Membrane-dependent coagulation reaction is independent of the concentration of phospholipid-bound substrate: Fluid phase factor X regulates the extrinsic system

(ligand competition/tissue factor/enzyme kinetics/ligand–receptor association/prothrombin fragment 1)

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ABSTRACT   Negatively charged phospholipids accelerate blood coagulation; one suggestion is that the binding of the γ-carboxyglutamic acid-containing zymogens to these lipid surfaces increases their effective concentration as substrates. Alternatively, the charged phospholipids could enhance the direct interaction of substrate with the catalytic complex, which is localized at the membrane surface. We distinguished the alternatives by using prothrombin fragment 1 to compete with the substrate for membrane binding sites without interfering with the direct enzyme–substrate interaction. In a tissue factor–factor VIIa system containing neutral phospholipids (to which the substrate does not bind), prothrombin fragment 1 had no significant effect on factor X activation (K_m, 877 ± 111 nM and 791 ± 103 nM, with and without prothrombin fragment 1, respectively). In contrast, in a system containing 30% phosphatidylserine, prothrombin fragment 1 displaced phospholipid-bound factor X, increasing the free factor X concentration and the reaction velocity in all 22 determinations. As the velocity increases correlated only with the free factor X concentration, we conclude that the free factor X concentration controls this reaction velocity. The K_m in the 30% phosphatidylserine system, calculated using free factor X concentration, was 41 ± 5 nM and 63 ± 9 nM, with and without prothrombin fragment 1, respectively. Thus, the negatively charged lipids decreased the intrinsic K_m by over 90%. The methodology employed should be applicable to ligand–receptor systems in which ligand binds nonspecifically to the membrane surface.

The specific binding of ligands to receptor molecules embedded in cell membranes mediates many biological functions. In coagulation the binding of zymogens (factors IX, X, and prothrombin) to enzymatic complexes localized on membranes regulates the rate of several reactions (for review, see ref. 1). Analysis of these reactions is complicated because the zymogens also bind nonspecifically to membrane surfaces, thus introducing uncertainty about the effective substrate concentration. The substrates can reach the enzymatic complexes either by bulk phase diffusion or by binding to the membrane surface and then "sliding" in the plane of the membrane. Although these mechanisms are not mutually exclusive, the dominant process will govern the substrate–enzyme association rate. While the theory of both processes has been extensively developed (2, 3), there have been few attempts to distinguish experimentally which mechanism regulates specific systems.

We have studied the activation of bovine coagulation factor X by tissue factor–factor VIIa complex. This complex is analogous to a receptor in several respects. Tissue factor is an integral membrane protein that requires insertion into

phospholipid vesicles for biological activity (4, 5). The formation of a 1:1 complex of the enzyme factor VIIa with tissue factor is essential for the activation of factor X (6). Factor X, upon binding to the enzyme–activator ("receptor") complex, is hydrolytically converted into factor Xa and an activation peptide. Because factor X binds to negatively charged phospholipids (7, 8), two pools of substrate exist: one free in solution and one membrane bound. To interpret kinetic data, one must know the concentration of substrate in the pool being hydrolyzed. If bulk phase diffusion dominates, then the concentration of free factor X will control the association rate of factor X with enzyme. If surface diffusion dominates, then the concentration of membrane-bound factor X will govern the rate of association with enzyme. Thus, the rate of factor X activation should directly depend on the concentration of one of the two factor X pools. These concentrations, however, are not independent; the concentration of free factor X determines the concentration of surface-bound factor X. In this study, we uncoupled the two pools by adding a reagent, prothrombin fragment 1, which competes with factor X for membrane binding sites but which we show does not interfere with the specific association of factor X with enzyme. Because prothrombin fragment 1 simultaneously decreases the concentration of surface-bound factor X and increases the concentration of free factor X, we were able to correlate reaction velocity with differing concentrations of each pool. We show that free factor X dominates the rate of factor X activation. Thus, the binding of substrate to membrane does not enhance substrate–enzyme association, and free factor X is the true substrate in the tissue-factor pathway of coagulation.

MATERIALS AND METHODS

Reagents. DEAE-Sephadex, Sepharose CL-2B, and Sephadex G-100 are products of Pharmacia. Affi-Gel 10 is the product of Bio-Rad. Phosphatidylcholine (PtdCho) from egg and phosphatidylserine (PtdSer) from bovine brain were obtained from Supelco (Bellefonte, PA). Dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone and n-octyl β-D-glucopyranoside were obtained from Calbiochem-Behring; Hepes was from Research Organics (Cleveland, OH). All other reagents were of reagent grade or better and were obtained from standard sources.

Buffers. Tris: 50 mM Trizma base, 100 mM NaCl, adjusted to pH 7.5 with HCl. Hepes: 10 mM Hepes, 150 mM NaCl, adjusted to pH 7.4 with NaOH. Where indicated, bovine serum albumin at 1 mg/ml was added.

Proteins. Protein concentrations were determined from absorbance at 280 nm using values for $A^{1\%}_{1\text{ cm}}$ of 12.9, 10.1, 9.6,

Abbreviations: PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; Gla, γ-carboxy-glutamic acid.
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and 14.4 for bovine factor VII (9), prothrombin fragment 1 (10), factor X (11), and prothrombin (10), respectively. Proteins were stored at −80°C.

Coagulation factors VII, X (12), and prothrombin were prepared from a barium citrate eluate of bovine plasma by chromatography on DEAE-Sephadex (13). Factor VII, which was purified to homogeneity by chromatography on an anti-factor VII immunoaffinity column (14), was converted to its more active two-chain derivative, factor VIIa, by factor Xa (13).

Prothrombin fragment 1 was prepared from prothrombin by incubating 200 mg of prothrombin with 250 units of bovine thrombin (Calbiochem-Behring; 2000 units/mg) in 125 ml of Tris buffer at 37°C for 40 hr. The reaction was stopped by adding diisopropylfluorophosphate to 1.5 mM. After 30 min, solid sodium citrate was added to 40 mM. The solution was cooled to 4°C and 20 ml of 1 M barium chloride was added dropwise. After 15 min, the mixture was centrifuged for 40 min at 17,000 × g at 4°C. The pellet was washed twice with 50 ml of 5 mM barium chloride and then dissolved in a minimum amount of 0.2 M EDTA, pH 7.5. After dialysis vs. 2 liters of Tris buffer, trace amounts of factor VII were removed by passage over a 50-ml anti-factor VII immunoaffinity column. The sample was concentrated to <5 ml on an Amicon PM10 membrane and then gel filtered on a Sephadex G-100 column (2.5 × 100 cm) using 500 ml of Hepes buffer. The final material was homogeneous by polyacrylamide gel electrophoresis (15) in NaDodSO₄ under nonreducing conditions; after reduction, two bands of similar molecular weight were present indicating probable loss of a small terminal peptide (16).

Purified bovine tissue factor (17) was reconstituted with PtdSer/PtdCho or PtdCho using n-octyl β-D-glucopyranoside (18) to solubilize the lipid and protein. The final tissue factor/phospholipid molar ratio was 1:100,000. Identically prepared vesicles (without tissue factor) were added to obtain the final phospholipid concentrations. Tissue factor is randomly oriented when inserted into phospholipid vesicles (19), thus tissue factor concentrations used for all calculations are 0.5 × [total tissue factor].
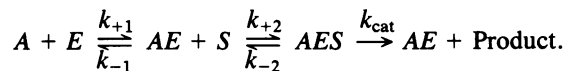
Radioactive Labeling. The carbohydrate moieties of factor X were reductively labeled with sodium boro[³H]hydride (New England Nuclear-Dupont; 5–15 Ci/mmol; 1 Ci = 37 GBq) using the method of Van Lenten and Ashwell (20), as described (21).

Phospholipids. Vesicles were either pure PtdCho or a mixture of PtdSer/PtdCho (30:70, wt/wt) and were prepared by mixing PtdSer and PtdCho in chloroform and drying under nitrogen. Solid n-octyl β-D-glucopyranoside at a weight ratio of 5.6:1 was added along with a tracer quantity of [¹⁴C]PtdCho (New England Nuclear-Dupont). The mixture was dissolved in 1 ml of Hepes buffer and dialyzed vs. three changes of 500 ml of the same buffer at 24°C for 72–96 hr. The vesicles were gel filtered on a 1.5 × 55 cm column of Sepharose CL-2B and eluted with Hepes buffer. The peak fractions, which were located by measuring light scattering at 280 nm, were pooled; the phospholipid concentration was determined from the ¹⁴C content.

Kinetic Assays. The rate of hydrolysis of factor X by tissue factor–factor VIIa complex was measured by a radioassay as described (21). Briefly, the radiolabeled activation peptide was extracted at appropriate intervals into 5% (wt/vol) trichloroacetic acid. The concentration of the peptide was determined by liquid scintillation spectroscopy. Each velocity determination consisted of seven time points counted in duplicate. Reaction mixtures contained 0.025 nM tissue factor, 500 μM phospholipid (either PtdCho or PtdSer/Ptd-Cho), 50 nM factor VIIa and various amounts of [³H]factor X. Pairs of samples with and without 6 μM prothrombin fragment 1 were assayed.

All components except factor VIIa were mixed together in Hepes/albumin buffer in the presence of 5 mM CaCl₂ and incubated for 30 min at 25°C, and the reaction was started by addition of factor VIIa. To prevent hydrolysis of factor X by factor Xa, 2 μM dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone was added. To test for the existence of an effect of prothrombin fragment 1 on the kinetics of factor X activation, analysis of the reaction pairs was performed using the Wilcoxon paired-sample test (22).

The hydrolysis of factor X (S) by tissue factor (A) and factor VIIa (E) is an essential activation reaction (6):

$$A + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AE + S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} AES \overset{k_{cat}}{\rightarrow} AE + \text{Product.}$$

When the concentration of enzyme is much greater than that of tissue factor and k_{-1}/k_{+1} (i.e., when the activator is saturated), we can model this system with an equation of the Michaelis–Menten form (23): velocity = $k_{cat} \times A_T \times S/(K2 + S)$, where $K2 = (k_{-2} + k_{cat})/k_{+2}$. We used 50 nM enzyme [K1 is 4.5 nM (19)] and have determined that increasing this concentration does not affect reaction velocities (data not shown). Kinetic parameters, k_{cat} and K_m, were obtained by fitting the above equation to the appropriate data using Marquardt's nonlinear least squares algorithm (24). The results are shown ± the standard error.

Binding Studies. Mixtures (0.1 ml in Hepes/albumin buffer) containing 0.025 nM tissue factor, 500 μM phospholipid, 2 μM dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone, 5 mM CaCl₂, and various amounts of [³H]factor X added alone or with prothrombin fragment 1 (6 μM) were incubated in cellulose propionate tubes for 30 min at 25°C. Then, the samples were centrifuged at 150,000 × g at 25°C for 20 min in a Beckman Airfuge. The phospholipid vesicles sediment accompanied by bound protein. Free factor X was assayed by removing a 25-μl aliquot from the supernatant and measuring its radioactivity. Total factor X was determined from identically prepared samples (buffer replacing phospholipid) that were not centrifuged. Bound is the difference between total and free.

Because vesicle sedimentation is never 100% complete, some of the bound factor X remains in the free fractions. We used the ¹⁴C contained in all our vesicle preparations to quantify sedimentation. Total phospholipid radioactivity was determined from triplicate, uncentrifuged samples (prepared without [³H]factor X). The fractional recovery, FR, was determined from the ¹⁴C content as follows:

$$FR = 1 - (\text{cpm in supernatant/total cpm}).$$

Typical fractional recoveries were >0.9. The following expressions were used to calculate corrected [factor X]_bound, [X_B], and [factor X]_free, [X_F], from the measured concentrations of bound and free factor X, [X_{B,m}] and [X_{F,m}]. The measured total factor X, [X_T], requires no correction.

$$[X_B] = [X_{B,m}]/FR.$$

$$[X_F] = [X_T] - [X_B].$$

The equilibrium dissociation constant, K_d, and the maximum binding capacity, n, were determined by fitting

$$[X_B] = n[\text{phospholipid}][X_F]/(K_d + [X_F])$$

to the corrected data using Marquardt's nonlinear least squares algorithm (24). The results are shown ± the standard error.

Control experiments to validate the binding method showed that equilibrium was established within the first 10 min of the incubation period. The binding of [³H]factor X was

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reversible, being completely abolished by addition of large excess of unlabeled ligand. Finally, in the absence of phospholipid, the proteins are not sedimented (data not shown).

RESULTS

Factor X Binding in the Presence and Absence of Prothrombin Fragment 1. Fig. 1 shows the binding of factor X to PtdSer/PtdCho vesicles as a function of free factor X. Comparing samples containing equivalent total factor X concentrations, one notes that addition of prothrombin fragment 1 decreases the concentration of bound factor X at all values of total factor X measured.

K_d and n for factor X binding were 0.52 ± 0.007 μM and 8.91 ± 0.12 μmol of factor X bound/mmol of PtdSer/PtdCho, respectively. The maximal binding capacity agrees quite well with reported values (8, 25–27). Reported K_ds range from 0.04 μM (26) to 2.5 μM (25); our determination is in excellent agreement with the majority of these values. The addition of 6 μM prothrombin fragment 1 resulted in the following apparent parameters for factor X binding: apparent K_d 1.43 ± 0.21 μM and n apparent 7.32 ± 0.29 μmol of factor X bound/mmol of PtdSer/PtdCho. These results are consistent with the hypothesis that prothrombin fragment 1 and factor X compete for phospholipid binding sites (i.e., a large effect on apparent binding affinity with minor effects on the maximal binding). Neither factor X nor prothrombin fragment 1 binds to pure PtdCho vesicles (data not shown).

Kinetic Effects upon Addition of Prothrombin Fragment 1. Paired reaction mixtures with and without prothrombin fragment 1 were assembled containing various total factor X concentrations, with all other constituents being constant. Fig. 2 shows the ratio of the reaction velocities in each pair (velocity with prothrombin fragment 1 divided by the velocity without prothrombin fragment 1) plotted vs. the total factor X concentration of each pair.

Using tissue factor reconstituted with PtdCho, we found that prothrombin fragment 1 had no significant effect on the rate of factor X activation. In 5 of 13 pairs the reaction velocity decreased with prothrombin fragment 1 (Wilcoxon paired-sample $T = 24$, $0.1 < P < 0.2$). The k_{cat} and K_m for factor X activation in this system are shown in Table 1. In accord with the Wilcoxon analysis, the parameters with and without prothrombin fragment 1 are essentially the same.
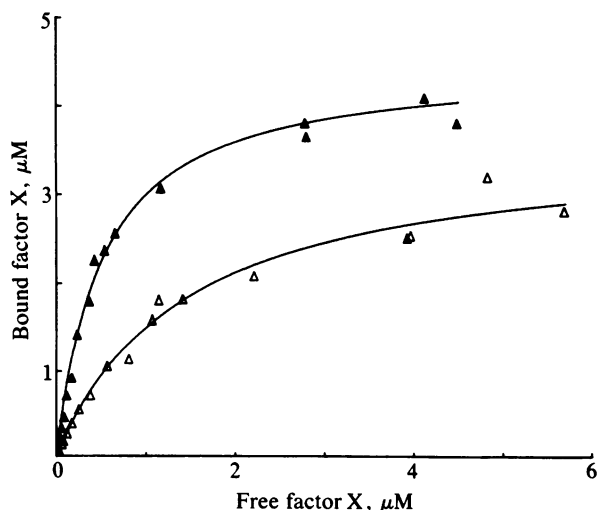

FIG. 1. Effect of addition of prothrombin fragment 1 on the binding of factor X to PtdSer/PtdCho vesicles. Upper fitted curve is for data in the absence of prothrombin fragment 1. Lower fitted curve is for data in the presence of 6 μM prothrombin fragment 1. ▲, Factor X alone; △, factor X and prothrombin fragment 1.
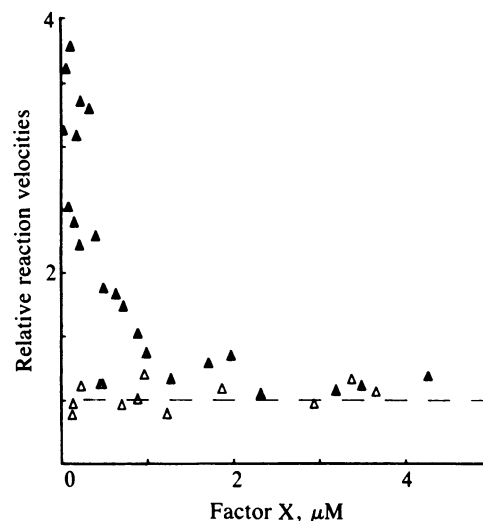

FIG. 2. Effect of prothrombin fragment 1 on factor X activation. Data from paired reactions are plotted as relative reaction velocities (determined in the presence of 6 μM prothrombin fragment 1 divided by that in the absence of prothrombin fragment 1) versus the total factor X concentration contained in each pair. The dashed horizontal line indicates a ratio of 1 (no effect of prothrombin fragment 1). △, Tissue factor is reconstituted with PtdCho. ▲, The PtdSer/PtdCho system.

Note that the kinetic parameters are expressed only in terms of free factor X concentration because factor X does not bind to the neutral PtdCho vesicles.

In contrast, when tissue factor reconstituted with PtdSer/PtdCho was used, the addition of prothrombin fragment 1 caused a velocity increase in all 22 pairs (Wilcoxon paired-sample $T = 0$, $P < 0.0001$).

Table 1 shows the kinetic parameters K_m and k_{cat} for the PtdSer/PtdCho system, calculated on the basis of either free or phospholipid-bound factor X concentrations. The parameters calculated using the free factor X concentration are essentially unaffected by prothrombin fragment 1.

DISCUSSION

We show a clear relationship between the concentration of factor X free in solution and reaction velocity in a system consisting of tissue factor, factor VIIa, factor X, phospholipid, and calcium ions. To make this determination, we "uncoupled" the distribution of substrate (factor X) between phospholipid-bound and free pools by using prothrombin fragment 1 to perturb factor X binding to lipid without affecting the kinetic apparatus, per se. Any kinetic effects of prothrombin fragment 1 thus rest solely upon changes in phospholipid-bound and free substrate concentrations. The advantage of this approach is that phospholipid concentration and composition are kept constant, thereby avoiding potential ambiguities of phospholipid-induced changes on the kinetic parameters.

We established that prothrombin fragment 1 has no effect on the kinetic apparatus by adding prothrombin fragment 1 to a tissue factor system containing PtdCho vesicles to which neither factor X nor prothrombin fragment 1 binds. Under these conditions, in which total substrate is equivalent to free substrate and there is no phospholipid-bound substrate to displace, prothrombin fragment 1 does not perturb the reaction velocity (Table 1). Therefore, we conclude that prothrombin fragment 1 has no effect on the fundamental kinetics of this reaction.

We next showed (Fig. 1) that prothrombin fragment 1 decreases the binding of factor X to the negatively charged

Table 1.   Kinetic parameters calculated using the concentration of bound and free factor X

Phospholipid	Fragment 1	Bound factor X		Free factor X	
		K_m, nM	k_{cat}, s^{-1}	K_m, nM	k_{cat}, s^{-1}
PtdSer/PtdCho	−	625 ± 103	6.6 ± 0.4	63 ± 9	5.8 ± 0.3
	+	108 ± 14	6.5 ± 0.2	41 ± 5	6.3 ± 0.2
PtdCho	−	NA	NA	791 ± 103	6.2 ± 0.3
	+	NA	NA	877 ± 111	6.7 ± 0.3

For each reaction velocity determination, bound and free factor X were calculated from:

$$[X_B] = \frac{1}{2}((R + K_d + [X_T]) - \{(R + K_d + [X_T])^2 - 4R[X_T]\}^{1/2})$$

and $[X_F] = [X_T] - [X_B]$, where R is n[PtdSer/PtdCho]. Fragment 1 is prothrombin fragment 1. The concentration of PtdSer/PtdCho was 0.5 mM. Because there is no binding to PtdCho vesicles, kinetic parameters were calculated only in terms of free substrate. NA, not applicable.

membrane surface in a tissue factor system reconstituted with PtdSer/PtdCho. Presumably, factor X binding decreases because prothrombin fragment 1 competes with factor X for sites on the vesicle surface. Protein–protein dimerization, another possible mechanism for affecting binding, is unlikely to be significant at the protein concentrations used (28). The phospholipid concentration, 0.5 mM, corresponds to 4.5 μM sites for factor X binding (total phospholipid × n). At this concentration of sites (nine times the K_d), most of the factor X is bound at the lower factor X concentrations. Any significant displacement of factor X from the phospholipid, therefore, causes a large increase in the free factor X concentration. The kinetic experiments, performed under the same conditions, are thus optimized to detect changes attributable to the free substrate pool.

From the binding data (Fig. 1) it is clear that prothrombin fragment 1 decreased the phospholipid-bound factor X and increased the free factor X. In each kinetic pair the velocity increased with prothrombin fragment 1; indeed, at low substrate concentrations the velocity rose 3- to 4-fold (Fig. 2). This observation is qualitatively consistent with free factor X controlling the rate of the reaction. To quantify the effects in terms of conventional kinetic parameters, we calculated the phospholipid-bound and free factor X concentrations for each substrate concentration (see Table 1) and determined the kinetic parameters using these concentrations. The K_m and k_{cat} determined using the free factor X concentration were unaffected by addition of prothrombin fragment 1. This quantitative agreement is further evidence that the free factor X concentration controls the reaction velocity and is consistent with the observation that prothrombin fragment 1 does not perturb the "intrinsic" K_m. Accordingly, the decrease in the K_m for the bound factor X using PtdSer/PtdCho is a direct consequence of the fact that free factor X regulates the reaction; prothrombin fragment 1 increases the concentration of free factor X at the expense of the bound fraction. Hence, upon the addition of prothrombin fragment 1 the K_m for the bound factor X will fall because of this relationship. We, therefore, conclude that the concentration of factor X in free solution controls the reaction rate in the tissue factor pathway.

We note, however, that the K_m derived using the concentration of free substrate in the PtdSer/PtdCho system is still considerably lower than that obtained using tissue factor reconstituted with PtdCho (Table 1). This means, in effect, that acidic phospholipids actually alter the intrinsic K_m of the reaction and that the acceleration observed in the presence of PtdSer is due to a change in the fundamental kinetic parameters rather than increased substrate concentration at the vesicle surface. This contrasts with the suggestion that rate accelerations are produced by localizing and concentrating substrates at the phospholipid surface (29–31).

The identity of the true substrate pool in other coagulation reactions remains controversial (30, 32–34) partially because potential phospholipid-dependent effects on the intrinsic K_m

have been ignored. Pusey and Nelsestuen (32) and van Rijn et al. (34) have concluded that free substrate controls the rate of prothrombin hydrolysis in the complete prothrombinase complex. These conclusions were based primarily on experiments in which substrate binding was perturbed by changing phospholipid composition or concentration and then relating these changes to effects on the apparent K_m. On the other hand, Nesheim et al. (30) in their computer model of prothrombin activation consider phospholipid-bound substrate to be the major contributor to hydrolysis. The assumptions of this model are that neither acidic phospholipids nor the enzyme activator factor Va induce a change in the intrinsic K_m.

It is generally accepted that γ-carboxyglutamic acid (Gla) residues are responsible for the binding of coagulation zymogens to negatively charged membranes (35, 36). If the binding of factor X to negatively charged membranes does not influence the activation rate (as shown above), what purpose do the Gla residues serve? The factor X coagulant protein from Russell's viper venom and factor VIIa cleave the same site on factor X (37, 38). Activation of factor X by factor X coagulant protein from Russell's viper venom requires calcium ions, but occurs in the absence of phospholipid; in this fully soluble system, intact factor X is activated much faster than factor X from which the Gla-domain has been removed (39). Thus, independent of any phospholipid binding capacity, the Gla domain is essential for the substrate factor X to be activated. Further, the Gla domain on factor VII is required for binding to tissue factor (A. Guha and Y.N., unpublished observation). Accordingly, there is no a priori reason to invoke phospholipid binding by substrate to explain Ca^{2+}- and Gla-mediated acceleration of blood coagulation. We speculate that a Gla-mediated, Ca^{2+}-induced conformational change (40) may render the cleavage site on factor X more accessible to the factor X coagulant protein from Russell's viper venom or factor VIIa.

The reaction of factor X with the tissue factor–factor VIIa complex is similar in many respects to the interaction of ligand with membrane-localized receptor molecules. In most membrane-associated receptor systems, the total surface area covered by receptor is a small fraction of the total membrane surface area (41). More likely than not a diffusing ligand will first collide with a nonreceptor site on the membrane. Because particles in solution move by a random walk, a ligand, having once collided with the membrane surface, has a high probability of repeat collisions (42) and more opportunities to encounter receptor. Note that the repeat collisions occur without any binding interaction between membrane and ligand and the movement of ligand is governed at all times by fluid phase diffusion parameters.

To use reaction velocity as an index of association rates, the tissue factor pathway must be diffusion controlled. The k_{cat}/intrinsic K_m measured in our system in the presence of negatively charged phospholipids is approximately 1 × 10^8 M^{-1}s^{-1}, which is close to theoretical maximal values for

bimolecular association rates (3). Thus, the tissue factor pathway appears to be a good model for ligand–receptor associations. In this regard, Wiegel and DeLisi (43) have published a theoretical treatment concluding that ligand–receptor association rates are not greatly enhanced by nonspecific binding of ligand to the membrane surface with subsequent surface diffusion. Berg (44) indicates that enhancements are possible under certain conditions. Our results support the assertion of Wiegel and DeLisi (43), and our methodology provides an experimental framework for testing other ligand–receptor systems.

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