High-molecular weight kininogen. A secreted platelet protein.

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Research Article

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High-Molecular Weight Kininogen

A SECRETED PLATELET PROTEIN

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ABSTRACT Human platelets were studied immunochemically to determine if they contain high-molecular weight kininogen. On crossed immunoelectrophoresis with total kininogen antisera (antisera that recognizes both high- and low-molecular weight kininogen) extracts of platelets contained total kininogen antigen. Platelet total kininogen antigen showed complete antigenic identity with plasma total kininogen and displayed the same electrophoretic migration as plasma total kininogen. Using antisera monospecific to high molecular weight kininogen, a competitive enzyme-linked immunosorbent assay (CELISA) was developed to directly measure platelet high-molecular weight kininogen. By CELISA, 27–101 ng of high molecular weight kininogen antigen per 10^8 platelets was quantitated in detergent-soluble lysates of washed human platelets from nine normal donors with a mean level of 60 ng±24/10^8 platelets. Plasma high-molecular weight kininogen, either in the platelet suspending medium or on the surface of the platelets, could only account for 5% of antigen measured in the solubilized platelets. On the CELISA, platelet high-molecular weight kininogen was immunochemically identical to plasma and purified high-molecular weight kininogen.

Platelet high-molecular weight kininogen was secreted from platelets after exposure to ionophore A23187 (3–15 μM), collagen (5–150 μg/ml), and thrombin (1.6 U/ml). Secreted platelet high-molecular weight kininogen did not become a part of the platelet Triton-insoluble cytoskeleton. On cross immunoelectrophoresis secreted platelet total kininogen antigen had a similar electrophoretic migration to plasma total kininogen. Thus, human platelets contain high-molecular weight kininogen that can be secreted from platelets and that may participate in plasma coagulation reactions.

INTRODUCTION

The role of platelets in hemostasis has been shown to extend beyond the formation of a platelet plug at the site of vessel injury and the contribution of phospholipid for coagulation reactions. Human platelets contain a number of coagulation factors: fibrinogen (1–3), Factor V (4–8), and Factor VIII-related antigen (9–11). These proteins are localized in platelet alpha granules (1, 2, 8–10) and are secreted from the platelet upon stimulation (3, 5–9, 11), presumably to participate in platelet-platelet, platelet-coagulation factor, and platelet-subendothelial cell interactions.

Studies by Walsh (12, 13) have indicated that washed platelets enhance reactions involving Factor XII and XI, and in the absence of Factor XII, collagen-stimulated platelets can activate Factor XI. Furthermore, platelets in the presence of kallikrein will promote the cleavage of ^{125}I-Factor XII and ^{125}I-Factor XI and these reactions are enhanced by the presence of high-molecular weight kininogen (HMWK)[1] (14).

This work has been presented, in part, at the American Federation of Clinical Research, Wash. DC, May 1980; Congress of the International Society of Hematology, Montreal, Canada, August 1980; the VIIIth International Congress on Thrombosis and Haemostasis, Toronto, Canada, July 1981; and the Fifth National Conference on Thrombosis and Hemostasis, American Heart Association, Dallas, TX, November 1982. This work has been published, in part, in abstract form. 1980. *Clin. Res.* **28:** 323*a*. (Abstr.); 1981. *Clin. Res.* **29:** 347*a*. (Abstr.); *Thromb. Hemostasis.* **41:** 96, 1981; and *Circulation.* **66:** II-296, 1982.

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[1] *Abbreviations used in this paper:* CELISA, competitive enzyme-linked immunosorbent assay; EID, electroimmunodiffusion; HMWK, high-molecular weight kininogen; LMWK, low molecular weight kininogen; PBS-Tween, 0.001 M sodium phosphate pH 7.4, 0.15 M NaCl containing 0.05% Tween-20; PGE_1, prostaglandin E_1; XIEP, crossed immunoelectrophoresis.

Moreover, Factor XI activity and antigen have been shown to be closely associated with washed platelets (15, 16) and Factor XI has been shown to bind to platelets only in the presence of HMWK (17).

To date each coagulation protein identified in platelets (fibrinogen, Factor V, Factor VIII-related antigen) function as both substrate and cofactor in plasma proteolysis. HMWK is the major substrate and cofactor of the contact phase of coagulation. By analogy to other platelet procoagulant proteins, HMWK might be expected to be present in platelets and thus contribute to the participation of platelets in early blood coagulation. This study presents immunochemical evidence for the presence of HMWK in platelets and its secretion following platelet activation with a divalent cationophore A23187, collagen, and thrombin.

METHODS

Materials. Hirudin grade IV, 2-deoxy-D-glucose, antimycin A, D-gluconic acid δ-lactone, Triton X-100, crystalline bovine serum albumin fraction V, radioimmunoassay grade bovine serum albumin, sodium pyruvate, Tween 20, prostaglandin E_1 (PGE_1) rabbit anti-goat whole immunoglobulin conjugated with alkaline phosphatase, and *p*-nitrophenylphosphate disodium were purchased from Sigma Chemical Co., St. Louis, MO. Ionophore A23187 was obtained from Calbiochem Behring Corp., American Hoechst Corp., San Diego, CA. Acid soluble calf skin collagen (6.3 mg/ml) was bought from Millipore Corp., Freehold, NJ. Human α-thrombin (5,128 U/mg) was obtained from Dr. J. Fenton, New York State Department of Health, Albany, NY. [^{14}C]5-Hydroxytryptamine (51.5 mCi/mmol), and Na ^{125}I (50 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Iodogen (chloroamide, 1,3,4,6-tetrachloro-3a-6a-diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, IL. Kaolin was obtained from Fisher Scientific Co., Pittsburgh, PA. Inosithin was purchased from Associated Concentrates, New York. Sodium barbital buffer, pH 8.8 was purchased from Gelman Sciences, Inc., Ann Arbor, MI. Agarose, highest electroendosmotic grade, was obtained from Marine Colloids, Inc., Rockland, ME. Imipramine was obtained from Ciba-Geigy Corp., Summit, NJ. All other materials were reagent grade.

Plasma and platelets. Total kininogen-deficient plasma (plasma-deficient in both HMWK and low-molecular weight kininogen [LMWK]) and platelets were donated by Mrs. M. Williams, Philadelphia, PA. HMWK-deficient plasma (Fitzgerald plasma) was a generous gift of Dr. A. Scicli, Henry Ford Hospital, Detroit, MI. Pooled normal human plasma (lot No. 120) was purchased from George King, Biomedical, Inc. Overland Park, KS. Platelet concentrates (≤72-h old) were generously supplied by the Penn-Jersey Regional Red Cross, Philadelphia, PA. Normal blood donors consisted of young males (age 21–40) with their written informed consent. Fresh blood was collected and platelet-rich plasma and platelet-poor plasma was prepared as previously described (18).

Preparation of HMWK and LMWK. HMWK was purified by two procedures. HMWK used for rabbit immunization purified by a modification (19) of the method of Habal and Movat (20) had a specific activity of 15 coagulant U/mg and was a single component of 120,000 D on sodium dodecyl sulfate gel electrophoresis (21). HMWK, used for goat immunization and competitive enzyme-linked immunosorbent assay (CELISA) studies, was prepared according to the procedure of Kerbiriou and Griffin (22). This preparation of HMWK was a single band of 120,000 M_r on sodium dodecyl sulfate gel electrophoresis (23) with a specific activity of 12.5–18 U/mg. LMWK was purified from plasma by a modification (24) of the method of Habal et al. (25). Purified LMWK was characterized as a homogenous protein that had negligible HMWK procoagulant activity but a positive immune precipitation reaction against antisera to total kininogen. On nonreduced sodium dodecyl sulfate gel electrophoresis it displayed a single band of 52,000 D.

Protein determinations were performed by the methods of Lowry et al. (26) and Bradford (27) using crystalline bovine serum albumin as the standard.

Purified HMWK was radiolabeled with Na^{125}I using Iodogen by the method of Fraker and Speck (28). Purified HMWK (50–300 μg in 0.2 M sodium phosphate, 1.0 M NaCl pH 7.5) was incubated with carrier-free Na^{125}I in a plastic vial precoated with Iodogen (2–4 μg) for 15–35 min on ice. The iodination reaction was stopped by the addition of sodium metabisulfite (60 μg/ml final concentration) and free ^{125}I was separated from protein-bound ^{125}I by gel-filtration on a 0.8 × 10-cm column of Sephadex G-50 equilibrated in 0.02 M sodium phosphate, 1.0 M NaCl, pH 7.5 containing 0.25% gelatin. The specific radioactivity of the protein varied from 1–8 μCi/μg. The radiolabeled protein retained > 95% of its procoagulant activity as well as its antigenic properties.

Antisera and antibodies. Antiserum to total kininogen (both HMWK and LMWK) was produced in rabbits and goats as previously reported (24). On immunodiffusion the antiserum formed a single precipitin arc against purified HMWK, LMWK, and Fitzgerald plasma and a double-precipitin arc against normal plasma, indicating that the antisera reacted with both LMWK and the antigenic sites to the unique portion of HMWK (light chain) that distinguishes it from LMWK (29). Moreover, an IgG fraction of this antiserum adsorbed with kaolin inhibited the HMWK procoagulant activity of normal plasma (24). Following adsorption, the antibody itself contributed no HMWK, prekallikrein, Factor XII or Factor XI coagulant activity and when incubated with normal plasma had no Factor XII, prekallikrein, or Factor XI inhibitory activity in any of these specific coagulant assays. Monospecific antisera to HMWK was produced by 26 sequential adsorptions of anti-total kininogen antisera with total kininogen-deficient (Williams) plasma (1 part plasma to 100 parts antisera) and purified LMWK (16 μg/ml of antisera). Both the antisera to total kininogen and HMWK did not cross-react with any of the preparations of bovine serum albumin used throughout these studies.

Functional and immunochemical assays. HMWK procoagulant activity was measured by a one-stage kaolin activated assay (30) using factor-deficient plasma as previously reported (31). Samples were compared against a daily standard curve from pooled normal human plasma diluted in buffered isotonic saline (0.1 M sodium barbital, 0.09 M NaCl in 14% 0.1 N HCl, pH 7.4). The final dilutions of plasma ranged from 1:40 to 1:40,000 and were linear on a log-log plot. 1 unit was defined as that amount in 1 ml pooled normal plasma.

Electroimmunodiffusion (EID) was performed according to the method of Laurell (32) on 74 × 50-mm glass plates covered with 7 ml of 1% agarose in sodium barbital buffer, pH 8.8, $\tau/2$ = 0.0375 containing 1% antiserum. Samples were placed in wells 2.5-mm diam and electrophoresis was performed for 16 h at 14°C and 150 V. Crossed immuno-

electrophoresis (XIEP) in the first dimension was performed on 84 × 94-mm glass plates for 3 h at 14°C and 250 V using sodium barbital buffer, pH 8.8 in the running chamber as well as in the 1% agarose gel. At completion of the first dimension, agarose strips containing the protein were cut out and placed on clean glass slides. Fresh agarose (1%) in sodium barbital buffer, pH 8.8 was poured containing antiserum and electrophoresis was performed as in the EID. All plates were soaked and stained as previously reported (33).

CELISA for HMWK. Plasma and platelet concentrations of HMWK were determined by CELISA for HMWK using a modification of the Engvall procedure (34). Goat anti-human HMWK was the primary antisera and rabbit anti-goat antibody conjugated with alkaline phosphatase was used as the secondary antibody. On day 1 100 ng of purified HMWK in 0.1 M Na_2CO_3 pH 9.6 was linked to the surface of polystrene cuvette wells (Gilford instrument Laboratories Inc., Oberlin, OH) by overnight incubation at 37°C. On the same day, incubation mixtures, in 1.5-ml conical polypropylene tubes (No. 72.690, Sarstedt, Inc., Princeton, NJ) precoated with 0.2% bovine serum albumin, were made containing the following: 0.2 ml of antigen (purified HMWK, plasma, or solubilized platelets) diluted in 0.001 M sodium phosphate pH 7.4, 0.15 M NaCl containing 0.05% Tween-20 (PBS-Tween) and 0.2 ml of a 1:200 or 1:500 dilution (depending on which batch of antisera was used) of goat anti-human HMWK antisera. These samples were incubated overnight at 37°C. On day 2 each antigen-linked cuvette well was washed three times with PBS-Tween and incubated for 1 h at 37°C with 0.2% radioimmunoassay grade bovine serum albumin. After washing, 0.2 ml from each incubation mixture was added to each cuvette well and incubated for 2 h at 37°C. At the conclusion of this incubation the rewashed wells were exposed to 0.2 ml of a 1:250 to 1:500 dilution (depending upon the batch of specific antisera used) in PBS-Tween of rabbit anti-goat antibody conjugated with alkaline phosphatase. After another 2.5-h incubation at 37°C, the washed cuvettes received sequentially timed additions of 0.4 ml of *p*-nitrophenylphosphate disodium (1 mg/ml) in 0.05 M Na_2CO_3, 1 mM MgCl, pH 9.8. At precise time intervals (10–20 min) after the addition of the substrate to each well, the amount of hydrolysis of the substrate in each well was either stopped with sequential additions of 0.4 ml of 2 M NaOH (final concentration 1 M NaOH) or was sequentially measured spectrophotometrically in a PR50 EIA Processor-Reader (Gilford Instrument Laboratories, Inc.) at 405 nm. A similar CELISA was also established with anti-total kininogen antisera at a primary dilution of 1:500.

The CELISA assay was standardized against purified HMWK. Purified single-band HMWK was diluted according to its protein concentration determined by Lowry (26) and Bradford (27) protein assays. The amount of HMWK antigen in one pool of normal human plasma (lot No. 120 George King, Biomedical, Inc.) was then compared with the purified HMWK diluted in total kininogen-deficient plasma on EID using anti-HMWK antisera. All subsequent dilutions of pooled normal plasma were based on the amount of HMWK antigen determined in the pool. Samples for the standard curve (purified HMWK, plasma) and unknowns (plasma, solubilized platelets) were both assayed at 10 different dilutions in triplicate. All data were analyzed on a TRS-80 model III computer (Tandy Corp., Forth Worth, TX). Raw optical density readings were averaged after the blanks were substracted. Outliers were defined as points with a >10% SD. A best-fit standard curve was determined by nonlinear regression. The best-fit standard curve was then entered into an iterative computer program that used a four-parameter logistic function and an optional weighting function for a sigmoidal line fit of the standard curve (35). The weighting function was determined by a multiple linear regression for each batch of antisera (35). This program yields a semilogarithmic graph in which the ordinate is the optical absorbance calculated in relative values from 0 to 1 and the abscissa is the absolute amount of antigen incubated with antisera. Unknown samples were determined by linear least-squares regression from the standard curve of the iterative program and the calculated slope of the regression line was an estimate of the specific activity of the sample (35). Test sample determinations that were considered valid were computed results that fell on the linear portion of the standard curve for the individual assay and were characterized by a regression analysis having p values < 0.05 in an F test on the determination of the line and in a t test against the null hypothesis that the slope of the line equals zero. Immunochemical identity of the slope of the linear portions of the standard curves of purified, plasma, and platelet HMWK was determined by two-tailed paired t testing and a modified linear regression analysis of Youden (36) where the null hypothesis states that the slope of the line determined equals one.

Preparation of platelet extract. Platelet extracts were prepared as previously reported (16) from platelet-rich plasma concentrates less than 72-h old and from fresh platelets. Platelets from platelet-rich plasma were separated from plasma by centrifugation, gel-filtration over Sepharose 2B, and recentrifugation. After solubilization of the platelet pellet with 0.2% Triton X-100, the supernatant was placed on a concanavalin A Sepharose column in order to obtain a partially purified platelet extract. The eluate after introduction of 1 M α-methylmannoside was collected and concentrated to 1/1,000th of the starting volume of platelet-rich plasma. The amount of plasma HMWK contaminating the platelet extract was estimated by determining the recovery of ^{125}I-HMWK, added to the starting platelet-rich plasma, in the final material.

Preparation of washed platelets. Fresh platelets were washed by a combination of albumin density gradient centrifugation and gel filtration (37). 8 ml of platelet-rich plasma was layered on a 2.5 ml discontinuous increasing albumin density gradient (10, 15, 20, 30, 40%) prepared according to the method of Walsh (38). A 40% stock solution of albumin was diluted with Hepes buffer (37, 39) to prepare the gradient. After centrifugation for 15 min at 180 *g* at 23°C in a GLC-2B centrifuge (DuPont Instruments, Wilmington, DE), the platelet layer was removed and gel-filtered (<1 ml platelet suspension per 10 ml gel) on 2.8 × 9.8-cm columns of Sepharose 2B in Hepes buffer. Void volume fractions were pooled. In the determination of all fresh platelet samples for supernatant and total HMWK values, blood for platelet-rich plasma was collected into anticoagulant containing PGE_1 (final concentration 1 μM) and these same platelets were gel-filtered with buffer also containing prostaglandin E_1 at the same concentration.

Platelet secretion studies. Freshly washed platelets were lysed either by freezing and thawing, four times after being diluted 1:3 with deionized water, or by adding 0.01 volume of 20% Triton X-100 (0.2%) for 30 min at 22°C. Platelets lysed by freezing and thawing were used for determination of HMWK procoagulant activity; platelets solubilized with Triton X-100 were used for HMWK antigen measurement. Washed intact platelets for secretion studies were incubated with [^{14}C]5-hydroxytryptamine for 30 min at 37°C and then treated with imipramine (2 μM) (40). Aliquots of platelets

used in secretion studies were also pretreated with metabolic inhibitors, antimycin A (15 μg/ml) and 2-deoxy-D-glucose (30 mM), to inhibit aerobic and anaerobic glycolysis (39). In addition, D-gluconic acid δ-lactone (10 mM) was added to the platelets treated with metabolic inhibitors to inhibit glycogenolysis (41). Platelet [^{14}C]5-hydroxytryptamine secretion was assessed by a modification (42) of the method of Jerushalmy and Zucker (43). Platelet lactic dehydrogenase loss was determined according to the method of Wroblewski and Ladue (44). Platelet alpha granule secretion was assessed by measurement of low-affinity platelet factor 4 by the method of Rucinski (45).

Platelet secretion studies with ionophore A23187 and collagen were performed in 3-ml aggregometer cuvettes (Payton Inst., Buffalo, NY) containing 1 ml of washed platelets (10^8 to 10^9 platelets/ml). After the addition of the agonist the cuvette was transferred to an aluminum block maintained at 37°C positioned on a magnetic stirrer (1,200 rpm). At precisely 10 min from the introduction of the stimulus, each cuvette was placed on ice. Aliquots (0.2 ml) of activated platelets were removed and placed in a microcentrifuge tube containing 0.05 ml of a 135 μM formaldehyde, 5 mM EDTA solution. The remaining 0.8 ml aliquots were placed in other microcentrifuge tubes without formaldehyde-EDTA. Each tube was centrifuged at 12,000 *g* in an Eppendorf centrifuge (Brinkman Instruments, Inc., Waterbury, CT) for 5 min at 25°C. Supernatants (0.1 ml) of platelet aliquots centrifuged with formaldehyde-EDTA were used for [^{14}C]5-hydroxytryptamine determinations. The supernatants of the 0.8 ml platelet aliquots were immediately frozen (−70°C) and used for HMWK procoagulant, low-affinity platelet factor 4, and lactic dehydrogenase determinations. Washed platelets for thrombin secretion studies were placed in 16 × 95-mm flat-bottomed polystyrene tubes (No. 62.492 Sarstedt, Inc.) in 5-ml aliquots. The tube was placed in a 37°C water bath with stirring at 1,200 rpm and thrombin (1.6 U/ml) was added. After 5 min the reaction was stopped by the addition of hirudin (2.5 U/ml). The supernatants of the 5.0 ml platelet aliquots were used to assay for HMWK antigen using the CELISA. Concomitantly, washed platelets were also used to prepare Triton-insoluble cytoskeletons according to the method of Tuszynski et al. (46).

Platelet agonist dose-response studies. Ionophore A23187 (stock solution 3 mM in dimethylsulfoxide) was used in concentrations of 1, 3, 5, 7, 10, 15 μM. Dimethylsulfoxide at a 0.5% concentration did not inhibit washed platelets' ability to respond to collagen (25 μg/ml) or ADP (5 uM), and human fibrinogen (1 mg/ml). Collagen was used in final concentrations of 1, 5, 25, 50, 100, 150 μg/ml. At these concentrations of collagen, the pH of the platelet reaction mixture remained constant in the range of 7.30–7.50. For each dose of agonist, three to four experiments were performed on platelets from different donors. All secretion studies were performed with a nonstimulated control. Percent secretion (or loss) was determined by the ratio of the supernatant of the agonist-treated specimen to the supernatant of the platelet lysate, after the value of the control supernatant was subtracted from both. All values expressed represent the percentage of the total amount of each constituent found in the platelet.

Indirect antibody consumption assay to determine platelet-associated HMWK. 7.5 ml of fresh platelets (1 × 10^9 platelets/ml) pretreated with 1 μM PGE_1 and prepared by albumin density gradient centrifugation and gel filtration, were incubated for 30min at 37°C with an equal volume of anti-total kininogen antisera. The antisera had previously been diluted 1:250 in PBS-Tween and centrifuged at 100,000 *g* for 30 min in a Beckman Model L3-50 ultracentrifuge (Beckman instruments Inc., Palo Alto, CA) to remove aggregates. A second 7.5-ml aliquot of identically washed platelets was centrifuged at 12,000 *g* (Eppendorf centrifuge) and the supernatant was incubated 1:1 with anti-total kininogen antisera prepared as described above. After a 2-min centrifugation at 12,000 *g* (Eppendorf centrifuge) the antisera adsorbed with platelets was compared with that adsorbed with supernatant on the CELISA using fixed amounts of purified HMWK antigen to determine a standard curve.

RESULTS

Characteristics of platelet extract. Platelet extracts were prepared in order to perform immunochemical studies on a sufficiently enriched platelet constituent preparation essentially free of plasma total kininogen contamination. To estimate the amount of plasma HMWK that might be contaminating the final platelet extract, tracer studies were performed with ^{125}I-HMWK added to the starting platelet-rich plasma (Table I). The final amount of the total ^{125}I-HMWK tracer in the entire α-methylmannoside eluate of the concanavalin A Sepharose chromatograph was ≦20 pg out of the 10 μg of ^{125}I-HMWK added or 0.0002% of the original amount of the radiolabel. This finding indicated that 1:500,000 of the original amount of plasma HMWK may be contaminating the final platelet extract.

Determination of platelet-associated plasma total kininogen and HMWK. The amount of plasma HMWK that could contaminate the platelet extract (1:500,000 of the starting value) can only describe a lower limit of possible plasma contamination. The presence of soluble tightly bound and nonexchange-

TABLE I
Plasma Contamination of Platelet Extract *

Material	Volume	Total cpm‡ (×10^9)	Yield§
	ml		%
Platelet-rich plasma	100	306	100
Platelets pre-gel-filtration	4	40	13.2
Detergent-solubilized platelet extract	1	0.00814	0.0027
Concanavalin A eluate	0.1	0.00065	0.0002

* 10 μg of ^{125}I-HMWK (sp act 1.39 μCi/μg) was added to 100 ml of platelet-rich plasma (10^9 platelets/ml). The platelets were washed by centrifugation, gel filtration, and centrifugation, and detergent-solubilized platelet material was placed on concanavalin A-Sepharose and eluated with α-methylmannoside. This eluate was concentrated and used for further studies.

‡ Amount given represents the total radioactivity at each step in the preparation of the platelet extract.

§ Determined from the ratio of the total counts per minute in each step to the total counts per minute of the platelet-rich plasma × 100.

able plasma HMWK on the platelet surface could possibly account for additional plasma contamination of the platelet extract. To evaluate this possibility an indirect antibody consumption assay was designed to compare antiserum adsorbed with platelets with antisera diluted with platelet supernatant on the CELISA. Total kininogen antisera was incubated with whole platelets to determine whether plasma HMWK tightly bound to platelets would adsorb the anti-total kininogen antisera's ability to detect fixed amounts of purified HMWK. Incubation of platelets under these conditions and centrifugation was associated with <1% lysis as determined by lactic dehydrogenase loss. As shown in Fig. 1 when antiserum adsorbed with platelets was incubated with purified HMWK an almost identical, parallel standard curve (two-tailed $P > 0.3$) was produced when compared with purified HMWK incubated with unadsorbed antisera. This result showed that incubation of whole platelets with anti-total kininogen antisera did not lead to a decrease in the titer of the antisera that could interact with purified HMWK. This finding indicated that essentially no plasma total kininogen was tightly bound to the platelet surface since the standard curve would have been shifted to the right if the titer of the platelet-adsorbed antisera was decreased.

The presence of platelet-associated plasma HMWK was also assessed by studies with ^{125}I-HMWK. ^{125}I-HMWK was introduced into platelet-rich plasma and the platelets were separated from the plasma by albumin density gradient centrifugation and gel filtration. As seen in Table II, the amount of ^{125}I-HMWK present in the supernatants of intact washed platelets was similar to the amount of ^{125}I-HMWK present in the supernatants of agonist-stimulated platelets and platelets solubilized by freezing and thawing. This finding indicated that none of the added ^{125}I-HMWK became soluble upon platelet stimulation. Thus, it is unlikely that plasma total kininogen or HMWK associated with the platelet surface membrane could be contaminating the solubilized extracts of washed platelets.

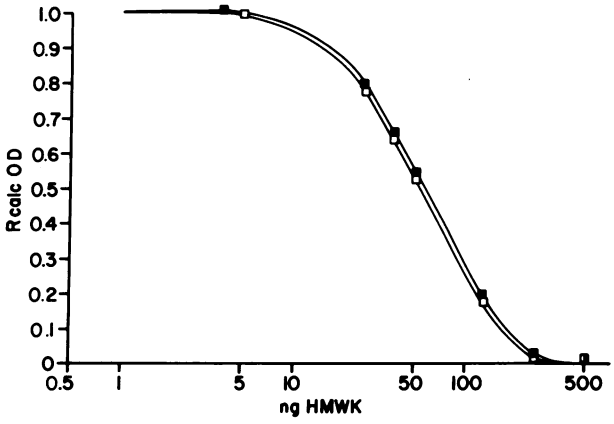

FIGURE 1 Competitive inhibition standard curve produced by purified HMWK with antiserum adsorbed with platelets and unadsorbed total kininogen antisera. Anti-total kininogen antisera were incubated with an equal volume of whole platelets or the supernatant of the platelet suspension. The platelet-adsorbed antisera and unadsorbed antisera were then incubated with equal amounts of purified HMWK to produce a standard curve using the CELISA (Methods). The standard curve produced by platelet-adsorbed antisera (■ - - -) is plotted along with the standard curve produced by unadsorbed antisera (□). In this assay the ordinate is expressed as relative absorbance (Rcalc OD) and the abscissa is the absolute amount of antigen incubated with antisera.

TABLE II
*^{125}I-HMWK Studies with Washed Platelets**

Material	Total cpm‡ ($\times 10^4$)	Recovery§ (%)
Platelet-rich plasma	286	100
Washed platelets	0.21	0.07
Supernatant intact washed platelets	0.079	0.028
Supernatant of platelets stimulated with A23187	0.077	0.027
Supernatant frozen and thawed platelets	0.084	0.029

* 2.2 μg of ^{125}I-HMWK (sp act 4.7 μCi/μg) was added to 8 ml of platelet-rich plasma and the platelets were washed by albumin density gradient centrifugation and gel filtration. The final washed platelets had a concentration of 662,000 platelets/μl.

‡ Amount given represents the total radioactivity present in 1 ml of the material.

§ Determined from the ratio of the total counts per minute in each material to the total counts per minute of the platelet-rich plasma × 100.

Immunochemical characterization of platelet total kininogen. Platelet total kininogen antigen in platelet extracts was detected on XIEP using rabbit anti-total kininogen antisera. Platelet extract either alone or mixed with normal human plasma, appeared to have an identical electrophoretic migration as total kininogen in normal human plasma (Fig. 2 A). These same results were obtained whether the platelet extract was prepared in the presence or absence of proteolytic inhibitors. On tandem XIEP (Fig. 2 B) platelet extract and normal human plasma showed complete identity. Using a different batch of adsorbed anti-total kininogen antisera (Fig. 2 C), the addition of platelet extract to normal human plasma produced an accentuation of the more anodal tail of the precipitin arc characteristic of normal human plasma. This portion of the XIEP migration of normal human plasma was identical to the more anodal migration on XIEP of purified HMWK. Although these studies show im-

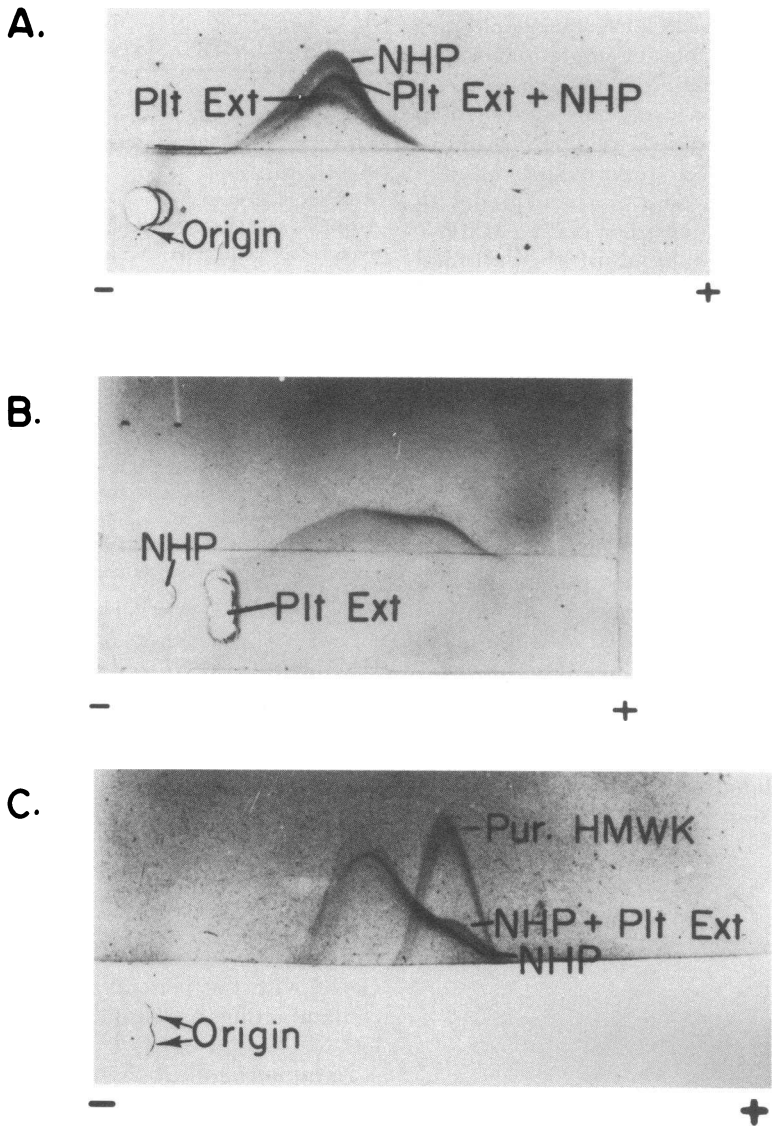

FIGURE 2 (A) XIEP of platelet extract. Rabbit anti-total kininogen antiserum was used to determine the XIEP characteristics of platelet total kininogen. Antiserum concentration of 2% in agarose was used to determine the electrophoretic migration of platelet extract (Plt Ext), platelet extract mixed with diluted (1:20) normal human plasma (NHP), and normal human plasma alone (1:20). Three separate XIEP were run at the same time and the images are superimposed by aligning the wells. 20 μl were added to each well. (B) Tandem XIEP. Normal human plasma (NHP) and platelet extract (Plt Ext) were placed on one plate in adjacent wells for electrophoresis in the first dimension. At the completion of the electrophoresis, the agarose strip containing both specimens were electrophoresed in the second dimension against antisera to total kininogen in the same concentration as in Fig. 2 A. (C) XIEP of platelet extract and purified HMWK. Antisera to total kininogen in a concentration of 4% in agarose was used to study the XIEP features of normal human plasma (NHP), normal human plasma mixed with platelet extract (Plt. Ext), and purified HMWK (Pur HMWK). Three separate XIEP were run at the same time and the images are superimposed for photography by aligning the wells.

munochemical identity between plasma and platelet total kininogen, they do not discriminate whether the kininogen antigen in platelets is only HMWK or a combination of HMWK or LMWK. The more cathodal portions of Figs. 2 B and 2 C could be compatible with the presence of platelet LMWK.

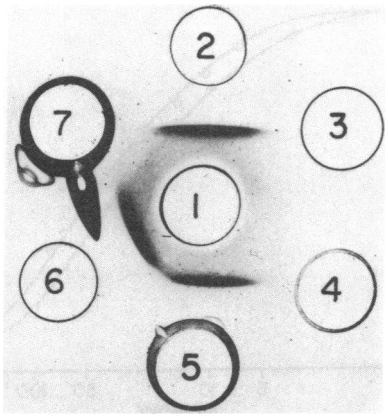

FIGURE 3 Immunodiffusion of antisera to HMWK. 19 μl of added antisera, plasma, or purified protein in their respective wells were allowed to diffuse through 1% agarose for 48 h at room temperature. After drying, the plate was stained with Coomassie Brillant Blue R-250. Well (1): antisera to HMWK, wells 2 and 5; pooled normal plasma, well 3; purified LMWK (360 μg/ml), well 4; total kininogen-deficient (Williams) plasma, well 6; purified HMWK (136 μg/ml), well 7; HMWK-deficient (Fitzgerald) plasma.

CELISA. A CELISA was developed to directly quantitate HMWK antigen in platelets. Monospecific antisera to HMWK was used in this assay (Fig. 3). This antisera gave a single precipitin arc that showed complete identity with pooled normal plasma and purified HMWK and no precipitin arc against HMWK-deficient plasma, total kininogen-deficient plasma, and purified LMWK. On the CELISA, the amount of measured optical density from hydrolysis of the substrate was inversely proportional to the amount of soluble antigen present in the incubation mixture (Fig. 4). In the CELISA, HMWK antigen in normal plasma was immunochemically identical giving a parallel standard curve in its linear portion with purified HMWK (two-tailed $P > 0.9$ on modified Youden analysis and >0.1 on paired t test) and with purified HMWK reconstituted into total kininogen-immunodeficient plasma (two-tailed $P > 0.5$ on modified Youden analysis and >0.1 on paired t test) (Fig. 4). Using purified HMWK and pooled normal plasma for standard curves the calculated coordinate of the midpoint had a CV of 7.7 and 8.6%, respectively. Using pooled normal plasma, which was determined to contained 80 μg HMWK antigen/ml for the standard curve, 10 individual normal plasmas had a mean value of HMWK antigen of 105 μg/ml, which was not significantly different ($P < 0.4$) than the mean value of 82 μg/ml of the same plasmas determined by EID. The interassay coefficient of variation of a single plasma sample assayed four times over a 1-mo period was 8.8%.

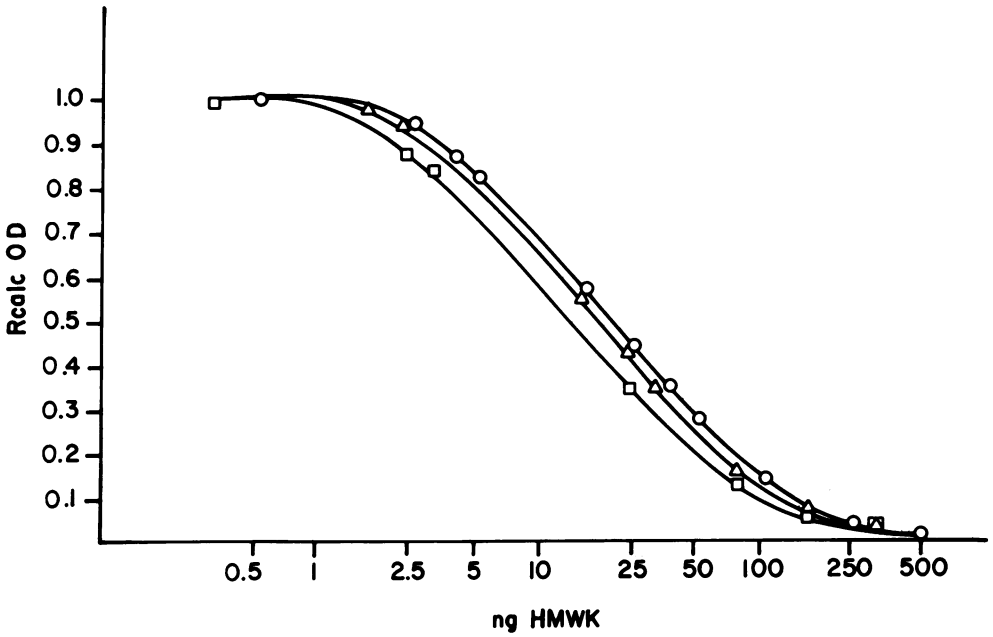

FIGURE 4 Standard competitive inhibition curve obtained with HMWK antisera on the CELISA. The ordinate is the relative absorbance (Rcalc OD) and the abscissa is the absolute amount of antigen incubated with antisera. Inhibition curve produced by purified HMWK (□); inhibition curve produced by pooled normal plasma (○); inhibition curve produced by total kininogen-immunodeficient plasma reconstituted with purified HMWK (△).

HMWK in washed platelets. Fresh platelets (10^9 platelets/ml), washed by albumin density gradient centrifugation and gel filtration, were used to directly determine the amount of platelet HMWK antigen by CELISA after detergent solubilization. ^{125}I-HMWK (2.2 μg with a sp act of 4.7 uCi/μg) was introduced into the platelet-rich plasma, incubated for 30 min, and traced through the washing procedure to estimate the amount of possible plasma contamination associated with these washed platelets (Table II). The final amount of the total ^{125}I-HMWK tracer in 1 ml of washed platelets (6.62×10^8 platelets/ml) was equal to 0.19 ng out of the 275 ng of the ^{125}I-HMWK added per milliliter of platelet-rich plasma or 0.07% of the original amount of the radiolabel. This finding indicated that for every milliliter of washed platelets, 56 ng of plasma HMWK or, if the ^{125}I-HMWK tracer was in equilibrium with the platelets and other HMWK, 8.45 ng HMWK/10^8 platelets would be present in the suspending medium if 1 ml plasma contained 80 μg HMWK. The equilibrium of the ^{125}I-HMWK tracer with platelet-rich plasma was assessed in additional experiments whereby the ^{125}I-HMWK tracer was incubated with the platelets in plasma for 1 min and 3 h. In those experiments there was only a 25% increase in the amount of the radiolabel associated with platelets at the longer incubation. In the CELISA, the amount of antigen directly measured was that which was present in 0.1 ml of washed platelets. Thus, only 5.6 ng or 0.85 ng/10^8 platelets of the HMWK antigen present in each assay of solubilized platelets could be from plasma HMWK contamination. To verify this estimate, the supernatants of washed platelets from the nine donors were directly assayed for HMWK antigen by CELISA. In nine individual donors the mean amount of HMWK antigen directly measured in the platelet supernatants was 2.8 ng±2.1/10^8 platelets (range 0.8–6.4 ng).

The detergent extract of washed platelets was studied for HMWK antigen. The amount of platelet HMWK antigen detected in all extracts had a mean value 60 ng±24/10^8 platelets (mean±SD) with a range of 27–101 ng/10^8 platelets. Platelet HMWK antigen was immunochemically indistinguishable from plasma HMWK antigen (Fig. 5). This immunochemical identity was indicated by the capacity of HMWK from solubilized platelets to produce complete competitive inhibition with an inhibition curve parallel in its linear portion to that produced by plasma HMWK (two-tailed $P > 0.8$ on a modified Youden analysis and two-tailed $P > 0.2$ on paired t test). In two separate experiments, platelets from a patient with a complete deficiency of plasma total kininogen had no detectable platelet HMWK antigen (i.e., <5 ng/10^9 platelets) on the CELISA.

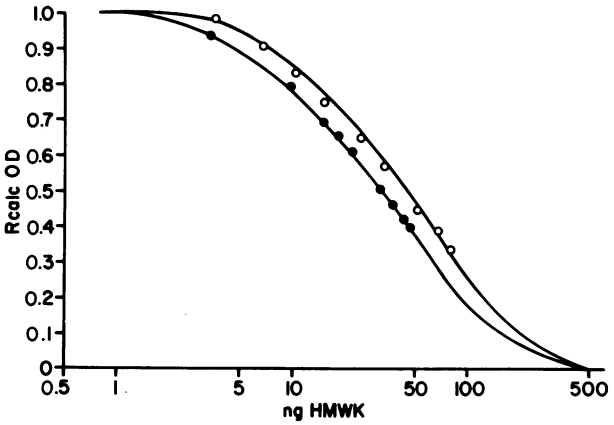

FIGURE 5 Competition inhibition CELISA comparing the detergent extract of washed human platelets with plasma HMWK. Inhibition curve produced by pooled normal plasma (○); inhibition curve produced by solubilized platelets (●). In the particular platelet extract analyzed, the concentration of HMWK antigen was 47 ng/10^8 platelets.

Platelet secretion of HMWK stimulated by the ionophore A23187. Washed fresh platelets secreted detectable HMWK after addition of 3 μM ionophore A23187 (Fig. 6 A). The maximum amount of secreted platelet HMWK was detected at 15 μM A23187. At higher doses of A23187 (data not shown), platelet HMWK secretion rose, but this higher amount of detectable HMWK was associated with increased amounts of platelet lysis, as determined by lactic dehydrogenase loss. At the concentrations of ionophore A23187 used, the amount of platelet lysis determined by lactic dehydrogenase assay was 0–5% (Fig. 6 A). With ionophore A23187, platelet HMWK secretion paralleled the secretion of the α-granule marker, low-affinity platelet factor 4, although not to the same extent (Fig. 6 A). Secretion of the dense body marker, 5-hydroxytryptamine, showed a similar concentration dependence.

The maximal secretion levels of each component were studied at 10 min after ionophore A23187 stimulation. Expressed as a percentage of the total platelet content, they were HMWK, 46% (SEM±2); 5-hydroxytryptamine, 66% (SEM±8); and low-affinity platelet factor 4, 67% (SEM±11) (Table III). The use of metabolic inhibitors showed that platelet HMWK secretion is an active process (Table III). The addition of D-gluconic acid δ-lactone, an inhibitor of glycogen phosphorylase (43) eliminated almost all secretion of the markers at the higher dose concentrations. At the maximum dose of ionophore A23187, metabolic inhibitors blocked almost 97% of platelet HMWK secretion, 88% of low-affinity platelet factor 4 secretion, and 91% of 5-hydroxytryptamine secretion.

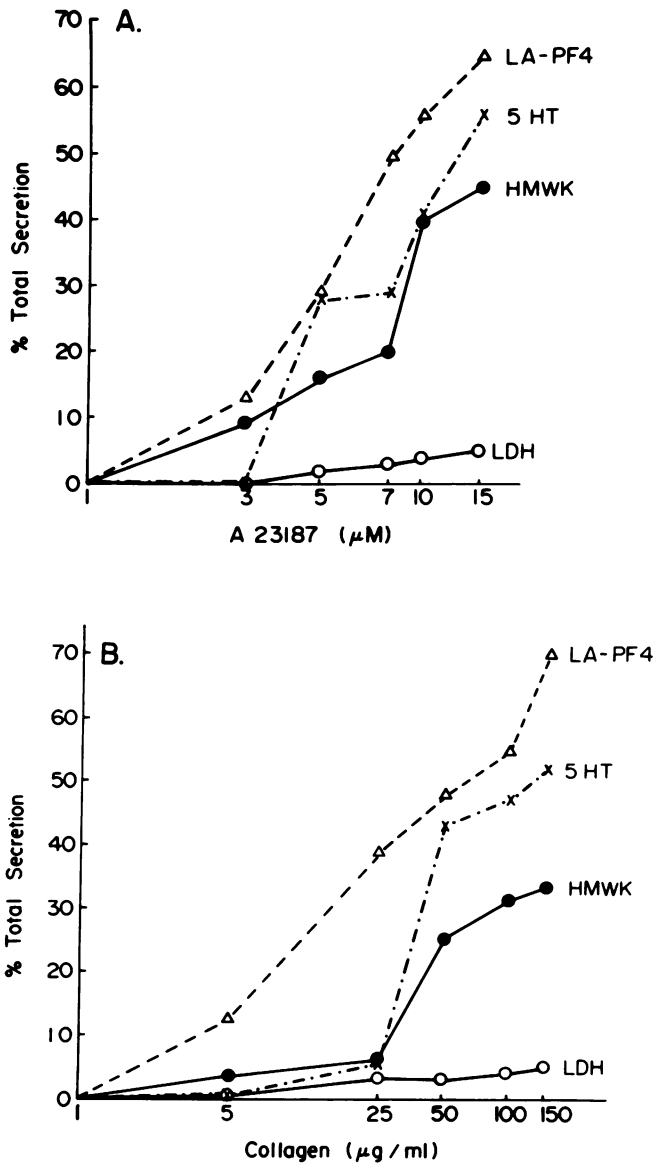

FIGURE 6 (A) Ionophore A23187-induced platelet secretion. Final concentration is plotted on the abscissa of the dose-response of agonist to percentage of total secretion, plotted on the ordinate. One representative experiment is shown of ionophore A23187 incubated with washed platelets for 10 min at 37°C stirred at 1,200 rpm. At a given dose in each experiment, assay of platelet HMWK, low-affinity platelet factor 4 (LA-PF_4) [^{14}C]5-hydroxytryptamine (5HT) and lactic dehydrogenase (LDH) were determined on the supernatants of activated platelets. HMWK secretion was determined by coagulant assay. (B) Collagen-induced platelet secretion. A dose-response curve of one representative experiment of collagen-induced secretion on washed platelets stirred at 1,200 rpm for 10 min at 37°C. All parameters are plotted as in Fig. 6 A.

Platelet secretion of HMWK by collagen. Platelet secretion studies with collagen were similar to those obtained when A23187 was used as the agonist. Platelet HMWK secretion was detectable at 5 μg/ml of collagen (Fig. 6 B). The maximal amount of platelet HMWK secretion was obtained at 150 μg/ml collagen. At collagen doses higher than this, platelet HMWK secretion, along with other platelet markers decreased. This drop in the amount of secretion apparently was due to a marked fall in the pH of the reaction mixture

TABLE III
*Percent Total Secretion at Maximal Agonist Doses**

Markers‡	Washed platelets§		Metabolic inhibitor-treated platelets‖	
	A23187¶	Collagen**	A23187¶	Collagen**
HMWK	46±2	32±12	3±2	12±6
LA-PF4	67±9	66±10	12±9	1±0.23
14C-5HT	66±8	43±13	9±3	0
LDH	5±2	6±13	1±0.63	1±0.6

* Platelet agonist was introduced into the cuvette and the platelet suspension was stirred at 1,200 rpm at 37°C. The total duration of time from the introduction of the stimulus till the end of stirring was 10 min. Values expressed were determined from the ratio of agonist-treated specimen over total amount present in freeze-thaw lysate after concomitant nonstimulated platelet controls were subtracted from both. Values represent the means±SEM of four experiments.

‡ LA-PF4, low-affinity platelet factor 4; [^{14}C]5HT, [^{14}C]5-hydroxytryptamine; LDH, lactic dehydrogenase. HMWK was determined by procoagulant assay. See Methods for each other assay procedure.

§ Washed platelets; albumin density gradient centrifugation and gel-filtered platelets.

‖ Metabolic inhibitor-treated platelets were platelets incubated with a mixture of antimycin A (15 μg/ml), 2-deoxy-D-glucose (30 mM), and D-gluconic acid δ-lactone (10 mM) for 30 min at 37°C.

¶ Ionophore A23187 was introduced in the cuvette in a final concentration of 15 μM.

** Collagen was introduced in the cuvette in a final concentration of 150 μg/ml.

from ~7.30 to ~7.00 and a rise in the lactic dehydrogenase level, suggesting platelet lysis. At the concentrations of collagen used, the amount of platelet lysis determined by lactic dehydrogenase assay was 0–6%.

Platelet secretion of HMWK by thrombin. The secretion of HMWK-related antigen from platelets after thrombin stimulation was assessed. Thrombin activation (1.6 U/ml) resulted in 63% secretion (a mean of two experiments) of platelet HMWK antigen as determined by CELISA. This result was not due to cell lysis since the lactic dehydrogenase loss from these platelets was <2%. In two simultaneously performed experiments, secreted platelet HMWK antigen did not become part of the Triton-insoluble cytoskeleton of thrombin-stimulated platelets. In a separate experiment 30 ml of washed platelets (1.20×10^8 platelets/ml) were stimulated with thrombin (1.6 U/ml) and stirred at 1,200 rpm at 37°C for 10 min. The supernatant was collected after centrifugation and concentrated to 1 ml. The thrombin-stimulated platelet concentrate was used for XIEP against anti-total kininogen antisera (Fig. 7). The thrombin-secreted platelet total kininogen antigen had the same electrophoretic mobility as plasma total kininogen antigen. This finding indicated that upon stimulation the released platelet total kininogen antigen was immunochemically similar to native plasma total kininogen and platelet total kininogen antigen.

DISCUSSION

The presence of HMWK and total kininogen in platelets is demonstrated in fresh washed platelets and in extracts prepared from platelets. Antigen from detergent-solubilized washed human platelets competitively inhibits an enzyme-linked immunosorbant assay specific for HMWK producing a curve parallel to that of plasma HMWK. These immunochemical characteristics indicate that the antigen within platelets shares all determinants expressed by plasma HMWK, and the antiserum reacts with these determinants with similar avidity. Platelets from nine individual donors contain HMWK and the mean level is 60 ng/10^8 platelets. Two independent approaches, ^{125}I-HMWK tracer studies and direct measurement of HMWK antigen in the platelet supernatants from each individual donor estimate that contaminating plasma HMWK could account for only 0.9–2.8 ng/10^8 platelets of the total amount of HMWK antigen measured in the aliquots of each platelet lysate determined by the CELISA. Therefore, plasma contamination could at most contribute only 5% of the level of HMWK detected in solubilized platelets. The measured value for platelet HMWK on the CELISA appears to represent intracellular platelet HMWK since total kininogen antigen is not detected on the platelet surface in an indirect antibody consumption assay. Moreover, activated or lysed washed platelets do not solubilize ^{125}I-HMWK, which was previously added to the platelet-rich plasma.

The presence of total kininogen antigen in prepared extracts of platelet concentrates is also shown. On XIEP, platelet total kininogen antigen is immunochemically identical to plasma total kininogen. Differences in the XIEP patterns of total kininogen antigen in Figs. 2 and 7 are the result of different batches of rabbit and goat antisera and do not reflect differences in total kininogen antigen. The XIEP patterns do not exclude the presence of LMWK in platelets. Independent studies by us have shown that functional prekallikrein or prekallikrein/kallikrein antigen is not associated with the platelet extract. Moreover, the XIEP patterns in Figs. 2 and 7 cannot be due to HMWK-prekallikrein complexes since this complex made with purified proteins has a less anodal migration using these antisera than the positions shown in the figures (data not shown).

The finding that platelet HMWK is secreted upon stimulation by ionophore A23187, collagen, or throm-

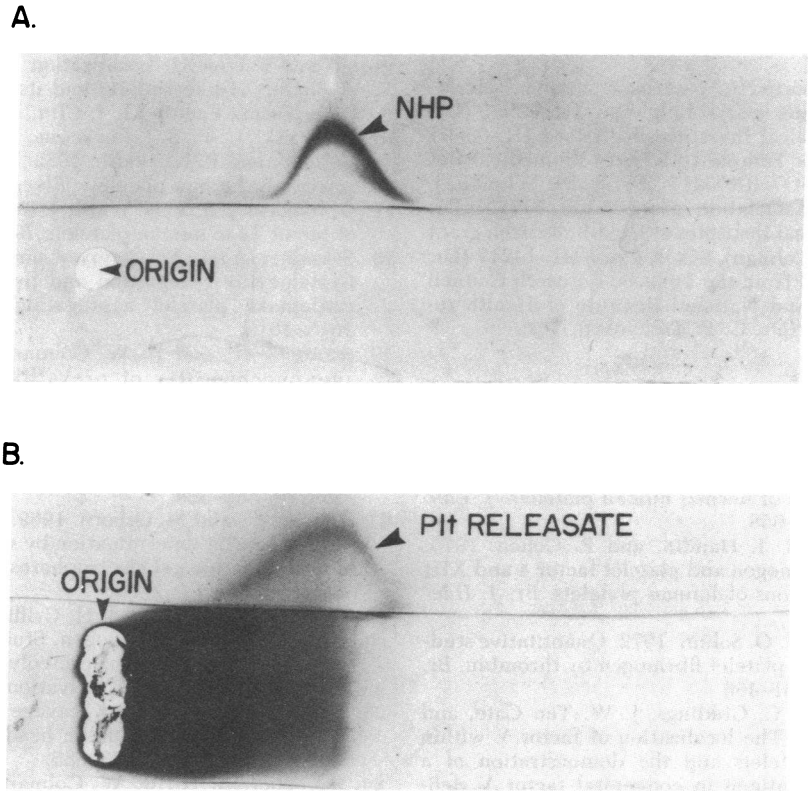

FIGURE 7 Thrombin-secreted platelet total kininogen antigen. (A) 20 μl of pooled normal plasma diluted 1:20 (NHP) and (B) 80 μl of a concentrated thrombin-stimulated platelet releasate (Plt releasate) were run on XIEP using anti-total kininogen antisera in a concentration of 2% in 1% agarose. (A) and (B) were performed simultaneously.

bin and that this secretion is an energy-requiring process without cell lysis is similar to the behavior of the other platelet procoagulant proteins e.g., fibrinogen (3), Factor VIII-related antigen (11), and Factor V (6–8). Immunochemically, secreted platelet total kininogen and plasma total kininogen show the same electrophoretic pattern. The results of low-dose stimulation of platelets with ionophore A23187 and collagen reveal that HMWK and low-affinity platelet factor 4 are secreted without concomitant release of serotonin. These data are consistent with the secretion pattern of α-granule constituents, such as platelet factor 4 and fibrinogen characterized by Kaplan et al. (47). However, platelet subcellular fractionation is needed to verify an α-granule localization of platelet HMWK.

The platelet HMWK level for 3×10^8 platelets/ml plasma is 180 ng, which constitutes 0.23% of the plasma level of 80 μg/ml. This value is less than that which has been determined for platelet fibrinogen (1.4%) (3), Factor V (14%) (48), and Factor VIII-related antigen 5–25%) (9, 10). However, since platelets are concentrated within the fibrin clot, local concentrations of secreted platelet HMWK may exceed physiologic plasma amounts. If platelets secrete their granules by exocytosis and platelet granules are about one-tenth the volume of platelets [the volume of 10^{11} platelets is 0.7 ml (49)], the concentration of platelet HMWK in granules at the platelet surface during secretion would be ~0.9 mg/ml for 10^8 platelets. Thus, like platelet Factor V, secretion may be a means of achieving high local concentrations at the platelet surface. This interpretation enhances its physiologic potential since platelet HMWK at this concentration may effectively promote the surface-dependent reactions of Hageman factor (50–52) and prevent the inactivation of contact phase proteases by their naturally occurring inhibitors (53).

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