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Research Article

Recent studies from our laboratory indicate that a high concentration of platelet-derived calcium-activated cysteine protease (calpain) can cleave high molecular weight kininogen (HMWK). On immunodiffusion and immunoblot, antiserum directed to the heavy chain of HMWK showed immunochemical identity with alpha-cysteine protease inhibitor--a major plasma inhibitor of tissue calpains. Studies were then initiated to determine whether purified or plasma HMWK was also an inhibitor of platelet calpain. Purified alpha-cysteine protease inhibitor, alpha-2-macroglobulin, as well as purified heavy chain of HMWK or HMWK itself inhibited purified platelet calpain. Kinetic analysis revealed that HMWK inhibited platelet calpain noncompetitively (Ki approximately equal to 5 nM). Incubation of platelet calpain with HMWK, alpha-2-macroglobulin, purified heavy chain of HMWK, or purified alpha-cysteine protease inhibitor under similar conditions resulted in an IC50 of 36, 500, 700, and 1,700 nM, respectively. The contribution of these proteins in plasma towards the inhibition of platelet calpain was investigated next. Normal plasma contained a protein that conferred a five to sixfold greater IC50 of purified platelet calpain than plasma deficient in either HMWK or total kininogen. Reconstitution of total kininogen deficient plasma with purified HMWK to normal levels (0.67 microM) completely corrected the subnormal inhibitory activity. However, reconstitution of HMWK deficient plasma to normal levels of low molecular weight kininogen (2.4 microM) did not fully correct the subnormal calpain inhibitory capacity […]

High Molecular Weight Kininogen Is an Inhibitor of Platelet Calpain

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Abstract

Recent studies from our laboratory indicate that a high concentration of platelet-derived calcium-activated cysteine protease (calpain) can cleave high molecular weight kininogen (HMWK). On immunodiffusion and immunoblot, antiserum directed to the heavy chain of HMWK showed immunochemical identity with alpha-cysteine protease inhibitor—a major plasma inhibitor of tissue calpains. Studies were then initiated to determine whether purified or plasma HMWK was also an inhibitor of platelet calpain. Purified alpha-cysteine protease inhibitor, alpha-2-macroglobulin, as well as purified heavy chain of HMWK or HMWK itself inhibited purified platelet calpain. Kinetic analysis revealed that HMWK inhibited platelet calpain noncompetitively ($K_i \simeq 5$ nM). Incubation of platelet calpain with HMWK, alpha-2-macroglobulin, purified heavy chain of HMWK, or purified alpha-cysteine protease inhibitor under similar conditions resulted in an IC_{50} of 36, 500, 700, and 1,700 nM, respectively. The contribution of these proteins in plasma towards the inhibition of platelet calpain was investigated next. Normal plasma contained a protein that conferred a five to sixfold greater IC_{50} of purified platelet calpain than plasma deficient in either HMWK or total kininogen. Reconstitution of total kininogen deficient plasma with purified HMWK to normal levels (0.67 μM) completely corrected the subnormal inhibitory activity. However, reconstitution of HMWK deficient plasma to normal levels of low molecular weight kininogen (2.4 μM) did not fully correct the subnormal calpain inhibitory capacity of this plasma. These studies indicate that HMWK is a potent inhibitor as well as a substrate of platelet calpain and that the plasma and cellular kininogens may function as regulators of cytosolic, calcium-activated cysteine proteases.

Introduction

The neutral calcium-activated cysteine proteases (calpains) are a group of cytosolic enzymes, distributed in many cells (1, 2), whose specific intracellular functions are not known. Recent studies indicate that these proteases are contained within platelets (3–6), and that they cleave the high molecular weight hemostatic cofactors, von Willebrand factor (7), fibrinogen (8), and factor V (9). Studies from our laboratory indicate that a platelet calcium-activated cysteine protease increases the surface-mediated activity of high molecular weight kininogen (HMWK)[1] as measured by coagulant assay (10). Furthermore, when nanomolar concentrations of ^{125}I-HMWK (120 kD) are incubated with platelet cytosol in the presence of Ca^{++}, the radiolabeled protein is proteolyzed into a 100-kD band as well as other cleavage products of smaller mass (10).

Recent studies suggest that the interaction of HMWK with tissue calpains may be more complex than simply serving as an additional substrate for platelet calpains. Studies by Ohkubo et al. (11) indicate that alpha-cysteine proteinase inhibitor—a major plasma protease inhibitor of cysteine proteases (12)—is identical in its amino acid sequence and immunochemical reactivity to plasma low molecular weight kininogen (LMWK). Moreover, Muller-Esterl et al. (13) and Sueyoshi et al. (14) have shown that kininogens functionally inhibit the cysteine proteases, papain and cathepsins B, G, H, and L. Because the synthesis of the plasma kininogens is controlled by one gene (15) and the heavy chain of HMWK is identical to the heavy chain of LMWK, both proteins should serve as inhibitors of cysteine proteases. Because the kininogens are inhibitors of papain and cathepsins (10), we hypothesized that both HMWK and LMWK could also be inhibitors of the calcium-requiring cysteine proteinases, the tissue calpains.

Alpha-cysteine protease inhibitor (LMWK) exists in plasma at a concentration of 2.4 μM (16). Alpha-2-macroglobulin, which has a plasma concentration of 3.5 μM (17), has also been described as an inhibitor of tissue calpains (1). In that HMWK has a plasma concentration of only 0.67 μM (18), its affinity for the calpains would have to be much greater than either LMWK or alpha-2-macroglobulin in order for it to serve as an important calpain inhibitor. Studies were initiated to determine the efficiency of HMWK as an inhibitor of platelet calpain and to determine its relative reactivity in both purified systems and plasma, as compared to alpha-cysteine protease inhibitor (LMWK) and alpha-2-macroglobulin. In this report, we present evidence that HMWK in plasma is a potent inhibitor of platelet calpain and that a new biological function for kininogens must be considered.

Methods

Materials. ^{14}C-methylated alpha-casein (0.82 μCi/mg) and Na ^{125}I (50 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Iodogen (Chloroamide, 1,3,4,6-tetrachloro-3alpha-6alpha-diphenylglycoluril was obtained from Pierce Chemical Co., Rockford, IL. Nitrocellulose paper, high and low molecular weight standards for polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS), and dithiothreitol (DTT) were purchased from Bio-Rad Corp., Richmond, CA. Polyethylene glycol (8,000) and kaolin were obtained from Fisher Scientific Co., King of Prussia, PA. Inosithin was purchased from Associated Concentrates, Woodside, NY. Sodium barbital buffer, pH 8.8, was purchased from Gelman Sciences, Inc., Ann Arbor, MI. Agarose (low electroendosmotic grade) was obtained from Marine Colloids, Inc., Rockland, ME. Rabbit anti-goat F(ab)$_2$ affinity-purified antibody adsorbed with normal human serum was purchased from Pel-Freez, Rogers, AR. Alpha-casein was purchased from Worthington Biochemical Corp., Freehold, NJ. Ultrogel AcA 34 was purchased from LKB Instruments, Rockville,

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1. *Abbreviations used in this paper:* DTT, dithiothreitol; HMWK, high molecular kininogen; LMWK, light molecular weight kininogen; PAGE, polyacrylamide gel electrophoresis.

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MD. E-64 *N*-[*N*-(L3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl] agmatine was purchased from Enzyme System Products, Livermore, CA. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, and were of the best reagent grade.

Plasmas and platelets. Pooled normal plasma (lot N10) was purchased from George King Biomedicals, Inc., Overland Park, KS. Total kininogen-deficient plasma (Williams plasma), i.e., plasma lacking in both HMWK and LMWK was donated by Mrs. M. Williams, Philadelphia, PA (19). HMWK-deficient plasma (19) (Fitzgerald plasma) was a generous gift of Dr. A. Scicli, Henry Ford Hospital, Detroit, MI. The value for total kininogen antigen was determined by electroimmunodiffusion (18) for the normal plasma and Fitzgerald plasma used in these studies and was found to be 352 μg/ml (4.92 μM) and 63 μg/ml (0.98 μM), respectively. Outdated (>72 h) normal platelet concentrates were donated by the Penn-Jersey Regional Red Cross, Philadelphia, PA.

Preparation of fresh platelet cytosol. 450 ml of fresh blood from one donor was collected into 73 mM citric acid, 3 mM trisodium citrate containing 2% dextrose (1:10) (ACD), from which platelet-rich plasma was obtained (20). After adjusting the pH to 6.5 with acid-citrate-dextrose, the platelet-rich plasma was washed twice by the centrifugation technique of Mustard et al. (21). The Tyrode's buffer, containing Ca^{++} (2 mM) and Mg^{++} (1 mM), had both apyrase and heparin (12.5 U/ml) in the first wash, and apyrase alone in the second wash. Apryase was prepared from potatoes by the method of Molnar and Lorand (22) and was titered so that only a minimal amount was used (prevention of second-wave platelet aggregation by ADP). The final washed pellet was resuspended in 1–2 ml of Hepes buffer (23) without bovine serum albumin and dextrose or 0.1 M Tris-Cl, 0.15 M NaCl, pH 7.4 (Tris-buffered saline) with or without 1 mM EDTA. These platelet preparations contained <0.05% red cells and <0.03% leukocytes (monocytes, granulocytes, and lymphocytes, combined). After making the platelet suspension hypotonic by addition of 0.5–1 ml of deionized water, the platelets were lysed by freezing (dry ice) and thawing (37°C) four times. The supernatants of the lysed platelets were collected, after centrifugation at 12,000 *g* in an Eppendorf centrifuge (Brinkmann Instruments, Inc., Westbury, NY), and ultracentrifuged at 100,000 *g* (model L3-50, Beckman Instruments, Inc., Palo Alto, CA) for 90 min at 4°C to collect platelet cytosol. Cytosol of lysed fresh platelets was used fresh, or stored at −70°C and used once after thawing. Assessment of cleavage of exogenous ^{125}I-HMWK by enzymes in platelet cytosol was performed on aliquots of the cytosol–^{125}I-HMWK mixture (25–50 μl) that were reduced by the addition of 2% β-mercaptoethanol at 100°C for 10 min. The samples were run on 7.5% SDS-PAGE (24) followed by autoradiography of the dried gel. Quantification of the extent of ^{125}I-HMWK cleavage was performed by densitometer scan of the autoradiograms using a SD3000 Spectrodensitometer (Schoeffel Instrument Division, Kratos, Inc., Westwood, NJ). The relative peak areas were quantified by weighing paper tracings of the peaks outlined by the recorder.

Functional assays. HMWK was assayed by one-stage kaolin-activated coagulant assay using total kininogen-deficient plasma as substrate, as previously reported (18). 1 U was defined as that amount in 1 ml of pooled normal plasma.

The calcium-activated cysteine protease (calpain) was measured by its ability to hydrolyze alpha-casein in a modified assay as described by Waxman and Krebs (25). The casein substrate was prepared such that its final concentration in the assay was 5 mg/ml in 0.05 M Tris-Cl, pH 7.5, containing 3 mM DTT. An assay volume of 200 μl containing the above substrate and sample was used. 25 μl of enzyme sample to be measured was added at 0° and then warmed to 25°C in a regulated water bath for 2–3 min. The hydrolysis reaction was started by adding 20 mM $CaCl_2$ (4 mM final concentration) and the samples were incubated for 60 min at 25°C with intermittent mixing. The addition of 20 mM EDTA instead of $CaCl_2$ served as a blank. The reaction was terminated by precipitation with 0.45 ml of ice-cold 4% perchloric acid and the samples were mixed and kept on ice prior to centrifugation at 12,000 *g*. The amount of alpha-casein hydrolysis was determined by the absorbance of the supernatant at 280 nm, after its EDTA-blank had been subtracted. 1 U/ml of enzyme activity was arbitrarily defined as the amount of enzyme that increased the absorbance at 280 nm 1.000 OD/h · ml. The hydrolysis of unlabeled alpha-casein was used only to monitor the enzyme during the purification procedure.

A modified ^{14}C-methylated alpha-casein radiometric assay (26) was employed to measure the effect of various substances on the calcium-activated cysteine protease. Enzyme at 25°C was added to a tube containing 0.05 M Tris-Cl, pH 7.5, DTT (3 mM), EDTA (2 mM), and potential inhibitors prior to a precise 2-min incubation. The residual enzymatic activity was then monitored with the ^{14}C-methylated alpha-casein. The addition of the EDTA during the enzyme–inhibitor interaction greatly reduced the autoinactivation of calpain. In studies with E-64, EDTA was excluded from the incubation mixture of the enzyme with inhibitor, because free Ca^{++} was essential to observe maximal inhibitory activity (27, 28). The radiometric assay was identical to the cold assay with the following exceptions: the assay of residual enzymatic activity included 5 mM Ca^{++}; unlabeled alpha-casein was present at 1 mg/ml, and ^{14}C-methylated alpha-casein (20,000 dpm), which was <1% of the total alpha-casein present, was included in the reaction tube. Background values for each incubation were obtained by substituting EDTA for calcium. After a 1-h incubation, the reaction and background samples were precipitated with perchloric acid (10% final concentration) in the presence of 1.25 g% bovine serum albumin. After precipitation and centrifugation, the supernatants (300–600 μl) were added to 10 ml of scintillation fluid (Liquicent, National Diagnostics, Somerville, NJ). After the background counts were subtracted from the total activity for each point, the percent inhibition for each potential inhibitor was determined by the formula, 1 minus the ratio of the enzyme activity in the inhibitor-treated sample to the enzyme activity in the absence of inhibitors multiplied by 100.

In studies to determine the kinetic mechanism of HMWK inhibition of calpain, hydrolysis of the substrate by enzyme in the absence or presence of HMWK proceeded for 10 min before precipitation with perchloric acid. The K_i of HMWK inhibition of platelet calpain was determined by a secondary plot of the slope of the curve of the double-reciprocal plot at each inhibitor concentration versus inhibitor concentration and by a computer program (29) using a TRS-80 model 4P (Tandy Corporation, Fort Worth, TX).

Proteins. HMWK was purified by a modified procedure (10) of Kerbiriou and Griffin (30) which included 0.2 M epsilon-aminocaproic acid in all buffers and 2 mM diisopropylflurophosphate added to the pooled material prior to each step. This preparation of HMWK on reduced 8% SDS-PAGE (24) was mostly a single band of 120 kD with >98% purity and had a specific activity of 12–20 U/mg. Purified HMWK was radiolabeled with ^{125}I using Iodogen, by a procedure previously reported (18).

The heavy (64 kD) and intermediate light chain (56 kD) of HMWK were purified by a modified procedure of Kerbiriou and Griffin (30). After a 16-h incubation of HMWK (120 kD) with purified human urinary kallikrein (a generous gift by Dr. Harry Margulies, University of South Carolina, Charleston, SC), the cleaved HMWK was alkylated with 120 mM iodoacetamide followed by reduction with 50 mM DTT. The sample was applied to a SP Sephadex column in 0.01 M sodium acetate, 0.08 M NaCl, pH 5.4. The alkylated and reduced heavy chain of HMWK was not absorbed to the cation exchanger. The 56-kD light chain was eluted with a linear NaCl gradient from 0.08 to 0.5 M. The specific activity of the purified 56-kD light chain of HMWK was 7.8 U/mg.

Purified alpha-cysteine protease inhibitor (1 mg/ml) was a generous gift of Drs. G. Salvesan and A. Barrett, Cambridge University, England. Purified Cl inhibitor, with a concentration of 1.02 mg/ml (9.7 μM) was prepared as previously reported (31). Purified alpha-1-antitrypsin (4 mg/ml, 64.5 μM) was a generous gift of Dr. Charles Glaser, Pacific Medical Center, San Francisco, CA (32). Partially purified alpha-2-macroglobulin was a generous gift of Dr. Marc Schapira, Hôpital Cantonal, Geneva, Switzerland. As previously reported, this alpha-2-macroglobulin was 90% pure with a concentration of 1.43 antigen U/ml (2.6 μM) (33). One antigen unit of alpha-2-macroglobulin is the amount of alpha-2-macroglobulin contained in 1 ml of pooled normal plasma (lot N10, George King Biomedicals).

Purification of platelet calpain. Platelet calcium-activated cysteine

protease (calpain) was purified from washed platelet concentrates suspended in 0.1 M Tris-Cl pH 7.5 containing 5 mM EDTA and 3 mM DTT. After sonication twice at 0° for 10 s on a Branson sonicator (Ultrasonics Inc., Plainview, NY), the lysate was ultracentrifuged at 100,000 *g* (model L3-50, Beckman Instruments, Inc.) for 60 min at 4°. The cytosol was then precipitated with polyethylene glycol (8,000), final concentration 3%. The resulting supernatant was reprecipitated with polyethylene glycol, final concentration 20%. The second pellet, resuspended in 0.05 M Tris-Cl, 0.05 M NaCl, pH 7.5, containing 5 mM EDTA, and 2 mM DTT, was applied to a DE-52 ion exchange column (2.6 × 21 cm); the enzyme was eluted with a linear salt gradient from 0.05 M NaCl to 0.5 M NaCl. Fractions containing the enzyme were concentrated by precipitation with 20% polyethylene glycol and resuspended in 0.05 M Tris-Cl, 0.5 M NaCl, containing 5 mM EDTA and 2 mM DTT prior to application on a reactive red agarose 120 column (0.8 × 40 cm). The enzyme was eluted with 0.05 M Tris-Cl, pH 7.5, containing 5 mM EDTA and 2 mM DTT. The final step of purification was gel filtration on a Ultrogel AcA 34 (55 × 0.8 cm) column in the same buffer (34).

The purified protein was analyzed on reduced SDS-PAGE (19) and on nondenaturing PAGE (35). The calcium concentrations at a given pH used for activation of purified platelet calpain were determined by having EDTA in the suspension buffer. Known quantities of calcium were added and the free calcium concentration was calculated by a computer program (36, 37) on a TRS-80 Model III (Tandy Corporation, Fort Worth, TX), using all known equilibrium constants for all of the ionic species in the calpain suspension buffer, 0.05 M Tris-Cl, pH 7.5, containing 4 mM EDTA and 10 mM $MgCl_2$.

Protein assays were performed by the methods of Bradford (38), Lowry et al. (39), and Ross and Schatz (40) using crystalline bovine serum albumin as the standard.

Immunochemical assays. Goat antibody and antiserum directed to the light chain of HMWK (18) and to both the heavy and light chains of HMWK (41) were prepared as previously reported (18, 41). Immunodiffusion in 1% agarose was performed as previously described (18, 41). The alpha-2-macroglobulin concentration of normal plasma, Fitzgerald plasma, and Williams plasma was determined by radial immunodiffusion (31, 33).

Immunoblots were performed by a modified technique of Towbin et al. (42). Samples for electroblotting, nonreduced or reduced with 2% β-mercaptoethanol, were run on an 8% SDS-PAGE, and the protein was subsequently transferred to nitrocellulose by electroblotting for 16 h at 4°C at 40 V using a running buffer of 25 mM Tris-Cl, 192 mM glycine, pH 8.3, containing 20% methanol. After electroblotting, the nitrocellulose was incubated with 4% radioimmunoassay grade bovine serum albumin in 0.01 M Tris-Cl, 0.15 M NaCl, pH 7.5, containing 1 mM EDTA on a rotating shaker at 23°C for 1 h. At the end of the hour, the bovine serum albumin solution was made 0.05% by the addition of Tween-20. After a 5-min incubation, anti-HMWK (18) or anti-total kininogen antibody (41) (i.e., antibody that recognizes both HMWK and LMWK) or antiserum was added to the bovine serum albumin solution with Tween-20 and incubated for 2 h on a rotating shaker at 23°C. After incubation, the paper was washed four times with 0.01 M Tris-Cl, 0.15 M NaCl, pH 7.5, containing 0.05% Tween-20. An affinity-purified ^{125}I-rabbit anti-goat antibody in the same buffer was incubated with the nitrocellulose paper on a rotating shaker for 2 h at 23°C. The rabbit anti-goat antibody was radiolabeled with ^{125}I using Iodogen by a technique previously reported for radiolabeling HMWK (18). After this, the nitrocellulose paper was washed four times in the same buffer containing 0.2% bovine serum albumin followed by two washes containing buffer without albumin. The nitrocellulose was stained with 0.1% amidoblack in 45% methanol and 7% acetic acid. After drying, autoradiography of the dried nitrocellulose was performed.

Results

Inhibition of cleavage of ^{125}I-HMWK by calpain in platelet cytosol by excess unlabeled HMWK. Platelet cytosol from 3.1 × 10^9 platelets/ml not pretreated with inhibitors produced proteolysis of the ^{125}I-HMWK (7.5 nM) with the appearance of bands at 100 and 75 kD (Fig. 1). Densitometer scan of a submaximally exposed autoradiogram revealed that 33% of the ^{125}I-HMWK was cleaved in the untreated platelet cytosol as compared with 6% cleaved ^{125}I-HMWK in the control incubation mixture. The majority of the cleaved ^{125}I-HMWK was in a distinct 100-kD band. However, when the platelet material was pretreated with either leupeptin (0.5 mM) or unlabeled HMWK (2 μM), the 100-kD band from ^{125}I-HMWK was not seen although the 75-kD band was still present. The leupeptin-treated platelet cytosol had 6% cleaved ^{125}I-HMWK, similar to the control; the unlabeled HMWK-treated platelet cytosol had 12% cleaved ^{125}I-HMWK. This latter finding suggested that in addition to being a substrate for platelet calpain, HMWK may also be an inhibitor.

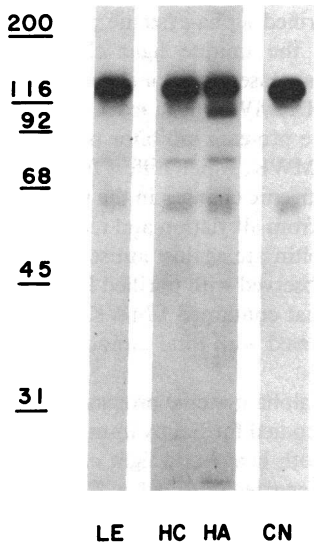

Figure 1. Inhibition of cleavage of ^{125}I-HMWK by calpain in platelet cytosol by unlabeled HMWK. A mixture of platelet cytosol (8.5 vol) from lysed platelets (3.1 × 10^9 platelets/ml) and 1.5 vol of 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, was incubated for 10 min at 37°C in the presence of 0.5 mM leupeptin (*LE*) or 2 μM unlabeled HMWK (*HC*), or left untreated (*HA*). At the end of the incubation, 7.5 nM of ^{125}I-HMWK was introduced into each aliquot (*LE, HC, HA*). After incubation for 1 h at 37°C, the reaction was stopped by the introduction of 4% SDS containing 2% β-mercaptoethanol and boiled for 10 min. Lane *CN* is a control incubation of ^{125}I-HMWK introduced into buffer. 25 μl from each sample was applied to an 8% SDS-PAGE for electrophoresis. After drying, autoradiography was performed for 24–60 h. The numbers to the left represent molecular weight standards in kilodaltons.

Immunochemical identity of alpha-cysteine protease inhibitor with plasma kininogens. Using antiserum directed against total kininogen (both HMWK and LMWK), purified alpha-cysteine protease inhibitor produced a precipitin arc that showed identity with HMWK (Fig. 2). However, HMWK showed an additional

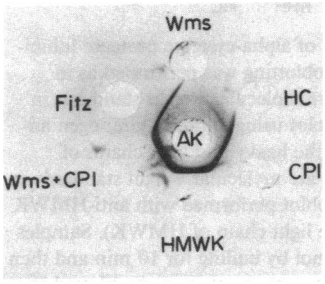

Figure 2. Immunodiffusion with antikininogen antisera. Plasma or purified proteins were added to wells (10–20 μl/well) in 1% agarose gel in sodium barbital buffer, pH 8.8, $\tau/2 = 0.0375$. Immunodiffusion was allowed for 24 h at 23°C. After 48 h of soaking in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4, the plate was dried and stained with 0.5% Coomassie brillant blue R-250 and 1.0% amidoblack in methanol-acetic acid H_2O (5:5:1). *AK*, 10 μl of antikininogen antiserum; *Wms*, 10 μl of total kininogen-deficient plasma (Williams plasma); *HC*, purified heavy chain of HMWK; *CPI*, 10 μg of purified alpha-cysteine protease inhibitor; *HMWK*, 25 μg of purified HMWK; *Wms + CPI*, 10 μl of total kininogen-deficient plasma and 10 μg of purified alpha-cysteine protease inhibitor; *Fitz*, 10 μl of HMWK-deficient plasma (Fitzgerald plasma).

spur of partial identity to the purified alpha-cysteine protease inhibitor, probably representing the unique light chain of HMWK. In turn, alpha-cysteine protease inhibitor showed partial identity to the heavy chain of HMWK. The extra spur of partial identity with alpha-cysteine protease inhibitor probably resulted from the light chain of LMWK, which differs from the light chain of HMWK, and/or antigenic changes in the purified heavy chain of HMWK resulting from alkylation and reduction in its preparation. A single precipitin arc against antiserum directed to plasma kininogen was observed with purified HMWK and Fitzgerald plasma (plasma that contained LMWK but no HMWK). No precipitin arc occurred with total kininogen-deficient plasma.

The immunoidentification of alpha-cysteine protease inhibitor with plasma kininogens was studied further by immunoblot (Fig. 3). Antiserum directed to both heavy and light chains of HMWK (*anti-kininogen Ab*) recognized purified and plasma HMWK (120 kD) as well as purified light chain of HMWK (56 kD) and purified alkylated and reduced heavy chain of HMWK (64 kD) (Fig. 3 *left*). Likewise, this antiserum recognized purified alpha-cysteine protease inhibitor which gave a broad band between 60 and 65 kD. Because there was some overlap of the band of alpha-cysteine protease inhibitor on this gel electrophoresis system with the purified 56-kD light chain of HMWK, studies were performed to determine if the light chain of HMWK contaminated the alpha-cysteine protease inhibitor preparation. Because the light chain accounts for all of the coagulant activity, the finding that this preparation of alpha-cysteine protease inhibitor had <0.01 U/ml coagulant activity indicated that the HMWK light chain was not present. The absence of the light chain of HMWK in the alpha-cysteine protease inhibitor preparation was confirmed by further immunoblot studies with antiserum uniquely directed to the light chain of HMWK (Fig. 3 *right*). Antiserum directed to the light chain of HMWK recognized purified HMWK, HMWK in normal plasma, and purified 56-kD light chain of HMWK. The purified heavy chains from either HMWK or alpha-cysteine protease inhibitor were not recognized by antiserum directed to the light chain of HMWK. These combined immunoblot studies indicated that purified alpha-cysteine protease inhibitor was immunochemically similar to the common heavy chain of HMWK and LMWK.

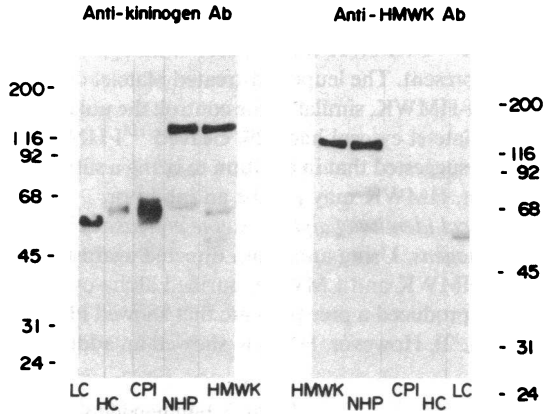

Figure 3. Immunoblot identification of alpha-cysteine protease inhibitor with plasma kininogens. Immunoblotting was performed as in Methods. The numbers on the *left* are molecular weight standards in kilodaltons referring to the immunoblot using antitotal kininogen antiserum (antiserum directed to both the heavy and light chains of HMWK). The numbers on the *right* are molecular weight standards in kilodaltons that refer to the immunoblot performed with anti-HMWK antiserum (antiserum directed to the light chain of HMWK). Samples were reduced in 2% β-mercaptoethanol by boiling for 10 min and then run on 8% SDS-PAGE. Protein on the gel was then electroblotted to nitrocellulose (see Methods). Immunoidentification was performed on nitrocellulose paper. *HMWK*, 0.25 μg of purified HMWK added to gel; *NHP*, 1 μl of normal human plasma; *CPI*, 1 μg of purified alpha-cysteine protease inhibitor; *HC*, 0.23 μg of reduced and alkylated purified heavy chain of HMWK; *LC*, 0.20 μg of purified 56-kD light chain of HMWK. After the immunoblot, the dried nitrocellulose paper was exposed to x-ray paper for autoradiography for ≥60 h. The present gel is one representative experiment of two.

Table I. Purification of Platelet Calpain

	Total activity	Total protein	Specific activity	Purification	Recovery
	U	*mg*	*U/mg*		%
Sonicated washed platelets	1,500	2,338	0.64	1	100
100,000 *g* supernatant	1,497*	1,271	1.18*	1.8	99
3% PEG‡ supernatant	1,346	990	1.36	2.1	90
20% PEG‡ resuspension	594	301	1.97	3.1	40
DE-52	580	62	9.35	14.6	39
Reactive red agarose	301	11.3	26.5	41.4	20
Ultrogel AcA 34	293	4.8	60.0	94	19.5

* The activity and specific activity in this fraction is uncorrected for the possible presence of the endogenous inhibitor, calpastatin (2).
‡ PEG, polyethylene glycol 8000.

Purification of platelet calpain. Human platelet calpain was purified from platelet concentrates 94-fold with a final yield of 19.5% (Table I). The exact degree of purification was difficult to assess in that an inhibitor, calpastatin, (2) is present in the sonicate. The specific activity of the enzyme was 60 U/mg. On nondenaturing 10% PAGE without SDS, this purified protein was 90% pure, as determined by densitometric scan of the stained gel (Fig. 4). On a 10% SDS-PAGE, 75% of the protein on densitometric gel scan was associated with a major band 80 at kD and a minor band at 30 kD (Fig. 4). Minor bands (<25% of the protein) were recognized at 140–160 kD. Both the 80- and 30-

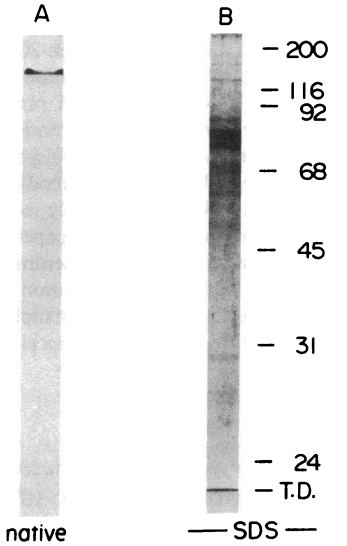

Figure 4. Gel electrophoresis of purified platelet calpain. (*A*) 10 μg of purified platelet calpain was applied to a 10% alkaline PAGE (native) and electrophoresed at 25 mA for 5 h at 4°C. (*B*) 10 μg of purified platelet calpain, after reduction with 2% β-mercaptoethanol and boiling for 10 min, was subjected to 10% SDS-PAGE (SDS) and electrophoresed at 30 mA for 4 h at 23°C. Both gels were stained with Coomassie blue. The numbers to the right of the gel represent molecular weight standards in kilodaltons.

kD bands comigrated with the fractions containing the protease activity on the gel filtration column.

Characterization of platelet calpain. The calcium dependence of the purified calpain was evaluated over a range of 10–1,300 μM free calcium (Fig. 5). The EC_{50} was 150 μM free Ca^{++} and activation was negligible below 100 μM (Fig. 5). Activation of the enzyme was maximal at 500 μM free calcium. The finding that the purified platelet calpain had the greatest activity at ~500 μM free calcium indicated that platelet calpain was a calpain II (2). In this study, magnesium was used to prevent a pH change owing to the liberation of H^+ from EDTA as calcium was added. However, when Mg^{++} was omitted, the pH changed only 0.15 U and the EC_{50} for Ca^{++} was similar.

Studies with inhibitors were performed to further characterize the enzyme as a calcium-activated cysteine protease (Table II). Both EDTA and EGTA, individually, at 0.55 mM completely neutralized the enzymatic activity, further indicating that it was specifically calcium-dependent. Sulfhydryl blocking agents, $HgCl_2$ (3 mM) and iodoacetamide (1 mM), as well as bacterial protease inhibitors, leupeptin (10 mM) and antipain (33 μM), were excellent inhibitors. In the presence of 0.88 mM free Ca^{++}, 0.80 μM E-64, an active site directed inhibitor, inactivated 96% of the enzyme's activity in 20 min. These investigations indicated that the enzyme had a free thiol group exposed by Ca^{++}, essential for its activity, similar to the requirement noted previously (27, 28). In contrast, little inhibition was found by the serine protease inhibitors, benzamidine (10 mM), phenylmethylsulfonylfluoride (1 mM), aprotinin (0.15 mM), and diisopropylfluorphosphate (5 mM). These studies support the conclusion in Fig. 5 that the enzyme is a calpain II.

Inhibition of platelet calpain by purified plasma proteins. The effect of various plasma proteins on the activity of platelet calpain was investigated (Table III). Purified plasma proteins, at a final concentration of 200 nM were incubated with platelet calpain. Alpha-cysteine protease inhibitor, as well as purified heavy chain of HMWK, inhibited platelet calpain 11% and 23%, respectively. Purified alpha-2-macroglobulin inhibited 29% of the platelet calpain activity whereas purified C1 inhibitor (214 nM), alpha-1-antitrypsin (3,200 nM), and purified 56-kD light chain of HMWK produced little inhibition. However, HMWK produced 93% inhibition of the enzyme activity. This latter finding suggested that HMWK, as well as alpha-cysteine protease inhibitor (LMWK) and alpha-2-macroglobulin, were plasma inhibitors of platelet calpain. Purified HMWK was a true inhibitor of platelet calpain. The presence of HMWK noncompetitively inhibited platelet calpain with an apparent K_i of 5.15 nM±0.61 (mean±SEM) (data not shown) as analyzed from a double-reciprocal Lineweaver–Burk plot.

Further studies were performed with purified proteins in order to approximate their relative inhibitory potency of platelet calpain (Fig. 6). HMWK, at 50% normal plasma concentration (333 nM), inhibited 95% of the enzyme activity whereas alpha-2-macroglobulin, at 50% normal plasma concentration (1.7 μM), and alpha-cysteine protease inhibitor, at normal plasma concentration (2.4 μM), only inhibited 72% and 58%, respectively, of the platelet calpain activity. Under these conditions, the IC_{50} of HMWK for platelet calpain was 36 nM. Unlike some calpain inhibitors which react with the free sulfhydryl group at the enzyme active site (27, 28), the ability of HMWK, at any concentration, to inhibit platelet calpain was not influenced by the

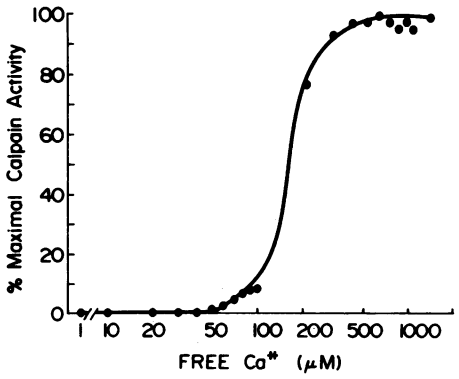

Figure 5. Calcium activation curve for platelet calpain. Purified platelet calpain was activated by the addition of increasing concentrations of free calcium from 10 to 1,300 μM in 0.05 M Tris-Cl, pH 7.5, containing 4 mM EDTA and 10 mM $MgCl_2$. Using the known equilibrium constants for each of the ionic species in this buffer at this pH (34), the concentration of the free calcium needed to activate the enzyme was calculated by a computer program (35).

Table II. Inhibition of Platelet Calpain

Inhibitor*	% inhibition
EDTA (0.55 mM)‡	100
EGTA (0.55 mM)‡	100
Leupeptin (10 μM)	96
Leupeptin (1 μM)	68
$HgCl_2$ (3 mM)	100
Iodoacetamide (1 mM)	91
Antipain (33 μM)	83
Phenylmethylsulfonyl Fluoride (1 mM)	16
Benzamidine (10 mM)	23
Aprotinin (0.15 mM)§	0
Diisopropylflurophosphate (5 mM)§	0

* Each inhibitor, at the concentration indicated in parenthesis, was incubated for 2 min with the purified platelet calpain at room temperature prior to assay in the presence of 2 mM EDTA.
‡ Values represent excess chelator (mM) after the enzyme was stabilized to prevent loss of activity during the 2-min incubation.
§ The inhibitor was preincubated for 10 min with the purified platelet calpain at 25°C.

Table III. Inhibition of Platelet Calpain by Purified Plasma Proteins

Protein*	% inhibition
Alpha-cysteine protease inhibitor	11
Heavy chain HMWK (64 kD)	23
Light chain HMWK (56 kD)	<2
High molecular weight kininogen	93
Alpha-2-macroglobulin	29
C1 inhibitor	<2
Alpha-1-antitrypsin	<2

* Each protein was tested at a concentration of 200 nM, except for alpha-1-antitrypsin which was 3,200 nM. Incubation with platelet calpain was for 2 min at 25°C.

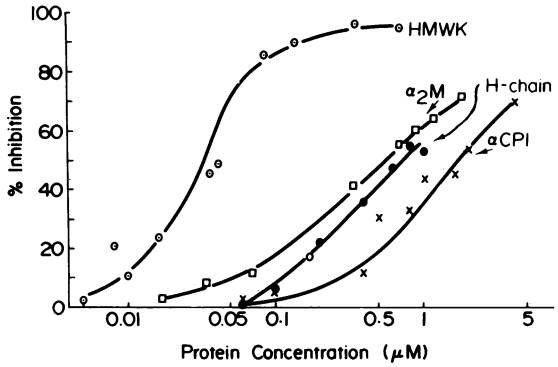

Figure 6. Comparison of purified HMWK, alpha-cysteine protease inhibitor and alpha-2-macroglobulin inhibition of platelet calpain. Purified platelet calpain in the presence of 2 mM EDTA was incubated with various concentrations of purified HMWK (⊙); purified alpha-2-macroglobulin (□), purified heavy chain of HMWK (●); or purified alpha-cysteine protease inhibitor (×) for 2 min at room temperature. Each point represents the mean of two to four experiments at each concentration with the purified protein.

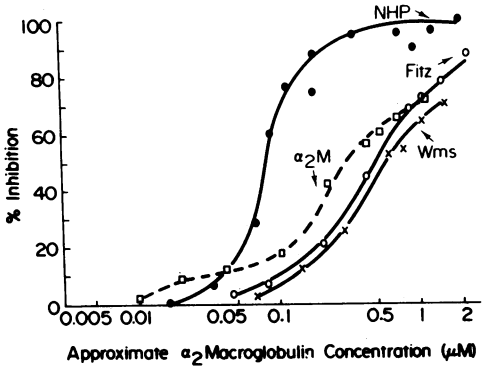

Figure 7. Comparison of plasma inhibition of platelet calpain. The approximate alpha-2-macroglobulin concentration in normal plasma, Fitzgerald plasma, and Williams plasma were determined by radial immunodiffusion and compared with purified alpha-2-macroglobulin. Dilutions of plasma were made such that the final approximate alpha-2-macroglobulin concentration were the same. Each dilution of the plasma, as well as purified alpha-2-macroglobulin at a similar concentration, was incubated with the same amount of platelet calpain in the presence of 2 mM EDTA for 2 min at 25°C. Each point represents the mean of two to five experiments at each approximate alpha-2-macroglobulin concentration with the purified protein or each plasma. (●) Normal human plasma; (□), alpha-2-macroglobulin; (○), Fitzgerald plasma; and (×), Williams plasma.

presence of free Ca^{++} in the incubation mixture. The IC_{50} for other purified plasma proteins was 500 nM for alpha-2-macroglobulin, 1,700 nM for alpha-cysteine proteinase inhibitor (LMWK), and 700 nM for isolated heavy chain of HMWK (identical to the heavy chain of LMWK). In contrast to HMWK, the ability of alpha-cysteine protease inhibitor (LMWK) to inhibit platelet calpain was increased twofold by the presence of free Ca^{++} in the incubation mixture. Because the 50% inhibitory concentration for HMWK resulted from only 5% of normal plasma concentration, and the IC_{50} for alpha-2-macroglobulin and heavy chain of HMWK were 15% and 30%, respectively, of normal plasma concentrations, purified HMWK was predicted to be at least threefold as potent an inhibitor of platelet calpain than either purified alpha-2-macroglobulin or alpha-cysteine protease inhibitor (LMWK) in plasma.

Inhibition of platelet calpain by plasma. Further studies were performed to assess the effect of HMWK on platelet calpain in a plasma environment (Fig. 7). Because alpha-2-macroglobulin has been shown to inhibit platelet calpain (1), we diluted normal human plasma, total kininogen-deficient plasma (Williams plasma), and high molecular weight kininogen-deficient plasma (Fitzgerald plasma) so that their alpha-2-macroglobulin concentration was 1,000 nM prior to studying each plasma's ability to inhibit the enzymatic activity of purified platelet calpain. At 1,000 nM alpha-2-macroglobulin concentration, normal plasma inhibited 97% of the caseinolytic activity of platelet calpain, whereas purified alpha-2-macroglobulin or Fitzgerald plasma at the same alpha-2-macroglobulin concentration inhibited only 72% of the activity of platelet calpain. Total kininogen-deficient plasma (Williams plasma) had a similar extent of platelet calpain inhibition (66%). However, the IC_{50} of normal human plasma fell on the curve at a concentration of alpha-2-macroglobulin that was equivalent to 80 nM, whereas the IC_{50} of purified alpha-2-macroglobulin, Fitzgerald plasma, and Williams plasma was between 300 and 600 nM alpha-2-macroglobulin concentration. These findings indicated that normal plasma must contain an additional inhibitor of platelet calpain other than alpha-2-macroglobulin and alpha-cysteine protease inhibitor (LMWK). Further studies showed that treatment of normal plasma with 0.2 M methylamine—a chemical inhibitor of alpha-2-macroglobulin (43)—did not change the IC_{50} of normal plasma on platelet calpain (data not shown). These findings indicated that alpha-2-macroglobulin was not the major inhibitor of this enzyme in plasma. Because both Williams and Fitzgerald plasmas displayed weaker inhibitory activity towards platelet calpain than normal plasma, the additional inhibitor, therefore, appeared to be HMWK inasmuch as it was the common missing factor in both plasmas.

To test this hypothesis, the inhibitory capacity of normal human plasma for platelet calpain was directly compared with the inhibitory activity of purified HMWK (Fig. 8) under similar

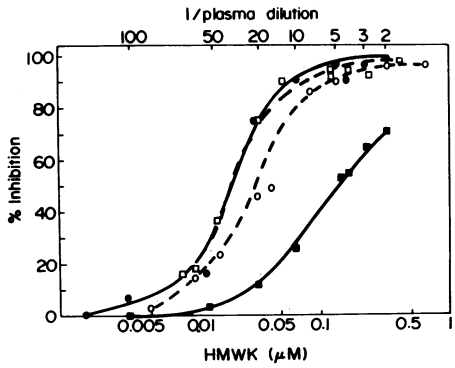

Figure 8. Effect of HMWK (purified or plasma) on inhibition of platelet calpain. The inhibition of platelet calpain by normal human plasma (●——●), purified HMWK (○ - - - ○), total kininogen-deficient plasma (Williams) reconstituted with 80 μg/ml (0.67 μM) of purified HMWK (□ - - - □) and total kininogen-deficient plasma alone (■——■) were compared. The concentration of purified HMWK (μM) is on the bottom abscissa, and the plasma dilutions (normal and total kininogen-deficient) are labeled on the top abscissa. Each point represents the mean of two to five determinations.

conditions. Purified HMWK was found to have an IC_{50} of 34 nM whereas normal human plasma had an IC_{50} of 23 nM, when calculated on the basis of its HMWK content. Likewise, when the IC_{50} of normal plasma was compared with an equal dilution of total kininogen-deficient plasma, we found that it had sixfold greater inhibitory activity than total kininogen-deficient plasma (Fig. 8). Total kininogen-deficient plasma that was reconstituted with purified HMWK (0.67 μM) displayed an IC_{50} of 20 nM, which was similar to what was observed with normal plasma. This correction of inhibitory activity contrasted with the minimal enhancement of inhibition when Fitzgerald plasma (1 μM concentration LMWK) was reconstituted with purified heavy chain of HMWK (LMWK) (data not shown) to normal levels of 2.4 μM (16). The IC_{50} on platelet calpain increased about twofold, equivalent to about a 1:10 to 1:20 dilution of plasma although the total concentration of kininogen (0.1–0.12 μM) contributing to the IC_{50} were similar. However, the IC_{50} of reconstituted Fitzgerald plasma with LMWK was twofold less by plasma dilution and threefold greater in kininogen concentration than the increased inhibitory capacity of reconstituted total kininogen-deficient plasma with 0.67 μM purified HMWK alone (Fig. 8). This study indicated that LMWK was not the major plasma inhibitor of platelet calpain. Therefore, these combined studies suggested that HMWK is the major plasma inhibitor of platelet calpain.

Discussion

The finding (Fig. 1) that the plasma kininogens interact with the calcium-activated neutral protease (calpain) suggests a new functional role for the proteins of the contact phase of plasma proteolysis. In support of the findings of Ohkubo et al. (11) and Muller-Esterl et al. (13), both plasma HMWK and LMWK show complete identity with alpha-cysteine protease inhibitor on immunodiffusion (Fig. 2). We have extended these studies using immunoblotting (Fig. 3) to show that the heavy chains of both HMWK and LMWK also were immunochemically similar to alpha-cysteine protease. The lack of intensity of plasma LMWK on the immunoblot of normal plasma with the antikininogen antiserum was expected in that this antiserum was raised by injection of purified HMWK and is mostly directed to the light chain of HMWK. It was also not surprising that an alkylated and reduced heavy chain of HMWK that was produced by the kallikrein cleavage of whole HMWK only showed partial immunochemical identity to native alpha-cysteine protease inhibitor (LMWK) and purified HMWK (Fig. 2), because the unique light chain of each kininogen form is not present in the heavy chain preparation. However, the effects of alkylation and reduction on the heavy chain of HMWK were such that they did not interfere with the ability of the purified molecule to inactivate purified platelet calpain, similar to the native LMWK itself (Fig. 6).

The purified platelet calpain is similar to what has been previously reported (5, 6), having two subunits, 80 and 30 kD, similar to that described in rat kidney (44). Unlike the findings of Sakon et al. (4), we found only one form of platelet calpain that responded to mM concentrations of free calcium, which would classify the enzyme as a calpain II (44). Our finding of a single platelet calcium-activated protease, which is best activated by millimolar concentrations of free calcium, are supported by the findings of Yoshida et al. (5) and Truglia and Stracher (6). However, there is a difference in the calculated absolute concentration of free calcium needed to activate platelet calpain in our studies, when compared to previous studies (5). We found optimal activation at 0.5 mM free Ca^{++}, contrasting with the optimum of 1 mM free Ca^{++} found in the study of Yoshida et al. (5). This difference can be explained by the methodology used to calculate the free Ca^{++} as well as the buffer, pH, and temperature whereby the study was performed. In agreement with our finding, however, Kitahara et al. (34) found a calpain II in porcine kidney with maximal activity at a free Ca^{++} concentration of 0.5 mM. Furthermore, similar to the observations of Yoshida et al. (5), we found that the purified enzyme autodigests when exposed to calcium. In our experiments, the addition of 2 mM EDTA to an incubation mixture prevented enzyme self-destruction during 0–10-min incubations.

The finding that HMWK is another plasma protein that can inhibit calpains indicates a third plasma protein, along with alpha-2-macroglobulin and LMWK, that could regulate these tissue-derived cytosolic enzymes (Figs. 6–8). This observation was predicted, because the heavy chain of HMWK and LMWK are identical in amino acid composition (15, 45) as well as immunochemical reactivity (41). However, an unexpected finding was that HMWK, on a molar basis, was a more potent inhibitor of platelet calpain than either alpha-2-macroglobulin, alpha-cysteine protease inhibitor (LMWK) or isolated heavy chain of HMWK, under the conditions employed (Fig. 6). Unlike some cysteine protease inhibitors (27, 28), free calcium was found to exert no influence on the ability of HMWK to inhibit platelet calpain. This finding suggests that the native conformation of HMWK contributes to its activity as an inhibitor.

The results from Figs. 7 and 8 indicate that HMWK is an important plasma inhibitor of platelet calpain. In experiments where normal plasma and plasmas congenitally deficient in total kininogen or HMWK were compared, we observed that plasma containing HMWK exhibited the greatest degree of inhibition of platelet calpain (Fig. 7). The finding that Fitzgerald plasma (plasma deficient in only HMWK) displayed greater inhibition of platelet calpain at low dilutions than either purified alpha-2-macroglobulin alone or Williams plasma (total kininogen-deficient plasma) is not surprising, because Fitzgerald plasma does contain some LMWK (Fig. 7). However, at higher dilutions, the inhibitory contribution of LMWK is lost because the concentration of LMWK in this plasma is subnormal at 63 μg/ml (0.98 μM). Although reconstitution of Fitzgerald plasma with purified heavy chain of HMWK to levels of LMWK in normal plasma (2.4 μM) led to a twofold increase in the calpain inhibitory capacity of this plasma, full inhibitory activity was not attained (Figs. 7 and 8). Finally, in reconstitution experiments, where purified HMWK was added to total kininogen-deficient plasma to achieve a normal level (0.67 μM), we observed inhibition of platelet calpain which was virtually identical to that obtained with normal plasma (Fig. 8). These combined studies indicate that HMWK is the major plasma inhibitor of platelet calpain, accounting for ~80–90% of the platelet calpain inhibitory capacity of plasma. Preliminary kinetic studies indicate that HMWK is a tight noncompetitive inhibitor of platelet calpain with an apparent K_i of 5 nM.

The structural basis by which HMWK [plasma concentration, 0.67 μM (18)] supercedes plasma alpha-2-macroglobulin [plasma concentration, 3.4 μM (17)] and LMWK [plasma concentration, 2.4 μM (16)] as an inhibitor of platelet calpain is not known. Preliminary studies from this laboratory indicate that HMWK is also a substrate for platelet calpain (10). Platelet cal-

pain can proteolyze HMWK into cleavage products of smaller mass and can increase the surface-mediated coagulant activity of the molecule 35-fold (10). Many protease inhibitors are also substrates for the enzymes they inhibit (46). These findings indicate that platelet calpain, in addition to its interaction with the heavy chain of HMWK, may alter the light chain of HMWK, the portion of the HMWK molecule which contains the coagulant activity of the molecule (12, 45, 47). Finally, although calcium is needed for optimal inhibition of small active site-directed inhibitors (27, 28) and LMWK, but not for the inhibitory activity of HMWK, the additional bulk of the light chain may exert steric effects to account for increased inhibition of HMWK compared to that of LMWK.

The finding that plasma kininogens are important regulators of tissue enzymes indicates a new function for the kininogens. However, because the calpains are cytosolic proteins, one must consider how these two differently located proteins may communicate under physiologic conditions. It is possible that the important form of kininogen that may regulate platelet calpain is that which is located within platelets (18). Because platelet HMWK is mostly a granule protein (10, 18) and platelet calpain is cytosolic, these proteins might interact when platelets are activated, at which time platelet granule contents might communicate with platelet cytosol. Both platelet and plasma kininogens could be important inhibitors of cytosolic platelet calpain subsequent to platelet cytolytic injury by mechanical trauma or complement activation. This latter notion is in accord with our current hypothesis (10) of the role of HMWK and other proteins of the contact phase of plasma proteolysis as mediators of defense reactions to injury and inflammation.

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