Functional Assembly of Intrinsic Coagulation Proteases on Monocytes and Platelets. Comparison Between Cofactor Activities Induced by Thrombin and Factor Xa

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Summary

Generation of coagulation factor Xa by the intrinsic pathway protease complex is essential for normal activation of the coagulation cascade in vivo. Monocytes and platelets provide membrane sites for assembly of components of this protease complex, factors IXa and VIII. Under biologically relevant conditions, expression of functional activity by this complex is associated with activation of factor VIII to VIIIa. In the present studies, autocatalytic regulatory pathways operating on monocyte and platelet membranes were investigated by comparing the cofactor function of thrombin-activated factor VIII to that of factor Xa-activated factor VIII. Reciprocal functional titrations with purified human factor VIII and factor IXa were performed at fixed concentrations of human monocytes, CaCl₂, factor X, and either factor IXa or factor VIII. Factor VIII was preactivated with either thrombin or factor Xa, and reactions were initiated by addition of factor X. Rates of factor X activation were measured using chromogenic substrate specific for factor Xa. The K₅₀ values, i.e., concentration of factor VIII at which rates were half maximal, were 0.96 nM with thrombin-activated factor VIII and 1.1 nM with factor Xa-activated factor VIII. These values are close to factor VIII concentration in plasma. The V₅₀, i.e., rates at saturating concentrations of factor VIII, were 33.3 and 13.6 nM factor Xa/min, respectively. The K₅₀ and V₅₀ values obtained in titrations with factor IXa were not significantly different from those obtained with factor VIII. In titrations with factor X, the values of Michaelis-Menten coefficients (Kₘ) were 31.7 nM with thrombin-activated factor VIII, and 14.2 nM with factor Xa-activated factor VIII. Maximal rates were 23.4 and 4.9 nM factor Xa/min, respectively. The apparent catalytic efficiency was similar with either form of factor VIII. Kinetic profiles obtained with platelets as a source of membrane were comparable to those obtained with monocytes. These kinetic profiles are consistent with a 1:1 stoichiometry for the functional interaction between cofactor and enzyme on the surface of monocytes and platelets. Taken together, these results indicate that autocatalytic pathways connecting the extrinsic, intrinsic, and common coagulation pathways can operate efficiently on the monocyte membrane.

Activation of the coagulation cascade is mediated by membrane-dependent protease complexes historically grouped into three different pathways: extrinsic, intrinsic, and common. The monocyte/macrophage, a central element in the inflammatory response, has been shown to provide appropriate membrane sites for efficient assembly of coagulation proteases. Kinetic and functional characteristics of monocyte-mediated reactions of the extrinsic and common coagulation pathways have been examined in detail before (1–7). In contrast, little information is available on kinetic characteristics of intrinsic pathway reactions mediated by monocytes. These reactions include autocatalytic loops that connect the extrinsic and common pathways and that, potentially, regulate fibrin deposition during inflammatory, neoplastic, atherosclerotic, and degenerative processes. The monocyte is the only blood cell type capable of promoting activation of both extrinsic and common pathways at biologically relevant rates. Therefore, to better understand the role of monocytes in coagulation, it is important to determine whether the autocatalytic coagulation loops can operate efficiently at the level of the monocyte membrane.

Activation of coagulation factor X via the intrinsic pathway

Parts of this study have been published in abstract form. (1990. FASEB (Fed. Am. Soc. Exp. Biol.) J. 4:2278a.)
Materials and Methods

Cell Isolation and Characterization. Human mononuclear cells were purified from peripheral blood using Ficoll-Hypaque as previously described (19). Mononuclear cells were further enriched for monocytes by centrifugation through hypotonic Percoll (48-50% Percoll in 1% NaCl), layered under isosmotic culture medium (M199 supplemented with 0.5% heat-inactivated lactalbumin hydrolysate, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were washed at least twice with 0.85% NaCl containing 0.1% EDTA, and at least once more with culture medium. Extensive washing was necessary to completely deplete the preparation of platelets. Purity of the cell preparation was determined by morphologic examination of cytospin preparations either stained with Wright's stain or processed histochemically for nonspecific esterase using α-naphthyl acetate as substrate (20). The cell preparations contained 85-95% monocytes, 5-15% lymphocytes, and ≈2% PMN.

Platelets separated during monocyte purification were processed like the monocyte-rich population and used for comparison in kinetic studies. Platelets were also isolated in a resting state by gel filtration (21, 22). For kinetic measurements, these platelets were fully activated with 1 μM ionophore A23187 for 5 min at 37°C as described before (23). At this concentration, A23187 was shown to induce maximal procoagulant activity in human platelets in the absence of cell lysis, as indicated by lack of lactate dehydrogenase release (23).

The platelet content of the monocyte population was monitored routinely by careful morphologic examination under light microscopy. In addition, representative preparations were processed for electron microscopy and immunostained with mAbs specific for platelet Ib/IIa glycoprotein (21).

Immunostaining and Electron Microscopy. Purified monocyte preparations were assayed ultrastructurally for whole platelet and platelet membrane fragment contamination by immunoblot cytochemistry. For these studies, cells were fixed in suspension with 2.5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.35), for 10-15 min and pelleted at 1,000 g for 2 min. The monocytes were then rinsed in Gey's buffer (Gibco Laboratories, Grand Island, NY) (pH 7.3), followed by three rinses in Gey's containing 0.1% glycine to quench free aldehydes and reduce nonspecific antibody binding. After rinsing once more with glycine-free Gey's buffer, 50 μl of murine monoclonal anti-GP IIb/IIIa antibody P2 (AMAC Inc., Westbrook, ME) was added to the monocyte at a final IgG concentration of 10 μg/ml. This antibody is specific for platelet membranes and shows no cross-reactivity with monocytes (21, and this report). Rabbit polyclonal antihuman lactoferrin antibodies (Organon-Teknika Corp., West Chester, PA) were used at a (total protein concentration of 100 μg/ml) as a control for nonspecific primary antibody binding. After a 45-min incubation at 22°C with the primary antibodies, cell pellets were rinsed three times in Gey's buffer, and 50-100 μl of a mixture of species-specific secondary immunogold probes (Janssen Life Science, Olen, Belgium) was incubated with the pellets for 30 min at 22°C. This secondary gold probe mixture consisted of 15 nM goat anti-mouse IgG and 5 nM goat anti-rabbit IgG (as an internal immunogold control) mixed 1:1 (vol/vol) and diluted 1:2 in Gey's buffer. The pellets were subsequently rinsed three times in Gey's buffer and once in 0.1 M cacodylate, and postfixed in 1% OsO4/0.1 M cacodylate for 20 min at 22°C.

Monocyte pellets were stained with uranyl acetate and lead citrate and observed on a transmission electron microscope (TEM)1 (model 400; Philips, Mahwah, NJ) at magnifications of 9,000-25,000. Platelet suspensions obtained during monocyte purification were also processed for TEM without subjecting the cells to immunostaining procedures.

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1 Abbreviation used in this paper: TEM, transmission electron microscope.
Coagulation Factors. Purified human factor VIII with sp act <3,000 U/mg of protein was obtained from Armour Pharmaceutical Company (Kankakee, IL). Purity and electrophoretic characteristics of this protein have been documented before (24, 25). 1 U of factor VIII is equivalent to factor VIII activity present in 1 ml of normal human plasma. Purified human factors X and IXa with sp act of 125–200 and 200 U/mg of protein, respectively, were obtained from Enzyme Research Laboratories Inc. (South Bend, IN). Factors X and IXa (factor IX activated with factor XIa) were electrophoretically and functionally pure as determined by SDS polyacrylamide electrophoresis and in coagulation tests. Factor Xa was generated from factor X using Russell's viper venom as previously described (26, 27). Concentrations of factor Xa and thrombin used for factor VIII activation were standardized routinely using specific chromogenic substrates S-2222 (N-Benzoyl-L-isoleucyl-L-glutamyl-L-arginine-p-nitroanilide hydrochloride) and S-2238 (H-D-Phenylalanyl-L-propanolyl-L-arginine-p-nitroanilide hydrochloride), respectively (Kabi Diagnostics, Helena Laboratories, Beaumont, TX). A rate of 0.05 absorbance U/min was obtained with 0.035 U/ml of thrombin. A rate of 0.006 absorbance U/min was obtained with 1 nM of factor Xa. Thrombin (2,720 National Institutes of Health (NIH) U/mg) was a generous gift from Dr. J. Fenton, The Albany Medical College of Union University, Albany, NY. For calculation of molar concentrations of factor VIII used in titration experiments 1 U/ml of this factor was equated to 1 nM (17).

Measurement of Factor X Activation Rates on Monocytes. Initial rates of factor X activation were measured using incubation mixtures containing cells, 2.8 × 10^6/ml; CaCl$_2$, 5 mM; and purified human factors IXa, VIII, and X at the concentrations indicated for each type of experiment. Factor VIII was fully converted to the cofactor form, factor VIIIa, by including either thrombin or factor Xa in the mixtures. Reagents were in Tris buffer 0.15 N NaCl pH 7.3 and reactions were initiated with the substrate factor X. After addition of substrate, 40-μl samples were taken every 30 sec for 3.5 min. Concentrations of factor Xa in each sample were determined with specific factor Xa chromogenic substrate, at 0.3 mg/ml in Tris buffer, 0.25 N NaCl pH 8.3 (27). Initial rates of chromogenic substrate hydrolysis were followed at 405 nM using a Vimax kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA).

Initial rates were calculated by linear regression of factor Xa concentration over time using graphic methods and the computer program Enzfitter (Elsevier-Biosoft, Cambridge, UK). Rates of factor Xa generation over time were also calculated by fitting the experimental progression curves to polynomial equations such as $[Xa]_t = a+bt+ct^2$, where $[Xa]_t$ is the concentration of Xa formed at time $t$. By differentiating the resulting equations, $d(Xa)/dt = b+2ct$, the initial rate is equivalent to the linear coefficient. Fitting of polynomial equations was done using the computer program Stat-View 512+ (Brain Power, Inc., Calabasas, CA). For functional titrations, factor VIII, factor IXa, or factor X concentrations were varied, while other components were kept constant. Initial rates were plotted against concentrations of either factor VIII, IXa, or X, and rectangular hyperbolas were fitted to the data using the computer program Enzfitter (Elsevier-Biosoft). For monocytes and centrifuged platelets, standard errors of kinetic parameters obtained in each fitting were 1–15% of the parameter value. Except where indicated, all experiments used to determine kinetic parameters were repeated at least three times.

For interpretation of these rate measurements, it was assumed that components of the factor X activating complex (calcium, factors IXa and VIIIa, and membrane) were at equilibrium, and that addition of substrate did not affect this equilibrium. This assumption is justified by results of experiments, described below, designed to determine optimal initial conditions. In these experiments prolonging preincubation periods before initiating reactions with substrate did not result in significant changes of subsequent initial rates.

Interpretation of initial rate measurements was done within the hypothetical framework of a one-enzyme, one-substrate, reaction, the basic mechanism of which can be represented as:

$$(IXa/VIIIa) + X \xrightarrow{k_1} (IXa/VIIIa).X \xrightarrow{k_2} Xa,$$

where, (IXa/VIIIa) is the membrane bound enzyme. This complex is formed by specific interactions between factor IXa and factor VIIIa on membrane phospholipids, in the presence of calcium ions. The expression (IXa/VIIIa).X represents the membrane-bound enzyme-substrate complex. In this scheme, binding of factor X to (IXa/VIIIa) is reversible, and $k_1$ and $k_2$ are the rate constants for the association and dissociation reactions. The rate constant of product formation from the substrate-enzyme complex is $k_2$. The membrane lipids, calcium and factor VIIIa, are considered to be essential activators. That is, the enzymatic component factor IXa does not exhibit biologically relevant activity unless it is bound to membranes in the presence of factor VIIIa and calcium (8). This was the case for the range of concentrations and observation intervals of the present experiments. During steady state, i.e., the period in which overall rates of product formation are linear, the concentration of (IXa/VIIIa).X is assumed to be constant, and the catalytic efficiency ($k_{cat}/K_m$) of the reaction is $k_2/(k_1+k_2)$.

Previous studies by others, using platelets (28), have established that bound factor IXa is a small fraction of the total amount in mixtures. Consequently, at the cell and reagent concentrations used in these studies, the substrate in the bulk phase was always at a great molar excess over concentration of bound functional factor IXa/VIIIa complexes. The term functional interaction is used to indicate reactions on the membrane that result in detectable rates of factor X activation under the specified conditions. Thus, possible molecular interactions between factors IXa, VIII, and X in the bulk phase are ignored since only a negligible amount of product is produced in the absence of cells.

Standardization of Initial Conditions. The reaction of interest in these studies was the activation of factor X by factor IXa/VIIIa complexes on monocytes. Therefore, it was important to demonstrate that the concentration of functional activating complexes remained constant for the duration of the rate measurements. Also, since one of the objectives of these studies was to functionally compare thrombin-activated factor VIII (factor VIIIa$_0$) to factor Xa-activated factor VIII (factor VIIIa$_{Xa}$), these preliminary experiments were designed to insure that activity of both forms of the cofactor was maximal during measurements. Additionally, since extensive proteolytic cleavages of factor VIII by both activators have been shown to be associated with loss of procoagulant activity (15), it was necessary to find the lowest concentration of activator, and the shortest activation times resulting in complete activation of the cofactor.

To determine the time required to achieve maximal activation of factor VIII, this factor at 1 U/ml was preincubated with cells, 2.8 × 10^6/ml; CaCl$_2$, 5 mM; factor IXa, 0.75 nM, and either 0.8 nM of factor Xa or 0.05 U/ml (0.45 nM) thrombin. Preincubation time was varied from 0–9 min in reaction mixtures containing factor Xa, and from 0–4 min in mixtures containing thrombin. After preincubation periods, reactions were initiated by addition of 140 nM of factor X, and the concentration of factor
Xa was determined in sequential samples with S-2222 chromogenic substrate, as indicated above. The length of presteady-state or lag time was estimated by extrapolation of the steady-state regression line to the abscissa in plots of factor Xa concentration versus time. Steady-state rates were determined from the slope of straight lines fitted to experimental points manually, and by using a computer program for regression analysis as indicated above.

Lag times were inversely correlated with the length of the preincubation period, and were much shorter for thrombin than for factor Xa. After these lags, rate of factor X activation was linear. Prolonging preincubation periods up to 9 min in mixtures with Xa, and up to 3 min in mixtures with thrombin did not change initial rates significantly. Thus, after 0.5 min of incubation with thrombin and 4 min with factor Xa, factor VIII activation was maximal and remained constant for at least 3 and 9 min, respectively. In all subsequent kinetic experiments, sampling of reaction mixtures was kept within these time limits.

The effect of either factor Xa or thrombin concentration on lag times was determined using reaction mixtures constructed as above, but with concentrations of factor Xa ranging from 0.2 to 1.6 nM, and concentrations of thrombin ranging from 0.05 to 0.65 U/ml (0.45-5.8 nM). Preincubation intervals were kept at 4 and 0.5 min, respectively. The length of the lag periods was inversely correlated with the concentration of activating enzyme. At physiologic concentrations of factor VIII, 1 U/ml, no lags were detected at any of the thrombin concentrations tested. Lags were also abolished with concentrations of factor Xa >0.3 nM. After lag periods, initial rates of factor X activation were the same regardless of the concentration of either factor Xa or thrombin used to preactivate factor VIII. Postlag linear rates of factor X activation in mixtures with 1 and 0.2 U/ml of factor VIIIa(0) were 25 and 8 nM factor Xa/min, respectively. In mixtures with factor VIIIa(0), rates were 6.6 and 2.3 nM factor Xa/min. Results of these factor VIII activation experiments in the presence of monocytes are very similar to results with platelets reported in detail by other investigators (17).

**Protein and Lipid Measurements.** Protein determinations were performed with reagent (Biorad Laboratories, Richmond, CA), using BSA as standard. Cell lipid extraction and lipid phosphorus determinations were performed as described before (29, 30). Mean lipid phosphorus content was 6.6 ± 0.57, and 5.1 ± 0.9 nmol for five paired samples with 10^6 monocytes and 10^7 platelets, respectively. Mean protein content was 29.04 ± 3.7 µg, and 7.7 ± 1.3 µg, respectively.

![Figure 1. Differential staining of human monocytes and platelets with anti-IIb/IIIa specific mAbs. Bound antibodies were detected with gold-labeled secondary antibodies (see Materials and Methods). Arrows point to a platelet that is positively stained. In contrast, monocyte membranes show no label. The proportion of whole, positively-stained platelets was 19/200, i.e., 9.5%. Picture also shows (arrow, top left) a typical example of the positively-stained membrane fragments, found associated with some of the cells. These fragments are ~0.18 µm in diameter, and have a surface area estimated to be <0.02% of the surface area of a typical monocyte of 15 µm diameter. Magnification = 18,900.](image-url)
Results

Determination of Monocyte Population Purity by Immunostaining and Electron Microscope Examination. The platelet membrane is known to express sites for very efficient activation of factor X by factor IXa/VIIIa (8). Since platelets are frequently coisolated with monocytes when using standard cell isolation procedures, it was essential to exclude significant contamination with either whole platelet or platelet membrane fragments of the monocyte preparation. This was achieved using immunogold staining with specific anti-G-PIIb/IIIa mAbs and electron microscope examination as explained in Materials and Methods. Fig. 1 illustrates the specificity of the immunogold staining for the platelet membrane. The monocytes were also examined for positively stained membrane fragments. 120 of 200 randomly-selected monocyte profiles, (60%) were negative for any associated positively-staining membrane, while the remaining 80 (40%) were associated with one or occasionally two fragments that measured ≈0.18 μm in diameter. From these measurements, we estimated that the surface of each one of these particles corresponds to <0.02% of the surface area of a typical monocyte of 15 μm diameter.

Figure 2. Functional titration of factors VIIIa and IXa on monocytes. Initial rates of factor X activation were measured as indicated in Materials and Methods and Table 1. Data are mean from three titration experiments with either factor VIIIa (○), or factor IXa (●).

The proportion of whole positively-stained platelets was 19/200, i.e., 9.5%, confirming routine observations with light microscopy.

Platelets separated from the mononuclear cells by differen-

Table 1. Factor X Activation by Factor IXa/VIIIa on Monocytes: Functional Titration with Factors VIIIa and IXa

| Factor VIII activator | Titration with factor VIIIa | Titration with factor IXa |
|----------------------|-----------------------------|--------------------------|
|                      | K1/2 (nM)                   | Vsat (FXa/min)            | K1/2 (nM) | Vsat (FXa/min) |
| Factor Xa            | 1.10 ± 0.14                 | 13.6 ± 2.1               | 1.02 ± 0.16 | 12.7 ± 1.3   |
| Thrombin             | 0.96 ± 0.18                 | 33.3 ± 1.9               | 0.64 ± 0.14 | 36.8 ± 3.6   |

* Initial rates of factor X activation were measured in reaction mixtures with fixed concentration of cells 2.8 × 10⁶/ml, CaCl₂, 5 mM, factor IXa, 0.75 nM, factor X, 140 nM, and factor VIII concentrations ranging from 4 to 0.031 U/ml (1 U/ml = 1 nM).

† Factor IXa was titrated in reaction mixtures prepared as above, except that factor VIII concentration was kept at 1 U/ml, and factor IXa concentration was varied from 0.12 to 15 nM.

‡ Factor VIII was activated with either factor Xa, 0.8 nM, or thrombin, 0.05 U/ml, as indicated in Materials and Methods.

§ Parameters K1/2 (concentration of either factor VIIIa or IXa at which initial rates were half of maximum) and Vsat (rates at saturating concentration of either factor VIIIa or IXa) were calculated by fitting data to rectangular hyperbolae using a computer program. Data are mean ± SE of three experiments, each with cells from a different donor.

Table 2. Factor X Activation by Factor IXa/VIIIa on Platelets Isolated by Centrifugation: Functional Titration with Factors VIIIa and IXa

| Factor VIII activator | Titration with factor VIIIa | Titration with factor IXa |
|----------------------|-----------------------------|--------------------------|
|                      | K1/2 (nM)                   | Vsat (FXa/min)            | K1/2 (nM) | Vsat (FXa/min) |
| Factor Xa            | 1.10 ± 0.15                 | 14.8 ± 8.6               | 0.70 ± 0.09 | 14.4 ± 1.2   |
| Thrombin             | 1.07 ± 0.26                 | 30.3 ± 4.0               | 0.79 ± 0.09 | 32.1 ± 2.4   |

* Initial rate measurements and calculation of parameter values were as indicated in Table 1. Platelet concentration was 28 × 10⁶/ml. Data are mean ± SE of three experiments, each done with platelets from a different donor.
tional centrifugation were also examined under TEM before the immunostaining procedure. By morphologic criteria, these platelets appeared partially activated as evidenced by characteristic shape changes and degranulation.

Factor X Activation by Factor IXa/VIIIa on Monocytes. Functional Titration with Factors VIIIa and IXa. To characterize the relationship between factor IXa and VIIIa concentration and initial rates of factor X activation, reaction mixtures were titrated with factor IXa and VIIIa. Factor X activation rates were measured at fixed concentrations of cells, $2.8 \times 10^6$/ml; CaCl$_2$, 5 mM; either factor IXa or factor VIII, and factor X, 140 nM. For titrations with factor IXa, concentrations were from 0.12 to 1 nM, and factor VIII was kept at 1 U/ml. For titrations with factor VIII, concentrations were from 0.03 to 4 nM, and factor IXa was kept at 0.75 nM. Factor VIII was activated optimally with either 0.8 nM factor Xa, or 0.05 U/ml (0.45 nM) thrombin added 4 and 0.5 min, respectively, before initiating reactions with factor X. Enzymatic mixtures with factor VIIIa(t) or factor VIIIa(xa) were prepared and assayed simultaneously to minimize experimental variability.

Rates of factor X activation increased with factor IXa and VIIIa concentration and approached a maximum asymptotically (Fig. 2). Apparent activation parameters are summarized in Table 1.

Factor X Activation by Factor IXa/VIIIa on Platelets. Functional Titrations with Factors VIIIa and IXa. Initial rate measurements and calculation of kinetic parameters were also performed in platelets. Reagent concentrations and experimental conditions were as indicated for monocytes; the platelet concentration in these experiments was 28 x 10$^6$/ml. Results in Tables 2 and 3 show that kinetic parameters on platelets, activated by either centrifugal force or by A23187, are similar to those obtained with monocytes.

The induction of factor IXa/VIIIa assembly sites on platelets by A23187 was investigated in cells from six different donors. In these experiments, concentrations were gel-filtered platelets, $28 \times 10^6$/ml; factor IXa, 0.8 nM; factor VIIIa, 1 nM, and factor X, 70 nM. Initial rates were measured in paired reaction mixtures preincubated for 5 min at 37°C in either the presence or absence of 1 μM A23187. Initial rates were 10.3 ± 4.2 and 0.65 ± 0.2 nM factor Xa/min on stimulated and resting platelets, respectively. Measured under identical conditions, the rates on platelets activated by centrifugation were 11.1 ± 2.6 nM factor Xa/min. Thus, although centrifuged platelets are partially activated by morphologic criteria, they appear to be fully activated with respect to procoagulant activity.

Kinetic Parameters of Factor X Activation on Monocytes and Platelets. Comparison between IXa/VIIIa(xa) and IXa/VIIIa(t) Complexes. The results from titrations with enzyme components indicate higher Vmax with factor VIIIa(xa) than with factor VIIIa(t), but similar K1/2 values. This suggested that the catalytic rate of enzymatic complexes in mixtures with factor VIIIa(xa) is higher than in mixtures with factor VIIIa(t).

![Figure 3. Factor X activation by factor IXa/VIIIa assembled on monocytes. Comparison between factor Xa-activated and thrombin-activated factor VIII. Initial rates of factor X activation were measured in reaction mixtures constructed as indicated in Materials and Methods. Factor VIII concentration was fixed at 1 U/ml, and factor X concentration was varied from 1.1 to 140 nM. Factor VIII was activated with either factor Xa (O) or thrombin ( ●). Data are from one of eight experiments, each with cells from a different donor. Mean and standard errors for these experiments are in Table 4.](image)

### Table 3. Factor X Activation by Factor IXa on Gel-filtered Platelets Activated with Ionophore: Functional Titration with Factors VIIIa, IXa, and X

| Factor VIII activator | Titration with factor VIIIa | Titration with factor IXa | Titration with factor X |
|-----------------------|-----------------------------|---------------------------|-------------------------|
|                       | K1/2 (VFXa/min)             | K1/2 (VFXa/min)           | Km (VFXa/min)           |
| Factor Xa             | 0.56                        | 18                        | 0.87                    | 23                      | 27                      | 22                      |
| Thrombin              | 0.76                        | 39                        | 0.43                    | 30                      | 64                      | 38                      |

Platelets were isolated by gel filtration and activated with 1 μM ionophore A23187. Concentrations, initial rate measurements, and kinetic parameters, determination were as indicated in Tables 1 and 2. Data are means of two experiments, each with platelets from a different donor.

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It was therefore interesting to compare the rate-concentration relationship of factor X activation with either factor VIIIa(t) or factor VIIIa(xa)-containing complexes. For these experiments, concentrations of factors VIII and IXa were fixed at 1 U/ml, and 0.75 nM, respectively. Factor X concentration was varied from 1.1 to 140 nM. Concentrations of cells and calcium were as indicated above. As in titration experiments with factors IXa and VIII, mixtures with either VIIIa(t) or VIIIa(xa) were prepared and assayed simultaneously. Initial rates were linear and increased with factor X concentration approaching asymptotically to a maximum (Fig. 3). Apparent kinetic parameters $K_m'$ and $V_{max}'$ were estimated by fitting data to the Michaelis-Menten equation using Enzfitter (Elsevier-Biosoft). The value of $K_m'$ was $14.2 \pm 3.0$ nM in mixtures with factor VIIIa(xa), and $31.7 \pm 5.6$ nM in mixtures with factor VIIIa(t). The corresponding values were $V_{max}'$ of $4.9 \pm 0.8$ and $23.4 \pm 4.1$. Differences in both $K_m'$ and $V_{max}'$ among each activator were consistent in eight experiments, each with cells isolated from different donors. Results with platelets were similar (Tables 2, 3, and 4).

**Discussion**

In the present studies we examined kinetics of factor X activation by components of the intrinsic coagulation pathway assembled on the membrane of blood monocytes and platelets. Functional titrations with highly purified human factors VIII and IXa were performed using reaction mixtures containing fixed concentrations of monocytes and of calcium and physiologic concentrations of factor X. Under these conditions, the concentration of factors VIII and IXa, giving half-maximal rates of activation, was similar to plasma concentration of factor VIII, and close to the recently reported dissociation constant for factor IXa interaction with platelet membranes (8). The $K_{1/2}$ and $V_{sat}$ values obtained in reciprocal titrations of factors IXa and VIIIa were equivalent within experimental error. These results are consistent with hypothetical assembly mechanisms proposed by other investigators (10), and suggest a 1:1 molecular ratio for the functional interaction between factors IXa and VIII on the platelet membrane.

The $K_{1/2}$ values of reactions with thrombin-activated factor VIII were similar to the $K_{1/2}$ of reactions with factor Xa-activated factor VIII. In contrast, $V_{sat}$ values and maximal rates of activation in factor X titration experiments were two- to fourfold higher in mixtures with factor VIIIa(t) than in mixtures with VIIIa(xa). Considering that titrations were performed under identical experimental conditions, these results also indicate that the $k_2$, i.e., $k_{cat}$, of factor IXa/VIIIa(t) complexes is two- to fourfold higher than that of factor IXa/VIIIa(xa) complexes. Since values of apparent $K_m$ were two- to threefold lower with factors IXa/VIIIa(xa) than with factors IXa/VIIIa(t), it follows that the apparent catalytic efficiencies ($k_{cat}/K_m$) of enzymatic complexes with either form of the cofactor are similar.

Higher activity of factor VIIIa(t) as compared with VIIIa(xa) in systems using artificial lipid and platelets has been reported by several laboratories (15–17). In these previous studies, comparative functional titrations with either form of the cofactor were not made, and, therefore, it was not determined whether the higher activity observed with VIIIa(t) resulted from differences in the $k_{cat}$ or from differences in the efficiency of functional interaction between factor VIII and IXa (17). Our results with monocytes and platelets indicate a difference in the $k_{cat}$ and in apparent $K_m$ for the two forms of the enzyme. In contrast, the efficiency of interaction with factor IXa is similar for both forms of factor VIII. Thus, with substrate fixed at nearly saturating concentration, the rate of factor Xa generation (normalized as a fraction of maximal rate) was the same for equivalent concentrations of each cofactor species.

The ability of monocytes to promote functional assembly of factor IXa/VIIIa does not appear to depend on monocyte...

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**Table 4. Kinetic Parameters of Factor X Activation on Monocytes and Platelets Isolated by Centrifugation: Comparison between IXa/VIIIa(t) and IXa/VIIIa(xa) Complexes**

| Factor VIII activator | Monocytes | Platelets |
|-----------------------|-----------|-----------|
|                       | $K_m'$    | $V_{max}'$ | $K_m'$    | $V_{max}'$ |
| Factor Xa             | 14.2 ± 3.0| 4.9 ± 0.8  | 17.9 ± 0.09| 13.4 ± 1.7 |
| Thrombin              | 31.7 ± 5.8| 23.4 ± 4.1 | 45.15 ± 7.6| 32.8 ± 6.9 |

*p = 0.004 (8)*

$K_m'$ and $V_{max}'$ (apparent $K_m$ and $V_{max}$) were calculated by fitting Michaelis-Menten equation to data using the computer program Enzfitter.

* Initial rates of factor X activation were measured in reaction mixtures prepared as indicated in Materials and Methods and Tables 1 and 2. Factor X was included at concentrations ranging from 1.1 to 140 nM. Monocytes were at 2.8 × 10⁶/ml, and platelets at 28 × 10⁶/ml.

The $K_{1/2}$ values of reactions with thrombin-activated factor VIII were similar to the $K_{1/2}$ of reactions with factor Xa-activated factor VIII. In contrast, $V_{sat}$ values and maximal rates of activation in factor X titration experiments were two- to fourfold higher in mixtures with factor VIIIa(t) than in mixtures with VIIIa(xa). Considering that titrations were performed under identical experimental conditions, these results also indicate that the $k_2$, i.e., $k_{cat}$, of factor IXa/VIIIa(t) complexes is two- to fourfold higher than that of factor IXa/VIIIa(xa) complexes. Since values of apparent $K_m$ were two- to threefold lower with factors IXa/VIIIa(xa) than with factors IXa/VIIIa(t), it follows that the apparent catalytic efficiencies ($k_{cat}/K_m$) of enzymatic complexes with either form of the cofactor are similar.

Higher activity of factor VIIIa(t) as compared with VIIIa(xa) in systems using artificial lipid and platelets has been reported by several laboratories (15–17). In these previous studies, comparative functional titrations with either form of the cofactor were not made, and, therefore, it was not determined whether the higher activity observed with VIIIa(t) resulted from differences in the $k_{cat}$ or from differences in the efficiency of functional interaction between factor VIII and IXa (17). Our results with monocytes and platelets indicate a difference in the $k_{cat}$ and in apparent $K_m$ for the two forms of the enzyme. In contrast, the efficiency of interaction with factor IXa is similar for both forms of factor VIII. Thus, with substrate fixed at nearly saturating concentration, the rate of factor Xa generation (normalized as a fraction of maximal rate) was the same for equivalent concentrations of each cofactor species.

The ability of monocytes to promote functional assembly of factor IXa/VIIIa does not appear to depend on monocyte...
activation or differentiation. Neither the isolation procedures nor exposure to reaction components resulted in detectable morphologic changes within the time interval of kinetic measurements reported here. Further, rates of factor X activation on freshly isolated monocytes were similar to those on monocytes stimulated in vitro with endotoxin (18).

The differential centrifugation procedures used in the present studies resulted in activation of platelets. This was evidenced by their morphologic appearance under TEM, and by the fact that no further activation was required for expression of functional factor IXa/VIIIa assembly sites. In contrast, platelets isolated by gel filtration did not promote functional assembly sites for the factor IXa/VIIIa complex (18). Thus it is possible that isolation procedures, i.e., differential centrifugation and density gradients, also affect the procoagulant activity of monocytes.

Subtle changes in the membrane i.e., exposure of phosphatidylserine (31), may occur without detectable morphologic manifestations. However, it is important to note that differential centrifugation through density gradients does not result in exposure of assembly sites on all blood cell types. Lymphocytes subjected to the same isolation procedures as were the monocytes did not promote functional assembly of factors IXa/VIIIa (18).

Monocytes and platelets are the only cells derived from bone marrow under physiologic conditions that are capable of promoting fast activation of factor X via intrinsic pathway components (18). Because of their abundance, platelets are likely to provide membrane sites for factor X activation during intravascular coagulation. However, at sites undergoing inflammatory, degenerative, and neoplastic disease, the monocyte is more prevalent than the platelet. Monocytes and macrophages express tissue factor during maturation in vivo and in vitro (1–5) and, like the platelet, support assembly of the prothrombinase reaction (6, 7). We now show that the efficiency of the functional interaction between factors IXa and either factor VIIIa(t) or VIIIa(xa) on the monocyte is similar to the efficiency of this reaction on platelets. Therefore, these results further indicate that monocytes can be important sources of membrane sites for functional assembly of the intrinsic pathway protease in the extravascular spaces. The demonstration of efficient activation of factor X by complexes with either thrombin or factor Xa-activated factor VIII also indicates that autocatalytic regulatory pathways connecting the extrinsic, intrinsic, and common coagulation pathways can operate on the monocyte surface.

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