).

In the present study, we report the competitive inhibition of hK1 by the cations Na^+, K^+, Ca^{2+} and Mg^{2+}, the major cations normally found in human urine, and already known to inhibit kallikreins at their physiological concentrations.[13] Additionally, the present study also describes the mixed inhibition of hK1 by Al^{3+}, which is a cation known to be toxic, especially for the brain.

MATERIALS AND METHODS

Chemicals

D-Val-Leu-Arg-pNA was purchased from Chromogenix A B (Italy). Sodium chloride, potassium chloride, calcium chloride, aluminium chloride, glycine, sodium hydroxyde, and bovine pancreatic trypsin inhibitor (BPTI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Enzyme

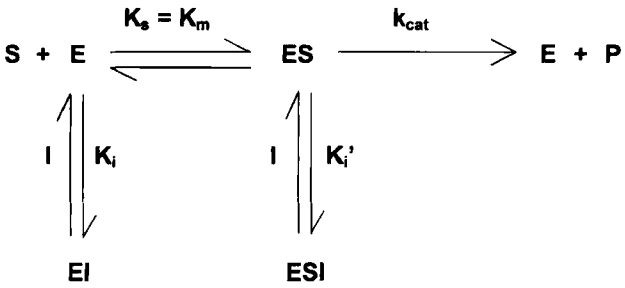
Homogeneous hK1 was purified in our laboratory from healthy male urine. In the enzymatic assays BPTI-titrated hK1 was used.[24]

Kallikrein Amidase Activity

Hydrolysis of D-Val-Leu-Arg-*p*NA was carried out spectrophotometrically at 410 nm, which monitors the release of *p*-nitroaniline (pNA) ($\varepsilon_{410} = 8800\,M^{-1}\,cm^{-1}$).[11,25] A 1 cm pathlength cuvette containing 50 µl of 47.9 nM hK1 (M_r 31000)[24] in 20 mM glycine-NaOH buffer, pH 9.0 and 100 µl of buffer or 100 µl of an adequate dilution of a stock solution (485 mM NaCl, 479 mM KCl, 174 mM $CaCl_2$, 186 mM $MgCl_2$ or 66 mM $AlCl_3$) in the same buffer, was placed in the thermostated cell holder compartment of a Shimadzu UV 160 A UV-Vis recording spectrophotometer (spectral band width 2 nm) at 37°C and pre-incubated for 5 min for temperature equilibration. The buffer and the cation solution were prepared with deionized water produced by a Milli-Q Water System (Millipore Corporation, Bedford, USA). The concentrations of Na^+ and K^+ were determined by Flame Spectrophotometry (Corning 400 Flame Photometer); Ca^{2+} was determined by the colorimetric method of *o*-cresolphthalein-complexone (Katal Biotecnológica Indústria e Comércio Ltda, Belo Horizonte, MG, Brasil); Mg^{2+} was determined by a colorimetric method with Xylidyl Blue II (Katal) and Al^{3+} by chloride determination by a spectrophotometric method with mercuric thiocyanate (Doles Reagentes e Equipamentos para Laboratórios Ltda, Goiânia, GO, Brasil). Then, 350 µl (7.1–42.9 µM) of D-Val-Leu-Arg-*p*NA in 20 mM glycine–NaOH buffer, pH 9.0 were added, and the increase of absorbance at 410 nm with time was continuously recorded for 3 min. The mixtures were incubated in quadruplicate. The slope of the time-dependent absorbance curve extrapolated to zero time was converted into µM min^{-1} of released *p*NA. The control contained 50 µl of the assay buffer instead of hK1. Total substrate concentration was determined from the amount of *p*NA released after complete hydrolysis by excess of bovine β-trypsin (b-β-TR).[24]

Treatment of Kinetic Data

The kinetic data for the inhibition of hK1 by sodium, potassium, calcium and magnesium cations can be described according to Cornish–Bowden[26] as competitive inhibition. On the other hand, the kinetic data for the inhibition of hK1 by aluminium cation can be described by the following scheme



which, according to Cornish–Bowden,[26] is the simplest formal mechanism for mixed inhibition where the inhibitor binds both to the free enzyme E to give a complex EI with dissociation constant K_i, and to the ES complex to give an unreactive ESI complex with dissociation constant K_i'.

The normalized initial rate v will be given by the following equation

$$\frac{v_i}{[E_o]} = v = \frac{k_{cat}\cdot[S]}{K_m\cdot(1+[I]/Ki) + [S]\cdot(1+[I]/Ki')} \quad (1)$$

According to Cornish–Bowden,[26] when linear mixed inhibition occurs, both k_{cat}^{app} and k_{cat}^{app}/K_m^{app} vary linearly with the inhibitor concentration.

Statistical Analysis of the Data

The kinetic parameters (K_m, K_m^{app}, k_{cat} and k_{cat}^{app}) in the absence and in the presence of sodium, potassium, calcium and magnesium cations were calculated based on unweighted non-linear regression analysis of the data fit to the appropriate Michaelis–Menten equation. The K_i values for sodium, potassium, calcium, and magnesium cations were determined by linear regression of the replots of K_m^{app} and k_{cat}^{app} vs [I]. The K_i value and the K_i' value for aluminium cation were determined by linear regression of the replots of K_m^{app}/k_{cat}^{app} *vs* [I] and of $1/k_{cat}^{app}$ *vs* [I], respectively.

RESULTS

The hK1-catalyzed hydrolysis of D-Val-Leu-Arg-*p*NA followed Michaelis–Menten kinetics for the assayed substrate concentration range (5–30 µM). The 1/v *vs* 1/[S] plot for the hydrolysis of D-Val-Leu-Arg-*p*NA (5–30 µM) catalyzed by hK1 (4.79 nM) in the absence and presence of NaCl (6.1–48.5 mM) is shown in Figure 1(A). The replot of the parameters

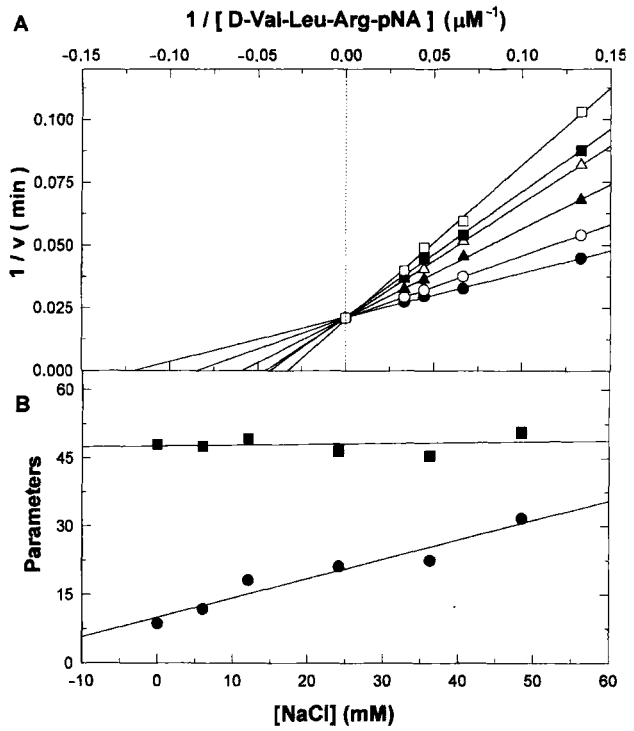


FIGURE 1 (A) The effect of NaCl on the amidase activity of hK1. Lineweaver-Burk plot for the hydrolysis of D-Val-Leu-Arg-*p*NA by hK1 in the absence (control) and in the presence of NaCl. Experimental conditions: 20 mM glycine-NaOH, pH 9.0 and 37°C, 3 min incubation. hK1 concentration: 4.79 nM. NaCl concentrations: closed circles, 0 mM; open circles, 6.1 mM; closed triangles, 12.2 mM; open triangles, 24.4 mM; closed squares, 36.6 mM, and open squares, 48.5 mM. Each point in the plot is the mean of quadruplicate determinations. (B) Replot of the kinetic parameters for the hydrolysis of D-Val-Leu-Arg-pNA by hK1 in the absence (control) and in the presence of NaCl. Symbols: closed squares: k_{cat}^{app}; closed circles: K_m^{app}. More details are described in MATERIALS AND METHODS.

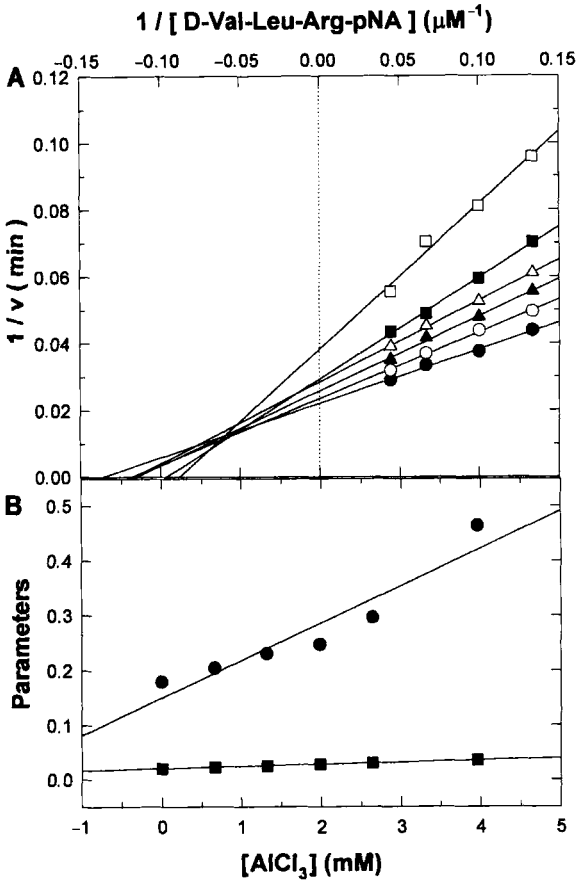


FIGURE 2 (A) The effect of $AlCl_3$ on the amidase activity of hK1. Lineweaver-Burk plot for the hydrolysis of D-Val-Leu-Arg-*p*NA by hK1 in the absence (control) and in the presence of $AlCl_3$. Experimental conditions: 20 mM glycine-NaOH, pH 9.0 and 37°C, 3 min incubation. hK1 concentration: 4.79 nM. $AlCl_3$ concentrations: closed circles, 0 mM; open circles, 0.7 mM; closed triangles, 1.3 mM; open triangles, 2.0 mM; closed squares, 2.6 mM, and open squares, 4.0 mM. Each point in the plot is the mean of quadruplicate determinations. (B) Replot of the kinetic parameters for the hydrolysis of D-Val-Leu-Arg-*p*NA by hK1 in the absence (control) and in the presence of $AlCl_3$. Symbols: closed circles: K_m^{app}/k_{cat}^{app}; closed squares: $1/k_{cat}^{app}$. More details are described in MATERIALS AND METHODS.

(k_{cat}^{app} and K_m^{app}) *vs* NaCl concentration is shown in Figure 1(B). Each point is the mean of 4 determinations. Statistical analysis of the data using the Graph Pad program at the 95% confidence level showed that the line for K_m^{app} has a slope that is significantly different from zero, whereas the line for k_{cat}^{app} has not. The statistical test for departure from linearity gave negative (non-significant) results for both lines. The straight lines obtained are consistent with linear competitive inhibition. Similar results were obtained with KCl, $CaCl_2$ and $MgCl_2$, respectively (data not shown). The 1/v *vs* 1/[S] plot for the hydrolysis of D-Val-Leu-Arg-*p*NA (5–30 μM) catalyzed by hK1 (4.79 nM) in the absence and in the presence of $AlCl_3$ (0.7–4.0 mM) is shown in Figure 2(A). The replots of the parameters ($1/k_{cat}^{app}$ and K_m^{app}/k_{cat}^{app}) *vs* $AlCl_3$ concentration is shown in Figure 2(B). Each point is the mean of 4 determinations. Statistical analysis of the data using the Graph Pad program at the 95% confidence level showed that the lines of (B) have slopes that are significantly different from zero, and the statistical test for departure from linearity gave negative (non-significant) results. The kinetic parameters for hK1-catalyzed hydrolysis of D-Val-Leu-Arg-pNA in the absence and in the presence of NaCl, KCl, $CaCl_2$, $MgCl_2$ and $AlCl_3$ are shown in Table I. The ionic radius,[27] ionic volume, charge density, and K_i (dissociation constant) for hK1 inhibition by cations are shown in Table II.

DISCUSSION

The identity of our preparation of hK1 with the human urinary kallikrein described by Geiger and Fritz[11] was indicated by the following concordances: molecular weight estimated by gel filtration and BPTI titration; isoelectric point; inhibition by diisopropylfluorophosphate, BPTI and benzamidine;

TABLE I Kinetic parameters for hK1

Experimental conditions	Inihibitor (mM)	K_m^{app} ± SE (μM)	k_{cat}^{app} ± SE (min^{-1})	k_{cat}^{app}/K_m^{app} ± SE ($min^{-1}\mu M^{-1}$)	K_i ± SE (mM)	K_i' ± SE (mM)
Control	0.0	8.6 ± 0.6	48.0 ± 1.50	5.6 ± 0.2	–	–
KCl[a]	12.4	14.1 ± 0.7	52.5 ± 1.6	3.7 ± 0.1	67 ± 13	–
	24.8	16.3 ± 1.1	52.6 ± 2.1	3.2 ± 0.1		
	37.2	19.8 ± 0.6	56.3 ± 1.2	2.8 ± 0.1		
	49.6	21.4 ± 2.2	55.3 ± 3.8	2.6 ± 0.1		
	74.4	22.5 ± 2.2	51.7 ± 3.5	3.0 ± 0.1		
NaCl[a]	6.1	11.8 ± 0.1	47.6 ± 0.2	4.0 ± 0.1	22 ± 1	–
	12.1	18.1 ± 1.0	49.3 ± 1.3	2.7 ± 0.1		
	24.2	21.2 ± 1.6	47.0 ± 5.9	2.2 ± 0.1		
	36.3	22.5 ± 2.1	45.6 ± 2.2	2.0 ± 0.1		
	48.5	31.7 ± 5.3	50.9 ± 5.0	1.6 ± 0.1		
$CaCl_2$[a]	1.1	8.7 ± 1.0	45.6 ± 2.6	5.3 ± 0.5	9.8 ± 1.0	–
	2.2	10.3 ± 1.1	47.0 ± 2.5	4.6 ± 0.3		
	4.4	12.1 ± 0.4	49.0 ± 0.9	4.1 ± 0.1		
	6.6	10.1 ± 1.1	41.8 ± 2.3	4.1 ± 0.3		
	8.7	14.4 ± 2.3	47.4 ± 4.4	3.3 ± 0.4		
	10.9	17.1 ± 0.6	50.0 ± 1.1	2.9 ± 0.1		
$MgCl_2$[a]	1.4	11.7 ± 0.8	50.5 ± .1.8	4.3 ± 0.3	4.9 ± 0.4	–
	2.9	13.3 ± 1.9	49.5 ± 4.0	3.7 ± 0.6		
	4.3	13.3 ± 1.3	44.4 ± 2.5	3.4 ± 0.4		
	5.0	15.9 ± 1.1	43.0 ± 1.7	2.7 ± 0.2		
$AlCl_3$[a]	0.7	8.9 ± 0.5	43.4 ± 1.0	4.9 ± 0.3	2.0 ± 0.4	5.4 ± 0.4
	1.3	9.1 ± 0.9	39.6 ± 1.5	4.3 ± 0.5		
	2.0	8.7 ± 0.4	35.3 ± 0.6	4.1 ± 0.2		
	2.6	9.9 ± 0.6	33.3 ± 0.8	3.4 ± 0.2		
	4.0	12.9 ± 2.4	27.8 ± 2.4	2.2 ± 0.5		

[a] 20 mM glycine-NaOH, pH 9.0, 37°C; hK1concentration: 4.79 nM.

and by its lack of inhibition by soybean trypsin inhibitor. Homogeneity of hK1 was proven by the detection of a single N-terminal amino acid residue of isoleucine (Laboratório de Bioquímica e Química de Proteínas da Universidade Nacional de Brasília, DF, Brazil) as already described.[11]

Proceeding with the characterization of the hK1 preparation it was decided to check the inhibition of its amidase activity by salts was examined, bearing in mind that, due to the presence of the carboxylate of Asp^{189} in its active site[28] or specificity pocket (S_1),[29] the cation may interact with the active site. Consequently the kinetic effects of chloride salts of the following cations: Na^+, K^+, Ca^{2+}, Mg^{2+} and Al^{3+} were studied. Some of these ions are important and could inhibit kallikreins at their physiological concentrations.[13] Additionaly, Al^{3+} cation is known to be toxic, especially for the brain; the neurological manifestations of Al^{3+} accumulation in renal-impaired humans when Al^{3+} is present in dialysis fluids, or is given parenterally, are characterized as dialysis encephalopathy syndrome.[19]

hK1 Inhibition by NaCl, KCl, $CaCl_2$ and $MgCl_2$

The inhibition of human tissue kallikrein activity by cations has been previously described. Thus, Lieberthal *et al.*[13] studied the effect of ions on the ability of purified human urinary kallikrein (hK1) to cleave its natural substrate (kininogen) as well as two synthetic substrates, Nα-*p*-tosyl-L-arginine [^{3}H]methyl ester and Pro-Phe-Arg [^{3}H]benzylamide. The authors observed that the kininogenase activity of kallikrein is markedly dependent on the concentration of cations *in vitro*. According to the authors, kininogenase activity is very low when measured in a low electrolyte buffer. The addition of cations to the reaction mixture increases activity up to 27-fold and

TABLE II Ionic radius, ionic volume, charge density, and K_i values for hK1 inhibition by cations

Cation	Ionic radius (Å)[27]	Ionic volume (Å^3)	Charge density (charge/ionic volume)	K_i (mM)
K^+	1.33	9.86	0.10	67 ± 13
Na^+	0.95	3.59	0.28	22 ± 1
Ca^{2+}	0.99	4.06	0.49	9.8 ± 1.0
Mg^{2+}	0.65	1.15	1.74	4.9 ± 0.4
Al^{3+}	0.50	0.52	5.73	2.0 ± 0.4

maximum activity was achieved with 100 mM sodium, 100 mM potassium, or 20 mM magnesium. Furthermore, the authors evaluated the effect of cations (Na^+, K^+, NH_4^+, Mg^{2+}, and Ca^{2+}) on the amidase and the 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, univalent and divalent cations inhibit the amidase and to a lesser extent the esterase activity of the enzyme. The authors determined the concentration of cation in the assay system that inhibits amidase activity of human urinary kallikrein (hK1) by 50% (Na^+ 52 mM, K^+ 47 mM, NH_4^+ 41 mM, Mg^{2+} 5.0 mM and Ca^{2+} 2.2 mM, respectively). The authors also observed that the esterase activity of human urinary kallikrein (hK1) is inhibited by both univalent and divalent cations. However, at comparable concentrations of cations, the esterase activity of kallikrein is inhibited to a lesser extent than amidase activity. Chao *et al.*[18] studied the inhibitory effects of sodium and other monovalent cations on purified *vs* membrane-bound kallikrein. The authors found that the enzymatic activities (Tos-Arg-OMe esterase activity and kininogenase activity) and the binding of the enzyme to a specific antibody (immunoreactivity) of human urinary kallikrein are inhibited by monovalent cations (Na^+ and K^+). However, the type of inhibition and its kinetic constants were not described by these authors.

In the present study the double-reciprocal plot for the hydrolysis of D-Val-Leu-Arg-*p*NA (5–30 mM) by hK1 (4.79 nM) in the absence and in the presence of NaCl (6.1–48.5 mM) indicated that the enzyme is competitively inhibited by Na^+ (Figure 1A). Preliminary data obtained in our laboratory showed the same degree of inhibition by Na^+ when pH 9.0 glycine-NaOH buffers 200, 20 or 2 mM, respectively, were used.

According to Plowman,[30] competitive inhibition can be linear, hyperbolic or parabolic. Thus, in order to clarify the type of inhibition of hK1 by Na^+, it was decided to replot the values of k_{cat}^{app} and K_m^{app} *vs* Na^+ concentration according to Plowman[30] (Figure 1B). The results obtained showed linear lines for k_{cat}^{app} with a slope not significantly different from zero and for K_m^{app} with a slope significantly different from zero, indicating that Na^+ is a linear competitive inhibitor of hK1 amidase activity in the concentration range tested. This result indicates that Na^+ binds to the Asp^{189} residue of the S_1 pocket,[29] thus hindering the binding of the substrate at the same S_1 subsite. Similar results were obtained with the cations K^+, Ca^{2+} and Mg^{2+} (data not shown). The data in Table I are consistent with competitive inhibition, since in the presence of NaCl, KCl and $CaCl_2$ the K_m^{app} values increase with inhibitor concentration, although k_{cat}^{app} values remain approximately constant. Concerning the $MgCl_2$ data for k_{cat}^{app} in Table I, although the values appear not to be constant but slightly decreasing, statistical analysis of the data using a Graph Pad program at the 95% confidence level showed that the respective line in the graph k_{cat}^{app} *vs* $MgCl_2$ has a slope not significantly different from zero (data not shown). No hint of a possible second binding site for these inhibitors can be discerned in the experimental data obtained.

Comparison of the K_i values for K^+ (67 ± 13 mM), Na^+ (22 ± 0.1 mM), Ca^{2+} (9.8 ± 1.0 mM) and Mg^{2+} (4.9 ± 0.4 mM) reveals that Mg^{2+} and Ca^{2+} are more potent hK1 inhibitors than Na^+ and K^+, respectively. The present results agree with previous observations regarding the ability of univalent and divalent cations to inhibit the amidase activity of hK1 by 50% in which it was found that divalent cations (Ca^{2+} and Mg^{2+}) were more potent as inhibitors than univalent cations (Na^+, K^+ and NH_4^+).[13]

Comparison of the K_i values for K^+ (67 ± 13 mM) and Na^+ (22 ± 0.1 mM) reveals that the sodium cation is 3-fold more potent as an inhibitor of the amidase activity of hK1. This result is in contrast to the reported data of Lieberthal *et al.*,[13] who found that sodium (53 mM) and potassium (47 mM) showed equal inhibitory potency (IC_{50}) towards amidase activity of human urinary kallikrein (hK1).

On the other hand, since the charge density of Na^+ (0.28 charge/ionic volume) (Table II) is larger than the charge density of K^+, (0.10) it would be reasonable to expect that Na^+ would interact better with the Asp^{189} of the S_1 subsite[29] of the active center of hK1,[27] yielding a more stable ionic binding than K^+.

Comparison of the effect of Na^+ on hK1 and thrombin reveals that with this cation, the behavior of these serine proteases is clearly different. Although hK1 is inhibited by Na^+, thrombin requires Na^+ for optimal catalytic activity.[31] According to Di Cera *et al.*,[31] all thrombin interactions are affected by the equilibrium between the slow and fast forms. The slow ⟶ fast transition is triggered by the binding of Na^+ and results in enhanced specificity towards a variety of synthetic and physiological ligands. The Na^+ effect on thrombin is an example of a general regulation mechanism for enzyme activity by monovalent cations first described for pyruvate kinase in 1942.[31] Subsequently it was demonstrated that another serine protease involved in blood coagulation, factor X_a, also cleavage chromogenic amide substrates with higher specificity in the presence of Na^+. Now the importance of Na^+ in protease regulation is firmly established and the study for analysis of similar effects in serine proteases in general is set. According to Di Cera *et al.*,[31] monovalent cations affect the properties of an enzyme in essentially two ways; by forming a ternary complex with the enzyme and substrate, in

which case the requirement for the monovalent cation is absolute and no substrate hydrolysis is observed in its absence or through allosteric activation induced by binding to a site distinct from the active site. This mode of action is documented in only a few K^+-activated enzymes, like pyruvate kinase, but seems to be preponderant among the Na^+-activated enzymes. The authors have demonstrated that some proteases involved in blood coagulation and the complement cascade require Na^+ for optimal catalytic activity, whereas proteases involved in digestive processes and fibrinolysis do not. The molecular origin of this difference is remarkably simple and provides one of the most important structure-function correlations ever reported for serine proteases or enzymes in general. When the primary structure and the catalytic activity of a number of serine proteases were studied, it was observed that those with Tyr^{225} or Phe^{225} were found to show maximal catalytic activity in the presence of Na^+. On the other hand, proteases with Pro^{225}, like trypsin and hK1, showed no discrimination among monovalent cations, which indicates the lack of specific binding interactions of Na^+, Li^+ or K^+. According to the authors, replacement of Tyr^{225} with Pro in thrombin results in the loss of discrimination among monovalent cations and allosteric regulation and stabilization of the anticoagulant slow form. Hence, residue 225 determines the Na^+-induced allosteric enhancement of catalytic activity in serine proteases of the chymotrypsin family.

Human tissue kallikrein (hK1), a Ser^{225} protease,[32,33] did not show Na^+ activation, which indicates the lack of the specific site for allosteric interaction with the cation. In contrast, hK1 is competitively inhibited by Na^+, which indicates that the cation may interact with the carboxylate oxygen of Asp^{189} in its active site.

Comparison of the K_i values of Ca^{2+} (9.8 ± 1.0 mM) and Mg^{2+} (4.9 ± 0.4 mM) reveals that the magnesium cation was a 2-fold more potent hK1 inhibitor than the calcium cation. This result is in disagreement with those by Lieberthal *et al.*[13] in which calcium (2.3 mM) was found to be a 2-fold better inhibitor of amidase activity of human urinary kallikrein (hK1) than magnesium (5.0 mM).

On the other hand, since the charge density of Mg^{2+}, (1.74 charge/ionic volume) (Table II) is larger than the charge density of Ca^{2+} (0.49), it would be reasonable to expect that Mg^{2+} would interact better with the anionic site of the active center of hK1[27] yielding a stabler ionic binding than Ca^{2+}.

The inhibition of hK1-catalyzed hydrolysis of D-Val-Leu-Arg-pNA by Na^+ and K^+ is in agreement with the previous report of Chao *et al.*[18] which found that the esterase and kininogenase activities, as well as the immunoreactivities of purified rat urinary kallikrein (rK1) and human urinary kallikrein (hK1), are inhibited by monovalent cations.

Bovine serum albumin (0.2%) protected the inhibition of hK1 by Na^+ and K^+. A similar result was also reported by Chao *et al.*[18]

It can be concluded that the observed inhibitory effect of ions on hK1 amidase activity is due to the cation since, as already reported,[13] the inhibitory effect of ions on the amidase activity of human urinary kallikrein (hK1) was clearly unrelated to an ionic strength effect and also to the nature of the anion; according to these authors, anion salts of the same cation (chloride, sulfate, acetate and phosphate) have similar inhibitory effects.

Comparison of the effect of calcium on hK1 and bovine trypsin (b-TR) reveals that with this cation, the behavior of these two serine proteinases[28] is clearly different: while b-TR is activated by calcium hK1 is, in contrast, inhibited by this cation.

According to Bode and Schwager,[34] calcium ions accelerate the conversion of trypsinogen to trypsin, prevent the formation of inert proteins during activation and protect trypsin against autolysis. Epstein *et al.*[35] reported that trypsinogen possesses two binding sites for Ca^{2+}; one on the hexapeptide cleaved on the conversion of trypsinogen to trypsin and the other on the trypsin moiety. It has been observed that the binding of Ca^{2+} to the enzyme inhibits its autodigestion and the formation of inert proteins.[34] Thus, calcium binding to trypsin has an important biological function.[35] On the other hand, the effect of Ca^{2+} on hK1 is characterized as a competitive inhibition where the apparent values of K_m increase in the presence of increasing concentrations of the cation, while the apparent values of k_{cat} remains approximately unchanged (Table I). Thus, while Ca^{2+} binds to trypsin at a different site from its active center, the cation binds to hK1 in the S_1 subsite of its active center which contains the Asp^{189} residue responsible for the anion site of its active site. In the literature, the effect of Ca^{2+} on trypsin is described only as an activator (up to 50 mM).

According to Sichler *et al.*,[36] the architecture of the S_1 subsite within serine proteases is highly conserved. One marked difference is localised at position 190, which is either Ser or Ala and serves as a fingerprint of the enzyme subfamilies. The enzymes of the blood coagulation cascade differ significantly in this position.[36] The executioners of the cascade, thrombin and factor X_a, have Ala^{190}, whereas the physiologic activators of factor X, factor VII_a and factor IX_a, have Ser^{190} conserved through different species. Porcine trypsin, porcine pancreatic kallikrein (pK1), rat pancreatic kallikrein (rK1) and hK1[32,33] have Ser^{190}.

According to Sichler *et al.*[36] from a structural point of view, the binding of Arg into the S_1 subsite differs

from Lys as only the former is able to form a direct ionic interaction with Asp^{189}, while the interaction of the shorter Lys side chain to Asp^{189} is water-bridged. Ser^{190} is positioned in the S_1 pocket to allow an additional hydrogen bond to a bound Arg or Lys, thus stabilizing either of the substrate residues. P_1-Arg is bound more tightly and therefore has a lower K_m value than P_1-Lys.[36] This conclusion is also supported by data reported by Sousa *et al.*[37] in which K_m values of $10.3 \pm 1.0\,\mu M$ and $165.0 \pm 92\,\mu M$ were found for the hK1-catalyzed hydrolysis of S-2266 (D-Val-Leu-Arg-*p*NA) and S-2251 (D-Val-Leu-Lys-*p*NA), respectively.

In the present study, it would be reasonable to expect the cations to interact strongly with the S_1 subsite of the active center of hK1. However, the values of K_i observed (in the mM range, Table I) do not concur with this reasoning. We may propose, as speculation, the following explanation:

(i) the binding of the cations to the Asp^{189}, at the botton of the S_1 pocket may be stabilized by an ionic interaction mediated by a water molecule thus weakening the binding. This kind of interaction would be similar to the binding of the side chain of Lys to the Asp^{189}, which is water-bridged.[36] On the other hand, the interaction of the cations to the Asp^{189} would differ from the binding of the side chain of Arg to Asp^{189}, which is a direct ionic interaction;[36]

(ii) when the cation binds to Asp^{189} there is no possibility of making an additional hydrogen bond between the cation and Ser^{190} similar to that occurring with the side chains of Arg and Lys, respectively. Thus, Ser^{190} probably does not participate in the interaction between the cations and the S_1 subsite of the active center of hK1.

In the present work we have studied the kinetics of the inhibition of hK1 amidase activity by Na^+, K^+, Ca^{2+} and Mg^{2+}, cations normally found in human urine. These results may have important physiological implications since, according to Lieberthal *et al.*[13] hK1 is thought to act within the distal tubular fluid where ionic composition varies widely.

hK1 Inhibition by $AlCl_3$

According to Zatta *et al.*,[20] as a phenomenon the interaction between Al(III) and biological systems is well documented, however, direct information on the molecular basis of Al^{3+} biological activity is rather poor.

The present work was undertaken to examine in depth the kinetics of the inhibition of hK1 amidase activity by Al^{3+} in order to identify the precise mechanism of inhibition and to determine an accurate inhibition constant (K_i).

The Lineweaver-Burk plot for the hydrolysis of D-Val-Leu-Arg-*p*NA (5–30 μM) by hK1 (4.79 nM) in the absence and in the presence of $AlCl_3$ (0.7–4.0 mM) (Figure 2A) indicating linear mixed inhibition.[25] In order to clarify further the inhibition type, we replotted the values of parameters ($1/k_{cat}^{app}$ and K_m^{app}/k_{cat}^{app}) *vs* $AlCl_3$ concentration, respectively, according to Cornish–Bowden[26] (Figure 2B). As $1/k_{cat}^{app}$ and K_m^{app}/k_{cat}^{app} vary linearly with inhibitor, linear mixed inhibition was indicated.

Comparison of the K_i values for hK1 inhibition by K^+, Na^+, Ca^{2+}, Mg^{2+} and Al^{3+} (67 ± 13 mM, 22 ± 1 mM, 9.8 ± 1.0 mM, 4.9 ± 0.4 mM, and 2.0 ± 0.4 mM, respectively), reveals that Al^{3+} is a better hK1 inhibitor than the monovalent and divalent cations.

Comparison of the K_i values for hK1 inhibition by K^+, Na^+, Ca^{2+}, Mg^{2+} and Al^{3+} (67 ± 13 mM, 22 ± 1 mM, 9.8 ± 1.0 mM, 4.9 ± 0.4 mM, and 2.0 ± 0.4 mM, respectively) with the values of charge density (charge/ionic volume) of K^+, Na^+, Ca^{2+}, Mg^{2+} and Al^{3+} (0.10, 0.28, 0.49, 1.74, and 5.73, respectively) (Table II) reveals that the K_i values decrease as the charge density of the cation increases.

On the other hand, the K_i' value for hK1 inhibition by Al^{3+} (5.4 ± 0.4 mM) reveals that Al^{3+} is also able to bind to a second binding site in hK1.

The fact that $K_i' > K_i$ indicates that Al^{3+} binds to a second binding site in the hK1 molecule with lower affinity than it binds to the S1 subsite of the hK1 active center.

The presence of a second binding site in glandular (tissue) kallikrein was proposed by Mares-Guia and Diniz.[28] We have already indicated, by kinetic studies, the presence of a second binding site in hK1 to which inhibitors and substrates are able to bind,[10,24,37] although the nature and position of this second binding site are not yet known.

Aluminium is not a physiological cation; on the contrary, it has been described as neurotoxic.[19] According to Yokel,[19] the bulk of brain Al^{3+} has been reported to be ~ 0.018 mM (wet weight) in normal humans, 0.10 mM in Alzheimer's disease brain, and 0.18 mM in dialysis encephalopathy dementia complex. Comparison of the K_i value for hK1 inhibition by Al^{3+} (2.0 ± 0.4 mM) with the values of bulk brain aluminium specified above, reveals that it is unlikely that the neurotoxic effects of Al^{3+} could be due to inhibition of brain hK1 unless the brain kallikreins have a much higher affinity for Al^{3+}, or the Al^{3+} concentration can be much larger in specific areas of the brain.

On the other hand, the mixed inhibition of hK1 by Al^{3+} found in the present study is in agreement with

the previous observation by Zatta *et al.*[20] that Al(III) is a mixed inhibitor of trypsin.

The authors studied the effects of $Al(lac)_3$ (aluminum lactate) and $Al(malt)_3$ (aluminum maltolate) on the esterase activities of trypsin and α-chymotrypsin, respectively and found that Al(III) inhibited the activity of both serine proteases. The inhibitory effects of $Al(lac)_3$ (0.1 M and 0.25 M) and $Al(malt)_3$ (0.125 M and 0.250 M) on the activity of trypsin was characterized as being of the mixed type. The K_i values for $Al(lac)_3$ are 0.88 ± 0.12 mM and 0.95 ± 0.13 mM, respectively, and the K_i values for $Al(malt)_3$ were 0.95 ± 0.09 mM and 0.48 ± 0.03 mM, respectively. On the other hand, the inhibitory effect of $Al(lac)_3$ (1.0 mM and 2.5 mM) on the activity of α-chymotrypsin was characterized as being of the uncompetitive type, while the inhibitory effect of $Al(malt)_3$ (0.1 mM and 0.25 mM) on the same enzyme was characterized as noncompetitive. The K_i values for $Al(lac)_3$ were 0.36 ± 0.09 mM and 0.24 ± 0.07 mM, respectively, while the K_i values for $Al(malt)_3$ were 0.47 mM for both $Al(malt)_3$ concentrations used.[20]

The presence of a second inhibitor binding site in hK1 seems to be clear and may have important implications for the physiological activity of this enzyme.

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