# Annexin V Inhibition of Factor IXa-Catalyzed Factor X Activation on Human Platelets and on Negatively-Charged Phospholipid Vesicles†

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ABSTRACT: Annexin V was found to inhibit factor IXa-catalyzed factor X activation on both thrombin-activated human platelets and artificial lipid vesicles containing phosphatidylserine, supporting previous observations of the importance of negatively-charged lipid in potentiating the reaction. Annexin V reduced the $V_{max}$ of factor X activation in factor IXa titrations on the platelet surface with an $IC_{50}$ of 4 nM in the absence of thrombin-activated factor VIII (factor VIIIa), and 4.5 nM in its presence, whereas there was no effect on the $EC_{50,FIXa}$. This noncompetitive inhibition is consistent with interference of recognition of the factor IXa binding site on the platelet, which was confirmed by equilibrium binding of [$^{125}$I]-factor IXa to thrombin-activated platelets where, in the absence of factor VIIIa and factor X, annexin V reduced the number of factor IXa binding sites/platelet from 610 to 320, without changing the $K_{d,app}$. In the presence of factor VIIIa and factor X, annexin V reduced the number of binding sites, but also raised the $K_{d,app}$. Although factor VIIIa improved the affinity of factor IXa for the lipid surface from $K_d$ ~60 nM in its absence to $K_d$ 1 nM in its presence, addition of annexin V to factor IXa titrations on lipid vesicles in the presence of factor VIIIa increased the $EC_{50,FIXa}$ with an $IC_{50}$ of 1.5 nM, without affecting the $V_{max}$. These data provide evidence that factor IXa, although requiring negatively-charged phospholipid for part of its binding site, is accommodated differently on platelets and on artificial vesicles.

Blood platelets provide a physiologic surface important for efficient assembly of enzymatic complexes involved in blood coagulation. The nature of that surface has proven difficult to ascertain. Since negatively-charged phospholipid vesicles can serve as an *in vitro* surface catalyst for factor X (FX)[1] activation and thrombin generation, it has been assumed that negatively-charged lipids are essential for enzymatic complex assembly *in vivo*. Elegant work by Bevers et al. (1982, 1983), demonstrating the activation-dependent appearance of negatively-charged phospholipids on the surface of platelets coincident with their ability to support thrombin generation addressed the problem that negatively-charged lipids are normally confined to the cytoplasmic face of cell membranes and would not therefore be available for coagulation events in the absence of cell rupture (Chap et al., 1977; Schick et al., 1976). However, high concentrations of strong platelet agonists were necessary for substantial exposure of negatively-charged lipids. In contrast, it has been demonstrated (Ahmad et al., 1989a) that FX activation by the enzyme FIXa in the presence of its cofactor FVIIIa is maximal on platelets activated by thrombin at 0.1 U/mL, a concentration of thrombin which resulted in only a small exposure of negatively-charged phospholipids (Bevers et al., 1982, 1983). Since the molar ratio of negatively-charged phospholipids in lipid vesicles used *in vitro* must approach 30−40% for optimal binding and surface catalysis (Beals et al., 1989), clearly a random appearance, following activation, of negatively-charged phospholipids representing a minor proportion of outer leaflet lipids and proteins cannot fully explain the formation of platelet binding sites for the enzymatic complex.

In order to determine whether differences exist between activated platelets and negatively-charged phospholipid vesicles in their ability to support FX activation, annexin V was used as an inhibitor of the negatively-charged lipid component of the reaction. Annexin V is a member of a family of related intracellular molecules with calcium and negatively-charged lipid binding properties (Huber et al., 1990a; Romisch & Heimberger, 1990). The family structure consists of four conserved domains, all of which bind calcium with a motif that is different from the more familiar E-F hand motif of calmodulin (Huber et al., 1990b). The functions of the members of this family are unknown, but are thought to include a role in endocytosis and exocytosis, vesicle fusion, and microvesiculation (Funakoshi et al., 1987). They are known to interfere with *in vitro* coagulation reactions (Reutlingspergen et al., 1988; Romisch et al., 1990), but since only extremely minute amounts have been found in plasma (Romisch et al., 1992), it is unknown whether this is physiologically relevant. Nonetheless, this phenomenon

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[1] Abbreviations: factor X (FX); activated factor X (FXa); recombinant human factor VIII (rFVIII); phosphatidylserine (PS); phosphatidylcholine (PC); phospholipid vesicles composed of PS and PC at a molar ratio of 1:3 (1:3 PS/PC vesicles); bovine serum albumin (BSA); polyacrylamide gel electrophoresis (PAGE); chromogenic substrate *N*-α-((benzyloxy)carbonyl)-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride (*N*-α-Cbo-D-Arg-Gly-Arg-pNA-2HCl) (S2765).

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can be used to explore the role of negatively-charged lipids in the support of coagulation reactions (Sugimua et al., 1994) with certain caveats.

Annexin V is known to self-associate on a lipid surface into trimers (Conche et al., 1992). At high concentrations, annexin V forms a meshwork on a lipid vesicle that deforms the spherical surface into planar facets (Mosser et al., 1991). There is no proof that annexin V interacts with extracellular proteins (Van Heerde et al., 1994), but deformation of the lipid membrane could have profound effects on the lipid environment of membrane proteins and, therefore, on protein function. For this reason, low concentrations of annexin V were used in an attempt to confine analyses to initial effects on lipid only.

In this study, FIXa and FVIIIa were titrated into FX activation reactions performed on both thrombin-activated platelets and artificial vesicles composed of phosphatidylserine (PS) and phosphatidylcholine (PC) at a molar ratio of 1:3 (1:3 PS/PC vesicles) at various annexin V concentrations to determine the effect that occupying lipid sites might have on the $V_{max}$ and the concentrations of enzyme and cofactor necessary for half-maximal FX activation. Equilibrium binding studies with normal human platelets and competition binding using radioiodinated FIXa were performed in the presence and absence of FVIIIa and FX, and in the presence and absence of annexin V, to correlate the effects of the lipid competitor on availability of enzyme binding sites with its effects on FX activation.

## EXPERIMENTAL PROCEDURES

*Platelet Preparation*. Ten parts of venous blood were collected into one part acid citrate dextrose anticoagulant (ACD: trisodium citrate·$2H_2O$ 12 mM; citric acid monohydrate 10 mM; dextrose 15 mM) and centrifuged at 200*g* at room temperature for 20 min. The recovered platelet-rich plasma to which was added apyrase to 0.25 U/mL (Sigma Chemical Co., St. Louis, MO) was incubated for 10 min at 37 °C before centrifugation at 800*g* for 20 min at room temperature. The platelet pellet, resuspended in a 15 mM HEPES-buffered Tyrodes solution (NaCl 0.13 M, KCl 3 mM, MgCl 1 mM, monosodium phosphate 0.4 mM) (buffer A), at pH 6.5, with bovine serum albumin (BSA) (2 mg/mL) and apyrase (0.25 U/mL), was incubated for 10 min at 37 °C before centrifugation for 10 min at 800*g*. The pellet, resuspended in the same buffer, was incubated for 10 min at 37 °C and chromatographed over Sepharose 2B-CL (Sigma Chemical Co., St. Louis, MO) equilibrated with buffer A at pH 7.2 containing BSA 2 mg/mL, as described previously (Ahmad et al., 1989a). The platelet peak was pooled and counted on a Coulter Counter (Coulter Corp., Hialeah, FL), and the gel-filtered platelets were stored at room temperature, with occasional agitation, and incubated at 37 °C for 5 min before an assay. Platelets were assayed within 3 h of gel filtration.

*1:3 PS/PC Phospholipid Vesicles*. Large unilamellar vesicles were prepared according to Mayer (Mayer et al., 1986). Porcine brain PS and L-α-dioleoyl-PC (Avanti Polar Lipids, Alabaster, AL), stored in chloroform at −20 °C, were mixed 1:3 in a clean glass test tube, and solvent was evaporated under nitrogen and replaced by benzene. Samples were lyophilized and then hydrated with buffer A, pH 7.2, over 30 min with occasional vortexing. Vesicles were disrupted by repeated freeze/thaw cycles before repeated extrusion through 0.1 μm polycarbonate filters (Costar Corp., Cambridge, MA) by nitrogen pressure through a custom-designed device as described by Hope et al. (1985). The resulting vesicles represented a 95−98% recovery of lipid as determined by phosphorus assay (Amador & Urban, 1972) and could be stored for months at 4 °C with no loss of activity. Data were collected using five different preparations of extruded vesicles. Each preparation of vesicles was titrated into the FXa generation assay to verify that the concentrations of lipid used remained limiting.

*Proteins*. FIX, FX, and FXIa were purchased from Enzyme Research Laboratories (South Bend, IN) dissolved in 20 mM Tris, 150 mM NaCl, 1 mM benzamidine. Purity was assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and protein concentration was confirmed by bicinchoninic acid protein assay (BCA) from Pierce Chemical Co. (Rockford, IL) and/or by clotting assay (Proctor & Rapaport, 1961). FIX (10 μM) was activated to FIXa by incubation with FXIa at a 50:1 molar ratio in 20 mM Tris, 150 mM NaCl, pH 7.8, containing $CaCl_2$ (5 mM) at 37 °C for 2 h, as described previously (Ahmad et al., 1989a), quick-frozen in small aliquots, and stored at −80 °C for no more than 30 days. Completion of activation was assessed by SDS−PAGE. FX was dialyzed free of benzamidine and occasionally absorbed with soybean trypsin inhibitor coupled to Affigel-15 beads (Biorad Laboratories Inc., Hercules, CA) to remove any possibly contaminating FXa. Recombinant human FVIII (rFVIII) was kindly provided by Baxter Healthcare Corp. (Duarte, CA), stored in 10 mM HEPES, 0.5 M NaCl, 5 mM $CaCl_2$, pH 6.5, and activity was determined by a one-stage clotting assay (Proctor & Rapaport, 1961). Human placental annexin V was kindly supplied by Dr. Kazuo Fujikawa, Department of Biochemistry, University of Washington, Seattle, WA. Human α-thrombin (Sigma Chemical Co., St. Louis, MO) was stored at 200 U/mL at −80 °C. The thrombin receptor hexapeptide SFFLRN-amide was synthesized using ((9-fluorenyl)methoxy)-carbonyl (FMOC) chemistry on an Applied Biosystems 430A synthesizer, and by reverse phase HPLC was purified to >99.9% homogeneity. All proteins were stored in small aliquots at −80 °C and thawed only once before use.

*FXa Generation Assay*. FXa generation was assayed by a modification of our previously published method (Ahmad et al., 1989b; Rawala-Sheikh et al., 1990). All proteins were diluted in 50 mM HEPPS, 175 mM NaCl, pH 8.1 (buffer B). For assay in the absence of FVIIIa, platelets or lipid vesicles in buffer A containing BSA 1 mg/mL were combined with annexin V or buffer B, FIXa at various concentrations, and $CaCl_2$ (5 mM) in microtiter wells. The plate was prewarmed at 37 °C before a further 5 min incubation with thrombin (0.1 U/mL), and the reaction was begun by addition of FX. After a 20 min incubation, the reaction was stopped by addition of EDTA (10 mM) and removal onto ice. For assay in the presence of FVIIIa, the rFVIII was added to the wells containing the surface, FIXa, and inhibitor or buffer. Activation of platelets was by thrombin receptor peptide at 20 μM for 4 min, before addition of thrombin at 0.1 U/mL for 1 min to activate the FVIII. The reaction was begun by addition of FX and stopped after 3 min by addition of EDTA and removal of the plate as above. Fluid phase reactions were performed

in the absence of FVIIIa, substituting buffer for a surface. Reactions were proven to be linear with respect to product formation under these conditions.

The FXa generated in all reaction mixtures was assayed for its rate of hydrolysis of chromogenic substrate *N*-α-((benzyloxy)carbonyl)-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride (*N*-α-Cbo-D-Arg-Gly-Arg-pNA-2HCl) (S2765) (AB Kabi Diagnostica, Stockholm, Sweden). Aliquots diluted into buffer B were prewarmed before addition of substrate to 300 $\mu$M, and the hydrolysis was followed kinetically on a ThermoMax microplate reader (Molecular Devices Corp., Menlo Park, CA) using the manufacturer's Softmax software. Reactions were assayed in duplicate at dilutions which assured initial rates of chromogenic substrate hydrolysis, and the rates of hydrolysis were converted to FXa concentration by comparison to a standard curve resulting from hydrolysis of S2765 by known concentrations of FX fully activated by Russell's Viper Venom (Sigma Chemical Co., St. Louis, MO). Data were collected into an Excel spreadsheet (Microsoft Corp., Redmond, WA) where velocity results were converted to nM FXa generated per minute. Fluid phase FX hydrolysis, which constituted no more than 10% of the total, was subtracted from total platelet- or phospholipid-potentiated FX activation performed in the absence of FVIIIa. Data from three to six experiments were averaged for each analysis.

Titration curves were generated by KalaidoGraph (Synergy Software, PCS Inc., Reading, PA) run on a Macintosh Quadra 900 (Apple Corp., Cupertino, CA), using a nonlinear least squares fit of data points to an equation for a rectangular hyperbola: $y = ax/(b + x)$. Inhibition curves were generated also on KalaidoGraph, comparing the fit of data points to a theoretical curve for simple competition: $y = a/(x + a)$. Data were fitted to Dixon plots by linear analysis, and the resulting slopes were replotted for further insight into the mechanism of inhibition as described by Segel (1975).

*Radiolabeling of FIX*. FIX was iodinated by the Iodogen (Pierce Chemical Co., Rockford, IL) method as reported previously (Tuszynski et al., 1980). Specific activity was determined from cpm/volume and from protein concentration, and was found to be in the range $(2-2.5) \times 10^6$ cpm/$\mu$g. [$^{125}$I]-FIX was activated as described above. SDS−PAGE of iodinated activated protein showed a single band at 45 kDa when electrophoresed under nonreducing conditions, and a heavy chain and a light chain at 28 and 18 kDa, respectively, when electrophoresed under reducing conditions (data not shown).

*Binding Studies Using [$^{125}$I]-FIXa*. Equilibrium binding studies were performed as reported previously (Ahmad et al., 1989a). Duplicate reaction mixtures containing gel-filtered platelets, thrombin (0.1 U/mL), varying concentrations of radiolabeled FIXa, and annexin V (5 nM), where desired, were incubated for 5 min at 37 °C. FVIII (2 U/mL) and FX (1.5 $\mu$M) were added when desired, and binding was initiated by addition of $CaCl_2$ (5 mM). After a 20 min incubation at 37 °C, the reaction was quenched by removal to ice and immediate centrifugation of aliquots through silicone oil for 5 min at maximum speed in a Beckman microcentrifuge. The pellets in the oil phase were snipped free and counted along with the supernatants in a Wallac $\gamma$-counter (Wallac Oy, Turku, Finland). Results in cpm were converted to amount of protein bound. Specific binding was determined by subtraction from total binding of nonspecific binding measured in the presence of a 100-fold excess of cold FIXa.

Competition binding studies were performed as reported previously (Ahmad et al., 1992). Duplicate reaction mixtures containing gel-filtered platelets, thrombin (0.1 U/mL), 3.5 nM [$^{125}$I]-FIXa, and varying concentrations of annexin V or cold FIXa were incubated for 5 min at 37 °C. After addition of FVIII (2 U/mL) and FX (1.5 $\mu$M), binding was initiated by addition of $CaCl_2$ (5 mM). After a 20 min incubation at 37 °C, the samples were handled as described above.

Equilibrium binding curves were analyzed by KalaidoGraph, using a nonlinear least squares fit to the equation $y = ax/(b + x)$, and by Scatchard analysis to yield a $V_{max}$, $K_{d,app}$, and total number of binding sites per platelet. Data points from competition experiments were compared by KalaidoGraph for fit to a theoretical equation for simple competition: $y = a/(x + a)$.

## RESULTS

Enzyme FIXa and cofactor FVIIIa were titrated into the FXa generation reaction in the presence and absence of annexin V to investigate differences between enzyme complex binding sites provided by the activated platelet surface and by artificial phospholipid 1:3 PS/PC vesicles. FIXa titrations performed in the absence of FVIIIa were expected to provide information about the direct binding of enzyme to each surface without the complications of additional contacts between enzyme and cofactor for stabilization of the enzymatic complex. Reaction curves generated were analyzed as reciprocal plots, Dixon plots, and replots of Dixon slopes to further investigate the effect of annexin V on the surface-supported reactions.

When annexin V, with its high affinity for negatively-charged lipid, was added to the FIXa titration reactions in the absence of FVIIIa, FX activation was inhibited on both activated platelets and on 1:3 PS/PC vesicles. Figure 1a shows the effect of different concentrations of annexin V on the platelet-supported FIXa titrations performed in the absence of FVIIIa. Figures 1b, 1c, and 1d show the effects of different concentrations of annexin V on FIXa titrations performed in the absence of FVIIIa on 25, 10, and 5 $\mu$M 1:3 PS/PC vesicles, respectively. These data provide additional strong evidence that negatively-charged lipid is necessary to sustain maximally effective enzyme complex formation and function, especially since the fluid phase FIXa-catalyzed activation of FX in the absence of FVIIIa was not affected by addition of annexin V (data not shown).

The reciprocal curves of data from reactions on the platelet surface (Figure 1a inset) intersect on the *x*-axis, showing that annexin V lowers the $V_{max}$ without affecting the $EC_{50,FIXa}$. Dixon plots of the data (Figure 2a) intersect as well on the *x*-axis, indicating an $IC_{50}$ of 4.0 nM (Table 1), and Dixon slopes replotted against enzyme concentration (Figure 2a inset) fall on a line that intersects the *y*-axis above 0. These are characteristics of a simple noncompetitive mechanism which lowers the $V_{max}$ without affecting the $EC_{50,FIXa}$, suggesting that annexin V does not compete for FIXa binding sites on activated platelets.

In FIXa titrations performed on the artificial vesicle surface, the effect of annexin V depended on the concentration of lipid used. Three rate-limiting concentrations of 1:3

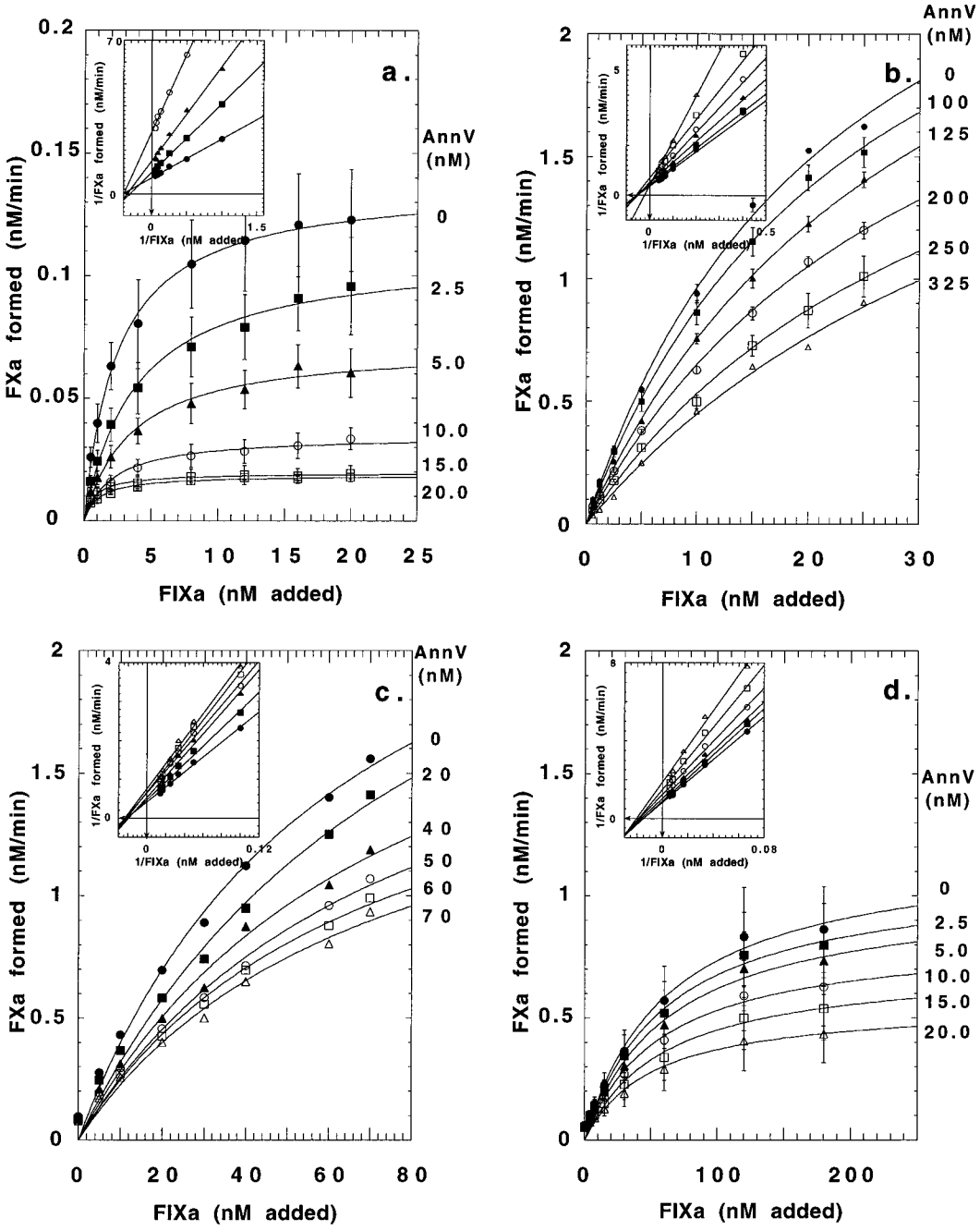


FIGURE 1: Annexin V inhibition of FX activation on platelets and on 1:3 PS/PC vesicles: FIXa titrations in the absence of FVIIIa. Washed and gel-filtered platelets (a: $3 \times 10^8$/mL) or 1:3 PS/PC vesicles (b: 25 $\mu$M, c: 10 $\mu$M, d: 5 $\mu$M) were incubated with various dilutions of FIXa and various dilutions of annexin V and calcium (5 mM), and activated for 5 min at 37 °C with thrombin 0.1 U/mL. Activation was begun by addition of FX (a: 500 nM, b, c, d: 250 nM). Reactions were stopped at 20 min by addition of EDTA. FXa formed was detected by hydrolysis of chromogenic substrate S2765 as described in the Experimental Procedures. Data from 5 different experiments (2 experiments at 10 $\mu$M) were analyzed by titration curves and by reciprocal plots (insets). (a) annexin V: 0 (●), 2.5 (■), 5 (▲), 10 (○), 15 (□), and 20 (△) nM. (b) annexin V: 0 (●), 100 (■), 125 (▲), 200 (○), 250 (□), and 325 (△) nM. (c) annexin V: 0 (●), 20 (■), 40 (▲), 50 (○), 60 (□), and 70 (△) nM. (d) annexin V: 0 (●), 2.5 (■), 5 (▲), 10 (○), 15 (□), and 20 (△) nM.

PS/PC vesicles were used to support FX activation: 25 $\mu$M (Figure 1b), 10 $\mu$M (Figure 1c), and 5 $\mu$M (Figure 1d). Reciprocal plots of the data shown as insets in Figures 1b, 1c, and 1d revealed that the mechanism of annexin V inhibition varied from raising the $EC_{50,FIXa}$ with little effect on the $V_{max}$ (25 $\mu$M) to lowering the $V_{max}$ with slight effects on the $EC_{50,FIXa}$ (5 $\mu$M) (Table 1). The Dixon plot of the FIXa titration data at 25 $\mu$M 1:3 PS/PC vesicles in the absence of FVIIIa (Figure 2b) indicates a competitive inhibition of FX activation, as does the replot of the Dixon slopes. This would be expected on a surface where only lipid sites are available and the high affinity of annexin V for negative lipid (Andree et al., 1990) makes it a good competitor for ionic interactions with the negatively-charged lipid surface. At an intermediate concentration of PL (10 $\mu$M) (Figure 1c), partial noncompetitive inhibition was observed, indicated by the shortened fanning of the reciprocal plots (Figure 1c inset), and suggesting a reduced efficiency of annexin V at higher concentrations.

In the presence of FVIIIa, annexin V was also an effective inhibitor of FX activation on both activated platelets and 1:3 PS/PC vesicles (Figures 3a and 3b). Annexin V inhibited the platelet-supported reactions by lowering the $V_{max}$ without affecting the $EC_{50,FIXa}$ (Figure 3a and inset) with an $IC_{50}$ of

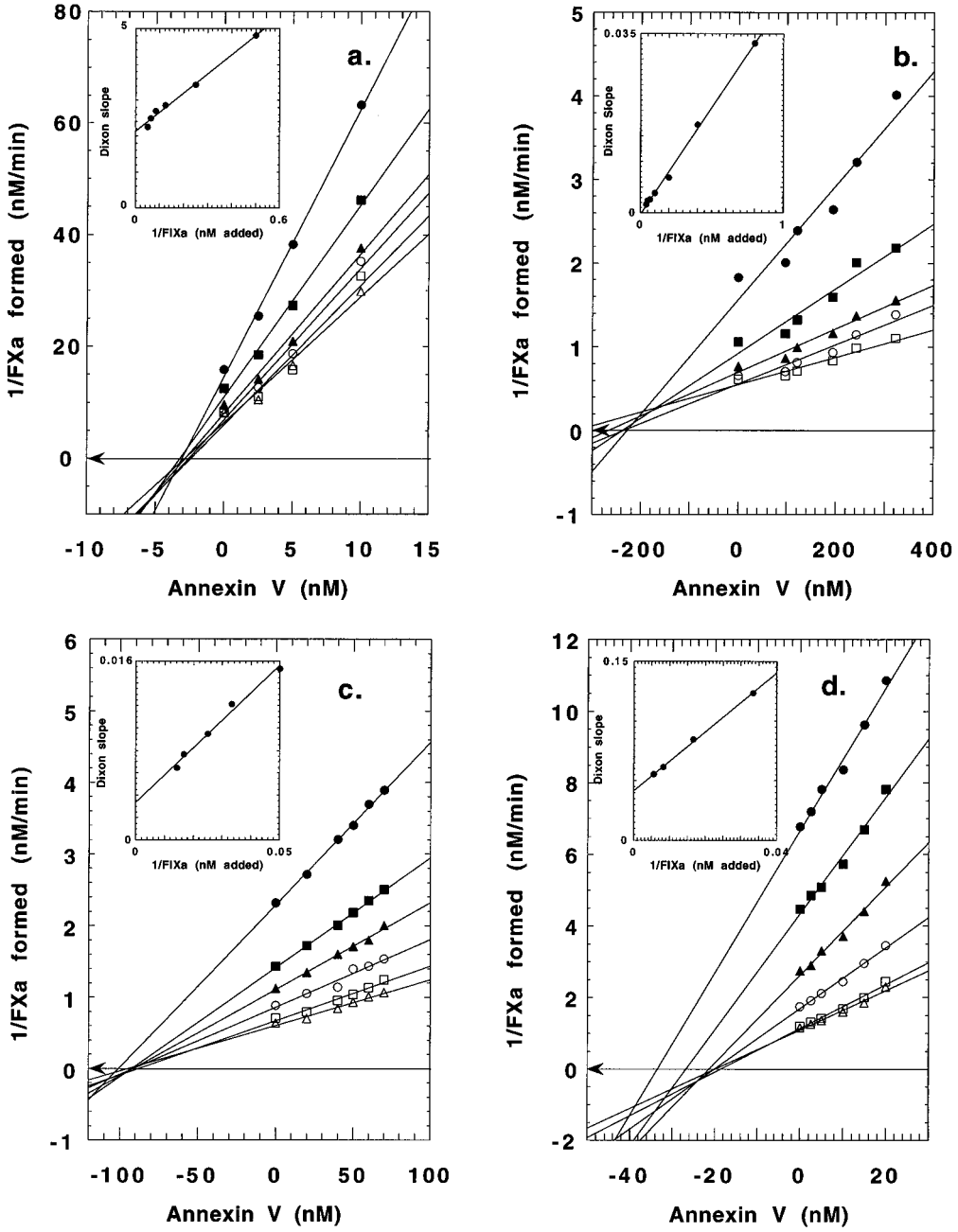


FIGURE 2: Annexin V inhibition of FX activation on platelets and on 1:3 PS/PC vesicles: FIXa titrations in the absence of FVIIIa. Data from reactions described in Figure 1 were analyzed by Dixon plots and by replots of the Dixon slopes (insets). FIXa concentrations were: (a) Platelets: 2 (●), 4 (■), 8 (▲), 12 (○), 16 (□), and 20 (△) nM. (b) 1:3 PS/PC vesicles 25 $\mu$M: 5 (●), 10 (■), 15 (▲), 20 (○), and 25 (□) nM. (c) 1:3 PS/PC vesicles 10 $\mu$M: 10 (●), 20 (■), 30 (▲), 40 (○), 60 (□), and 70 (△) nM. (d) 1:3 PS/PC vesicles 5 $\mu$M: 7.5 (●), 15 (■), 30 (▲), 60 (○), 120 (□), and 180 (△) nM.

4.5 nM (Table 2) and with the same noncompetitive mechanism (Figure 3c and inset) as seen in the absence of FVIIIa. On 1:3 PS/PC vesicles in the presence of FVIIIa, annexin V, with an $IC_{50}$ of 1.5 nM (Table 2), inhibited FX activation by raising the $EC_{50,FIXa}$ while leaving the $V_{max}$ unaffected (Figure 3b inset). This, the Dixon plot and the replot of the Dixon slopes (Figure 3d and inset) indicate a competitive mechanism of inhibition, in contrast to the noncompetitive mechanism seen on activated platelets (compare Figures 3c and 3d and insets). Although the presence of FVIIIa appeared to provide a protein anchor that improved the affinity of FIXa for artificial vesicles from 60 nM (Table 1) to 1 nM (Table 2), annexin V still competed with FIXa for a lipid binding site.

To study the effect of annexin V on specific binding of [$^{125}$I]-labeled FIXa to platelets, equilibrium binding studies were carried out in the presence and absence of saturating concentrations of FX and thrombin-activated FVIII, with or without 5 nM annexin V. In the presence of annexin V (Figure 4a), there was a significant reduction in the specific binding of [$^{125}$I]-FIXa to activated platelets in both the presence and absence of FVIIIa (2 U/mL) and FX (1.5 $\mu$M), as compared to the equilibrium binding curves obtained in the absence of annexin V. When the equilibrium binding data shown in Figure 4a were subjected to Scatchard analysis, we obtained straight lines (data not shown), indicating the presence of a single class of binding sites for FIXa in the presence and absence of the inhibitor. The affinity and

Table 1: Effect of Annexin V on FXa Generation on Activated Platelets and on 1:3 PS/PC Vesicles in the Absence of FVIIIa[a]

| FIXa titration − FVIIIa | | | | | |
|---|---|---|---|---|---|
| platelets ($3 \times 10^8$/mL) | | | 1:3 PS/PC (25 $\mu$M) | | |
| AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FIXa}$ (±SE) | AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FIXa}$ (±SE) |
| 0 | 0.114 (0.023) | 2.436 (0.339) | 0 | 3.63 (0.31) | 25.50 (3.59) |
| 2.5 | 0.076 (0.026) | 2.828 (1.068) | 100 | 3.32 (0.27) | 29.73 (3.93) |
| 5 | 0.053 (0.011) | 2.748 (0.975) | 125 | 3.12 (0.02) | 31.00 (1.00) |
| 10 | 0.027 (0.005) | 2.686 (0.268) | 200 | 2.81 (0.25) | 33.00 (3.89) |
| 15 | 0.013 (0.006) | 0.448 (0.184) | 250 | 2.74 (0.65) | 40.50 (10.52) |
| 20 | 0.005 | 0.215 | 325 | 1.95 (0.60) | 40.00 (7.00) |
| $IC_{50}$ | 4 nM | | $IC_{50}$ | 200 nM | |
| 1:3 PS/PC (10 $\mu$M) | | | 1:3 PS/PC (5 $\mu$M) | | |
| AnnV (nM) | $V_{max}$ | $EC_{50,FIXa}$ | AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FIXa}$ (±SE) |
| 0 | 2.38 | 57.68 | 0 | 1.07 (0.15) | 57.80 (5.91) |
| 20 | 2.23 | 60.18 | 2.5 | 0.98 (0.15) | 58.30 (3.81) |
| 40 | 1.83 | 54.78 | 5 | 0.89 (0.14) | 58.00 (6.94) |
| 50 | 1.73 | 57.43 | 10 | 0.71 (0.15) | 50.40 (5.09) |
| 60 | 1.48 | 51.93 | 15 | 0.64 (0.15) | 52.93 (8.90) |
| 70 | 1.47 | 55.63 | 20 | 0.56 (0.17) | 49.87 (2.62) |
| $IC_{50}$ | 90 nM | | $IC_{50}$ | 22 nM | |

[a] $V_{max}$ and $EC_{50}$ determinations were derived from titration curves generated from reaction rates at various FIXa concentrations as described in the Experimental Procedures. Means were calculated from the derived data from 2−5 separate experiments and standard errors (SE) determined from 3−5 separate experiments. $IC_{50}$s were determined from the *x*-axis intercepts of Dixon plots and replots of Dixon slopes.

stoichiometry of FIXa binding in both the absence and presence of 5 nM annexin V were determined in three separate experiments, the means (±SE) of which are given in Table 3. The presence of annexin V at 5 nM reduced the number of FIXa binding sites available on the activated platelet surface in the absence of FVIIIa and FX from 600 sites/platelet to 337 sites/platelet, without affecting the $K_{d,app}$ which remained at ∼3.0 nM (Table 3). Thus the demonstration of noncompetitive inhibition in the kinetic studies in the absence of FVIIIa is consistent with the observations from the equilibrium binding studies that the number of FIXa binding sites is progressively decreased at increasing annexin V concentrations without any effect on FIXa binding affinity.

The presence of FVIIIa and FX, both at saturating concentrations, had no effect on the number of binding sites for FIXa, but resulted in a decrease in the $K_{d,app}$ to 0.75 ± 0.07 nM (Table 3). When annexin V (5 nM) was added to the equilibrium binding reactions in the presence of FVIIIa and FX, the total number of FIXa binding sites was lowered from 610 ± 55 to 309 ± 42 sites/platelet, supporting the kinetic findings that annexin V eliminates some of the platelet binding sites for FIXa. However, the Scatchard analysis indicated that annexin V also raised the $K_{d,app}$ from 0.75 nM to 6.4 nM, which is 2-fold the binding constant measured in uninhibited binding reactions in the absence of FVIIIa (Table 3). There was no corresponding increase seen in $EC_{50,FIXa}$ calculated from the kinetic reactions (Table 2).

We also studied the ability of annexin V to compete with FIXa by incubating thrombin-stimulated platelets with [$^{125}$I]-FIXa and various concentrations of either unlabeled FIXa or annexin V in the presence of FVIIIa and FX. When the residual binding of [$^{125}$I]-FIXa was determined (Figure 4b), it was apparent that excess unlabeled FIXa or annexin V prevented >95% of [$^{125}$I]-FIXa binding. As calculated from the competition curves presented in Figure 4b, the concentration of FIXa required for half-maximal inhibition of [$^{125}$I]-FIXa binding in the presence of FVIIIa and FX was 0.5 nM, and the $IC_{50}$ of annexin V was 3.5 nM.

A careful examination of the competition curve plotting relative residual [$^{125}$I]-FIXa bound against annexin V concentration reveals a nonconformity to the theoretical curve for simple competition (Figure 4b). A cooperative effect, presumably caused by the self-association of annexin V (Conche et al., 1992), makes annexin V a progressively more effective inhibitor with increasing concentration. This cooperative effect was seen as well in competition curves of the kinetic data. At concentrations of annexin V beyond that necessary for 70% inhibition, the simple mechanisms seen at lower concentrations become more complex, with nonlinear Dixon plots and replots.

To determine if the increase in $K_{d,app}$ caused by annexin V in the equilibrium binding studies performed in the presence of FVIIIa and FX was attributable to an effect of annexin V on FVIIIa binding, FVIIIa was titrated into the FX activation reactions on both surfaces in the presence of increasing annexin V concentrations (Figure 5). The $V_{max}$ of the reaction on platelets (Figure 5a and inset) was inhibited proportionally by increasing amounts of inhibitor, indicating a reduction in the number of enzymatic complexes without an effect on the function of remaining complexes, but the amount of FVIIIa necessary for a half-maximal reaction, 1.7 U/mL, remained unchanged (Table 2). This and the Dixon plot and the replot of Dixon slopes (Figure 5c and inset) indicate that annexin V inhibited the effects of FVIIIa titrations into the FX activation reaction on the activated platelet surface by a simple noncompetitive mechanism.

Annexin V was found to have a similar kinetic effect in titrations of FVIIIa performed on 1:3 PS/PC vesicles (Figure 5b and inset). Annexin V inhibited the effects of FVIIIa on 1:3 PS/PC vesicles by lowering the $V_{max}$ without changing the concentration of FVIIIa required for half-maximal reaction rate (3.5 U/mL) (Table 2). The Dixon plot and the replot of Dixon slopes (Figure 5d, and inset) indicate a mixed noncompetitive mechanism of inhibition, in comparison to the simple noncompetitive mechanism seen with platelets.

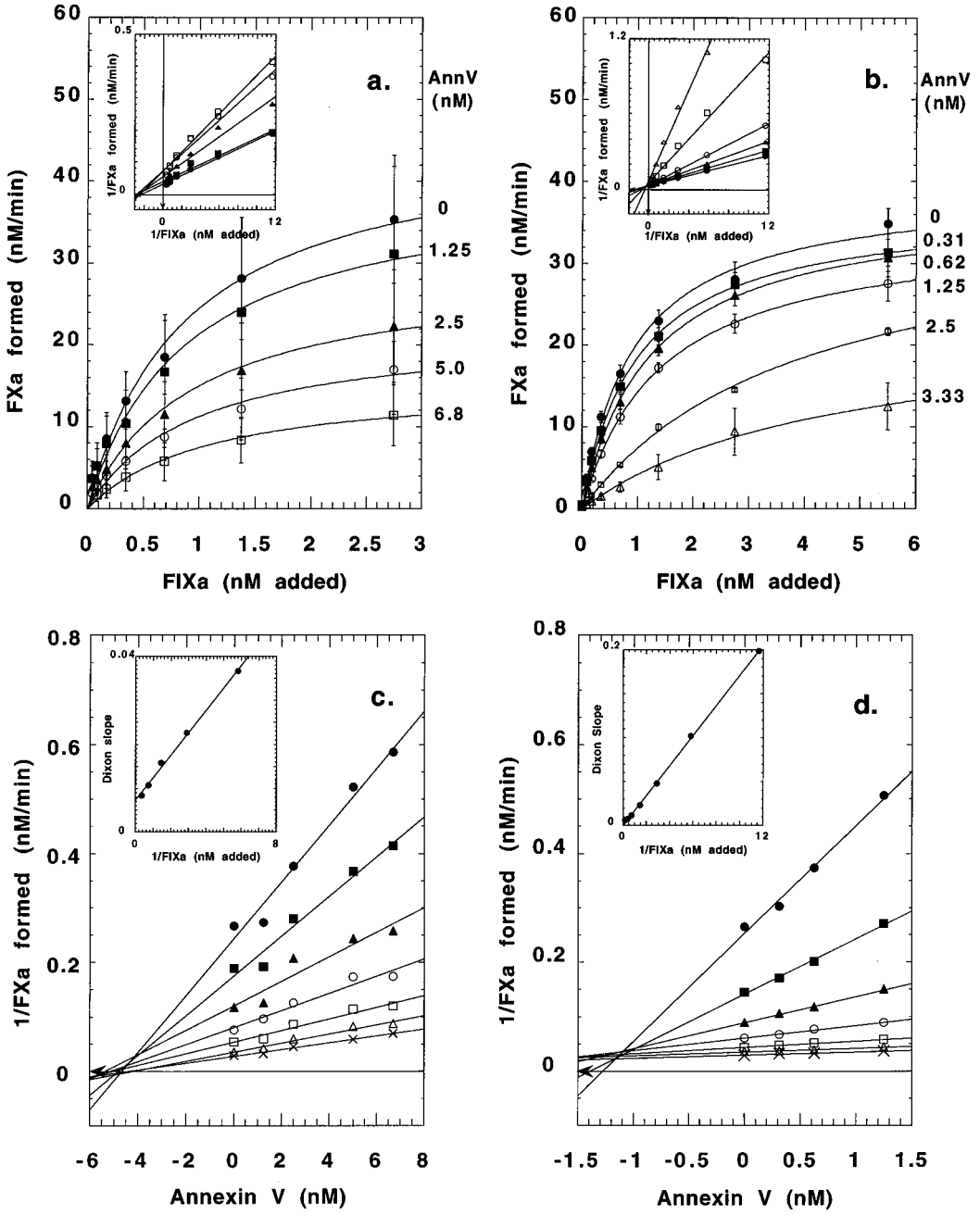


FIGURE 3: Annexin V inhibition of FX activation on platelets and on 1:3 PS/PC vesicles: FIXa titrations in the presence of FVIIIa. Washed and gel-filtered platelets (a and c: $5 \times 10^7$/mL) or 1:3 PS/PC vesicles (b and d: 0.5 $\mu$M) were incubated with various dilutions of FIXa, various dilutions of annexin V, rFVIII (6 U/mL), and calcium (5 mM), and activated for 4 min at 37 °C with thrombin receptor peptide (20 $\mu$M) before addition of thrombin (0.1 U/mL) for 1 min to activate the rFVIII. Activation was begun by addition of FX (a and c: 1000 nM, b and d: 250 nM). Reactions were stopped at 3 min by addition of EDTA. FXa formed was detected by hydrolysis of chromogenic substrate S2765 as described in the Experimental Procedures. Data from 3−5 different experiments were analyzed by titration curves (a and b), by reciprocal plots (a and b insets), by Dixon plots (c and d), and by replots of Dixon slopes (c and d insets). (a) annexin V: 0 (●), 1.25 (■), 2.5 (▲), 5 (○), and 6.8 (□) nM. (b) annexin V: 0 (●), 0.31 (■), 0.62 (▲), 1.25 (○), 2.5 (□), and 3.33 (△) nM. (c) FIXa: 0.043 (●), 0.086 (■), 0.172 (▲), 0.344 (○), 0.688 (□), 1.375 (△), and 2.75 (×) nM. (d) FIXa: 0.086 (●), 0.172 (■), 0.344 (▲), 0.688 (○), 1.375 (□), 2.75 (△), and 5.5 (×) nM.

A comparison of the $IC_{50}$ or half-maximal inhibitory effect of annexin V on the various components of the activation reaction might reveal differences in the surface contact sites for the enzyme complex. This is valid where the surfaces potentiate the reaction to the same extent, i.e., where the number of platelets and concentration of phospholipid vesicles used produces the same $V_{max}$, implying an equivalent number of enzymatic complexes. Annexin V inhibited FX activation in FVIIIa titrations performed with $5 \times 10^7$ activated platelets/mL (Figure 5a) with an $IC_{50}$ of 1.6 nM (Table 2). FVIIIa titrations performed with 0.5 $\mu$M 1:3 PS/PC vesicles which produced the same $V_{max}$ (Figure 5b) were inhibited by annexin V with an $IC_{50}$ of 1.7 nM (Table 2). The similarity of the $IC_{50}$s for both surfaces suggests that annexin V is equally potent at interfering with FVIIIa enhancement of enzyme activity on both surfaces. In the FIXa titrations, since the $V_{max}$ in the presence of FVIIIa on 1:3 PS/PC vesicles was the same as that calculated for the reaction on platelets (Figures 3a and 3b), $5 \times 10^7$ platelets should contain the same number of enzyme assembly sites as 0.5 nmol of vesicles. Dixon plots of the FIXa titrations (Figures 3c and 3d) yielded an $IC_{50}$ of 4.5 nM for the annexin

Table 2: Effect of Annexin V on FXa Generation on Activated Platelets and on 1:3 PS/PC Vesicles in the Presence of FVIIIa[a]

| FIXa titration + FVIIIa | | | | | |
|---|---|---|---|---|---|
| platelets ($5 \times 10^7$/mL) | | | 1:3 PS/PC (0.5 $\mu$M) | | |
| AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FIXa}$ (±SE) | AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FIXa}$ (±SE) |
| 0 | 39.13 (3.00) | 1.10 (0.17) | 0 | 38.83 (1.88) | 0.92 (0.06) |
| 1.25 | 34.49 (4.33) | 1.56 (0.12) | 0.312 | 37.17 (2.81) | 1.01 (0.10) |
| 2.5 | 25.18 (4.45) | 1.26 (0.08) | 0.625 | 38.07 (3.83) | 1.28 (0.26) |
| 5 | 17.45 (6.01) | 1.28 (0.04) | 1.25 | 34.87 (3.24) | 1.46 (0.23) |
| 6.8 | 18.55 (5.92) | 1.04 (0.12) | 2.5 | 38.00 (0.50) | 4.26 (0.29) |
| 10 | 15.80 (1.50) | 0.83 | 3.3 | 22.00 | 4.50 |
| $IC_{50}$ | 4.5 nM | | $IC_{50}$ | 1.5 nM | |
| FVIIIa titration | | | | | |
| platelets ($5 \times 10^7$/mL) | | | 1:3 PS/PC (0.5 $\mu$M) | | |
| AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FVIII}$ (±SE) | AnnV (nM) | $V_{max}$ (±SE) | $EC_{50,FVIII}$ (±SE) |
| 0 | 15.80 (1.64) | 1.74 (0.15) | 0 | 15.31 (1.20) | 3.50 (0.28) |
| 1.25 | 12.00 (2.88) | 1.67 (0.03) | 0.312 | 14.53 (1.60) | 3.53 (0.30) |
| 2.5 | 7.98 (1.78) | 1.58 (0.10) | 0.625 | 12.90 (1.63) | 3.81 (0.16) |
| 5 | 4.63 (0.35) | 1.48 (0.06) | 1.25 | 10.14 (0.85) | 3.50 (0.56) |
| 6.8 | 2.43 (0.39) | 1.48 (0.09) | 2.5 | 5.16 (0.76) | 2.51 (0.18) |
| $IC_{50}$ | 1.6 nM | | $IC_{50}$ | 1.7 nM | |

[a] $V_{max}$ and $EC_{50}$ determinations were derived from titration curves generated from reaction rates at various FIXa or FVIIIa concentrations as described in the Experimental Procedures. Means (±standard errors) were calculated from the derived data from 3−5 separate experiments. $IC_{50}$s were determined from the *x*-axis intercepts of Dixon plots and replots of Dixon slopes.

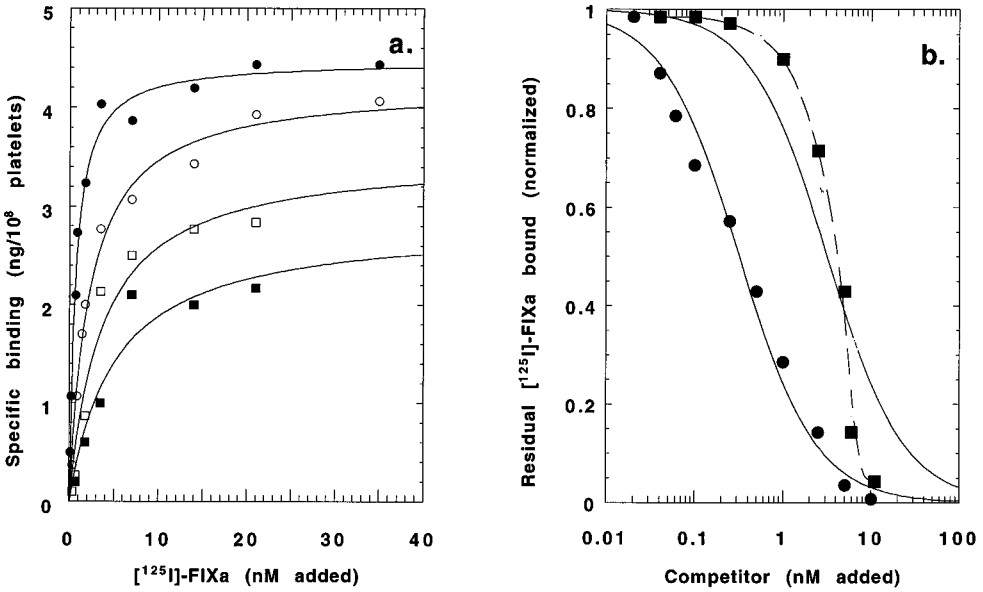


FIGURE 4: Annexin V inhibition of [$^{125}$I]-FIXa binding to platelets. (a) Equilibrium binding: Gel-filtered platelets ($3.5 \times 10^8$/mL) were incubated at 37 °C with human α-thrombin (0.1 U/mL), $CaCl_2$ (5 mM), and [$^{125}$I]-FIXa in the absence or presence of thrombin-activated FVIII (2 U/mL), FX (1.5 $\mu$M), and/or annexin V (5 nM). Binding was determined as described in the Experimental Procedures. Specific binding was calculated by subtraction of nonspecific binding determined in the presence of excess unlabeled FIXa (0.4 $\mu$M) from total binding. The results shown represent specific binding data for FIXa in the absence (●) and presence (■) of FVIIIa and FX, compared with specific binding data for FIXa in the presence of annexin V, in the absence (○) and presence (□) of FVIIIa and FX. Data points represent the means of duplicate values from three separate experiments. (b) Competitive binding: Gel-filtered platelets ($3.5 \times 10^8$/mL) were incubated for 20 min at 37 °C with human α-thrombin (0.1 U/mL), $CaCl_2$ (5 mM), [$^{125}$I]-FIXa (3.5 nM), various concentrations of unlabeled FIXa or annexin V in the presence of thrombin-activated FVIII (U/mL), and FX (1.5 $\mu$M) as described above. Binding was determined as described in the Experimental Procedures. Specific maximal binding (1.0) was determined as described above and used to normalize results. Shown are the means of 3 separate experiments performed in duplicate representing residual FIXa binding in the presence of unlabeled FIXa (●), compared to residual FIXa binding in the presence of annexin V (■). Solid lines represent computer-generated curves fitting the data to an equation for competitive binding: $y = a/(x + a)$. Dotted line represents interpolation through data points.

V effect on the platelet reaction and an $IC_{50}$ of 1.5 nM for the annexin V effect on the reaction supported by 1:3 PS/PC vesicles (Table 2). This suggests that the enzyme assembly sites on the vesicles were more sensitive to the presence of annexin V than the corresponding sites on activated platelets, although the cofactor contact sites were not.

## DISCUSSION

Annexin V was used in these studies to investigate the nature of enzymatic complex formation for the intrinsic pathway activation of blood coagulation FX. It has been well-established that artificial phospholipid vesicles containing negatively-charged phospholipids can substitute for

Table 3: Effect of Annexin V on the Binding of [$^{125}$I]-FIXa to Thrombin-Activated Human Platelets[a]

| ligand | FVIIIa and FX | binding sites/platelet | app $K_d$ (nM) |
|---|---|---|---|
| [$^{125}$I]-FIXa | absent | $600 \pm 48$ | $3.5 \pm 0.31$ |
| | present | $610 \pm 55$ | $0.75 \pm 0.07$ |
| [$^{125}$I]-FIXa + annexin V (5 nM) | absent | $337 \pm 50$ | $3.0 \pm 0.26$ |
| | present | $309 \pm 42$ | $6.4 \pm 1.23$ |

[a] Binding parameters shown are the mean (±SE) calculated from three separate experiments, each performed with platelets obtained from a different normal subject.

physiologic cell surfaces in potentiating the reaction, by decreasing the half-maximal enzyme and substrate concentrations required (van Dieijen et al., 1981, 1985). Activation of blood platelets has been found to be accompanied by exposure of negatively-charged phospholipids, suggesting their importance in providing the appropriate surface binding sites (Bevers et al., 1982, 1983). If the exposure of negatively-charged phospholipids were the sole determinant of FX activation complex binding, the affinity of annexin V for negatively-charged lipid should equally affect FX activation on either activated platelets or 1:3 PS/PC vesicles.

In the present study, differences between the ability of activated platelets and of negatively-charged phospholipid vesicles to support FX activation by FIXa are clearly evident when comparing the $EC_{50,FIXa}$ in the absence of the cofactor FVIIIa. The concentration of FIXa necessary to sustain half-maximal FX activation on platelets was 2.5 nM, consistent with previous observations (Ahmad et al., 1989a), whereas on 1:3 PS/PC vesicles, the $EC_{50,FIXa}$ was ~60 nM, indicating that the affinity of FIXa for phospholipid in the absence of a protein environment is 25-fold less than its affinity for the activated platelet surface (Table 1). This observation strongly suggests that components in addition to phospholipids in the platelet membrane must confer higher affinity to the binding of FIXa in the absence of FVIIIa.

In the presence of FVIIIa, this difference in the $EC_{50,FIXa}$ was eliminated. Only 1.0 nM FIXa on platelets, and 1.0 nM FIXa on 1:3 PS/PC vesicles, were sufficient for half-maximal activity (Table 2), representing a 2.5-fold improvement in affinity of FIXa for the platelet surface, and a 60-fold increase in affinity of FIXa for the phospholipid surface. When FVIIIa was titrated into reactions on both surfaces, it was found that the concentration required for half-maximal activity ($EC50_{FVIIIa}$) on platelets (1.7 U/mL) was similar to that required on 1:3 PS/PC vesicles (3.0−3.5 U/mL) (Table 2). Thus, there is a kinetic indication that FVIIIa can be accommodated almost as well by a purely phospholipid environment as it is by the activated platelet surface, and that, in its presence, FIXa is accommodated as well by the purely lipid environment as by the activated platelet surface.

The fact that increasing concentrations of annexin V inhibited FX activation on both platelets and artificial vesicles supports the hypothesis that negatively-charged lipids comprise an important component for enzyme complex association with a biological surface. However, an analysis of the mechanism of inhibition revealed important differences between the artificial lipid vesicles and activated platelets. On thrombin-activated platelets, annexin V caused a pattern of simple noncompetitive inhibition whether FVIIIa or FIXa was titrated into the activation reaction (Figures 1a, 2a; 3a, 3c; and 5a, 5c). There was no suggestion of either competition for binding or change in affinity of either FVIIIa or FIXa for its binding sites until higher annexin V concentrations were used, i.e., 2-fold above the $K_d$ for annexin V binding to activated platelets (Thiagarajan & Tait, 1990). In contrast, on 1:3 PS/PC vesicles, annexin V produced a pattern of competitive inhibition of FX activation when FIXa was titrated into the reaction, determined by the ability of the inhibitor to increase the $EC_{50,FIXa}$ for the reaction without affecting the $V_{max}$ (Figures 1b and 2b, 3b and 3d). Thus, annexin V competed with the enzyme for surface binding sites on artificial vesicles where only negative and neutral lipids are available for binding.

On 1:3 PS/PC vesicles in the absence of FVIIIa, the concentration of annexin V binding sites appeared to affect the mechanism of inhibition, with 25 $\mu$M lipid producing a pattern of mixed competitive inhibition and 5 $\mu$M lipid producing a pattern of mixed noncompetitive inhibition (Figures 2b and 2d). The $IC_{50}$'s for inhibition of the reaction varied with the three concentrations of lipid used (Table 1). If the $K_{d,app}$ for annexin V binding to lipid vesicles is $10^{-10}$−$10^{-11}$ M (Andree et al., 1990), then the concentrations of annexin V used in these experiments, 0.31−200 nM, should have been saturating for the binding of annexin V to limiting lipid sites. However, if the amount of lipid was in excess for the binding of annexin V, while it was still rate-limiting for the FX activation reactions, then the bulk phase concentration of annexin V could have been much lower than expected from the amount added, resulting in failure to saturate annexin V binding sites. As a consequence, the apparent $IC_{50}$ for annexin V would be overestimated; however, occupation of fewer annexin V binding sites might reduce annexin V cooperative effects (Conche et al., 1992; Mosser et al., 1991) and favor monomer interaction with its binding sites, thus presenting truer effects on binding sites for components of the FX activating complex. The $IC_{50}$ for annexin V inhibition of FX activation on platelets in the absence of FVIIIa was the same (4 nM) as that on platelets in the presence of FVIIIa (4.5 nM) even though 6 times the number of platelets were used in the former reaction. Thus, the platelets were not in excess either for the binding of annexin V or for FX activation. Although any $K_{d,app}$ for annexin V must include the effects of self-association, on platelets, phospholipid binding sites for annexin V ($K_{d,app}$ 7 nM; Thiagarajan & Tait, 1990) are discontinuous due to the presence of membrane proteins, affecting the ease of self-association and favoring monomer interactions with binding sites.

In kinetic studies, annexin V addition to the platelet-supported reactions did not result in competition for surface-enhanced FIXa activity reflected in changes in $EC_{50,FIXa}$, but resulted rather in a reduction of $V_{max}$; hence the platelet binding sites for the enzyme FIXa appear to be affected by, but different from, platelet binding sites for annexin V. The results of our equilibrium binding studies with [$^{125}$I]-FIXa (Figure 4) support the interpretation of our kinetic studies as noncompetitive inhibition since, in the absence of FVIIIa, annexin V reduced the number of binding sites without affecting the $K_{d,app}$ for FIXa. These results can be interpreted in several ways: (1) the decrease in the $V_{max}$ is caused by annexin V interference with another element of the enzyme complex, perhaps with substrate interaction with the surface;

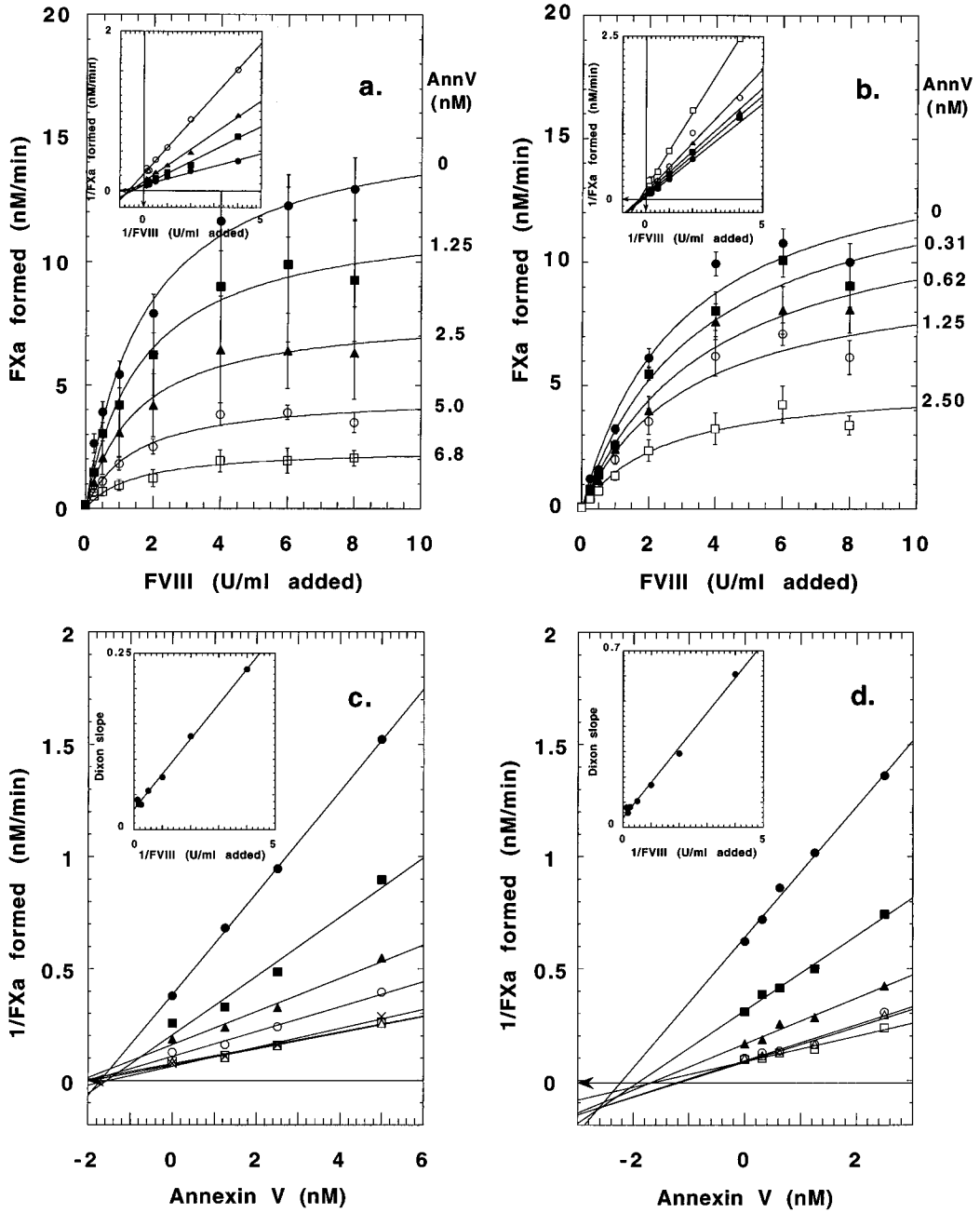


FIGURE 5: Annexin V inhibition of FX activation on platelets and on 1:3 PS/PC vesicles: FVIII titrations. Washed and gel-filtered platelets (a and c: $5 \times 10^7$/mL) or 1:3 PS/PC vesicles (b and d: 0.5 $\mu$M) were incubated with FIXa (500 pM) and various dilutions of annexin V, in the presence of various dilutions of rFVIII and calcium (5 mM), and incubated for 5 min at 37 °C with thrombin receptor peptide (20 $\mu$M). FVIII was converted to FVIIIa by addition of thrombin 0.1 U/mL for 1 min. Activation was begun by addition of FX (a and c: 500 nM; b and d: 250 nM). Reactions were stopped at 3 min and assayed as described above. Data combined from 3−4 different experiments were analyzed by titration curves (a and b), by reciprocal plots (insets a and b), by Dixon plots (c and d), and by replots of Dixon slopes (insets c and d). (a) annexin V: 0 (●), 1.25 (■), 2.5 (▲), 5 (○), and 6.8 (□) nM. (b) annexin V: 0 (●), 0.312 (■), 0.625 (▲), 1.25 (○), and 2.5 (□) nM. (c and d) FVIII: 0.5 (●), 1 (■), 2 (▲), 4 (○) 6 (□), and 8 (△) U/mL.

(2) formation of trimers of annexin V (Conche et al., 1992) might sterically hinder FIXa from binding to a site proximal to bound annexin V, or the activated platelet surface might bind annexin V differently than does the solely lipid surface; (3) the platelet surface provides both protein and lipid components of a FIXa binding site and protein components could dictate which lipid configurations are available for protein binding.

Considering the first possibility, when equilibrium binding of [$^{125}$I]-FIXa was performed in the presence of FVIIIa and FX, annexin V not only reduced the number of binding sites but also produced changes in the $K_{d,app}$ that could be attributed to interference with either FVIIIa or FX binding or both. FX binding to thrombin-activated platelets has been shown to be inhibited by annexin V (Scandura et al., 1996), and the resulting reduction of the surface-associated substrate pool would be expected to contribute to the decrease in $V_{max}$. We have observed a mixed pattern of annexin V inhibition in titrations of FX into the FX activation reaction[2] with the decrease in $V_{max}$ accompanied by increases in $K_{m,app}$, supporting the suggestion of a reduction of the surface-associated substrate pool. Equilibrium studies of FVIIIa binding to

[2] F. S. London, and P. N. Walsh, unpublished observations (1996).

platelets could help to clarify whether annexin V has the same effect on FVIIIa binding as on FIXa binding and FX binding. In the kinetic experiments reported here, annexin V appeared to inhibit FVIIIa enhancement of the FX activation reaction on both surfaces by a noncompetitive mechanism, without affecting the affinity of FVIIIa for either surface. This is consistent with the evidence that there is a hydrophobic component of the interaction of phospholipids with FVIIIa as well as with FVa (Gilbert & Baleja, 1995; Kalafatis et al., 1994), whereas annexin V is thought to interact with the surface of phospholipids without any penetration into the hydrocarbon chain region. Using a lipophilic probe, 1-azidopyrene, Kalafatis et al. (1994) found that while FV was labeled upon incubation with PC vesicles, annexin V was not, whereas both annexin V and FV incorporated the label in the presence of PS/PC (25:75), supporting the conclusion that FV, unlike annexin V, can interact hydrophobically as well as electrostatically with phospholipid.

Addressing the second possibility, we have tried to avoid the complications accompanying trimer formation of annexin V by analyzing the effects of low concentrations of inhibitor, below the $K_d$ of annexin V for the platelet surface (7 nM). Platelet membrane proteins might affect the binding of annexin V to platelets, although there is no evidence that annexin V binds directly to membrane proteins. Proteins released from platelets upon activation might also affect the binding of annexin V to the platelet surface, e.g., FV or FVIII. However, if FVIII, taken up by platelets and released upon platelet activation, were to affect annexin V binding to platelets, the cofactor-enhanced reactions on phospholipid vesicles, upon addition of annexin V, should have been inhibited by the same mechanism as were the activated platelets. This was not the case. Rather our results from the FVIIIa titrations suggest that there is no kinetic competition between FVIIIa and annexin V for the same binding site. FV was not added to the lipid vesicle-supported reactions; however, FVIII and FV appear to bind phospholipid analogously (Gilbert & Baleja, 1995). Therefore, competition or interference between FV and annexin V would not be expected. The presence of BSA in both reactions assured that nonspecific protein interactions were identical for both surfaces. Since at the concentrations of annexin V used we observed on phospholipid vesicles a noncompetitive mode of inhibition of FVIIIa titration while FIXa titrations were inhibited competitively, but on platelets both titrations were inhibited by a noncompetitive mechanism, we feel that any differences in the binding of annexin V to either surface were not responsible for differences in inhibition mechanism noted.

Our results suggest that activated platelets and negatively-charged phospholipid vesicles provide different environments for the assembly of the intrinsic FX activation complex. The nature of the difference can be attributed to the greater complexity of the platelet surface as compared to the artificial vesicle surface. On the vesicle surface, lipid molecules directly involved in binding proteins of the activation complex are diffusionally directed into a configuration which forms a binding site. The lipid constituents of phospholipid vesicles are constrained in their diffusional mobility chiefly by the charge and bulk of their polar heads, and the saturation of their fatty acid side chains (Quin & Chapman, 1980). Lipids exposed on the surface of the cell membrane are additionally constrained in their lateral mobility by their interactions with integral membrane proteins and the restraining influence of cytoskeletal components (Kinnunen et al., 1994). Hence, one would expect less diffusionally directed assembly of binding sites on the platelet surface, and more involvement of platelet proteins in forming the binding site or in binding coagulation proteins directly.

Finally, we must acknowledge that 1:3 PS/PC phospholipid vesicles, although used as standard negatively-charged lipid surfaces for the last twenty years, do not mimic the architecture of lipids available on the surface of activated platelets. It can be extrapolated from the studies of Bevers et al. (1982, 1983) that thrombin-activated platelets present for hydrolysis by exogenous phospholipase A2 and sphingomyelinase ~76% neutral lipids (SM=PC) and 24% procoagulant lipids (PS 4%, phosphatidylethanolamine (PE) 20%). Few studies have used this combination in artificial vesicles; however, Gilbert and Arena (1995, 1996) found that vesicles of cholesterol/PC/PS/PE (20:56:4:20) were less effective at 1 mM $CaCl_2$ in supporting FX activation than vesicles containing 25 mol % PS, even when the amount of PS in both preparations was the same, suggesting that it is the density of PS available on the surface that determines the efficiency of FX activation on artificial lipid vesicles. It would be dangerous to equate any *in vitro* choice of lipids with the physiological surface available on the platelet since nothing is known of the fatty acid composition of activation-exposed lipids and hence of the fluidity of lipid molecules interacting with coagulation proteins. Nothing is known about the ability of integral membrane proteins to regulate either the classes of lipids constituting a binding site, their mobility, or their architecture. Indeed, our use of vesicles containing lipids extracted from platelets (representing, therefore, both inner and outer leaflet lipids of all membranes) yielded a higher $EC_{50,FIXa}$ in the presence of FVIIIa (2.7 nM) (data not shown) than use of 1:3 PS/PC vesicles (1 nM). Also, annexin V inhibited FIXa titrations using vesicles of extracted platelet lipids with the same competitive pattern as seen in reactions using 1:3 PS/PC vesicles, and at a similar $IC_{50}$ (1.2 nM and 1.5 nM, respectively) for equimolar phospholipid concentrations (data not shown). We can therefore deduce that it is either the specific configuration of lipid or the protein on the platelet surface that provides the difference in FIXa binding.

The results of these experiments are consistent with, although not proof of, a binding site for FIXa on the activated platelet surface containing both negatively-charged phospholipid and protein. We have detected with ligand blotting techniques protease-sensitive activation-dependent surface expression of FIXa binding protein(s) in platelet membrane preparations (London & Walsh, 1990). This (these) membrane protein(s) may comprise receptors associated with aminophospholipids both of which are exposed in response to specific agonists (e.g., thrombin or the thrombin receptor peptide) to facilitate the assembly of the FX-activating complex on the platelet membrane. This possibility is being actively investigated in our laboratory.

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