Receptors for High Molecular Weight Kininogen on Stimulated Washed Human Platelets†

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ABSTRACT: Binding of human high molecular weight kininogen to washed human platelets was studied by measuring platelet-associated radiolabeled ligand in pellets of centrifuged platelets. High molecular weight kininogen was bound to stimulated platelets in the presence of $ZnCl_2$ in a specific and saturable manner. Calcium ions potentiated ligand binding but did not substitute for zinc ions. Optimal binding of high molecular weight kininogen occurred near the plasma concentrations of both zinc and calcium ions. Scatchard analysis yielded 24 200 binding sites for high molecular weight kininogen with an apparent dissociation constant of 20 nM. These studies show that stimulated human platelets can bind many high molecular weight kininogen molecules with high affinity and suggest that the platelet surface may potentially serve as an important site for localizing the initial reactions of the plasma kinin-forming and intrinsic coagulation pathways.

The initiation of intrinsic coagulation in vitro occurs as a complex series of reactions involving at least four plasma proteins: factor XII, prekallikrein, high molecular weight kininogen (HM_rK),[1] and factor XI (Griffin & Cochrane, 1976; Wiggins et al., 1977; Meier et al., 1977; Davie et al., 1979; Mandle et al., 1976; Thompson et al., 1977; Cochrane et al., 1973). These reactions involve the assembly and interactions of these proteins on negatively charged surfaces, culminating in the HM_rK-dependent activation of factor XI.

Early investigations suggested that platelets play a role in the propagation of the intrinsic coagulation pathway (Walsh, 1972a,b). Stimulated platelets were shown to support activation of factor XI by two mechanisms, only one of which required exogenous factor XII. Recent studies using washed platelets and highly purified proteins confirmed and extended these observations (Walsh & Griffin, 1981). Activation of factor XI in the presence of stimulated platelets was dependent not only upon factor XIIa or kallikrein but also upon the presence of the nonenzymatic cofactor HM_rK. One possible mechanism whereby HM_rK might promote platelet-dependent activation of factor XI would involve the binding of HM_rK to a specific receptor on the platelet surface. Platelet-bound HM_rK might then act as a factor XI or XIa receptor itself or as a component of such a receptor, as factor Va does for factor Xa on the platelet surface (Kane et al., 1980; Tracy et al., 1981). Alternatively, after binding to the platelet, HM_rK might interact with factor XI or XIa bound to a separate receptor. A quantitative study of the binding of HM_rK to platelets was undertaken as part of an assessment of the potential contributions of platelets to the intrinsic coagulation and kinin-forming pathways. This study presents evidence that HM_rK is specifically bound with high affinity to the surface of washed stimulated human platelets.

Materials and Methods

All chemicals used were the best grade commercially available. Carrier-free $Na^{125}I$, ^{51}Cr-labeled sodium chromate, and 5-hydroxy[β-^{14}C]tryptamine creatinine sulfate were obtained from Amersham Corp. Methyl silicone oil 1.0 (DC200) and Hi Phenyl silicone oil 125 (DC550) were supplied by William F. Nye Speciality Lubricants (New Bedford, MA). Ultrol HEPES and crystallized BSA were obtained from Calbiochem Behring Corp. Aspirin and adenosine 5′-diphosphate (grade III) were purchased from Sigma and dissolved just prior to use. Prostaglandin E_1 from Sigma was stored as a 1.0 mg/mL solution in 95% ethanol at −70 °C.

Buffers. "Calcium-free" HEPES–Tyrode's buffer (Timmons & Hawiger, 1978) consisted of 138 mM NaCl 2.7 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 3.5 mM HEPES, 5.5 mM dextrose, and 3.3 mM $NaH_2PO_4 \cdot H_2O$, pH 6.5 or 7.35 (see below), containing 1 mg/mL BSA. This buffer was filtered through a 0.45-μm Millipore filter before use. $CaCl_2$, $ZnCl_2$, and tetrasodium ethylenediaminetetraacetic acid were dissolved in water and filtered through a 0.1-μm Millipore filter prior to use.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Weber et al. (1972). Samples were prepared as described previously (Walsh & Griffin, 1981).

Clotting Assays. Clotting assays of all coagulant proteins except α-thrombin were determined in kaolin-activated partial thromboplastin time tests (Bouma & Griffin, 1977) in appropriate deficient plasmas (George King Biochemicals, Overland Park, KS). One clotting unit is defined as the activity found in 1 mL of pooled normal human plasma, except for α-thrombin. The activity of α-thrombin was measured in a thrombin time assay (Fletcher et al., 1959) and was recorded in NIH units. One NIH unit of thrombin clots 0.5 mL of normal human plasma in 15 s (Seegers & Smith, 1942).

Proteins. Prekallikrein (Kerbiriou et al., 1980) and factor XII (Griffin & Cochrane, 1976) were isolated from fresh human plasma as previously described. Specific activities were 20 and 80 clotting units/mg, respectively. HM_rK was purified according to Kerbiriou & Griffin (1979) as a single polypeptide chain with a specific activity of 14 clotting units/mg. The kinin-free double-chain disulfide-linked form of HM_rK was prepared from the single-chain form as described by these authors. Human α-thrombin was the kind gift of Dr. Annie Bezeaud. Fibrinogen was prepared as previously described (Doolittle et al., 1967). Ovomucoid trypsin inhibitor, oval-

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; HM_rK, high molecular weight kininogen; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, tetrasodium ethylenediaminetetraacetic acid; ADP, adenosine 5′-diphosphate.

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bumin, and potato apyrase (grade III) were obtained from Sigma, and soybean trypsin inhibitor was from Calbiochem Behring Corp. These were used without further purification.

Radiolabels. HM_rK, BSA, and ovalbumin were radiolabeled with ^{125}I by using the Chloramine T technique (McConahey & Dixon, 1966) to specific radioactivities of 10–40 $\mu Ci/\mu g$. Radiolabeled single-chain HM_rK migrated as a single band on SDS–PAGE whereas radiolabeled double-chain HM_rK migrated as two bands on SDS–PAGE in the presence of 2-mercaptoethanol. The bands corresponded to M_r 110 000 for single-chain HM_rK and to M_r 55 000 and 65 000 for double-chain HM_rK. Radiolabeled ovalbumin migrated as a single band of M_r 43 000 and ^{125}I-labeled BSA as a single band of M_r 65 000.

Preparation of Washed Platelets. Blood was obtained from normal healthy adult human donors and mixed with $^1/_6$th volume of acid citrate dextrose. The citrated blood was centrifuged at 160*g* to obtain platelet-rich plasma. This was collected and centrifuged at 660*g*. The platelet pellet was gently resuspended in calcium-free HEPES–Tyrode's buffer, pH 6.5, and then gel filtered over Sepharose CL-2B in an 8 × 2.7 cm column that had been equilibrated with calcium-free HEPES–Tyrode's buffer, pH 7.35 (Tangen et al., 1971). Platelets were counted electronically (Coulter Electronics, Hialeah, FL). When ^{125}I-labeled BSA or ^{125}I-labeled factor XI was added as a tracer to platelet-rich plasma, it was found that less than 0.06% of either of these proteins was retained in the platelet eluates of the gel filtration column (Walsh & Griffin, 1981).

Measurements of Sedimentability of Washed Platelets. Washed platelets were centrifuged at 800*g* for 15 min and resuspended at 4×10^8 per mL in calcium-free HEPES–Tyrode's buffer, pH 6.5. One milliliter of platelets (1×10^9 per mL) was incubated for 30 min at 37 °C with 50 μCi of ^{51}Cr-labeled sodium chromate (Amersham). The labeled platelets were gel filtered as described above. Aliquots were incubated with buffer or thrombin (0.5 unit/mL) for 20 min at 37 °C. Platelets were separated from buffer by a modification of the method of Feinberg and co-workers (Feinberg et al., 1974). Aliquots were centrifuged through 170 μL of a mixture of silicone oils (4 volumes of DC550:1 volume of DC200) in microsediment tubes (Sarstedt). The tips containing the sediments were amputated by using wire cutters, and the sediments and supernatants were counted separately in a Micromedic Systems Model 4/600 γ counter. More than 86% of the ^{51}Cr radioactivity sedimented, giving this figure as the lower limit of platelet sedimentability. Less than 4% difference was observed between thrombin-treated and untreated platelets.

Measurement of Aqueous Volume Trapped during Platelet Centrifugation. Platelets were incubated with ^{125}I-labeled BSA for 30 min at 37 °C and centrifuged over silicone oil as described above. The fraction of radiolabeled BSA that sedimented was assumed to correspond to the fraction of aqueous volume that sedimented, since the buffer contained 1 mg/mL BSA. This trapped volume was calculated to represent less than 8 fL per platelet, or less than 0.2% of the total volume in a typical binding experiment.

Binding Assays. All experiments were performed at 37 °C without stirring. In studies of the time course of binding of ^{125}I-labeled HM_rK, 500–1000 μL of prewarmed platelets [$(2–3) \times 10^8$/mL] in calcium-free HEPES–Tyrode's buffer, pH 7.35, was incubated in a 1.5-mL plastic Eppendorf centrifuge tube with mixtures of radiolabeled and unlabeled HM_rK, $ZnCl_2$, $CaCl_2$, or buffer. At various times after the addition of α-thrombin, ADP, or buffer, 120-μL aliquots were removed and centrifuged in tubes containing 150 μL of silicone oil as described above. In some experiments, the platelets were preincubated with platelet inhibitors. In other experiments, 205 μL of prewarmed washed platelets was incubated with a mixture of ^{125}I-labeled and unlabeled HM_rK, $ZnCl_2$, $CaCl_2$, or buffer and ADP, α-thrombin, or buffer. An aliquot (170 μL) of the reaction mixture was removed after 20 min and treated as described above to determine bound radioactivity.

Release Assays. Washed platelets [$(2–3) \times 10^8$ per mL] were incubated 60 min at 37 °C with 5-hydroxy[^{14}C]tryptamine creatinine sulfate (50–60 $\mu Ci/\mu mol$) at 0.25 μCi/mL washed platelets. A final volume of 205 μL of platelets was incubated with varying amounts of unlabeled HM_rK, $ZnCl_2$, $CaCl_2$, or buffer and ADP, α-thrombin, or buffer. After 20 min at 37 °C, 170 μL was withdrawn and centrifuged at 10000*g*. A 120-μL sample of the supernatant of this aliquot was removed and added to 8 mL of Betaphase scintillation fluid (WestChem Products). Radioactivity was measured in a Beckman LS230 β counter. Total radioactivity was determined by using platelets which had been incubated with 2% Triton X-100 for 180 min at 37 °C and treated as above. Background radioactivity was measured by using platelets incubated with buffer only. Total and released radioactivities were adjusted by subtracting the background radioactivity from each. Percent release was calculated as

$$\% \text{ release} = 100\frac{\text{adjusted release}}{\text{adjusted total}}$$

Control Binding Studies with Erythrocytes. Human erythrocytes were prepared essentially as described (Kabayashi & Levine, 1983) except that the resuspension buffer used was calcium-free HEPES–Tyrode's buffer pH 7.35, containing 1 mg/mL BSA. Washed erythrocytes at 2.5×10^8 per mL were incubated with mixtures of radiolabeled and unlabeled HM_rK, $ZnCl_2$, $CaCl_2$, or buffer, and thrombin or buffer at 37 °C. At various times, aliquots were centrifuged over silicone oil as described above to determine bound radioactivity.

Calculation of Binding Constants and Number of Sites. Binding data were analyzed according to the method of Scatchard (1949). Points were the averages of triplicate determinations. Nonspecific binding, apparent dissociation constants, and numbers of binding sites were calculated by using the data-fitting program LIGAND (Munson & Rodbard, 1980) on a Hewlett-Packard 9836 computer.

Results

Time Course of Binding ^{125}I-HM_rK to Platelets. For measured of the binding of ^{125}I-HM_rK to platelets, washed platelets were incubated at 37 °C with a mixture of unlabeled and ^{125}I-labeled HM_rK in the presence or absence of $ZnCl_2$, $CaCl_2$, and thrombin (Figure 1). The incubation mixture was sampled at various times, and aliquots were centrifuged through a silicone oil barrier to separate the platelets from the unbound proteins. In the presence of 25 μM $ZnCl_2$ and 2.0 mM $CaCl_2$, stimulated platelets bound 25–30 ng of HM_rK per 10^8 platelets. Maximum binding was reached in approximately 20 min, declining slightly thereafter. Binding required both zinc and calcium ions. Unstimulated platelets bound only small amounts of HM_rK, perhaps representing a small subpopulation of platelets which became activated during the washing procedure. Similar binding kinetics were observed if 0.5 mM $ZnCl_2$ was used in the absence of $CaCl_2$ (data not shown). In this case, binding was also dependent upon platelet stimulation, but not upon the inclusion of calcium ions (see below).

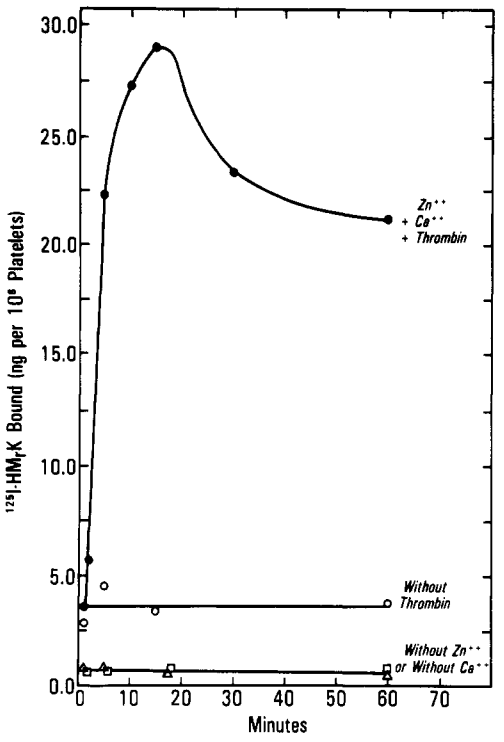

FIGURE 1: Binding of ^{125}I-HM$_r$K to platelets. Platelets were incubated unstirred at 37 °C with 200 ng/mL ^{125}I-HM$_r$K and 0.1 unit/mL α-thrombin or buffer with or without 25 μM $ZnCl_2$ and 2.0 mM $CaCl_2$. At the indicated times, aliquots were removed and centrifuged as described under Materials and Methods.

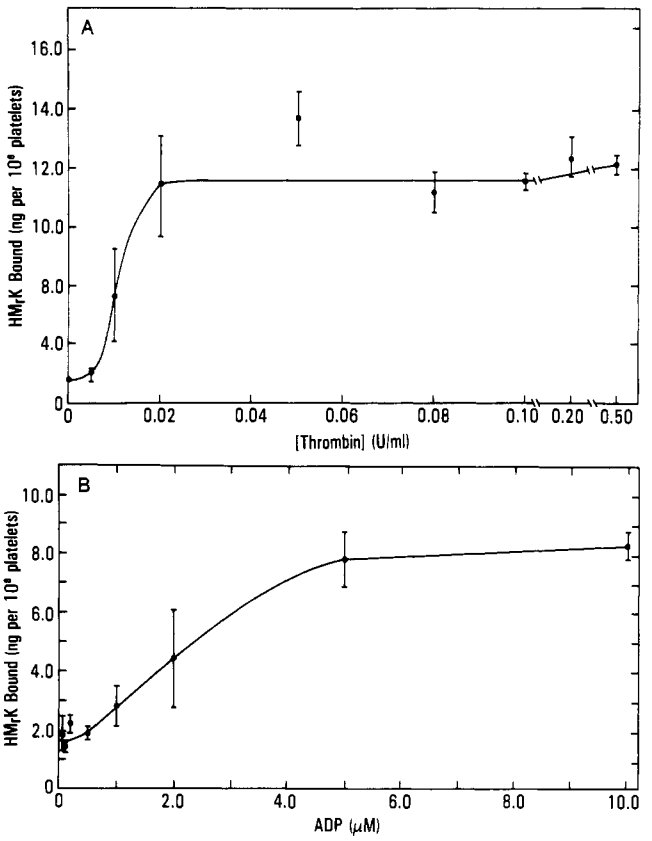

FIGURE 2: Effect of platelet stimulation on binding of ^{125}I-HM$_r$K. Platelets were incubated for 20 min at 37 °C with 200 ng/mL ^{125}I-HM$_r$K, 0.5 mM $ZnCl_2$, and the indicated concentrations of thrombin (A) or ADP (B). Binding was determined as described under Materials and Methods.

If buffer alone was substituted for platelets, 0.5% of the radiolabel was recovered in the sediment zone in the microcentrifuge tube. Similar low levels of radioisotope (≤0.5%) were found in centrifuge tube tips in experiments in which ^{125}I-labeled ovalbumin or ^{125}I-labeled HM$_r$K that had lost its procoagulant activity during labeling was used as ligand in platelet binding assays (data not shown). Thus, HM$_r$K is bound to stimulated platelets in a manner dependent upon the presence of the divalent zinc and calcium cations.

Effect of Concentration of Platelet Activator. The effect of varying the concentration of the thrombin or ADP used to stimulate the platelets was studied (Figure 2). Maximal binding of HM$_r$K to platelets was observed at approximately 0.02–0.03 unit/mL thrombin or 5 μM ADP. These values are similar to activator concentrations, yielding maximal binding of other ligands to their receptors on stimulated platelets (Marguerie et al., 1979; Plow & Marguerie, 1980).

Effect of $ZnCl_2$ on Dense Granule Release. To determine whether $ZnCl_2$ exerted its effect by inhibition or enhancement of the platelet release reaction, platelets labeled with 5-hydroxy[^{14}C]tryptamine were incubated with $ZnCl_2$ (0.5 mM) or buffer, ^{125}I-labeled or unlabeled HM$_r$K, and various concentrations of thrombin. After 20 min at 37 °C, aliquots were assayed for release of ^{14}C or binding of ^{125}I as described under Materials and Methods. In the presence of $ZnCl_2$, binding and release of the respective radiolabeled molecules showed similar, although not identical, dependencies on thrombin concentration (Figure 3A). Only background levels of binding of ^{125}I-HM$_r$K were observed in the absence of $ZnCl_2$. Figure 3B shows a comparison of release of 5-hydroxy[^{14}C]tryptamine in the presence or absence of $ZnCl_2$. There was no significant difference in release at various concentrations of thrombin. Thus, it may be concluded that while $ZnCl_2$ has a large enhancing effect on the binding of HM$_r$K, this is not due to an alteration of the release reaction.

Table I: Comparison of Association of ^{125}I-HM$_r$K with Platelets and Erythrocytes[a]

cell (2.5 × 10^8 per mL)	ng bound per 10^8 cells
platelets	21.5
erythrocytes	3.7

[a] Platelets or erythrocytes (2.5 × 10^8 cells/mL) were incubated 20 min at 37 °C with 200 ng/mL ^{125}I-HM$_r$K, 0.1 unit/mL thrombin, 25 μM $ZnCl_2$, and 2.0 mM $CaCl_2$ and then centrifuged as described under Materials and Methods.

Effect of Platelet Inhibition. ^{125}I-HM$_r$K binding was studied by using platelets which had been preincubated with buffer, aspirin, apyrase, or prostaglandin E_1 for 20 min at 37 °C. Binding to ADP-stimulated platelets is shown in Figure 4. Apyrase or prostaglandin E_1 reduced binding to the background observed in the absence of $ZnCl_2$. In the presence of aspirin, the final extent of binding was reduced 30–50%. In thrombin-stimulated platelets, only prostaglandin E_1 treatment completely inhibited binding; apyrase treatment reduced the binding somewhat less, and aspirin treatment gave no significant reduction (data not shown).

Binding of ^{125}I-Labeled HM$_r$K to Erythrocytes. To show that the binding observed was specific for platelets, and was not due to some common property of cell membranes, binding of HM$_r$K to washed erythrocytes was studied (Table I). Equal numbers of erythrocytes and platelets were used. Although the erythrocyte is a much larger cell than the platelet, and therefore presumably has a higher surface area, 6 times as much HM$_r$K bound in the platelet samples as in the erythrocyte samples. The binding to platelets was $ZnCl_2$, $CaCl_2$, and thrombin dependent, whereas that to erythrocytes was independent of these reagents and did not increase with time

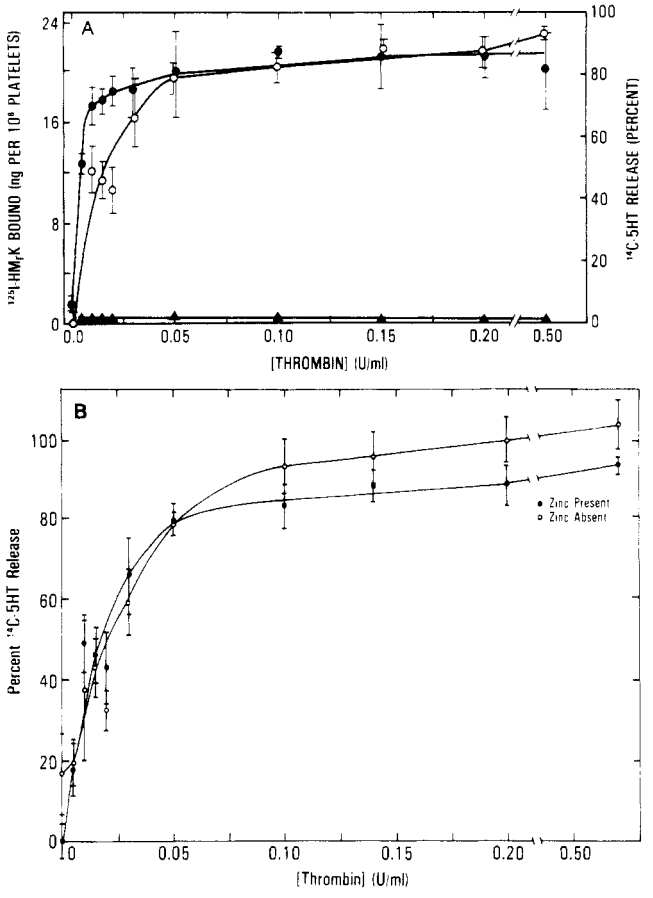

FIGURE 3: Effect of thrombin and zinc ions on binding of ^{125}I-HM$_r$K to platelets and on dense granule release. Platelets were preloaded with 5-hydroxy[^{14}C]tryptamine and incubated 20 min at 37 °C with various concentrations of thrombin, 0.5 mM $ZnCl_2$ or buffer, and HM$_r$K. ^{125}I-HM$_r$K was used for the binding measurements, and unlabeled HM$_r$K was used for the measurements of ^{14}C release. (A) Comparison of the effect of thrombin on ^{125}I-HM$_r$K binding (●) and 5-hydroxy[^{14}C]tryptamine release (○) in the presence of $ZnCl_2$. ^{125}I-HM$_r$K binding in the absence of $ZnCl_2$ (▲) is shown. (B) Effect of 0.5 mM $ZnCl_2$ on the release of 5-hydroxy[^{14}C]tryptamine: zinc ions present (●); zinc ions absent (○).

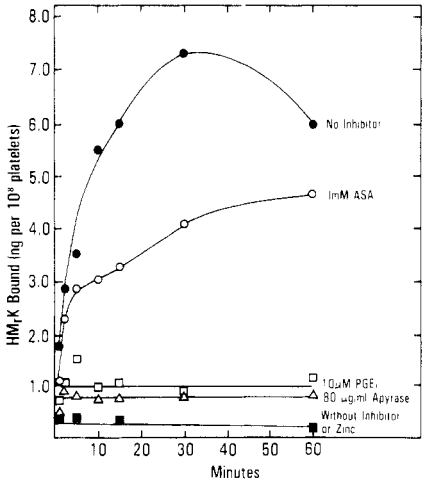

FIGURE 4: Binding of ^{125}I-HM$_r$K to platelets preincubated with platelet inhibitors. Platelets were preincubated 20 min at 37 °C with buffer (■, ●), 1.0 mM aspirin (ASA) (○), 10 μM prostaglandin E_1 (□), or 80 μg/mL apyrase (△). At 0 min, 5 μM ADP, 200 ng/mL ^{125}I-HM$_r$K, and either 0.5 mM $ZnCl_2$ (●, ○, □, △) or buffer (■) were added. At the indicated times, 120-μL aliquots were removed and centrifuged.

of incubation (data not shown). Thus, the zinc ion dependent binding of HM$_r$K appears to be specific for platelets.

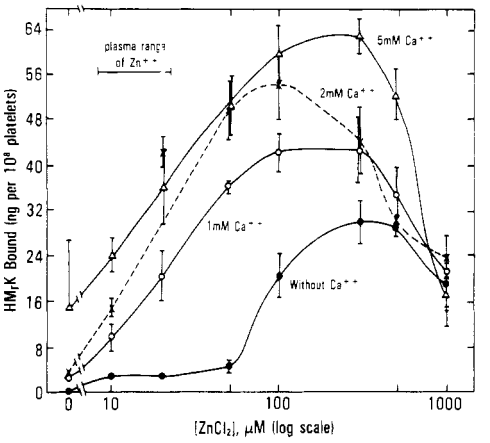

FIGURE 5: Effect of calcium ions on binding of ^{125}I-HM$_r$K to platelets at various concentrations of zinc ions. Platelets were incubated at 37 °C with the indicated concentrations of $ZnCl_2$, ^{125}I-HM$_r$K (225 ng/mL), thrombin (0.1 unit/mL), and $CaCl_2$ at 0 (●), 1 (○), 2 (×), or 5 mM (△). After 20 min, aliquots were removed, and the amount of ^{125}I-HM$_r$K binding was determined.

Table II: Competition for Binding of ^{125}I-HM$_r$K with Various Proteins[a]

competing protein	% control binding	competing protein	% control binding
none	100	factor XII	87
single-chain HM$_r$K	3.5	prekallikrein	93
		ovomucoid trypsin inhibitor	103
kinin-free HM$_r$K	7.5	ovalbumin	101
soybean trypsin inhibitor	79		

[a] Platelets were incubated with 25 μM $ZnCl_2$, 2.0 mM $CaCl_2$, 0.1 unit/mL thrombin, and 200 ng/mL ^{125}I-HM$_r$K mixed with buffer or 60 μg/mL samples of various proteins. After 20 min, samples were centrifuged. Binding of ^{125}I-HM$_r$K was compared to control binding in the absence of competing proteins.

Effect of Calcium Ions on Zinc Ion Dependent Binding. Binding of HM$_r$K to thrombin-stimulated platelets as a function of the concentration of $ZnCl_2$ was studied in the presence of concentrations of $CaCl_2$ from 0 to 5 mM (Figure 5). At all concentrations of $CaCl_2$, binding displayed a marked dependence on the concentration of $ZnCl_2$. Binding was maximal between 100 and 500 μM $ZnCl_2$ at all concentrations of $CaCl_2$ and declined at higher concentrations of $ZnCl_2$. There was also a dependence on $CaCl_2$ concentration, with maximum binding occurring at 2 mM. The major effect of increasing concentrations of $CaCl_2$ was to lower the threshold of the $ZnCl_2$ effect from 100 μM in the absence of $CaCl_2$ to 10–20 μM at 2–5 mM $CaCl_2$. Thus, calcium ions appear to enhance the effect of low concentrations of zinc ions without substituting for them. The horizontal bar shows the range of zinc ion concentration in plasma as determined by atomic absorption (Henry, 1979). At plasma levels of calcium ions, namely, 2 mM (Henry, 1979), considerable HM$_r$K binding was observed.

Specificity of HM$_r$K Binding to Platelets. To determine whether binding of HM$_r$K to platelets was specific, the binding of ^{125}I-labeled HM$_r$K mixed with unlabeled HM$_r$K or with other proteins was studied (Table II). Platelets were incubated with 25 μM $ZnCl_2$, 2.0 mM $CaCl_2$, thrombin, and various proteins mixed with 0.2 μg/mL ^{125}I-labeled HM$_r$K at 37 °C for 20 min. Either single-chain or double-chain forms of HM$_r$K (60 μg/mL) inhibited the binding of the radiolabel by >95%. This indicates that unlabeled HM$_r$K successfully competed with ^{125}I-labeled HM$_r$K for binding. Factor XII,

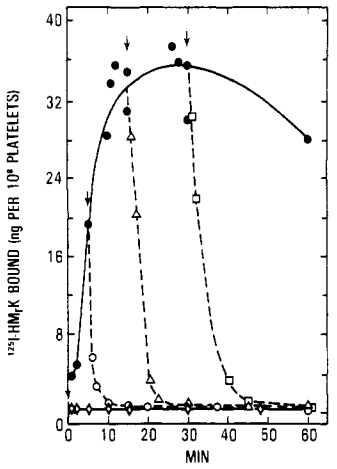

FIGURE 6: Reversibility of ^{125}I-HM$_r$K binding to platelets. The time course of ^{125}I-HM$_r$K (200 ng/mL) binding to thrombin (0.1 unit/mL) stimulated platelets was determined in the presence of 25 μM $ZnCl_2$ and 2.0 mM $CaCl_2$ (●). At the times indicated by the arrows, 60 μg/mL unlabeled HM$_r$K was added to the reaction mixture, and the dissociation of ^{125}I-HM$_r$K was followed. Additions were made at 0 (◇), 5 (○), 15 (△), or 30 min (□).

prekallikrein, soybean trypsin inhibitor, ovalbumin, or ovomucoid trypsin inhibitor failed to compete for the binding of ^{125}I-HM$_r$K to stimulated platelets. This suggests that the binding site for HM$_r$K on the platelet surface is specific for HM$_r$K.

Reversibility of HM$_r$K Binding to Platelets. In order to analyze binding parameters by the method of Scatchard (1949), it is necessary that binding reach equilibrium, as this is one of the assumptions behind the derivation of the Scatchard equation. The reversibility of the binding of HM$_r$K to its platelet receptor in the presence of 25 μM $ZnCl_2$ and 2.0 mM $CaCl_2$ was examined by measuring the dissociation of bound ^{125}I-labeled HM$_r$K when high concentrations (60 μg/mL) of unlabeled HM$_r$K were added (Figure 6) to the reaction mixture. If 60 μg/mL unlabeled HM$_r$K was present at time zero, >98% of the binding of the radiolabel was prevented. If the unlabeled HM$_r$K was added at times up to 30 min after platelet stimulation, >98% of the bound radiolabel was dissociated. This suggests that the binding is at equilibrium and does not become irreversible with time. When the binding of ^{125}I-labeled HM$_r$K was studied in the presence of 0.5 mM $ZnCl_2$ in the absence of $CaCl_2$, a similar rapid dissociation was caused by addition of 1.4 mM EDTA up to 30 min after platelet stimulation by thrombin (data not shown). This suggests that zinc ions must be present continuously in order to maintain the HM$_r$K bound to stimulated platelets.

Saturability of HM$_r$K Binding Sites. To determine the number and affinity of HM$_r$K binding sites on washed stimulated platelets, binding of ^{125}I-labeled HM$_r$K was examined as a function of HM$_r$K concentration (Figure 7A). On the basis of the data-fitting computer program LIGAND (Munson & Rodbard, 1980), the nonspecific binding at each concentration of HM$_r$K was calculated to be 2.0 ± 0.3%. This low background of nonspecific binding was subtracted from the total bound radioactivity to obtain the specific binding. Saturation of binding was observed at 10–15 μg/mL HM$_r$K, with approximately 380 ng bound per 10^8 platelets in the presence of thrombin, 2 mM calcium ions, and 25 μM zinc ions. Since binding was at equilibrium (Figure 6), these data were subjected to a Scatchard analysis assuming a single class of binding sites and a straight line was obtained (Figure 7B). Analysis of the specific binding using LIGAND yielded an apparent dissociation constant for HM$_r$K of 20.4 ± 2.9 nM,

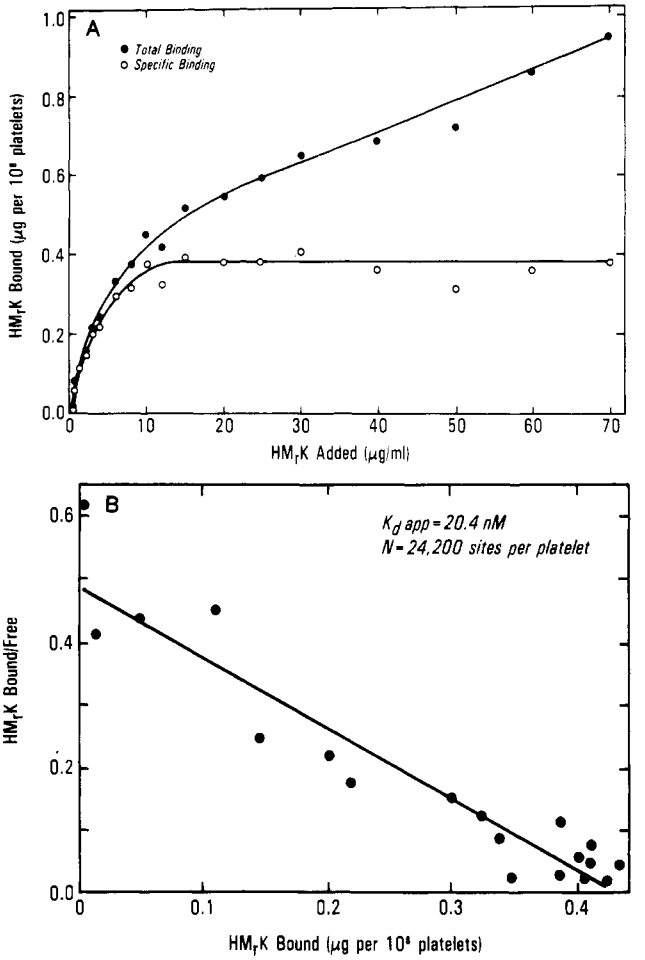

FIGURE 7: HM$_r$K concentration dependence of ^{125}I-HM$_r$K binding to platelets. Platelets were incubated with 25 μM $ZnCl_2$, 2.0 mM $CaCl_2$, 0.1 unit/mL thrombin, and mixtures of ^{125}I-labeled and unlabeled HM$_r$K at various concentrations. The amount bound was determined at 20 min. (A) Saturation of binding of HM$_r$K to thrombin-stimulated platelets in the presence of $ZnCl_2$ (25 μM) and $CaCl_2$ (2 mM) (calculation by LIGAND computer program). (●) Total binding; (○) specific binding: (B) Scatchard analysis of the data shown in (A) (calculation by LIGAND computer program). The line is a best fit of data for triplicate samples. Nonspecific binding, apparent dissociation constants, and number of binding sites were fitted as parameters by using the LIGAND program (Munson & Rodbard, 1980).

representing 24 200 ± 1900 binding sites per platelet. This apparent dissociation constant is 30-fold lower than the plasma concentration of HM$_r$K which is approximately 640 nM. When the data were modeled as two classes of binding sites, the results essentially converged to the one-site model.

In other experiments using platelets from two other donors, these parameters were 29.8 ± 5.6 and 18.5 ± 2.5 nM for the apparent dissociation constant, representing 21 000 ± 2200 and 32 200 ± 2700 binding sites per platelet, respectively.

Discussion

Specific binding of HM$_r$K to stimulated washed human platelets is demonstrated in the studies presented here. Platelets bind HM$_r$K in a time-dependent, reversible, saturable, and specific manner. Specificity is shown by the fact that saturating levels of unlabeled HM$_r$K effectively compete for binding of radiolabeled HM$_r$K, whereas other proteins fail to compete (Table II). When binding data are subjected to Scatchard analysis assuming a single class of binding sites, it is calculated that stimulated platelets possess approximately 24 000 sites for HM$_r$K with an apparent dissociation constant of 20 nM. This constant is far below the plasma concentration

of HM_rK, 640 nM, and thus it may be expected that the receptors would be saturated at plasma concentrations of HM_rK. Platelets must be stimulated by thrombin or ADP to bind significant amounts of HM_rK. Data from studies with platelets inhibited by prostaglandin E_1 (PGE_1), apyrase, or aspirin (Figure 4) suggest that only functionally active platelets express receptor activity.

One particularly unexpected observation in the studies reported here involves the role of zinc ions in platelet interactions with HM_rK. Under all conditions tested to date, binding of HM_rK to stimulated platelets is dependent on the presence of $ZnCl_2$ in the reaction mixture. $MgCl_2$ was present in all studies. In the absence of calcium ions, optimal binding occurs between 100 and 500 μM $ZnCl_2$. In the presence of physiologic amounts of calcium ions, i.e., 2–5 mM $CaCl_2$, the concentration of zinc ions required for optimal binding of HM_rK decreases to 20–50 μM. Calcium ions therefore potentiate zinc-dependent binding of HM_rK to platelets without being able to substitute for zinc ions. The concentrations of calcium and zinc ions in plasma are 2.0–2.5 mM and 10–25 μM, respectively (Henry, 1979). Thus, near-optimal binding of HM_rK may be expected at plasma concentrations of these metal ions. Zinc and calcium ions are essential not only for initial binding of HM_rK but also for retaining bound HM_rK since EDTA totally reverses HM_rK binding. Similar zinc and calcium ion dependencies are observed for the HM_rK-dependent binding of factor XI to stimulated platelets[2] (Greengard et al., 1983). Factor XI and HM_rK reversibly form noncovalent complexes in solution and on artificial surfaces (Thompson et al., 1977; Wiggins et al., 1977), and HM_rK acts as a nonenzymatic cofactor in the activation of factor XI by factor XIIa on surfaces (Griffin & Cochrane, 1976). The HM_rK-dependent binding of factor XI (Greengard et al., 1983) and of factor XIa (Sinha et al., 1984) to stimulated platelets suggests that a similar assembly may form at loci provided by specific platelet membrane sites.

There are several possible functions for zinc ions in the binding of HM_rK to the platelet surface. One attractive possibility for the function of zinc ions in HM_rK binding would involve a direct role in the actual interaction of the HM_rK molecule with its platelet receptor. HM_rK is known to possess a histidine-rich region (Han et al., 1975, 1976) which is involved in its interaction with negatively charged surfaces (Scicli et al., 1979). Zinc ions readily bind to the imidazole side chain of histidine residues in proteins such as carboxypeptidase A (Hartsuck & Lipscomb, 1971), thermolysin (Matthews et al., 1972), and carbonic anhydrase (Chlebowski & Coleman, 1976). The interaction of HM_rK with its platelet receptor may involve zinc ion complexes similar to the calcium ion complexes supposedly formed between the negatively charged carboxyl groups of γ-carboxyglutamic acid residues of vitamin K dependent proteins and the negatively charged phosphoryl groups of phospholipids. A less likely possibility is that zinc ions could directly affect basic platelet functions. Zinc ions do not affect thrombin-stimulated platelet aggregation[3] or dense granule release (Figure 3B). The effect of zinc ions on other platelet reactions is unknown, and it is possible that zinc ions either promote or inhibit one or more platelet reactions without affecting dense granule release.

It has recently been reported that kininogen-related antigens, presumably both HM_rK and low molecular weight kininogen, are contained in platelets and released by platelet stimulation (Kerbiriou & Larrieu, 1984; Schmaier et al., 1983). Platelets can release 60 ng of HM_rK per 10^8 platelets (Schmaier et al., 1983). Under the conditions of the experiments reported here, this would amount to 0.15 μg/mL HM_rK, a concentration 2 orders of magnitude below that needed to saturate HM_rK receptors. Therefore, platelet-derived HM_rK would not be expected to influence significantly the binding of HM_rK to stimulated platelets under the conditions employed in our studies.

While the role of zinc ions in the interaction of HM_rK with platelets is not clear, there are other examples of the involvement of divalent cations in the binding of plasma proteins to platelet receptors. Extensive binding of factors XI and XIa to platelets requires zinc ions[2] (Greengard et al., 1983), although low levels of factor XIa bind to platelets in the apparent absence of zinc ions (Sinha et al., 1984). Fibrinogen binds optimally when the concentration of $CaCl_2$ is 1 mM, with half as much bound if 1 mM $MgCl_2$ is substituted (Marguerie et al., 1980). Miletich and co-workers (Miletich et al., 1977) reported that factor Xa did not bind to platelets in the absence of calcium ions. Plow & Ginsberg (1981) found that EDTA and EGTA inhibited the binding of fibronectin to platelets but did not investigate the role of added metal ions. Other divalent cations are also possibly involved in the ristocetin-dependent binding of von Willebrand factor to platelets, since EGTA effectively inhibited its binding, whereas high concentrations of calcium, magnesium, or EDTA had little effect (Kao et al., 1979). EDTA and EGTA inhibited the binding of von Willebrand factor to platelets stimulated by ADP, thrombin, or ADP and epinephrin (Ruggeri et al., 1983; Fujimoto et al., 1982), suggesting that divalent cations are involved in this binding. Metal ions also have specific molecular effects on platelet function, aside from their well-established effects on platelet aggregation. The complex of platelet surface glycoproteins IIb and IIIa, which is required for fibrinogen binding, is held together by divalent cations (Kunicki et al., 1981; Hagen et al., 1982; Gogstad et al., 1982). Platelets also contain a calcium-dependent protease (Phillips & Jakabova, 1977) whose substrates include actin binding protein and P235 (band 2 protein) and which may have a role in the cytoskeletal changes that accompany platelet activation (Phillips & Jakabova, 1977; White, 1980; Fox et al., 1983).

The addition of zinc ions to the list of divalent cations mediating platelet functions should provide new insights into the molecular mechanisms of the binding of important plasma proteins to human platelets.

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Registry No. ADP, 58-64-0; Zn, 7440-66-6; Ca, 7440-70-2; thrombin, 9002-04-4; serotonin, 50-67-9.

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