High Molecular Weight Kininogen Binds to Unstimulated Platelets

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Abstract

Studies were performed to determine if the unstimulated platelet membrane has a site for high molecular weight kininogen (HMWK) binding. ^{125}I-HMWK bound to unstimulated platelets. Zn^{++} was required for ^{125}I-HMWK binding to unstimulated platelets and binding was maximal at 50 μM Zn^{++}. Neither Mg^{++} nor Ca^{++} substituted for Zn^{++} in supporting ^{125}I-HMWK binding to unstimulated platelets, and neither ion potentiated binding in the presence of 50 μM zinc. ^{125}I-HMWK competed with equal affinity with HMWK for binding, and excess HMWK inhibited ^{125}I-HMWK–platelet binding. Only HMWK, not prekallikrein, Factor XII, Factor XI, Factor V, fibrinogen, or fibronectin inhibited ^{125}I-HMWK–platelet binding. ^{125}I-HMWK binding to unstimulated platelets was 89% reversible within 10 min with a 50-fold molar excess of HMWK. Unstimulated platelets contained a single set of saturable, high affinity binding sites for ^{125}I-HMWK with an apparent dissociation constant of 0.99 $nM\pm0.35$ and 3,313 molecules/platelet±843. These studies indicate that the unstimulated external platelet membrane has a binding site for HMWK that could serve as a surface to modulate contact phase activation.

Introduction

The contact phase of plasma proteolysis consists of the zymogens, Factor XII, and prekallikrein (PK),[1] and the cofactor, high molecular weight kininogen (HMWK). Initiation of contact proteolysis is believed to be due to the binding of Factor XII to negatively charged surfaces where autoactivation of Factor XII occurs, converting it to an active serine protease (1–11). Nonphysiologic substances with a negative surface charge that activate Factor XII include kaolin, celite, glass, dextran sulfate, and ellagic acid (12). The physiologic activator(s) of the system is still unknown. Proposed substances have included crude collagen (13), sulfatides (14, 15), and glycosaminoglycans (16).

Platelets have been proposed as a negatively charged surface for contact activation. Platelets contain a form of HMWK (17) and platelet HMWK is expressed on the external platelet mem-

brane after thrombin activation (18). This latter finding suggests that the platelet membrane has a site for HMWK binding. Investigations by Greengard and Griffin (19) have indicated that HMWK specifically binds to activated platelets. If HMWK only binds to activated platelets, then the participation of platelets in contact activation could never be a primary event in intrinsic coagulation. It would only result as a consequence of platelet activation and thrombin formation. Alternatively, if it were shown that platelets that have not been activated by exogenous agonists, which we will call unstimulated platelets, contain a high affinity receptor for HMWK, then platelets could be a surface to modify intrinsic coagulation. A study was initiated to determine whether unstimulated platelets possess a binding site for HMWK. The results of this investigation show that a specific, high affinity binding site for HMWK is present on the unstimulated platelet surface.

Methods

Materials. Human alpha thrombin (3,209 NIH U/mg) was obtained from Dr. J. Fenton, New York State Department of Health, Albany, NY. ^{125}I-Na (50 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Iodogen (chloroamide, 1,3,4,6-tetrachloro-3 alpha, 6 alpha-diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, IL. N-butyl-phthalate was obtained from Fisher Scientific Co., Pittsburgh, PA. Chelex 100 was purchased from Bio-Rad Laboratories, Richmond, CA. Apyrase was prepared by the method of Molnar and Lorand (20), and stored at −70°C until used. Heparin was obtained from Elkins-Sinn, Inc., Cherry Hill, NJ. Purified Factor XI (600 $\mu g/ml$) was a gift from Dr. Peter Walsh; Factor XII (68 $\mu g/ml$) and Factor V (1 mg/ml) were obtained from Dr. Robin Pixley and Dr. Robert Colman; and fibrinogen (17 mg/ml) and fibronectin (1.3 mg/ml) were donated by Dr. Andrei Budzynski; all of Temple University, Philadelphia, PA. Apiezon oil (a mixture of silicon oils) was obtained from Apiezon Products Limited, London, England. All other materials were obtained from Sigma Chemical Co., St. Louis, MO and were the best grade commercially available.

Plasma and platelets. Pooled normal plasma (lot N 10) was purchased from George King Biomedical, Inc., Overland Park, KS. Total kininogen-deficient plasma (plasma deficient in both HMWK and low molecular weight kininogen) and platelets were donated by Mrs. M. Williams, Philadelphia, PA. Prekallikrein-deficient plasma was donated by Dr. Charles Abiligard, University of California at Davis. Normal donors were young males and females (age 21–45 yr) receiving no medication, who had given their written informed consent. Fresh blood was collected and platelet-rich plasma and platelet-poor plasma were prepared as previously described (21).

Proteins. HMWK was purified using a modified method (18) of Kerbiriou and Griffin (22) which included 0.2 M epsilon amino caproic acid in all buffers and 2 mM diisopropylfluorophosphate added to the pooled material before each step. This preparation of HMWK on a 7.5% polyacrylamide gel with sodium dodecyl sulfate (SDS) on reduced gel electrophoresis (23) was mostly a single band of 120,000 molecular weight (mol wt) with >98% purity (Fig. 1) and with a specific activity of 12–20 U/mg. In some experiments a partially cleaved preparation of HMWK was used which contained 70% single-chain HMWK (120,000 mol wt) and 30% two-chain HMWK (64,000 and 47,000 mol wt), as determined from densitometer scans (SD 3000 Spectrodensitometer; Schoeftel In-
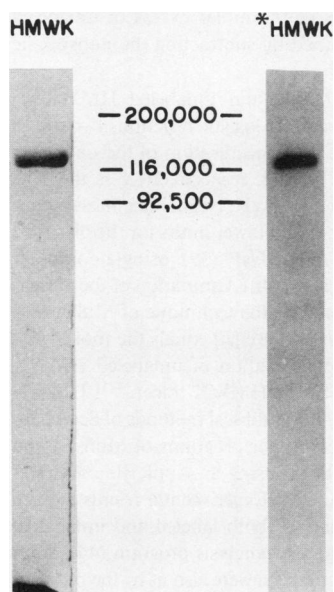
1. *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; HMWK, high molecular weight kininogen; PADGEM, platelet activation-dependent granule-external membrane; PGE_1, prostaglandin E_1; PK, prekallikrein.

Figure 1. Polyacrylamide gel electrophoresis SDS of HMWK. Gel on left (HMWK) represents 10 μg of purified HMWK stained with Coomassie Blue R-250. Gel on right (*HMWK) represents ^{125}I-HMWK autoradiogram after gel electrophoresis in SDS. The gels represented were run in an acrylamide concentration of 7.5 in the presence of SDS after the sample was reduced by boiling for 10 min with 2% β-mercaptoethanol. The numbers in the center represent molecular weight standards.

strument Division, Kratos, Inc., Westwood, NJ) of autoradiograms of the protein after it had been electrophoresed on reduced polyacrylamide gels in SDS.

Purified HMWK was radiolabeled with ^{125}I-Na using Iodogen by the method of Fraker and Speck (24) under conditions previously described (17). Purified HMWK (100–250 μg) in 0.02 M Tris-HCl, 1.0 M NaCl, pH 8.0 was incubated with equal amounts of ^{125}I-Na (1 mCi) and sodium iodide carrier in a polypropylene tube chilled on ice. The starting ratio of total iodide/HMWK was 2:1. This mixture was transferred to another propylene tube that had been precoated with 4 μg of Iodogen and incubated for 30–60 min on ice. The reaction was stopped by the addition of sodium metabisulfite (final concentration, 50 μg/ml) 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 Tris-Cl, 1.0 M NaCl, pH 8.0, containing 0.25% gelatin. The specific radioactivity of the protein varied from 1 to 7 μCi/μg and >70% of the molecules of HMWK were iodinated. The radiolabeled protein was >95% trichloroacetic acid–precipitable and retained >95% of its procoagulant activity as well as its antigenic properties. ^{125}I-bovine serum albumin (BSA) was prepared by a similar procedure using Iodogen (24).

PK was purified by the method of Scott et al. (25). Protein determinations were performed by the method of Bradford (26) using crystalline BSA as the standard.

Functional and immunochemical assays. HMWK procoagulant activity was measured by a one-stage kaolin activation assay (27) using total kininogen-deficient plasma as substrate. Samples were compared against a daily standard curve from pooled normal human plasma diluted 1/10 to 1/1,000 with 0.01 M Tris 0.15 M NaCl, pH 7.4. One unit was defined as that amount in 1 ml of pooled normal plasma. HMWK antigen was assayed by electroimmunodiffusion using monospecific antisera to the light-chain of HMWK as described previously (17). Total platelet HMWK was measured by a competitive enzyme-linked immunosorbent assay (ELISA), performed as previously published (17). PK coagulant activity (Fletcher factor) was measured by a modification of the activated partial thromboplastin time using PK-deficient plasma as substrate as previously described (28). 1 U of coagulant activity was defined as the amount present in 1 ml of normal pooled plasma. Radial immunodiffusion (29) using goat antisera monospecific to human PK/kallikrein was used to quantitate PK antigen as previously described (30).

Preparation of washed platelets. Platelet-rich plasma (21) was obtained from citrated human blood and gel filtered on a Sepharose 2B column (31) equilibrated in calcium-free Tyrode's buffer (0.135 M NaCl, 2.7 mM KCl, 11.9 mM NaHCO$_3$, 0.36 mM NaH$_2$PO$_4$, pH 7.35) with Hepes (14.7 mM), BSA (1 mg/ml), and dextrose (3.5 mg/ml) (32). Unless stated

otherwise in the text, this buffer, which is called Hepes Tyrode's buffer, also contained 2 mM Mg^{++}. Platelets were counted electronically (Coulter Electronics, Inc., Hialeah, FL). Before and after gel filtration, all platelet preparations were studied for their ability to respond to ADP (1–5 μM) when stirred in an aggregometer (Chronolog Corp., Havertown, PA). Fibrinogen (final concentration, 200 μg/ml) was added to the gel-filtered platelets before the addition of the agonist, ADP. Only platelets that responded to ADP (≤5 μM) with both primary and secondary wave platelet aggregation in platelet-rich plasma and ADP (≤5 μM) and human fibrinogen (200 μg/ml) after gel filtration were used in the binding studies. In experiments with prostaglandin E$_1$ (PGE$_1$)-treated platelets, blood was collected into anticoagulant containing 2 μM PGE$_1$ and the platelet-rich plasma was gel-filtered in buffer and on Sepharose 2B which was equilibrated with 2 μM PGE$_1$. After gel filtration, the washed platelets were tested to respond to 5 μM ADP in the presence of 200 μg/ml fibrinogen in an aggregometer. If the platelets responded to ADP, an additional treatment with PGE$_1$ (final concentration, 2 μM) was given to the washed platelets before initiating binding studies.

Platelets were also prepared by the centrifugation technique of Mustard et al. (33). Nine parts blood were collected into one part anticoagulant consisting of 73 mM citric acid, 3 mM trisodium citrate containing 2% dextrose and platelet-rich plasma was obtained (21). After adjusting the pH of the platelet-rich plasma to 6.5 with citric acid, the platelet-rich plasma was washed twice by centrifugation. The Tyrode's buffer containing Ca^{++} (2 mM) and Mg^{++} (1 mM) had both apyrase and heparin (12.5 U/ml) in the first wash and apyrase alone in the second wash. Apyrase was titered so that the minimal amount necessary to prevent second-wave platelet aggregation with ADP was used. The final washed platelets were resuspended in Hepes Tyrode's buffer (32).

On scanning electron microscopy, washed platelets from both techniques had some pseudopodia. In this manuscript, the term unstimulated platelets refers to platelets which did not receive exogenous agonists.

The HMWK content of washed platelets was studied by the techniques previously reported (17). Briefly, washed platelets were solubilized with 0.5% Triton X-100 for 30 min at 23°C. The platelet lysates were then studied for their HMWK content by a competitive ELISA (17).

Immunofluorescence of washed platelets. Immunofluorescence staining of thrombin-treated or buffered-treated platelets was performed by the technique of Wencel-Drake et al. (34). Briefly, gel-filtered platelets were left untreated or stimulated with thrombin (0.1 U/ml) in the presence of Gly-Pro-Arg-Pro (0.4 mM) to prevent fibrin formation and platelet aggregation (35). Aliquots of each platelet preparation were fixed with 2% paraformaldehyde on ice for 1 h. Unreacted aldehyde was blocked with NH$_4$Cl–Tris-buffered saline, pH 7.4, and the cells were permitted to settle on polylysine-coated glass coverslips. In some cases, the cells were treated with 0.1% Triton X-100 for 3 min to render them permeable to antibody before staining. The permeable or intact cells were then incubated for 20 min with either specific antibody or preimmune rabbit serum. The cells were rinsed with Tris-buffered saline containing 0.1% radioimmunoassay grade BSA and stained for 20 min with a fluorescein-labeled goat antirabbit IgG (Sigma Chemical Co.). The platelets were viewed at a magnification of 400 with an Olympus BH$_2$ microscope (Olympus Corp., New Hyde Park, NY) equipped with a 100-W mercury lamp for phase contrast and epifluorescence microscopy with a chromatic splitter and 0515 barrier filter and photography using 3M ISO 1000 film. Identical photographic exposure, development, and printing times were used to depict immune and nonimmune (control) immunofluorescence. Antiplatelet glycoprotein antiserum (36) used in the immunofluorescence was the generous gift of Dr. Alan Gewirtz, Temple University, Philadelphia, PA. Antiserum to the platelet activation-dependent granule-external membrane (PADGEM) protein (37, 38) was the generous gift of Dr. Barbara Furie and Dr. Cindy Berman, New England Medical Center, Boston, MA. The PADGEM protein is a platelet alpha granule membrane protein which only becomes expressed on the external membrane of activated platelets (37, 38).

Atomic absorption spectroscopy. Atomic absorption spectroscopy was performed in a Perkin-Elmer Mass Spectrophotometer (Model 4000 AA, Perkin-Elmer, Corp., Norwalk, CT). For determination of magnesium,

a stock standard solution of 83 mM magnesium was prepared and serial dilutions from 41.6 to 0.58 μM were prepared and used as the standard curve. A 285.2-nm wavelength and a 0.7-nm slit width were used. For zinc determination, a stock standard solution of 16.6 mM of zinc was made and serial dilutions from 33 to 0.6 μM were prepared. The wavelength was 214 nm and the slit width was 0.7 nm. For the determination of calcium, a stock solution of 25 mM was prepared in 0.01% lanthanum chloride and serial dilutions from 200 to 6.25 μM were prepared. The wavelength was 423 nm and the slit width was 0.7 nM. For all ion determinations a nitrous oxide-acetylene flame was used. Deionized water was used for a blank setting at 0.000. Hepes Tyrode's buffer with or without added magnesium (2 mM final concentration) and BSA (2 g/liter) and dextrose (1 g/liter) were prepared. The levels of Zn^{++}, Mg^{++}, and Ca^{++} were determined for both buffers (Hepes Tyrode's without and with magnesium) before and after passage through a 1×5 cm Chelex 100 column. Before passage through the column, the concentration of Zn^{++} was 3 μM; Ca^{++}, 12 μM; and Mg^{++}, 50 μM—in the absence of added magnesium. After passage through the column, the concentration of each of the divalent cations was ≤ 1 μM (the limit of sensitivity of atomic absorption spectroscopy).

Binding experiments. In all binding experiments platelets were at a final concentration of 2×10^8/ml. In a typical binding experiment 200–300 μl of gel-filtered platelets in Hepes Tyrode's buffer, pH 7.35 were incubated at 37°C without stirring in a 1.5 ml polypropylene centrifuge tube (Sarstedt, Inc., Princeton, NJ) with radiolabeled HMWK and additions of a total volume of 250–350 μl. After incubation of appropriately expanded volumes, 50-μl aliquots were removed (in triplicate) for each experimental point and centrifuged at 9,650 g at 23°C in a microfuge (Model B; Beckman Instruments, Inc., Fullerton, CA) through a 200-μl mixture of silicon oils (one part Apiezon/nine parts N-butyl-phthalate) in polypropylene microsediment tubes with narrow bore extended tips (Sarstedt, Inc.) for 2 min at room temperature. After the supernatant was carefully removed, the tips containing the pellet were amputated and counted in a Rack gamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Unless otherwise stated, all binding assays were performed on washed platelets with additions that were incubated for 10–15 min before centrifugation through oil.

Measurement of trapped aqueous volume and nonspecific binding. Platelets were incubated with ^{125}I-labeled HMWK BSA for 50 min at 37°C and centrifuged over a mixture of silicone oils as described above. The fraction of the radiolabeled BSA that sedimented was assumed to correspond to the fraction of aqueous volume that sedimented, since the buffer contained 1 mg/ml BSA. The fraction of the radiolabeled BSA that appeared in the pellet was <0.2% of the total volume in a typical binding experiment.

Nonspecific binding was determined in simultaneously performed experiments with identical amounts of ^{125}I-HMWK and additions in the presence of 50-fold or 100-fold molar excess of unlabeled HMWK. If a 50-fold excess of unlabeled HMWK was used, nonspecific binding was ≤ 0.5% of the radioactivity added. Nonspecific binding was ≤ 0.4% of added radioactivity with a 100-fold molar excess of unlabeled HMWK.

Characterization of platelet-bound ^{125}I-HMWK. The form of ^{125}I-HMWK bound to platelets was assessed by solubilizing platelet pellets with bound ^{125}I-HMWK in 4% SDS containing 0.5 mM leupeptin. After reduction of the solubilized platelet pellet and its supernatant with 2% β-mercaptoethanol, the samples were applied to an 8% polyacrylamide gel in SDS for electrophoresis. The structure of platelet-bound ^{125}I-HMWK was determined by performing autoradiography on the dried gel. Leupeptin was added to the 4% SDS to prevent proteolysis of the platelet-bound ^{125}I-HMWK by platelet calpains during the solubilization (18).

Expression and calculations of binding experiments. Calculation of bound HMWK was based on the specific activities of the radiolabeled ligand, and results were expressed as nanograms of HMWK bound per 10^8 platelets. In concentration dependence experiments, total binding was the amount of ^{125}I-HMWK bound in the absence of a 50-fold molar excess of unlabeled ligand, while nonspecific binding was the amount of

^{125}I-HMWK bound in the presence of the molar excess of unlabeled ligand. "Specific" binding was obtained by subtracting the nonspecific binding from the total binding.

Competitive binding of ^{125}I-HMWK and unlabeled HMWK to platelets were analyzed by a four-parameter logistic function, $Y = d + (a - d)/(1 + X/c)^b$, where X and Y are the concentration of the unlabeled HMWK and radiolabeled HMWK bound, respectively; c is the 50% inhibitory concentration by unlabeled HMWK; b is the steepness factor; and a and d are the extrapolated upper and lower limits for the observed value of Y, when X is 0 or infinite, respectively (39), using a computer program developed by Canellas and Karu (40). Estimation of the affinity constant of the binding was performed by the technique of Muller (41) using the formula $K_a = 8/3([I_t] - [T_t])$ where $[I_t]$ equals the molar concentration of the 50% inhibitory concentration of unlabeled HMWK and $[T_t]$ is the molar concentration of ^{125}I-HMWK tracer. ^{125}I-HMWK platelet binding was also analyzed by the graphical methods of Scatchard (42), and Klotz (43) as well as the computer programs of Munson and Rodbard (44) and Brass and Shattil (45) using an Apple IIe computer (Apple Computer Corp., Cupertino, CA). Experimental results at each concentration of the total dose of ligand (both labeled and unlabeled) and bound radioligand were fed into a preanalysis program (44). After calculating the amount of free ligand the data were also fit by the program developed by Brass and Shattil (45). When using this latter program, evaluable results were those with a calculated error of <0.02% and a generated curve that intersected all the data points. A group paired t test was used to determine if binding data from various experiments were different.

Results

Binding of ^{125}I-labeled HMWK to unstimulated and thrombin-activated platelets. The requirement for platelet activation to demonstrate HMWK binding to platelets was ascertained by measuring the amount of ^{125}I-HMWK-platelet binding to platelets activated by variable concentrations of thrombin (Fig. 2). In the absence of thrombin, 4.6 ng ^{125}I-HMWK/10^8 platelets were bound. This binding to unstimulated platelets was specific because in the presence of a 50-fold molar excess of unlabeled HMWK, the nonspecific binding was <30% of the total binding.
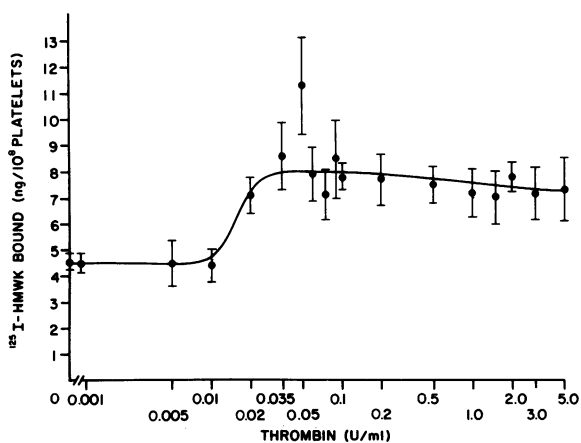
Figure 2. The effect of thrombin concentration on ^{125}I-HMWK binding to platelets. Gel-filtered platelets (2.2×10^8/ml) in Hepes Tyrode's buffer, pH 7.35, were incubated at 37°C with ^{125}I-labeled HMWK (1 μg/ml) in the presence of Zn^{++} (50 μM) and thrombin at the concentrations indicated. After 10 min incubation, the amount of ^{125}I-HMWK bound to the platelets was determined as indicated in Methods. The results presented are the mean±SEM of seven separate experiments and the plotted data represent total binding.

After thrombin activation of platelets (0.001–0.01 U/ml), there was no significant increase ($P > 0.05$) in the amount of ^{125}I-HMWK that bound to platelets over unstimulated platelets when platelets were incubated with radioligand for 10 min. However, at concentrations of thrombin ≥ 0.02 U/ml, there was a significant increase ($P < 0.01$) in the amount of ^{125}I-HMWK bound to that seen with unstimulated platelets and that at the lower doses of thrombin. These results indicated that ^{125}I-HMWK substantially bound to both unstimulated and activated platelets.

The ability of ^{125}I-HMWK to bind to platelets in the absence of exogenous activation was further studied by three independent approaches. In the first approach, binding of ^{125}I-HMWK to platelets prepared by gel filtration was compared with binding to platelets prepared by the centrifugation technique of Mustard et al. (33). Binding of ^{125}I-HMWK to unstimulated platelets washed by either technique increased over time with a similar kinetic pattern (data not shown). At 10 min, 3.0 ng and 2.9 ng ^{125}I-HMWK were bound per 10^8 platelets for gel-filtered platelets and centrifugation-washed platelets, respectively. The amount of ^{125}I-HMWK bound to platelets washed by either technique was not significantly different ($P > 0.25$) at all time points (1–50 min). This study indicated that the ability of ^{125}I-HMWK to bind to unstimulated platelets was not a function of the technique used to prepare the platelets.

As a second approach to determine that ^{125}I-HMWK was binding to unstimulated platelets, a series of experiments was performed comparing binding of ^{125}I-HMWK to gel-filtered platelets prepared in the absence or presence of 2 μM PGE$_1$. In these experiments, ^{125}I-HMWK bound equally to the two preparations of gel-filtered platelets. At 10 min, 3.7 ng ^{125}I-HMWK/ 10^8 platelets± 1.1 (mean$\pm$SD) bound to gel-filtered platelets and 3.4 ng ^{125}I-HMWK/10^8 platelets± 0.6 bound to PGE$_1$-treated gel filtered platelets. These results were not demonstrated to be significantly different ($P > 0.15$).

We performed immunofluorescence studies on unstimulated and activated platelets using antiserum directed to the PADGEM antigen (37, 38) to determine if the gel-filtered platelets used in the binding studies were activated. When we used Triton X-100 permeabilized platelets, both an anti-platelet glycoprotein antibody and the PADGEM antibody produced positive immunofluorescence that diffusely stained the platelets. When gel-filtered platelets were activated with 0.1 U/ml thrombin, a rim pattern of immunofluorescence was seen with the PADGEM antibody. However, when gel-filtered platelets were fixed after washing without permeabilization with Triton X-100 or without treatment with thrombin, no immunofluorescent staining was detected when the platelets were reacted with the PADGEM antibody. This study indicated that the gel-filtered platelets used in the present studies were unstimulated. These combined investigations indicated that the washed platelets used in these binding studies were unstimulated platelets.

Effect of divalent cations on ^{125}I-HMWK binding to unstimulated platelets. In order to establish the optimal conditions for ^{125}I-HMWK-platelet binding, studies were carried out to determine the divalent cation requirements for ^{125}I-HMWK binding to unstimulated platelets (Fig. 3). Binding of ^{125}I-HMWK to platelets increased with time at all concentrations of Zn^{++} and was greatest at any time point with a concentration of 50 μM added Zn^{++}. However, binding of ^{125}I-HMWK to platelets in the presence of 50 μM Zn^{++} at any time point was not significantly greater than that seen at 25 or 100 μM added zinc ($P \geq 0.10$ and $P \geq 0.05$, respectively). The amount of ^{125}I-HMWK
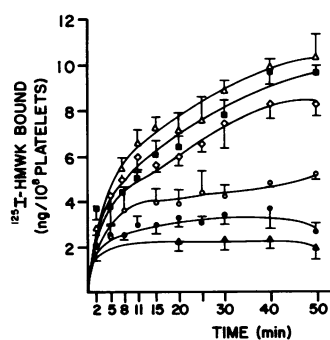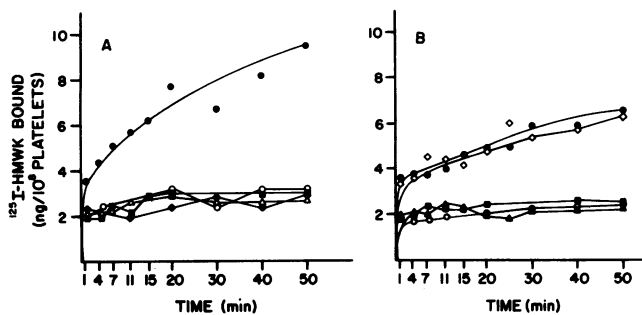

Figure 3. Effect of Zn^{++} concentration on ^{125}I-HMWK binding to unstimulated platelets. Time course experiments: Gel-filtered platelets (2.2 × 10^8/ ml) in Hepes Tyrode's buffer, pH 7.35, were incubated at 37°C with ^{125}I-labeled HMWK (1 μg/ml) for variable time periods in the presence of varying concentration of Zn^{++}: 0 μM, ●; 25 μM, ■; 50 μM, △; 100 μM, ◇; and 200 μM, ○. Nonspecific binding (NS) was measured in the presence of a 50-fold molar excess of unlabeled HMWK and 50 μM Zn^{++} (▲). At designated time points, samples were removed and ^{125}I-HMWK-platelet binding was determined as indicated in Methods. The plotted data are the mean±SEM of six experiments and represent total binding.

bound with 200 μM added ZnCl$_2$ was significantly less ($P < 0.001$) than the amount bound with any of the above concentrations of zinc. The amount of ^{125}I-HMWK bound in the presence of 50 μM Zn^{++} and a 50-fold molar excess of unlabeled HMWK was not significantly different from the amount bound with no added ZnCl$_2$ ($P > 0.05$). Passage of the buffer through Chelex 100 resin did not decrease the level of binding seen when no zinc was added. These findings indicated that there is an optimal concentration of Zn^{++} (25–100 μM) which results in maximal binding of ^{125}I-HMWK to unstimulated platelets. These concentrations of zinc correspond to the physiological levels of zinc in plasma (46).

We performed further studies to determine the contribution of the divalent cations, calcium and magnesium, on ^{125}I-HMWK binding to unstimulated platelets. Calcium ion alone could not support ^{125}I-HMWK binding to platelets. Using platelets washed in the presence of 2 mM Mg^{++}, Ca^{++} in a concentration from 0.5 to 7.0 mM did not contribute to any statistically significantly greater ^{125}I-HMWK platelet binding to unstimulated platelets in the presence of 50 μM Zn^{++} than zinc alone ($P \geq 0.1$). Additional studies were performed to determine whether magnesium ion contributed to ^{125}I-HMWK platelet binding. When we used platelets washed in the absence of magnesium, the level of ^{125}I-HMWK platelet binding in the presence of 2 mM Mg^{++} alone was not significantly different than that found in the presence of 50 μM zinc and a 50-fold molar excess of unlabeled HMWK ($P \geq 0.30$). Moreover, binding of ^{125}I-HMWK to unstimulated platelets in the presence of 50 μM Zn^{++} and 2 mM Mg^{++} was not significantly greater than the binding seen in the presence of zinc alone ($P \geq 0.30$). However, binding of the radiolabeled ligand in the presence of zinc was significantly greater than any binding in the absence of zinc ($P < 0.005$). These combined studies suggest that neither calcium nor magnesium contribute to the ability of ^{125}I-HMWK to bind to unstimulated platelets.

The combined effect of Zn^{++}, Ca^{++}, and Mg^{++} on ^{125}I-HMWK platelet binding to unstimulated platelets was studied further (Fig. 4). In Fig. 4 A, platelets were prepared in the presence of 2 mM Mg^{++} and ^{125}I-HMWK platelet binding was performed in the presence of 2 mM Ca^{++}. In these experiments, specific binding of ^{125}I-HMWK to unstimulated platelets only occurred in the presence of zinc ion. When zinc was excluded from the

Figure 4. Effect of chelators of divalent cations on HMWK binding to unstimulated platelets. Gel-filtered platelets (2.2 × 10⁸/ml) in Hepes Tyrode's buffer were prepared in the presence (*A*) or absence (*B*) of 2 mM Mg^{++} and incubated at 37°C for 2–50 min with ^{125}I-labeled HMWK (1 μg/ml) and various combinations of divalent cations. In each experiment where an addition was made the concentration of Zn^{++} was 50 μM; Ca^{++}, 2 mM; and Mg^{++}, 2 mM. At the designated time points, ^{125}I-HMWK-platelet binding was determined as indicated in Methods. In *A*: — ● —, Zn^{++}, Ca^{++} and Mg^{++} (total); — ■ —, Zn^{++}, Ca^{++}, Mg^{++} plus EDTA (10 mM); — △ —, Zn^{++}, Ca^{++}, Mg^{++} plus EGTA (10 mM); — ♦ —, Ca^{++}, Mg^{++} alone but no Zn^{++}; and — ○ —, Zn^{++}, Ca^{++}, Mg^{++} in the presence of 50 μg/ml unlabeled HMWK (nonspecific). The data plotted are the mean of three experiments with platelets washed in the presence of Mg^{++} and represent total binding. In *B*, — ● —, Zn^{++}, Ca^{++} and Mg^{++} (total); — ◇ —, Zn^{++} alone; — ■ —, Zn^{++} plus EDTA (10 mM); — ▲ —, Ca^{++}, Mg^{++} alone but no Zn^{++}; and — ○ —, Zn^{++}, Ca^{++}, Mg^{++} in the presence of 50 μg/ml unlabeled HMWK (nonspecific). The plotted data are the mean of six experiments with platelets washed in the absence of Mg^{++} and represent total binding.

divalent cation mixture, or EDTA (10 mM) or EGTA (10 mM) was included in the reaction mixture, the level of binding was not any different than that seen when binding was performed in the presence of a 50-fold molar excess of unlabeled HMWK. In Fig. 4 *B*, platelets were prepared in the absence of Mg^{++}. These washed platelets with no added Mg^{++} aggregated to 5 μM ADP in the presence of 200 μg/ml human fibrinogen. In these experiments, specific ^{125}I-HMWK binding to unstimulated platelets again only occurred when Zn^{++} was present. Moreover, the level of ^{125}I-HMWK-platelet binding with zinc alone was not significantly different than that seen with Zn^{++} in the presence of added calcium and magnesium ions ($P \geq 0.50$). In the absence of zinc or in the presence of 10 mM EDTA, the level of ^{125}I-HMWK-platelet binding was not any different than that seen with zinc in the presence of a 50-fold molar excess of unlabeled HMWK. These combined studies indicated that Zn^{++} is the sole divalent cation requirement for ^{125}I-HMWK binding to unstimulated platelets.

Specificity of ^{125}I-HMWK platelet binding to unstimulated platelets. Three approaches were utilized to establish that binding of ^{125}I-HMWK to the unstimulated platelet was specific. First, it was determined that the observed interaction of ^{125}I-HMWK with platelets was not due to radioiodination of the HMWK. Gel filtered platelets were incubated in the presence of mixtures of ^{125}I-HMWK and unlabeled HMWK at a constant total concentration of HMWK. As the proportion of radiolabeled HMWK to unlabeled HMWK increased, a linear relationship was observed in the percentage of ^{125}I-HMWK in the mixtures and the amount bound (Fig. 5). The linear correlation coefficient of *r* > 0.98 indicated that the labeled and nonlabeled HMWK interacted with the platelet with the same apparent affinity.
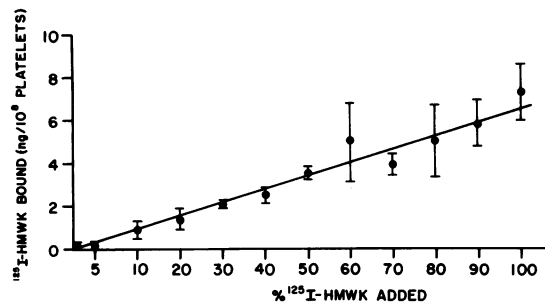
Figure 5. Relative binding of ^{125}I-labeled HMWK and unlabeled HMWK to unstimulated platelets. Gel-filtered platelets (2.2 × 10⁸/ml) in Hepes Tyrode's buffer, pH 7.35, were incubated for 10 min at 37°C with Zn^{++} (50 μM) and various proportions of ^{125}I-labeled and unlabeled HMWK, keeping the total concentration of HMWK constant at 1 μg/ml. Binding was determined as detailed in Methods and binding of ^{125}I-labeled HMWK was plotted as a function of the fraction of the total made up of added ^{125}I-HMWK. The plotted data are mean±SEM of three experiments.

Since <2% of the added ^{125}I-HMWK was bound by the platelets, we attempted to verify that the observed binding was not limited to a small population of the added ^{125}I-ligand. A constant amount of ^{125}I-HMWK (1 μg/ml) was added to platelets in the absence or presence of a 50-fold molar excess of unlabeled HMWK. After 50 min at 37°C, the unbound fraction of the ^{125}I-HMWK was transferred to a second platelet suspension and incubated for 50 min. The second unbound fraction was then transferred a third time. No decrease in the percent of the ligand bound to platelets was observed from the first to third transfer. Therefore, the observed specific binding of ^{125}I-HMWK to unstimulated platelets did not appear to be limited to a small subpopulation of radiolabeled HMWK molecules. Platelet-bound ^{125}I-HMWK from solubilized platelets on autoradiograms of reduced polyacrylamide gels in SDS was identical to the added ^{125}I-HMWK ligand.

As a second approach to validate specificity of ^{125}I-HMWK-platelet binding, we assessed the capacity of unlabeled HMWK, related contact phase proteins, and unrelated coagulation proteins to inhibit the platelet interaction with ^{125}I-HMWK. As shown in Table I, unlabeled HMWK inhibited the binding of the radiolabeled ligand by >79%, which confirmed previous estimates of nonspecific binding of <30% of total binding. In 10 experiments using at least five different preparations of labeled ligand, the level of nonspecific binding was 17±3% (mean±SEM). Binding of ^{125}I-HMWK was not inhibited by a 25–100-fold molar excess of PK, Factor XI, Factor XII, Factor V, fibronectin, or fibrinogen regardless of whether the competitor and the ^{125}I-HMWK incubated 10 or 50 min after simultaneous introduction to the platelets.

A third approach to assess the specificity of ^{125}I-HMWK binding to inactivated platelets was to examine the effect of increasing concentrations of unlabeled HMWK on the binding of ^{125}I-HMWK when the dose (7.45±0.29 nM) of the radioligand was held constant (Fig. 6). Unlabeled HMWK competitively inhibited the binding of radiolabeled HMWK. The mean±SEM concentration of unlabeled HMWK required for 50% competition inhibition was 0.71±0.35 μg/ml (6.06 nM±2.9), which gave a calculated apparent dissociation constant (K_d) of 1.57±0.1 nM (41). This value was similar to the calculated K_d (0.52 nM) determined from the mean of each point in all experiments used

Table I. Specificity of ^{125}I-HMWK Binding to Unstimulated Platelets*

Protein competitor‡	Amount of ^{125}I-HMWK bound pl§	HMWK bound as a percent of control
None	11.4	100
Unlabeled HMWK (50)	2.45	21
Prekallikrein (100)	10.69	94
Factor XI (25)	8.58	75
Factor XII (100)	7.75	68
Factor V (50)	15.2	133
Fibronectin (50)	9.74	85
Fibrinogen (50)	8.95	79

* ^{125}I-labeled HMWK (1 μg/ml) was incubated for 50 min at 37°C with gel-filtered platelets (2 × 10^8/ml) in Hepes Tyrode's buffer in the presence of Zn^{++} (50 μM) and Ca^{++} (2 mM) and various proteins.
‡ Each competitor was added in the molar excess (which is given by the value in parenthesis) to the final concentration of the ^{125}I-HMWK.
§ Values presented are the means of 2–3 experiments with different platelet donors.

together to determine a competition inhibition curve and to calculate the midpoint of this curve by a four-parameter logistic function (40) (Fig. 6). These values were in reasonable agreement with a value of 1.34±0.07 nM determined by computer fit of a high affinity site using a one-site model (44, 45). When a two-site model was used, a high affinity binding site was characterized as having an apparent K_d of 0.70±0.15 nM.

Reversibility of ^{125}I-HMWK binding to unstimulated platelets. To study the reversibility of binding, we incubated gel-filtered platelets with ^{125}I-HMWK, and after 10 min and 20 min, added a 50-fold molar excess of unlabeled HMWK. After the addition of the unlabeled ligand, rapid dissociation of bound ligand was observed (Fig. 7). When the unlabeled HMWK was added after 10 min of incubation, 89% of the bound radioligand was displaced within 1 min; when unlabeled HMWK was added after 20 min of incubation, 79% of the bound radioligand was displaced within 1 min. No further reversal of binding was observed
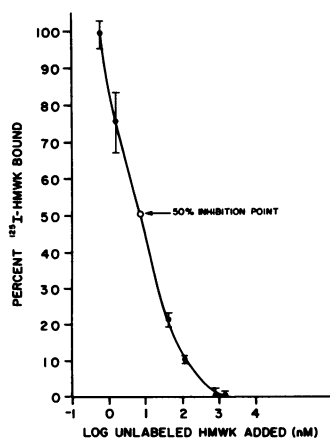

Figure 6. Comparison of the ability of nonlabeled HMWK to competitively inhibit ^{125}I-HMWK binding to platelets. ^{125}I-HMWK (0.89 μg/ml [7.45 nM]) were incubated with gel-filtered platelets (2.2 × 10^8/ml) in Hepes Tyrode's buffer for 10 min at 37°C in the absence or presence of the indicated concentration of unlabeled HMWK. In the presented figure, the data were fit to a competition inhibition curve generated by a computer program using a four-parameter logistic function (ref. 40) which calculates the values on the ordinate into relative values between 0 and 100%. The figure presented is the mean±SEM of the points derived from three experiments where the concentration of the added ^{125}I-HMWK was similar.
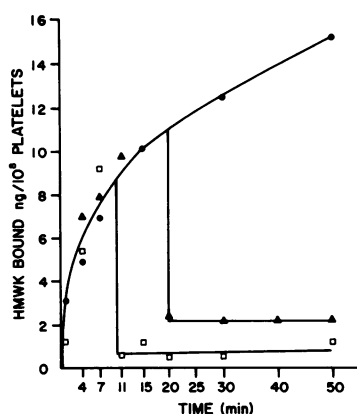

Figure 7. Reversibility of HMWK binding to unactivated platelets. Gel-filtered platelets (2.2 × 10^8/ml) in Hepes Tyrode's buffer were incubated for 50 min at 37°C in the presence of Zn^{++} (50 μM), Ca^{++} (2 mM), and ^{125}I-labeled HMWK (1 μg/ml). At 10 min (—□—) and 20 min (—▲—) a 50-fold molar excess of unlabeled HMWK was added. The binding was determined at the indicated time points as detailed in Methods. The plotted data are one experiment of three and represent total binding in the presence of no added unlabeled ligand (—●—) or with the previously indicated additions.

when the incubations with the unlabeled HMWK were extended to 50 min. When a 50-fold molar excess of unlabeled HMWK was added after a 50-min incubation, only 15% of the radiolabeled ligand was displaced (data not shown).

Two types of experiments were performed to determine that the association of ^{125}I-HMWK with unstimulated platelets was one of binding and not incorporation or ingestion. Firstly, reversibility binding experiments were performed at 37 and 4°C. At both temperatures, the extent of association of the radiolabeled ligand to platelets was the same. When a 50-fold molar excess of unlabeled HMWK was introduced to platelets that were incubated with ^{125}I-HMWK for 20 min at 37 and 4°C in simultaneous but separate experiments, the amount of the radioligand that dissociated from the platelets was 80% for both incubations. Secondly, studies were performed to determine whether total kininogen-deficient platelets (17) ingested plasma HMWK. Total kininogen-deficient platelets were washed and an aliquot of the washed platelets was incubated in normal plasma (88 μg/ml HMWK) for 1.5 h at 37°C. After the incubation, the normal plasma-treated total kininogen-deficient platelets were rewashed. Both samples of the washed total kininogen deficient platelets were then studied for their total HMWK content. After incubation in normal plasma, the total kininogen-deficient platelets had a HMWK content of <3.4 ng/10^8 platelets, a value below the lower limit of the assay and identical to the washed total kininogen-deficient platelets before incubation in normal plasma. These combined studies indicated that the association of HMWK with platelets was one of binding and not incorporation or ingestion.
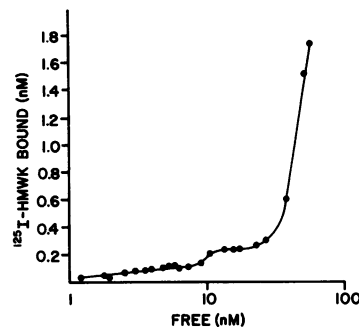
Saturability of ^{125}I-HMWK binding to unstimulated platelets. Since ^{125}I-HMWK binding to nonactivated platelets appeared to reach equilibrium at 10 to 20 min, the number and affinity of HMWK binding sites on unstimulated platelets were determined as a function of HMWK concentration. Increasing concentrations of ^{125}I-HMWK were added to platelets in the presence of Zn^{++} (50 μM) and Ca^{++} (2 mM) and in the presence or absence of a 50-fold molar excess of unlabeled ligand. Fig. 8 is one representative experiment of six. As the concentration of added ^{125}I-HMWK increased, the level of binding increased. At lower concentrations of added ^{125}I-HMWK (<1 μg/ml added) nonspecific binding was <20% of the total binding. At concentrations of added ^{125}I-HMWK >1 μg/ml (8.3 nM), the percent of non-
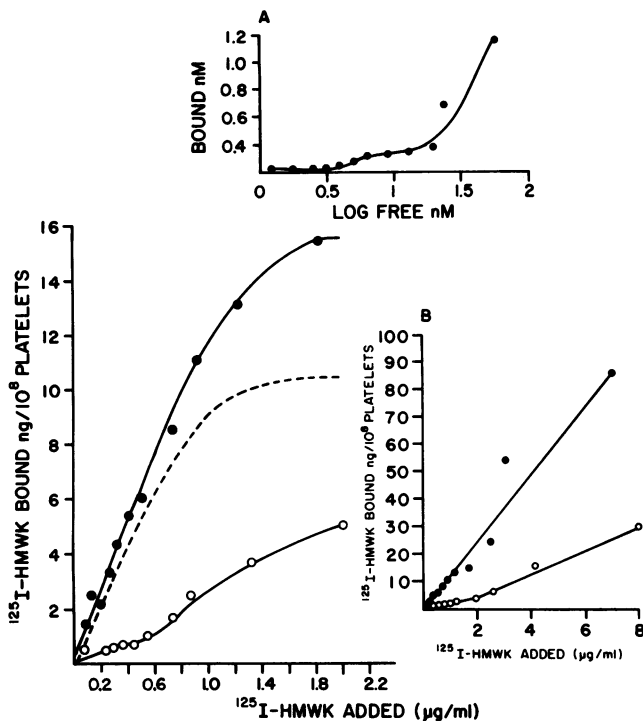
Figure 8. Concentration-dependent binding of ^{125}I-HMWK to platelets. Platelets at a concentration of $2.2 \times 10^8/ml$ in Hepes Tyrode's buffer were incubated with increasing concentrations of ^{125}I-HMWK in the presence of Zn^{++} (50 μM) and Ca^{++} (2 mM) at 37°C for 15 min. ^{125}I-HMWK-platelet binding was determined as indicated in Methods. Total binding (— ● —) and nonspecific binding (— ○ —) were directly determined and specific binding (- - - -) was calculated by subtracting nonspecific binding from total binding. Inset *A* represents a plot of bound ^{125}I-HMWK (nM) on the ordinate vs. log free (nM) HMWK on the abscissa. The line running through the points represents a manual graph of the computer fitted data (50). Inset *B* represents the total range of added and bound ^{125}I-HMWK in this experiment from which the saturable binding curve was derived.

specific binding increased to be a higher percentage of the total. Fig. 8, inset *B*, the total range of added and bound ^{125}I-HMWK for this experiment is given. Although points determined up to 2 $\mu g/ml$ (16.6 nM) of added ^{125}I-HMWK appeared to give a curve that approached a plateau, point values > 2 $\mu g/ml$ (16.6 nM) of added ^{125}I-HMWK did not demonstrate a leveling-off of binding. The points from this concentration-dependent experiment were then plotted on a bound-vs.-log free graph (Fig. 8, inset *A*). The sigmoid curve depicted on this graph delineated one binding site which was saturated at ~10 nM (1.2 $\mu g/ml$) of added ^{125}I-HMWK. Using the graphical method of Scatchard (42), the saturable binding site appeared to be one of high affinity with an apparent K_d of 1.39 nM. Confirmation of this interpretation of the graphical representation of the experimental data was obtained by computer (44, 45) analysis of the same experiment. Whether a one-site or two-site model for calculation was used, one high affinity site was described with an apparent K_d of 0.44 nM and 1,600–1,700 binding sites per platelet. Thus, there was reasonable agreement between the methods of analysis.

Fig. 9 is a graph of the computer fitted points from six individual experiments on unstimulated platelets with concentrations between 1 and 100 nM of free ^{125}I-HMWK. As can be seen, a sigmoid curve was described with a plateau at ~12 nM.

Figure 9. Computer-fitted binding isotherm of ^{125}I-HMWK platelet binding to unstimulated platelets. Computer-fitted data points from six experiments performed at 10 min with a concentration of free ^{125}I-HMWK between 1 and 100 nM were plotted on the abscissa and the bound ^{125}I-HMWK (nM) was plotted on the ordinate. The line running through the points is manually drawn through the computer-fitted values.

This plateau characterized one saturable binding site with an apparent K_d of 0.46 ± 0.19 nM (mean$\pm$SD) and $2,198 \pm 1,036$ molecules per platelet (range, 1,626–3,855 molecules per platelet). Whether a one-site or two-site model was used for calculating the data, this high affinity binding site (apparent K_d, 0.46 nM and 0.38 nM, respectively) was described. This finding validated the description of the high affinity binding site. Using the graphical method of Scatchard, the mean$\pm$SD of the apparent K_d for these same six experiments was 0.99 ± 0.34 nM with $3,313 \pm 843$ molecules per platelet.

Discussion

The demonstration that HMWK specifically binds to unstimulated platelets suggests that the platelet surface may contain a discrete receptor for HMWK on its external membrane. Three approaches were used to show that the platelets used in the binding studies were unstimulated. Firstly, ^{125}I-HMWK-platelet binding was similar when platelets were washed by centrifugation in the presence of inhibitors as when they were gel filtered. Secondly, ^{125}I-HMWK platelet binding was the same to gel-filtered platelets prepared in the presence or absence of 2 μM PGE_1. Lastly, gel-filtered platelets did not express the PADGEM antigen on their surface by immunofluorescence. The specificity of ^{125}I-HMWK-platelet binding is shown by a number of approaches. Trapping of unbound HMWK between cells in the pellet is excluded by ^{125}I-albumin studies which show only 0.2% of radioactivity with the pellet vs. $\geq 1.0\%$ for the ^{125}I-HMWK-platelet interaction. ^{125}I-HMWK-platelet binding in the presence of ≥ 50-fold molar excess of unlabeled HMWK is <30% of the total binding. Moreover, radiolabeled HMWK competes with equal affinity with unlabeled HMWK for platelet binding (Fig. 5) and excess unlabeled HMWK competitively inhibits ^{125}I-HMWK-platelet binding (Fig. 6). Finally, only unlabeled HMWK, not PK, Factor XII, Factor XI, Factor V, fibronectin, or fibrinogen (Table I), is able to block ^{125}I-HMWK-platelet binding.

Binding of HMWK to unstimulated platelets has an essential divalent cation requirement of zinc (Figs. 3 and 4). Neither calcium nor magnesium contribute anything to the binding of ^{125}I-HMWK to unstimulated platelets since in the absence of these divalent cations, but in the presence of zinc ion, ^{125}I-HMWK specifically binds to platelets (Fig. 4 *B*). Using unstimulated platelets, the optimal Zn^{++} concentration for ^{125}I-HMWK platelet binding is 50 μM (Fig. 3), which is the plasma concentration of zinc (46). Zinc concentrations greater than 200 μM inhibit ^{125}I-HMWK binding to unstimulated platelets (Fig. 3). This

finding explains why Greengard and Griffin (19), using 500 μM zinc ion, did not see substantial binding of ^{125}I-HMWK to unstimulated platelets. Since Zn^{++} has not been shown to be an essential divalent cation for binding of other proteins to platelets, the effect of zinc in HMWK-platelet binding may be on the HMWK molecule and not on the platelets. 50 μM zinc ion induces a conformational change of bovine HMWK and its light chain as measured by scanning absorption spectrophotometry (47). Zinc ions (50–100 μM) also accelerate PK and Factor XII activation by Factor XIIa and kallikrein, respectively, in the presence of sulfatides and HMWK (47).

HMWK binding to unstimulated platelets is characterized by one high affinity, saturable site with an apparent K_d of 0.46 ±0.19 nM by computer fitting of the experimental points in six experiments (Fig. 9) (44, 45). The estimated K_d determined from the midpoint of fitting the points of the competition inhibition curves from three experiments (Fig. 6) on a four-parameter logistic function (40) and using the midpoint to determine the apparent K_d of binding (41) is found to be similar (K_d, 0.52 nM). Secondly, employing a two-site model for computer analysis of the competition inhibition experiments (45), this high affinity site has an apparent K_d of 0.70 nM, which is in agreement with the values found in the concentration-dependent binding experiments. Using the graphical methods of Scatchard (42) to analyze the experimental data of this high affinity site on unstimulated platelets, the mean of the apparent K_d in six experiments is found to be similar, 0.99±0.35 nM.

HMWK binding to unstimulated platelets may have a role in the activation of Factor XI. Plasma Factor XI circulates in complex with plasma HMWK (48) and plasma HMWK's adsorption to surfaces is coordinated with the presence of Factor XIIa (49). Since plasma HMWK functions as a cofactor for the surface activation of Factor XII, PK and Factor XI (50), HMWK bound to platelets could provide a locus for these reactions. Given the affinity and number of binding sites for Factor XI (apparent K_d, 10 nM, 1,500 sites/platelet) (51) and Factor XIa (apparent K_d, 3.25 nM, 225 sites/platelet) (52), HMWK binding to unstimulated platelets could provide a sufficient number of platelet sites to support the binding of these proteins to platelets. Since the apparent K_d for HMWK-platelet binding is similar to that determined for Factor XI- or XIa-platelet binding, and both of these proteins require HMWK in order to bind to platelets, HMWK (plasma or platelet) may be the platelet receptor for these proteins. Coordinate binding studies are needed to clarify whether HMWK is the platelet receptor for Factors XI and XIa. In conclusion, the finding that ^{125}I-HMWK binds with high affinity to the unstimulated platelet suggests that in vivo, the platelet surface may function as a physiological negatively charged surface for the localization of the proteins of the Hageman factor pathways. It remains for further studies to determine whether contact activation on the platelet surface promotes or inhibits intrinsic coagulation or platelet activation.

Acknowledgments

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