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Binding of Coagulation Factor XI to Washed Human Platelets†

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ABSTRACT: The binding of human coagulation factor XI to washed human platelets was studied in the presence of zinc ions, calcium ions, and high molecular weight kininogen. Significant factor XI binding occurred at physiological levels of these metal ions when high molecular weight kininogen was present. Binding required platelet stimulation and was specific, reversible, and saturable. Scatchard analysis of the binding yielded approximately 1500 binding sites per platelet with an apparent dissociation constant of approximately 10 nM. Since the concentration of factor XI in plasma is about 25 nM, this suggests that in plasma factor XI binding sites on stimulated platelets might be saturated. Calcium ions and high molecular weight kininogen acted synergistically to enhance the ability of low concentrations of zinc ions to promote factor XI binding. The similarity between the concentrations of metal ions optimal for factor XI binding and those optimal for high molecular weight kininogen binding, as well as the ability of high molecular weight kininogen to modulate these metal ion effects, implies that factor XI and high molecular weight kininogen may form a complex on the platelet surface as they do in solution and on artificial negatively charged surfaces.

The process of contact activation of intrinsic coagulation has been the subject of intensive investigation in recent years. A large body of data has been developed concerning the events leading to the activation of factor XI, involving the proteins factor XII, prekallikrein, and high molecular weight kininogen (HM_rK)[1] [reviewed in Cochrane & Griffin (1982) and Griffin & Cochrane (1979)]. It has been shown that these proteins are assembled on negatively charged surfaces and form a contact factor complex capable of generating factor XIa activity on the surface. The studies from which this model was derived employed a variety of artificial or nonphysiological negatively charged surfaces such as kaolin, celite, glass, ellagic acid, sulfatides, or dextran sulfate. Although such studies have been useful in elucidating the molecular interactions involved in contact activation, questions about the physiological sites of the initiation of intrinsic coagulation remain open. The finding that crude preparations of collagen support contact activation of plasma (Niewiarowski et al., 1964, 1965; Wilner et al., 1968; Cochrane et al., 1972a,b) suggests the possibility that exposed subendothelium may be one such site. However, it was proposed by Walsh (1972a) that the activated platelet surface may also serve as locus for these reactions. Clotting assays showed that activated platelets could substitute for artificial negatively charged surfaces in promoting factor XI activation by the contact system (Walsh, 1972a–d). More recently, in experiments using highly purified contact factors and well-washed platelets, activated platelets in the presence of high molecular weight kininogen were shown to promote the proteolytic activation of factor XI by factor XIIa, as well

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[1] Abbreviations: HM_rK, high molecular weight kininogen; M_r, molecular weight; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; $K_d$app, apparent dissociation constant; Con A, concanavalin A; QAE, diethyl(2-hydroxypropyl)aminoethyl.

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as the activation of factor XII by kallikrein (Walsh & Griffin, 1981). This suggested that stimulated platelets were able to support the same assembly of contact factors as is observed on artificial negatively charged surfaces. Intriguingly, it was also observed that in the presence of the cofactor high molecular weight kininogen, kallikrein was able to stimulate factor XI activation in the apparent absence of factor XII (Walsh & Griffin, 1981). In this study, it was demonstrated that the proteolytic cleavage products of activated factor XI associated with the platelets. Recently, it was determined that high molecular weight kininogen binds specifically with high affinity to stimulated platelets (Greengard & Griffin, 1984) and that this binding requires the presence of zinc ions. This observation is consistent with the idea that a complex of contact factors is assembled on the platelet surface. The present study was undertaken to examine the interaction of factor XI with platelets in order to determine whether such an assembly may indeed occur. Evidence for specific high-affinity binding of factor XI to the surface of stimulated human platelets is presented here. This binding of factor XI to platelets is shown to be dependent upon zinc ions and calcium ions, and the cofactor high molecular weight kininogen is shown to act synergistically with calcium ions to enhance the effect of low concentrations of zinc ions.

Materials and Methods

All chemicals were the best grade commercially available. Carrier-free $Na^{125}I$ was obtained from Amersham Corp., and ^{125}I-labeled Bolton and Hunter reagent was obtained from New England Nuclear. Methyl silicone oil (1.0 DC 200) and Hi-Phenyl silicone oil (125 DC 550) were obtained from William F. Nye Specialty Lubricants (New Bedford, MA). Cohn fraction V bovine serum albumin (BSA) and Ultrol HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid] were from Calbiochem-Behring Corp. Iodogen was from Pierce Chemical Co.

Buffers. "Calcium-free" HEPES–Tyrodes 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 $NaH_2PO_4 \cdot H_2O$ and was titrated to pH 6.5 or 7.3 (see below). BSA (1.0 mg/mL) was added, and the buffer was filtered over a Millipore filter (0.45-μm pore size).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) by the method of Weber and co-workers (Weber et al., 1972). Samples were prepared as described previously (Greengard & Griffin, 1984).

Clotting Assays. Clotting activities 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). One clotting unit is defined as the activity found in 1 mL of pooled normal human plasma for all coagulant proteins except for α-thrombin. One unit of α-thrombin was defined as the amount that clots 0.5 mL of normal human plasma in 15 s in a standard thrombin time test. The prothrombin in 1 mL of normal human plasma can generate approximately 30 units of thrombin activity.

Proteins. Prekallikrein (Kerbiriou et al., 1980) and factor XII (Griffin & Cochrane, 1976) were isolated from fresh human plasma as described previously. Specific activities were 20 and 80 clotting units/mg, respectively. Kallikrein was prepared from prekallikrein as reported by Bouma et al. (1980). High molecular weight kininogen was purified according to Kerbiriou and Griffin (1978) as a single polypeptide chain with a specific activity of 14 clotting units/mg. The kinin-free two-chain disulfide-linked form was prepared from the single-chain form as described by these authors. Factor XI (250 clotting units/mg) was purified according to the method of Bouma and Griffin (1977) except that the QAE-Sephadex and Con A–Sepharose chromatographic steps were omitted. Partially purified factor XI obtained after DEAE-Sephadex and SP-Sephadex chromatography was applied to a column of Sepharose to which HM_rK had been cross-linked according to the instructions of the manufacturer (Pharmacia, CNBr–Sepharose). Factor XI was eluted as described by Bouma et al. (1981). Factor XI was activated to factor XIa by β-factor XIIa as previously described (van der Graaf et al., 1983). Human α-thrombin was the kind gift of Dr. Annie Bezeaud.

Radiolabels. Factor XI was radiolabeled with ^{125}I-labeled Bolton and Hunter reagent (Bolton & Hunter, 1973) to a specific activity of 30 μCi/μg. This corresponds to 1.0 atom of ^{125}I per mole of factor XI polypeptide chain. Bovine serum albumin was radiolabeled with ^{125}I by using Chloramine T (McConahey & Dixon, 1966) to a specific activity of 9.5 μCi/μg. Both radiolabeled proteins migrated as single bands on SDS–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. The bands correspond to M_r 80 000 for factor XI and M_r 65 000 for bovine serum albumin. In the absence of 2-mercaptoethanol, factor XI migrated as a single band corresponding to M_r 160 000.

Preparation of Washed Erythrocyte and Platelet Suspensions. Platelets were prepared as described (Greengard & Griffin, 1984). Briefly, platelet-rich plasma was obtained from citrated human blood and washed as follows: platelet-rich plasma was centrifuged, and the platelets were resuspended in calcium-free HEPES–Tyrodes buffer, pH 6.5, and gel filtered on column of Sepharose 2B equilibrated in calcium-free HEPES–Tyrodes buffer, pH 7.3 (Tangen et al., 1971). Platelets were counted electronically (Coulter Electronics, Hialeah, FL). When ^{125}I-labeled bovine serum albumin or ^{125}I-labeled factor XI was added as a tracer, it was found that less than 0.06% of these proteins was retained with the platelet eluates (Walsh & Griffin, 1981). Erythrocytes were prepared as described before (Greengard & Griffin, 1984) and were suspended in HEPES–Tyrodes buffer, pH 7.3.

Binding Experiments. All incubations were performed at 37 °C without stirring the reaction mixture. Platelets were prewarmed and incubated at a concentration of (2–3) × 10^8/mL in calcium-free HEPES–Tyrodes buffer, pH 7.3, in a 1.5-mL Eppendorf plastic centrifuge tube with a mixture of radiolabeled and unlabled factor XI, divalent cations, platelet stimuli, and HM_rK or other proteins. At various times after the addition of the platelet stimulus, aliquots were removed (110 μL in time course experiments, 170 μL in other experiments) and centrifuged through a mixture of silicone oils as described (Greengard & Griffin, 1984). Unless otherwise stated, total binding is shown, uncorrected for any nonsaturable component. More than 86% of the platelets were sedimented under these conditions (Greengard & Griffin, 1984).

Competition Experiments. Platelets were incubated with $ZnCl_2$ (25 μM), $CaCl_2$ (2.0 mM), thrombin (0.10 unit/mL), HM_rK (5.0 μg/mL), and ^{125}I-factor XI (0.025 μg/mL) and then mixed with 6.0 μg/mL samples of various unlabeled proteins or buffer. After 20 min, samples were centrifuged. Binding of ^{125}I-factor XI was compared to the control binding in the absence of competing proteins.

Calculations of Binding Constants and Number of Sites. Binding data were analyzed according to the method of

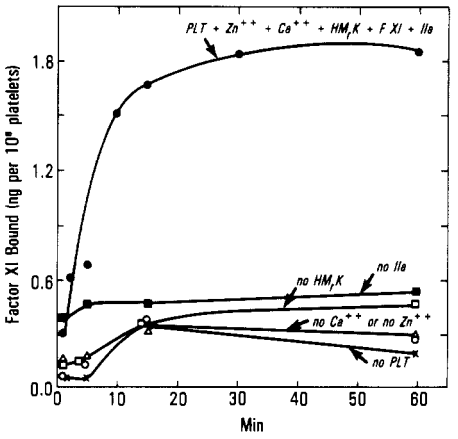

FIGURE 1: Binding of ^{125}I-factor XI to platelets. Platelets were incubated without stirring at 37 °C with ^{125}I-factor XI (50 ng/mL), HM_rK (5 μg/mL) or buffer, $ZnCl_2$ (25 μM) or buffer, $CaCl_2$ (2.0 mM) or buffer, and thrombin (0.1 unit/mL) or buffer. At the indicated times, aliquots were removed and centrifuged as described under Materials and Methods.

Scatchard (1949). Points were the averages of triplicate determinations. "Nonspecific" binding, apparent dissociation constants, and numbers of binding sites were calculated by using the nonlinear data-fitting computer program "LIGAND" of Munson and Rodbard (1980) on a Hewlett-Packard 9836 computer. Different concentrations of total ligand were made by mixing increasing amounts of unlabeled protein with a constant amount of radiolabeled protein. The "nonspecific" binding was calculated by assuming that it represented an infinitely nonsaturable component of the total binding (Munson & Rodbard, 1980).

RESULTS

^{125}I-Factor XI Binding to Platelets. To determine whether ^{125}I-labeled factor XI binds to platelets, washed platelets were incubated unstirred at 37 °C with a mixture of unlabeled and ^{125}I-labeled factor XI in the presence or absence of 25 μM $ZnCl_2$, 2.0 mM $CaCl_2$, human HM_rK, and human α-thrombin (Figure 1). The incubation mixture was sampled at varying time intervals, and aliquots were centrifuged through silicone oil barriers to separate platelets from unbound proteins. Binding to stimulated platelets in the presence of HM_rK, $ZnCl_2$, and $CaCl_2$ reached a plateau in approximately 20 min. If either $ZnCl_2$ or $CaCl_2$ was omitted, a much lower amount of binding was observed. Since omission of HM_rK from the incubation mixture also decreased binding to this background level, it may be inferred that HM_rK acts as a cofactor in the interaction of factor XI with stimulated platelets as it does in the assembly of the contact factor complex on artificial negatively charged surfaces (Wiggins et al., 1979; Thompson et al., 1977). The requirement for zinc and calcium ions suggests that the mechanism for the binding of factor XI is, at least in part, similar to that observed for HM_rK binding to stimulated platelets (Greengard & Griffin, 1984). A preparation of ^{125}I-factor XI radiolabeled by using a variation of the Iodogen technique (Fraker & Speck, 1978) which had lost more than 95% of its procoagulant activity failed to bind under these conditions (data not shown).

Stimulation of platelets was essential for maximal binding of factor XI to platelets as the experiment shown in Figure 2 demonstrates. Maximum binding was observed when platelets were stimulated with approximately 0.05 unit/mL thrombin. This binding was inhibited by 60% when 80 μg/mL apyrase was present (data not shown), suggesting an ADP-dependent mechanism of receptor expression.

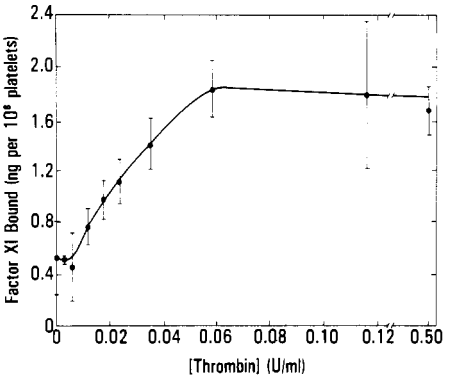

FIGURE 2: Effect of thrombin on binding of ^{125}I-factor XI to platelets. Platelets were incubated for 20 min at 37 °C with ^{125}I-factor XI (50 ng/mL), $CaCl_2$ (2.0 mM), $ZnCl_2$ (25 μM), HM_rK (1.5 μg/mL), and the indicated concentrations of thrombin. Binding was determined as described under Materials and Methods.

Table I: Comparison of Association of Factor XI with Platelets and Erythrocytes[a]

cell	$ZnCl_2$	$CaCl_2$	HM_rK	thrombin	factor XI in the pellet (ng/10^8 cells)
platelets	+	+	+	+	2.41 ± 0.06
erythrocytes	+	+	+	+	0.40 ± 0.21
platelets	−	+	+	+	0.11 ± 0.01
erythrocytes	−	+	+	+	0.07 ± 0.02
platelets	+	−	+	+	0.27 ± 0.05
erythrocytes	+	−	+	+	0.13 ± 0.02
platelets	+	+	−	+	0.30 ± 0.02
erythrocytes	+	+	−	+	0.06 ± 0.02
platelets	+	+	+	−	0.89 ± 0.07
erythrocytes	+	+	+	−	0.47 ± 0.06
no cells	+	+	+	+	0.40

[a] Platelets or erythrocytes (2.6 × 10^8 cells/mL) were incubated 20 min at 37 °C with ^{125}I-factor XI (50 ng/mL) with or without HM_rK (4 μg/mL), $CaCl_2$ (2.0 mM), $ZnCl_2$ (25 μM), and thrombin (0.1 unit/mL). Samples were centrifuged as described under Materials and Methods. A plus sign indicates the presence of a component in the incubation mixture; a minus sign indicates the absence of a component. A control without cells and all other components was performed to determine the amount of radiolabeled factor XI that sedimented in the absence of cells.

Platelet Dependence of Factor XI Binding. The experiments described above suggest that zinc ions mediate the interaction of factor XI with the platelet surface. In order to determine whether this is a receptor-mediated interaction, it was first necessary to demonstrate that binding is specific for platelets. The interaction of factor XI with platelets and with erythrocytes is compared in Table I. An equal concentration of either platelets or erythrocytes was incubated with the indicated metal ions and proteins. In the presence of zinc ions, calcium ions, HM_rK, and thrombin, 6-fold more factor XI was present in the platelet pellet than in the erythrocyte pellet although the "trapped volume" associated with erythrocytes is much greater than that associated with platelets. Platelets required the presence of HM_rK, zinc ions, calcium ions, and a platelet agonist (i.e., thrombin) in order to bind factor XI, whereas only a small amount of factor XI was sedimented in the trapped volume associated with the erythrocytes. This nonspecific association was relatively unaffected by the presence of thrombin, HM_rK, or divalent cations.

Effect of Calcium Ions and High Molecular Weight Kininogen on the Zinc Ion Dependent Binding of Factor XI to Stimulated Platelets. In order to ascertain the optimal conditions for factor XI binding to platelets, the effects of different

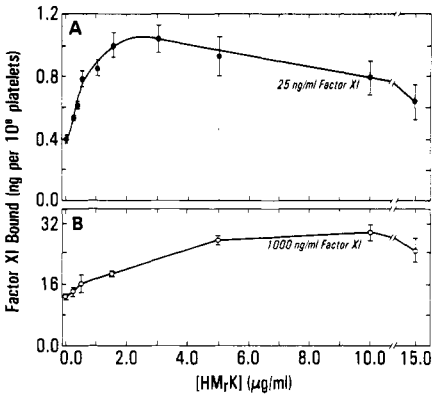

FIGURE 3: Effect of HM_rK on binding of ^{125}I-factor XI to platelets. Platelets were incubated for 20 min at 37 °C with ^{125}I-factor XI, $CaCl_2$ (2.0 mM), $ZnCl_2$ (25 μM), varying amounts of HM_rK, and thrombin (0.15 unit/mL). ^{125}I-Factor XI concentration was (A) 25 or (B) 1000 ng/mL. Binding was determined as described under Materials and Methods.

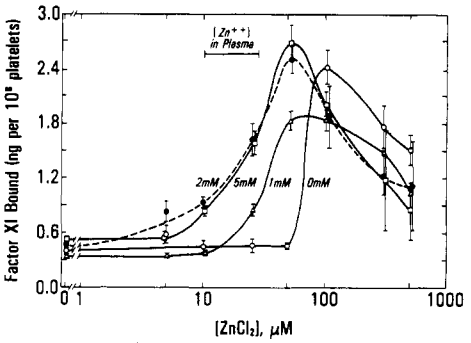

FIGURE 4: Effect of $CaCl_2$ on ^{125}I-factor XI binding to platelets in the presence of HM_rK at various concentrations of $ZnCl_2$. Platelets were incubated at 37 °C with the indicated concentrations of $ZnCl_2$, HM_rK (5 μg/mL), thrombin (0.1 unit/mL), ^{125}I-factor XI (25 ng/mL), and $CaCl_2$ at 0 (○), 1 (△), 2 (●), or 5 mM (□). After 20 min, aliquots were removed, and the amount of ^{125}I-factor XI binding was determined.

concentrations of $CaCl_2$ and HM_rK on factor XI binding were determined. Figure 3 shows the effect of different concentrations of HM_rK on factor XI binding to thrombin-stimulated platelets at 2.0 mM $CaCl_2$ and 25 μM $ZnCl_2$. Binding was measured at two different concentrations of factor XI. At 25 ng/mL factor XI, a plateau was obtained between approximately 2 and 5 μg/mL HM_rK (figure 3A). More HM_rK was required for maximal binding of 1000 ng/mL factor XI, with the plateau occurring at 5–10 μg/mL HM_rK (figure 3B). Calcium ions have been shown to potentiate the ability of low concentrations of zinc ions to promote the binding of HM_rK to platelets (Greengard & Griffin, 1984). In the presence of HM_rK, calcium ions also potentiate the binding of factor XI (Figure 4). Optimal conditions for factor XI binding (Figure 4) were achieved at 2 mM $CaCl_2$ or greater, in agreement with the conditions yielding optimal HM_rK binding (Greengard & Griffin, 1984). In contrast, the effect of calcium ions on factor XI binding in the absence of added HM_rK was inhibitory (Figure 5). In the absence of calcium ions or HM_rK, a maximum of 1.8 ng of factor XI per 10^8 platelets was bound at 300–500 μM $ZnCl_2$. When calcium ions were present, however, there was a concentration-dependent inhibition of the ability of zinc ions to promote factor XI binding. This difference in the effect of calcium ions on factor XI binding vs. HM_rK binding could be explained if the role of calcium ions is to promote the binding of HM_rK to a platelet receptor which, in turn, promotes the subsequent binding of factor XI. Figure 6 demonstrates the synergistic effect of calcium ions

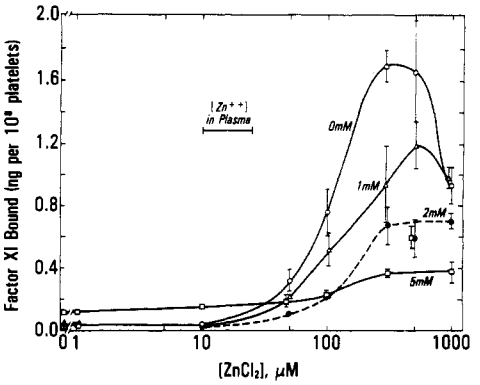

FIGURE 5: Effect of $CaCl_2$ on ^{125}I-factor XI binding to platelets in the absence of HM_rK at various concentrations of $ZnCl_2$. Platelets were incubated at 37 °C with the indicated concentrations of $ZnCl_2$, ^{125}I-factor XI (25 ng/mL), thrombin (0.10 unit/mL), and $CaCl_2$ at 0 (○), 1 (△), 2 (●), or 5 mM (□). After 20 min, binding was measured as described under Materials and Methods.

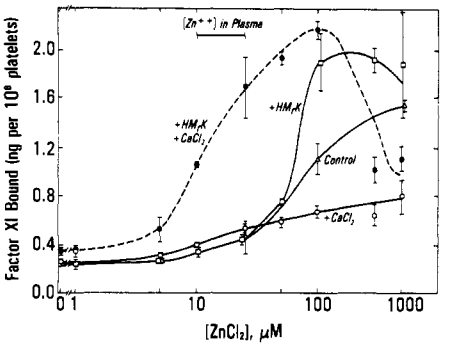

FIGURE 6: Synergistic effect of HM_rK and calcium ions on binding of ^{125}I-factor XI to platelets. Platelets were incubated at 37 °C with the indicated concentrations of $ZnCl_2$, $CaCl_2$ (2.0 mM) or buffer, HM_rK (5 μg/mL) or buffer, thrombin (0.1 unit/mL), and ^{125}I-factor XI (27 ng/mL). After 20 min, aliquots were removed, and the amount of ^{125}I-factor XI binding was determined.

and HM_rK on the binding of factor XI. The binding of factor XI to platelets in the absence of added HM_rK was inhibited by the presence of 2.0 mM $CaCl_2$, even in the presence of high concentrations of $ZnCl_2$. In the presence of added HM_rK but the absence of added zinc ions, the same maximum amount of factor XI was bound as in the absence of calcium ions in the experiment shown in Figure 5, namely, 1.8 ng per 10^8 platelets at 300–500 μM $ZnCl_2$. However, when sufficient $CaCl_2$ and HM_rK were both added to the reaction mixture, there was both an increase in the maximum amount of factor XI bound per platelet and a potentiation of the ability of low concentrations of zinc ions to promote the binding of factor XI to the platelets. This is consistent with the hypothesis that factor XI binds to the platelet surface as a complex with platelet-associated HM_rK.

Specificity of Factor XI Binding. In order to evaluate the specificity of the zinc ion dependent factor XI interaction with platelets, the ability of various proteins to compete for binding sites was studied. Radiolabeled factor XI (25 ng/mL) was mixed with buffer or 6 μg/mL aliquots of other proteins in the presence of 5 μg/mL HM_rK, 25 μM $ZnCl_2$, 2 mM $CaCl_2$, and 0.10 unit/mL thrombin. Unlabeled factor XI competed away 78% of the radiolabeled molecules. Surprisingly, even prekallikrein, a molecule which shares with factor XI the ability to bind to HM_rK, failed to compete for factor XI binding to the platelets (110% of control). Factor XIa, however, competed with ^{125}I-factor XI as efficiently as did unlabeled factor XI. Binding of ^{125}I-factor XI was unaffected by factor IX, ovalbumin, soybean trypsin inhibitor, or ovomucoid

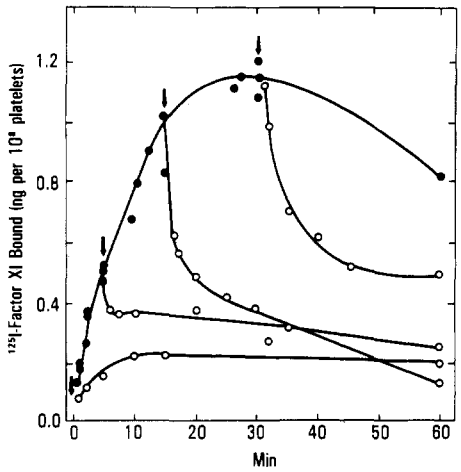

FIGURE 7: Reversibility of ^{125}I-factor XI binding to platelets. The time course of ^{125}I-factor XI (25 ng/mL) binding to thrombin-stimulated (0.15 unit/mL) platelets was determined at 37 °C in the presence of $ZnCl_2$ (25 μM) and $CaCl_2$ (2.0 mM) (●). At the times indicated by the arrows, 6.0 μg/mL unlabeled factor XI was added to the incubation mixture, and the binding of ^{125}I-factor XI was measured (○). Additions of unlabeled factor XI were made at 0, 5, 15, and 30 min.

trypsin inhibitor (100% ± 10% of control).

Reversibility of Factor XI Binding. Figure 7 shows the ability of unlabeled factor XI present in excess (240-fold) to dissociate bound ^{125}I-labeled factor XI. Unlabeled factor XI was added at times 0, 5, 10, and 30 min. When the unlabeled ligand was added at time zero, a small amount of radiolabeled factor XI was bound, amounting to 20% of the control binding. When the addition was made at 5 or 15 min, dissociation of bound radiolabel was observed, with 30–40% of the total being removed during the first minute, followed by a much slower further dissociation. When the addition was made at 30 min, the first phase occurred much more slowly, requiring 5 min for the first 40% of removal, and with a much lower final extent of dissociation.

Saturability of Factor XI Binding. The data in Figure 7 indicate that factor XI binding to platelets is to a large extent reversible and is therefore amenable to Scatchard analysis. Binding of various concentrations of factor XI to thrombin-stimulated platelets was observed in the presence of 4 μg/mL HM_rK, 25 μM $ZnCl_2$, and 2 mM $CaCl_2$. Figure 8A shows the saturability of this binding (solid circles). Nonspecific binding, as calculated from competition of unlabeled and radiolabeled factor XI using the LIGAND computer program (Munson & Rodbard, 1980), was subtracted at each point (open circles). The results show that saturation was achieved at about 18 nM, or 70% of plasma concentration. A Klotz plot (plot not shown) of these data is sigmoidal, confirming that by this criterion as well that saturation has been reached (Klotz, 1983). When the saturation data were fitted to a Scatchard analysis by the LIGAND computer program, an apparent dissociation constant of 9.6 ± 2.2 nM for a total of 1530 ± 390 binding sites was calculated (figure 8B). A second binding curve performed in the presence of 1.5 μg/mL HM_rK achieved saturation at the same factor XI concentration, although with only 75% as much factor XI bound (data not shown). When the data were subjected to analysis by the LIGAND program, similar numbers were obtained for factor XI binding with $K_d$app = 13.4 ± 4.2 nM and 1070 ± 410 binding sites.

DISCUSSION

A specific physical interaction of factor XI with platelets has been described in the studies presented here. Binding of factor XI to platelets requires platelet stimulation and zinc ions. Calcium ions and the protein cofactor HM_rK also play critical roles in the binding of factor XI. In the absence of added HM_rK, calcium ions inhibit the ability of zinc ions to support factor XI binding. In the absence of calcium ions, HM_rK promotes factor XI binding only at very high concentrations of zinc ions (Figure 6). Together, however, calcium ions and HM_rK act synergistically to enhance factor XI binding at physiologic concentrations of zinc ions.

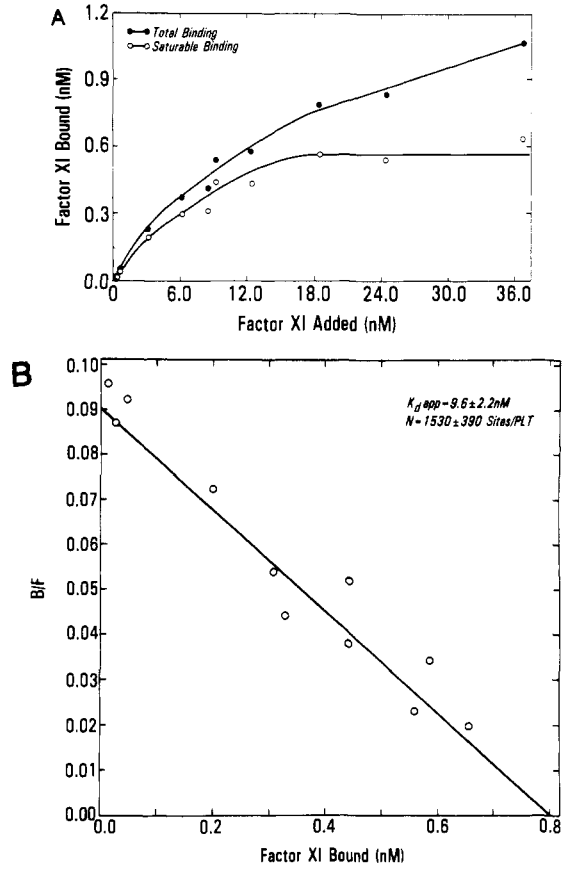

FIGURE 8: Factor XI concentration dependence of ^{125}I-factor XI binding to platelets. Platelets were incubated at 37 °C with $ZnCl_2$ (25 μM), $CaCl_2$ (2.0 mM), thrombin (0.15 unit/mL), HM_rK (4.0 μg/mL), and mixtures of ^{125}I-labeled and unlabeled factor XI at various concentrations. Binding was determined at 20 min. (A) Amount of factor XI bound at different input concentrations: (●) total binding; (○) specific binding. (B) Scatchard analysis of the data shown in (A). The line is a best fit of data from triplicate samples. Nonspecific binding, apparent dissociation constants, and number of binding sites were fitted as parameters using the LIGAND computer program (Munson & Rodbard, 1980). 1 nM factor XI (160 ng/mL) under these conditions equaled 64 ng per 10^8 platelets.

It has previously been shown that similar amounts of calcium ions act in a similar manner to enhance the ability of these concentrations of zinc ions to promote the binding of HM_rK to stimulated platelets (Greengard & Griffin, 1984). The data in the current study, therefore, are consistent with a model in which factor XI binds to HM_rK which in turn is bound to receptors on the platelet surface. Coordinate binding studies will be required to determine the actual stoichiometry of binding.

There are interesting parallels between the binding of factor XI to platelets and the binding of factor Xa. Factor Xa binding is enhanced by the presence of factor Va on the platelet surface (Kane et al., 1980; Tracy et al., 1979, 1981). Factor Va is a nonenzymatic cofactor in the activation of prothrombin by factor Xa (Davie & Fujikawa, 1975; Suttie & Jackson,

1977; Colman & Weinberg, 1976). Similarly, HM_rK is a nonenzymatic cofactor required for the activation of factor XI by α-factor XIIa in the presence of artificial negatively charged surfaces (Cochrane & Griffin, 1976; Griffin & Cochrane, 1979). HM_rK is required for the binding of factor XI to washed platelets (Figure 1). Since HM_rK itself binds to the platelet under these conditions, it may be concluded that HM_rK comprises an element of the binding site for factor XI on the platelet surface, as factor Va appears to do for factor Xa (Tracy et al., 1979, 1981; Kane et al., 1980; Miletich et al., 1978a,b). In contrast to the binding of factor Xa, an enzyme, to the unstimulated platelet (Kane et al., 1980; Tracy et al., 1979, 1981), the ligand in the present study is the factor XI zymogen, which binds optimally to stimulated platelets. Since the ligand is a zymogen, it is difficult to correlate its binding directly with physiological activity. However, the studies of Walsh and Griffin (1981) suggest that factor XI can be activated on the platelet surface.

The specificity of factor XI receptors is demonstrated by the failure of other proteins except for factor XIa to compete with radiolabeled factor XI for binding sites. The fact that prekallikrein fails to compete for these sites is especially interesting in light of the fact that prekallikrein and factor XI compete for HM_rK in solution (Thompson et al., 1977, 1979). One explanation for this observation might be that binding to its platelet receptor alters the properties of HM_rK to increase its affinity for factor XI relative to prekallikrein, perhaps due to the divalent nature of factor XI. Further studies will be necessary to clarify this point.

Recently, it was reported that factor XIa binds to the platelet in a specific manner in the absence of added zinc ions (Sinha et al., 1984). The number of binding sites per platelet at saturation for factor XIa was only 250, considerably below the 1500 reported for factor XI in the presence of zinc and calcium ions. Additionally, it was reported that factor XI did not compete with factor XIa for binding sites (Sinha et al., 1984). The relationship of these factor XIa binding sites (Sinha et al., 1984) to the zinc-dependent factor XI binding sites described here remains to be determined. The data reported here, however, show that factor XIa competes with ^{125}I-factor XI for binding sites at least as efficiently as does unlabeled factor XI in zinc ion dependent binding to the platelet. In fact, recent studies of zinc ion dependent binding to stimulated human platelets showed that factor XI and factor XIa compete for the same sites but that the affinity of factor XIa is 4–8 times greater than that of factor XI (Greengard, 1985).[2]

The data presented here support the hypothesis that the surface of stimulated platelets may provide at least one locus for the initiation of contact activation. Platelets are known to provide such a site for the assembly of the prothrombinase complex (Kane et al., 1980; Miletich et al., 1977, 1978b). In addition, the observation reported previously (Greengard & Griffin, 1984) and here that zinc ions play a critical role in the assembly of these contact proteins on the platelet surface raises the interesting question of a wider role for these metal ions in platelet physiology and in contact activation reactions.

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[2] J. S. Greengard and J. H. Griffin, unpublished results.

Registry No. Zn, 7440-66-6; Ca, 7440-70-2; α-thrombin, 62046-50-8; factor XI, 9013-55-2.

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Kinetics of Interaction of C1 Inhibitor with Complement $C1\bar{s}$[†]

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ABSTRACT: The kinetics of inhibition of the complement serine protease, $C1\bar{s}$, by its only known inhibitor, C1 inhibitor, have been measured by a variety of methods. One method continuously monitors the loss of esterolytic activity with a synthetic substrate coupled to a chromogen while another monitors the formation of a stable (covalent) complex by high-pressure size-exclusion chromatography under dissociating conditions. Additional methods employ fluorescence probes to follow the formation of bimolecular complexes but are not expected to distinguish between covalent product and noncovalent (reversible) intermediates. There was good agreement between rate constants obtained by the various methods over a broad range of inhibitor concentrations, suggesting that noncovalent intermediates do not accumulate to a significant extent. The reaction appears to be pure second order with a bimolecular rate constant of 6.0×10^4 M^{-1} s^{-1} at 30 °C, independent of Ca^{2+}, and an activation energy of 11.0 kcal/mol. The rate increases up to 35-fold in the presence of heparin which was shown to bind to all three components (enzyme, inhibitor, and complex) with similar affinity (K_d = 2.0–3.3 μM). The fluorescent probe 1,1′-bis(anilino)-4,4′-bi(naphthalene)-8,8′-disulfonate [bis(ANS)] bound to the complex with K_d = 0.26 μM under conditions where the individual components had little affinity for the dye, consistent with the generation of one or more hydrophobic binding sites on the protein surface during complex formation.

Human C1 inhibitor (C1-Inh)[1] performs a pivotal function in the regulation of the complement system in that it is the only circulating inhibitor known to react with the activated complement proteases, $C1\bar{r}$ and $C1\bar{s}$ (Sim et al., 1979; Ziccardi, 1981; Cooper, 1985). The resulting 1:1 complexes are stable to heat and SDS but can be dissociated with hydroxylamine, suggesting the formation of a covalent ester linkage between the components (Harpel & Cooper, 1975). Although the kinetics of these reactions have been investigated in two different laboratories, there is a 40-fold difference between the bimolecular rate constants reported for the reaction with $C1\bar{s}$ (Sim et al., 1980; Nilsson & Wiman, 1983). There is also uncertainty as to the mechanism of the reaction, in particular with respect to the importance of a reversibly associated intermediate complex whose subsequent conversion to a stable (covalent) product may be rate limiting under some conditions [see also Salvesen et al. (1985)]. Part of the confusion could be due to the different methods employed. Sim et al. (1980) measured the rate of formation of a heat-stable complex by SDS–PAGE and found their data to be consistent with simple second-order kinetics up to about 70% completion of the reaction. Nilsson and Wiman (1983) measured the rate of disappearance of enzyme activity by a spectrophotometric method and emphasized a two-step mechanism. In an effort to clarify the kinetics of this important reaction, we have measured its rate by several methods covering a broad range of reactant concentrations. It is shown that the rate of the initial bimolecular reaction, as measured by loss of enzymatic activity and by changes in fluorescence intensity and polarization of extrinsic fluorescent probes, is almost indistin-

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; bis(ANS), 1,1′-bis(anilino)-4,4′-bi(naphthalene)-8,8′-disulfonate; C1-Inh, C1 inhibitor or C1 inactivator; DTDP, 4,4′-dithiodipyridine; FITC, fluorescein isothiocyanate; HBS, 0.02 M Hepes/0.15 M NaCl, pH 7.4; HPSEC, high-pressure size-exclusion chromatography; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, 0.02 M Tris/0.15 M NaCl, pH 7.4; Z-Lys-sBzl, thiobenzyl ester of benzyloxycarbonyllysine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; kDa, kilodalton(s); PBS, phosphate-buffered saline.

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