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# Inhibition of rat tissue kallikrein gene family members by rat kallikrein-binding protein and α1-proteinase inhibitor

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The regulation of tissue kallikrein activity by plasma serine proteinase inhibitors (serpins) was investigated by measuring the association rate constants of six tissue-kallikrein family members isolated from the rat submandibular gland, with rat kallikrein-binding protein (rKBP) and α1-proteinase inhibitor (α1-PI). Both these serpins inhibited kallikreins rK2, rK7, rK8, rK9 and rK10[1] with association rate constants in the $10^3$–$10^4$ $M^{-1}\cdot s^{-1}$ range, whereas only 'true' tissue kallikrein rK1 was not susceptible to α1-PI. This results in slow inhibition of rK1 by plasma serpins, which could explain why this kallikrein is the ony member of the gene family identified so far that induces a transient decrease in blood pressure when injected in minute amounts into the circulation.

Kallikrein; Serpin; Proteinase inhibitor; Kallikrein-binding protein

## 1. INTRODUCTION

The kallikrein multigene family in the rat, designated RNOKLKx [1], includes at least 13 members [2], whose six protein products have now been clearly identified and correlated with their respective genes [3]. These proteins all have proteolytic activity, but their biological functions remain for the most part unknown. They are also structurally closely related, but recent data have shown that they possess different substrate specificities [4–6] and tissue distributions [7,8], indicating that they may be involved in physiological processes other than those depending on the release of kinins from kininogens. Rat tissue kallikreins also have different susceptibilities to heterologous inhibitors [3], which suggests that, physiologically, each one may be specifically regulated.

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*Abbreviations:* Z, benzyloxycarbonyl; -MCA, 7-amido-4-methylcoumarin; pNPGB, *p*-nitrophenyl-*p*'guanidinobenzoate; Abz, *o*-aminobenzoyl; EDDnp, ethylenediamine 2-4-dinitrophenyl; Tos-Arg-OMe, *p*-tosyl-arginyl-methylester; α1-PI, α1-proteinase inhibitor; rKBP, rat kallikrein-binding protein

[1]Kallikreins are abbreviated according to the rules defined at the Gene Nomenclature Workshop during the Kinin'91 meeting held in Munich, September 8–14, 1991 [1]. The most common names in use until recently were: tissue kallikrein, tonin, proteinase A or kallikrein k7, kallikrein k8, SEV or KLP-S3, antigen γ or kallikrein k10 or T-kininogenase, for rK1, rK2, rK7, rK8, rK9 and rK10, respectively [1].

Kallikrein-binding proteins (KBP), which are structurally related to inhibitors of the serpin family, have recently been found in human and rat plasma [9–12] but their precise physiological importance remains to be elucidated. Rat KBP is not a potent or specific plasma inhibitor of the 'true' tissue kallikrein rK1 [10], which agrees with the kininogenase activity of this proteinase in plasma. However there is no information as to the effects of plasma proteinase inhibitors on other members of the tissue kallikrein family. None of these proteinases has vasoactive properties comparable to those of rK1. This could be due to rapid inhibition by plasma proteinase inhibitors and especially by serpins. We have therefore examined the kinetics of inhibition of six members of the rat tissue kallikrein family by homologous KBP and α1-PI.

## 2. MATERIALS AND METHODS

### 2.1. *Enzymes*

Rat tissue kallikreins rK1, rK2, rK7, rK8, rK9, rK10 were purified from submandibular gland homogenates as described previously [4–6]. Bovine trypsin was purchased from Boehringer.

### 2.2. *Substrates*

The substrate with intramolecularly quenched fluorescence Abz-Phe-Arg-Ser-Arg-EDDnp was a gift from Dr. E. Prado (Escola Paulista, Sao Paulo, Brazil), Z-Phe-Arg-MCA was from Bachem and Tos-Arg-OMe from Sigma.

### 2.3. *Inhibitors*

Rat α1-PI was purified from inflammatory serum by thiol disulfide interchange [13] and Cibacron blue chromatography to remove serum albumin [14]. rKBP was purified as described previously [10]. Aprotinin was purchased from Boehringer.

2.4. *Titrations*

Bovine trypsin was first titrated using *p*-nitrophenyl-*p'*-guanidinobenzoate as described by Chase and Shaw [15]. This enzyme was then used to determine the reactive site concentration of all inhibitors, i.e. aprotinin, rat KBP and rat α1-PI. Rat KBP and α1-PI were prepared as stock solutions of 14 μM and 21 μM, respectively. Aprotinin was used as an active site titrant for all kallikreins except rK2 and rK9, which were titrated with pNPGB [6]. Kallikreins were assayed at 30°C in 0.1M Tris-HCl buffer pH 8.5, 1 mM EDTA, whereas trypsin was assayed in 0.1M Tris-HCl pH 8.0, 50 mM calcium chloride. All other experimental conditions were as described previously [16].

2.5. *Rate constants for association*

Rate constants for association ($k_{on}$) were monitored under second order conditions by allowing equimolar concentrations of proteinase and inhibitor to react for various periods before adding substrate and immediately recording to residual activity. Individual concentrations of reactants are given in Table I. The final concentrations of proteinases and inhibitors were in the $10^{-7}$–$10^{-8}$ molar range for kallikreins and around $10^{-9}$ molar for trypsin. All proteinases mixtures were incubated for 2–30 min before adding the appropriate substrate. Under second order conditions, the integrated equation of association is:

$$1/E = k_{on} \cdot t + 1/Eo$$

where Eo is the total enzyme concentration, and E the free enzyme concentration at time $t$. Plots of 1/E versus time are linear with a slope corresponding to $k_{on}$ [17] provided the reverse reaction is negligible.

The rK1-α1-PI interaction was also measured under pseudo-first order conditions, using a twenty fold molar excess of inhibitor (0.18 μM) over enzyme (9 nM). Other experimental conditions were as before.

## 3. RESULTS AND DISCUSSION

Previous studies on the kinin-releasing properties of rat kallikrein gene family enzymes have shown that rK1, but none of the other kallikrein family members, could induce a transient decrease in the blood pressure of anesthesized rats upon injection of amounts as small as 1 μg into the circulation [16]. Kallikrein rK10, however, releases bradykinin from purified rat high-$M_r$-kininogen in vitro [18] (Gutman et al., unpublished results). The failure of rK10 to release kinin in vivo when used under the same experimental conditions as rK1 [16], could be because this proteinase is inactivated faster than is rK1. Only circulating autoantibodies [19] and α1-PI in human serum [20] have been reported to modulate kallikrein activity, in addition to the recently described kallikrein-binding protein [9–12]. Sequence data have shown that this KBP, which is a negative marker of inflammation in the rat, corresponds to rat thyroid hormone-regulated protein [21], growth hormone-regulated proteinase inhibitor, also reported as SPI-2 [22–24], and could be the rat homologue of mouse contrapsin [25]. Human kallikrein-binding protein appears to be related to, but different from protein C inhibitor, a polyvalent heparin-dependent inhibitor of serine proteinases [26,27].

Whether or not the modulation of tissue kallikrein activity by rKBP and related inhibitors of the serpin family is of physiological relevance has not been investigated so far. We therefore determined the rate constants of association of rat KBP and rat α1-PI with rat tissue kallikreins rK1, rK2, rK7, rK8, rK9 and rK10, and bovine trypsin, as a first step in a study of the physiological relevance of rKBP towards tissue kallikreins.

Reactive site titrated inhibitors and kallikreins were used at 1:1 molar ratio to determine association rate constants. Though the mechanism of inhibition by rat KBP is not known, it seems reasonable to assume that it is similar to that of α1-PI, which is structurally related, and belongs to the same family. This agrees with the fact that the reaction with KBP follows second order kinetics almost to completion, and that the binding to tissue kallikrein results in the formation of SDS-stable complexes [9], both results indicating a tight complex between the proteinase and its serpin inhibitor. The extreme stability of these complexes, prevents accurate determination of $K_i$ values [28]. Association rate constants ($k_{on}$) therefore are the most representative kinetic parameters describing proteinase-serpin interactions.

Table I

Association rate constants ($k_{on}$) for the interaction between α1-PI or rKBP and six rat tissue kallikrein family members and bovine trypsin

| Protease (final molarity) | Substrate (final molarity) | Inhibitor | |
|---|---|---|---|
| | | α1-PI | rKBP |
| Trypsin ($3.5\cdot10^{-9}$ M) | Z-Phe-Arg-MCA ($1.2\cdot10^{-5}$ M) | $(6.6 \pm 1)10^6$ | |
| ($3.5\cdot10^{-9}$ M) | | | $(1.2 \pm 0.2)10^6$ |
| rK1 ($4.5\cdot10^{-8}$ M) | Z-Phe-Arg-MCA ($1.2\cdot10^{-5}$ M) | no inhibition | $(2.5 \pm 0.6)10^3$ |
| ($4.5\cdot10^{-8}$ M) | | | |
| rK7 ($5.5\cdot10^{-8}$ M) | Z-Phe-Arg-MCA ($2.5\cdot10^{-6}$ M) | $(6.1 \pm 0.4)10^4$ | |
| ($6.6\cdot10^{-8}$ M) | | | $(5 \pm 0.8)10^4$ |
| rK8 ($2\cdot10^{-7}$ M) | Z-Phe-Arg-MCA ($1.6\cdot10^{-5}$ M) | $(1.1 \pm 0.1)10^4$ | |
| ($2\cdot10^{-7}$ M) | | | $(1.7 \pm 0.3)10^4$ |
| rK10 ($5.3\cdot10^{-8}$ M) | Z-Phe-Arg-MCA ($2.5\cdot10^{-6}$ M) | $(1.2 \pm 0.3)10^4$ | |
| ($1.7\cdot10^{-7}$ M) | | | $(3.7 \pm 0.5)10^3$ |
| rK2 | Abz-Phe-Arg-Ser-Arg-EDDNP | | |
| ($8.6\cdot10^{-8}$ M) | ($6\cdot10^{-7}$ M) | $(1.2 \pm 0.4)10^4$ | |
| ($1.7\cdot10^{-7}$ M) | | | $(7.8 \pm 1)10^3$ |
| rK9 ($2.7\cdot10^{-8}$ M) | Tos-Arg-OMe ($2\cdot10^{-3}$ M) | $(2.1 \pm 0.7)10^3$ | |
| ($3.7\cdot10^{-9}$ M) | | | $(8 \pm 0.6)10^3$ |

Experiments were carried out at 30°C, in 0.1 M Tris-HCl buffer pH 8.5, 1 mM EDTA for all kallikreins and in 0.1 M Tris-HCl buffer, pH 8.0, 50 mM calcium chloride for trypsin. Results ($M^{-1}\cdot s^{-1}$) are the means of at least two experiments.

The results in Table I show that all the proteinases were inhibited, though not rapidly, by both inhibitors, with the notable exception of rK1, which is not susceptible to α1-PI.

The lack of rK1 inhibition by α1-PI, was confirmed using under pseudo-first order conditions with respect to proteinase to attempt to increase the rate of inhibition. Even with the twenty-fold molar excess of α1-PI over rK1 used, there was no significant inhibition of rK1.

The resistance of rK1 to inhibition by α1-PI may be explained by its substrate specificity, which differs from that of other rat kallikreins and of human tissue kallikrein, hK2 [1]. rK1 releases bradykinin from rat kininogens after cleavage at two P1 Arg sites (nomenclature of Schechter and Berger [29]), whereas its human homologue, which is inhibited by α1-PI, though rather slowly, cleaves kininogen after Met and Arg residues to release Lys-bradykinin. The susceptible bond (P1–P1') in both rat and human α1-PI is Met-Ser [30]. The restricted specificity of rat kallikrein rK1 compared to that of other members of the gene family [3], might render it unable to accommodate a P1 Met residue, and so prevent it from being inhibited by this inhibitor. In contrast to α1-PI, the susceptible bond in rKBP is thought to be Lys-Ser [21,22], which is more favourable for interaction with trypsin-related proteinases.

Another characteristic feature of kallikrein inhibition by serpin inhibitors is the rather slow and similar rate at which the binding occurs whatever the kallikrein used. The rate constants values are all within about one order of magnitude, and are about 100 times lower than that for bovine trypsin.

This low rate of binding raises the question of the biological relevance of the phenomenon. The inhibition of kallikreins by serpins in the circulation should follow pseudo first order kinetics, since the inhibitors are in large molar excess over proteinases. Under these conditions the half time for association is:

$$t_{0.5} = 0.693 / k_{on}[I_o]$$

The plasma physiological concentrations $[I_o]$ of α1-PI (50–70 μM) and rKBP (6–8 μM) (Chao, J., unpublished results) give pseudo first order constants ($k_{on}[I_o]$) of 0.1 to 1 $s^{-1}$ for those kallikreins which are inhibited by both inhibitors. The half-life of association in plasma would therefore be 1 to 10 s, and about 40 s for rK1, which may be enough to modulate kallikrein activities, but not to ensure immediate control of these activities [31]. With regards to rK10 which has kininogenase properties in vitro but not in vivo, the half-life for association with serpins (essentially α1-PI) would be about 1 s, which is significantly less than the half-time required for KBP to inhibit rK1. This difference is even more significant during inflammation, since α1-PI concentration increases under these conditions, while KBP decreases [10]. Nevertheless, the rate at which rK10 is inhibited by serpin inhibitors cannot ensure immediate and complete control of its activity; thus the presence of other potent and thus far unidentified circulating inhibitor(s) preventing rK10 but not rK1 from being a physiological kinin-releasing proteinase cannot be eliminated.

The proteinase of the tissue kallikrein family have different substrate specificities [4–6]. This is probably due to their extended substrate binding site, which confers a specificity to subsites other than S1, which preferentially accomodates Arg residues in most tissue kallikreins. This explains why several members of the kallikrein family, which do not exhibit kinin-releasing activities, may have specific activities in other tissues or secretions where they happen to be present [7]. The rate of KBP and α1-PI binding to tissue kallikreins found here suggests, that these inhibitors can be physiologically relevant in maintaining the activity of such proteinases within a range of concentration compatible with their biological function [31]. The fact that spontaneously hypertensive rats have low level of circulating KBP also suggests that the modulation of rK1 activity by this inhibitor is biologically significant [32].

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