High Molecular Weight Kininogen Inhibits Thrombin-Induced Platelet Aggregation and Cleavage of Aggregin by Inhibiting Binding of Thrombin to Platelets

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In this study we show that high molecular weight kininogen (HK) inhibited α-thrombin–induced aggregation of human platelets in a dose-dependent manner with complete inhibition occurring at plasma concentration (0.67 μmol/L) of HK. HK (0.67 μmol/L) also completely inhibited thrombin-induced cleavage of aggregin (M_r = 100 Kd), a surface membrane protein that mediates adenosine diphosphate (ADP)-induced shape change, aggregation, and fibrinogen binding. The inhibition of HK was specific for α- and γ-thrombin–induced platelet aggregation, because HK did not inhibit platelet aggregation induced by ADP, collagen, calcium ionophore (A23187), phorbol myristate acetate (PMA), PMA + A23187, or 9,11-methano derivative of prostaglandin H_2 (U46619). These effects were explained by the ability of HK, at physiologic concentration, to completely inhibit binding of ^{125}I-α-thrombin to washed platelets. As a result of this action of HK, this plasma protein also completely inhibited thrombin-induced secretion of adenosine triphosphate, blocked intracellular rise in Ca^{2+} in platelets exposed to α- and γ-thrombin, inhibited thrombin-induced platelet shape change, and blocked the ability of thrombin to antagonize the increase in intracellular cyclic adenosine monophosphate (cAMP) levels induced by iloprost. Because elevation of cAMP is known to inhibit binding of thrombin to platelets, we established that HK did not increase the intracellular concentration of platelet cAMP. Finally, HK did not inhibit enzymatic activity of thrombin. To study the role of HK in the plasma environment, we used γ-thrombin to avoid fibrin formation by α-thrombin. Platelet aggregation induced by γ-thrombin was also inhibited by HK in a dose-dependent manner. The EC_{50} (concentration to produce 50% of the maximum rate of aggregation) of γ-thrombin for washed platelets was 7 nmol/L and increased to 102 nmol/L when platelets were suspended in normal human plasma. The EC_{50} for platelet aggregation induced by α-thrombin in plasma deficient in total kininogen was 40 nmol/L. When supplemented with HK at plasma concentration (0.67 μmol/L), the EC_{50} increased to 90 nmol/L, a value similar to that for normal human plasma. These results indicate that (1) HK inhibits thrombin-induced platelet aggregation and cleavage of aggregin by inhibiting binding of thrombin to platelets; (2) HK is a specific inhibitor of platelet aggregation induced by α- and γ-thrombin; and (3) HK plays a role in modulating platelet aggregation stimulated by α-thrombin in plasma.

ON THE BASIS of the previous studies from our laboratory and others, we have proposed that there are two pathways that can lead to exposure of the fibrinogen receptor and platelet aggregation.[1] The first is adenosine diphosphate (ADP)-dependent and is involved in aggregation by ADP,[2] epinephrine,[3] collagen,[4] and thromboxane A_2.[5] Binding of ADP to a single surface membrane protein, aggregin (M_r = 100 Kd), distinct from glycoprotein (GP) IIIa,[6] causes a conformational change facilitating binding of fibrinogen to the platelet GP IIb/IIIa complex.[2] This process is inhibited by 5′-p-fluorosulfonylbenzoyl adenosine (FSBA), an affinity analogue of ADP, which covalently modifies aggregin.[7] A second way of exposing fibrinogen receptors necessary for platelet aggregation is ADP-independent and involves proteolysis of aggregin. Direct proteolysis of aggregin by chymotrypsin[2] and calpain[8] leads to platelet aggregation when incubation mixtures containing platelets and these proteases are treated with fibrinogen. Thrombin (2 nmol/L) aggregates platelets in an ADP-independent manner by increasing intracellular Ca^{2+}, $[Ca^{2+}]_i$, which activates calpain.[8] This process requires binding of thrombin to the high-affinity receptors.[9] The ensuing cleavage is indirect because it does not take place in isolated membranes and requires metabolic energy.[8] Moreover, aggregin is cleaved to peptides of molecular weight less than 1 Kd. This mode of cleavage of aggregin is atypical of the known limited proteolytic action of thrombin on other substrates, including GP V.[10] We have recently provided evidence for the involvement of calpain in this digestion because thrombin induces calpain expression on the external face of the platelet membrane.[11] Therefore, we examined whether high molecular weight kininogen (HK), a major plasma protease inhibitor of calpain(s),[12] which also binds reversibly to platelets,[13] inhibited platelet aggregation and cleavage of aggregin induced by thrombin by inhibiting calpain expressed on platelet surface.

The study shows that HK inhibited platelet aggregation by thrombin and not by most other agonists. However, we found that HK inhibited the previously mentioned thrombin-induced platelet responses by inhibiting binding of thrombin to intact washed platelets. Other thrombin-induced platelet responses, eg, secretion, shape change, and intracellular increase in calcium ions, were also inhibited by HK. Furthermore, the action of thrombin was also modulated in the plasma environment by HK.

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MATERIALS AND METHODS

Materials. FSBA and [^{3}H]FSBA were prepaired by the method of Colman et al.[14] The radiolabeled FSBA had a specific radioactivity of 20 Ci/mol. FSBA concentration was determined spectrophotometrically as described previously.[2] Bovine serum albumin (BSA), D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone (PPACK), hirudine, ADP, and phorbol myristate acetate (PMA) were obtained from Sigma (St Louis, MO). Prestained molecular weight standards were purchased from Bethesda Research Laboratories (Bethesda, MD) and consisted of the following: myosine (heavy chain, 200 Kd), phosphorylase b (92.5 Kd), BSA (68 Kd), ovalbumin (43 Kd), α-chymotrypsinogen (25.7 Kd), β-lactoglobulin (18.4 Kd), and cytrochrome C (12.3 Kd). Iloprost was a gift from Dr Barry Ashby (Temple University School of Medicine, Philadelphia, PA). U46619 was kindly provided by Dr S. Niewiarowski of the Physiology Department of Temple University. A23187 was obtained from Calbiochem (San Diego, CA). α-Thrombin (3,200 NIH U/mg protein) and γ-thrombin (8.44 NIH U/mg protein) were generous gifts from Dr John W. Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany). Throughout the text the word "thrombin" signifies α-thrombin. When γ-thrombin was used in experiment(s), it is so stated. Fibrinogen was obtained from Kabi (Stockholm, Sweden). Collagen was purchased Hormon Chemie (Munich, Germany). HK-deficient plasma was purchased from George King Biomedicals, Inc (Overlook Park, KS). Plasma deficient in total kininogen was obtained by direct donation with informed consent from M. Williams, Philadelphia, PA. Normal human plasma was obtained from the blood of normal healthy donors with the informed written consent, and the HK content of their plasma was analyzed by kaolin-activated coagulant assay as reported previously.[15] One unit of HK represents that amount in 1 mL of pooled normal plasma.

Platelet isolation and washing. Platelet-rich plasma (PRP) was prepared by differential centrifugation (120*g*, 30 minutes, 23°C) of fresh whole human blood drawn into acid/citrate/dextrose (citric acid 0.079 mol/L, sodium citrate 0.085 mol/L, and dextrose 0.180 mol/L) in a ratio of 1 mL of anticoagulant solution per 9 mL of blood. Platelets used in aggregation studies were washed and their suspensions prepared by the modification of the method of Mustard et al.[16] Modification included incubation of PRP with prostaglandin E_1, hirudine and apyrase, and two successive washes of platelets with Tyrode-albumin buffer containing hirudine and apyrase, and apyrase alone. Platelets used in thrombin-induced cleavage studies were prepared by a modification of the method of Mills et al.[17] Modifications included washing of the platelets with Tyrode-albumin buffers devoid of BSA. Incubation time during washing steps was reduced to 15 minutes.

Platelet labeling with FSBA. Chemical modification of platelets by FSBA or [^{3}H]FSBA was performed as reported previously.[2]

Platelet shape change and platelet aggregation. Platelet shape change and aggregation in the presence of agonists and/or inhibitors were performed at 37°C under constant stirring (1,100 rpm) conditions in a Chronolog (Lumi) aggrometer (Havertown, PA). The total volume of the incubation mixture in the sample cuvette was 500 μL and the platelet concentration was adjusted to 1×10^8/mL and 5×10^8/mL for the shape change and aggregation, respectively. Platelet shape change or aggregation was initiated by the addition of an agonoist to the sample cuvette. Shape change and aggregation data presented in this report represent the decrease or increase in the light transmission of platelet suspension with shape change and aggregation, respectively. Light transmission was calibrated in terms of millivolts of current signal set arbitrarily to a scale of 100.

Protein assay. Protein assays were performed according to the method of Lowry et al,[18] using BSA as the standard.

Platelet membrane isolation, extraction, and electrophoresis. [^{3}H]FSBA-labeled membranes were isolated from [^{3}H]FSBA-labeled platelets by the glycerol-lysis method of Barber and Jamieson,[19] with the modification that lysis buffer and sucrose solution also contained diisopropylfluorophosphate (10 mmol/L), benzamidine (102 mmol/L), soybean trypsin inhibitor (23 μmol/L), pepstatin A (0.15 mmol/L), leupeptin (0.5 mmol/L), and N-cbz-glutamyltyrosine (0.18 mmol/L). The isolated membranes were solubilized and dialyzed as reported previously.[2] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and measurement of radioactivity in gel slices was performed as described by Figures et al.[2] Molecular weights were determined by coelectrophoresis of prestained protein standards.

Cleavage of aggregin in [^{3}H]FSBA-labeled intact platelets. [^{3}H]FSBA-labeled intact platelets in the presence or absence of HK were incubated with an agonist at 37°C for 30 minutes. Following incubation, the cells were sedimented and suspended in a Tyrode-albumin buffer without Ca^{2+} as reported previously.[2] [^{3}H]FSBA-labeled membranes were prepared, solubilized, and subjected to SDS-PAGE as reported previously.[2]

Purification of HK. HK was purified by the method of Kerbiriou and Griffin,[20] with the modification that 0.2 mol/L ε-aminocaproic acid was included in all buffers and 2 mmol/L diisopropylflurophosphate was added to the pooled material before each step. HK on reduced SDS-PAGE was predominantly a single band with a molecular mass of 120 Kd with ≥98% purity, and with a specific activity of 12 to 20 coagulant U/mL.

Measurement of intracellular Ca^{2+}. Concentrations of intracellular Ca^{2+} were measured spectrophotometrically using quin-2/AM (AM = acetoxymethyl ester) fluorophore as previously described.[8]

Measurement of adenosine triphosphate (ATP) release. ATP release from the dense granules of platelets following exposure to agonists and/or inhibitors was measured by the commercial firefly-luciferase assay (Sigma). The assay was calibrated with a solution of ATP of known concentration by measuring percent light transmission in the absence of any agonist/inhibitor.

Measurement of cyclic adenosine monophosphate (cAMP) in human platelets. PRP, apyrase, and hirudin[8] were incubated with 1 mmol/L aspirin for 20 minutes at 23°C. Platelets were then isolated and washed as described by Puri et al.[8] Washed platelets (1×10^8 platelets/100 μL) were incubated in the presence or absence of HK (0.67 μmol/L) with each agonist (see Table 1). The reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid and the reaction mixture cooled in ice for 5 minutes. The precipitated protein was separated by centrifugation (10,000*g*, 5 minutes) at 4°C in a Fisher Microcentrifuge Model 235-A (Pittsburgh, PA). The amount of cAMP in 10 to 20 μL of the supernatants was measured by a commercial protein binding assay (Diagnostic Product Corp, Los Angeles, CA).

^{125}I-α-thrombin binding to human platelets. ^{125}I-α-thrombin was prepared by the iodogen procedure described by Tandon et al.[21] The efficiency of labeling of thrombin was determined by paper chromatography in 85% methanol. After chromatography the radioactivity in the regions corresponding to the solvent front and the point of sample application was determined. The efficiency of labeling was obtained by multiplying the fraction of radioactivity remaining at the point of application by 100. It was found to be 63.3%. ^{125}I-thrombin retained fully its ability to clot fibrinogen and to hydrolyze the synthetic substrate, H-D-phenylalanine-pipecolyl-L-arginine-p-nitroanilide (S2238; Kabi). The radioiodinated thrombin showed essentially one band when subjected to SDS-PAGE. The specific radioactivity of the radioiodinated thrombin was 1.65

μCi/μg. The binding of ^{125}I-thrombin was performed in a total volume of 200 μL containing 5 × 10^8 platelets, and the incubation time was 10 minutes at 23°C. After washing, platelets were suspended in the binding buffer containing 136 mmol/L and Tris-HCl, pH 7.35. Cell-bound thrombin in the absence and presence of HK (350 mol/L excess) in the incubation mixture (100 μL) was separated from the unbound thrombin by centrifugation (10,000g, 5 minutes) through 100 μL of silicon oil mixture (Dow Corning) (William F. Nye, Inc, New Bedford, MA) (200:500::2:5). The supernatent containing the unbound thrombin was removed by suction, and the cell pellet containing ^{125}I-thrombin bound to platelets was excised and the radioactivity assayed in an LKB 80,000 γ-counter. Nonspecific thrombin binding to platelets was identically determined in the presence of a 50-fold excess of unlabeled α-thrombin.

RESULTS

Effect of HK on platelet aggregation and cleavage of aggregin induced by thrombin. The inhibition of α-thrombin–induced aggregation of washed unmodified platelets and FSBA-modified platelets and FSBA-modified platelets by HK was concentration-dependent (Fig 1). HK, at normal plasma concentration (80 μg/mL or 0.67 μmol/L,[12] completely prevented platelet aggregation initiated by α-thrombin (Fig 1, inset). We have previously shown that aggregin is completely cleaved after incubation with α-thrombin.[8] We now find that HK (0.67 μmol/L) completely inhibited cleavage of aggregin after exposure of [^{3}H]FSBA-labeled platelets to thrombin (2 nmol/L) (Fig 2). [^{3}H]FSBA-labeled

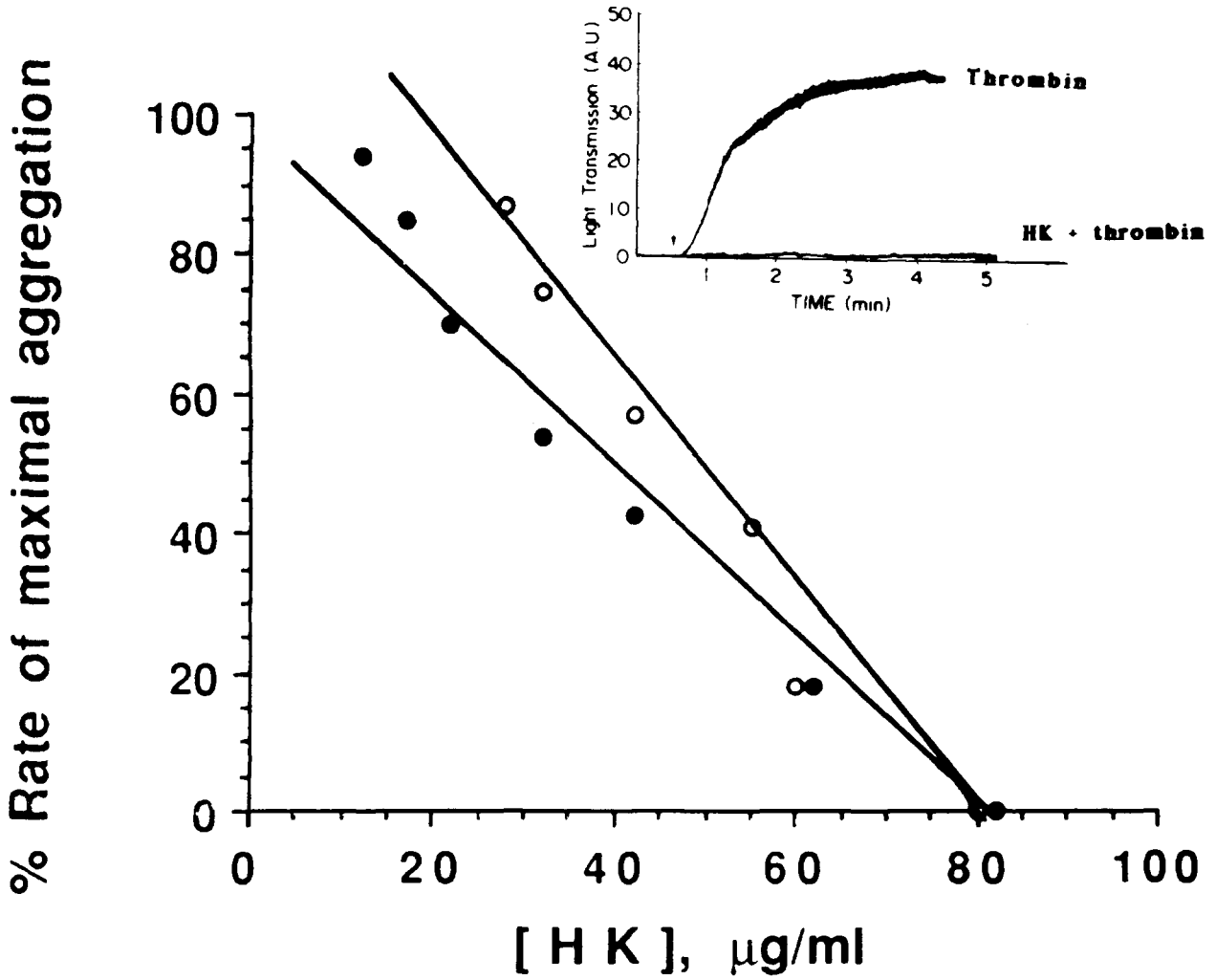

Fig 1. Concentration-dependent inhibition of α-thrombin–induced aggregation of washed and FSBA-modified platelets by HK. Platelets were modified by FSBA as described in Materials and Methods. Washed (●) or FSBA-modified (○) platelets (5 × 10^8/mL) were incubated with various concentrations of HK in the presence of Ca^{2+} (1 mmol/L) for 1 minute at 37°C. Aggregation was started by the addition of α-thrombin (2 nmol/L). The rates of platelet aggregation induced by α-thrombin in the presence of HK are shown as percent of the control (taken as 100%) in the absence of HK. The presence of Zn^{2+} (50 μmol/L)[28] or its absence in the incubation mixtures had no effect on the ability of HK to inhibit thrombin-induced platelet aggregation. Each point represents the mean of triplicate experiments with a maximum variation of 15%. The inset shows tracings of thrombin-induced platelet aggregation. The control platelets (5 × 10^8/mL) were first incubated with Ca^{2+} (1 mmol/L) for 1 minute at 37°C and the aggregation initiated by the addition of α-thrombin (0.2 U/mL). In a similar experiment, platelets (5 × 10^8/mL) were preincubated with HK (0.67 μmol/L) and Ca^{2+} (2 mmol/L) for 1 minute at 37°C before initiating aggregation by α-thrombin.

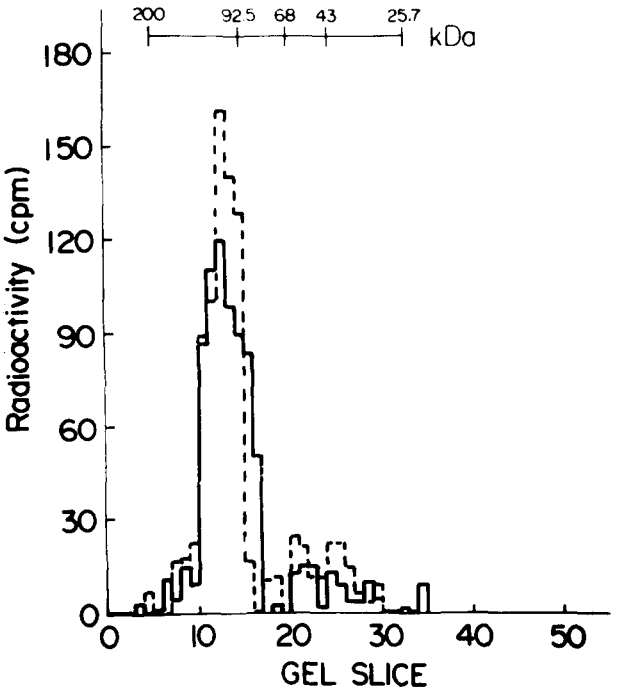

Fig 2. Effect of HK on thrombin-induced cleavage of aggregin. Washed platelets were labeled with [^{3}H]FSBA as described in Materials and Methods. [^{3}H]FSBA-labeled platelets (5×10^9/3.5 mL) were incubated with HK (77 μg/mL) in the presence of Ca^{2+} (1 mmol/L) for 10 minutes at 37°C. They were then treated with α-thrombin (2 nmol/L) at 37°C for 30 minutes followed by the addition of 10 nmol/L PPACK to inhibit thrombin. A control incubation mixture in the absence of thrombin was treated identically. Membranes obtained from the untreated and thrombin-treated platelets were solubilized, dialyzed, and subjected to SDS-PAGE (see Materials and Methods). The distribution of radioactivity in the gels corresponding to platelets treated with only HK (—) and those treated with HK followed by thrombin (----) are illustrated.

aggregin was completely cleaved by thrombin (data not shown) when the radiolabeled platelets were incubated with thrombin at 37°C for 30 minutes without stirring or for 3 minutes with constant stirring, a condition under which aggregation would normally occur.[8] At the plasma concentration, HK did not inhibit the amidolytic activity of either α- or γ-thrombin as ascertained by chromogenic assay.

Effect of HK on platelet aggregation induced by various agonists. HK (0.67 μmol/L) completely inhibited α- and γ-thrombin–induced platelet aggregation and also partially inhibited A23187 + Ca^{2+}–induced platelet aggregation (50% of control). HK did not inhibit ADP-, collagen-, PMA-, PMA + A23187-, and U46619-induced platelet aggregation.

Cleavage of aggregin in [^{3}H]FSBA-labeled platelets in the presence of various agonists. Collagen, A23187, PMA, PMA + A23187, and U46619 were completely ineffective in bringing about the cleavage of aggregin in the radiolabeled platelets. However, the presence of Ca^{2+} in incubation mixtures containing A23187 led to significant cleavage (67%) of aggregin. HK (0.67 μmol/L) partially blocked the cleavage of aggregin induced by A23187 + Ca^{2+}.

Effect of HK on binding of thrombin to platelets. To test the possibility that HK might prevent thrombin from interacting with its receptors, we investigated the binding of ^{125}I-thrombin to washed intact platelets. The total binding was concentration-dependent with saturation observed at 1 nmol/L. Nonspecific binding at a 50-fold mol/L excess of unlabeled thrombin was 30% to 40% of total binding at concentrations higher than 0.5 nmol/L. The binding observed with HK did not differ from the nonspecific binding. HK (350-fold mol/L excess) completely inhibited binding of ^{125}I-thrombin to washed intact platelets (Fig 3).

Effect of HK on platelet cAMP levels. Increase in cAMP levels has been shown to inhibit binding of α-thrombin.[22] Therefore, we investigated whether the inhibitory effect of HK could be attributed to the effect of HK on intracellular platelet cAMP concentration. The cAMP in intact unstimulated platelets was not detectable (Table 1), in agreement with a previously reported value of only 1.6 pmol cAMP/10^9 platelets.[23] Iloprost, an analogue of prostaglandin I_2 and a known stimulator of adenylate cyclase, markedly increased the platelet cAMP levels in the presence of papaverine, a cAMP phosphodiesterase inhibitor.[24] We found that HK at plasma levels (0.67 μmol/L) did not affect the ability of iloprost and papaverine to increase the platelet cAMP levels (Table 1). Thrombin (2 nmol/L) inhibited by 90% the intracellular rise in cAMP levels induced by iloprost (Table 1). HK (0.67 μmol/L) blocked the ability of thrombin to antagonize the increase in intracellular cAMP levels induced by iloprost.

Effect of HK on thrombin-induced secretion of ATP. Thrombin (2 nmol/L) alone caused secretion of 5.2 μmol/L ATP (Table 2) from washed intact platelets. Thrombin-induced ATP secretion was inhibited by HK (0.067 to 0.67 μmol/L) in a concentration-dependent manner and was complete at physiologic concentration (0.67 μmol/L) (Table 2).

Effect of HK on the thrombin-induced increase in intracellular levels of Ca^{2+}. When platelets were incubated with α-thrombin, an elevation of intracellular calcium ($[Ca^{2+}]_i$) corresponding to almost 1 μmol/L was observed (Table 3). Preincubation of platelets with HK (0.67 μmol/L) for 1 minute completely blocked the thrombin-induced increase in $[Ca^{2+}]_i$. Addition of HK to the incubation mixture containing thrombin and platelets after 10, 20, and 30 seconds led to an increase in $[Ca^{2+}]_i$ by 23%, 39%, and 68%, respectively (experiments 3, 4, and 5, Table 3). These results suggest that once a certain number of receptors on the platelet surface are occupied by thrombin, and with the increase in $[Ca^{2+}]_i$ being so rapid, HK was unable to reverse this effect. HK (0.67 μmol/L) also completely inhibited the increase in $[Ca^{2+}]_i$ induced by γ-thrombin (data not shown).

Effect of HK on thrombin-induced platelet shape change. A very small increase in $[Ca^{2+}]_i$ is associated with platelet shape change.[25] We found that thrombin, at a concentration as low as 0.031 nmol/L, induced platelet shape change (Table 4). Maximum shape change was observed with 0.5 nmol/L thrombin. Preincubation of platelets with HK (0.33 μmol/L) for 30 seconds blocked thrombin (0.5 nmol/L)-

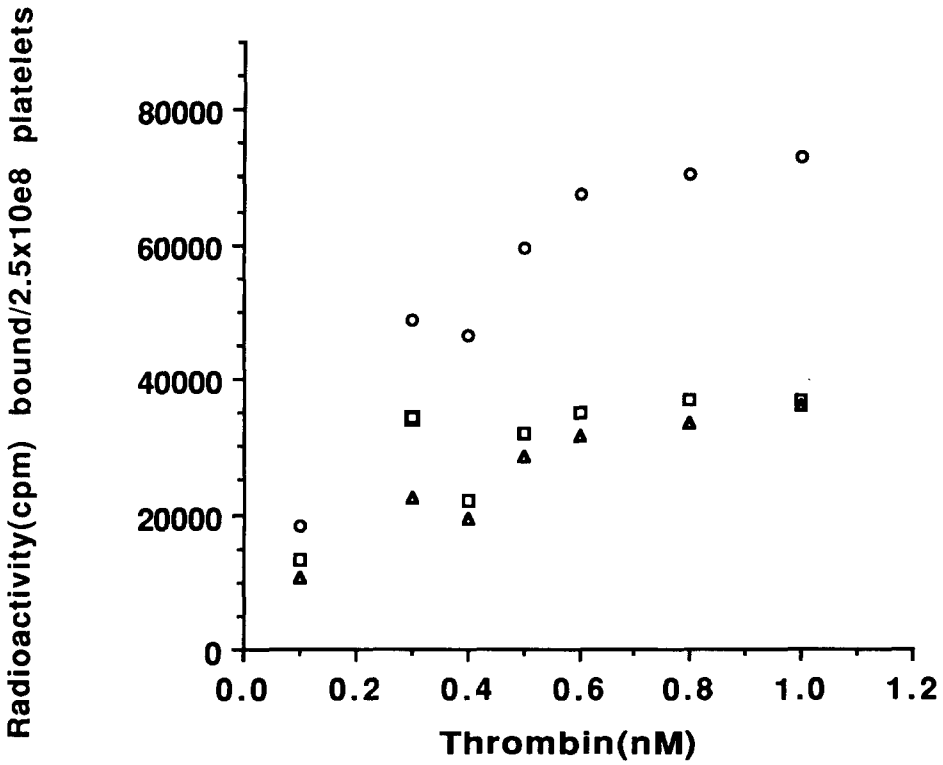

Fig 3. Effect of HK on the binding of ^{125}I-thrombin to intact platelets. ^{125}I-thrombin was prepared as described in Materials and Methods. Platelets were incubated with radiolabeled thrombin (0.1 to 1.0 nmol/L) in the absence (○) or presence of 350-fold mol/L excess HK (□), and total bound radioactivity was determined. Nonspecific binding of the radiolabeled thrombin to platelets (△) was determined under identical conditions in the presence of a 50-fold mol/L excess of unlabeled thrombin. Data are expressed (average of two experiments) as the radioactivity (cpm) bound per 2.5 × 10^8 platelets versus concentration of thrombin.

induced shape change by almost 90% (experiment 9, Table 4).

Inhibition of thrombin-induced platelet aggregation by HK in plasma. To define the role of HK in the plasma environment, we made use of plasma with a varying amount of HK. Because fibrinogen is present in the various plasmas used, we used γ-thrombin, an autolytic derivative of α-thrombin, because the former has little or no clotting activity.[26] HK inhibited γ-thrombin–induced platelet aggregation in a dose-dependent manner (Fig 4), with complete inhibition at 70 μg/mL (0.59 μmol/L). The IC_{50} (0.24 μmol/L) of HK for platelet aggregation stimulated by γ-thrombin was only slightly lower than the IC_{50} for α-thrombin (0.32 nmol/L) (Fig 1). HK had no effect on the amidolytic activity of γ-thrombin.

The effect of γ-thrombin on thrombin-induced platelet aggregation in various plasmas is shown in Fig 5. The EC_{50} of γ-thrombin (concentration to produce 50% of maximum rate of aggregation) was 7 nmol/L in washed platelets (curve 1, Fig 5) and 102 nmol/L, a 14.5-fold increase, when the same platelets were suspended in pooled normal plasma (curve 6, Fig 5). When washed platelets were suspended in total kininogen-deficient plasma, the EC_{50} of γ-thrombin to induce platelet aggregation was 40 nmol/L (curve 2, Fig 5) and was comparable with the EC_{50} value of 50 nmol/L for the plasma deficient only in HK (curve 3, Fig 5). When plasma deficient in both kininogens was supplemented with HK (0.67 μmol/L) at the plasma concentration, the EC_{50} of γ-thrombin increased to 90 nmol/L (curve 4, Fig 5), a value closer to that for normal human plasma. These results show that HK plays a significant role in modulating thrombin activity in plasma.

Table 1. Effect of HK on Intracellular Platelet cAMP Levels

Experiment	Additions	Concentration	pmol cAMP/10^8 Platelets
1	None		0
2	Iloprost + papaverine	5 μmol/L and 2 mmol/L	417 ± 5.7
3	HK	0.67 μmol/L	0
4	HK + iloprost + papaverine	0.67 μmol/L, 5 μmol/L, and 2 mmol/L	431 ± 12.9
5	Thrombin	0.002 μmol/L	0
6	Thrombin + iloprost + papaverine	0.002 μmol/L, 5 μmol/L, and 2 mmol/L	41.2 ± 5.8
7	HK + thrombin + iloprost + papaverine	0.67 μmol/L, 0.002 mmol/L, 5 μmol/L, and 2 mmol/L	427 ± 21.3

Intracellular cAMP in intact washed platelets in the absence (experiment 10) and presence of HK (experiment 3) was measured as described in Materials and Methods. Platelets were then treated with iloprost and papaverine in the absence (experiment 1) and presence of HK (experiment 4) before determination of cAMP. Platelets were then treated with thrombin alone (experiment 5), and thrombin and iloprost plus papaverine (experiment 6), and cAMP levels were determined. Finally, platelets were preincubated with HK and then treated with thrombin followed by iloprost plus papaverine, and cAMP levels were determined. The results are expressed as mean ± SE. Experiments were performed in triplicate.

Table 2. Effect of HK of Thrombin-Induced Secretion of ATP

Experiment	HK (μmol/L)	ATP Released (μmol/L)	% Inhibition of ATP Secretion
1	0	5.20	0
2	0.067	4.38	15.8
3	0.135	3.98	23.5
4	0.270	3.40	34.7
5	0.380	2.52	51.6
6	0.670	0	100

Platelets (2×10^8/500 μL) were treated with buffer + thrombin (2 nmol/L), and ATP release measured by the firefly-luciferase procedure as described in Materials and Methods (experiment 1). Platelets (2×10^8/500 μL) were then preincubated with increasing concentrations of HK (experiments 2 through 6) at 37°C for 1 minute followed by a fixed concentration of thrombin (2 nmol/L), and ATP release was determined.

DISCUSSION

We recently showed that cleavage of aggregin in [^{3}H]FSBA-labeled platelets as well as unmodified platelets during platelet aggregation induced by thrombin is indirectly mediated by the intracellularly activated calcium-dependent cysteine protease, calpain.[8] Platelet aggregation and cleavage of aggregin by α-thrombin are also mediated by the high-affinity thrombin receptors on the platelet surface.[9] Because HK was previously shown to be the most potent plasma inhibitor of platelet calpain,[12] we postulated that HK, at plasma concentration (0.67 μmol/L), might inhibit thrombin-induced platelet aggregation and cleavage of aggregin. In fact, HK inhibited thrombin-induced platelet aggregation of both unmodified and FSBA-modified platelets in a dose-dependent manner, with complete inhibition occurring at a plasma concentration of 0.67 μmol/L. HK, at normal plasma concentration (0.67 μmol/L), also completely inhibited thrombin-induced cleavage of aggregin in [^{3}H]FSBA-modified platelets. HK did not inhibit amidolytic activity of α- and γ-thrombin.

We then found that HK inhibited binding of ^{125}I-thrombin to washed intact platelets (Fig 3). This is consistent with our previous findings that cleavage of aggregin requires occupancy of high-affinity receptors by thrombin.[9] Therefore, we examined whether or not HK inhibited other thrombin-induced platelet responses. We found that HK neither increased platelet cAMP levels nor did it affect the ability of iloprost to increase platelet cAMP levels. HK also blocked the ability of thrombin to antagonize adenylate cyclase receptor linked to that enzyme. Thrombin-induced ATP secretion by the platelet dense granules was inhibited by HK in a concentration-dependent manner as well as by a thrombin-induced increase in $[Ca^{2+}]_i$ and shape change.

Table 4. Effect of HK on Thrombin-Induced Rate of Platelet Shape Change

Experiment	Addition	Concentration (nmol/L)	Rate of Shape Change (mV/min)	% Maximal of Rate of Shape Change
1	Thrombin	2	233	100
2	Thrombin	1	233	100
3	Thrombin	0.5	233	100
4	Thrombin	0.25	223	95.7
5	Thrombin	0.125	133	57.1
6	Thrombin	0.0625	77	32.9
7	Thrombin	0.0313	40	17.2
8	HK	335	0	0
9	(1) HK, 30	335		
	(2) Thrombin	0.5	27	11.4

Platelets (2×10^8/mL) were treated with different concentrations of thrombin (experiments 1 through 7) to determine the optimum concentration of the protease necessary to give maximum shape change. HK itself did not cause platelet shape change (experiment 8). Shape change was then determined in platelets preincubated with HK (670-fold mol/L excess) followed by treatment with thrombin (experiment 9). The small degree of shape change observed in this case may be because of residual thrombin that have escaped neutralization by HK during the time period of incubation (30 seconds).

HK did not inhibit platelet aggregation induced by the agonists that function by ADP-dependent mechanisms, eg, ADP,[2] collagen,[3] and U46619,[4] or by the ones that function by mechanisms involving activation of protein kinase C and/or modulation of protein kinase C activity, eg PMA,[27] and PMA + A23187,[28] respectively.

The calcium ionophore A23187 is known to affect platelet shape change, aggregation, and secretion (reference 29, and other references cited therein). However, HK did not inhibit platelet aggregation induced by A23187 in the absence of added calcium. On the other hand, in the presence of external Ca^{2+} (5 mmol/L), A23187 has been shown previously to activate platelet calpain and cleave membrane and surface GPs.[30] Consistent with these findings are our results that show that HK partially inhibited platelet aggregation and cleavage of aggregin induced by A23187 + Ca^{2+}. Therefore, HK has the potential of inhibiting platelet aggregation induced by agonist(s) that increase intracellular Ca^{2+} high enough to activate platelet

Table 3. Effect of HK on the Increase in Intracellular Calcium, $[Ca^{2+}]_i$, Induced by α-Thrombin

Experiment	First Addition	Concentration (μmol/L)	Second Addition	Concentration (μmol/L)	$[Ca^{2+}]_i$ (μmol/L)
1	α-Thrombin	0.002	—	—	0.979
2	HK	0.67	α-Thrombin	0.002	0.000
3	α-Thrombin	0.002	After 10 s, HK	0.67	0.228
4	α-Thrombin	0.002	After 20 s, HK	0.67	0.383
5	α-Thrombin	0.002	After 30 s, HK	0.67	0.670

Platelets (2×10^8/mL) were treated with thrombin (experiment 1) and increase in $[Ca^{2+}]_i$ determined by the quin-2/AM procedure described in Materials and Methods. Platelets were preincubated with a plasma concentration of HK at 37°C for 1 minute followed by thrombin (experiment 2) and the increase in $[Ca^{2+}]_i$ was determined. In the next three experiments (3 through 5), platelets were treated with the same concentration of thrombin, but HK was added at different intervals after the addition of protease. Data are presented as the mean of duplicate measurements.

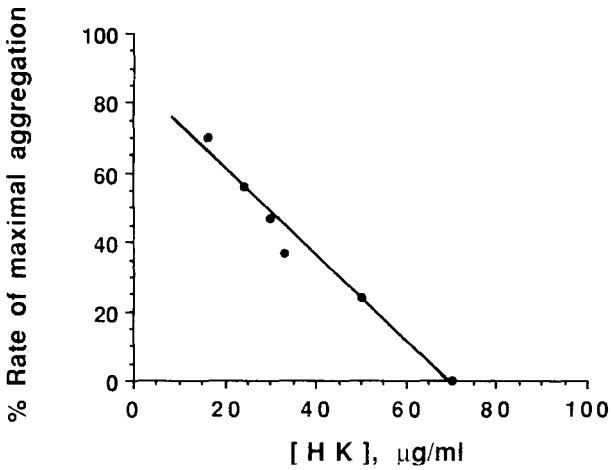

Fig 4. Concentration-dependent inhibition of γ-thrombin–induced aggregation of washed platelets by HK. Washed platelets (5 × 10^8/mL) were preincubated with various concentrations of HK and Ca^{2+} (1 mmol/L) and aggregation initiated by the addition of γ-thrombin (25 nmol/L). The rates of aggregation in the presence of HK are expressed as percent of the control (taken as 100%) in the absence of HK. Each point represents an average of triplicate experiments with a maximum variation of 15%.

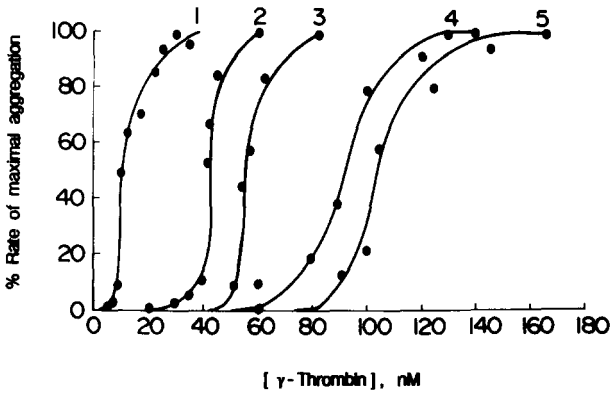

Fig 5. Effect of kininogen on γ-thrombin–induced platelet aggregation in plasma. Washed platelets (5 × 10^8/mL) (curve 1) were incubated with Ca^{2+} (1 mmol/L) for 1 minute at 37°C and aggregation started by various amounts of γ-thrombin. The rates of aggregation are expressed as percent of the control (taken as 100%) in the absence of HK for each concentration of γ-thrombin tested. Total kininogen-deficient plasma (curve 2), HK-deficient plasma (curve 3), total kininogen-deficient plasma supplemented with HK (0.67 μmol/L) (curve 4), and normal human plasma (curve 5) were diluted with three parts of washed platelets and γ-thrombin–induced aggregation was monitored as described above. The total platelet concentration in each case was 5 × 10^8/mL. Each data point represents a mean of two values with a maximum error of 15%.

calpain and translocate it onto the outer side of the membrane. Although thrombin-induced platelet aggregation proceeds by this mechanism[8,9] and HK inhibits it, it does so by inhibiting binding of thrombin to platelets, an event that precedes and prevents the activation of calpain.

Finally, we evaluated the significance of HK as a modulator of the effects of thrombin on platelets in plasma. To study HK in plasma, we used γ-thrombin, which can stimulate platelets but does not convert fibrinogen into fibrin.[26] Crouch and Lapetina[31] showed that γ-thrombin is nearly as potent as α-thrombin in eliciting platelet activation. The EC_{50} of γ-thrombin to stimulate platelet aggregation in pooled normal plasma (102 nmol/L) is almost 15 times that for washed platelets (7 nmol/L) (Fig 5). The finding that EC_{50} of γ-thrombin for HK-low molecular weight kininogen (LK)–deficient plasma (40 nmol/L) is close to that for plasma deficient only in HK (50 nmol/L), and that the EC_{50} of γ-thrombin for HK-LK–deficient plasma reconstituted with 0.67 μmol/L HK (90 nmol/L) is close to that of the normal pooled plasma (102 nmol/L) (Fig 5), suggests that HK is a modulator of thrombin-induced platelet aggregation in plasma. The difference between the potency of γ-thrombin for total kininogen-deficient plasma and plasma deficient only in HK (Fig 5) is consistent with preliminary findings of other investigators[32] that LK may also contribute, though less significantly, to the inhibition of thrombin-induced platelet aggregation in plasma. It is likely that LK, which has the identical heavy chain to that of HK, also inhibits binding of thrombin to platelets. The possibility that antithrombin III (in the absence of heparin) modulates thrombin-induced platelet aggregation in normal human plasma is less likely because antithrombin III has been previously shown to inhibit thrombin activity by less than 15% in 5 minutes,[33] while the rate of thrombin-induced platelet aggregation reaches a maximum value in less than 1 minute. The concentration of antithrombin III in various plasmas may be different, but the reconstitution data presented in Fig 5 show that HK alone can shift the dose-response curve for platelet aggregation by γ-thrombin without altering the concentration of antithrombin III.

In summary, we conclude that (1) HK is a specific inhibitor of thrombin-induced platelet aggregation; (2) HK inhibits thrombin-induced platelet aggregation by inhibiting binding of thrombin to platelets; and (3) HK plays an important role in modulating thrombin-induced platelet activation in human plasma.

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