Coordinate Binding of Factor Va and Factor Xa to the Unstimulated Platelet*

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The interaction of Factor Xa and Factor Va at the platelet surface was investigated by direct, simultaneous binding measurements of both proteins to platelets and by immunochemical and kinetic techniques. Binding measurements of 125I-Factor Xa and 125I-Factor Va to unstimulated platelets indicate that the amount of Factor Xa bound is proportional to the amount of Factor Va bound. At saturating concentrations of Factor Xa, the ratio of platelet-bound Factor Xa to platelet-bound Factor Va was unity. At saturating levels of Factor Va (1.3 × 10^{-10} M), 2300 molecules of Factor Xa are bound to the platelet, whereas at an equivalent concentration of unactivated Factor V, only 800 molecules of Factor Xa are bound. In the absence of exogenous Factor V or Va, thrombin-treated washed platelets bound only 200 Factor Xa molecules per platelet, suggesting that under these conditions, platelet Factor Va is the limiting component. Monovalent Fab fragments of burro antiovine Factor V inhibit, to the same degree, the rate of thrombin generation and the binding of Factor Va and Factor Xa to the platelet surface. Anti-Factor V Fab decreased the extent of Factor Va and Factor Xa binding equivalently. When the interaction of Factor Xa with platelets is modeled as Factor Xa binding to platelet-bound Factor Va, double reciprocal plots are linear, yielding a stoichiometry of 4.0 × 10^{-10} M. These sites most likely represent the Factor Xa binding sites involved in the function of the prothrombinase complex at the platelet surface.

Coagulation Factor Va is an essential nonenzymatic cofactor in Factor Xa-catalyzed prothrombin activation (1-3). Factor Va, Ca^{2+}, phospholipid, and the enzyme, Factor Xa, comprise the functional catalytic unit commonly referred to as the prothrombinase complex (1). Phospholipids are required, presumably to provide the surface upon which catalysis occurs (1, 4, 5) and Ca^{2+} promotes the binding of Factor Xa and prothrombin to the phospholipid surface (6-8). Details of the extent of participation of Factor Va in the prothrombinase complex have only recently been appreciated. Studies concerning the pronounced influence of Factor Va on the rate of prothrombin activation (9), its interaction with well defined phospholipid vesicles (5), and its interaction with other components of the prothrombinase complex (10, 11) have produced much information concerning the participation of Factor Va in the functioning and assembly of the prothrombinase complex. The combined results of these studies suggest a model (12) in which prothrombinase is composed of a phospholipid-bound complex of Factor Va and Factor Xa with Ca^{2+} participating in the binding interactions required for the proper assembly of the catalyst. Factor Va and Factor Xa interact stoichiometrically (1:1), with an apparent dissociation constant of 7 × 10^{-16} M (9). Factor Va provides the binding site for Factor Xa in a manner analogous to its postulated role in the assembly of the prothrombinase complex at the platelet surface (13-18).

There are numerous reports in the literature which suggest that Factor Va is the receptor for Factor Xa at the platelet surface. The majority of these studies have been done with Factor Xa binding to human platelets (13, 14, 17, 18). The requirement of Factor Va for Factor Xa binding has been inferred from the reduced Factor Xa binding to platelets from patients with varying degrees of congenital Factor V deficiency (14, 17). The reduced binding could be corrected by the addition of thrombin-treated platelet supernatants, which presumably add Factor Va back to the deficient system. Furthermore, a spontaneously occurring anti-Factor V autoantibody has been shown to specifically block Factor Xa binding to normal human platelets with a concomitant decrease in thrombin generation, indicating that platelet-bound Factor Xa is essential for prothrombin activation at the platelet surface (13).

We have been investigating the role of Factor Va and platelets in prothrombin activation in the bovine system since the purity and characterization of bovine Factor Va (19-21) and bovine Factor X are well established (22-24). In addition, Dahlback and Stenflo (15) have characterized bovine Factor Xa platelet binding, which will catalyze prothrombin activation at the platelet surface. They report approximately 200 to 400 molecules of Factor Xa bound per platelet with an apparent dissociation constant of 1.9 × 10^{-16} M when thrombin-activated platelets are the source of Factor Va in their system. We have studied the binding of Factor V and Factor Va to bovine platelets (16). Our studies indicate that both Factor V and Factor Va bind tightly to platelets. Factor Va appears to interact with platelets significantly better than its procofactor, Factor V, in that it binds to a class of platelet sites which will not accommodate Factor V. There are approximately 800 to 900 high affinity Factor Va binding sites with a $K_d$ of $4.0 	imes 10^{-10} M$. Factor V and Factor Va share another class of lower affinity binding sites, $K_d = 3.0 	imes 10^{-7} M$. Platelet activation is not required for, nor has any effect on, the binding of either Factor V or Factor Va to platelets. Hence, the binding sites for both Factor V and Factor Va are present on the surface of platelets.

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unstimulated platelets. Kane et al. (18) proposed that the Factor Va binding sites are most likely present on the surface of unstimulated platelets. We recently showed that Factor Va bound to unstimulated platelets. Therefore, when Factor Va binding measurements are made with platelets providing the Factor V source, the requirement for thrombin activation reflects conversion of Factor V to Factor Va and not expression of the Factor Xa binding sites, as previously postulated (13–15). However, a direct relationship between Factor Va and Factor Xa binding to the platelet has not been established.

In order to determine whether Factor Va is the receptor for Factor Xa on the surface of unstimulated platelets, we made simultaneous binding measurements of both Factor Va and Factor Xa to unstimulated platelets. Binding data evidence, as well as immunochemical and kinetic evidence, will be presented which strongly suggests that Factor Va is the Factor Xa receptor at the platelet surface.

**EXPERIMENTAL PROCEDURES**

Materials

Free basic, bovine serum albumins, fatty-free bovine serum albumins and IgE-reagent [from 1-(4-ethylamino)-2-(1H-imidazol-4-yl)propan-2-ol (alpha-ethylated)] were obtained from Sigma. Factor Va (thrombin-activated Factor V) was obtained from Histo-Biologische Institute, Athens, Greece. Anti-Factor Va antisera were obtained from J.B. Biddle Co., and sodium dodecyl sulfate (SDS) was from Pierce. The thrombin inhibitor, danarylglycine-N-[(3-ethyl-1,5-pentanediyl)amide] (10APA) was prepared as described by Neihelm and coworkers (9). Other reagents and chemicals were of analytical grade.

Results

Factor Xa Binding to Platelets in the Presence of Exogenous Factor V and Factor Va—The binding of Factor Xa to unstimulated platelets, in the presence of varying amounts of Factor Va, was measured as a function of Factor Xa concentration. The amount of Factor Xa added to the reaction mixtures ranged from 2.7 × 10⁻⁷ M to 3.1 × 10⁻⁶ M (1.5 ng/ml to 170 ng/ml). Ca²⁺ (2.5 mm) was present in all reaction mixtures. When Factor Va (thrombin-activated Factor V) was included in the reaction mixtures, 30 μM DAPA was also added to inhibit any thrombin-catalyzed alterations of the platelets. The concentration dependence of specific Factor Xa binding in these experiments, following a 30-min incubation, is shown in Fig. 1, in which the molecules of Factor Xa bound per platelet are plotted versus Factor Xa added. Nonspecific binding due to isotope entrapment was determined in parallel reaction mixtures containing a 100-fold molar excess of unlabelled Factor Xa. Specific Factor Xa binding was obtained by subtraction of nonspecifically bound [³²P]-Factor Xa to platelets from the total [³²P]-Factor Xa bound. The binding of [³²P]-Factor Xa in the presence of 1.52 × 10⁻⁷ M (0.5 ng/ml) Factor Va (closed circles, solid line) and 0.62 × 10⁻⁷ M (2 ng/ml) Factor Va (open circles, solid line) is compared to the [³²P]-Factor Xa binding in the presence of 1.52 × 10⁻⁷ M (0.5 ng/ml) Factor Va. The results were obtained with 10APA in all experiments. This substrate concentration was selected to prevent any thrombin-catalyzed alterations of the platelets. The major peak of radioactivity corresponded to the protein band of monoclonal antibody 10APA, consistent with the presence of Factor Xa bound to platelets. The binding of Factor Xa to platelets in the presence of Factor Va was determined by subtraction of nonspecifically bound [³²P]-Factor Xa from total [³²P]-Factor Xa binding. The binding of [³²P]-Factor Xa to platelets was determined by subtraction of radioactive counts from total [³²P]-Factor Xa binding. The [³²P]-Factor Xa binding was determined by subtraction of radioactive counts from total [³²P]-Factor Xa binding. The [³²P]-Factor Xa binding was determined by subtraction of radioactive counts from total [³²P]-Factor Xa binding. The [³²P]-Factor Xa binding was determined by subtraction of radioactive counts from total [³²P]-Factor Xa binding.

^1 Portions of this paper (including "Experimental Procedures," Figs. 1 and 2, and the "Appendix") are presented in miniprint as prepared by the author. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014. Request Document No. 80M-1508, cite author($, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

^2 The abbreviations used are: NaDodSO₄, sodium dodecyl sulfate; DAPA, dansylarginine-N-[3-(ethyl-1,5-pentanediyl)amide; RVX-X, Factor X activator from Russell's viper venom.
binding of $^{125}$I-Factor Xa to thrombin-activated platelets (closed circles, dashed line) without any added Factor Va. As Fig. 1 indicates, Factor Xa binding was greatly enhanced in the presence of added Factor Va when compared to the Factor Xa binding to thrombin-activated platelets. Factor Xa binding was dependent upon the Factor Va concentration, since the binding obtained with $6.06 \times 10^{-9}$M Factor Va (open circles, solid line) was greater than that observed with $1.52 \times 10^{-9}$M Factor Va (closed circles, solid line). The addition of $1.52 \times 10^{-8}$M Factor Va to a reaction mixture containing $3 \times 10^{-9}$M Factor Xa resulted in the binding of 500 Factor Xa molecules per platelet as compared to 1300 Factor Xa molecules bound when $6.06 \times 10^{-9}$M Factor Va is added. This observation is in marked contrast to the 200 molecules of Factor Xa bound to thrombin-activated platelets in the absence of any added Factor Va when, presumably, the only source of Factor Va was that which was associated with the platelet following thrombin activation.

Similar binding studies were also conducted in experiments in which $1.39 \times 10^{-9}$M prothrombin (0.1 mg/ml) was added in order to determine whether prothrombin had any effect on Factor Xa binding in the presence of added Factor Va. Again, DAPA (30 $\mu$m) was also included. No significant differences in Factor Xa binding were observed when prothrombin was present in the reaction mixtures. The binding of Factor Xa to unstimulated platelets was also measured in the presence of added Factor V in order to determine whether Factor V would promote a platelet-Factor Xa interaction. DAPA (30 $\mu$m) was included in all of the reaction mixtures to reduce the likelihood of conversion of Factor Xa to thrombin-activated platelets. The results of specific $^{125}$I-Factor Xa binding measurements to unstimulated platelets in the presence of $1.21 \times 10^{-9}$M Factor V (squares) and Factor Va (circles), following a 30-min incubation, are shown in Fig. 2. The Factor Xa binding data obtained with added Factor V indicate that the addition of Factor V, at a concentration well below its physiological concentration, will promote the binding of Factor Xa to platelets, but at a greatly reduced level from that observed with added Factor Va. The addition of Factor V resulted in approximately 800 molecules of Factor Xa bound per platelet, whereas with the addition of Factor Va, 2300 Factor Xa molecules were bound per platelet. These results are consistent with earlier data obtained with Factor V and Factor Va binding to unstimulated platelets (16). In those studies, it was shown that Factor Va interacts with platelets significantly better than its procofactor, Factor V. As many as 3500 Factor Va molecules would bind per platelet, in marked contrast to approximately 800 Factor V molecules bound per platelet.

**Simultaneous Binding Measurements of Factor Xa and Factor Va to Unstimulated Platelets**—Initial experiments suggested that Factor Xa binding to platelets was directly related to Factor Va binding. Therefore, double label binding experiments were conducted using $^{125}$I-Factor Xa and $^{131}$I-Factor Va in order to make simultaneous measurements of the binding of both ligands to unstimulated platelets. Initial experiments measured binding at a fixed, saturating concentration of Factor Va in order to assess the dependence of Factor Xa binding on the Factor Va concentration. Six different platelet reaction mixtures were used which contained $4.5 \times 10^{-9}$M (250 ng/ml) $^{125}$I-Factor Xa and either no added Factor Va or $3.03 \times 10^{-10}$M, $5.76 \times 10^{-10}$M, $1.13 \times 10^{-9}$M, $1.68 \times 10^{-9}$M, or $2.62 \times 10^{-9}$M $^{131}$I-Factor Va (100 ng/ml to 865 ng/ml). Prothrombin ($1.39 \times 10^{-9}$M, 0.1 mg/ml), $2.5 \text{mM} \text{Ca}^{2+}$, and 30 $\mu$m DAPA were also included in the reaction mixtures. The time course of binding of both $^{125}$I-Factor Xa and $^{131}$I-Factor Va at the various Factor Va concentrations was determined by making binding measurements at 0.5, 1, 2, 4, 7, 10, and 15 min after the addition of both ligands to the reaction mixture. Nonspecific binding was determined in parallel reaction mixtures containing a 100-fold molar excess of unlabelled Factor Xa and Factor Va. The time course of specific Factor Xa (squares) and Factor Va (circles) binding to unstimulated platelets at a Factor Va concentration of $2.62 \times 10^{-9}$M is shown in Fig. 3. The time course of binding of $^{125}$I-Factor Xa in the absence of added Factor Va (triangles) is also shown. The data shown in Fig. 3 indicate that both Factor Xa and Factor Va appear to bind to the platelet at approximately the same rate. Initial binding of both ligands was very rapid with equilibrium being achieved at approximately 7 to 12 min. The 350 molecules of Factor Xa bound in the absence of added Factor Va may be promoted by endogenous platelet-associated Factor V or Factor Va, since bioassay and radioimmunoassay quantitation of Factor Va associated with these platelets indicated approximately 420 Factor V molecules were present per platelet. However, a less specific Factor Xa-platelet interaction occurring at relatively high Factor Xa concentrations cannot be ruled out. The data shown in Fig. 3 for the

**Fig. 3.** Simultaneous binding measurements of $^{125}$I-Factor Xa and $^{131}$I-Factor Va to unstimulated platelets as a function of time. • —•, Binding of Factor Xa, $4.5 \times 10^{-9}$M (250 $\mu$g/ml); ▲—▲, binding of Factor Va, $2.62 \times 10^{-9}$M (0.86 $\mu$g/ml); A—A, binding of Factor Xa with no added Factor Va. Nonspecific binding due to isotope entrapment was determined in parallel reaction mixtures containing a 100-fold molar excess of unlabelled Factor Va and Factor Xa. Specific binding of both ligands was determined by subtraction of nonspecific binding from total binding. The specific radioactivities of $^{125}$I-Factor Xa and $^{131}$I-Factor Va were 1700 cpm/ng and 171 cpm/ng, respectively.
The highest Factor Va concentration employed in these studies are representative of the time course of binding measurements made with the four other Factor Va concentrations. That is, the time course of binding for Factor Xa and Factor Va is independent of the Factor Va concentration under the conditions utilized. The only observed difference was that the amount of Factor Va and Factor Xa bound per platelet varied with the Factor Va concentration.

The Factor Xa and Factor Va binding data for each different time interval were examined as a function of Factor Va concentration. The data obtained after a 15-min incubation period are shown in Fig. 4 in which the molecules of Factor Xa bound per platelet (closed circles, solid lines) and molecules of Factor Va bound per platelet (closed circles, dashed line) are plotted versus the Factor Va concentration. As the Factor Va concentration increases, Factor Va binding and Factor Xa binding increase in a coincident manner, indicating that the binding of Factor Xa to platelets is directly related to the binding of Factor Va.

The data shown in Fig. 4 were then expressed as molecules of Factor Xa bound per platelet versus molecules of Factor Va bound per platelet (Fig. 5). The data are linear, and the slope of the line provides the relationship between Factor Xa and Factor Va bound to the platelet surface. We determined by linear regression analysis that the ratio of molecules of Factor Xa to molecules of Factor Va bound per platelet was 0.91. Thus, after a 15-min incubation, both ligands were present on the platelet surface in a 1:1 ratio, i.e., for each molecule of Factor Va bound, a molecule of Factor Xa was bound.

The binding data for each different time interval were then analyzed in the same manner, and the results are presented in Table I. At every time interval chosen, the ratio of molecules of Factor Xa bound to molecules of Factor Va bound is approximately 1. These data indicate that the binding of Factor Xa to platelets is dependent on the Factor Va concentration. Furthermore, when a saturating concentration of Factor Xa is employed, that is, a concentration high enough to saturate all available binding sites, Factor Xa and Factor Va are bound to the platelet in a 1:1 molar ratio.

The simultaneous binding of [125I]-Factor Xa and [131I]-Factor Va to unstimulated platelets was also studied as a function of the concentrations of both ligands. Five [125I]-Factor Xa concentrations were used: 0.7 nM, 1.4 nM, 2.2 nM, 3.0 nM, and 4.2 nM (40, 80, 120, 165, and 235 ng/ml of Factor Xa, respectively).

Table I

<table>
<thead>
<tr>
<th>Time of binding measurement</th>
<th>Molecules Factor Xa bound/molecules Factor Va bound</th>
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<tbody>
<tr>
<td>min</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.04</td>
</tr>
<tr>
<td>1</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>0.78</td>
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<tr>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>7</td>
<td>0.70</td>
</tr>
<tr>
<td>10</td>
<td>0.91</td>
</tr>
<tr>
<td>15</td>
<td>0.91</td>
</tr>
</tbody>
</table>

The binding of [125I]-Factor Xa at these concentrations was studied at eight [131I]-Factor Va concentrations which varied from 0.1 nM to 1.43 nM (33 ng/ml to 475 ng/ml). The final concentration of Factor Va present was sufficient to saturate the high affinity Factor Va binding sites previously observed (16). Factor Xa binding measurements were also made without added Factor Va. All binding measurements were made following a 15-min incubation. The reaction mixtures again contained 1.39 × 10⁻⁶ M prothrombin, 2.5 mM Ca²⁺, and 30 μM DAPA. The data obtained indicated that Factor Xa binding was dependent upon both the Factor Xa and Factor Va concentrations, with saturation being obtained when either ligand was limiting. When the binding data obtained at each Factor Xa concentration were analyzed in terms of molecules of Factor Xa bound per platelet versus molecules of Factor Va bound per platelet, the data were linear and are depicted in Fig. 6. The nine data points shown in each panel represent the amount of platelet-bound Factor Xa (vertical axis) and platelet-bound Factor Va (horizontal axis) for each of the concentrations of Factor Va added to the reaction mixtures ranging from 0 to 1.43 × 10⁻⁶ M. Added Factor Xa was held constant at the concentration shown in each panel. Examination of the data indicates that at a fixed Factor Xa concentration, the amount of Factor Xa bound to platelets increases.

Fig. 5. Determination of the ratio of molecules of Factor Xa bound per platelet to molecules Factor Va bound per platelet following a 15-min incubation as a function of Factor Va concentration. Linear regression analysis of the data produced a slope equal to 0.91 molecule of Factor Xa bound to a molecule of Factor Va bound per platelet.

Fig. 4. Simultaneous binding measurements of [125I]-Factor Xa and [131I]-Factor Va to unstimulated platelets as a function of Factor Va concentration. Binding measurements were made following a 15-min incubation. The [125I]-Factor Xa concentration was held constant at 4.5 × 10⁻⁹ M. •—••, Binding of Factor Xa; •—•, binding of Factor Va. Nonspecific binding corrections were made as described in Fig. 3. The specific radioactivities of the labeled proteins were also as in Fig. 3.
Factor Va-dependent Factor Xa Binding

Table II

Ratio of the molecules of Factor Xa and Factor Va bound to unstimulated platelets as a function of Factor Xa concentration

Measurements of \(^{125}\)I-Factor Xa and \(^{131}\)I-Factor Va binding to unstimulated platelets were made following a 15-min incubation of reaction mixtures containing varying amounts of both ligands. Five concentrations of Factor Xa were used: 0.7 nM, 1.4 nM, 2.2 nM, 3.0 nM, and 4.2 nM (40 ng/ml to 230 ng/ml). The binding of \(^{125}\)I-Factor Xa at these concentrations was determined as a function of eight \(^{131}\)I-Factor Va concentrations which varied from 0.1 nM to 1.43 nM (33 ng/ml to 475 ng/ml). The data were corrected for nonspecific binding as described in Fig. 3. The data shown represent the numerical values obtained for the slopes shown in Fig. 6.

<table>
<thead>
<tr>
<th>(10^6 \times [\text{Xa}])</th>
<th>Molecules of Factor Xa/molecules of Factor Va</th>
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<tbody>
<tr>
<td>A</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>2.2</td>
</tr>
<tr>
<td>D</td>
<td>3.0</td>
</tr>
<tr>
<td>E</td>
<td>4.2</td>
</tr>
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</table>

The data indicate that as the Factor Xa concentration is increased, the molar ratio of platelet-bound Factor Xa to platelet-bound Factor Va approaches 1. At lower, nonsaturating concentrations of Factor Xa, more Factor Va is bound to the platelet than Factor Xa. As the Factor Xa concentration is increased, more Factor Xa binding sites are filled and the ratio of bound Factor Xa to bound Factor Va increases. Finally, as was shown above, when Factor Xa was present at a high enough concentration to saturate all available platelet binding sites, then Factor Xa and Factor Va were platelet bound in a 1:1 molar ratio.

When the binding interaction of Factor Xa to platelets is modeled as Factor Xa binding to platelet-bound Factor Va, the process may be represented by an equation (the complete derivation is detailed under "Methods") which expresses a linear relationship between the molar ratio of platelet-bound Factor Xa to platelet-bound Factor Va under the conditions utilized. The slopes obtained from the data are listed in Table II. The data indicate that as the Factor Xa concentration is increased, the molar ratio of platelet-bound Factor Xa to platelet-bound Factor Va approaches 1. At lower, nonsaturating concentrations of Factor Xa, more Factor Va is bound to the platelet than Factor Xa. As the Factor Xa concentration is increased, more Factor Xa binding sites are filled and the ratio of bound Factor Xa to bound Factor Va increases. Finally, as was shown above, when Factor Xa was present at a high enough concentration to saturate all available platelet binding sites, then Factor Xa and Factor Va were platelet bound in a 1:1 molar ratio.

The double reciprocal plot of the data shown in Table II should be linear, with the slope of the line equal to the dissociation constant for Factor Xa binding to its platelet receptor and the vertical axis intercept equal to the binding stoichiometry of this interaction. The double reciprocal plot is shown in Fig. 7. The apparent dissociation constant obtained is \(6 \times 10^{-5}\)M with a binding stoichiometry of 1.04. These values are in excellent agreement with those previously reported by Nesheim et al. (9). Using a phospholipid vesicle model system, they obtained nearly identical dissociation constants for both Factor Xa and Factor Va binding interactions of \(7.1 \times 10^{-5}\)M and \(7.3 \times 10^{-5}\)M in which the amount of both ligands bound to platelets was determined from reaction mixtures containing a fixed amount of \(^{125}\)I-Factor Xa at the concentration shown and varying amounts of \(^{131}\)I-Factor Va (0.1 nM to 1.43 nM). Binding measurements were made following a 15-min incubation. The amount of Factor Xa bound per platelet (vertical axis) and Factor Va bound per platelet (horizontal axis) represents the eight different Factor Va concentrations used. Nonspecific binding corrections were made as described in Fig. 3. The specific radioactivities of \(^{125}\)I-Factor Xa and \(^{131}\)I-Factor Va were 575 cpm/ng and 260 cpm/ng, respectively.
contaminating traces of Factor V.

The binding of Factor Xa to platelets was modeled as Factor Xa binding to platelet-bound Factor Va and can be described by Equation 1 previously detailed under “Methods.” Accordingly, the ratio of molecules of platelet-bound Factor Va to platelet-bound Factor Xa is plotted as a function of nominal Factor concentration. The plot gave a dissociation constant of $6 \times 10^{-6}\text{M}$ and a binding stoichiometry of 1.04 molecules of platelet-bound Factor Va bound per molecule of bound Factor Xa.

$10^{-10}\text{M}$, respectively, and binding stoichiometries of 0.61 and 0.87 (mole/mole), respectively.

Kinetic Determinations of the Binding Interaction of Factor Va with Platelets—The binding data obtained by measuring the amount of radiolabeled Factor Va and Factor Xa bound to platelets permit calculation of the apparent dissociation constant and the stoichiometry of Factor Xa platelet binding, as detailed in this paper, and of Factor Va platelet binding, as detailed previously (16). Our earlier work indicated that Factor Va appeared to interact with two distinct platelet sites, with apparent dissociation constants of $4 \times 10^{-10}\text{M}$ and $3 \times 10^{-8}\text{M}$, respectively. There were approximately 900 to 900 high affinity sites and as many as 3500 lower affinity sites. The Factor Xa binding data presented in this paper strongly suggest that Factor Va is the platelet receptor for Factor Xa. Therefore, it is imperative to know whether the previously determined platelet binding sites for Factor Va are functional sites and, therefore, involved in the Factor Xa-catalyzed activation of prothrombin.

Experiments were performed in which the influence of Factor Va on the rate of prothrombin activation was taken to reflect Factor Va binding to a site on the platelet surface and thereby forming the receptor for Factor Xa. Prothrombin activation was monitored continually by fluorometry using the fluorescent compound DAPA as described by Nesheim et al. (9). Prothrombin activation mixtures contained $1.39 \times 10^{-6}\text{M}$ prothrombin, $2.5 \text{mM Ca}^{2+}$, $5 \times 10^{-6}\text{M}$ Factor Xa, $3 \times 10^{-6}\text{M}$ DAPA, $3.0 \times 10^{9}$ platelets/ml, and varying amounts of Factor Va. Therefore, saturating concentrations of Factor Xa, prothrombin, and calcium were held fixed and the Factor Va concentration varied from 0.17 nM to 2.85 nM (56 ng/ml to 940 ng/ml). When further addition of Factor Va produced no additional increase in the velocity of thrombin formation, it was presumed that the functional platelet sites were saturated with Factor Va. Velocities less than the saturation values were considered directly proportional to the extent of available sites filled. The ratio of observed velocity to the limit velocity allowed calculation of the fraction of sites bound and the fraction of sites free. A double reciprocal plot was constructed according to the equation detailed by Nesheim et al. (9), which expresses a linear relationship between the reciprocal of the fraction of sites free and the Factor Va concentration divided by the fraction of sites bound. The derivation of this equation is detailed under “Methods” (Equation 2). The assumption was made that a kinetically discernible Factor Va-Factor Xa interaction, independent of the platelets, was not occurring. The saturation curve of prothrombin activation in the presence of varying concentrations of Factor Va is shown in Fig. 8. The rate of thrombin generation is plotted versus the Factor Va concentration. The double reciprocal plot is shown as an inset in which the reciprocal of free Factor Va platelet sites is plotted versus the Factor Va concentration divided by the fraction of bound Factor Va platelet sites. The apparent dissociation constant calculated from the slope is equal to $1.6 \times 10^{-9}\text{M}$, and the number of functional Factor Va platelet binding sites calculated from the intercept is equal to 915. A repetitive experiment in which a different source of bovine platelets was employed gave nearly identical results, $K_d = 2.2 \times 10^{-9}\text{M}$, $n = 927$. These values are very similar to the values obtained previously by equilibrium binding studies of radiolabeled Factor Xa ($K_d = 4 \times 10^{-9}\text{M}$, $n = 800$ to 900) which represent the Factor Va exclusive high affinity sites.

For comparative purposes, the binding parameters of the prothrombinsase components obtained in this work and in previously published work are shown in Table III. These values which describe a functional prothrombinsase catalyst are all very similar. The similarity of these values which were obtained using either a phospholipid vesicle model system, unstimulated platelets, or thrombin-activated platelets is consistent with a platelet-Factor Xa-Factor Xa interaction in which Factor Va bound to platelets forms the receptor to which Factor Xa binds.

Antibovine Factor V IgG-Fab Inhibition of Factor Va and Factor Xa Platelet Binding and Factor Xa-catalyzed Prothrombin Activation—Monovalent IgG-Fab fragments of prothrombin in 22°C, 0.02 M tri-HCl, 0.15 M NaCl, 1.39 $\times 10^{-6}\text{M}$ prothrombin, $3 \times 10^{-6}\text{M}$ DAPA, 2.5 mM Ca$^{2+}$, and $3 \times 10^{9}$ platelets/ml, pH 7.4. Reaction mixtures containing varying concentrations of Factor Va were initiated with 5 $\times 10^{-6}\text{M}$ Factor Xa and initial velocities of thrombin formation were obtained from recorded data. Results are expressed as the amount of thrombin generated per minute as a function of nominal Factor Va concentration. The double reciprocal plot obtained from the saturation curve is shown as an inset. The reciprocal of the fraction of free Factor Va platelet sites is plotted as a function of the nominal Factor Va concentration divided by the fraction of bound Factor Va platelet sites. The calculated apparent dissociation constant ($K_d$) and number of Factor Va platelet binding sites were $1.5 \times 10^{-10}\text{M}$ and 915, respectively.

For these experiments, prothrombin and Factor Xa had been passed over Sepharose-bound anti-Factor V antibody to remove contaminating traces of Factor V.

![Figure 7](Image URL)  
**FIG. 7.** Double reciprocal plot of the binding of Factor Xa to unstimulated platelets derived from equilibrium binding measurements of radiolabeled Factor Va and Factor Xa to platelets. The binding of Factor Xa to platelets was modeled as Factor Xa binding to platelet-bound Factor Va and can be described by Equation 1 previously detailed under “Methods.” Accordingly, the ratio of molecules of platelet-bound Factor Va to platelet-bound Factor Xa is plotted as a function of nominal Factor concentration. The plot gave a dissociation constant of $6 \times 10^{-6}\text{M}$ and a binding stoichiometry of 1.04 molecules of platelet-bound Factor Va bound per molecule of bound Factor Xa.

![Figure 8](Image URL)  
**FIG. 8.** Factor Va platelet binding as monitored by the kinetics of prothrombin activation. Activation conditions: 22°C, 0.02 M tri-HCl, 0.15 M NaCl, 1.39 $\times 10^{-6}\text{M}$ prothrombin, $3 \times 10^{-6}\text{M}$ DAPA, 2.5 mM Ca$^{2+}$, and $3 \times 10^{9}$ platelets/ml, pH 7.4. Reaction mixtures containing varying concentrations of Factor Va were initiated with 5 $\times 10^{-6}\text{M}$ Factor Xa and initial velocities of thrombin formation were obtained from recorded data. Results are expressed as the amount of thrombin generated per minute as a function of nominal Factor Va concentration. The double reciprocal plot obtained from the saturation curve is shown as an inset. The reciprocal of the fraction of free Factor Va platelet sites is plotted as a function of the nominal Factor Va concentration divided by the fraction of bound Factor Va platelet sites. The calculated apparent dissociation constant ($K_d$) and number of Factor Va platelet binding sites were $1.5 \times 10^{-10}\text{M}$ and 915, respectively.
Factor Va-dependent Factor Xa Binding

Table III

<table>
<thead>
<tr>
<th>Methoda</th>
<th>Varied Component</th>
<th>Fixed Component</th>
<th>Apparent ( K_d )</th>
<th>Stoichiometryb</th>
<th>No. Factor Va binding sites per platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium(^1) binding</td>
<td>Va</td>
<td>Unstimulated platelets</td>
<td>4.0 ( \pm ) 2.1</td>
<td>837 ( \pm ) 48</td>
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<tr>
<td>Kinetics</td>
<td>Va</td>
<td>II, Xa, unstimulated platelets</td>
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<td>915</td>
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<td>Va</td>
<td>II, Xa, unstimulated platelets</td>
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<td>Equilibrium binding</td>
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<td>II, unstimulated platelets</td>
<td>8.0</td>
<td>1.04</td>
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<tr>
<td>Kinetics(^c)</td>
<td>Xa</td>
<td>Activated platelets</td>
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<tr>
<td>Kinetics(^e)</td>
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<td>II, PCPS, Va</td>
<td>7.1</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Method by which the apparent dissociation constant (\( K_d \)), stoichiometry, and number of Factor Va binding sites per platelet were determined.

\(^b\) Expressed as Xa/Va or Va/Xa.

\(^c\) Tracy et al. (16).

\(^d\) Dahlback and Stenflo (15).

\(^e\) Nesheim et al. (9).

Phosphatidylycholine-phosphatidylserine vesicles.

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**DISCUSSION**

Simultaneous binding measurements of radiolabeled Factor Va and Factor Xa to unstimulated platelets, as well as immunochromical and kinetic studies, have enabled us to show: 1) that the Factor Xa receptor sites involved in platelet-mediated prothrombin activation are present on the surface of unstimulated bovine platelets; 2) that Factor Va and Factor Xa interact with the platelet with a well defined stoichiometry (1 mol/mol) and high affinity; and 3) that the high affinity Factor Va platelet sites previously described by equilibrium binding experiments most likely represent Factor Xa binding sites which are involved in the function of the prothrombinase complex at the platelet surface. We have shown that Factor Xa platelet binding is concentration-dependent with respect to both Factor Va and Factor Xa and may be saturated when either ligand is limiting. The amount of Factor Xa bound to platelets increases in proportion to the amount of Factor Va bound to platelets, and the ratio of platelet-bound Factor Xa to platelet-bound Factor Va is unity when saturating concentrations of Factor Xa are employed. Monovalent Fab fragments prepared from burro antiovine Factor V antibody inhibit the coordinate Factor Va-Factor Xa platelet binding and prothrombin activation to the same degree. The Fab-mediated inhibition of Factor Va binding is correlated on a molar basis with the decrease in Factor Xa binding. These results strongly suggest that Factor Va serves as a ligand for Factor Xa.
as the binding site for Factor Xa on the surface of unstimulated platelets.

When the interaction of Factor Xa with platelets is modeled as Factor Xa binding to platelet-bound Factor Va, the apparent binding parameters obtained from equilibrium binding data indicate that Factor Xa and platelet-bound Factor Va interact with a stoichiometry of 1.04 (mole of Factor Va/mole of Factor Xa). A dissociation constant for this interaction of $6 \times 10^{-9}$ was obtained, and this value is in good agreement with the calculated $K_d$ for Factor Xa binding to bovine platelets as reported by Dahlbäck and Stenflo (15). Similar binding parameters have been reported by Nesheim et al. (9), who examined the Factor Va-Factor Xa interaction by studying the kinetics of prothrombin activation using defined phospholipid vesicles. The apparent $K_d$ obtained with the lipid model system was $7.5 \times 10^{-9}$, with a binding stoichiometry of 0.61 to 1.2 mole of Factor Xa bound/mole of Factor Va bound, adding additional support to the notion that Factor Va is the Factor Xa platelet receptor.

Kinetic determinations of the functional Factor Va platelet binding sites indicate that there are approximately 900 such sites with $K_d = 2 \times 10^{-9}$ M. This number is nearly identical with that which describes Factor Va exclusive, high-affinity binding sites derived from equilibrium binding measurements ($K_d = 4 \times 10^{-9}$, $n = 800$ to 900) and suggests that the high affinity Factor Va binding sites are the Factor Xa binding sites which are responsible for prothrombin activation at the platelet surface. Thus, all of the binding parameters describing the interaction of Factor Va and Factor Xa with platelets, either obtained by equilibrium binding measurements or inferred from kinetics, indicate that Factor Va and Factor Xa interact stoichiometrically (1:1) with an apparent $K_d$ of from $2 \times 10^{-9}$ to $6 \times 10^{-9}$ M. Factor Va provides the binding site for Factor Xa at the platelet surface with approximately 900 functional sites present per platelet.

We have been able to assess the interaction of Factor Va and Factor Xa with platelets by direct equilibrium binding measurements and by kinetic assessment of their functional platelet binding sites. It should be noted that a great many more Factor Va and Factor Xa molecules will bind per platelet than are functionally observed by prothrombin activation. As many as 2000 molecules of both ligands will bind per platelet; however, kinetic experiments suggest that platelets possess only 900 catalytically functional sites. The reduced number of functional sites from total observed binding sites may reflect the binding of prothrombin to the platelet-bound Factor Va-Factor Xa complex. With the phospholipid vesicle system, it has been shown that the partitioning of prothrombin to the catalytic surface is governed by its binding to lipid (9). In the platelet system, prothrombin must bind in the vicinity of the Factor Va-Factor Xa complex in order to serve as a substrate for Factor Xa. Therefore, the 900 functional Factor Va-Factor Xa sites may represent sites at which the necessary "lipo" is available to accommodate prothrombin binding.

The Factor Xa platelet binding characteristics reported here are consistent with those obtained by Dahlbäck and Stenflo (15), who characterized bovine Factor Xa binding to thrombin-activated platelets if the following points are considered. They reported approximately 300 Factor Xa binding sites per platelet, with a $K_d = 1.9 \times 10^{-9}$ M. We also found that when washed platelets are thrombin-activated, approximately 200 Factor Xa molecules are bound per platelet at saturating concentrations of added Factor Xa, indicating that platelet Factor Va is the limiting component. These results are not surprising, since we have previously reported that there are between 400 and 800 molecules of Factor V associated with a washed platelet (16). Therefore, under the conditions employed in both of these studies, the washed platelets do not provide enough Factor Va to saturate all of the available Factor Va platelet binding sites. Most likely, thrombin activation was required to activate Factor V to Factor Va, rather than to expose the putative, cryptic Factor Xa binding sites. The number of Factor Xa binding sites obtained may reflect simply the amount of Factor V associated with the washed platelets used by Dahlbäck and Stenflo (15).

Our results with the bovine system cannot be extrapolated to human Factor Xa platelet binding characteristics. However, the results obtained by other investigators with a heterologous human-bovine system appear phenomenologically similar. Initially, Miletich et al. (13) reported that thrombin-activated washed platelets possess 200 to 400 Factor Xa binding sites with a $K_d = 3 \times 10^{-11}$ M. Kane et al. (18) have shown that bovine Factor Va will promote the binding of human Factor Xa to human, unstimulated platelets, indicating that Factor Xa binding sites are on the surface of unstimulated platelets. The requirement for thrombin activation using washed human platelets most likely reflects the conversion of Factor V to Va rather than the exposure of the Factor Xa binding sites. However, little can still be said about the number of Factor Xa binding sites on human platelets, since saturating concentrations of Factor Xa were not employed in their studies.

Our results indicate that unactivated Factor V will promote the binding of Factor Xa to platelets, but at a reduced level from that observed for added Factor Va. The Factor Xa binding observed in the presence of added Factor V cannot be attributed to Factor Va contamination in the Factor V preparation or activation of the Factor V to Factor Va. A 20% Factor Va contamination could account for the results, but this was not the case. In addition, DAPA was present in excess such that less than 0.1% of any thrombin generated would have been available to activate the Factor V to Factor Va. As many as 900 molecules of Factor Xa will bind per platelet in the presence of Factor V at a concentration well below its physiological concentration. This observation is consistent with our previously published data which showed that Factor V will function as a cofactor in the Factor Xa-catalyzed activation of prothrombin, but only at 0.25% of the rate observed with Factor Va (9).

This postulated Factor V-Factor Xa platelet interaction may be of physiological significance, since one would predict that Factor V is always associated with unstimulated platelets at plasma concentrations of Factor V (16, 37). At a site of vascular injury, thrombin may be produced with Factor V, rather than Factor Va, serving initially as the cofactor. Factor V already associated with the platelet membrane would then be thrombin-activated to Factor Va, resulting in: 1) its binding more tightly to the platelet surface (16); and 2) its ability to greatly enhance the rate of Factor Xa-catalyzed prothrombin activation at the site of injury.

The binding of Factor V and Factor Va at the platelet surface may explain the mechanical fixation and localization of the blood coagulation process at a site of vascular injury. Factor Xa would be fixed at the site of platelet adhesion and aggregation by virtue of its interaction with platelet-bound Factor V and/or Factor Va.

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APPENDIX

Derivation of an Expression for the Interaction of Factor Xa with Platelets

If the interaction of factor Xa with the plasma surface is described as Factor Xa binding to platelet-bound Factor Va (PVa), the following equation can be applied:

\[ \text{Factor Xa} + \text{PVa} \rightarrow \text{Complex} \]

The dissociation constant for this process can be expressed as in equation 2.

\[ K_D = \frac{[\text{Factor Xa}][\text{PVa}]}{[\text{Complex}]} \]

or,

\[ K_D = \frac{[\text{Factor Xa}]}{[\text{PVa}][\text{factor Xa bound}]} \]

where \( f \) and \( b \) are equal to the fraction of free and bound factor Xa in the system. PVa, the concentration of platelet-bound Factor Va, is not measured; however, factor Xa can be expressed in terms of total Factor Xa bound to platelets, PVa, and normal factor Xa concentration, [FA].

This relationship is shown in equation 3.

\[ [\text{Factor Xa}][\text{PVa}] = [\text{Factor Xa}][FA] \]

In which \( a \) represents the binding stoichiometry of factor Xa to Factor Va. Substitution of equation 4 into equation 3 gives

\[ \frac{[\text{Factor Xa}]}{[FA]} = \frac{[\text{PVa}]}{[FA]} \]

or, upon rearrangement

\[ [\text{Factor Xa}] = \frac{[FA]}{a} \]

The term 1/2f was approximately 1.0 at all times since experimental conditions were chosen such that the fraction of free factor Xa varied from 0.20 to 0.57. Under our experimental conditions, equation 7 can be replaced, without significant error, by equation 8 in which 1/2f = 1.

\[ [\text{Factor Xa}] = \frac{[FA]}{a} \]

Equation 7 expresses a linear relationship between the reciprocals of normal concentration of Factor Xa and the ratio of total Factor Xa and factor Xa bound to platelets. The slope is equal to the apparent dissociation constant for the binding of factor Xa to its platelet receptor, factor Va, and the intercept with the ordinate is equal to the stoichiometry of binding.