Nitrophorin-2: A Novel Mixed-Type Reversible Specific Inhibitor of the Intrinsic Factor-X Activating Complex†

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ABSTRACT: Nitrophorin-2 (NP-2), isolated from salivary glands of the blood-sucking insect *Rhodnius prolixus*, has been shown to be a specific inhibitor of the intrinsic factor X-(FX)-activating complex. The inhibitory effect of NP-2 is most potent in the presence of both FVIIIa and phospholipids (artificial phospholipid vesicles or activated human platelets). Detailed kinetic analyses of the inhibitory mechanism of NP-2 demonstrated a decrease in both V_{max} and K_m of activated FIX-(FIXa)-catalyzed FX activation in the presence of FVIIIa and phospholipid vesicles, characteristic of a hyperbolic mixed-type reversible inhibitor. NP-2 exhibits a higher binding affinity for the enzyme−substrate complex, i.e., FIXa/FVIIIa/Ca^{2+}/phospholipids/FX complex ($K_i' = 6.2$ nM) than for the enzyme complex, i.e., FIXa/FVIIIa/Ca^{2+}/phospholipids ($K_i = 16.5$ nM). The same inhibitory kinetic mechanism is valid in platelet-mediated FIXa-catalyzed FX activation ($K_i' = 5.9$ nM and $K_i = 12.6$ nM, respectively). The fact that NP-2 increases the concentrations (EC_{50}) of FIXa, FVIIIa, and phospholipid vesicles required for half-maximal rates of FX activation suggests that NP-2 interferes with the functioning of all three major components of the intrinsic FX-activating complex. NP-2 was found to inhibit FX activation when either phospholipids or FVIIIa are present, but not in the absence of both factors. Taken together, we conclude that NP-2 is a unique, potent, and highly specific inhibitor of the intrinsic FX-activating complex that inhibits FIXa bound either to the phospholipid or activated platelet surface or to the cofactor FVIIIa by interfering with the assembly of FX-activating complex on these surfaces.

Hematophagous animals have evolutionally developed various mechanisms to counter the coagulation of the host during blood sucking. The anticoagulant property of nitrophorin-2 (NP-2)[1] or prolixin-S, a salivary gland protein of the blood-sucking insect *Rhodnius prolixus*, was first described three decades ago (*1*). A crude extract of salivary glands was able to prolong the kaolin-cephalin clotting time without affecting the prothrombin time, suggesting that NP-2 is an inhibitor of the intrinsic blood coagulation pathway. On the basis of clotting assays, it was suggested that NP-2 is a factor VIII (FVIII) inhibitor (*1*). This ∼19.7 kDa salivary anticoagulant protein has recently been purified and characterized as an inhibitor for FIXa-catalyzed FX activation (*2*−*4*). However, the mechanism by which NP-2 inhibits this reaction has not been determined.

Activation of blood coagulation FX is a critical step in hemostasis because of its unique position at the intersection point of the intrinsic and extrinsic pathways (*5*). Evidence of the importance of the intrinsic FX activation pathway is provided by the severe bleeding disorders hemophilia A and B due to deficiencies of FVIII and FIX, respectively. Although the molecular basis of the FX-activating complex has not been fully elucidated, it has been commonly accepted that the surface-bound enzyme FIXa in the presence of its cofactor FVIIIa and Ca^{2+} proteolytically converts FX to its activated form FXa (*6*). Negatively charged phospholipid vesicles have been recognized as such a surface in in vitro studies (*7*), while in vivo, the activated platelet membrane has been implicated as the physiological locus supporting the assembly of the FX-activating complex by the intrinsic mechanism (*8*−*10*). Alternatively, FX can be converted to FXa by the extrinsic FX-activating complex which consists of FVIIa, tissue factor, Ca^{2+}, and a phospholipid surface exposed following tissue injury (*11*−*13*).

In the present study, we have investigated the kinetic mechanism by which NP-2 specifically inhibits FX activation by FIXa/FVIIIa/phospholipids complex. Ultimately, a complete understanding of the inhibitory mechanism may help

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[1] Abbreviations: NP-2, nitrophorin-2; FIXa, activated factor IX; FX, factor X; rFVIII, recombinant human factor VIII; PS, L-α-phosphatidyl-L-serine; PC, L-α-dioleoylphosphatidylcholine; RVV, Russell's viper venom; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; S-2765, chromogenic substrate *N*-α-benzyloxycarbonyl-D-arginyl-glycyl-arginyl-*p*-nitroaniline; S-2302, chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline; S-2366, chromogenic substrate H-D-prolyl-phenyl-arginyl-*p*-nitroaniline; S-2238, chromogenic substrate H-D-Phe-pipecolyl-arginyl-*p*-nitroaniline; EI, enzyme−inhibitor; ESI, enzyme−substrate−inhibitor; ES, enzyme−substrate.

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to elucidate the mechanism by which the FX-activating complex is assembled on the surface of phospholipid vesicles or activated platelets.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Human blood coagulation proteins α-thrombin, FIXa, FX, FXIa, FXIIa, kallikrein, and the FX-activating enzyme from Russell's viper venom (RVV) were purchased from Enzyme Research Laboratory (Southbend, IN). Recombinant human factor VIII (rFVIII) was kindly provided by Baxter Healthcare Corp. (Duarte, CA). NP-2 was purified from the salivary glands of *R. prolixus* as previously described (*3*). Phospholipase C, *bacillus cereus*, was purchased from Calbiochem (LaJolla, CA). Bovine brain L-α-phosphatidyl-L-serine (PS) and L-α-dioleoylphosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Birmingham, AL). Chromogenic substrates *N*-α-benzyloxycarbonyl-D-arginyl-glycyl-arginyl-*p*-nitroaniline (S-2765), L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline (S-2366), H-D-prolyl-phenyl-arginyl-*p*-nitroaniline (S-2302), and H-D-Phe-pipecolyl-arginyl-*p*-nitroaniline (S-2238) were obtained from Chromogenics (Mölndal, Sweden). All other materials were analytical grade or the highest grade commercially available.

Concentration and Activity of Proteins. Protein concentrations were determined by measuring light absorption at 280 nm using published extinction coefficients. The amidolytic activities of purified human FXa, FXIa, kallikrein, FXIIa, and α-thrombin were measured using chromogenic substrate-based assays. The cleavage of chromogenic substrates S-2765 (for FXa), S-2366 (for FXIa), S-2302 (for FXIIa and kallikrein), or S-2238 (for α-thrombin) by these proteases was monitored in a V_{max} kinetic microplate reader from Molecular Devices Corp. (Menlo Park, CA) at 405 nm for 10 min at 37 °C using appropriate standard curves for each enzyme. The active-site concentration of FIXa was determined by titration with antithrombin III of known concentrations (*14*).

Preparation of Human Platelets. Venous blood was collected from healthy donors in acid citrate dextrose (71 mM citric acid, 85 mM trisodium citrate, and 111 mM dextrose) anticoagulant (7:1 volume ratio) and centrifuged at 200*g* for 20 min to yield platelet-rich plasma. Platelets were isolated using a modified sequential albumin density-gradient method (*15*) followed by gel filtration on a Sepharose 2B column equilibrated in calcium free 4-(2-hydroxyethyl)-1-piperazine-ethane-sulforic acid (Hepes)/Tyrode's buffer (126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $NaH_2PO_4 \cdot H_2O$, 5.6 mM dextrose, 15 mM HEPES, 0.1% bovine serum albumin, pH 7.4). The isolated platelets were counted electronically using a model ZM Coulter counter (Coulter Electronics, Hialeah, FL) and diluted with HEPES/Tyrode's buffer to desired concentrations.

Preparation of Phospholipid Vesicles. Phospholipid vesicles (PS/PC in a 1:3 molar ratio) were prepared by high-pressure nitrogen extrusion through a 0.1 μm Nucleopore polycarbonate membrane (*16*, *17*). The recovery of resulting vesicles was measured by phosphorus assay (*18*).

Measurement of FX Activation by Chromogenic Assays. FX activation was measured in three different assays as follows.

(1) FX Activation by RVV. FX (1 μM) and RVV (50 nM) were incubated in the presence of $CaCl_2$ (5 mM) at 37 °C for varying times up to 3 h ($t_{1/2}$ = 10 min) during which time >95% FX had been activated. The final product FXa was diluted to various concentrations (0.1−10 nM) to which the chromogenic substrate S-2765 (350 μM) was added. The cleavage rate of S-2675 was measured at 405 nm for 10 min at 37 °C using a V_{max} kinetic microplate reader. A linear calibration curve was generated.

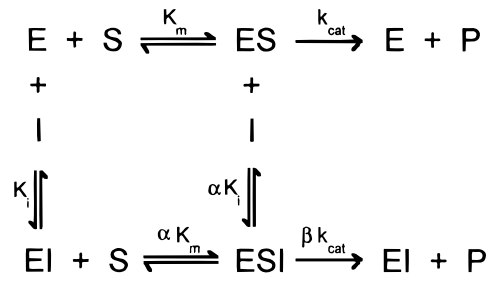
(2) FX Activation by FVIIa/Tissue Factor. FX activation by FVIIa was initiated by adding varying amounts of FX (10−250 nM) to a mixture containing FVIIa (0.5 nM), $CaCl_2$ (5 mM), and thromboplastin, which contains phospholipids and rabbit brain tissue factor. The reaction was stopped after 5 min by adding EDTA (10 mM). The FXa generated was determined by chromogenic assay using S-2765 and quantitated as described above.

(3) FX Activation by FIXa. Experiments examining FIXa-catalyzed FX activation were conducted using the method established in our laboratory with several modifications (*10*). Experiments were carried out at 37 °C either in the presence or absence of a surface (PS/PC or platelets) and cofactor (FVIII). In the presence of a phospholipid surface (0.5 μM PS/PC or 5×10^7/mL platelets), purified human FX at various concentrations (5−150 nM) was incubated with $CaCl_2$ (5 mM) and FVIII (15 units/mL). After human α-thrombin (0.1 unit/mL) was added to activate FVIII and platelets for 1 min, FIXa (0.25 nM) was added to initiate the FX activation. The reaction was stopped by adding ice-cold EDTA (10 mM) after 3 min. Hirudin (2.5 units/mL) was also added to neutralize thrombin. FXa generation was determined by amidolytic assay and quantitated using a standard curve of known concentrations of FXa. FX activation was also conducted in the absence of FVIII (FIXa 10 nM, PS/PC 20 μM, or platelets 1×10^8/mL, and FX 10−300 nM) or in the absence of a surface (FIXa 10 nM in the presence or absence of 30 units/mL FVIII). The reaction was carried out for 3−5 min in the presence of FVIII and 30−60 min in the absence of FVIII. All reactions were conducted at 37 °C in HEPES/Tyrode's buffer (pH 7.4) unless otherwise stated.

Study of the Inhibitory Effect of NP-2 and Its Specificity. First, experiments were carried out to study the time course of FX activation by the intrinsic FX-activating complex in the presence and absence of NP-2. Varying concentrations of NP-2 (2−50 nM) were added to a mixture containing FIXa (20 pM), FVIIIa (15 units/mL), $CaCl_2$ (5 mM), and PS/PC (0.5 μM). The reaction was started by adding FX (100 nM). Aliquots were removed at varying time points and added to EDTA (10 mM) containing buffer. FXa activity was measured by a chromogenic assay using S-2765 (350 μM). Experiments were also conducted to determine the time course of FX activation in the presence of either cofactor (FVIIIa or PS/PC) or in the absence of both cofactors. Under these conditions, higher concentrations of FIXa (20 nM), FX (200 nM), FVIII (100 units/mL), and PS/PC (20 μM) were utilized.

Experiments were done to further determine the inhibitory capacity of NP-2 in the presence or absence of phospholipids and FVIIIa. Under each condition, various concentrations of NP-2 (10−600 nM) were added to the mixture containing a fixed amount of FIXa (0.25 nM or 10 nM in the presence

Scheme 1



or absence of FVIIIa, respectively) with or without FVIIIa (15 units/mL) or PS/PC (0.5 μM or 10 μM in the presence or absence of FVIIIa, respectively) before the reaction was started by the addition of FX (100 nM). FX activation was monitored for 3 min in the presence of FVIIIa or 30 min in the absence of FVIIIa. Residual activity was plotted as a function of NP-2 concentration. To study the specificity of the NP-2 inhibitory effect, varying concentrations of FX (5−100 nM) were incubated with RVV (1 nM) or FVIIa/TF (0.5 nM) in the absence or presence of NP-2 (250 nM) for 1 h to determine if NP-2 has inhibitory effects on these alternative FX activation reactions. NP-2 was also tested to determine whether it affects another surface-dependent reaction, i.e., prothrombin activation. Briefly, a fixed amount of prothrombin (10 nM) was incubated with $CaCl_2$ (5 mM), PS/PC (0.5 μM), or platelets (5×10^7/mL) in the presence or absence of NP-2 (250 nM) and in the presence or absence of thrombin-activated FV (0.1 nM). The reaction was initiated by the addition of FXa (0.1 nM) and stopped by EDTA (10 mM) after 10 min. Thrombin generation rate was determined by measuring cleavage of S-2238 (300 μM) at 405 nm for 10 min using a microplate reader.

Kinetic Analysis of the Inhibition by NP-2 of FIXa-Catalyzed FX Activation. Data obtained from the inhibition studies of NP-2 on FIXa-catalyzed FX activation were fit to the following general scheme for enzyme-catalyzed reactions including mixed-type inhibition (*19*, *20*) (Scheme 1).

E, S, and I denote enzyme (FX-activating complex), substrate (FX), and inhibitor (NP-2), respectively. Michaelis−Menten parameters, K_m and V_{max}, for FIXa-catalyzed FX activation under different conditions (in the presence or absence of FVIII and in the presence or absence of phospholipid vesicles or platelets) were determined by nonlinear least-squares regression analysis according to the Michaelis−Menten equation $V = V_{max}K_m/(K_m + [S])$ using the computer program KaleidaGraph 3.0 run on a Macintosh Quadra 900 Computer (Apple Computer, Inc., Cupertino, CA). The turnover rate (k_{cat}) can be calculated by equation $k_{cat} = V_{max}/E$, where E is the enzyme concentration. The parameter α is a factor by which K_m changes when the inhibitor occupies the enzyme. K_i and K_i' ($K_i' = \alpha K_i$) are inhibition constants, i.e., the dissociation constants of the enzyme−inhibitor (EI) and enzyme−substrate−inhibitor (ESI) complexes, respectively. The key characteristic for a mixed-type inhibitor is that the ESI complex, in addition to the enzyme−substrate complex (ES), can also break down to products, but at a lower rate determined by the value of the factor β. Thus, at saturating concentrations of inhibitor, the rate of substrate turnover does not decrease to zero. The inhibition parameters (α, β, K_i, and K_i') were determined by the specific velocity plot method described by Baici (*20*)

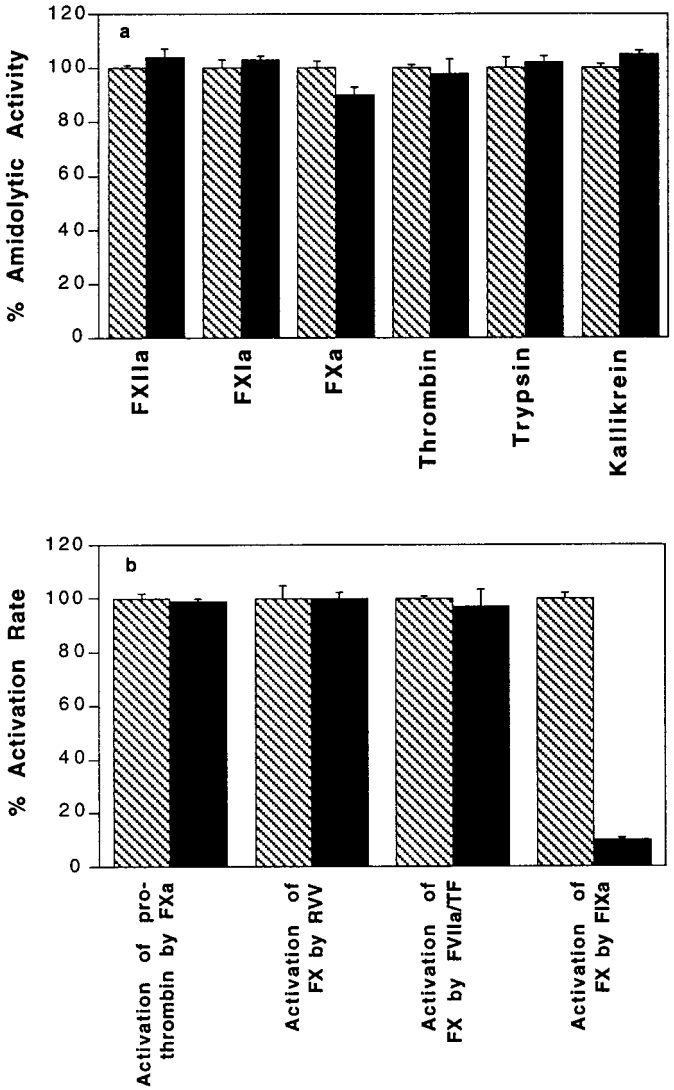


FIGURE 1: Specificity of NP-2 inhibitory effect. (a) Amidolytic activities of various proteases (5 nM) were measured by their ability to cleave appropriate chromogenic substrates in the absence (shaded bar) or presence (solid bar) of NP-2 (2.5 μM). (b) Activation of prothrombin by FXa and activation of FX by RVV, FVIIa/TF, or FIXa/FVIIIa/surface in the absence (shaded bar) or presence (solid bar) of NP-2 (200 nM) were carried out as described under Experimental Procedures. Results shown are means ± SEM of four observations.

based on the following general inhibition velocity equation.

$$\frac{V_o}{V_i} = \frac{1 + \frac{[I]}{K_i}}{1 + \frac{\beta[I]}{K_i'}} + \frac{[I]\left(\frac{1}{K_i'} - \frac{1}{K_i}\right)}{1 + \frac{\beta[I]}{K_i'}} \frac{[S]}{K_m + [S]'} \qquad (1)$$

where $[S]/([S] + K_m)$ is defined as the specific velocity; V_o/V_i is a ratio of uninhibited initial velocity and inhibited final velocity; and [S] and [I] are concentrations of the substrate and the inhibitor, respectively.

RESULTS

Specificity of the Anticoagulant Effect of NP-2. Kinetic analyses using appropriate peptidyl chromogenic substrates showed that NP-2 at concentrations of up to 2.5 μM had no direct inhibitory effects on the amidolytic activities of 5 nM

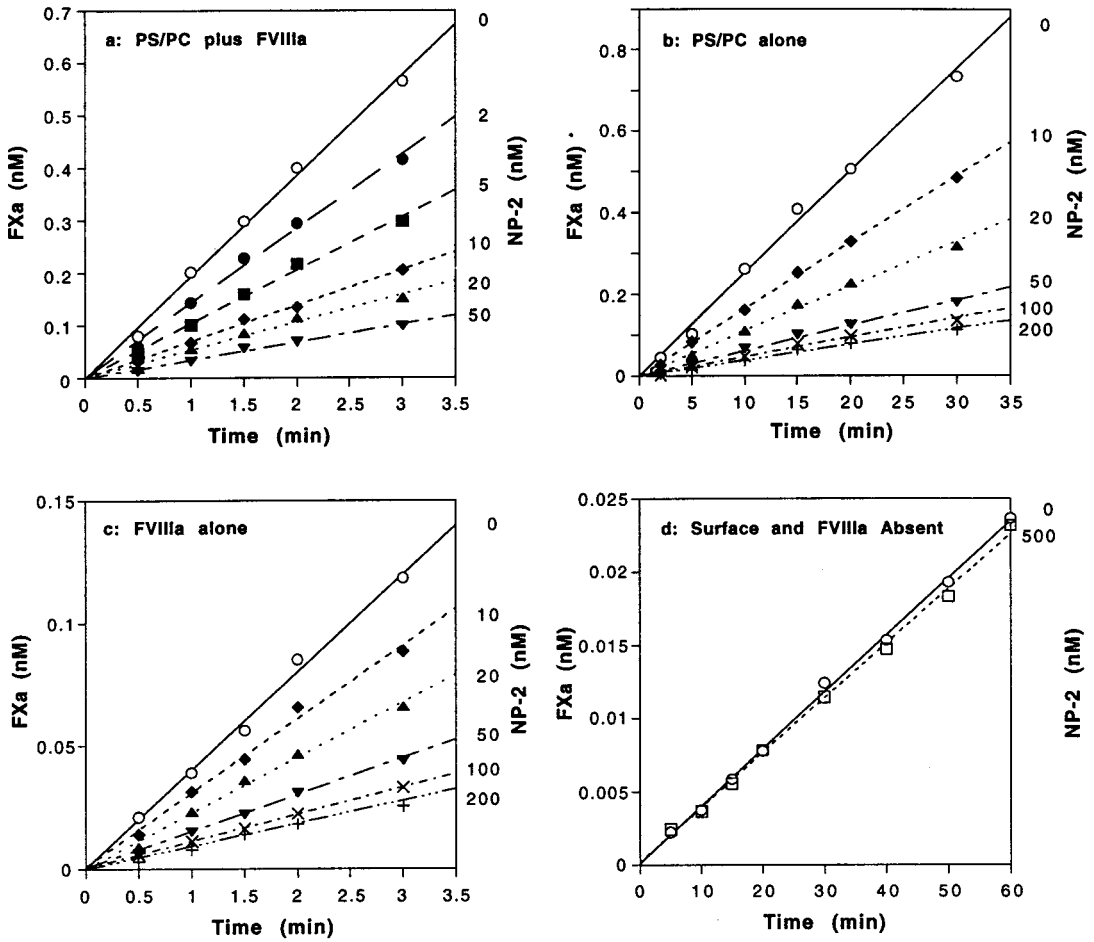


FIGURE 2: Time courses of inhibition by NP-2 of FX activation. (a) FX activation was conducted in the presence of both FVIIIa and phospholipid vesicles. Varying concentrations of NP-2 (2−50 nM) were added to a mixture containing FIXa (20 pM), FVIIIa (15 units/mL), $CaCl_2$ (5 mM), and PS/PC (0.5 μM). The reaction was started by adding FX (100 nM). Aliquots were removed at varying time points and added to EDTA (10 mM) containing buffer. FXa activity was determined by measuring the cleavage rate of S-2765 (350 μM). Experiments were also conducted (b) in the presence of either phospholipids alone or (c) FVIIIa alone and (d) in the absence of both phospholipids and FVIIIa. Under these conditions, FIXa (20 nM), FX (200 nM), FVIIIa (30 units/mL), and PS/PC (20 μM) were utilized.

FXIIa, kallikrein, FXIa, FXa, or thrombin (Figure 1a). At 200 nM, NP-2 did not inhibit FX activation by RVV, or FX activation by the extrinsic pathway component FVIIa in the presence of tissue factor, or prothrombin activation by FXa in the presence of FVa (Figure 1b). To rule out the possibility that NP-2 inhibits coagulation by simple binding to the phospholipid surface and competition with binding of coagulation factors, the experiments examining prothrombin activation were intentionally carried out at low concentrations of phospholipids (0.5 μM), FXa (0.1 nM), FVa (0.1 nM), and prothrombin (10 nM). No inhibitory effect of high concentrations of NP-2 (250 nM) was observed in either the absence or the presence of FVa. In contrast, 200 nM NP-2 resulted in 90% inhibition of FIXa-catalyzed FX activation in the presence of FVIIIa and phospholipids (Figure 1b). We also carried out SDS−PAGE analysis of NP-2 inhibition of FX activation by FIXa. FX activation by FIXa in the presence of FVIIIa and phospholipid vesicles to generate α-FXa and β-FXa is time dependent (data not shown). NP-2 (50 nM) prolonged the transition from FX to FXa, in confirmation of the results shown in Figure 1 (data not shown). Thus NP-2 is a specific intrinsic pathway inhibitor of FIXa-catalyzed FX activation.

Inhibition by NP-2 of FX Activation by the Intrinsic FX-Activating Complex. The time course of FX activation by FIXa under different conditions was examined in the presence of increasing concentrations of NP-2 (Figure 2). Concentration-dependent inhibitory effects of NP-2 on FX activation were observed in the presence of both FVIIIa and phospholipid vesicles (Figure 2a), in the presence of phospholipids alone (Figure 2b) and in the presence of FVIIIa alone (Figure 2c), but not in the absence of phospholipids and FVIIIa (Figure 2d). Thus, the inhibitory effect of NP-2 on FIXa-catalyzed FX activation is either surface-dependent or FVIIIa dependent, and NP-2 does not affect FIXa activity in solution.

Additional inhibition studies have been conducted to ascertain the potency of the NP-2 effect on FX activation by FIXa. NP-2 did not effectively inhibit solution-phase FX activation by FIXa in the absence of FVIIIa, whereas partial inhibition by NP-2 (IC_{50} = 50 nM) was observed when FVIIIa was present (Figure 3a). This demonstrates that NP-2 does not significantly affect FIXa activity in solution unless FVIIIa is present, in confirmation of the results shown in Figure 2. To rule out the possibility that our protein preparations may contain traces of phospholipid contamination, we pretreated FIXa, FX, and FVIII with phospholipase C (5 units/mL). We found that such treatment did not affect the rate of the FVIIIa-mediated FX activation in the absence of a surface (data not shown). In contrast, similar treatment

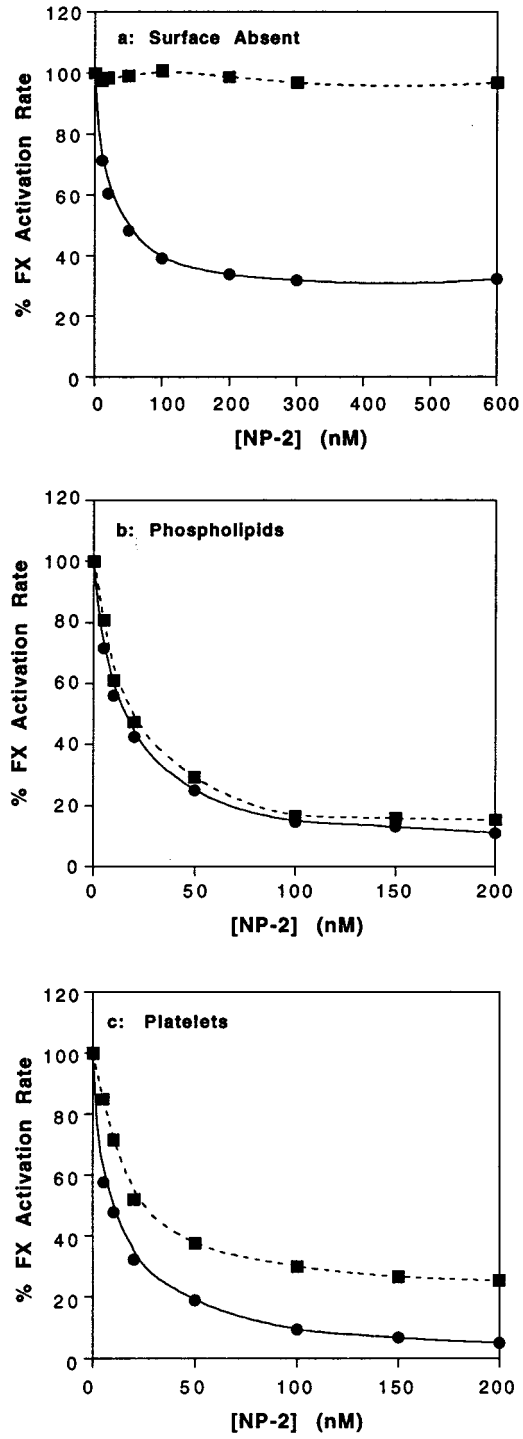


FIGURE 3: Concentration-dependent inhibition by NP-2 on FIXa-catalyzed FX activation. FX activation was performed in (a) the absence of a membrane surface; (b) in the presence of phospholipid vesicles; or (c) in the presence of activated platelets as described in Experimental Procedures. In each case, FVIIIa (15 units/mL) was either present (●) or absent (■). Varying concentrations of NP-2 (10−600 nM) were added into a mixture containing FX (150 nM) and $CaCl_2$ (5 mM). In some experiments, FVIII (preactivated by 0.1 unit/mL thrombin), PS/PC (0.5 or 20 μM, in the presence of FVIIIa and PS/PC or PS/PC alone, respectively), or activated platelets (5×10^7/mL) were added. FIXa (0.25 or 10 nM in the presence or absence of FVIIIa, respectively) was added to start FX activation. The reaction was carried out at 37 °C for 3 min (in the presence of FVIIIa) or 30 min (in the absence of FVIIIa) and stopped by addition of 10 mM EDTA. The amount of FXa generated was measured by a chromogenic assay described in the Experimental Procedures. Each plot represents average data from three to eight independent experiments.

of PS/PC vesicles eliminated the surface-dependent enhancement of FIXa-catalyzed FX activation (data not shown). This supports the concept that FVIIIa provides a surface-like function in the absence of phospholipids (*21*).

The presence of a phospholipid surface further potentiates the inhibitory effect of NP-2 on FIXa-catalyzed FX activation. In the presence of PS/PC vesicles (0.5 or 10 μM in the presence or absence of FVIIIa, respectively), NP-2 inhibited FX activation in an FVIIIa-independent manner with an IC_{50} of 19 nM in the absence of FVIIIa and 15 nM in the presence of FVIIIa (Figure 3b). When activated platelets (5×10^7/mL) were used as surface, the concentration-dependent effect of NP-2 was more potent in the presence of FVIIIa with an IC_{50} of 9 nM, compared to an IC_{50} of 26 nM in the absence of FVIIIa (Figure 3c). We therefore conclude that either FVIIIa or phospholipids or both are required for the activity of NP-2 as a potent inhibitor of FIXa-catalyzed FX activation.

Kinetic Mechanism by Which NP-2 Inhibits FIXa-Catalyzed FX Activation. The effects of NP-2 on FIXa-catalyzed FX activation in the presence or absence of surfaces (either phospholipid vesicles or activated platelets) and in the presence or absence of FVIIIa were determined in experiments in which substrate (FX) concentration was varied (Figure 4). At increasing NP-2 concentrations, families of hyperbolic curves were obtained in the presence of both FVIIIa and phospholipid vesicles (Figure 4a) or activated platelets (Figure 4c), with phospholipids alone (Figure 4e), or with FVIIIa alone (Figure 4g). The values of K_m and V_{max} obtained from these experiments were plotted as a function of NP-2 concentration (Figure 4, panels b, d, f, and h). Increasing concentrations of NP-2 result in decreases in both K_m and V_{max} of FX activation in the presence of both phospholipid surfaces and FVIIIa (Figure 4, panels b and d) or either factor (phospholipids or FVIIIa) (Figure 4, panels f and h).

To provide an initial analysis of the mechanism of inhibition in the presence of phospholipid vesicles and FVIIIa, double reciprocal plots of the data in Figure 4a were constructed (Figure 5a). It is apparent from the resulting linear plots that NP-2 is neither a purely competitive nor a purely noncompetitive inhibitor (Figure 5a). Segal has provided several methods of analysis for those inhibitors whose mechanisms cannot be determined by conventional plots (*19*). One method suggested is to replot the slopes and intercepts on the *y*-axis from the double reciprocal plot as a function of the inhibitor concentration (Figure 5b). Our data result in hyperbolic curves. Dixon plots of the same sets of data are also hyperbolic (Figure 5c). All these analyses indicate that the inhibitory behavior of NP-2 conforms to a hyperbolic, mixed-type inhibition, in which the inhibitor binds more tightly to the enzyme−substrate complex than to the enzyme, thereby decreasing the K_m (i.e., increasing the apparent affinity for substrate) and k_{cat} (Scheme 1). The values of α, β, K_i, and K_i' can be calculated using Baici's specific velocity plot method (*20*). The specific velocity (defined under Experimental Procedures) was plotted versus the ratio of V_o/V_i at different concentrations of NP-2 according to eq 1 (Figure 6a). These curves will be linear regardless of the mechanism and will intercept on the two ordinate axes at abscissa values 0 (defined as *a*) and 1 (defined as *b*). Inhibition parameters can be determined from

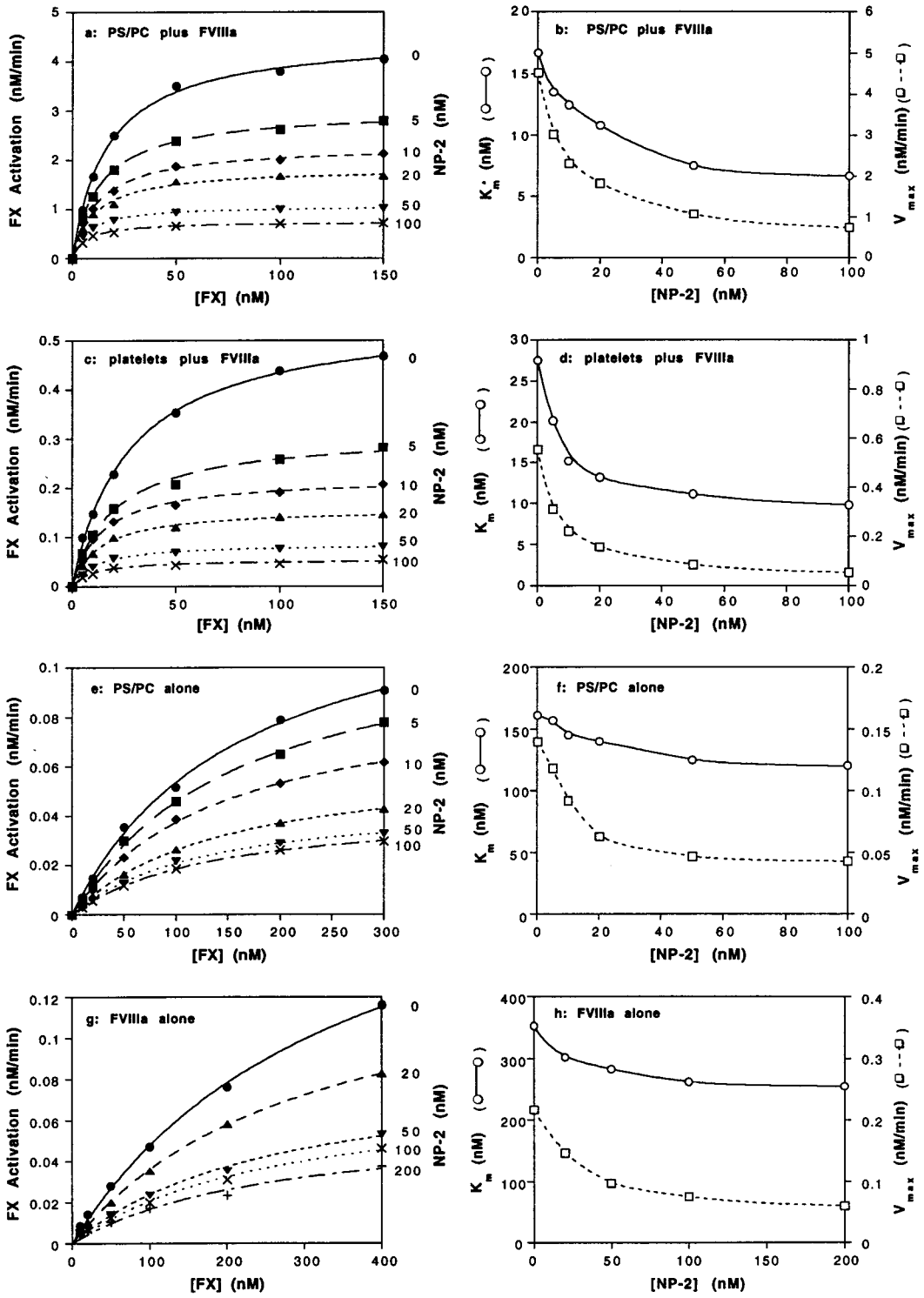


FIGURE 4: Inhibitory effect of NP-2 on the substrate (FX). (a) FX activation by FIXa in the presence of FVIIIa and PS/PC; (c) FVIIIa and platelets; (e) PS/PC alone; or (g) FVIIIa alone were measured. Varying concentrations of NP-2 [(●) 0 nM, (■) 5 nM, (◆) 10 nM, (▲) 20 nM, (▼) 50 nM, (×) 100 nM, (+) 200 nM] were added into a mixture containing FX (5−300 nM), PS/PC (0.5 or 20 μM in the presence or absence of FVIIIa, respectively) or activated platelets (5×10^7/mL), in the presence or absence of FVIIIa (15 units/mL). FIXa (0.25 nM when FVIIIa and PS/PC or FVIIIa and platelets were present, 20 nM when FVIIIa or PS/PC alone were present) was added to start the FX activation. Reactions were stopped by adding cold EDTA (10 mM) at 3 min in the presence of FVIIIa or 30 min in the absence of FVIIIa. K_m (○) and V_{max} (□) were determined under each condition by nonlinear least-squares regression analysis and were shown in panels b, d, f, and h. Each plot represented average data from seven to 11 independent experiments.

a replot (Figure 6b). The kinetic mechanism was revealed to be the same under different conditions, i.e., in the presence or absence of either PS/PC vesicles or platelets and in the presence or absence of FVIIIa (Table 1). The values of α and β in the presence of platelets are smaller than those with phospholipids, suggesting that NP-2 is a more potent inhibitor of platelet-mediated FX activation compared to the PS/PC-mediated reaction. FVIIIa further potentiates NP-2 inhibition of platelet-mediated FX activation. In contrast, FVIIIa does not have a significant effect on NP-2 inhibition when FX activation is PS/PC mediated. Our data show that NP-2 exhibits higher binding affinity to the ES complex, i.e., FIXa/

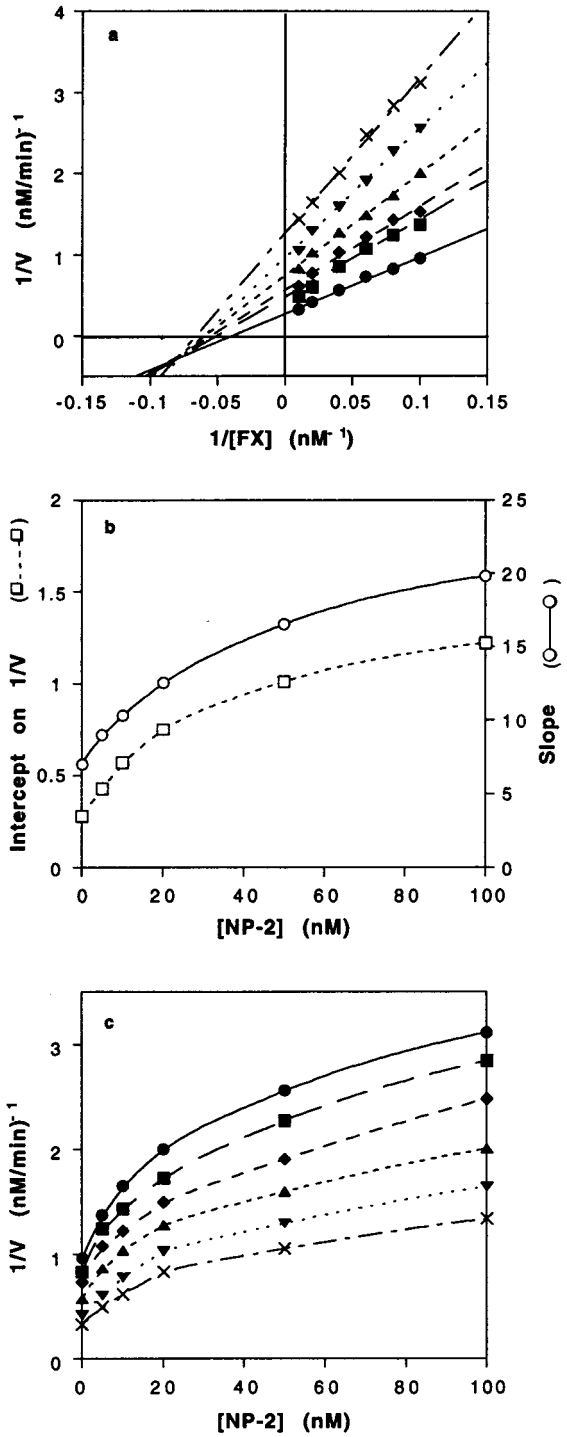


FIGURE 5: Determination of the kinetic mechanism by which NP-2 inhibits FIXa-catalyzed FX activation. Kinetic analysis of FX activation in the presence of both PS/PC and FVIIIa was presented as an example. (a) Lineweaver−Burk plots of data presented in Figure 4a. Varying concentrations of NP-2 [(●) 0 nM, (■) 5 nM, (◆) 10 nM, (▲) 20 nM, (▼) 50 nM, (×) 100 nM] were used. (b) Slopes (○) and intercepts (□) on the *y*-axis from the double reciprocal plot were plotted as a function of NP-2 concentration. (c) Dixon plot of the same sets of data shown in Figure 4a. The concentrations of FX used were as follows: 10 nM (●), 12.5 nM (■), 16.7 nM (◆), 25 nM (▲), 50 nM (▼), and 100 nM (×).

FVIIIa/Ca^{2+}/phospholipids/FX complex ($K_i' = 6.2$ nM) than to the enzyme complex, i.e., FIXa/FVIIIa/Ca^{2+}/phospholipids ($K_i = 16.5$ nM). Data obtained from platelet studies ($K_i' = 5.9$ nM, $K_i = 12.6$ nM) are in excellent agreement with those determined using phospholipid vesicles. We suggest that

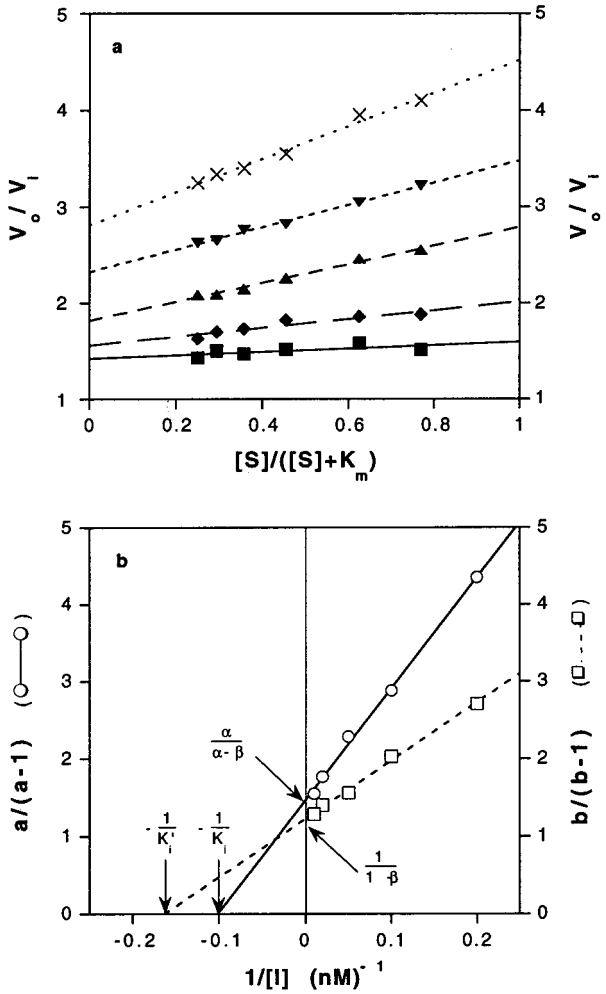


FIGURE 6: Determination of inhibition parameters for NP-2 using a method of specific velocity plot described by Baici (*20*). (a) The ratio of FX activation rate in the absence of NP-2 to the rate in the presence of varying concentrations of NP-2 [(●) 5 nM, (◆) 10 nM, (▲) 20 nM, (▼) 50 nM, (×) 100 nM] was plotted as a function of the specific velocity [S]/([S] + K_m) based on eq 1. (b) Replot of two sets of intercepts on the ordinate axes of Figure 6a at abscissa value 0 (defined as *a*) and 1 (defined as *b*) results in the resolution of α, *β*, K_i, and K_i'.

NP-2 is an inhibitor specific to FIXa bound to the phospholipid or platelet surface, especially in the presence of the cofactor FVIIIa. Although the enzyme complex which can be inhibited by NP-2 may also include a FIXa/FVIIIa or a FIXa/phospholipid complex, the inhibition under these conditions is not as potent as in the presence of both FVIIIa and phospholipids (Table 1).

NP-2 Effect on Each Component of the Intrinsic FX-Activating Complex. The foregoing analysis shows that NP-2 is a specific and potent hyperbolic mixed-type inhibitor of FX activation by FIXa. Data from our kinetic studies show that NP-2 exerts its effect by binding to both the enzyme complex (FIXa/FVIIIa/surface) and to the enzyme−substrate complex (FIXa/FVIIIa/FX/surface). This has prompted us to further investigate the NP-2 effect on the binding of each component of the FX-activating complex. Therefore, the effects of various concentrations of NP-2 on the rate of FX activation were examined in experiments in which the concentrations of the components of the enzyme complex were varied, i.e., FIXa (Figure 7a), phospholipids (Figure 7c), or FVIIIa (Figure 7e). The values of both EC_{50} and

Table 1: Kinetic Parameters of NP-2 Inhibition on FIXa-Catalyzed FX Activation[a]

	K_m (nM)	k_{cat} (min^{-1})	α	β	K_i (nM)	K_i' (nM)
PS/PC alone[b]	130.6 ± 9.7	0.013 ± 0.001	0.56 ± 0.10	0.19 ± 0.04	15.5 ± 2.4	10.1 ± 2.4
PS/PC plus FVIIIa[c]	25.5 ± 3.5	15.54 ± 1.5	0.23 ± 0.05	0.14 ± 0.09	16.5 ± 1.5	6.2 ± 2.2
platelets alone[d]	113.4 ± 12.4	0.004 ± 0.002	0.12 ± 0.08	0.023 ± 0.007	23.3 ± 3.2	7.3 ± 3.2
platelets plus FVIIIa[e]	26.1 ± 2.8	2.76 ± 0.24	0.07 ± 0.03	0.035 ± 0.004	12.6 ± 1.4	5.9 ± 1.3
FVIIIa alone[f]	353.2 ± 20.4	0.009 ± 0.001	0.57 ± 0.08	0.21 ± 0.05	21.6 ± 4.2	15.2 ± 4.1

[a] Footnotes b−f are the experimental conditions for Table 1 (see Experimental Procedures for more details). [b] FIXa 10 nM, PS/PC 20 μM, FX 10−300 nM. [c] FIXa 0.25 nM, PS/PC 0.5 μM, FVIIIa 15 units/mL, FX 5−150 nM. [d] FIXa 10 nM, platelets 1×10^8/mL, FX 10−300 nM. [e] FIXa 0.25 nM, platelets 5×10^7/mL, FVIIIa 15 units/mL, FX 5−150 nM. [f] FIXa 10 nM, FVIIIa 30 units/mL, FX 10−400 nM.

V_{max} were then plotted as a function of NP-2 concentration, where EC_{50} is the concentration of FIXa, phospholipid vesicles, or FVIIIa required for half-maximal rates of FX activation. We found that NP-2 increases the EC_{50} for FIXa (Figure 7b), phospholipid vesicles (Figure 7d), and FVIIIa (Figure 7f) and decreased the rate of FX activation in a concentration-dependent manner under all experimental conditions. Similar results were obtained when activated platelets replaced phospholipid vesicles (data not shown). This suggests that NP-2 interferes with the binding of all the major components of the intrinsic FX-activating complex. Taken together, we conclude that NP-2 effectively binds to the enzyme, FIXa, only in the presence of phospholipid surfaces and/or FVIIIa (Figure 7), thereby preventing the assembly of a productive complex.

DISCUSSION

In the present studies, we have characterized a unique insect anticoagulant protein, NP-2 or prolixin-S. NP-2 exhibited no inhibitory effect on the amidolytic activities of various serine proteases including thrombin, FVIIa, FXIIa, FXIa, FXa, and kallikrein. The specificity of the NP-2 effect is also demonstrated by its inability to inhibit two other surface-dependent reactions: prothrombin activation by the FXa/FVa/phospholipid complex and FX activation by the FVIIa/TF complex. The anticoagulant effect of NP-2 is highly specific for FIXa-catalyzed FX activation.

Our studies have revealed a complicated kinetic mechanism of inhibition by NP-2 of FX activation. This has led to the conclusion that NP-2 is a reversible, mixed-type, hyperbolic inhibitor of FIXa-catalyzed FX activation (Scheme 1). In the absence of NP-2, FIXa forms an enzyme complex with its cofactor FVIIIa on a phospholipid surface (*5*−*7*, *9*−*11*). This enzyme complex converts the substrate FX to FXa. NP-2 decreases V_{max} and k_{cat} and, to a lesser degree, decreases K_m. Our data show that NP-2 binds both to the enzyme complex (FIXa/FVIIIa/surface) to form the EI complex and to the ES complex (FIXa/FVIIIa/surface/FX) to form the ESI complex, but the binding to the latter is more efficient ($K_i > K_i'$, $\alpha < 1$). When the concentration of NP-2 is much higher than K_i', the enzyme is effectively converted into modified forms of the EI and ESI complex. This is reflected in a decrease in the apparent K_m. The turnover rate of the FX/FXase/NP-2 (ESI) complex was much lower than that of the FX/FXase (ES) complex, reflected by a decrease in the apparent V_{max} of the inhibited reaction ($\beta < 1$). To our knowledge, NP-2 is so far the first specific and potent anticoagulant protein whose kinetic mechanism has been identified as a hyperbolic, mixed-type inhibition.

The molecular mechanism by which NP-2 mediates specific inhibition of FIXa-catalyzed FX activation is unknown, but the present studies provide important insights to the identification of the component(s) of the intrinsic FX-activating complex, which is the target of the inhibitory effect of NP-2. The inhibitory effect of NP-2 was observed in the presence of either FVIIIa or a membrane surface (PS/PC vesicles or activated platelets), but not in the absence of both cofactors. NP-2 is most effective and potent as an inhibitor when all the major components of the intrinsic FX-activating complex (the enzyme FIXa, the cofactor FVIIIa, a membrane surface PS/PC or platelets, and the substrate FX) are present. In the absence of both a membrane surface and FVIIIa, NP-2 failed to inhibit FIXa-catalyzed FX activation even at inhibitor concentrations (600 nM, Figure 3a) 100-fold higher than the K_i' (~6 nM) for inhibition of the ES complex in the presence of FVIIIa and either phospholipids or platelets.

One possible interpretation of this result is that NP-2 fails to interact with either FIXa or FX in solution, but that FIXa undergoes a conformational alteration upon exposure to a phospholipid surface and/or FVIIIa that results in the exposure of a high-affinity binding site for NP-2. To evaluate this possibility further, kinetic analyses were carried out to investigate the interaction between NP-2 and each component of the FX-activating complex. Our results demonstrate that the extent of inhibition by NP-2 is a function of the concentrations of FX, FIXa, FVIIIa, and phospholipids added to the reaction mixture (Figures 4 and 7). In the presence of both FVIIIa and phospholipids, or activated platelets, NP-2 decreased the V_{max} and increased the EC_{50} for FIXa (Figure 7, panels a and b), PS/PC vesicles (Figure 7, panels c and d), and FVIIIa (Figure 7, panels e and f). The increase of EC_{50} for FIXa and FVIIIa by NP-2 can be explained by a diminished affinity of FIXa, FVIIIa, or FIXa/FVIIIa for the FX-activating complex on platelets or phospholipids. An increase in the EC_{50} for phospholipid vesicles (or for activated platelets, data not shown) suggests that NP-2 (possibly by binding to allosterically modified FIXa) might decrease the affinity of FIXa for the surface, thus rendering it a less favorable locus for the assembly of the FX-activating complex. The observation that FX activation by RVV or FVIIa/TF is not inhibited by NP-2 (Figure 1) implies that NP-2 does not interact with the enzyme cleavage site on FX since both RVV and FVIIa promote cleavage of the FX molecule at the same site as does FIXa (*22*−*24*). Although FX activation by FIXa was observed in the presence of either FVIIIa or phospholipid surface alone, NP-2 functions most potently when all components of the FX-activating complex are present. This is consistent with the conclusion that simultaneous complex formation of FVIIIa, phospholipids (or platelets), and FX with FIXa is critical for optimal FX activation.

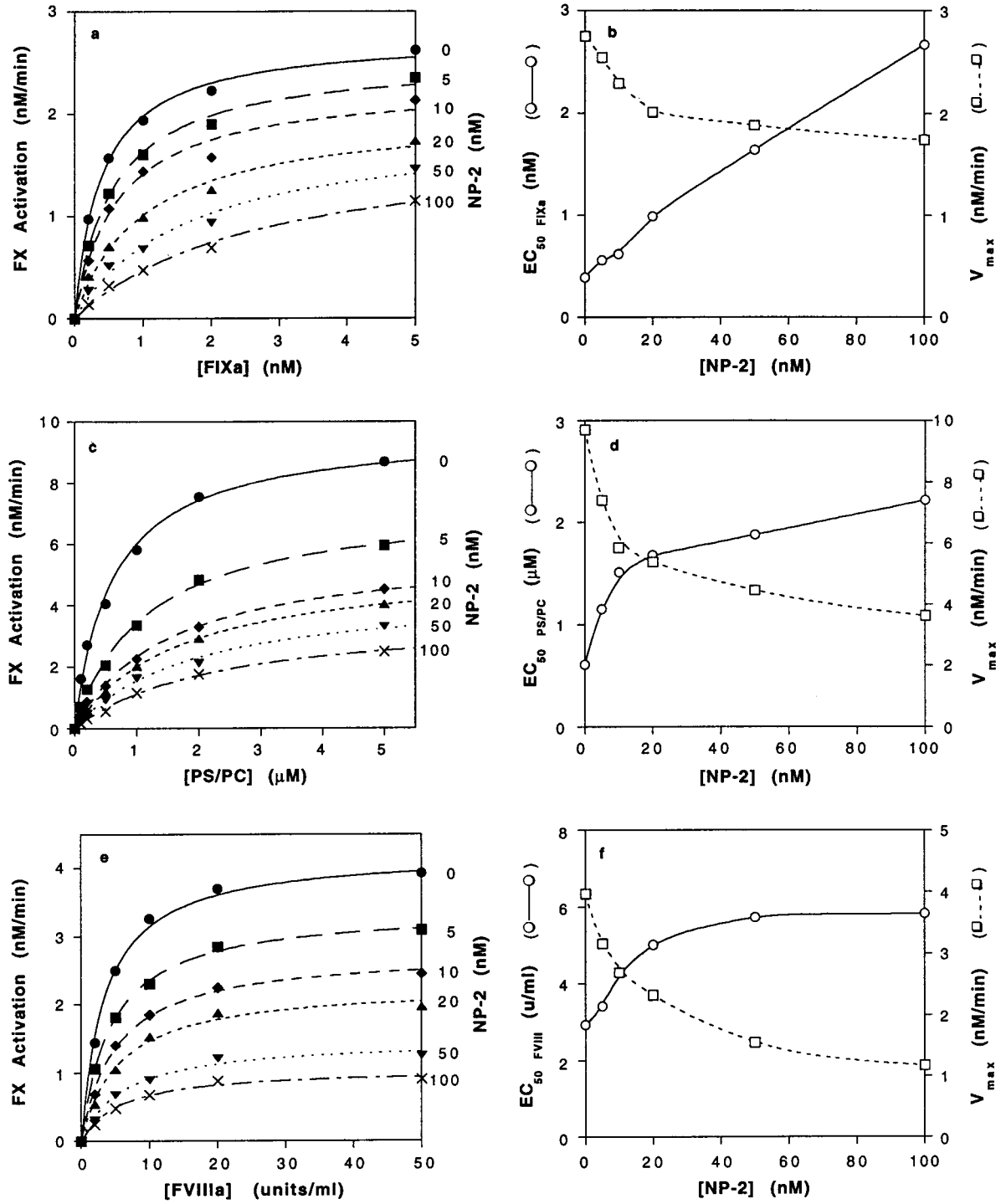


FIGURE 7: Effect of NP-2 on each component of the FX-activating complex. Inhibition by NP-2 on FX activation by FIXa was examined in the presence of FVIIIa and PS/PC in experiments in which one component of the enzyme complex (FIXa, PS/PC, or FVIIIa) was varied. Varying concentrations of NP-2 [(●) 0 nM, (■) 5 nM, (◆) 10 nM, (▲) 20 nM, (▼) 50 nM, (×) 100 nM] were added to mixtures in which the concentration of FIXa (panels a and b), PS/PC (panels c and d), or FVIIIa (panels e and f) was individually varied. FX 150 nM was used in each experiment. Other conditions were as follows: FVIIIa 15 units/mL (panels a and c), PS/PC 0.5 μM (panels a and e), FIXa 0.25 nM (panels c and e). Values EC_{50} (○) and V_{max} (□) are plotted in panels b, d, and f. Each plot represents average data from three to five independent experiments.

Similarities between the mechanism of FX activation and that of prothrombin activation have been suggested by studies of many investigators. Both reactions involve formation of an enzyme complex in which the presence of surfaces (phospholipid vesicles or activated platelets) and a cofactor (FVIIIa or FVa) dramatically enhance the catalytic efficiency (*25*, *26*). However, differences are also suggested by several lines of evidence (*10*, *27*, *28*). Our studies of inhibition of NP-2 on FX activation have further addressed these differences by demonstrating that NP-2 fails to inhibit prothrombin activation.

Although there is ample evidence that complex direct interactions of membrane-bound cofactor, enzyme, and substrate occur (*29*−*34*), the exact molecular mechanisms involved in the assembly of the FX-activating complex are not fully understood. The similar inhibitory effect of NP-2

in the presence of PS/PC and platelets is consistent with the view that the exposure of aminophospholipids on the external membrane of platelets is necessary for FX activation (*35*). Studies on the assembly of the FX-activating complex on the activated platelet surface demonstrate the existence of 500−600 FIXa binding sites/platelet, about half of which are shared with the zymogen FIX and the other half of which are specific for FIXa, occupancy of which is associated with a 20 million-fold acceleration of the catalytic efficiency (k_{cat}/K_m) of FX activation (*8*−*10*). The affinity and stoichiometry of FIXa binding to activated platelets ($K_d = \sim 2.5-3.0$ nM, $n = \sim 500$ sites/platelet) (*9*) are similar to those for the cofactor, FVIII ($K_d = \sim 3.0$ nM, $n = \sim 450$ sites/platelet) (*26*). In the presence of FVIII and FX, the affinity of FIXa binding to platelets is increased ~5-fold ($K_d = \sim 0.5$ nM) (*9*), whereas in the presence of active-site inhibited FIXa and FX, the affinity of FVIII binding to platelets is similarly increased (*36*). Activated platelets also expose a shared, high capacity ($n \sim 20\,000$ sites/platelet), low affinity ($K_d = \sim 470$ nM) binding site for FX and prothrombin (*37*), which is important for enhancing rates of FX activation on the platelet surface (*38*), and the presence of active-site inhibited FIXa and FVIII generate a specific, lower capacity ($n = \sim 1\,200$ sites/platelet), higher affinity ($K_d = \sim 10$ nM) FX binding site, occupancy of which we believe represents the assembly of the ES complex on the platelet surface (*39*). Therefore, it is clear that the assembly of the FX-activating complex on the platelet surface involves the activation-dependent exposure of binding sites for all three components, the enzyme (FIXa), the cofactor (FVIIIa), and the substrate (FX), and that the binding affinity of each component is affected by the presence of the other two components. It is also clear from our kinetic analyses that NP-2 binds reversibly to the FIXa/FVIIIa/surface complex and to the FIXa/FVIIIa/surface/FX complex. The most likely target for NP-2 binding is FIXa that has undergone allosteric modification by interaction with a phospholipid or platelet surface and/or FVIIIa (since NP-2 is unable to inhibit FX activation by FIXa in solution). Further studies will be required to elucidate the precise molecular mechanism of this unique, reversible, specific, mixed-type, hyperbolic inhibitor of FIXa-catalyzed FX activation. The actual mechanism of NP-2 inhibition may involve an even more complicated process than we have presented here. Our studies indicate that the activity of NP-2 is most potent in the presence of all components of the FX-activating complex. The high specificity and potency of the NP-2 effect on FX activation also raises the potential of NP-2 as an antithrombotic agent.

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