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Ca²⁺ Switches the Effect of PS-containing Membranes on Factor Xa from Activating to Inhibiting: Implications for Initiation of Blood Coagulation

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Abstract

Calcium (Ca²⁺) plays a pivotal role in cellular and organismal physiology. The Ca²⁺ ion has an intermediate protein-binding affinity, thus it can serve as an on/off switch in regulation of different biochemical processes. The serum level of ionized Ca²⁺ is regulated with normal ionized Ca²⁺ being in the range from 1.10 to 1.29 mM. Hypocalcaemia (free Ca²⁺ < 1.1mM) in critically ill patients is commonly accompanied by hemostatic abnormalities, ranging from isolated thrombocytopenia to complex defects such as disseminated intravascular coagulation, commonly thought to be due to insufficient functioning of anticoagulation pathways. A small amount of Factor Xa (fXa) produced by Factor VIIa and exposed tissue factor is key to initiating blood coagulation by producing enough thrombin to induce later stages of coagulation. FXa must bind to phosphatidylserine (PS)-containing membranes to produce thrombin at a physiologically significant rate. In this work, we show that overall fXa activity on PS-containing membranes is sharply regulated by a “Ca²⁺ switch” centered at 1.16 mM, below which fXa is active and above which fXa forms inactive dimers on PS-exposing membranes. Our data lead to a mathematical model that predicts the variation of fXa activity as a function of both calcium and membrane concentrations. Because the critical Ca²⁺ concentration is at the lower end of the normal plasma ionized Ca²⁺ concentration range, we propose a new regulatory mechanism by which local Ca²⁺ concentration switches fXa from an intrinsically active form to a form requiring its cofactor (fVa) to achieve significant activity.

Keywords

Factor Xa; Phosphatidylserine; Thrombin; Prothrombin; Calcium; Factor VIIa

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1. Introduction

Due to its unique coordination chemistry, the Ca^{2+} ion has an intermediate binding affinity for many proteins. This property makes Ca^{2+} an ideal on/off switch that plays a pivotal role in many essential cellular processes. Calcium has only one oxidation state, and it is nontoxic even at high concentrations. In plasma, roughly 45 percent of total Ca^{2+} exists as physiologically active free ions, historically referred to as “ionized calcium” in clinical chemistry (1). The serum level of ionized Ca^{2+} is closely regulated with a normal ionized calcium concentration in the range of 1.1–1.3 mM (4.5–5.6 mg/dL) (2,3). Plasma Ca^{2+} levels are critical in several clinical situations. Low plasma ionized Ca^{2+} concentration (hypocalcaemia) in medical, surgical, and trauma patients ranges from 60% to 85% (4). The mean value of ionized calcium concentration in a heterogeneous cohort of “critically ill” patients was 1.11 mM (5). Hemostatic abnormalities, ranging from isolated thrombocytopenia to complex defects, such as disseminated intravascular coagulation (DIC), are common in critically ill patients (6). DIC is proposed to result from insufficient physiologic anticoagulation pathways, although the exact etiology is not defined (7). In addition, increased concentrations of phosphatidylserine (PS)-containing membranes were also found to be correlated with enhanced coagulation in different patients, for example circulating cell-derived microparticles may enhance coagulation activation in sickle cell disease (8). A direct consequence of PS expression on endothelial micro particles (EMPs) results from the ability of PS to bind and activate coagulation factors, conferring a procoagulant potential to EMPs (8). In addition to PS exposure, EMPs harbor tissue factor (TF), the initiator of the extrinsic coagulation pathway. It was demonstrated previously that fXa is produced exclusively by TF-fVIIa complex (extrinsic pathway) in the initiation of coagulation (9). EMPs are detectable in normal human blood and are increased in patients with coagulation abnormalities (10). Elevated plasma levels of EMPs with exposed phosphatidylserine (PS) and tissue factor have a procoagulant potential and have been reported to be present in thrombosis (11). Elevated EMPs were also found within occluded coronary arteries (12). These observations have suggested a link between ionized Ca^{2+} and the availability of PS-exposing microparticles in regulating blood coagulation; however, the biochemical nature of this link is as yet undefined. Based on extensive in vitro analysis, we suggest that the link occurs early in the blood coagulation process, during initiation, when the local Ca^{2+} concentration precisely controls the activity of fXa on a PS-containing membrane as a result of Ca^{2+} -triggered fXa dimer formation. We suggest that this regulation by Ca^{2+} is a previously unrecognized anti-coagulant mechanism whose malfunction might explain many of the clinical observations referenced herein.

While binding to PS-containing membranes has long been known to enhance fXa activity in the presence of Ca^{2+} (13,14), the ability of PS-containing membranes to inhibit fXa activity was only recently noted and shown to reflect formation of fXa dimers on PS-containing membranes at 4 mM Ca^{2+} (15). The fXa dimer is 10^6 - to 10^7 -fold less active in prothrombin activation than monomeric fXa (16). The opposite was true at 1 mM Ca^{2+} where PS-containing membranes promoted fXa activity (15). This finding presents two questions: first, does fXa dimerization help regulate fXa activity just after its production by TF-fVIIa complex and before cofactor, factor Va (fVa), appears later in the coagulation process; and,

second, how might Ca^{2+} and PS-containing membranes contribute to this regulation. To answer these questions, we must resolve the behavior of a multi-variant system (three principle components are variable: fXa, Ca^{2+} , and PS-containing membranes) whose equilibria cannot be described by a simple closed-form expression. We describe the system in terms of a series of linked equations related by mass preservation conditions to answer these questions. This problem was treated using MatLab as documented elsewhere for a limited number of Ca^{2+} and membrane concentrations (15). To expand this treatment to a range of Ca^{2+} , lipid, and fXa concentrations that might have physiological relevance, we required knowledge of the heretofore poorly characterized variation with Ca^{2+} concentration of some key thermodynamic and enzymatic parameters: 1] the dissociation constant of fXa from a membrane surface (K_d^{Xa}), 2] the dissociation constant of fXa dimer on a surface ($K_{d,\sigma}^{Xa2}$), 3] the activity of a fXa monomer in solution ($k_{cat}/K_M)_{Xa\text{ free}}$, and 4] the activity of a fXa monomer on a PS-containing membrane ($k_{cat}/K_M)_{Xa}$. With knowledge of these parameters, we have developed a predictive model of how Ca^{2+} and membrane concentration might combine to precisely regulate fXa activity in the initiation phase of blood coagulation. Of physiological significance, this regulation is qualitatively insensitive to the presence of 0.6 mM Mg^{2+} .

2. Experimental Procedures

Materials

Human fXa, DEGR-Xa, prothrombin, and prethrombin 2 (Pre 2: the catalytic domain of prothrombin with the $\text{R}^{322}\text{-I}^{323}$ bond intact) were obtained from Hematologic Technologies Inc. (Essex Junction, VT). Pre 2 purity was checked by running it on a 10% PAGE Gel, where it showed a single band without any contamination of Frag1.2. Factor Xa purity was verified by a standardized active site assay (17). The factor Xa-specific substrate N-2-benzyloxycarbonyl-D-arginyl-L-arginine p-nitroanilide dihydrochloride (S-2765) and thrombin (IIa)-specific substrate H-D-phenylalanyl-L-pipecolyl-L-arginine p-nitroanilide dihydrochloride (S-2238) were purchased from DiaPharma (W. Chester., OH). Calcium chloride was reagent grade from Fisher Chemicals. 1,2-dioleoyl-3-sn-phosphatidylcholine (DOPC) and porcine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). Concentrations of the phospholipids were established by inorganic phosphate determination (18) and purity was tested by thin layer chromatography. All other chemicals were ACS reagent grade or the best available grade; all solvents were HPLC grade.

Methods

Preparation of Phospholipid Vesicles—Small unilamellar vesicles (SUVs) composed of DOPC and PS in the molar ratio 75:25 were prepared in 50 mM Tris, 150 mM NaCl, 0.6% PEG, pH 7.4 (buffer A) as described previously(19,20).

Measurement of the Kinetics of Active Site Formation Using Synthetic

Substrate—Human Pre2 activation to thrombin was followed with synthetic substrate, S-2238, which detects formation of both thrombin and meizothrombin (active thrombin precursor (21)). We used 500 nM Pre2 as the substrate because it does not limit free

membrane surface (15), which, if it occurred would complicate interpretation of our results. Pre2 conversion to thrombin involves cutting the same peptide bond (R^{273} - T^{274} in the catalytic domain) that is cut in converting prothrombin to meizothrombin, which determines the rate of active site formation *in vitro* in the presence of membranes (22) or in the presence of a soluble fXa activating form of PS (23). Pre2 activating mixtures contained Pre2, 75/25 DOPC/PS SUVs at a desired total lipid concentration, and fXa in buffer A and different concentrations of free Ca^{2+} , at 37°C. The activation reaction was quenched at several time intervals (1, 2, 3, 4, 5, and 6 min) with 0.11 mg/mL (final concentration) soybean trypsin inhibitor (24). Aliquots of 5 μ L of the quenched reaction mixture collected at different times were transferred into sample wells of a 96-well assay plate (Becton Dickinson, Oxnard, CA). The wells already contained 105 μ L Buffer A at 37 °C. An aliquot of 35 μ L of the substrate S-2238 (0.47 mg/mL with 20 mM $CaCl_2$ at 37 °C) was added to this mixture. Following a brief mixing time (4 s), the time-dependence of absorbance at 405 nm was recorded using a microplate reader (Versamax; Molecular Devices Corp., Sunnyvale, CA) so as to obtain the initial rate of S-2238 hydrolysis. Because Pre2 lacks the Ca^{2+} - and membrane-binding Gla (γ -decarboxy glutamic acid) domain of prothrombin, it should approach the membrane-bound prothrombinase complex from solution rather than from the membrane. To be sure that the influence of Ca^{2+} was similar with the physiological substrate, we also performed identical experiments using 500 nM prothrombin as substrate. In addition to ~ 1 mM free Ca^{2+} , human plasma contains 0.6 mM Mg^{2+} , which plays a role in other proteolytic reactions of blood coagulation. In order to ask whether Mg^{2+} influenced the Ca^{2+} effect reported here, we included 0.6mM Mg^{2+} in the buffer for prothrombin activation measurements to mimic as closely as possible physiologic conditions. The rate of thrombin plus meizothrombin active site formation was determined by comparing the initial rate of S-2238 hydrolysis to a standard curve generated using thrombin that was active-site titrated with p-nitrophenyl p'-guanidinobenzoate (Sigma Chemical Co.) (25). The initial rates (R) of Pre2 or prothrombin activation were determined from plots of active site concentration versus time, which were linear in the time range examined (0–6 min). Kinetic data were analyzed according to the Michaelis-Menten model, with k_{cat} and K_M being the usual kinetic constants in this formalism. For monomer fXa in solution, the R was linear in both fXa and Pre2 or prothrombin concentrations ($[Xa]$ and $[P2]$, respectively), demonstrating the second-order nature of prothrombin or Pre2 activation by monomer fXa within the Michaelis-Menten treatment. The rates of Pre2 or prothrombin activation by fXa in solution (R_{free}), by monomer on a membrane (R_1), and by fXa dimer on a membrane (R_2) are taken as second order rate constants (k_{cat}/K_M) for each of these fXa species.

Fluorescence measurements—The fluorescence intensity of DEGR-Xa was recorded at 23°C with a Spex® FluoroLog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ), using an excitation wavelength of 340 nm (band pass 2 nm) and emission wavelength of 550 nm (4-nm band pass). The excitation shutter was kept closed except during data collection to avoid photo-degradation of the dansyl fluorophore. Before every fluorescence titration, 100nM DEGR-Xa in buffer A at 23°C was incubated for ~50 min in a cuvette so as to condition the cuvette (26). After this initial incubation, the cuvette was rinsed with 2 mL of the buffer and fresh sample (2mL) was added to the conditioned cuvette for measurements. SUVs (25/75 PS/DOPC) were added in up to 4 μ L aliquots, with a maximum volume

dilution of 5%. We corrected for dilution by normalizing to the emission intensity of DEGR-Xa titrated by buffer alone. Fluorescence intensity was recorded ~2–3 min after each addition. Results were averaged as described previously (15,26).

2.1 Modeling the Dependence of Total fXa Activity on $[Ca^{2+}]$ and $[PL]$

The dependence of R on $[Ca^{2+}]$ and $[PL]$ was modeled using the previously published fXa dimerization model (15), but incorporating $[Ca^{2+}]$ -dependent parameters determined here. The equations for these calculations are all published (15) so we describe here only an overview of the calculation. The initial rate (R) (Figure 1 inset) is expressed as the sum of three rates:

$$R \left([Ca^{2+}] \right) = [Xa_{free}] \cdot R_{free} + [XaM] \cdot R_1 + [Xa_2M] \cdot R_2. \quad (1)$$

In this expression, R_{free} , R_1 , and R_2 are second order rate constants for fXa monomer in solution, for fXa monomer on a membrane, and for fXa dimer on a membrane, respectively. $[Xa_{free}]$, $[XaM]$, and $[Xa_2M]$ are the $[Ca^{2+}]$ -dependent concentrations of these species that we have shown previously can be calculated using two parameters: the $[Ca^{2+}]$ -dependent equilibrium constants $K_d^{Xa} \left([Ca^{2+}] \right)$ and $K_{d,\sigma}^{Xa_2} \left([Ca^{2+}] \right)$. Calculating the observed rate requires three additional parameters: the Ca^{2+} -dependent rates of IIa formation by each of the fXa species: $R_{free}([Ca^{2+}])$, $R_1([Ca^{2+}])$, and $R_2([Ca^{2+}])$. Since prediction of all these parameters was not possible based only on the experimental data shown in Figure 1 inset, we determined the unknown equilibrium constants and rates based on the experimental data in Figures 2 and 3, respectively, as described next.

2.2 $[Ca^{2+}]$ -Dependence of fXa Binding to, and Dimer Formation on, Membranes

These were obtained with fluorescently labeled fXa (DEGR-Xa) from data shown in Figure 2A. The equations and procedures for obtaining these constants at each $[Ca^{2+}]$ were documented previously (15). Just as described for activity of fXa in Equation 1, the fluorescence intensity of DEGR-Xa results from three species:

$$F \left([Ca^{2+}] \right) = [Xa_{free}] \cdot F_{free} + [XaM] \cdot F_1 + [Xa_2M] \cdot F_2, \quad (2)$$

where the F values are relative (to F_{free}) fluorescence intensities for DEGR-Xa in solution, on a membrane, and in a dimer on a membrane, respectively. These,

$K_{d,\sigma}^{DEGR-Xa_2} \left([Ca^{2+}] \right)$, and $K_d^{DEGR-Xa} \left([Ca^{2+}] \right)$ were adjusted to fit titration curves such as shown in Figure 2A at seven different Ca^{2+} concentrations to obtain the four undefined parameters using MatLab 6.5, as described previously (15), with fits shown in Figure 2A and parameter values in Figures 2B and 2C.

2.3 $[Ca^{2+}]$ -Dependence of fXa Activity in Solution

Sabharwal et al. previously suggested that fX contained three classes of Ca^{2+} binding sites: two high affinity ($K_d \sim 100 \pm 30 \mu M$), four of intermediate affinity ($K_d \sim 450 \pm 100 \mu M$) and five to six low affinity sites ($K_d \sim 2 \text{ mM}$). Furthermore, based on subtracting the

binding curve of decarboxylated fX (Gla residues converted to Glu) from that of the native fX, these authors also suggested a positive cooperativity in binding of Ca^{2+} to the intermediate affinity sites in the Gla domain of human fX (27). The data in this study were insufficient to establish firmly the number of sites that exhibit cooperative Ca^{2+} binding to the fXa Gla domain. In order to describe our data we also needed a reliable estimate of this parameter, which we call N_H because it corresponds to a Hill coefficient. Therefore, we globally fit our data (Figure 3A) along with the equilibrium dialysis data of Sabharwal et al. (inset to Figure 3A) to reexamine this issue. We used the model, originally proposed by Sabharwal et al. (27) that assumed three classes of binding sites: two classes of sites with independent binding of Ca^{2+} to fXa and a class of intermediate-affinity sites presumably located the Gla domain that cooperatively bind roughly two Ca^{2+} as described with a Hill term:

$$r = N_1 \cdot \frac{[\text{Ca}^{2+}]}{K_1 + [\text{Ca}^{2+}]} + N_2 \cdot \frac{[\text{Ca}^{2+}]}{K_2 + [\text{Ca}^{2+}]} + N_H \cdot \frac{[\text{Ca}^{2+}]^n}{K_H^n + [\text{Ca}^{2+}]^n}, \quad (3)$$

$$\left(\frac{k_{cat}}{K_M} \right)_{\text{Xa free}} = A_1 \cdot \frac{[\text{Ca}^{2+}]}{K_1 + [\text{Ca}^{2+}]} + A_2 \cdot \frac{[\text{Ca}^{2+}]}{K_2 + [\text{Ca}^{2+}]} + A_H \cdot \frac{[\text{Ca}^{2+}]^n}{K_H^n + [\text{Ca}^{2+}]^n}, \quad (4)$$

where r is the total number of bound Ca^{2+} ions, N_1 , N_2 , and N_H are the numbers of tight, loose, and intermediate (cooperative) sites, respectively, N_3 is the total number of intermediate sites with N_H being the number of these that are cooperatively linked, and K_1 , K_2 , and K_H are corresponding dissociation constants. The parameters that result from this process are given and discussed in the text.

3. Results and Discussion

3.1 $[\text{Ca}^{2+}]$ - and Membrane-dependence of fXa Proteolytic Activity

In order to examine the influence of Ca^{2+} on the response of fXa to PS-containing membranes, we have determined the proteolytic activity of human fXa towards human prothrombin2 (Pre2) and prothrombin as substrates at different Ca^{2+} concentrations and increasing total phospholipid concentrations ([PL]). The inset to Figure 1 shows the initial rates (R) of Pre2 activation by fXa in the presence of increasing concentrations of PS-containing membranes plotted relative to the rate in the absence of membranes (R_0), i.e., $R - R_0$ *versus* phospholipid concentration [PL], at several Ca^{2+} concentrations (0.77 mM – ●, 1.09 mM – ■, 1.12 mM – ◆, 1.16 mM – ▼, and 2.31 mM – ▲). These curves were roughly linear over the membrane concentrations considered (0–15 μM), although this linearity is lost at higher membrane concentrations (15). Factor Xa activity increased with increasing membrane concentration for 0.77 mM – ● and 1.09 mM – ■ Ca^{2+} but decreased at higher Ca^{2+} concentrations (Figure 1 inset). As a simple measure of this effect, we plot the slopes ($(R - R_0)/[PL]$) of the inset curves (and other such curves not shown) as a function of Ca^{2+} concentration in the main plot of Figure 1 (filled squares ■□). The experiments reveal a remarkably cooperative dependence of ($R - R_0)/[PL]$ on free Ca^{2+} concentration with the

midpoint of the cooperative rise exactly at the lower limit of the normal range (shaded region) of Ca^{2+} concentration in human plasma (1.10–1.29 mM). The proteolytic activity of fXa therefore is clearly promoted by its binding to PS-containing membranes at Ca^{2+} concentrations below those in plasma, but is inhibited at higher Ca^{2+} concentrations.

It has been demonstrated that Mg^{2+} as well as Ca^{2+} play a role in stabilizing the native conformation of coagulation factor IX. Mg^{2+} ions at physiological concentrations (~0.6 mM) markedly enhance the biological activities of factor IX (28), and low Mg^{2+} is consequently a significant contributor cardiovascular diseases (29). It is also reported that in the presence of 1.3 mM Ca^{2+} (just above normal plasma range), the addition of 0.6 mM Mg^{2+} leads to approximately a 5-fold faster rate of fX activation by fVIIa–soluble TF (sTF) (30), but that at low Ca^{2+} concentration, Mg^{2+} can alter the activity and structure of the fVIIa–sTF complex (31). These data suggest that both Mg^{2+} and Ca^{2+} are important in regulating coagulation and anticoagulation. Because of this, we performed prothrombin activation measurements at a physiological concentration (0.6 mM) of Mg^{2+} so as to mimic physiological conditions. The slopes of plots of initial rates of prothrombin activation with respect to lipid concentration ($(R-R_0)/[PL]$) in the presence of 0.6 mM Mg^{2+} at four Ca^{2+} concentrations are shown as open squares ($\square$) in Figure 1. These measurements confirm that the remarkably cooperative dependence of ($R-R_0$)/[PL] on free Ca^{2+} concentration occurs at both physiological and zero Mg^{2+} concentrations, and show that the midpoint of the cooperative rise with decreasing Ca^{2+} concentration is little influenced by the presence of physiological Mg^{2+} concentrations, nor is the inhibitory influence of membranes at higher Ca^{2+} concentrations. However, the positive influence of membranes at low concentration of Ca^{2+} in the presence of Mg^{2+} and the physiological substrate (prothrombin) ($\square\square$) was roughly three times that seen with Pre2 as the substrate without Mg^{2+} ($\blacksquare\square$). This could be due to inclusion of Mg^{2+} but most likely reflects the use of prothrombin as a substrate, since it approaches fXa from the membrane rather than from solution, as does Pre2. In this regard, a two-fold increase in activity of the prothrombinase complex is reported when prothrombin approaches from the membrane as compared to when it approached from solution (20).

We ask now why PS-containing membranes inhibit fXa at Ca^{2+} concentrations at and above those reported in human plasma but activate it below this Ca^{2+} concentration? Our *hypothesis is that this phenomenon can be quantitatively explained by formation of fXa inactive dimers on the membrane surface at higher Ca^{2+} concentrations.* To test this hypothesis, we use the previously introduced model of fXa dimer formation on a membrane surface (15) to calculate the concentrations of free and bound monomer fXa and membrane-bound dimer fXa and use these to predict fXa activity as a function of Ca^{2+} concentration. In order to do this, we required thermodynamic and kinetic parameters that describe both the equilibria between these species and their activities at various Ca^{2+} concentrations. These reflect three simultaneously occurring, calcium dependent and opposing phenomena that are addressed in the next two sections:

1. abrupt formation of inactive fXa dimers at $[\text{Ca}^{2+}] \approx 1$ mM (Figure 2B),
2. reduced fXa binding to lipid membranes as $[\text{Ca}^{2+}]$ decreases (Figure 2 C), and

3. a sigmoidal increase in activity of fXa in solution centered at $[\text{Ca}^{2+}] \approx 1 \text{ mM}$ (Figure 3A) and an abrupt increase in activity fXa monomer on a membrane $[\text{Ca}^{2+}] \approx 1 \text{ mM}$ (Figure 3B).

3.2 $[\text{Ca}^{2+}]$ -dependence of fXa Membrane Binding and Dimer Formation on a Membrane

A decrease in DEGR-Xa fluorescence at low $[\text{Ca}^{2+}]$ (0.77, and 1.04 mM) (Figure 2A) could be fitted with a simple binding model, i.e., by assuming that fXa binds to membranes as a monomer with no further self-association. Above $[\text{Ca}^{2+}] = 1.04 \text{ mM}$, a quantitative description of this system required our published surface dimerization model (15), which requires the dissociation constant for fXa binding to a membrane (K_d^{Xa}) as well as its surface dimerization dissociation constant ($K_{d,\sigma}^{Xa2}$) on a membrane at different Ca^{2+} concentrations, as described in Methods Section 2.2. These were obtained by fitting the titration curves recorded in Figure 2A to the dimerization model as described in detail previously (15). Analysis of these titration curves (Methods Section 2.2) yielded the Ca^{2+} concentration dependences of the DEGR-Xa surface dimerization constant ($K_{a,\sigma}^{DEGR-Xa2}$) on a membrane (Figure 2B) and of the dissociation constant for DEGR-fXa monomer binding to a membrane ($K_d^{DEGR-Xa}$; Figure 2C). The strong dependence of the dimerization constant on Ca^{2+} concentration is consistent with our previous observation that water-soluble-PS-stimulated dimerization of bovine fXa occurs in solution in a strongly Ca^{2+} -dependent fashion (32), although the discontinuity at $[\text{Ca}^{2+}] \approx 1$ (Figure 2C) was not detected in solution. If we used the dimer model to fit the data for $[\text{Ca}^{2+}] = 0.77$, and 1.04 mM, the best fit was obtained with $K_{a,\sigma}^{DEGR-Xa2} \approx 0$, suggesting that DEGR-Xa dimerization on the membrane surface is very weak at 0.77 and 1.04 mM Ca^{2+} , explaining both why these data were well described by a simple absorption isotherm. This results in the discontinuity at $[\text{Ca}^{2+}] \approx 1 \text{ mM}$ in Figure 2B. We used an x-shifted hyperbolic function (described in the legend for Figure 2) to empirically describe this functionality. We earlier identified by mass spectroscopy a Lys residue (270 in the Fung system) that is buried in the dimer interface and another (414) that becomes exposed in the dimer (16). Based on the best available MD model for fXa (Lee Pedersen, personal communication), both of these Lys are at the edge of the cleft that contains the active site triad (see Figure 7 of (16)). Thus, the surface of fXa near the active site is significantly altered in the dimer. The fluorescence of DEGR-Xa is very sensitive to dimer formation (26) as is the fluorescence polarization of a tri-peptide -linked fluorescein (FEGR) that undergoes self-fluorescence-resonance-energy-transfer (self-FRET) (15). Both DEGR and FEGR label the active site and significantly limit dimer formation on a membrane as well as in solution (15,16). These observations suggest that the free energy of DEGR-Xa dimer formation is significantly reduced by the presence of the probe near the dimer interface, as is confirmed by the surface dimer association constant for fXa at 3.85 mM Ca^{2+} being larger than that for DEGR-Xa by a factor of approximately $5 \cdot 10^4$ (15). In order to adjust for this, we scaled the surface dimer dissociation constant of DEGR-Xa at 3.85 mM Ca^{2+} to that determined previously (15) for fXa at 3.85 mM Ca^{2+} , resulting in $K_{a,\sigma}^{Xa2} \simeq 5 \cdot 10^4 K_{a,\sigma}^{DEGR-Xa2}$. We assumed this relationship holds at all Ca^{2+} concentrations. The validity of this assumption is supported by how well it accounts for the data in Figure 1.

Most estimates of the dissociation constant for fXa binding to a membrane are distorted by dimer formation on the membrane, so the range of K_d^{Xa} reported in the literature is quite broad (15). Since DEGR-Xa dimerization is much weaker than fXa dimerization (15,16), and since there is no indication that DEGR-Xa binds significantly differently from fXa to a membrane, we assumed $K_d^{Xa} = K_d^{DEGR-Xa}$. Again, the validity of this assumption is confirmed by the ability of our calculations to account for the data in Figure 1 (see Section 3.4). $K_d^{Xa} = K_d^{DEGR-Xa}$ is plotted in Figure 2C and was represented by an empirical linear function in order to carry our calculations as a function of Ca^{2+} concentration:

$$K_d^{Xa} \left([Ca^{2+}] \right) = k \cdot [Ca^{2+}] + n, \text{ with } n=101 \text{ nM, and } k=58 \text{ nM/mM } Ca^{2+} \quad (5)$$

3.3 $[Ca^{2+}]$ -dependence of fXa Activity in solution

Having determined these equilibrium constants, the only quantities needed for the determination of $(R-R_0)/[PL]$ as a function of both Ca^{2+} and membrane concentrations are the activities of fXa monomer in solution (R_{free}) and on a membrane (R_1), and of the fXa dimer on a membrane (R_2) as a function of Ca^{2+} concentration. The rate of Pre2 proteolysis by fXa monomer in solution ($R_{free}([Ca^{2+}]) = (k_{cat}/K_M)Xa_{free}$) was measured as described in Methods and is plotted in Figure 3A versus Ca^{2+} concentration. The lag in the fXa activity versus $[Ca^{2+}]$ at low concentrations of Ca^{2+} indicates that cooperative binding of Ca^{2+} to a number of sites is involved in regulation of fXa activity in solution. In order to describe these data, we needed to know more about the cooperative nature of Ca^{2+} binding to fXa in solution. As described in Methods 2.3, we used published equilibrium dialysis data for $^{45}Ca^{2+}$ -binding to fX (27) (reproduced in the inset to Figure 3A) to provide this information and globally fit these data along with our rate data shown in Figure 3A, using the model of Sabharwal *et al.* to account for Ca^{2+} binding (Methods 2.3). When all parameters of the model were allowed to adjust freely, the global fit yielded: two tight sites ($N_1 = 2$) with dissociation constant $K_{d1} = 130 \mu M$, four to five low affinity sites ($N_2 = 4-5$) with $K_{d2} = 2.4 \text{ mM}$, and three to four cooperative intermediate affinity sites ($N_H = 3-4$) with $K_{dH} = 1 \text{ mM}$ and a Hill coefficient initially estimated to equal to the total number of intermediate affinity sites ($N_H = N_3$). However, the activity parameters obtained from this fit were poorly defined, so we had to reduce the number of free parameters. We used the K_d values reported by Sabharwal *et al.* (27) for the two ($N_1 = 2$) independent tight sites ($K_{d1} \sim 100 \mu M$) and for 5–6 (N_2) low affinity sites $K_{d2} (\sim 2 \text{ mM})$, and the number of cooperative sites was set to preselected integer values $N_H = 2, 3, 4, 5$, with full cooperativity assumed ($N_H = N_3$). In this way, we obtained a global fit that described the data well (solid lines in Figure 3A and 3 inset) for $N_H = 3$, $N_1 = 1$ to 2, $K_{dH} = 980 \pm 90 \mu M$, and adjusted activities A_1 , A_2 , and A_H . These lead to a total Ca^{2+} binding stoichiometry of 9–11. The number of linked sites is somewhat different than suggested by Sabharwal *et al.* (27) (~ 2) who estimate N_3 as 4 using an independent-site approximation, but estimated N_H and K_{dH} indirectly using a linked-site model applied to hypothetical data obtained by subtracting the binding isotherm for decarboxylated fXa from that of intact fXa. The fact that our global fit predicted only one tight site as opposed to the two estimated by Sabharwal *et al.* may result from our activity as data playing a significant role in determining N_1 , which presumably could reflect

the single site in the catalytic domain that is reported to be critical for activity (33,34). Another tight site in the N-terminal epidermal growth factor-like (EGFn) domain influences catalytic domain conformation and indirectly activity when the EGFn domain is chemically linked to the Gla domain (35). The dissociation constant we estimated for the tight site ($K_{d,1} = 43 \pm 27 \mu\text{M}$) is somewhat tighter than that estimated for the EGFn site ($120 \mu\text{M}$ (35)). The activities (k_{cat}/K_M) associated with soluble fXa species having Ca^{2+} binding to different fXa sites were defined through fitting the data to obtain the best fit shown as the solid line in Figure 3A: $A_1 = 0 \pm 7 \text{ M}^{-1}\text{s}^{-1}$, $A_2 = 0 \pm 34 \text{ M}^{-1}\text{s}^{-1}$, and $A_H = 53 \pm 15 \text{ M}^{-1}\text{s}^{-1} = R_{\text{free}}$ in Equation 1. In this context, we could speculate that one Ca^{2+} binds to the catalytic domain to effect activity, one binds tightly to the EGFn-Gla pair to alter the site in the catalytic domain, and possibly one more binds moderately to the Gla domain to influence the EGFn-Gla site. This should result in a cooperative influence of Ca^{2+} on activity of soluble fXa, as observed (Figure 2A).

This left only the activity of membrane-bound fXa to determine. We have previously shown that the activity of the fXa dimer on a membrane is insignificant compared to the monomer rate (15), so we assumed that $R_2 \text{Ca}^2 \approx 0$ at all Ca^{2+} concentrations. As a result, dimer formation obscures any measurement designed to determine the activity of membrane-bound fXa monomer. Thus, the dimer model (15) was used to obtain the concentrations of membrane-bound fXa monomer and dimer at different membrane concentrations, and these were used to fit experimental activities (some of which are plotted in Figure 1 inset) by adjusting the activity of membrane-bound fXa in order to obtain $[(k_{cat}/K_M)_{\text{Xa}} = R_1(\text{Ca}^{2+})]$ at each Ca^{2+} concentration. Factor Xa dimerization constants and membrane-binding constants required for this calculation were fixed at values determined as described here from the data in Figures 2 and 3. The resulting membrane-bound monomer activities (R_1) are plotted in Figure 3B versus $[\text{Ca}^{2+}]$. Consistent with Ca^{2+} being required for both fXa activity (Figure 3A) and membrane binding (Figure 2B), activity decreased with decreasing Ca^{2+} concentration. The discontinuity in Figure 3B is due to the fact that fXa dimerization is so weak at and below 1.04 mM Ca^{2+} (see discontinuity in Figure 2B) that the dimer model was unreliable in describing data at and below 1.04 mM Ca^{2+} . This functionality was described mathematically as documented in the legend to Figure 3.

3.4 Quantitative Description of Data in Figure 1

In order to test our use of straight lines to approximate $(R-R_0)/[PL]$ (as plotted as $\blacksquare$ $\square$ in Figure 1), we calculated the derivative $(R-R_0)/[PL]$ numerically with MatLab using the dimer model with parameters obtained in Results 3.2 and best fit values of R_1 as obtained in Results 3.3 (Figure 3B). These calculated values at each Ca^{2+} concentration (open circles in Figure 1) nicely overlap with those obtained from straight lines through $R-R_0$ *versus* $[PL]$ plots ($\blacksquare$ $\square$). The fact that the experimental and calculated values agreed so well at each Ca^{2+} concentration strongly supports our hypothesis and also justifies the linear approximation used to obtain experimental $(R-R_0)/[PL]$ estimates.

Next, to obtain the solid line through the derivatives in Figure 1 as approximated by the straight line method ($\blacksquare$ $\square$) and as calculated using the dimer model ($\square$), we used all the Ca^{2+} dependent parameters obtained in Results 3.2 and 3.3 to calculate the concentrations of

all fXa species expected as a function of $[Ca^{2+}]$ and $[PL]$. These concentrations were used to calculate the initial rates (R) of Pre2 activation by fXa using Equation 1, and this was numerically differentiated using MatLab to obtain $(R-R_0)/[PL]$. The fact that the calculated $(R-R_0)/[PL]$ so well described the entire experimental data set presents a powerful argument for the validity of the functional $[Ca^{2+}]$ -dependences of parameters illustrated by the lines in Figures 2 and 3. In addition, it strongly supports the validity of our hypothesis. The dramatic decrease in $(R-R_0)/[PL]$ at roughly 1 mM Ca^{2+} is explained in part by the fact that fXa activity appears to depend on occupancy of one or more of three linked, intermediate-strength Ca^{2+} sites and in part by the fact that binding of a soluble form of phosphatidylserine (PS) to a regulatory site in the the EGF_N-Gla domain pair is highly sensitive to $[Ca^{2+}]$ (17,36,37). Interestingly, Sabharwal *et al.* suggest that the linked Ca^{2+} binding sites identified by them (27) are in the Gla domain, although one tight Ca^{2+} site is in the Gla-EGF_N pair apparently regulates the conformation of the catalytic domain (35), wherein occurs the tight Ca^{2+} site linked to activity (33,34). All this suggests that linked Ca^{2+} and PS binding sites located near to or in the membrane interfacial region are key to regulating fXa dimerization and thus fXa activity at the Ca^{2+} concentration found in plasma.

Finally, we tested whether the dimer model presented here could also account for influence of Ca^{2+} on the activity of fXa towards the physiological substrate prothrombin in the presence of Mg^{2+} , which occurs in plasma at 0.6 mM. Data obtained under these conditions are shown as open squares in Figure 1. The dimer model was used to calculate the dotted line through these data obtained with prothrombin in the presence of Mg^{2+} , but with the fXa monomer activity increased two-fold and the fXa dimerization association constant increased four-fold in the presence of Mg^{2+} . As noted in Section 3.1, the increased activity is consistent with prothrombin approaching membrane-bound fXa from the membrane surface both reasonable expectations based on earlier reports (28,38). This result thus demonstrates that the "Ca²⁺ switch" we report persists for the physiological substrate in the presence of physiological Mg^{2+} , but with the magnitude of the switch somewhat enhanced.

3.5 A Quantitative Model for Regulation of fXa activity by $[Ca^{2+}]$ and PL

Quantitative prediction of the Ca^{2+} -dependence of $(R-R_0)/[PL]$ in Figure 1 and the fact that our calculations are based on modeling fXa dimerization on a membrane (15) allows us to calculate the anticipated variation of either $(R-R_0)/[PL]$ or the initial rate of thrombin formation with both Ca^{2+} and membrane concentration. The calculated rate of prothrombin activation is shown as a 3D plot in Figure 4A (3rd dimension shown by color variation). Figures 4C and D show the variation of the derivatives $(R-R_0)/[Ca^{2+}]$ and $(R-R_0)/[PL]$ with both $[PL]$ and $[Ca^{2+}]$. Figure 4C illustrates and extends the results in Figure 1 to other lipid concentrations by predicting that at all but the very lowest membrane concentrations, a slight increase in $[Ca^{2+}]$ in the physiological range results a decidedly negative effect on the rate of thrombin generation due to fXa dimer formation increasing with Ca^{2+} concentration. Similarly, Figure 4D predicts that an increase in membrane concentration negatively impacts the rate of thrombin generation at low membrane concentrations and all Ca^{2+} concentrations above the lower limit of the physiological range. This effect is reduced significantly with increasing membrane concentration due to dilution of fXa on the membrane surface, leading to reduced formation of inactive fXa dimers.

4. Possible Physiological Significance of Findings

The modern “Cell-Based” model for blood coagulation envisions four stages of the process, all of which occur on different cellular membranes (39,40). In this model, the “initiation” event is viewed as primarily reflecting damage to fibroblasts in the sub-endothelial layer of blood vessel walls. This damage exposes plasma to both Tissue Factor (TF) and factor VIIa on the surface of fibroblasts and leads to production of endothelial and other “microparticles” that expose phosphatidylserine (PS) that is normally maintained on the inner, cytoplasmic leaflet of endothelial or fibroblast cells. It is reported that TF-bearing MPs play a potentially important role in pathological processes leading to thrombosis as well as in normal hemostasis (41). The small amount of fXa produced by the TF-VIIa complex has almost no activity in solution but its activity is enhanced by ~1000-fold if bound to PS-containing membranes (42). At this point, little factor Va is available, and “amplification” is purported to occur when platelets bind to endothelial cells and begin to release platelet factor Va from alpha granules. Factor Va is the amplifying cofactor of fXa, so this leads to increased production of thrombin. Finally, “propagation” results from platelet activation by thrombin, resulting in release of platelet microparticles that expose PS and platelet-associated fVa, thus leading to a burst of thrombin formation, ultimately supporting formation of the fibrin gel needed for a stable clot.

The results presented here apply primarily to the “initiation phase” when little or no factor Va is present. In vitro, factor Xa dimerization and its subsequent inactivation occur at certain surface densities of fXa on PS-containing membranes. Physiological levels of fX in plasma are 0.1–0.2 μM (43), but only about 10 pM fXa is activated (44) during the initiation phase by TF-fVIIa. This is approximately 10^5 times less than the concentration we used in our experiments in order to attain measurable rates of thrombin generation. However total lipid concentration of TF-bearing MPs is also much lower than total lipid concentration used in our experiments. MPs are hardly detectable in the peripheral blood of healthy individuals, whereas elevated levels of procoagulant MPs are often associated with thrombotic propensity (45). Plasma concentration of TF-bearing endothelial-derived MPs in healthy controls is around 100/ μl , whereas it can increase above 1000/ μl in patients experiencing pregnancy loss (46). If we take into account that MP size ranges from 100 to 1000 nm (47,48), then total lipid concentration in vivo from TF-bearing MPs is around 0.02 to 0.2 nM in healthy individuals and 0.2 to 2 nM in pregnancy loss patients. This is about 10^5 times less for healthy and 10^4 times less for pregnancy loss patients than the total lipid concentration in our in vitro experiments. Thus, the ratio of fXa to MP plasma concentration in normal plasma is about what used in our experiments but is roughly 10-fold reduced in pregnancy loss patients. Because we maintained physiological fXa to lipid ratios, it is reasonable to expect that we achieved surface densities of fXa on PS-containing membranes similar to densities present in endothelial microparticles in healthy individuals *in vivo*. It is not unreasonable to project that fXa might form inactive dimers in plasma of healthy individuals, but gets diluted on MP membranes in pregnancy loss or other patients suffering from thrombotic disease. This dilution of fXa on MPs could then lead to appearance of active fXa membrane-bound monomers that can promote thrombotic disease.

While the concentrations of both fXa and endothelial MPs at the wound site is not known, it is expected to be much greater than in plasma. If the fXa to lipid ratio remains comparable to that seen in plasma, we expect from our results that the local concentration of Ca^{2+} free at the wound site will be very important in regulating the initiation phase of the clotting process. This is illustrated in Figure 4A, wherein we plot the two-dimensional dependence of initial rate of thrombin formation on lipid and Ca^{2+} concentration as calculated using the parameters obtained from fitting the data of Figure 1. To appreciate the significance of the predictions of Figure 4A, we must consider how calcium concentration varies at the site of a wound. In thrombi within the carotid artery of an anesthetized pig, free $[\text{Ca}^{2+}]$ drops to a level well below plasma levels within 20 minutes of wound formation and then recovers within 40 minutes to plasma levels (49). The local drop in free $[\text{Ca}^{2+}]$ in the wound is associated with release of cytoplasm, where resting concentration of $[\text{Ca}^{2+}]$ is normally maintained in the range of 10–100 nM (50). While local Ca^{2+} concentration drops in the early wound, Mg^{2+} concentration remains 0.6 mM (28). However, as demonstrated here, the presence of 0.6 mM Mg^{2+} does not interfere with but accentuates the Ca^{2+} switch. Our results suggest that a drop in local $[\text{Ca}^{2+}]$ allows fXa bound to PS-exposing endothelial MPs to produce low levels of thrombin, perhaps sufficient to trigger initial platelet binding to damaged endothelial cells and to release platelet fVa, producing the transition to “propagation” phase. Figure 4A predicts a dramatic increase in the rate of thrombin formation with increasing membrane concentration at sub-plasma levels of Ca^{2+} (as indicated with the vertical arrow in lower left corner of the figure). However, at least some fraction of endothelial microparticles must escape into plasma, where their appearance not only correlates with thrombotic disease but where they may also perform other, some useful and some harmful, functions (8). A portion of these microparticles will expose some PS and present the deleterious possibility of prothrombin activation outside the region of the early wound. Figure 4A shows that fXa dimerization at and above the lower limit of Ca^{2+} normal plasma range (≥ 1.1 mM) provides a mechanism for neutralizing the prothrombin-activating ability of fXa bound to low concentrations of PS-exposing microparticles. However, Figure 4A also illustrates how a ten-fold drop in phospholipid concentration at plasma Ca^{2+} concentrations (e.g., from ~ 12 to $1.2 \mu\text{M}$ in our in vitro studies) can lead to a dramatic increase in the rate of thrombin production. The dramatic fashion in which the thrombin-generating ability of PS-bound fXa is modulated over a very narrow range of Ca^{2+} concentration suggests that the Ca^{2+} gradient between initial wound fluids and plasma might act as an important “ Ca^{2+} switch” in permitting but then confining clot initiation to the site of the wound (see Figure 4B).

Conclusion

Experimental data alone show that the response of the total fXa activity to increased concentration of lipid membranes decreases sharply at the lower limit of normal plasma ionized calcium range, suggesting the existence of a sharp limit in plasma ionized calcium concentration, at which survival and wellbeing of critically ill patients might be drastically affected. This effect is explained here by taking into account four simultaneously occurring, calcium-dependent, but opposing phenomena: 1) fXa binding to lipid membranes increases only slightly with $[\text{Ca}^{2+}]$ (Figure 2C), 2) formation of inactive fXa dimers at $[\text{Ca}^{2+}]$ greater than but not less than ~ 1 mM (Figure 2B), 3) sigmoidally increasing activity of fXa in

solution around $[Ca^{2+}] \sim 1$ mM (Figure 3A), and 4) the dramatic increase in activity of fXa monomer on a membrane at $[Ca^{2+}] \sim 1$ mM (Figure 3B). No one of these phenomena alone can explain the dramatically sigmoidal Ca^{2+} -dependence of sensitivity of the ability of fXa to activate Pre2 in the presence of PS-containing membranes (Figure 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

S-2238	H-D-phenylalanyl-L-pipecolyl-L-arginin-p-nitroanilid dihydrochlorid (synthetic substrate for thrombin)
S-2765	N- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginin-p-nitroanilid dihydrochlorid (synthetic substrate for factor Xa)
DOPC	1,2-dioleoyl-3-sn-phosphatidylcholine
PS	phosphatidylserine (porcine brain in this study)
Pre2	prethrombin 2 (catalytic domain of prothrombin)
IIa	thrombin
fXa	Factor Xa
Tris	tris(hydroxymethyl)aminomethane
PEG 8000	poly(ethylene glycol)
SUV	small unilamellar vesicle
DEGR	Dansyl-GLU-GLY-ARG-chloromethylketone
FEGR	Fluorescein-GLU-GLY-ARG-chloromethylketone
DEGR-Xa	irreversibly inactivated Factor Xa by alkylation of the active site histidine residue with DEGR

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Summary Statement

Phosphatidylserine-membrane-triggered factor Xa dimer formation is a sharp sigmoidal function of $[Ca^{2+}]$. This “calcium switch” occurs just below plasma calcium concentrations. This introduces a previously unrecognized anti-coagulant mechanism whose malfunction early in clot initiation might explain many clinical observations.

Figure 1. The effect of membrane concentration ([PL]) on the overall factor Xa (fXa) activity (Ca^{2+} switch effect)

Main plot: The derivative of initial rate of thrombin formation (from Pre2 or prothrombin) ($R-R_0$)/ $[\text{PL}]$ is plotted versus Ca^{2+} concentration. The filled squares (■) are the derivatives of initial rate *versus* $[\text{PL}]$ plots obtained with Pre2 as a substrate (examples shown in the Inset) as approximated from straight lines drawn through the data in each plot. The open circles (○) are average derivatives calculated for each $R-R_0$ *versus* $[\text{PL}]$ plot using the fXa dimer model (15) with parameters obtained here (Results 2.2 and 2.3) as described in the text. The open squares (□) are derivatives (determined by the straight line method) of $R-R_0$ *versus* $[\text{PL}]$ plots obtained at the indicated Ca^{2+} concentrations with 500 nM prothrombin as substrate and under more physiological conditions (0.6 mM magnesium). The curves drawn through the symbols are smooth curves generated with the dimer model using parameters shown in Figures 2 B&C and 3A&B, as explained in Results 2.3 and 3.3. The dotted line through the open squares is simulated with the dimer model, showing that the “ Ca^{2+} switch effect” can also be observed in presence of physiological concentration of Mg^{2+} . Shaded area represents physiological concentration of plasma ionized (free) calcium (2,3).

Inset: The differences of the initial rates ($R-R_0$) of thrombin (IIa) formation in presence of PS-containing membranes (R) and in absence of the membranes (R_0) by fXa as a function of membrane concentration ($[\text{PL}]$) was determined at 37°C using the thrombin substrate S-2238. The concentration of substrate Pre2 was 0.5 mM in buffer with different Ca^{2+} concentrations (0.77 mM – ●, 1.09 mM – ■, 1.12 mM – □, 1.16 mM – ▼, and 2.31 mM – ▲). Solid lines are fits of the initial rates *versus* $[\text{PL}]$ using a linear function.

Figure 2. Analysis of DEGR-Xa membrane binding and dimerization using phosphatidyl-serine-containing lipid membranes as titrant

A) Fluorescence intensity of 100 nM DEGR-Xa (scattering and background subtracted) in a buffer with different CaCl₂ concentration (0.77 mM–Δ, 1.04 mM–○, 1.16 mM–◇, 1.54 mM–▽, and 3.08 mM–□) at 23°C as a function of increasing concentration of 25% PS/DOPC membranes. Data were fitted to the dimerization model (15), as described in the Materials and Methods (solid lines) to yield surface dimerization constants $K_{a,\sigma}^{DEGR-Xa2}$ and membrane dissociation constants $K_d^{DEGR-Xa}$ for each CaCl₂ concentration.

B) Surface association dimerization constants ($K_{a,\sigma}^{DEGR-Xa2}$) of DEGR-Xa. We used an x shifted hyperbola to empirically describe the dependence of dimer association constant versus Ca²⁺ concentration:

$$K_{a,\sigma}^{DEGR-Xa2}([Ca^{2+}]) = \begin{cases} 0, & [Ca^{2+}] \leq [Ca^{2+}]_0 \\ \frac{a([Ca^{2+}] - [Ca^{2+}]_0)}{b + ([Ca^{2+}] - [Ca^{2+}]_0)}, & [Ca^{2+}] \geq [Ca^{2+}]_0 \end{cases}, \quad (1)$$

where $[Ca^{2+}]_0$ was 1.04 mM, a (the asymptotic limit) was $30 \cdot 10^8$ dm²/mol, while b (the curvature) was 1.13 mM.

C) Dissociation constants for DEGR-Xa binding to SUVs ($K_d^{DEGR-Xa}$) obtained from fitting the data shown in A, using the dimerization model as described in (15).

Figure 3.

Activity of fXa in solution or in the presence of phosphatidylserine-containing lipid membranes as a function of Ca^{2+} concentration.

A) Main plot: Activity of Pre2 activation (apparent $(k_{cat}/K_M)_{Xa_{free}} \approx R_{free}([Ca^{2+}])/[Pre2]$) by fXa in solution as a function of Ca^{2+} concentration in the absence of lipid membranes. Sigmoidal curve indicates a cooperative response. **Inset:** Equilibrium dialysis data of Ca^{2+} binding to fXa using ^{45}Ca obtained by Sabharwal et al. (27). Both data sets were globally fitted with a model taking into account three classes of binding sites: two classes of sites with independent binding of N_1 and N_2 Ca^{2+} ions to fXa and a class of sites with cooperative binding of N_H Ca^{2+} ions, described with a Hill term. The global fit yielded: two tight sites ($N_1=2$) with $K_d=130 \mu M$ four to five low affinity sites with ($K_d=2.4$ mM, $N_H = 4-5$), and three to four ($N_2 = 3-4$) intermediate affinity sites with $K_d=1$ mM and positive, infinite cooperativity (Hill coefficients being equal to number of sites).

B) Activity of fXa monomer in the presence of PS-containing SUVs as calculated from the data shown in Figure 1 inset, using the dimerization model as described in (15), with assumptions described in the Supplement. We used a continuous function to empirically describe the dependence of fXa surface bound monomer activity versus Ca^{2+} concentration:

$$\left(\frac{k_{cat}}{K_M}\right)_{X_a}([Ca^{2+}]) = \begin{cases} k \cdot [Ca^{2+}], & 0.77mM < [Ca^{2+}] \leq [Ca^{2+}]_0 \\ \frac{a([Ca^{2+}] - [Ca^{2+}]_0)}{b + ([Ca^{2+}] - [Ca^{2+}]_0)}, & [Ca^{2+}] \geq [Ca^{2+}]_0 \end{cases}, \quad (2)$$

where $[Ca^{2+}]_0$ was again set to 1.04, while the following parameters were obtained by the fit with Equation 2: $a = (6.6 \pm 0.7) \cdot 10^3 M^{-2} s^{-1}$, $b = (0.3 \pm 0.1) mM$ and $k = (57 \pm 31) \cdot 10^3 M^{-2} s^{-1}$, as shown in Figure 3B.

Figure 4.

A Variation of the initial rate of thrombin formation (R) by fXa as a function of membrane ([PL]) and calcium ([Ca²⁺]) concentrations, as calculated using the fXa dimer model and the Ca²⁺-dependent parameters determined in the main text (Results 2.3 and 3.3). **B:** Cartoon illustrating the physiological significance of the plot in Frame A in regulating fXa during the initiation phase of blood coagulation. **C:** Variation with Ca²⁺ and membrane ([PL]) concentrations of the derivative of the initial rate of thrombin formation by fXa with respect to ionized (free) Ca²⁺ concentration as calculated using the dimer model. **D:** Variation with Ca²⁺ and membrane ([PL]) concentrations of the derivative of the initial rate of thrombin formation by fXa with respect to membrane ([PL]) concentration as calculated using the dimer model. The vertical lines in frames A, C, and D demarcate the range of physiological concentration of plasma ionized (free) calcium.