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Kinetics of inhibition of platelet calpain II by human kininogens

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The plasma kininogens, high-molecular-mass and low-molecular-mass kininogens, are the most potent plasma inhibitors of platelet calpain. We explored the kinetic mechanisms for kininogen inhibition of calpain by comparing calpain inactivation by human high-molecular-mass kininogen (HK) and human low-molecular-mass kininogen (LK). With a [^{14}C]methylated α-casein substrate, the inhibition of calpain by HK did not follow classic Michaelis–Menten kinetics. With the use of a fluorogenic assay with the dipeptide substrate for calpain, 3-carboxypropionyl-leucyltyrosine 7-(4-methyl)coumarylamide, the inhibition by HK and LK fitted a kinetic model of tight-binding inhibition. LK was found to be a non-competitive inhibitor of platelet calpain with a K_i of 2.7 nM. HK showed mixed non-competitive inhibition of calpain with a K_i of 2.3 nM in the absence of substrate and K_i of 0.71 nM in the presence of saturating substrate, almost 4-fold tighter than LK. Proteolysis of HK by plasma and tissue kallikreins did not influence its ability to inhibit calpain. Digestion of the HK light chain by Factor XIa also did not alter its calpain-inhibitory function. These studies indicate that the kininogens are tight-binding non-competitive inhibitors of platelet calpain, the inhibitory domain in each case being mainly on the heavy chain. The light chain of HK appears to influence its kinetic behaviour.

INTRODUCTION

Calpains I and II are Ca^{2+}-activated neutral cysteine proteinases that differ in their requirement for Ca^{2+} (Murachi *et al.*, 1981). The EC_{50} values are respectively 2 μM and 150 μM for the two enzymes, but they have similar substrate-specificities. These enzymes are present in all mammalian cells but are absent from normal plasma (Murachi, 1983). Calpain, which is found in platelets in high concentrations (4.5 μg/10^8 platelets) (Schmaier *et al.*, 1990), has a molecular mass of 110 kDa, and consists of two subunits of 80 kDa and 29 kDa when reduced on SDS/PAGE. These enzymes are heat-labile (Inomata *et al.*, 1984), and exhibit conformational alterations and autolysis when exposed to Ca^{2+} ions (Tsuji *et al.*, 1981).

The most potent plasma inhibitor of calpain is high-molecular-mass kininogen (HK) (Schmaier *et al.*, 1986*a*), which will form a stoichiometric complex with calpain, as seen on non-denaturing PAGE in the presence of Ca^{2+} (Ishiguro *et al.*, 1987). HK is a multifunctional protein that also serves as the cofactor of the intrinsic blood-coagulation pathway and binds to platelets (Gustafson *et al.*, 1986), neutrophils (Gustafson *et al.*, 1989) and endothelial cells (Schmaier *et al.*, 1988). HK is a source of bradykinin after cleavage by a number of proteinases (Scott *et al.*, 1984, 1985), including calpain, when the enzyme is in slight molar excess (Higashiyama *et al.*, 1986). Low-molecular-mass kininogen (LK), similarily to HK, is also a potent inhibitor of calpain, but has been reported to require higher concentrations for equivalent extents of inhibition as compared with HK (Schmaier *et al.*, 1986*a*). The α_2- and α_1-cysteine-proteinase inhibitors are identical with kinin-free kininogens (Ohkubo *et al.*, 1984; Müller-Esterl *et al.*, 1985). Both HK and LK have identical heavy-chain portions, which consist of three homologous segments (domains 1, 2 and 3), (Salvesen *et al.*, 1986; Ishiguro *et al.*, 1987). HK and LK differ from one another by the length and sequences of their light chains, which diverge at a point 12 residues *C*-terminal beyond the bradykinin moiety (Takagaki *et al.*, 1985). The heavy chains of kininogens contain the primary determinants that inhibit platelet calpain (Schmaier *et al.*, 1987). To resolve an apparent discrepancy between the calpain-inhibitory potency of each form of human kininogens, we have conducted a detailed kinetic examination of the inhibition of platelet calpain by each kininogen to determine whether the heavy chain is the sole determinant of this activity.

MATERIALS AND METHODS

Materials

Total kininogen-deficient plasma was donated by Mrs. Williams, Philadelphia, PA, U.S.A. (Colman *et al.*, 1975). [^{14}C]Methylated α-casein was purchased from New England Nuclear, Boston, MA, U.S.A. Suc-Leu-Tyr-NH-Mec was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents and chemicals were of the best commercial grade available.

Purified proteins

Calpain was purified from sonicated fresh human platelets from a 100000 *g* supernatant (cytosol) by poly(ethylene glycol) precipitation, anion-exchange chromatography and gel filtration as previously described (Schmaier *et al.*, 1986*a*). During the purification, the presence of calpain was monitored by α-casein hydrolysis. One unit of calpain activity was defined as the amount of enzyme that increased the absorbance at 280 nm by 1.0 A_{280} unit/h per ml.

Human HK was purified from fresh plasma by a modification (Schmaier *et al.*, 1986*b*) of the procedure of Kerbiriou & Griffin (1979). This preparation of HK on reduced SDS/PAGE was essentially a single band of 120 kDa with greater than 98% purity and has a specific activity of 12.5 units/mg (Schmaier *et al.*, 1986*b*). HK was radiolabelled with Na^{125}I with the use of Iodogen (Schmaier *et al.*, 1983). Human LK was prepared by an affinity chromatography on carboxymethyl-papain–Sepharose followed by anion-exchange chromatography (Anastasi *et al.*, 1983; Gounaris *et al.*, 1984; Johnson *et al.*, 1987), and was a single band of 68 kDa with greater than 95% purity. Purified heavy chains and light chains of HK were prepared as previously

Abbreviations used: HK, high-molecular-mass kininogen; LK, low-molecular-mass kininogen; Suc-, 3-carboxypropionyl-; -NH-Mec, 7-(4-methyl)coumarylamide; -NH-Np, 4-nitroanilide.

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reported (Schmaier *et al.*, 1987). Human plasma kallikrein was prepared by activation of purified prekallikrein by Factor XII fragments (Scott *et al.*, 1979), and was a doublet of 88 kDa and 85 kDa on non-reduced SDS/PAGE. The specific activity was 14.5 μmol/min per mg with D-Pro-Phe-Arg-NH-Np as substrate. Factor XIa was prepared from Factor XI activated by Factor XIIa (Scott *et al.*, 1985), and was a single band of 160 kDa on non-reduced SDS/PAGE with a specific activity of 203 coagulant activity units/mg. Human urinary kallikrein was kindly given by Dr. Julie Chao (Medical University of South Carolina, Charleston, SC, U.S.A.), and was a single component of 25 kDa on non-reduced SDS/PAGE.

Polyclonal antibody to the light chain of HK was prepared by intramuscular injection into a goat of 250 μg of purified light chain of HK in complete Freund's adjuvant; 3 weeks later a second intramuscular injection of the same constituents was made. Then 1 week later blood was drawn for the preparation of serum. Antibody to the light chain of HK was purified from the serum by precipitation by 50%-saturated $(NH_4)_2SO_4$ followed by affinity chromatography on 1 ml of CNBr-activated Sepharose 4B coupled with 5 mg purified HK. Bound antibody was eluted with 0.2 M-glycine, followed by dialysis into 0.15 M-NaCl/0.01 M-Tris/HCl buffer, pH 7.4. The specificity of this affinity-purified antibody to the light chain of HK was shown by competitive e.l.i.s.a. experiments. The competitive e.l.i.s.a. was performed as previously reported (Schmaier *et al.*, 1983). In this assay, the anti-(HK light chain) antibody was consumed by kininogen antigen in normal plasma but not by total kininogen-deficient plasma (Williams) or HK-deficient plasma (Fitzgerald). Fitzgerald plasma was generously given by Dr. A. Scicli (Henry Ford Hospital, Detroit, MI, U.S.A.).

HK coagulant activity

The coagulant activity of human HK was determined as previously described (Colman *et al.*, 1975) by using a modification of the activated partial thromboplastin time with total kininogen-deficient plasma as substrate.

Cleavage of HK by kallikrein and Factor XIa

HK was incubated with plasma kallikrein and urinary kallikrein for 24 h at 37 °C at substrate/enzyme molar ratios of 25:1 and 10:1 respectively. A 60:1 molar ratio of HK to Factor XIa was used, and incubation was for 96 h to ensure complete proteolysis. A 10-fold molar excess of soyabean trypsin inhibitor was added to plasma kallikrein in all reactions at the end of the incubation period to stop the proteolytic reaction. The degree of cleavage of HK was analysed on SDS/10%-PAGE.

Functional assays of platelet calpain

The inhibition of calpain by various concentrations of different kininogens or cleaved HK used the following protocol. Calpain activity was monitored by the hydrolysis of Suc-Leu-Tyr-NH-Mec (Schmaier *et al.*, 1987). Portions (25 μl) of enzyme and buffer or inhibitor were added to a four-sided rectangular quartz cuvette at 25 °C containing 1 mM-substrate in 60 mM-Tris/HCl buffer, pH 7.5, containing 2.5 mM-dithiothreitol, 2.5% (v/v) dimethyl sulphoxide and 5 mM-$CaCl_2$. The rate of substrate hydrolysis was continuously recorded by a Perkin–Elmer LS-5 fluorescence spectrophotometer connected to an R100 chart recorder. The absorbance excitation maximum of substrate occurred at 380 nm (5 nm slit width) and emission response at 450 nm (10 nm slit width). Calpain activity was calibrated relative to the equivalent amount of enzyme that generated 0.1 units/ml, as measured with α-casein hydrolysis. Calpain activity (0.1 unit/ml) is equal to substrate hydrolysis generating a fluorescent response with a rate of emission increase of 30 mm/min at 200 mV sensitivity. The calpain casein-hydrolytic assay was performed as previously described (Schmaier *et al.*, 1986*a*) with [^{14}C]methylated α-casein.

Immunoprecipitation of calpain–HK complexes

Immunoprecipitation with staphylococcal Protein A (Pansorbin; Calbiochem, La Jolla, CA, U.S.A.) was performed to ascertain whether calpain was complexed with HK, with the use of HK and antibody to the light chain of HK. A 12 μg portion of purified HK in the presence of 60 ng of ^{125}I-HK was incubated for 1 h at room temperature in the absence or in the presence of 12 μg of purified platelet calpain II. These samples, as well as 12 μg of purified platelet calpain II alone, were then each incubated overnight at 4 °C with 7 μg of the anti-(HK light chain) antibody. The next day the incubations were made 10% (w/v) with respect to washed staphylococcal Protein A. The staphylococcal Protein A was washed in 0.19 M-NaCl/0.05 M-Tris/HCl buffer, pH 7.4, containing 6 mM-EDTA, 2.5% (w/v) Triton X-100, 0.1% SDS and 100 units of aprotinin/ml. After the staphylococcal Protein A had been washed four times in this buffer, it was resuspended in the same buffer containing 0.2% BSA. After 2 h incubation with the staphylococcal Protein A, the immunoprecipitates were washed five times in the above buffer followed by two washes with the same buffer without Triton X-100. The pellets were resuspended in sample buffer containing 2% (v/v) 2-mercaptoethanol. After boiling for 5 min, the eluted material was subjected to SDS/8%-PAGE. Protein bands were stained with Coomassie Blue. The dried gel was also studied by autoradiography.

Kinetics

Enzyme in the absence or in the presence of HK was incubated with increasing amounts of [^{14}C]methylated α-casein for 10 min at 25 °C. The inhibition of calpain by HK was determined by a double-reciprocal plot. The kinetics of calpain inhibition by kininogen was also analysed by a model for tight binding inhibition (Cha, 1976). These kinetic determinations with the fluorescent substrate were performed at 25 °C in the presence of increasing concentrations of competing substrate to retard the very high rates of calpain inhibition by kininogen and to minimize any autolysis of the calpain in the presence of Ca^{2+}. In these latter experiments the rates of calpain inactivation were recorded continuously over a 15–20 min interval. The observed rates of inhibition by calpain were calculated from the change in the slope of the lines determined by measuring the tangents drawn at 1 min intervals from the inhibition curves. Preincubation of calpain with HK or LK in the absence of substrate did not alter the rate of hydrolysis.

Kinetic determinations of enzyme interaction with inhibitor in the absence of substrate were also measured at short time intervals by assaying residual calpain activity. In these experiments the residual calpain activity was measured after calpain and kininogen were mixed, and samples were subsampled at increasing time intervals into a cuvette containing $CaCl_2$ (5 mM) with the substrate Suc-Leu-Tyr-NH-Mec at various concentrations. In all experiments the inhibition of calpain by either HK or LK was too rapid to measure under first-order conditions (greater than 10-fold inhibitor excess). Rates of calpain inactivation were therefore determined in all cases by second order association rate kinetics with inhibitor in slight excess to enzyme to prevent digestion of kininogen (Schmaier *et al.*, 1986*b*). The association rate constants were calculated according to the integrated second-order rate equation (Gigli *et al.*, 1970):

$$k''t = \frac{1}{[I]-[E]} \cdot \ln\left(\frac{[E]([I]-[EI])}{[I]([E]-[EI])}\right)$$

where [E] is the enzyme concentration, [I] is the inhibitor concentration and [EI] is the concentration of the enzyme–inhibitor complex, assuming a 1:1 stoichiometry. Since the stoichiometry of HK to enzyme may be 1:2 (Ishiguro *et al.*, 1987), the rates of inactivation were recalculated on that basis, but were unaffected. The residual activity (free enzyme) at 60 s intervals was subtracted from that of the total enzyme, and this gives the concentration of the enzyme–inhibitor complex. The dissociation rate-constants (k' or α) were determined from the formula $\alpha = \beta[I]$ by plotting the slope of the rate of inhibition (β) or the association rate constant (k'') in each reaction versus time (t) multiplied by the inhibitor concentration. The slope of the lines were fitted by linear regression. The rate constants determined without substrate are designated with a subscript 0, e.g. α_0, β_0.

Data analysis

All data computations and formulae were solved with the use of a programmable TI-59 calculator (Texas Instruments, Dallas, TX, U.S.A.) and incorporating the linear-regression subprogram contained on the master library module.

SDS/PAGE

SDS/10%-PAGE was used to monitor protein purifications and the results of cleavage experiments and were performed by the method of Laemmli (1970). All proteins on SDS/PAGE were run along with low-molecular-mass and high-molecular-mass markers (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Apparent differences in the molecular-mass markers indexed on the individual lanes presented in the Figures are due to variations in photographic enlargements from gel to gel.

RESULTS

Inhibition of platelet calpain II by kininogens

HK and LK at various concentrations were incubated with a single concentration of platelet calpain (9 nM) and assayed for residual calpain activity with the fluorimetric dipeptide substrate Suc-Leu-Tyr-NH-Mec (1 mM). Human HK was a potent inhibitor of calpain (IC_{50} 4.5 nM). Human LK (IC_{50} 7 nM) was of similar potency to HK. Attempts were made to reverse the inhibition of calpain by HK. The calpain/HK incubation mixtures were subjected dilutions as high as 100-fold. Calpain activity was measured at intervals over a 2 h period, but no change in activity was found. To ascertain whether calpain–HK complexes could be dissociated, calpain/HK incubation mixtures were studied to determine if antibody to the light chain of HK could immunoprecipitate human platelet calpain II in complex with HK (Fig. 1). Anti-HK antibody was able to bring down human platelet calpain II by immunoprecipitation of its complex with HK, as indicated by the presence of an 80 kDa Coomassie Blue-staining band. This band was specific for calpain in complex with HK because the antibody to HK incubated with purified platelet calpain alone did not immunoprecipitate the calpain. Since the stoichiometry of calpain to HK is 2:1 (Ishiguro *et al.*, 1987), the amount of HK immunoprecipitated by the antibody must have been below the Coomassie Blue-staining sensitivity of the gel. In order to confirm that assessment, ^{125}I-HK was added to the calpain/HK incubation mixture. As seen on autoradiography (Fig. 1), the antibody to HK immunoprecipitated ^{125}I-HK. These studies indicate a tight enzyme–inhibitor complex, which did not dissociate with multiple washings and dilution, and which was immunoprecipitated by an antibody to the inhibitor.

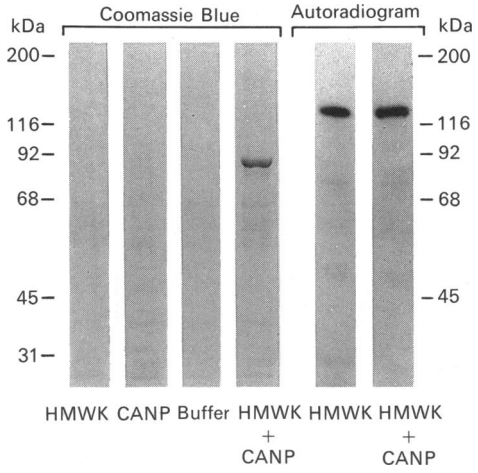


Fig. 1. Immunoprecipitation of the complex of calpain and HK

HK (12 μg) and ^{125}I-HK (60 ng) alone or incubated with 12 μg of purified platelet calpain II were incubated overnight at 4 °C with 7 μg of an affinity-purified antibody to the light chain of HK. After precipitation with staphylococcal Protein A, the samples were subjected to SDS/PAGE. Protein on the gel was detected by Coomassie Blue staining (on the left) and autoradiography (on the right). HMWK represents HK and ^{125}I-HK alone; CANP represents purified platelet calpain alone; Buffer is an incubation of antibody alone; HMWK + CANP represents the incubation of the complex of HK and ^{125}I-HK with purified platelet calpain. The numbers to the left and right of the gel represent the positions of molecular-mass standards.

Calpain inhibition by various cleaved HK preparations

Studies were performed to determine whether cleaved HK was as good an inhibitor of calpain as intact HK. Human HK, as a single component, was 120 kDa on SDS/PAGE under reducing conditions. Three kinds of cleaved HK were studied. As previously described (Maier *et al.*, 1983), urinary kallikrein digested HK by cleaving two bonds to produce on reduced SDS/PAGE, a heavy chain (65 kDa) and a light chain (56 kDa) (Fig. 2 inset). As previously reported (Scott *et al.*, 1984), plasma kallikrein cleaved a third bond in the light chain of HK to release an 8 kDa peptide to result in a 65 kDa heavy chain and a shorter 45 kDa light chain. In agreement with our previous studies (Scott *et al.*, 1985), Factor XIa cleaved HK to result in two polypeptides, at 75 kDa and 45 kDa. When HK is further cleaved by Factor XIa, some of the 75 kDa polypeptide (an intermediate) persisted in addition to the heavy chain (65 kDa) and light chain components. Further, Factor XIa-cleaved HK contains some degradation products of the light chain at 40 kDa and several products below 18 kDa. When each of these cleaved preparations of HK was tested at multiple concentrations for its inhibitory ability on calpain, no important differences in calpain-inhibitory potencies were observed (Fig. 2). All IC_{50} values of these kininogens were between 3.0 and 4.5 nM.

Measurement of K_i for calpain inhibition by HK with casein as a substrate

We next investigated the kinetic mechanism(s) for HK inhibition of calpain with the protein substrate [^{14}C]methylated α-casein. The double-reciprocal plot of α-casein hydrolysis versus substrate concentration at three HK concentrations is presented in Fig. 3. The Lineweaver–Burk plot shows that HK is a non-competitive inhibitor of platelet calpain with a K_i of 0.52 ± 0.061 nM (means ± S.E.M., $n = 9$). However, a secondary plot of the slope of the inhibition graph versus inhibitor concentration was not linear, suggesting that Michaelis–Menten

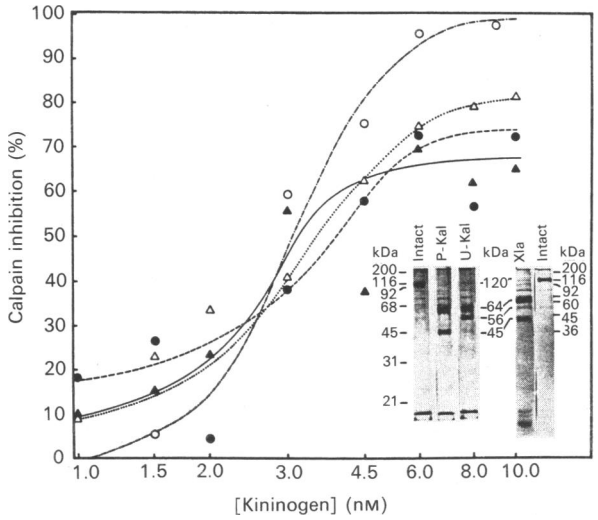


Fig. 2. Effect of proteolytic cleavage of HK on its ability to inhibit platelet calpain

HK was cleaved by Factor XIa (XIa, ○), urinary kallikrein (U-Kal, △) or plasma kallikrein (P-Kal, ▲) were compared with intact HK (Intact, ●) to determine their ability to inhibit platelet calpain. The inset depicts a reduced SDS/10%-PAGE of these protein preparations used in these inhibition experiments. The conditions for cleavage of HK are presented in the Materials and methods section. The numbers on the left and right sides of the gel are positions of apparent molecular-mass standards. The numbers in the centre of the gel show migratory positions of intact HK and HK cleaved by plasma kallikrein.

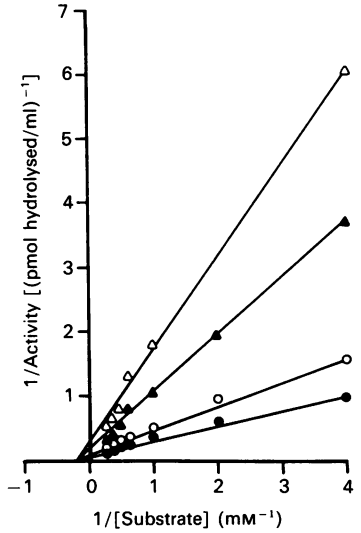


Fig. 3. Lineweaver–Burk analysis of the inhibition of calpain by HK with A-casein as substrate

Calpain at 9 nM was incubated without (●) or with HK at 4.5 nM (○), 9 nM (▲) and 13.5 nM (△) for 15 s at 25 °C, then subsamples were withdrawn and added to increasing substrate concentrations and the assay was performed as described in the Materials and methods section. The reciprocal of the rate of α-casein hydrolysis for each inhibitor concentration was plotted against the reciprocal of the substrate concentration.

kinetics is the wrong type of analysis for this study. Since HK is a tight-binding inhibitor with a high affinity for cysteine proteinases such as papain (Salvesen *et al.*, 1986), we applied the kinetic method of Cha (1976).

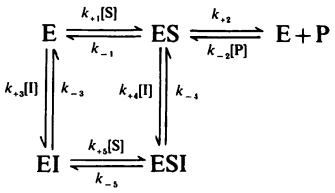
Determination of K_m

The K_m of the fluorogenic substrate for cleavage by calpain was determined in three separate experiments at pH 7.5 and 25 °C. Lineweaver–Burk plots resulted in a mean value of 0.34 ± 0.23 mM (means ± S.E.M., $n = 3$). This value for the K_m of platelet calpain II is in good agreement with the previously reported value of 0.4 mM (Crawford, 1987) for the K_m of chicken calpain II on the same substrate.

Determination of the apparent dissociation rate constant α (k′)

Calpain inhibition by HK and LK was too rapid for measurement under first-order dissociation conditions; therefore these reactions were performed under second-order association conditions. Enzyme activity (9 nM) was monitored in the presence of various concentrations of inhibitor and substrate as a function of time. A very rapid inhibition of calpain by kininogen was observed in the absence of substrate. This finding necessitated increasing both the enzyme and inhibitor concentrations 5-fold to permit sufficient sensitivity for measurement of the residual activity in time-dependent incubations. The calculated values of α for HK and LK appear in Tables 1 and 2 respectively.

The values of α for HK appear to be dependent on substrate concentration, suggesting a deviation from the mechanism for competitive inhibition. A general inhibition mechanism is represented by:



where $k'' = \beta = (k_{+3} + k_{+4}[S]/K_m)/(1 + [S]K_m)$, $K_i = k_{-3}/k_{+3}$ and $K_i' = k_{-4}/k_{+4}$.

The α values obtained from LK are reasonably constant, but the values for HK appear to show a dependence on substrate concentration. The average calculated value of α in the presence of substrate for LK is 12.79 ± 1.52 mM (means ± S.E.M., $n = 42$).

Association rate constant

The values for the association rate constants in the presence of (β) and absence (β_0) substrate were calculated from the slope of the plot of calpain inhibition versus time, as indicated in the Materials and methods section, and also appear in Table 1 for HK and Table 2 for LK. Note that the values of β for HK are effectively lowered by increasing substrate concentrations whereas the values for LK are much less affected, if affected at all.

Diagnostic plot for non-competitive inhibition

The reciprocals for the values of β for HK (Fig. 4) and LK (Fig. 5) were plotted against the substrate concentration, and also their theoretical lines for competitive inhibition based on a K_m of 0.34 mM and the assumption that $k_{+4} = 0$. The dotted lines appearing on either side of the dashed lines represent that statistical limits of 1 S.D. allowed for the competitive-inhibition model. The continuous line in Fig. 4 is the weighted linear-regression fit for the HK data obtained by using the weights of $[(n-2)/(\text{S.E.M.})^2]$ in accordance with Cha (1976). The plotted line for LK does not fit a linear plot, but is a good fit for a rectangular hyperbola; therefore LK is noncompetitive. HK at low values of $1/\beta$ approximates a linear plot, but it curves upward at higher values of $1/\beta$ (Fig. 4). Since the actual line is not within the 95% confidence limits for the theoretical line, HK does not strictly conform to the requirements for competitive inhibition (Cha, 1976).

Table 1. Kinetic parameters for HK

The Table lists estimated kinetic values (means ± S.E.M.) for the inhibition of platelet calpain by HK, where S is substrate (Suc-Leu-Tyr-NH-Mec), n is the number of determinations, $\alpha = k'$, β is the association rate constant and $\Delta\beta$ is the change in the association rate constant as influenced by the contribution of substrate.

[S] (mM)	n	$10^{-2} \times \alpha$ (s^{-1})	$10^{6} \times \beta$ ($M^{-1} \cdot s^{-1}$)	$10^{6}/\beta$ ($M \cdot s$)	$10^{6} \times \Delta\beta$ ($M^{-1} \cdot s^{-1}$)	$10^{-7}/\Delta\beta$ ($M \cdot s$)
0	10	44.754 ± 2.288	7.567 ± 0.296	0.132 ± 0.360	0	∞
0.25	6	2.565 ± 0.635	3.907 ± 0.697	0.256 ± 0.060	3.660 ± 0.661	2.732 ± 0.718
0.50	8	2.708 ± 0.625	3.122 ± 0.598	0.320 ± 0.087	4.445 ± 0.598	2.250 ± 0.988
1.00	11	1.672 ± 0.123	1.653 ± 0.014	0.605 ± 0.446	5.914 ± 0.443	1.691 ± 0.169
1.50	11	1.033 ± 0.077	0.971 ± 0.086	1.030 ± 0.484	6.596 ± 0.272	1.516 ± 0.139
2.00	11	0.791 ± 0.073	0.683 ± 0.084	1.464 ± 0.592	6.884 ± 0.264	1.453 ± 0.130
2.50	7	0.735 ± 0.082	0.483 ± 0.063	2.072 ± 0.532	7.084 ± 0.200	1.412 ± 0.089
3.00	5	0.517 ± 0.093	0.330 ± 0.083	3.032 ± 0.795	7.237 ± 0.261	1.382 ± 0.117

Table 2. Kinetic parameters for LK

The table lists estimated kinetic values (means ± S.E.M.) for the inhibition of platelet calpain by LK. Symbols are as defined in Table 1.

[S] (mM)	n	$10^{-2} \times \alpha$ (s^{-1})	$10^{6} \times \beta$ ($M^{-1} \cdot s^{-1}$)	$10^{6}/\beta$ ($M \cdot s$)	$10^{6} \times \Delta\beta$ ($M^{-1} \cdot s^{-1}$)	$10^{-7}/\Delta\beta$ ($M \cdot s$)
0	6	10.967 ± 1.330	4.680 ± 0.733	0.214 ± 0.041	0	∞
0.25	6	1.361 ± 0.106	0.946 ± 0.071	1.058 ± 0.238	3.734 ± 0.227	2.678 ± 0.197
0.50	8	1.606 ± 0.427	1.058 ± 0.099	0.945 ± 0.297	3.622 ± 0.312	2.761 ± 0.297
1.00	6	1.375 ± 0.373	1.083 ± 0.116	0.923 ± 0.732	3.597 ± 0.371	2.780 ± 0.350
1.50	5	1.448 ± 0.580	0.898 ± 0.128	1.113 ± 0.634	3.782 ± 0.406	2.644 ± 0.356
2.00	7	1.057 ± 0.425	0.658 ± 0.110	1.519 ± 0.396	4.022 ± 0.347	2.486 ± 0.342
2.50	6	1.309 ± 0.539	0.856 ± 0.123	1.169 ± 0.616	3.824 ± 0.390	2.615 ± 0.355
3.00	6	0.861 ± 0.316	0.621 ± 0.083	1.609 ± 0.684	4.059 ± 0.263	2.464 ± 0.209

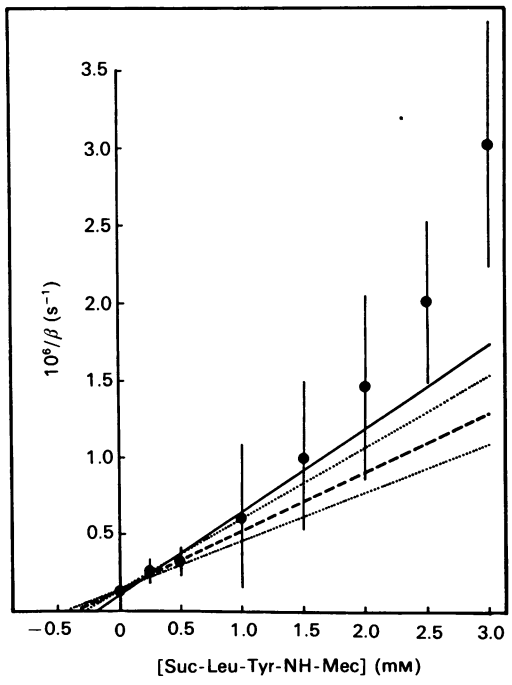


Fig. 4. Plot of the reciprocal of the association rate constant for inhibition of platelet calpain by HK as a function of substrate concentration

The continuous line was fitted by linear regression to these experimental data points by using the weighting function of $[(n-2)/(\text{S.E.M.})^2]$ for the data computed in Table 1. Vertical bars are standard errors for the experimental points. The dashed line indicates the expected theoretical result if the inhibition mechanism was competitive. The dotted lines on either side of the theoretical fit is at 1 S.D. for comparison with the experimental results.

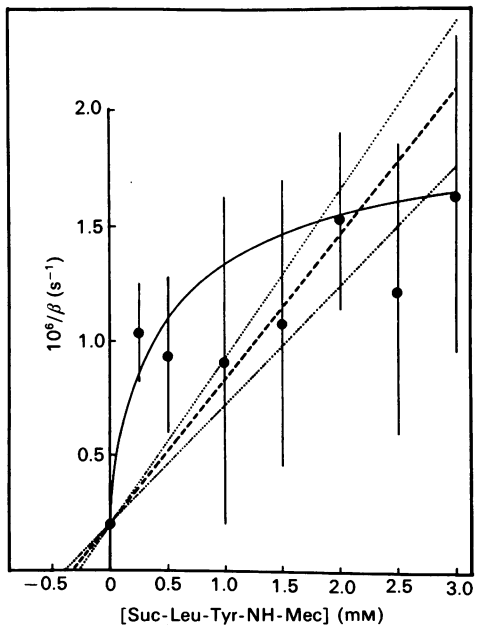


Fig. 5. Plot of the reciprocal of the association rate constant for inhibition of platelet calpain by LK as a function of substrate concentration

The continuous line indicates the best fit for a hyperbola for this data computed in Table 2. Vertical bars indicate the standard errors for these data. The dashed line indicates the expected theoretical result for competitive inhibition. The dotted lines on either side of the theoretical fit allow for 1 S.D. for comparison with the experimental results.

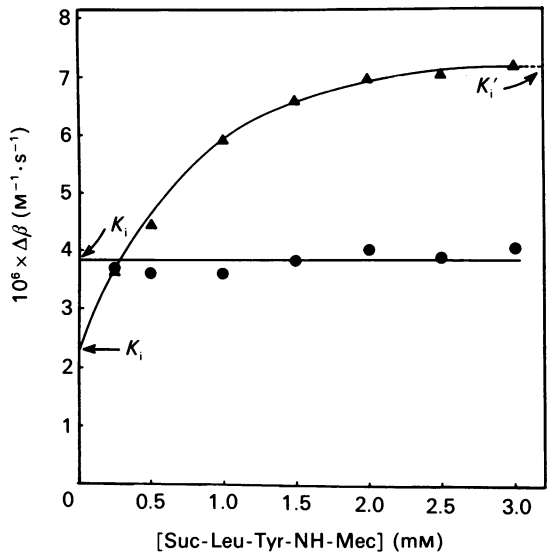


Fig. 6. Henderson plot of the change in slope ($\Delta\beta$) for platelet calpain inhibition by HK as a function of substrate

The ▲ symbols represent the values for HK from Table 1 and the ● symbols the values for LK from Table 2 plotted against the substrate concentrations. K_i is the value in the absence of substrate and K_i' is the value determined when the substrate is present. The line generated for LK indicates non-competitive inhibition. The plotted curve for HK indicates competitive inhibition for the values determined for low substrate concentrations. The flat line at high substrate concentration indicates that the inhibition becomes non-competitive as the substrate concentrations are increased (mixed non-competitive).

HK diagnostic plot

To further define the mechanisms of inhibition of calpain by HK, plot of $\Delta\beta$ versus [S] was constructed (Fig. 6). This plot is analogous to the diagnostic plot of slope versus substrate used by Henderson (1972). Competitive inhibition ($k_{+4} = 0$, $K_i = \infty$) is modelled by a straight line with a positive slope. Neither HK nor LK fits this model. Non-competitive inhibition is represented by straight line with a slope = 0 with $k_{+4} \neq 0$ and $k_i = K_i'$. LK fits this model. Mixed non-competitive inhibition is represented by a curve concave downward with a changing slope ($k_{+4} \neq 0$, $K_i \neq K_i'$). HK fits this model.

Calculation of K_i for LK and HK

From the values presented for α and β in Table 2, the calculation of K_i for LK is:

$$K_i = \frac{\alpha}{B_0} = \frac{1.28 \times 10^{-2}}{4.68 \times 10^6} = 2.7 \text{ nM}$$

The standard error of estimation is calculated by the formula:

$$\text{S.E.}(K_i) = [\text{S.E.M. } (\alpha)/\alpha] + [\text{S.E.M.}(B_0)/B_0]$$

Thus for LK the $\text{S.E.}(K_i) = K_i[(0.15/1.28) + (0.73/4.6)] = \pm 0.28$ nM.

For HK (Table 1) there are two values. $K_i = \alpha/\Delta\beta_0$, where α is the extrapolated value at [S] = ∞ and $\Delta\beta_0$ is the value extrapolated at [S] = 0 from Fig. 6, and is equal to $0.517 \times 10^{-2}/2.25 \times 10^6 = 2.3$ nM, with S.E. = ± 0.18 nM. K_i' is equal to $\alpha/\Delta\beta_\infty$, where α is the value at a saturating value of [S] determined for Fig. 6 and is equal to $0.517 \times 10^{-2}/7.237 \times 10^6 = 0.71$ nM. Thus for HK S.E. = ± 0.22 nM.

DISCUSSION

The discovery that the plasma kininogens are potent inhibitors of platelet calpain (Schmaier *et al.*, 1986*a*) indicates that these plasma proteins could modulate effects of calpain in the vascular compartment. Although we have found that HK (and probably LK) are localized in platelet α-granules (Schmaier *et al.*, 1983), the total contribution of HK from platelets (50 ng/10^8 platelets) is small relative to the amount of calpain (4500 ng/10^9 platelets) (Schmaier *et al.*, 1990). In fact, excess platelet calpain is capable of cleaving HK and increasing its coagulant activity (Schmaier *et al.*, 1986*b*). In contrast, in normal plasma HK (80 μg/ml), LK (160 μg/ml) and α_2-macroglobulin (2.5 mg/ml) are always present in excess of platelet calpain.

In a previous study we found that HK (Schmaier *et al.*, 1986*a*) is a more potent inhibitor of calpain than is LK or α_2-macroglobulin. These studies were performed with α-casein, a substrate that does not allow the performance of continuous rate assays. We therefore repeated the comparison between HK and LK using a dipeptide fluorescent substrate. In the present study we again find that both HK and LK have potent calpain-inhibitory function, but the IC_{50} values differ by less than 2-fold between the two kininogens. The difference, less than we previously reported (Schmaier *et al.*, 1986*a*), may be due to the more accurate quantification possible with a fluorescent substrate and a continuous rate assay. In addition, the previous preparation of LK (obtained as a gift from Dr. A. Barrett) was kinin-free. Perhaps proteolysis occurring during isolation or storage disrupted other structural components required for calpain inhibition. Our present preparations of LK contain kinin and showed no degradation products smaller than the native molecule.

Both HK and LK inhibit calpain, and thus their heavy chains must contain the structural information necessary for cysteine-proteinase inhibition. A study by Ishiguro *et al.* (1987) indicated that the molar ratio of HK to calpain II to give complete inhibition was 1:2, whereas for LK or isolated heavy chain the stoichiometry was 1:1. This observation was based on inhibitory capacity of kininogen and also by showing similar stoichiometry for forming non-covalent complexes on non-denaturing polyacrylamide gels. Our qualitative immunoprecipitation studies support these data, since antibody to HK that immunoprecipitated HK–calpain complex brought down more calpain than HK antigen (Fig. 1). The heavy chains of HK and LK are identical, and therefore the differences may lie in the conformation conferred by interaction with their respective light chains. To test this hypothesis, we subjected HK to cleavage with three different naturally occurring proteinases, each of which produces different light chains (Fig. 2 inset). Digestion with urinary kallikrein removes only bradykinin, leaving the entire 56 kDa light chain (Maier *et al.*, 1983). Plasma kallikrein removes another 8 kDa peptide from the *N*-terminal end of the light chain, yielding a 45 kDa light chain (Scott *et al.*, 1984). Factor XIa cleaves the light chain to smaller derivatives and in the process destroys the coagulant activity (Scott *et al.*, 1985). In the present study limited proteolytic digestion of HK permitted enough of the light chain to remain intact to preserve its modest contribution to calpain inhibition. Complete digestion would have resulted in, at the most, a 4-fold reduction in potency. None of these modifications in the light chain of HK appears to alter its calpain-inhibitory function appreciably (Fig. 2). A small change in the potency might be missed with the use of this method of determining the IC_{50}. These results contrast with those obtained by Higashiyama *et al.* (1986), who found kinin-free and fragments-1-and-2-free HK had greater papain-inhibitory function. However, that study used papain as a target

proteinase, and the inhibitory capacity of kininogens is markedly affected by differences in the proteinases targeted for inhibition.

We have, in the present study, carefully analysed the kinetics of inhibition of platelet calpain II by HK and LK. Initial studies used casein as a substrate (Fig. 3). The applicability of the Michaelis–Menten kinetics were tested by a re-plot of the slope of the inhibition curves from the Lineweaver–Burk plot against the concentration of HK. The resulting plot was non-linear (Morrison & Walsh, 1987). Previous studies of the cysteine proteinases papain and human cathepsins H and L showed that the inhibition by α_1-cysteine-proteinase inhibitor (LK) followed a tight-binding mechanism (Gounaris *et al.*, 1984). Furthermore, the active site of calpain shows a high degree of sequence similarity to that of papain (Ohno *et al.*, 1984). The kinetic analysis proposed by Cha (1976) was therefore chosen as a model of tight-binding inhibition. For further studies, this approach was facilitated by the use of a fluorescent substrate, allowing for continuous rate monitoring. Application of this method of analysis presented some difficulties. The kininogens are fast-reacting inhibitors of calpain. The short times observed for inhibition mandated performance of the kinetics under second-order association rate conditions. However, to prevent hydrolysis of HK by calpain, we had to perform the kinetics in inhibitor excess, but under the 10-fold excess necessary for classical inhibition measurements. These requirements yielded a narrow range for the kinetic determinations. If these inhibitors are reversible, this could not be detected by dilution. It is conceivable that, after dissociation, autolysis of the active enzyme occurred despite our precautions to avoid this reaction. However, the ability to electrophorese the complex without dissociation under non-denaturing conditions (Ishiguro *et al.*, 1987) and to immunoprecipitate the complex argues against easy reversibility (Fig. 1).

LK conformed to the example of non-competitive inhibition. Derived α (dissociation rate constant) and β values (K'') for LK were independent of substrate and relatively constant (Table 2). Moreover, a typical rectangular hyperbola resulted in a reciprocal plot of $1/\beta$ versus [S] (Fig. 5). The values of α for HK, however, showed dependence on substrate concentration, and thus differed from LK (Table 1). Likewise, the values of β were affected by substrate. The reciprocal plot (Fig. 4) did not give a typical rectangular plot for non-competitive inhibition; it deviated at higher substrate concentrations from the linear plot expected for competitive inhibition. Thus rigorous statistical analysis does not allow us to conclude that it is solely competitive under these conditions. Using a diagnostic plot of $\Delta\beta$ versus [S], we found that LK was non-competitive, whereas HK exhibited mixed non-competitive inhibition, indicative of HK having a different (competitive) affinity for the enzyme alone (K_i) than for the (non-competitive) enzyme–substrate complex (K_i'). The K_i for HK (2.3 nM) is comparable with that of LK (2.8 nM), reflecting their common heavy chain. However, the K_i' for HK determined in the presence of substrate is 0.71 nM, in close agreement with the results for inhibition of calpain with bovine HK in the presence of substrate, which was reported as 0.8 nM (Crawford, 1987). The tighter binding of calpain to HK than to LK in the presence of substrate may be due to a direct interaction with the light chain of HK or an additional binding site on the heavy chain available in HK, not LK, as the result of conformational changes in the heavy chain caused by the different light chains. The 1:2 (HK to calpain) stoichiometry for HK compared with 1:1 for LK may be occurring as a consequence of similar considerations. It should be noted that the comparison of HK and LK in the presence of substrate was performed with intact inhibitor protein. Therefore the influence of cleavage of the kininogens on the kinetic parameters of that reaction has not yet been tested.

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