High Molecular Weight Kininogen: Localization in the Unstimulated and Activated Platelet and Activation by a Platelet Calpain(s)

By Alvin H. Schmaier, Paulette M. Smith, A. David Purdon, James G. White, and Robert W. Colman

High mol wt kininogen (HMWK), the major cofactor-substrate of the contact phase of coagulation, is contained within and secreted by platelets. Studies have been performed to localize platelet HMWK in both the unstimulated and activated platelet and to ascertain the effect of platelet enzymes on HMWK itself. On platelet subcellular fractionation, platelet HMWK was localized to α-granules, and enzymes on HMWK itself. On platelet subcellular fractionation, platelet HMWK was localized to α-granules, and activated platelets as well as the releasate from these stimulated platelets had 17 ng HMWK antigen/10^9 platelets present, respectively. The anti-HMWK antibody consumption by activated normal platelets was specific for membrane-expressed platelet HMWK, since activated platelets from a patient with total kininogen deficiency did not adsorb the anti-HMWK antibody. Enzymes in the cytosolic fraction of platelets cleaved 125I-HMWK (mol wt 120,000) into a mol wt 100,000 polypeptide as well as smaller products at mol wt 74,000, mol wt 62,000, mol wt 47,000, and a few components below mol wt 45,000. No cleavage products were observed when DFP and leupeptin were present. The cleavage of HMWK was specifically prevented by inhibitors of calcium-activated cysteine proteases (leupeptin, N-ethylmaleimide, iodoacetamide, and EDTA) but not by inhibitors of serine proteases (DFP, benzamidine, soybean trypsin inhibitor, or aprotinin). Platelet cytosol increased the coagulant activity of exogenous purified HMWK with maximum HMWK coagulant activity (35-fold) occurring within ten minutes of exposure to platelet cytosol. Treatment of platelet cytosol with leupeptin prevented the increase in the coagulant activity of exogenous HMWK. These studies indicate that activated platelets express platelet HMWK on their external membrane and platelet enzymes can cleave and increase the coagulant activity of exogenous HMWK.

MATERIALS AND METHODS

Materials. Antiserum to fibrinogen was purchased from Atlantic Antibodies, Scarborough, Me. Nagarse was purchased from the Enzyme Development Corp, New York. Human α thrombin (3209 NIH U/mg) was a kind gift of Dr J. Fenton, New York State Department of Health, Albany, NY. Na[125]I (50 mCi/mmol) was obtained from New England Nuclear, Boston. Iodogen (chloroamide, 1,3,4,6-tetrachloro-3 a-6 a-diphenyl glycoluril) was obtained from the Thrombosis Research Center and the Hematology/Oncology Section, Department of Medicine, Temple University School of Medicine, Philadelphia; and the Departments of Pediatrics and Laboratory Medicine, University of Minnesota School of Medicine. Supported in part by NHLBI Clinical Investigator’s Award No. HL00694 and American Heart Association grant-in-aid No. 81725, American Heart Association Allegheny Mountain Pennsylvania Chapter, to A.H.S.; and grant No. HL24365 from the National Institutes of Health and grant No. 1420 from The Council Of Tobacco Research to R.W.C.

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from Pierce Chemical Co, Rockford, Ill. Standards for mol wt determination on polyacrylamide gels in sodium dodecyl sulfate (SDS) were purchased from BioRad Corp, Richmond, Calif. Antiserum to bradykinin was a generous gift of Dr David Proud, Johns Hopkins University, Baltimore, Md. All other reagents were obtained from Sigma Chemical Corp, St Louis, Mo.

**Plasma and Platelets.** Pooled normal plasma (lot No. 120 and N10) was purchased from George King Biomedical, Inc, Overland Park, Kan. These lots of plasma were previously determined to contain 80 and 88 𝜇g/mL, respectively, of HMWK antigen by comparison of the plasma antigen to purified HMWK reconstituted into total kininogen-deficient plasma.5 HMWK antigen was assayed by electroimmunoassay using monospecific antiserum to the light chain of HMWK.5 Total kininogen-deficient (ie, deficient in both HMWK and low mol wt kininogen) plasma6 and platelets5 were donated by Mrs M. Williams, Philadelphia, Pa. Platelets from a patient (R.K.) with the gray platelet syndrome7 was directly donated to this laboratory. This patient had a plasma HMWK level of 63 𝜇g/mL as determined by electroimmunoassay.5 Normal donors, who provided written informed consent, were young men and women (ages 21 to 40) on no medication. Fresh blood was collected, and platelet-rich plasma and platelet-poor plasma were prepared as previously described.8 In the experiments with the platelet calcium-activated protease (Calpain), supernatants of lysed platelets refers to a 12,000 g supernatant of frozen and thawed platelets. In these same experiments, cytosols of lysed platelets refers to a 100,000 g supernatant of frozen and thawed platelets. Elsewhere in the text, use of the term supernatant of platelets simply refers to the soluble material obtained after centrifugation.

**Preparation of HMWK.** HMWK was purified from fresh plasma using a modified procedure of Kerbiriou and Griffin9 that included 0.2 mol/L of ε-amino caproic acid in all buffers and 2 mmol/L of diisopropylfluorophosphate (DFP) added to the pooled HMWK prior to each step in the purification procedure. ε-Amino caproic acid was added to the buffers of the purification to prevent the possibility of plasmolin proteolysis of the HMWK during the purification procedure.10 This preparation of HMWK, reduced on 7.5% polyacrylamide gel electrophoresis (PAGE) with SDS,11 was a single band of mol wt 120,000. The specific activity of the purified HMWK varied from 12 to 20 U/mg. HMWK coagulant activity was measured by a one-stage kaolin-activated assay using total kininogen-deficient plasma as substrate6 as described previously.5 In brief, samples were compared against a daily standard curve from pooled normal human plasma diluted in 0.01 mol/L of Tnis-HCl, pH 7.4, and was homogenized twice in a French pressure cell9 at 500 lb/sq in with one centrifugation in between so that only the unbroken cells were subjected to the second homogenization. Five milliliters of the combined homogenate was set aside, and 20 mL were used for fractionation. Differential centrifugation of the homogenate resulted in the isolation of four fractions: the F1, which consists of unbroken platelets and large fragments, is a 1,000 g × 22-minute pellet; F2, which contains platelet granules and mitochondria, is a 12,000 g × 20-minute pellet; F3, which contains platelet membranes, is a 100,000 g × 60-minute pellet; and F4, which is platelet cytosol, is a 100,000 g supernate. The granule fraction, F1, was then divided in half and one half was applied to a 0.8 to 2.0 mol/L sucrose discontinuous step gradient in 0.2 mol/L increments.12 After centrifugation at 100,000 g for 60 minutes, five distinct zones were collected: zone A, 0.25 to 1.0 mol/L of sucrose; zone B, 1.0 to 1.3 mol/L of sucrose; zone C/D, 1.3 to 1.8 mol/L of sucrose; zone E, 1.8 to 2.0 mol/L of sucrose; and zone F, the pellet. Radiolabeled HMWK tracer experiments were performed to determine that the measured HMWK in the granule fractions arose from those fractions and was not merely soluble plasma HMWK attached to or trapped in the granule material. Prior to assay, fractions F3 and A through E of the F2 were dialyzed against 0.01 mol/L of Heps, 1 mmol/L of EDTA, pH 7.4, lyophilized, and resuspended in 5 mL of the dialysis buffer. Fractions were treated with 0.5% Triton X-100 prior to assay. HMWK antigen was assayed by CELISA using antiserum directed to the light chain of HMWK.5 The CELISA for HMWK used to determine the amount of HMWK in the total and each fraction of the subcellular fractionation by platelet homogenate and was performed identically as previously described.5 A standard competitive inhibition curve was obtained with normal plasma and plotted by a computer-fitted four-parameter logistic function.13 The amount of HMWK in each part of the subcellular fractionation was obtained from the calculated slope of a linear regression generated by multiple dilutions of the unknown sample.13 β-N-acetylglucosaminidase was assayed by the technique of Day et al.14 Fibrinogen was assayed by electroimmunoassay.15 Platelet serotonin was assayed by the technique of Weissbach et al.16 Only preparations that had a recovery >60% for total protein, HMWK, fibrinogen, β-N-acetylglucosaminidase, and serotonin were used in the analysis. The "relative specific amounts" of each marker in the preparations were calculated from the ratio of the "specific amount" of the marker in the fraction (amount of antigen or activity/mass of total protein) (F1 through F4; A through P) to the specific amount of the marker in the total platelet homogenate or F3 fraction, respectively. The percentage of distribution of each protein in each fraction was determined by the ratio of the area under the bar graph for each fraction (relative specific amount times total protein) to the area of the sum of all fractions in either the differential centrifugation or sucrose density ultracentrifugation. Studies on the total content of platelet HMWK in gray platelets

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and a concomitant normal control were done on blood anticoagulated with 3.8 g/dL sodium citrate containing PGE, (final concentration 1 μmol/L). After the platelet-rich plasma was separated, washed platelets were obtained by the combined techniques of albumin density gradient centrifugation and gel filtration with buffer containing 1 μmol/L of PGE, performed as previously reported.4 PGE, was added to the anticoagulant and gel filtration buffer to prevent loss of platelet HMWK during the washing procedure. The washed platelets were solubilized by the addition of Triton X-100 (0.5% final concentration) at room temperature for 30 minutes. The total platelet HMWK in these samples was assayed by CELISA performed identically as was previously reported.3

Localization of platelet HMWK in the Activated Platelet. Studies were performed to determine if platelet HMWK was detectable on the surface of activated platelets. Four hundred fifty milliliters of fresh blood from two donors was collected into 73 mmol/L of citric acid, 3 mmol/L of trisodium citrate containing 2% dextrose (1:10) (ACD), and platelet-rich plasma was obtained. After the pH was adjusted to 6.5 with 1 mol/L of citric acid, the platelet-rich plasma was washed twice by the centrifugation technique of Mustard.22 The Tyrode's buffer containing CaCl2 (2 mmol/L) and MgCl2 (1 mmol/L) had both apyrase and heparin (12.5 U/mL) in the first wash and apyrase alone in the second wash. Apyrase was prepared from potatoes by the method of Molnar and Lorand23 and was titered so that the minimal amount necessary to prevent secondary wash platelet aggregation with ADP was used. The final washed platelets were resuspended in Hepes buffer24 to a total volume of 10.5 mL. In four experiments, the platelet concentration ranged from 1.75 to 8.8 × 10^9 platelets per milliliter. These platelet preparations contained <0.05% red cells and <0.03% leukocytes (monocytes, granulocytes and lymphocytes, combined). The washed platelets were then divided into three aliquots, each of 3.5 mL. Two aliquots received PGE, (final concentration 1 μmol/L) and were incubated for 40 minutes at 37°C. The third aliquot was made to 0.4 mmol/L with Gly-Pro-Arg-Pro in order to prevent fibrin polymerization and platelet aggregation of activated platelets25 and was placed in a flat-bottomed polystyrene tube in a 37°C water bath. Thrombin (0.5 U/mL) was introduced, and the reaction was allowed to proceed for ten minutes without stirring. Immediately after the ten-minute incubation with thrombin, the contents of the tube were layered on a 2.5-mL discontinuous albumin density gradient (10%, 15%, 20%, 30%, 40%) prepared as previously reported.3 After centrifugation at 2,000 rpm for 25 minutes at 23°C in a GLC-2B centrifuge (DuPont Instruments, Wilmington, Del), 3.5 mL of activated platelet supernatant was separated from the activated platelets, which formed a layer over the 40% albumin cushion. The platelets were resuspended in 3.5 mL of Hepes buffer containing 0.4 mmol/L of Gly-Pro-Arg-Pro. A second platelet aliquot was centrifuged for four minutes at 12,000 g in an Eppendorf centrifuge (Brinkmann Instruments, Inc, Waterbury, Conn), and the supernatant was collected. Thus, four samples were collected: an aliquot of intact, unstimulated platelets, supernatant from unstimulated platelets, thrombin-activated platelet supernatant, and thrombin-activated platelets.

The platelet samples were prepared for use in a modified CELISA for HMWK as an indirect antibody consumption assay. Because membrane-expressed platelet HMWK would not be soluble to allow a direct determination of its presence, and solubilization of activated platelets would liberate intracellular platelet HMWK, an experimental design was developed to determine if surface-expressed platelet HMWK could be detected when whole platelets were incubated with the antisera directed to HMWK. Conditions for this antibody consumption assay for surface-expressed platelet HMWK were developed so that intraplatelet HMWK (that which was neither secreted nor expressed on the external membrane) would not interfere. The four platelet specimens were mixed with an equal volume of monospecific anti-HMWK antisera (final concentration 1:500). This antisera had previously been diluted 1:250 in 0.01 mol/L of sodium phosphate pH 7.4, 0.15 mol/L of NaCl containing 0.05% Tween-20 (PBS-Tween), and was also made 1:250 with total kininogen-deficient plasma. Before the prepared antisera was mixed with the platelet material, the antisera was ultracentrifuged at 100,000 g for 90 minutes in a Beckman Model L3-50 ultracentrifuge (Beckman Instruments, Inc, Palo Alto, Calif) to remove any aggregates that might be present. The total kininogen-deficient plasma was added to the incubation mixture to prevent nonspecific association of goat anti-HMWK IgG to human platelet Fc receptors by the addition of excess human IgG. After the four platelet samples were combined with the antisera, each mixture was incubated for one hour at 37°C. In preliminary experiments, a one-hour incubation of purified HMWK or HMWK in normal plasma was sufficient to achieve > a 90% interaction of soluble antigen and antibody when compared to a two- to three-hour incubation at 37°C. At completion of the incubation, each specimen was centrifuged at 12,000 g in an Eppendorf centrifuge, and the supernatants were collected. Aliquots for lactic dehydrogenase determination26 were taken from each of the four supernatant specimens to be compared to the amount of lactate dehydrogenase lost by solubilization of intact platelets by addition of 0.5% Triton X-100.

Four antibody samples per experiment were then compared by a CELISA assay for HMWK using known amounts of purified HMWK antigen to determine the slope of the competitive inhibition curve produced by each batch of platelet-adsorbed or platelet supernatant-adsorbed antisera. The competitive inhibition curves generated on the CELISA by the four aliquots of adsorbed antisera were produced by the method of incubation of samples as previously reported.3 However, since the aim of the assay was to determine whether the titer of the antisera would be decreased (consumed) by incubation with platelets or their supernatants, the final competitive inhibition curves were analyzed by nonlinear regression to determine the slope of the competitive inhibition curve produced by antisera adsorbed with each of the aliquots of platelet material. In all experiments, the differently adsorbed aliquots of antisera were reacted with the same amounts of purified HMWK (1 to 125 ng). The data were plotted as the concentration of the purified HMWK used to determine the slope of the competitive inhibition curve of the adsorbed antisera on the abscissa v the optical density at 405 nm on the ordinate. The measured value of this assay was the slope of the competitive inhibition curve. Statistical comparison of the differences of the slopes of the competitive inhibition curves was performed by grouped paired T tests.

Quantitation of the amount of platelet HMWK expressed on the surface of the platelet was obtained by comparing the values of the slopes produced by each sample of platelet-adsorbed or platelet supernatant-adsorbed antisera with a standard curve of the anti-HMWK antisera adsorbed with known concentrations of purified HMWK. In these latter experiments, the anti-HMWK antisera was adsorbed by purified HMWK at various concentrations (20 to 2,000 ng/mL) for one hour at 37°C. After incubation, the adsorbed antisera was interacted with purified HMWK to determine the adsorbed antisera's competitive inhibition curve. These slopes were also calculated by nonlinear regression. The data from these latter experiments were plotted as concentration of purified HMWK used to adsorb the antisera on the abscissa v the slope of the resultant competitive inhibition curve on the ordinate. The amount of HMWK (plasma or platelet) associated with the surface of the platelet or in its suspending medium was estimated by comparing the slopes of the competitive inhibition curve produced by the antisera adsorbed with
the platelet material with the slopes of the competitive inhibition curves produced by known concentrations of purified HMWK. The final results were calculated based on the number of platelets in each experiment and were expressed as nanogram of HMWK per 10⁸ platelets.

**Platelet proteolysis and activation of purified HMWK.** The effect of supernatants and cytosols of platelet lysates on purified exogenous [125I]-HMWK and nonradioabeled HMWK was studied. Platelets from 450 mL of blood were washed by the centrifugation technique of Mustard, and the final washed pellet was resuspended in 1 to 2 mL of Hepes buffer without bovine serum albumin and dextrose, or in 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4 (TBS) with or without 1 mmol/L of EDTA. After the platelet suspension was made hypotonic by addition of 0.5 to 1 mL of deionized water, the platelets were lysed by freezing and thawing four times on dry ice and at 37 °C. The supernatants of the lysed platelets were collected after centrifugation at 12,000 g in an Eppendorf centrifuge. In certain experiments, the supernatant of the platelet lysate was ultracentrifuged at 100,000 g for 90 minutes at 4 °C to collect a platelet cytosol. Supernatant and cytosol of lysed platelets were used to study the effect of platelet enzymes on [125I]-HMWK and exogenous HMWK. In certain experiments, the platelet lysate or cytosol was preincubated with cysteine or serine protease inhibitors for 10 to 30 minutes prior to the addition of the HMWKs. In experiments in which the platelet cytosol was prepared in the presence of 1 mmol/L of EDTA, the reaction was begun by the addition of 2.5 mmol/L of CaCl₂. All incubation mixtures of the platelet material and the HMWKs were performed at 37 °C. Determination of cleavage of exogenous [125I]-HMWK by platelets was performed by reducing a 25 to 50-μL aliquot of the platelet supernatant–cytosol-[125I]-HMWK mixture with 2% β-mercaptoethanol and adding it to a 7.5% polyacrylamide gel with SDS for electrophoresis. Detection of changes in the [125I]-HMWK was made by performing autoradiography on the dried gel. Determination of changes in activity of exogenous purified HMWK was made by coagulant assay of the HMWK-platelet supernatants or cytosol mixtures. Bradykinin release from exogenous HMWK added to platelet cytosol was measured by radioimmunoassay performed as previously reported.

**RESULTS**

**Localization of platelet HMWK in the unstimulated platelet.** Platelet subcellular fractionation studies were performed to determine the platelet location of platelet HMWK in the resting platelet. Six aliquots of washed platelets were evaluated for the subcellular localization of platelet HMWK by differential centrifugation (Fig 1). In these six preparations, the recovery (mean ± SEM) of total protein was 76% ± 3.2%; of HMWK antigen, 92% ± 9.5%; of fibrinogen antigen, 79% ± 14%; and of β-N-acetylglucosaminidase activity, 88% ± 5.8%. Radiolabeled HMWK tracer studies for the differential centrifugation separation were performed by the introduction of the ligand into the platelet homogenate after platelet lysis by the French Press. In these studies, 95.5% of the tracer was recovered in the F₁ cytosol fraction, 3.8% of the tracer was found in the F₂ membrane fraction, 0.4% of the tracer was found in the F₃ fraction, and only 0.3% of the tracer was localized to the F₄ granule/mitochondria fraction. When washed, lysed platelets were subjected to differential centrifugation, the largest amount (46% of the total antigen), as indicated by the area under the bar graph, of platelet HMWK antigen was found in the 12,000 g pellet (fraction F₂) (Fig 1). This localization of platelet HMWK to the F₂ fraction, a fraction previously characterized as enriched in platelet granules and mitochondria, was similar to the subcellular localization found for two known platelet granule constituents, fibrinogen and β-N-acetylglucosaminidase.

In an attempt to separate the components of the granule fraction further, the F₂ was subjected to sucrose density ultracentrifugation (Fig 2). In six experiments, the recovery (mean ± SEM) of total protein was 88% ± 2.6%; of HMWK antigen, 92% ± 7.8%; of fibrinogen antigen, 96% ± 10.5%; of β-N-acetylglucosaminidase, 80% ± 2.5%; and 98% ± 4.5% of serotonin. Radiolabeled HMWK tracer studies were also performed for the sucrose density gradient centrifugation separation with the presence of the ligand in the F₂ fraction. In these studies, 68% of the tracer remained in the soluble A fraction, 24% of the tracer was found in the C/D fraction, 4% of the tracer was found in the B fraction, 2.4% of the tracer was in the E fraction, and 1.5% of the tracer was trapped in the P fraction. Most platelet HMWK antigen entered the gradient (ie, was localized to fractions B through P, which indicated that it was in granules), and was found to be present in the largest amount, as indicated by the area under the bar graph, in the C/D and E fractions (Fig 2), fractions enriched with platelet α-granules. The C/D fraction
mean SEM and the SEM is represented by the bars (T) of six density ultracentrifugation. The data plotted represent the content of platelet HMWK.

100,000 g mol/L of sucrose; E: 1.8 to 2.0 mol/L of sucrose; F, the mol/L of sucrose; B, 1 to 1.3 mol/L of sucrose; C/D: 1.3 to 1.8 platelet syndrome7 were studied for their total content of granules, platelets from a patient (R.K.) with the gray platelet syndrome27'28 f3-N-acetylglucosaminidase, a lysosomal granule marker enzyme, was spread throughout the gradient, and serotonin, a dense granule constituent, was concentrated in the 100,000 g pellet (P). These combined studies suggested that platelet HMWK was mostly a platelet α granule protein in the unstimulated platelet.

To confirm independently that most platelet HMWK in the unstimulated platelet was localized to platelet α-granules, platelets from a patient (R.K.) with the gray platelet syndrome7 were studied for their total content of platelet HMWK. These platelets contained 16 ng/10⁶ platelets total platelet HMWK, whereas a concomitant normal platelet control contained 52 ng/10⁶ platelets. Comparing the value of total platelet HMWK in gray platelets with the normal control and nine previously determined values5 for total platelet HMWK in normal platelets (60 ng ± 24/10⁶ platelets), the gray platelets contained only 28% of the content of platelet HMWK.

Localization of platelet HMWK in the activated platelet. Studies were initiated to determine whether platelet HMWK became expressed on the external platelet membrane after activation. Platelets, in an unstimulated or activated state, along with their respective suspension buffers, were compared using a modified CELISA for HMWK antigen as an indirect antibody consumption assay. Anti-HMWK antisera was adsorbed with unstimulated or activated platelets and their supernatants to determine whether the titer of free anti-HMWK antibody (initial dilution 1:500) would be decreased. The detection of a decrease in the titer of the specific antibody was determined by a change in the slope of a competitive inhibition curve produced by the adsorbed antisera from each platelet aliquot when subsequently reacted with the same amounts of purified HMWK.

In preliminary experiments, competitive inhibition curves produced by increasing dilution (1/500, 1/800, 1/1,100, and 1/1,500) of anti-HMWK antisera incubated with identical amounts of purified HMWK resulted in a flattening (decrease in the slope) and a shift to the left of the competitive inhibition curve as the dilution of anti-HMWK antisera was increased from 1/500 to 1/1,500 (data not shown). The relationship between the change in the calculated slope of the competitive inhibition curve produced by anti-HMWK antisera adsorbed with exposed HMWK antigen was studied in a quantitative manner (Fig 3). Anti-HMWK antisera initially accounted for 65% of the total platelet HMWK, but only 44% of the total protein found in the F₂ fraction. Although 24% of the soluble £¹²Ç¹-HMWK tracer was found in the C/D fraction, the amount of trapped soluble HMWK in this fraction was really only 0.072% of the total HMWK measured since the F₂ fraction only contained 0.3% of the soluble HMWK. This finding by the radiolabeled tracer studies indicated that most of the HMWK antigen measured in the C/D fraction must have been derived from platelet granules. This localization of platelet HMWK was similar to that of platelet fibrinogen, a known platelet α-granule constituent.27 28 β-N-acetylglucosaminidase, a lysosomal granule marker enzyme, was spread throughout the gradient, and serotonin, a dense granule constituent, was concentrated in the 100,000 g pellet (P). These combined studies suggested that platelet HMWK was mostly a platelet α granule protein in the unstimulated platelet.

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diluted 1:500 produced a competitive inhibition curve with a calculated slope by nonlinear regression of 0.9. Incubating the anti-HMWK antisera initially diluted 1:500 in separate aliquots with increasing concentrations of purified HMWK (20 to 2,000 ng/mL) for one hour at 37 °C resulted in competitive inhibition curves with reduced calculated slopes (0.77 to 0.06) (Fig 3). This study indicated that the slope of a competitive inhibition curve produced by antisera adsorbed under the identical conditions could be used to calculate the amount of exposed HMWK antigen indirectly.

Unstimulated and activated platelets and their supernatants were incubated with anti-HMWK antisera initially diluted 1:500. In one representative experiment (Fig 4), the competitive inhibition curves produced by antisera adsorbed with the supernatant of unstimulated platelets (slope 0.34) and unstimulated platelets (slope 0.33) themselves gave parallel and almost superimposable curves as previously reported. The competitive inhibition curve produced by antisera adsorbed with the activated platelet supernatant was flattened with a decreased slope (0.20) when compared with that produced by the supernatant of unstimulated platelets (Fig 4). Because platelet HMWK is secreted by thrombin-activated platelets, this finding indicated that secreted platelet HMWK adsorbed and decreased the titer of the anti-HMWK antibody. The competitive inhibition curve produced by activated platelets (slope 0.26) was similar to the curve that characterized the material released by platelets (Fig 4). Antisera adsorbed with activated platelets showed a competitive inhibition curve that had a reduced slope and was left-shifted when compared with unstimulated platelets. This adsorption of the antisera by activated platelets could not be owing to platelet HMWK lost from cell lysis, because the amount of lactic dehydrogenase loss under the conditions of the experiments was <5%. This result suggested that some of the platelet HMWK, in addition to being secreted, must also have been expressed on the external membrane of the activated platelet.

In four experiments, the slopes of the competitive inhibition curves produced by each of the four platelet aliquots in each experiment were calculated by nonlinear regression. Anti-HMWK antisera adsorbed by unstimulated platelets and unstimulated platelet supernatant produced competitive inhibition curves with calculated slopes (mean ± SEM) of 0.37 ± 0.015 and 0.37 ± 0.011, respectively. The slopes produced by antisera adsorbed by unstimulated platelets and supernatants from unstimulated platelets in each experiment were not significantly different (P > .45). Anti-HMWK antisera adsorbed by activated platelets and activated platelet supernatant produced competitive inhibition curves with calculated slopes (mean ± SEM) of 0.22 ± 0.027 and 0.21 ± 0.017, respectively. The slope produced by antisera adsorbed by activated platelets and their supernatants was not significantly different (P > .4). However, the slopes produced by supernatants from activated platelets and activated platelets themselves were significantly decreased (P < .0125, P < .005, respectively) when compared to unstimulated platelets. This finding indicated that both specimens had adsorbed the antibody with expressed platelet HMWK. Because the slope of the competitive inhibition curve produced by adsorbed antisera is directly related to the concentration of HMWK available to consume the specific antibody (Fig 3), an estimate of the amount of HMWK associated with each of the platelet aliquots was calculated based on the number of platelets in each experiment. In the four experiments, unstimulated platelets and their supernatant had a mean of 4.9 ng HMWK/10^8 platelets and 4.2 ng HMWK/10^8 platelets, respectively, associated with the material. Alternatively, activated platelets and their supernatant had a mean of 17.3 ng HMWK/10^8 platelets and 17.2 ng HMWK/10^8 platelets, respectively, associated with the aliquots. The increased amount of HMWK made available from the activated platelet specimens must have been derived from the intraplatelet pool of HMWK. The similarity in the decrease in the slopes in the competitive inhibition curves and the calculated amount of available platelet HMWK between the activated
platelets and their supernatant indicated that the amount of platelet HMWK expressed on the external platelet membrane after platelet activation is similar to the amount of platelet HMWK secreted from activated platelets.

It is possible that the antibody consumption by thrombin-activated normal platelets was not specifically due to expressed platelet HMWK, but rather due to alterations on the platelet surface as result of thrombin stimulation. In order to evaluate this possibility, the competitive inhibition curves produced by antibody adsorbed by unstimulated and thrombin-activated total kininogen-deficient platelets, as well as the activated deficient platelet supernatant, were compared (inset, Fig 4). Adsorption of the anti-HMWK antisera by unstimulated total kininogen-deficient platelets, thrombin-activated total kininogen-deficient platelets, and activated deficient platelet supernatant produced competitive inhibition curves with calculated slopes of 0.71, 0.78, and 0.77, respectively. These slopes were similar to the competitive inhibition curve produced by unadsorbed antisera and, when corrected for the number of platelets in the experiment, gave values for available HMWK <1 ng HMWK/10^8 platelets—the lower limit of sensitivity of the assay. These data indicated that the consumption of the anti-HMWK antibody by activated platelets was specific for the surface expression of platelet HMWK.

Platelet proteolysis of purified 125I-HMWK. Studies were initiated to determine if platelet enzymes have any effect on the structure of purified HMWK. Initial studies were performed on supernatants of frozen and thawed platelets in the presence or absence of DFP (5 mmol/L) and leupeptin (0.5 mmol/L). Platelets were lysed by freezing and thawing as described in the Materials and Methods Section. As shown in Fig 5 (right), uninhibited supernatants of platelet lysates (2.1 x 10^9 platelets per milliliter) produced increasing extent of proteolysis of exogenous purified 125I-HMWK as a function of the lysate concentration. The initial proteolysis of the untreated samples, seen best with platelet lysate supernatants, were washed and resuspended in 3 mL of wash buffer and deionized water (2:1) and were lysed by freezing and in thawing; the supernatant from 2.1 x 10^9 platelets per milliliter was divided in half. One-half of the supernatant was incubated for 30 minutes at room temperature in the presence or absence of DFP (5 mmol/L) plus leupeptin (0.5 mmol/L). After incubation, five 100-μL samples were made of both the inhibitor-treated and untreated samples consisting of 10%, 20%, 40%, 60%, or 100% of platelet lysate in (TBS) 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4. At the conclusion of the incubation, 125I-HMWK (1.25 nmol/L) (specific radioactivity 3.3 μCi/μg) was added to each aliquot, and the mixture was incubated for ten minutes at 37 °C. At the conclusion, all samples were frozen at −70 °C. For the gel, 25 μL from each aliquot was reduced with 2% β-mercaptoethanol and applied directly to a 7.5% polyacrylamide gel in sodium dodecyl sulfate for electrophoresis; after drying, autoradiography was performed. SM represents 3.75 ng of the 125I-HMWK starting material applied directly to the gel. CN represents an incubation in which purified 125I-HMWK (1.25 nmol/L) was added to platelet buffer and handled identically to the platelet samples. The number to the right of the gel represents mol wt standards.

![Fig 5. Platelet proteolysis of purified 125I-HMWK.](image)

Fig 5. Platelet proteolysis of purified 125I-HMWK. Platelets were washed and resuspended in 3 mL of wash buffer and deionized water (2:1) and were lysed by freezing and in thawing; the supernatant from 2.1 x 10^9 platelets per milliliter was divided in half. One-half of the supernatant was incubated for 30 minutes at room temperature in the presence or absence of DFP (5 mmol/L) plus leupeptin (0.5 mmol/L). After incubation, five 100-μL samples were made of both the inhibitor-treated and untreated samples consisting of 10%, 20%, 40%, 60%, or 100% of platelet lysate in (TBS) 0.1 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4. At the conclusion of the incubation, 125I-HMWK (1.25 nmol/L) (specific radioactivity 3.3 μCi/μg) was added to each aliquot, and the mixture was incubated for ten minutes at 37 °C. At the conclusion, all samples were frozen at −70 °C. For the gel, 25 μL from each aliquot was reduced with 2% β-mercaptoethanol and applied directly to a 7.5% polyacrylamide gel in sodium dodecyl sulfate for electrophoresis; after drying, autoradiography was performed. SM represents 3.75 ng of the 125I-HMWK starting material applied directly to the gel. CN represents an incubation in which purified 125I-HMWK (1.25 nmol/L) was added to platelet buffer and handled identically to the platelet samples. The number to the right of the gel represents mol wt standards.

Nature of the Platelet Protease(s) That Cleaves 125I-HMWK. The nature of the platelet protease(s) that produced cleavage of 125I-HMWK was studied further. Platelet cytosol made from 1.0 x 10^9 platelets per milliliter was prepared and, in individual aliquots, was pretreated with a wide variety of serine and cysteine protease inhibitors (Fig 6). The addition of leupeptin (0.5 mmol/L), iodoacetamide (0.5 mmol/L), N-ethylmaleimide (0.5 mmol/L) and EDTA (10 mmol/L) prevented the proteolysis of 125I-HMWK into a mol wt 100,000 band as well as multiple bands of lower mol wt by platelet cytosol. However, pretreatment of platelet cytosol with DFP (5 mmol/L), soybean trypsin inhibitor (100 μg/mL), benzamidine (5 mmol/L), or aprotinin (1,000 KIU), did not. This finding indicated that a divalent cation dependent cysteine protease(s) [Calpain(s)] from the platelet cytosol was mostly responsible for cleavage of exogenous 125I-HMWK into a mol wt 100,000 band along with the appearance of other lower mol wt bands.

Activation of HMWK by a platelet protease. Although a platelet divalent cation dependent cysteine protease was responsible for cleavage of HMWK, the significance of this cleavage was unclear. To study whether changes in the HMWK molecule altered its activity, purified HMWK in a final concentration of 3 U/mL (12.7 U/mg) was added to platelet supernatants pretreated with leupeptin or left untreated (Table 1). As seen in Table 1, platelet lysate alone
the untreated platelet supernatant exhibited sevenfold increased specific activity. Because the purified HMWK was added in a final concentration of about 3 U/mL, the increased activity measured in the untreated specimen must have been due to “activation” of the exogenous HMWK by a leupeptin-sensitive protease. In separate experiments not shown in Table 1, leupeptin in a concentration of 0.1 mmol/L and iodoacetamide (0.5 mmol/L) also inhibited the increase in exogenous HMWK coagulant activity by platelet cytosols.

**Time course of activation of HMWK by a platelet protease.** The activation of exogenous purified HMWK by platelet cytosol was studied in kinetic experiments. A 100,000 g cytosol of lysed platelets (8 × 10⁸ platelets per milliliter) containing 1 mmol/L of EDTA was prepared in the presence or absence of leupeptin (Fig 7). After the addition of calcium (2.5 mmol/L), the coagulant activity of exogenous purified HMWK (final concentration of 2 U/mL or 12.5 U/mg before the addition of calcium) in the untreated platelet cytosol rose rapidly to 70 U/mL or 437 U/mg within ten minutes. This result indicated a 35-fold increase in coagulant activity. At longer incubations, the activity decreased, but at two hours the coagulant activity was 20 U/mL, ten times the original activity. No significant increase (2 to 4 U/mL) in the coagulant activity of exogenous HMWK added to the leupeptin-treated platelet cytosol was noted during the six-hour incubation.

Additional studies were performed to determine if bradykinin was released simultaneously with the cleavage of ¹²⁵I-HMWK and the increase in exogenous HMWK coagu-
lant activity. Under conditions similar to the coagulant activity studies of exogenous HMWK added to platelet cytosol, no bradykinin release was measurable. Because the possible amount of bradykinin released from the added HMWK could have been as much as 100-fold greater than the sensitivity of the radioimmunoassay to measure bradykinin, additional studies were performed to determine whether platelet cytosol contained an enzyme(s) that degraded bradykinin. It was found that platelet cytosol contained a DFP-sensitive enzyme that degraded exogenous bradykinin. Less than 10% of added bradykinin could be recovered from platelet cytosol, indicating that any released bradykinin by a calpain cleavage of HMWK in platelet cytosol would be rapidly proteolyzed.

DISCUSSION

The studies presented here indicate two potential pathways, perhaps interrelated, by which the interaction of HMWK with platelets may assume significance in the proteolytic reactions involved in contact phase activation. Platelet HMWK is expressed on the activated platelet surface and a platelet calcium-dependent cysteine protease(s) [Calpain(s)] is responsible for cleavage and increased coagulant activity of the HMWK molecule. Because platelet HMWK has been shown to be secreted from metabolically active platelets, it was not surprising that the largest amount of the protein was found in the granule fractions enriched with α-granules in the unstimulated platelet (Figs 1 and 2). By radiolabeled HMWK tracer studies, the amount of HMWK found in the Fg granule fraction must have been derived from the platelet granules themselves, since only 0.3% of a soluble HMWK tracer was attached to or trapped in the granule fraction. Furthermore, the amount of HMWK localized to the C/D α granule fraction must also have been derived from the platelet granules, since only 0.072% of the total soluble [125I]HMWK was found to be associated with this fraction. The subcellular fractionation studies were confirmed by a study on the total amount of platelet HMWK in platelets from a patient with the gray platelet syndrome.7 These platelets contained 16 ng/10^8 platelet HMWK, which is 28% of normal.

The finding that platelet HMWK is expressed on the external membrane of activated platelets (Fig 4) suggests one mechanism whereby a platelet form of a protein, which exists in the platelet in smaller quantities than in plasma, could be important. The expression of the platelet form of the protein on the activated platelet surface may be the means by which higher local concentrations of the protein may be achieved on the platelet surface than in plasma. The validity of the modified CELISA as a quantitative indirect antibody consumption assay used in this study to determine the amount of membrane-expressed platelet HMWK is shown by comparison of the present results with our previously reported data. In our previous study, we directly determined the amount of HMWK in supernatants of washed platelets to contain 2.8 ng HMWK/10^8 platelets. In the present study, using the curve in Fig 3, we calculated that the supernatants of washed unstimulated platelets contain 4.2 ng HMWK/10^8 platelets. In addition, in the previous study, we estimated that washed platelets themselves have about 5 ng HMWK/10^8 platelets associated with the platelet suspension. In the present experiments, we estimate that washed platelet suspensions contain 4.9 ng HMWK/10^8 platelets. The similarity between these quantitative studies indicate that assessments as to the amount of platelet HMWK expressed on the activated platelet surface are valid.

In the present experiments, it was estimated that an amount of the total platelet HMWK equal to that which was secreted also was expressed on the activated platelet surface (Fig 4). This assessment is derived from the finding that activated platelets and their supernatant adsorbed similar amounts of the antibody to HMWK, as indicated by the similar decrease in the slopes of the competitive inhibition curves in Fig 4. Comparing the calculated slopes of the competitive inhibition curves produced by activated platelets and their supernatants in four experiments with the slope of the competitive curves produced by the antisera adsorbed with variable concentrations of purified HMWK (Fig 3), the amount of platelet HMWK expressed on the platelet surface and simultaneously secreted from platelets was ~17 ng/10^8 platelets. Comparing this value with the normal value of total platelet HMWK (60 ng/10^8 platelets), ~28% of the total amount of platelet HMWK becomes secreted and independently expressed on the membrane of the activated platelet. This value for the amount of secreted platelet HMWK is lower than that (63%) which we previously reported for thrombin-activated platelets. It is possible that the difference resulted from the threefold greater concentration of thrombin used for induction of the secretion reaction in the latter experiments. The presence of HMWK on the platelet surface suggests that the platelet, through the expression of its HMWK, may be an in vivo negatively charged surface for the regulation of the contact proteolytic system. Further studies are needed to determine if membrane-expressed platelet HMWK exists there in a high local density.

The finding that a platelet protease(s) cleaves HMWK (Figs 5 and 6) is not surprising, since calcium-requiring platelet proteases have been demonstrated to cleave other hemostatic cofactors: factor V, platelet fibrinogen, and von Willebrand factor. In these studies with HMWK, a 100,000 mol wt cleavage product of HMWK along with lower mol wt bands are recognized (Figs 5 and 6). Because the cleavage of 125I-HMWK consistently resulted in a 100,000 mol wt band and was inhibited by agents known to inhibit platelet calpain(s), but was unaffected by several other serine protease inhibitors that do not inhibit platelet calpain (Fig 6), it is likely that the cleavage of 125I-HMWK observed in this study is a direct effect of calpain(s) and is not mediated through a secondary protease that is activated by calpain. Previous studies with purified proteins and in plasma indicated that kallikrein cleaves HMWK into heavy chains with an intermediate (56,000 mol wt) light chain.4 Similar cleavage of HMWK occurs with factor XIa, although the initial cleavage of HMWK results in a 74,000 mol wt band. The finding that a platelet cysteine protease(s) (Fig 6) is responsible for an additional 100,000 mol wt cleavage product of
HMWK would explain why this cleavage intermediate has not been recognized with purified plasma enzymes or in plasma when activated by a surface. The extent of proteolysis of $^{125}$I-HMWK seen in these studies was probably dependent upon the amount of platelets that was used to prepare the cytosol. When there was a high concentration of platelet enzymes (Fig 5), both the 120,000 mol wt band and the 100,000 mol wt band of $^{125}$I-HMWK were completely proteolyzed.

The finding that platelet calpain(s) can increase the specific coagulant activity of HMWK (Table 1, Fig 7) suggests that HMWK may be an important substrate for platelet calpains. The mechanism whereby platelet calpains increase the coagulant activity of HMWK is presently unknown. Because multiple bands are formed by platelet calpain cleavage of $^{125}$I-HMWK, it is not clear which cleavage product is responsible for the measured increased specific coagulant activity of HMWK. In a previous study, our group showed that kallikrein-cleaved HMWK had increased surface binding activity, and the amount of HMWK that adsorbed to an artificial surface was directly related to the expression of HMWK coagulant activity. That study suggests that proteolysis of HMWK, which exists as a procofactor, allows for increased surface-mediated activity. In that previous study, it was not possible to make a direct measurement of the specific coagulant activity of the kallikrein-cleaved HMWK such as was performed in the present investigation since prekallikrein is in the substrate plasma of the coagulant assay and when activated on a surface must produce similar cleavages during the one-stage assay procedure. However, both studies suggest that proteolysis of the HMWK can augment its activity. The present investigation supports the hypothesis that HMWK exists as a procofactor. Additional studies with purified platelet calpain and HMWK or its light chains are needed to determine whether the 100,000 mol wt band or some other cleavage product appearing at the same time is responsible for the increased HMWK coagulant activity. Furthermore, since platelet cytosols contain an enzyme(s) that degrades bradykinin, neither was it possible to determine whether platelet calpain(s) releases bradykinin. Further studies are needed with purified platelet calcium-activated cysteine protease(s) on purified intact HMWK to confirm whether this enzyme(s) releases bradykinin.

The interaction between HMWK and platelet calpains may be more than fortuitous. The recognition that HMWK is a substrate for platelet calpain has been corroborated by recent studies. Evidence by Ohkubo et al. and Muller-Esterl et al. indicate that $\alpha$-2-thiol proteinase inhibitor—a major plasma proteinase inhibitor of cysteine proteases—is identical to plasma low mol wt kininogen. Because the synthesis of the plasma kininogens is controlled by one gene, the heavy chain of HMWK is identical to the heavy chain of low mol wt kininogen. HMWK, which differs from low mol wt kininogen by the presence of a light chain that contains its coagulant activity, may be both a substrate and inhibitor of platelet calpains. Because it is well recognized that protease inhibitors are also substrates, further studies are required to determine if purified HMWK inhibits purified platelet calpain.

Several laboratories have reported the presence of a calcium-dependent platelet protease(s) which cleaves platelet membrane proteins, alters endothelial prostaglandin production, cleaves factor $V$, fibrinogen, and von Willebrand factor. At least two calcium-dependent cysteine proteases are ubiquitously distributed in tissues, Calpain I and Calpain II, which differ in their requirement for activation by calcium; Calpain I requires $\mu$mol/L concentrations of calcium and Calpain II requires $\mu$mol/L concentrations. To date, these proteases have been purified only from the cytosol fraction of the tissue from which it is derived. How these calpains become available to alter their substrates, which are either on the platelet surface or contained within platelet granules, is unknown. However, these substrates may become altered by platelet calpains when platelets are disrupted by mechanical injury or complement-mediated cytolysis. This later notion would be consistent with our current hypothesis on the importance of HMWK. Because a deficiency of HMWK is not associated with any specific disease state, but the protein becomes altered to participate in the consequences of many disease states, HMWK should be considered a pathophysiologic mediator. HMWK in plasma or platelets could serve to inhibit platelet calpain(s) directly by its heavy chain and to neutralize the consequences of calpain's action indirectly by the formation of an activated cofactor form. In conclusion, these studies indicate that activated platelets express platelet HMWK on their surface that may be available to promote contact phase proteolysis on or at the platelet surface, and that platelets themselves contain a calpain protease(s) that can cleave HMWK and increase its coagulant activity and that might accelerate contact phase activation.

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High molecular weight kininogen: localization in the unstimulated and activated platelet and activation by a platelet calpain(s)

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