The Role of Electrostatic Interactions in the Assembly of the Factor X Activating Complex on both Activated Platelets and Negatively-Charged Phospholipid Vesicles†

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ABSTRACT: Factor X was activated by factor IXa on the surface of either activated platelets or artificial lipid vesicles in the presence of different NaCl concentrations. The V_{max} of reactions using platelets was optimal at physiologic [NaCl] both in the absence and in the presence of factor VIIIa. In contrast, the V_{max} of reactions using vesicles decreased with increasing [NaCl] in the absence of factor VIIIa, and increased with increasing [NaCl] when cofactor was present. In the absence of factor VIIIa, the $EC_{50_{FIXa}}$, although stable to changes in [NaCl] in platelet-supported reactions, was found to increase significantly as [NaCl] increased in vesicle-supported reactions and correlated with the decreased V_{max}. Thus, in contrast to platelet-supported reactions, enzyme interaction with negatively-charged vesicles was highly dependent upon electrostatic interactions. In the presence of factor VIIIa, the $EC_{50_{FIXa}}$ of vesicle-supported reactions decreased with increasing [NaCl], indicating that interactions between FIXa and FVIIIa can increase enzyme affinity when fewer ionic interactions are favored. The $EC_{50_{FVIIIa}}$ was insensitive to changes in [NaCl] on both surfaces. The $K_{m_{app}}$ derived from platelet-supported titrations of factor X was lowest just above physiological [NaCl], whereas on vesicles $K_{m_{app}}$ was minimal at the lowest [NaCl] tested. Thus, the direct interaction of factor X and factor IXa with the artificial lipid surface is highly dependent upon ionic interactions with the negatively-charged polar heads of phospholipids. However, the interaction of factor IXa and factor X with the activated platelet surface must rely both on electrostatic interactions with lipid and on other interactions provided by surface proteins.

Both the intrinsic and extrinsic factor X (FX)[1] activation complexes and the prothombinase complex, centrally important to formation of a hemostatic clot, have long been studied in vitro and are known to be powerfully potentiated by the presence of a surface containing negatively-charged phospholipid (Papahadjopoulos et al., 1962; Bangham, 1961; Marcus & Spaet, 1958). The ability of artificial vesicles containing 20−30% phosphatidylserine (PS) to substitute completely for activated cells of the vasculature and the inability of neutrally-charged phosphatidylcholine (PC) to support clot formation have led to the assumption that electrostatic interactions between charges of the polar heads of lipids with protein components of the enzymatic complexes are the basis for interaction of complex components with the surface. Work by Zwaal and Hemker (Bevers et al., 1982, 1983) correlating activation-dependent exposure of negatively-charged lipids on platelets with competency for surface catalysis of thrombin formation has lent support to the identification of negatively-charged lipids as the platelet binding sites for components of these enzymatic complexes.

All vitamin K-dependent coagulation proteins containing γ-carboxylated glutamyl (GLA) residues have been shown to bind to negatively-charged lipid vesicles (Papahadjopoulos & Hanahan, 1964), and most have been shown to bind to activated platelets (Walsh, 1994; Ahmad et al., 1989a). It was originally proposed (Nelsestuen, 1984) that the calcium ions known to associate with the GLA regions of the proteins were responsible for direct bridging with negative charges on the lipids. Although it is now known from the crystal structure of prothrombin (Soriano-Garcia et al., 1992) that calcium ions form a skeletal framework for the three-dimensional conformation of the GLA domain, it is still possible that one or two calcium ions perpendicular to the conformational axis coordinate with lipid charges. However, it is more likely that interaction with negative charges of the lipid also involves amino acid side chains (Ahmad et al., 1994) and that apolar amino acid side chains also interact hydrophobically with lipid moieties (Zhang & Castellino, 1994; Christiansen et al., 1995). The cofactors FVa for prothrombinase activity and FVIIIa for F-X activation are

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[1] Abbreviations: FX, factor X; PS, phosphatidylserine; PC, phosphatidylcholine; GLA, γ-carboxylated glutamic acid; ACD, acid citrate dextrose; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; 1:3 PS/PC vesicles, vesicles composed of a 1:3 molar ratio of PS/PC; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BCA, bicinchoninic acid protein assay; S-2765, chromogenic substrate *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride; FVIII, recombinant human FVIII; FMOC, 9-fluorenylmethoxycarbonyl; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid.

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thought to interact hydrophobically as well as electrostatically with negatively-charged lipid surfaces (Gilbert & Baleja, 1995; Kalafatis et al., 1994).

When FIXa binds to artificial vesicles or activated platelets in the absence of its cofactor FVIIIa, it activates FX slowly (Rawala et al., 1990). If FIXa interacted in an identical manner with the negatively-charged lipid molecules of both the artificial lipid surface and the activated platelet surface, then varying NaCl concentrations to enhance or disrupt electrostatic interactions should affect FIXa-directed FXa generation equally on both surfaces, especially in the absence of FVIIIa. In this study, the enzyme FIXa, the cofactor FVIIIa, and the substrate FX were titrated separately into the F-X activation reactions at different NaCl concentrations which varied closely around physiologic levels so as not to interfere with the integrity or molecular interactions of the surfaces themselves. Data are presented on the effect of these [NaCl] changes on the reaction rates, the $K_{m_{app}}$, the $EC_{50_{FIXa}}$, and cofactor concentration required for half-maximal activity, and on the catalytic efficiency of the complexes on either surface.

EXPERIMENTAL PROCEDURES

Platelet Preparation. Venous blood was collected into acid citrate dextrose (ACD) anticoagulant (12 mM trisodium citrate·$2H_2O$, 10 mM citric acid monohydrate, and 15 mM dextrose) and centrifuged at 200*g* at room temperature for 20 min. The recovered platelet-rich plasma, to which apyrase (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.25 unit/mL, 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 *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered Tyrodes solution (0.13 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 0.4 mM monosodium phosphate) (buffer A), pH 6.5, with bovine serum albumin (BSA) (2 mg/mL) and apyrase (0.25 unit/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.) equilibrated with buffer A containing 2 mg/mL BSA, as described previously (Ahmad et al., 1989). 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.

Phospholipid Vesicles Composed of a 1:3 Molar Ratio of PS/PC (1:3 PS/PC). Large unilamellar vesicles were prepared according to Mayer et al. (1986). Porcine brain PS and L-α-dioleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), stored in chloroform at −20 °C, were mixed 1:3 mole per mole 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 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 two different preparations of lipid 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 Laboratory (Southbend, IN) and dissolved in 20 mM Tris, 150 mM NaCl, and 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 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., 1989), 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, if capable of cleaving *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride (*N*-α-Cbo-D-Arg-Gly-Arg-*p*NA-2HCl) (S-2765) in the absence of FIXa, treated with soybean trypsin inhibitor coupled to Affigel-15 beads (Biorad Laboratories Inc., Hercules, CA) to remove contaminating FXa. Recombinant human FVIII (rFVIII) was kindly provided by Baxter Healthcare Corp. (Duarte, CA) and stored in 10 mM HEPES, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 6.5, and the concentration was determined by a one-stage clotting assay (Proctor & Rapaport, 1961). Human α-thrombin (Sigma Chemical Co.) was stored at 200 units/mL at −80 °C. The thrombin receptor hexapeptide SFLLRN-amide was synthesized using 9-fluorenylmethoxycarbonyl (FMOC) chemistry on an Applied Biosystems 430A synthesizer and reverse-phase HPLC-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, 1989; Rawala-Sheikh, 1990). All proteins were diluted in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (HEPPS), pH 8.1 (buffer B). For assay in the absence of FVIIIa, platelets or lipid vesicles in buffer A containing 1 mg/mL BSA were diluted 1:2 with FIXa at various concentrations, $CaCl_2$ (5 mM), and dilutions of 1 M NaCl in microtiter wells. The plate was prewarmed at 37 °C before a further 5 min incubation with thrombin (0.1 units/mL), and the reaction was begun by addition of the indicated concentrations 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 NaCl dilutions. Platelets were activated by incubation with thrombin receptor peptide (20 μM) for 4 min at 37 °C. Thrombin was added to the incubation mixtures to 0.1 unit/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 A with 1 mg/mL BSA for a surface. Reaction rates were linear with respect to product formation under these conditions.

The FXa generated in all reaction mixtures was assayed for its rate of hydrolysis of the chromogenic substrate S-2765 (AB Kabi Diagnostica, Stockholm, Sweden). Aliquots diluted into buffer B containing 175 mM NaCl were prewarmed before addition of substrate to 300 μM, 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 linear 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 S-2765 by known concentrations of FX fully activated by Russell's Viper Venom (Sigma Chemical Co.). Data were collected into an Excell spreadsheet (Microsoft Corp., Redmond, WA) where velocity results were converted to nanomolar FXa generated per minute. Rates of fluid phase F-X activation, which were consistently less than 10% of surface-supported rates, were subtracted from total platelet- or phospholipid-potentiated F-X activation performed in the absence of FVIIIa. Data from three to five experiments were averaged for each analysis.

Analysis of the Data. 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 mean data points to an equation for a rectangular hyperbola: $y = Ax/(B + x)$ with A representing V_{max} and B representing the half-maximal concentration of the titrant. For comparative figures, data derived from reactions run at various concentrations of NaCl were divided by data derived from identical reactions performed at 0.15 M NaCl. Means, standard deviations, and standard errors were calculated from similarly run experiments. Kinetic parameters, $K_{m_{app}}$, and EC_{50} values were subjected to standard linear regression analysis to assess any trends in relation to NaCl concentration. A value of the regression parameter (slope) that was statistically different from zero ($p < 0.05$), as determined by the Student's t test, is indicative of a significant or, at $p < 0.01$, a highly significant dependency on NaCl concentration.

In previous work, a linear correlation was found between the quantity of [^{125}I]-FIXa bound/platelet in the presence of increasing [^{125}I]-FIXa added[2] and the rate of FXa generated in the presence of the same amount of FIXa added. This allows determinations of the turnover number (k_{cat}) for bound FIXa molecules. Since fluid phase FXa generation was unaffected by the changes in [NaCl] used in these studies, the k_{cat} was presumed to be unaffected by [NaCl] changes, and thus the rate at saturating substrate concentration was assumed to reflect the amount of FIXa bound. The V_{max} of surface-supported reactions at various [NaCl] was converted (using the linear relationship of FIXa bound and rate of FXa formed) to the amount of FIXa associated with the surface, or the concentration of enzymatic complexes on either platelets or vesicles. Catalytic efficiency (k_{cat}/K_m) was defined as the ratio of the turnover of bound FIXa at a particular [NaCl] to the amount of FX required to be added for half-maximal activity under those conditions.

[2] London, F., Ahmad, S. S., & Walsh, P. N. (1996) (submitted for publication).

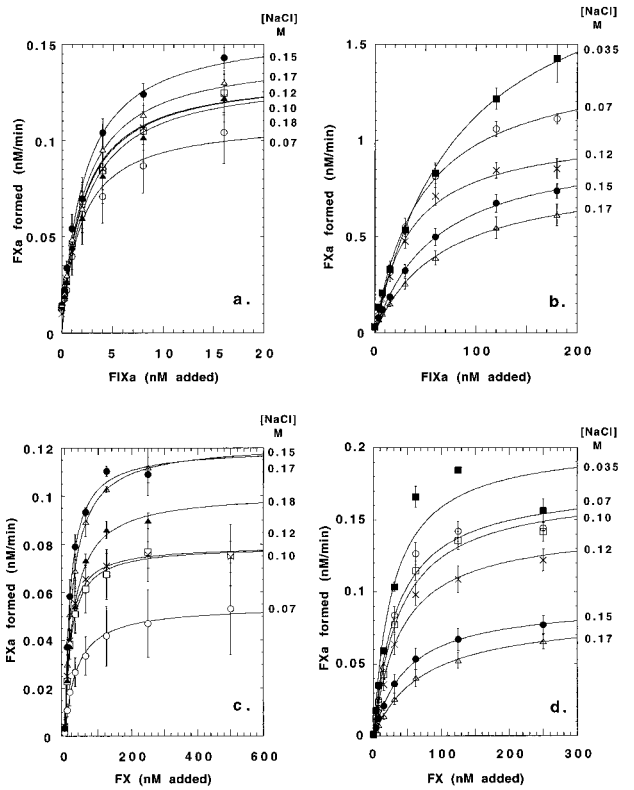

FIGURE 1: Enzyme and substrate titration curves of F-X activation supported by activated platelets or by 1:3 PS/PC vesicles in the absence of FVIIIa. Gel-filtered platelets (3×10^8/mL) (a and c) in buffer A or 5 μM 1:3 PS/PC vesicles (b and d) in buffer A were incubated with various concentrations of FIXa (a and b) or 5.5 nM FIXa (c and d) in buffer B, 5 mM $CaCl_2$, various dilutions of 1 M NaCl, and α-thrombin (0.1 unit/mL) and incubated at 37 °C for 5 min before addition of various concentrations of FX (c and d) or FX at 500 nM (a) or 250 nM (b) in buffer B. Incubation of reaction mixtures continued at 37 °C for 20 min, and the FXa generated was determined as described under Experimental Procedures. Data points on curves represent means and standard errors from four experiments run identically. NaCl concentrations: (■) 0.035 M, (○) 0.07 M, (□) 0.10 M, (×) 0.12 M, (●) 0.15 M, (△) 0.17 M, (▲) 0.185 M.

RESULTS

To investigate the requirements for electrostatic interactions in the activation of FX, purified FIXa, FVIIIa, and FX were titrated separately into F-X activation reactions under conditions of varying NaCl concentration. Increasing [NaCl] should compete for protein−protein or protein−surface salt bridges. Since variations in [NaCl] from 0.075 to 0.19 M did not affect the amount of [^{14}C]serotonin secreted in response to thrombin at 0.1 units/mL (data not shown), platelet activation was presumed to be unchanged in the presence of these concentrations of NaCl. Fluid phase F-X activation involves direct interactions of FX and FIXa only. Since fluid phase FX titration curves (as well as FIXa titration curves) generated at [NaCl] from 0.075 to 0.19 M were superimposable (data not shown), the k_{cat} was determined to be unaffected by these changes in [NaCl], and any inhibition of FXa generation was presumed to result from interference either with protein−protein or with protein−surface interactions. Surface-supported reactions contained sufficient platelets to potentiate FXa generation well above fluid phase activity and yet to remain fairly low in the linear range of platelet titration reactions. Lipid concentrations were chosen to mimic levels of activity in platelet-containing reactions

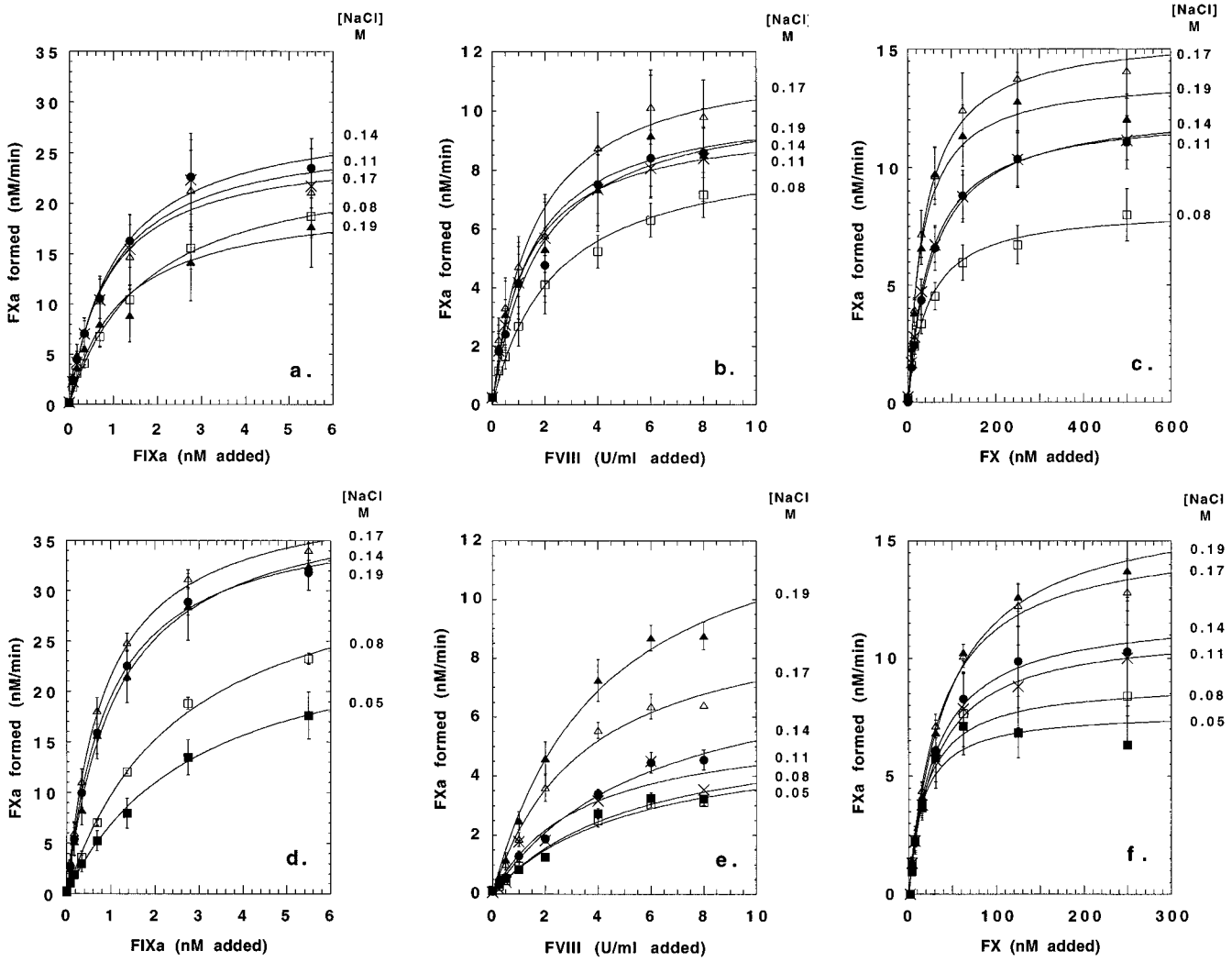

FIGURE 2: Enzyme, cofactor, and substrate titration curves of F-X activation supported by activated platelets or by 1:3 PS/PC vesicles in the presence of FVIIIa. Gel-filtered platelets (5×10^7/mL) (a, b, and c) or 0.5 μM 1:3 PS/PC vesicles (d, e, and f) in buffer A were incubated with various concentrations of FIXa (a and d) or FIXa at 500 pM (b, c, e, and f) in buffer B, 5 mM $CaCl_2$, various concentrations of rFVIII (b and e) or rFVIII at 6 units/mL (a, c, d, and f), various dilutions of 1 M NaCl, and 20 μM thrombin receptor peptide. The reaction mixtures were incubated at 37 °C for 4 min before adding α-thrombin to 0.1 unit/mL to activate the rFVIII 1 min prior to adding various concentrations of FX (c and f) or FX at 500 nM (a and b) or 250 nM (d and e). After a 3 min incubation at 37 °C, FXa generated in reaction mixtures was determined as described under Experimental Procedures. Data points on curves represent means and standard errors from 3−5 experiments run identically. NaCl concentrations: (■) 0.05 M, (□) 0.08 M, (×) 0.11 M, (●) 0.14 M, (△) 0.17 M, (▲) 0.19 M.

yet also remained low in the linear range of lipid titration reactions. Since the reaction rates at these levels of surface additions were the same, it is assumed that they represent the same number of enzymatic complexes bound.

A general survey of results of all titrations revealed that the platelet-dependent activation of FX was sensitive to [NaCl] above and below physiological. Figure 1a and Figure 1c, which show respectively the results of FIXa and FX titrations in the absence of FVIIIa, and Figure 2a,b,c, which show respectively the results of FIXa, FVIIIa, and FX titrations performed in the presence of FVIIIa, show that optimal platelet-supported FXa generation occurred in almost all cases at physiologic [NaCl]. A variation of 40 mM on either side resulted in suboptimal rates, except with cofactor titration (Figure 2b) and substrate titration in the presence of FVIIIa (Figure 2c) where V_{max} continued to increase at greater than physiological [NaCl]. In contrast, the response of the 1:3 PS/PC vesicle-supported reactions to variations of [NaCl] depended on the presence or absence of FVIIIa. In its absence, enzyme titrations (Figure 1b) and substrate titrations (Figure 1d) showed decreased rates of vesicle-supported FXa generation as [NaCl] was increased from 0.035 to 0.17 M. In the presence of FVIIIa, increasing [NaCl] from 0.05 to 0.19 M increased the maximum rate of F-X activation resulting from FIXa titrations (Figure 2d), from FVIIIa titrations (Figure 2e), or from FX titrations (Figure 2f). These comparisons can be seen more clearly when data are normalized relative to those obtained at 0.15 M NaCl. Figure 3a presents normalized V_{max} data from platelets, and Figure 3b shows normalized V_{max} data from 1:3 PS/PC vesicles in the absence of FVIIIa. These clearly demonstrate the different effects of varying [NaCl] on the V_{max} of these surface-supported reactions.

A detailed examination of the changes in kinetic parameters produced by changes in NaCl concentration in the absence of FVIIIa could reveal differences in the mode of contact of enzyme or substrate with either surface without the complications of cofactor interactions. Table 1 presents $EC_{50_{FIXa}}$ and $K_{m_{app}}$ data from reactions performed in the absence of FVIIIa on both surfaces. On activated platelets,

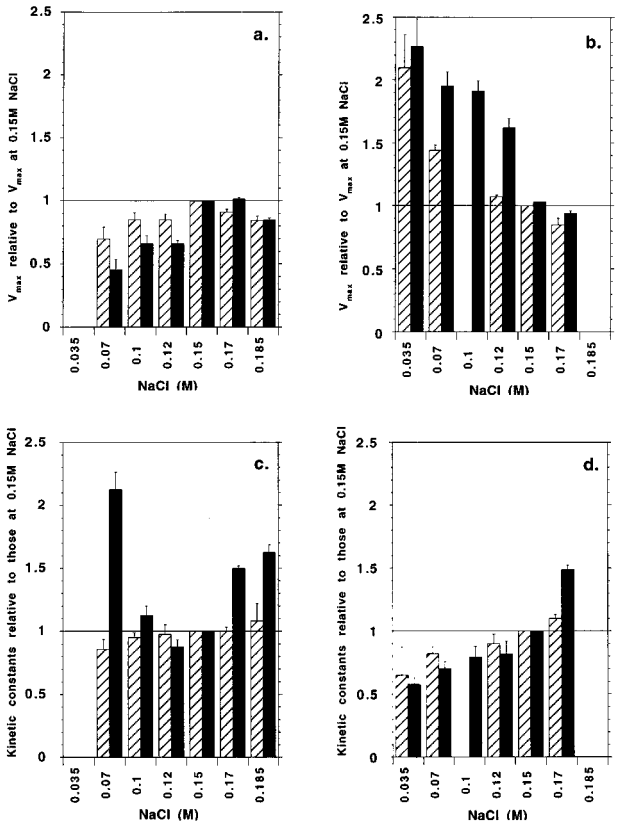

FIGURE 3: V_{max} and kinetic parameters derived from F-X activation supported by activated platelets or by 1:3 PS/PC vesicles, performed in the absence of FVIIIa at various concentrations of NaCl and normalized with respect to those derived from FX activations performed at 0.15 M NaCl. Data on V_{max} (a and b) and data on enzyme and substrate concentrations required for half-maximal reaction rates (c and d), derived from enzyme and substrate titrations shown in Figure 1, were normalized with respect to data obtained from reactions performed in 0.15 M NaCl and are plotted against their respective NaCl concentrations. Bars represent the means and standard errors of four experiments. Reactions supported by thrombin-activated platelets (a and c); reactions supported by 1:3 PS/PC vesicles (b and d). Hatched bars, FIXa titrations; solid bars, FX titrations.

the increasing [NaCl] corresponded with no significant change in the $EC_{50_{FIXa}}$, whereas $K_{m_{app}}$ varied inversely with V_{max}. Thus, the rate of the reaction was dependent upon the affinity of the substrate for the platelet surface, which was optimum at physiologic salt concentration. In contrast, on the negatively-charged vesicle surface, the decrease in rate observed with increasing [NaCl] corresponded with highly significant increases in both $EC_{50_{FIXa}}$ ($p < 0.01$) and $K_{m_{app}}$ ($p < 0.01$). Here the rate of reaction depended upon the affinity of both the enzyme and the substrate for the lipid surface which was decreased as increasing [NaCl] interfered with electrostatic interactions. These differences in the two surfaces can be seen clearly in Figure 3c,d, where data on kinetic parameters obtained in the absence of FVIIIa at different [NaCl] were normalized to those obtained at 0.15 M NaCl.

Table 2 presents EC_{50} and $K_{m_{app}}$ from the FVIIIa-enhanced F-X activations under conditions of varying NaCl concentration. The $EC_{50_{FVIIIa}}$ was unaffected by changes in [NaCl] on both surfaces although twice the amount of FVIIIa was required on lipid vesicles as on activated platelets. The $EC_{50_{FIXa}}$ was increased both on activated platelets and on 1:3 PS/PC vesicles at NaCl concentrations well below physiologic (0.05−0.1 M) and correlates with decreased V_{max}. This trend is opposite to that noted for phospholipid vesicles in the absence of FVIIIa (see Table 1).

Table 1: Effect of Changes in [NaCl] on Kinetic Parameters of FXa Generation in the Absence of FVIIIa[a]

[NaCl] (M)	$EC_{50_{FIXa}}$ (nM) ± SE	$V_{max_{FIXa}}$ (nM/min) ± SE	$K_{m_{app}}$ (nM) ± SE	$V_{max_{FX}}$ (nM/min) ± SE
		Platelets (3×10^8/mL)		
0.035				
0.07	2.59 ± 0.57	0.111 ± 0.008	33.9 ± 3.2	0.05 ± 0.001
0.10	2.43 ± 0.46	0.135 ± 0.018	17.7 ± 1.6	0.08 ± 0.002
0.12	2.32 ± 0.35	0.135 ± 0.006	14.9 ± 1.4	0.08 ± 0.002
0.15	2.37 ± 0.42	0.159 ± 0.008	16.6 ± 1.5	0.12 ± 0.003
0.17	2.36 ± 0.48	0.145 ± 0.008	23.2 ± 1.3	0.12 ± 0.002
0.185	2.67 ± 0.66	0.134 ± 0.010	26.2 ± 2.1	0.10 ± 0.002
		1:3 PS/PC Vesicles (5 μM)		
0.035	54 ± 6.7[b]	2.08 ± 0.097	28.5 ± 9.4[b]	0.20 ± 0.02
0.07	68 ± 3.6[b]	1.43 ± 0.041	34.7 ± 6.4[b]	0.18 ± 0.01
0.10			39.2 ± 5.6[b]	0.17 ± 0.008
0.12	74 ± 4.3[b]	1.06 ± 0.044	40.5 ± 5.7[b]	0.14 ± 0.007
0.15	83 ± 5.8[b]	0.99 ± 0.039	49.4 ± 2.6[b]	0.09 ± 0.002
0.17	91 ± 8.2[b]	0.84 ± 0.043	73.5 ± 4.8[b]	0.08 ± 0.002
0.185				

[a] FIXa and FX were titrated into F-X activation reactions in the presence of varying [NaCl] as described under Experimental Procedures. From titration curves of the data, maximum rates and half-maximal concentrations of FIXa and FX were determined. Data represent the means ± SE of 4 separate experiments. [b] There is a highly significant ($p < 0.01$) dependency of EC_{50} value on [NaCl].

In the presence of FVIIIa, the $K_{m_{app}}$ increased directly with V_{max} as [NaCl] was increased up to physiologic concentration in the activated platelet system, and beyond physiologic concentration in the 1:3 PS/PC vesicle-supported reactions (Table 2). More substrate was required when interaction between cofactor and enzyme optimized complex formation and function.

We calculated the concentration of enzymatic complexes formed at different [NaCl] on both surfaces. Using the results of binding experiments relating the amount of FIXa bound per platelet to the amount of FIXa added,[2] we found a linear correlation between the reaction rates arising from different FIXa concentrations in the presence of excess FX and the amount of FIXa bound at those concentrations, in reactions containing 0.15 M NaCl. From this relationship, we could then calculate the k_{cat} of bound FIXa and determine the catalytic efficiency (i.e., k_{cat}/K_m). Based on the lack of effect of [NaCl] changes on fluid phase reactions, we assumed that the k_{cat} is unaffected by changes in [NaCl]. Therefore, the V_{max} of F-X activation reactions performed at different [NaCl] was assumed to reflect the number of holoenzyme complexes, consisting of FIXa bound to the surface, or of FIXa and FVIIIa bound to the surface, assembled under those conditions. We could therefore calculate the amount of FIXa bound at different [NaCl] and the $k_{cat}/k_{m_{app}}$ ratio (catalytic efficiency) at different [NaCl].

On activated platelets, the k_{cat} was calculated as 0.73 min^{-1} in the absence of FVIIIa, and 670 min^{-1} in its presence. On 1:3 PS/PC vesicles, the k_{cat} was 0.725 min^{-1} in the absence of FVIIIa, and 670 min^{-1} in its presence. Thus, the k_{cat} was calculated to be improved 1,000-fold on both surfces by the presence of FVIIIa.

Figure 4 presents the results of calculations converting the V_{max} for FX titrations at each [NaCl] to the concentration of FIXa bound, with Figure 4a,c representing the absence of

Table 2: Effect of Changes in [NaCl] on Kinetic Parameters of FXa Generation in the Presence of FVIIIa[a]

[NaCl] (M)	$EC_{50_{FIXa}}$ (nM) ± SE	$V_{max_{FIXa}}$ (nM/min) ± SE	$EC_{50_{FVIIIa}}$ (nM) ± SE	$V_{max_{FVIIIa}}$ (nM/min) ± SE	$K_{m_{app}}$ (nM) ± SE	$V_{max_{FX}}$ (nM/min) ± SE
			Platelets (5×10^7/mL)			
0.05						
0.08	1.73 ± 0.19	24.7 ± 1.13	2.38 ± 0.34	8.9 ± 0.48	45.5 ± 5.8	8.3 ± 0.31
0.11	0.98 ± 0.17	27.2 ± 1.68	1.30 ± 0.08	9.7 ± 0.21	51.5 ± 1.8	12.4 ± 0.13
0.14	1.06 ± 0.14	29.1 ± 1.35	1.76 ± 0.33	10.3 ± 0.69	57.8 ± 2.8	12.6 ± 0.19
0.17	0.89 ± 0.15	25.0 ± 1.48	1.56 ± 0.31	12.0 ± 0.77	39.3 ± 3.4	15.7 ± 0.38
0.19	1.19 ± 0.31	20.5 ± 1.98	1.43 ± 0.30	10.6 ± 0.67	33.3 ± 5.1	13.9 ± 0.57
			1:3 PS/PC Vesicles (0.5 μM)			
0.05	2.97 ± 0.32	27.3 ± 1.45	5.49 ± 1.84	5.8 ± 1.0	16.1 ± 5.0[b]	7.7 ± 0.66
0.08	2.57 ± 0.24	34.8 ± 1.50	5.20 ± 1.29	5.4 ± 0.68	20.1 ± 4.7[b]	8.9 ± 0.60
0.11			3.38 ± 1.73	5.8 ± 1.3	30.7 ± 3.1[b]	11.2 ± 0.36
0.14	0.96 ± 0.05	39.6 ± 0.90	5.93 ± 1.24	8.3 ± 0.92	30.9 ± 3.5[b]	12.0 ± 0.43
0.17	0.87 ± 0.05	40.2 ± 0.76	3.50 ± 0.63	9.7 ± 0.76	36.2 ± 4.3[b]	15.3 ± 0.60
0.19	1.14 ± 0.07	38.1 ± 0.66	4.18 ± 0.76	14.1 ± 1.2	44.7 ± 4.9[b]	16.7 ± 0.64

[a] FIXa, FVIIIa, and FX were titrated into F-X activation reactions in the presence of varying [NaCl] as described under Experimental Procedures. From titration curves of the data, maximum rates and half-maximal concentrations of FIXa, FVIIIa, and FX were determined. Data represent the means ± SE of 3−5 separate experiments. [b] There is a highly significant ($p < 0.01$) dependency of EC_{50} value on [NaCl].

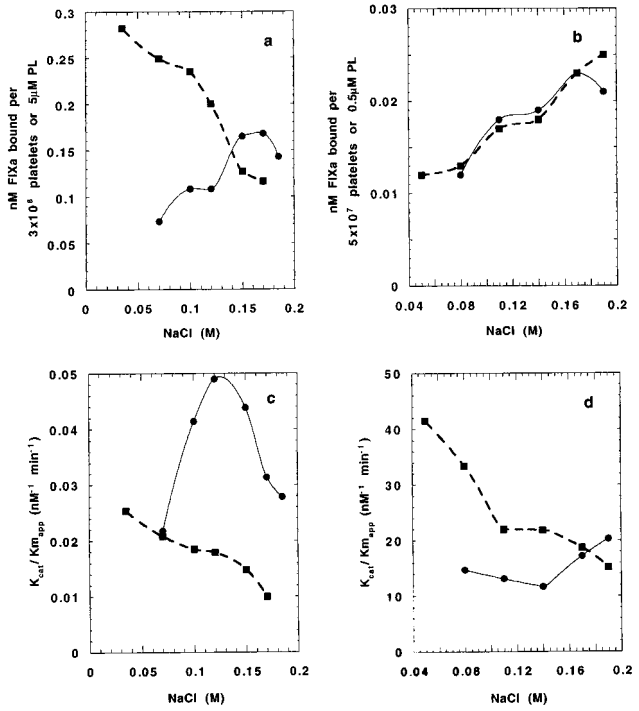

FIGURE 4: FIXa calculated to be bound to activated platelets or to 1:3 PS/PC vesicles at different [NaCl] and catalytic efficiencies in the presence and absence of FVIIIa. The V_{max} data derived from FX titrations performed in the presence of activated platelets (●) (a and c, 3×10^8/mL; b and d, 5×10^7/mL) or 1:3 PS/PC vesicles (■) (a and c, 5 μM; b and d, 0.5 μM) at various [NaCl] were used to calculate the amount of FIXa bound (a and b) to the respective surfaces using the linear correlation between the amount of [^{125}I]-FIXa bound at a given concentration of [^{125}I]-FIXa added[2] and the rate of F-X activation at excess FX by that same concentration of FIXa added. Catalytic efficiency (c and d) was calculated from the ratio of the k_{cat} (V_{max}/FIXa bound) to the $K_{m_{app}}$ data derived from FX titrations performed at different [NaCl]. Panels a and c represent calculations using data from reactions performed in the absence of FVIIIa. Panels b and d represent calculations using data from reactions performed in the presence of FVIIIa (6 units/mL).

FVIIIa and Figure 4b,d representing the presence of FVIIIa. The results support those derived from $EC_{50_{FIXa}}$ analysis of FIXa titration curves. The amount of FIXa bound to the activated platelet surface increased with increasing [NaCl] and was optimal at physiologic [NaCl] in the absence and presence of FVIIIa (Figure 4a,b). In contrast, on 1:3 PS/PC vesicles, the binding of FIXa in the absence of FVIIIa (Figure 4a) was optimal at low [NaCl] and decreased with increasing [NaCl]. In the presence of FVIIIa (Figure 4b), the amount of FIXa bound to the lipid vesicles increased with increasing [NaCl]. The catalytic efficiency of the complex on platelets, in the presence and absence of FVIIIa, was optimal at physiological [NaCl] (Figure 4c,d). In contrast, the catalytic efficiency of the complex on 1:3 PS/PC vesicles, in the presence and absence of FVIIIa, was optimal at low [NaCl] and decreased as [NaCl] was increased toward physiological (Figure 4c,d).

DISCUSSION

Varying the concentration of NaCl in the surface-supported enzymatic conversion of FX to FXa would be expected to affect intermolecular electrostatic interactions. As [NaCl] increases, the additional ions compete for charges engaged in intermolecular salt bridges. If the nature of the interaction of enzyme components with the activated platelet surface was identical with the nature of their interaction with artificial lipid vesicles, then FXa generation on both surfaces should be identically affected by manipulation of the ionic environment. The NaCl concentration was carefully varied, between 0.075 and 0.19 M for platelets and between 0.035 and 0.19 M for 1:3 PS/PC vesicles, with the goal of achieving a slight disruption or enhancement of charge−charge interactions without a disruption of protein−protein interactions involving hydrogen bonding, van der Waals forces, and hydrophobic interactions. The same NaCl concentrations were found not to affect secretion of serotonin by platelets stimulated by thrombin at 0.1 unit/mL; hence, it is assumed that platelet activation and membrane events relying on it are not grossly affected by these changes in [NaCl]. Varying NaCl concentrations also had no effect on fluid phase enzymatic reactions. Therefore, we considered whether variations in the kinetics of F-X activation reflect the effects of [NaCl] on the interactions of the protein components of the FX activating complex with one another or with the respective surface provided.

In this study, F-X activation supported by 1:3 PS/PC vesicles has been found to be more sensitive than that supported by activated platelets to small disruptions of

electrostatic interactions achieved by varying NaCl concentrations by ~50% around physiological NaCl concentration. Increasing [NaCl] in the absence of cofactor caused opposite changes in the V_{max} of reactions on the two surfaces. The V_{max} of reactions on artificial vesicles decreased with increased [NaCl] while the V_{max} of reactions on activated platelets increased with increased [NaCl] up to physiologic salt concentrations and then decreased slightly at higher [NaCl].

Changes in reaction rates of the enzyme complex in the absence of cofactor most likely reflect changes in surface−protein interactions, especially since fluid phase enzymatic activity was unaffected by these variations in NaCl concentration in assays performed under the same conditions, suggesting that enzyme−substrate interactions were not affected. Inspection of changes in kinetic parameters caused by changes in NaCl concentration in the absence of cofactor suggests differences in the mode of interaction of enzyme and substrate with the two surfaces.

Electrostatic interactions appeared to play an important role in the interactions of both substrate and enzyme with the artificial surface in the absence of FVIIIa, since both the $K_{m_{app}}$ and the $EC_{50_{FIXa}}$ increased as V_{max} decreased with increasing [NaCl]. On the phospholipid vesicle in the absence of FVIIIa, only the polar heads, the glycerol backbone, and perhaps a portion of the hydrocarbon chains are available for interaction with the enzyme and the substrate. Binding of both to lipid vesicles has been found to be mediated by their respective GLA domains which have 62% sequence identity (Ichinose & Davie, 1994). It has been suggested that the interaction of GLA domains with negatively-charged phospholipid is electrostatic in nature (vanDieijen et al., 1981). There is also evidence that apolar residues on the amino terminus of the GLA domain of both protein C (Zhang & Castellino, 1994; Christiansen et al., 1995) and FIXa (Ahmad et al., 1994) contribute to the binding of these respective proteins to phospholipid vesicles (protein C) and to platelets (FIXa). The present results support the importance of electrostatic interaction with the phospholipid vesicle surface since greater rates and affinities were achieved at lower NaCl concentrations which favored greater electrostatic interactions. If hydrophobic interactions represent <50% of the binding energy, disruption of electrostatic interactions could still inhibit protein/lipid binding even at an ionic strength where hydrophobic interactions would be favored.

It has recently been suggested (Gilbert & Arena, 1996) that the consequence of enzyme/surface interaction for F-X activation in the absence of FVIIIa may be a conformational change in the enzyme leading to the enhanced k_{cat} observed on surfaces. It is possible that this molecular change either in enzyme or in substrate is favored by low [NaCl] on phospholipid vesicle surfaces.

The presence of cofactor FVIIIa is known to confer a 3−5-fold increase in the affinity of FIXa for the platelet surface (Ahmad et al., 1989) and a 60−80-fold increase in the affinity of FIXa for the artificial vesicle surface (Tables 1 and 2) (vanDieijen et al., 1985).[2] It is clear, therefore, that the interaction between enzyme and cofactor supplements the contact between the enzyme and the surface. The major outcome of that interaction is a large increase in the turnover number resulting in rates of F-X activation 3 orders of magnitude higher than in the absence of FVIIIa. The present study showed that, in the artificial vesicle-supported reactions, the presence of FVIIIa reversed the dependence of FXa generation on electrostatic interactions of the enzyme with the surface. V_{max} increased as electrostatic interactions were disrupted with increasing [NaCl]. The increased V_{max} correlated with a decrease in the $EC_{50_{FIXa}}$. This must indicate a hydrophobic intermolecular interaction of FIXa with FVIIIa that supplements electrostatic and hydrophobic interactions with the surface, decreasing the high degree of dependence on electrostatic interactions seen in the absence of FVIIIa. The increased V_{max} did not correlate with changes in $EC_{50_{FIVIIIa}}$, indicating that the V_{max} changes were not the result of changes in cofactor affinity or effectiveness.

Rates of F-X activation in the presence of activated platelets were less sensitive to small disruptions of electrostatic interactions than rates obtained in the presence of 1:3 PS/PC vesicles. Optimal rates occurred at physiological [NaCl] in the absence and presence of FVIIIa. In the absence of FVIIIa, the decreases in V_{max} on either side of physiological [NaCl] were correlated with inverse changes in the affinity of the substrate but not with changes in enzyme affinity. In the presence of FVIIIa, the decreased V_{max} at 0.1 M NaCl correlated with a decrease in enzyme affinity and not with changes in cofactor affinity. Thus, the affinity of substrate in the absence of cofactor and the affinity of enzyme in its presence appeared weakened at less then physiologic [NaCl] where electrostatic interactions would be favored. This suggests that more of the binding energy of enzyme and substrate interaction with the activated platelet surface, both in the presence and in the absence of FVIIIa, is contributed by nonelectrostatic forces than is the case on lipid vesicles. The activated platelet surface contains negatively-charged phospholipids exposed from the internal leaflet of the membrane bilayer upon activation (Bevers et al., 1983). If the enzyme or substrate interacted only with randomly exposed lipid, their mode of interaction with lipid should be identical on activated platelets and on phospholipid vesicles. The activated platelet surface, however, also provides membrane proteins which, if conferring an additional binding site or regulating the architecture of exposed lipid, could provide opportunities for hydrophobic interactions with exposed lipid or for hydrogen bonding and hydrophobic interactions with adjacent protein. The loss of optimal rates of F-X activation on either side of physiological NaCl concentration is consistent with the hypothesis that on the activated platelet, although part of the binding energy is conferred by electrostatic interactions with phospholipids, part is conferred by hydrophobic interactions with the surface, perhaps with surface proteins.

There is controversy over whether fluid phase substrate or surface-bound substrate serves as the true substrate pool for the enzyme. It has recently been shown that the surface of activated platelets presents FX binding sites that are shared with prothrombin and that competition for platelet FX binding sites by excess prothrombin inhibits FXa generation on the activated platelet surface (Scandura et al., 1996; Scandura & Walsh, 1996). This suggests that the affinity of FX for the platelet surface and diffusion of surface-bound substrate to the enzyme complex are limiting in the reaction, especially at high catalytic rates where adjacent surface-bound substrate is rapidly depleted and the affinity of the FX for the surface determines the availability of substrate from the true substrate pool. In this scenario, the $K_{m_{app}}$ derived from these surface-supported reactions measures not

the affinity of the substrate for the enzyme but rather the affinity of the substrate for the surface and the diffusion of the substrate from these surface-available pools to the enzyme. The effect of [NaCl] changes on the $K_{m_{app}}$ derived in the absence of FVIIIa suggests that on both surfaces, electrostatic interactions play an important role in both these activities. On platelets, higher [NaCl] where electrostatic interactions are disrupted produces increases in the $K_{m_{app}}$, but $K_{m_{app}}$ is also increased at the lowest [NaCl] where electrostatic interactions are favored. $K_{m_{app}}$ is lowest around physiologic [NaCl], suggesting that both electrostatic and nonelectrostatic forces contribute to the ease with which the substrate interacts with the surface. On phospholipid vesicles, in the absence of cofactor, the derived $K_{m_{app}}$ is lowest at the lowest [NaCl] tested where electrostatic forces are favored, and rises significantly as [NaCl] is increased ($p < 0.01$), suggesting that either the affinity of the FX for the lipid vesicle surface or its ability to diffuse along the surface to the enzyme is more sensitive to disruption of electrostatic interactions than is FX interaction with the activated platelet surface. In the presence of FVIIIa, at high catalytic rates where substrate conversion is 1000-fold greater than in the absence of FVIIIa, increasing [NaCl] on the activated platelet surface caused the $K_{m_{app}}$ to increase, but at 0.17 M NaCl where V_{max} was optimal, $K_{m_{app}}$ was decreased (see Table 2). On the contrary, the increasing rates of artificial vesicle-supported reactions with increasing [NaCl] up to 0.19 M, the highest concentration tested, were accompanied by parallel increases in $K_{m_{app}}$. This difference in results might reflect differences either in the binding sites for FX or in its diffusion rate on the different surfaces.

When the V_{max} data from FX titrations were converted to the concentration of FIXa complexes bound, the results corroborated the analysis of kinetic parameters (Figure 4). Although the presence of FVIIIa increased the amount of FIXa bound to 1:3 PS/PC vesicles at physiologic [NaCl], the efficiency of the enzyme complex was still best at [NaCl] where electrostatic interactions would be favored, because the efficiency is driven by the interactions of FX with the surface.

When considering the effect of varying [NaCl] on FXa generation, we questioned the effect of ionic strength on FVIIIa association with the surface and on cofactor subunit assembly. On both surfaces, the $EC_{50_{FVIIIa}}$ was insensitive to changes in NaCl concentration ($p > 0.05$), in agreement with reports that FVIIIa interacts, at least in part, hydrophobically with the negatively-charged phospholipid surface (Gilbert & Baleja, 1995). The association of heavy and light chains of FVIII is known to be both hydrophobic and electrostatic (Fay, 1988). The association of the A2 subunit of FVIIIa with the A1 subunit has been found to be primarily electrostatic with dissociation occurring at slightly alkaline pH (Lollar & Parker, 1990) and with association inhibited by increases in [NaCl] from 0.05 to 0.2 M (Fay et al., 1991). This was confirmed by Persson et al. (1995), who found that association of subunit A2 to immobilized heavy and light chains decreased as [NaCl] was increased from 0.1 to 0.2 M, although the off rates were not much affected. In the present study, increasing [NaCl] in FVIIIa-containing reactions improved the reaction rates and the V_{max}'s, corroborating that FVIIIa added to reactions containing increased [NaCl] does not dissociate into A2 and A1/A3C1C2 subunits, leading to loss of functional cofactor activity.

It is possible to explain the inhibitory effect of low [NaCl] in reactions containing FVIIIa as the result of favoring electrostatic interactions between proteins, suggesting that cofactor and enzyme or cofactor and substrate interact primarily hydrophobically. The small increase in enzyme affinity attributable to the presence of FVIIIa in platelet-supported reactions ($K_{d_{app}}$ ~2.5 nM reduced to ~1 nM) and the large effect of FVIIIa on enzyme affinity in vesicle-supported reactions are unaffected by [NaCl]. Even the change at the lowest [NaCl] is not equivalent to the change associated with the presence of cofactor. Therefore, it is unlikely that the NaCl-induced changes in V_{max} result from changes in cofactor−enzyme interactions which affect enzyme affinity.

The chief effect of FVIIIa on F-X activation is a major increase in the k_{cat}. In this study, the V_{max} is increased 2-fold at increased [NaCl] where more apolar interactions are favored, even though this increase represents a minuscule proportion of the 1000-fold increase in k_{cat} associated with the presence of FVIIIa. It is possible that these small reaction rate changes reflect the effect of increased ionic strength on cofactor-improved k_{cat}. Reaction rates on both surfaces, lipid vesicles and platelets, are similarly inhibited by low [NaCl] in the presence of FVIIIa. Reactions performed at high [NaCl], however, display different characteristics on the two surfaces, with vesicle-supported reaction rates responding favorably to the highest concentrations of NaCl used, while activated platelet-supported reactions display decreased activity at the highest NaCl concentrations. This suggests that the change in rates with changes in [NaCl] is mediated partly through surface−protein interactions which are different on 1:3 PS/PC vesicles and platelets.

It would be difficult to select an artificial vesicle composition to mimic the specific lipid milieu on the surface of activated platelets. A lipid analysis of extracted platelet membranes provides little information about the classes of lipids exposed, or about their fatty acid composition or fluidity, even though it has been demonstrated that the fluidity of lipids influences their ability to potentiate procoagulant activity (Higgins et al., 1985). It is possible to extract information about the proportion of lipids available after activation of platelets from the studies of Bevers et al. (1982, 1983), which used phospholipase A2 and sphingomyelinase to hydrolyze the platelet surface lipids. Although control platelets had available to the enzymes about 90% neutral lipids [60% sphingomyelin (SM), 31% PC] and 9% procoagulant lipids [1−2% PS, 8% phosphatidylethanolamine (PE)], those activated with thrombin (0.2−2 units/mL) exposed 76% neutral lipids (SM = PC) and 24% procoagulant lipids (4−5% PS, 20% PE). It is therefore clear that unilamellar vesicles of PC/PS (75:25) represent not only a standard lipid preparation for coagulation studies but also a reasonable analogue of thrombin-stimulated platelets.

Gilbert and Arena (1995, 1996) used vesicles of cholesterol/PC/PS/PE (20:56:4:20) to investigate the kinetics of FVIIIa-enhanced F-X activation. Their data showed that at 1 mM $CaCl_2$ and low lipid concentrations, vesicles containing 25 mol % PS were 10-fold more effective than those containing PS/PE (4:20), although at higher lipid concentration (125 μM) there was only a 2-fold difference. Their data also showed that using 5-fold the amount of PS/PE vesicles to equal the final concentration of PS in 25 mol % PS vesicles still did not result in equivalent activity, suggesting that the

density of PS and not its bulk concentration determines its efficacy. We could in future experiments compare the standard vesicle preparation with vesicles which include PS/PE (5:20), although there is still not enough information available about fatty acid composition or the architecture of binding site domains on platelets to design an artificial vesicle system that would truly mimic the platelet lipid surface.

The results of this study are in agreement with results obtained using the calcium and negatively-charged lipid binding protein annexin V to inhibit FXa generation on activated platelets and on 1:3 PS/PC vesicles.[2] The studies with annexin V suggest that activated platelets offer the enzyme FIXa the possibility of interaction both with negatively-charged phospholipid, available also on artificial phospholipid vesicles, and with proteins available on the platelet surface. The current study also suggests that FX may interact differently with the platelet surface than with an artificial phospholipid vesicle. Upon activation, platelet membranes present specific, saturable sites for interaction with both the enzyme (FIXa) and the substrate (FX), consisting in part of phospholipids and in part, perhaps, of proteins.

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