Unsaturated Phospholipid Acyl Chains Are Required To Constitute Membrane Binding Sites for Factor VIII†

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ABSTRACT: Membranes containing phosphatidyl-L-serine (PS) and phosphatidylethanolamine (PE) greatly enhance the function of the enzymatic cofactor factor VIII. The mechanisms of enhanced function involve condensation of enzyme (factor IXa), activated cofactor (factor VIIIa), and substrate (factor X) at a common location and, most dramatically, activation of the assembled enzyme−cofactor complex. We asked whether unsaturated phospholipid (PL) acyl chains are necessary to constitute factor VIII binding sites or to activate the factor VIIIa−factor IXa complex. We found that membranes composed of saturated, dimyristoyl phospholipids had 20-fold fewer factor VIII binding sites and that these sites supported less than 5% normal activity of the factor VIIIa−factor IXa complex. Thrombin-activated factor VIII bound to a similar number of membrane sites, and thrombin activation did not reduce the affinity for saturated membranes more than 2-fold so that the loss of functional activity is due to a requirement of the factor VIIIa−factor IXa complex for unsaturated acyl chains that exceeds the requirement for factor VIII binding alone. Replacement of dimyristoyl-PS, -PE, or -PC individually with the corresponding unsaturated phospholipid restored 75%, 60%, and 15%, respectively, of factor VIII binding sites but less than 10% of factor VIIIa−factor IXa activating activity. Lyso-PS did not support binding of factor VIII or function of the factor VIIIa−factor IXa complex even when PE and phosphatidylcholine contained unsaturated acyl chains. We conclude that the *sn*-2 acyl chain of PS and unsaturated phospholipid acyl chains are chemical requirements for constitution of fully functional factor VIII binding sites on phospholipid membranes.

Factor VIII (antihemophilic factor) is a plasma glycoprotein which functions as an enzymatic cofactor for the serine protease, factor IXa, on PS-containing[1] membranes [for review, see (*1*)]. The complex of activated factor VIII with factor IXa on a membrane efficiently cleaves the zymogen, factor X, converting it to the active form. Activated factor X then catalyzes cleavage of prothrombin to thrombin, the chief effector molecule in hemostasis. The importance of the factor VIIIa−factor IXa complex is illustrated by hemophilia, a disease in which deficiency of factor VIII or factor IX causes life-threatening bleeding. Although phospholipid membranes exert a critical regulatory role for the factor VIIIa−factor IXa complex, the chemical moieties of phospholipid membranes that constitute factor VIII binding sites and determine activation of the factor VIIIa−factor IXa complex have been only partially characterized.

The factor VIII gene directs synthesis of a single polypeptide chain of approximately 280 kDa (*2*−*4*). The factor VIII molecule has a repeating domain structure A1-A2-B-A3-C1-C2 in which the A domains are homologous with a repeating domain of ceruloplasmin, the highly glycosylated B domain lacks homology to known proteins, and the C domains are homologous with a domain of milk fat globule proteins (*5*, *6*) and with a PS binding slime mold lectin (*7*). Single-chain factor VIII is cleaved in the Golgi apparatus to yield a heterodimer consisting of a "heavy chain" composed of the A1-A2-B fragment and a "light chain" consisting of the A3-C1-C2 fragment (*8*). In plasma, factor VIII binds to von Willebrand factor which prevents factor VIII from binding to membranes and premature proteolytic degradation [for review see (*9*)]. In the course of blood coagulation, factor VIII is cleaved by thrombin to the active form, factor VIIIa (*10*), which no longer binds to von Willebrand factor and is free to bind to phospholipid membranes (*11*). The activated factor VIII molecule is a heterotrimer consisting of the A1 domain, the A2 domain, and the light chain (*12*, *13*). The A2 and A3 domains interact with factor IXa (*14*, *15*) while the C-terminal portion of the C2 domain interacts with vWf prior to activation (*16*) and with PS-containing membranes following release from von Willebrand factor (*17*, *18*). Activated human factor VIII is unstable because the A2 domain dissociates with a half-life of approximately 2 min (*19*). However, when factor IXa binds to factor VIIIa, it increases the affinity of the A2 domain for the protein complex, thus contributing to formation of an enzyme complex that is stable for many minutes (*20*).

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[1] Abbreviations: PS, phosphatidyl-L-serine; PE, phosphatidylethanolamine; PC, phophatidylcholine; Chol, cholesterol; DMPS, dimyristoyl-PS; DMPE, dimyristoyl-PE; DMPC, dimyristoyl-PC; PL, phospholipid.

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Factor VIII binds to PS-containing membranes with high affinity (*21*) and with remarkable specificity (*11*). High-affinity binding is mediated by a multistep binding process in which the first step is probably electrostatic interaction between the membrane binding motif of factor VIII and exposed PS (*22*). The specificity of the membrane binding interaction is illustrated by the failure of other plasma PS binding proteins to compete with factor VIII for membrane binding sites (*11*). Membrane binding is mediated in part by stereoselective interaction of factor VIII with *O*-phospho-L-serine, the ionic headgroup of PS (*23*). High-affinity membrane binding of factor VIII requires PS content ≥8%, a condition rarely, if ever, encountered on membranes of viable cells. However, if PE is included in the membranes, as little as 1% PS is sufficient to induce formation of high-affinity factor VIII binding sites (*24*). Thus, the external leaflets of undifferentiated cells (*25*), and mature, stimulated platelets (*26*) have the phospholipid compositions predicted to provide specific, high-affinity factor VIII binding sites.

The importance of membrane binding for function of factor VIII in the factor VIIIa−factor IXa membrane complex is best illustrated by comparing the enzymatic parameters in the presence and absence of phospholipid membranes. PS-containing membranes increase the affinity of factor VIIIa for factor IXa by approximately 20-fold and increase the affinity of the factor VIIIa−factor IXa complex for the substrate factor X by approximately 80-fold. However, the largest effect of PS-containing membranes is on the k_{cat} of the assembled factor VIIIa−factor IXa complex which increases 6000-fold when bound to PS-containing membranes (*27*). Thus, it appears that PS-containing membranes act as allosteric activators of the factor VIIIa−factor IXa complex. The likely role of phospholipid structures in allosteric activation of the factor VIIIa−factor IXa complex is illustrated by the capacity of soluble PS molecules with short acyl chains to partially activate the enzyme complex at submicellar concentrations (*28*). Thus, while prior investigation indicates the importance of specific phospholipid headgroup moieties required to support binding of factor VIII, it has not interrogated the importance of acyl chain structures which reside in the hydrophobic core of the membrane.

Factor VIII is homologous with factor V, another enzymatic cofactor of blood coagulation (*29*). Both proteins share the same repeating domain structure and, following proteolytic activation, function as membrane-bound cofactors for serine proteases [for review, see (*1*)]. Factor Xa, the product of the factor VIIIa−factor IXa complex, serves as the enzyme in the prothrombinase complex composed of membrane-bound factor Va assembled with factor Xa. The two proteins differ from each other in that factor V circulates in plasma primarily as a free molecule at a 100-fold higher concentration than factor VIII. Factor V also binds to membranes that contain a lower mole fraction of PS than that required by factor VIII (*21*) and apparently binds via the amino-terminal portion of the C2 domain (*30*) rather than the C-terminal portion homologous with the membrane binding motif of factor VIII. Recent reports indicate that enzymatic function of the prothrombinase complex (membrane-bound factor Va−factor Xa) is slowed 25-fold on membranes with saturated acyl chains (*31, 32*). For this enzyme complex, saturated acyl chains alter the preferred prothrombin peptide bond recognized and the rate of cleavage. The apparent requirement for unsaturated acyl chains to support an allosteric change near the catalytic triad of factor Xa helps to motivate our study of unsaturated acyl chains and the factor VIIIa−factor IXa complex.

EXPERIMENTAL PROCEDURES

L-Oleoyllyso-PS, bovine brain PS, dioleoyl-PS, 1-stearoyl-2-oleoyl-PS, PE from egg yolk, dimyristoyl-PE, PC from egg yolk, and dimyristoyl-PC were from Avanti Polar Lipids (Alabaster, AL). Recombinant human factor VIII was a gift from D. Pittman of Genetics Institute, Cambridge, MA. Human factor IXa, factor X, and factor Xa were from Enzyme Research Laboratories (South Bend, IN).

Preparation and Evaluation of Proteins. The purity of proteins was evaluated by SDS−PAGE with silver staining. All preparations used in these studies exhibited only the bands corresponding to those previously attributed to the respective proteins with the exception of a single contaminant band that comigrated with bovine albumin standards. This contaminant was present as approximately 5% of the total protein for the factor IXa, factor X, and factor Xa preparations. Factor X was contaminated by factor Xa at a level of approximately 1 part per 2000 as judged by the rate of development of the chromogenic substrate S-2765 (Helena, Beaumont, TX). Therefore, stock factor X was incubated with 19 μM dansyl-Glu-Gly-Arg-$CClH_2$ (Calbiochem, San Diego, CA) for 90 min at RT and dialyzed against 0.14 M NaCl, 0.05 M Tris/HCl, pH 7.5, yielding a product that did not cleave S-2765 at a rate above buffer at the highest concentrations used in these experiments. Recombinant human factor VIII was activated by thrombin as described (*19, 27*). This factor VIIIa had activity, at a concentration of 0.1 nM, ranging from 30 to 50% of factor VIII activity when activated by thrombin in the presence of factor IXa and phospholipid. The loss in activity was attributed to dissociation of the A2 domain from the factor VIIIa complex during the approximately 2 min interval between dilution of factor VIIIa and initiation of the reaction in keeping with the measured decay half-life of 2 min (*19*). Factor VIII was labeled with fluorescein-maleimide as previously described (*11, 33*). The protein concentration of factor VIII was determined using a Micro-BCA Assay (Pierce, Rockford, IL) using bovine albumin as a standard. All proteins were aliquoted into fractions for single usage, flash-frozen in liquid nitrogen, and stored at −80 °C until use.

Lipospheres and Phospholipid Vesicles. Phospholipid vesicles were synthesized by sonication in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) under argon until the suspension was visually clear. Phospholipid concentration was determined by phosphorus assay (*34*). Vesicles were used fresh, or 1 mL aliquots were quick-frozen in liquid nitrogen, stored at −80 °C, and thawed at 37 °C. Storage at 4 °C prior to incubation with microspheres did not exceed 1 day. Glass microspheres of 1.6 μm nominal diameter (Duke Scientific, Palo Alto, CA) were cleaned, size-restricted, and covered with a phospholipid bilayer as previously described (*11*) except that Tween 80 was omitted from the wash buffer and sonicated vesicles of 100% egg PC, 10 μM, were included. Membranes supported by glass microspheres (lipospheres) were stored at 4 °C and used within 8 h of synthesis.

Flow Cytometry Binding Assay. Flow cytometry was performed on 25 μL aliquots of 100 μL samples with an approximate liposphere concentration of 1×10^6/mL using a Becton Dickinson FACScan. Data acquisition was triggered by 90° light scatter with all photomultipliers in the log mode. Noise was reduced during analysis by eliminating events with forward and side scatter values different from those characteristic of the lipospheres. Mean log fluorescence was converted to linear fluorescence for values depicted in the figures. Only experiments in which the fluorescence histogram indicated a log-normal distribution, as judged by inspection, were analyzed quantitatively. For competition binding experiments, various concentrations of saturated vesicles were incubated with factor VIII for 10 min prior to the addition of lipospheres. After 10 min, liposphere-bound factor VIII was measured by flow cytometry.

Analysis of Binding Data. For simple binding experiments, e.g., Figure 1A, data were fitted to the standard binding equation by nonlinear least-squares analysis using the software FitAll v 4.0. The assumptions were that factor VIII recognizes a single class of binding sites and that the fraction of factor VIII bound to lipospheres can be ignored in calculating the free factor VIII concentration. The second assumption is justified by the low concentration of phospholipid associated with 1×10^6 lipospheres/mL, 80 nM (*11*), which is not predicted to bind more than 10% of free factor VIII under experimental conditions. For competition binding experiments (Figures 1B and 2B), free factor VIII, $VIII_f$, in the presence of saturated phospholipid vesicles was calculated from liposphere-bound factor VIII using the relationship:

$$[VIII_f] = \frac{BK_D}{1 - B}$$

where B is the fraction of occupied binding sites on lipospheres and K_D is the dissociation constant describing binding of factor VIII to the unsaturated phospholipid membrane on lipospheres. B was determined by comparing fluorescence readings to the maximal fluorescence projected from nonlinear least-squares curve-fitting and the same batch of fluorescein-labeled factor VIII and unsaturated phospholipid for the liposphere bilayers. The concentration of factor VIII bound to vesicles of saturated phospholipids was obtained from experimental data using the relationship:

$$VIII_b = VIII_t - VIII_f$$

These results were compared to predicted bound factor VIII from the relationship:

$$[VIII_b] = \{K_{D\ sat} + [VIII_t] + [PL]/n - \sqrt{(K_{D\ sat} + [VIII_t] + [PL]/n)^2 - 4[VIII_t][PL]/n}\}/2$$

where $K_{D\ sat}$ characterizes binding to saturated bilayers, the phospholipid/binding site stoichiometry is n, and the concentration of saturated phospholipid is [PL]. Data from two experiments with different factor VIII concentrations were simultaneously fitted by eye using Microsoft Excel v 5.0.

Factor Xase Assay. Factor Xase activity was measured with a two-step amidolytic substrate assay. Phospholipid was mixed with a reaction mixture containing 0.025 nM factor IXa, and 65 nM factor X in 0.15 M NaCl, 0.2 wt %/v bovine serum albumin, 50 mM trizma-HCl, pH 7.8. The reaction was started by adding Ca^{2+} and factor VIIIa at final concentrations of 1.5 mM and 5 nM, taking care that ≤ 2 min elapsed from dilution of concentrated factor VIIIa until reaction initiation. After 5 min at 25 °C, the reaction was stopped by diluting the mixture 1:0.8 with 16 mM EDTA, and factor Xa activity was determined immediately in a thermostated kinetic microtiter plate reader (Molecular Devices, Menlo Park, CA) at 25 °C using 0.1 mM S-2765. A standard curve was prepared using pure factor Xa. The results displayed in the figures are means of duplicates from a representative experiment.

RESULTS

We hypothesized that unsaturated phospholipid acyl chains are constituents of factor VIII binding sites. To test this hypothesis, we compared binding of fluorescein-labeled factor VIII to membranes containing phospholipids with synthetic, saturated acyl chains vs mixed acyl chains of biologic origin (Figure 1A). While factor VIII bound saturably and with high affinity to membranes supported by glass microspheres (lipospheres) with a composition of 4% PS, 20% PE, 76% PE of biologic origin, binding to lipospheres with the same phospholipid composition but with all myristoyl acyl chains was reduced more than 80%. Nonlinear, least squares curve-fitting indicated that the number of factor VIII binding sites was greatly reduced but that the affinity of factor VIII for these sites remained approximately the same (Table 1). To confirm that the reduction in number of binding sites reflects an effect of saturated acyl chains on native membrane structure rather than altered structure related to support of membranes by glass microspheres, we performed competition binding experiments. Binding of factor VIII to sonicated vesicles of biologic or dimyristoyl lipids was detected as decreased free factor VIII available to bind to lipospheres. Vesicles of biologic phospholipids competed more than 90% of bound factor VIII from lipospheres at a concentration of 4 μM phospholipid, consistent with the stoichiometry of 180 phospholipid molecules/binding site that we have previously measured (data not shown). In contrast, when factor VIII was incubated with sonicated vesicles of dimyristoyl phospholipids (Figure 1B) prior to addition of lipospheres, much higher concentrations of sonicated vesicles were required to bind significant fractions of factor VIII. The smooth lines indicate fitted curves assuming a K_D of 17 nM and 3300 phospholipid molecules/binding site. Deviation from these curves for phospholipid concentrations >32 μM is justified by noting that at these concentrations many large vesicle aggregates are detected by flow cytometry and rationalizing that vesicle aggregates display fewer factor VIII binding sites per phospholipid molecule because some sites are blocked by other vesicles. The 95% reduction in the number of factor VIII binding sites present on vesicles of saturated phospholipids vs vesicles of biologic phospholipids confirms that unsaturated phospholipids are necessary for efficient formation of factor VIII binding sites.

To determine whether decreased binding of factor VIII correlated to decreased function as a cofactor in the factor Xase complex, we evaluated the capacity of sonicated phospholipid vesicles of the same compositions to support activity (Figure 1C). In preliminary experiments, we identi-

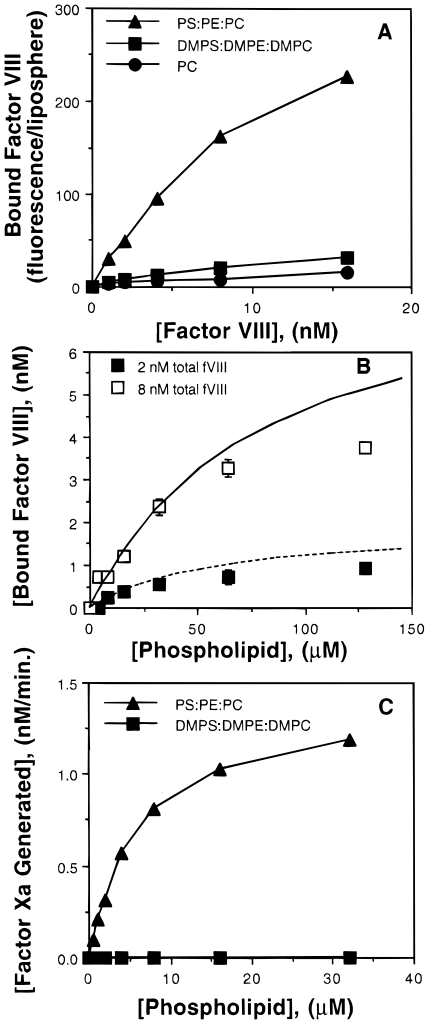

FIGURE 1: Effect of acyl chain saturation on binding of factor VIII to membranes with 4% PS. (A) Fluorescein-labeled factor VIII at the indicated concentrations was incubated with liposphere containing 4% PS and either biologic, unsaturated phospholipids (▲) or synthetic, saturated phospholipids (■). As a control, liposphere with membranes of PC (●) were utilized. Bound factor VIII was measured by flow cytometry. The apparent number of factor VIII binding sites was reduced on membranes of saturated phospholipid, approaching fluorescence associated with the PC liposphere. (B) Binding of 2 nM (■) or 8 nM fluorescein-labeled factor VIII (□) to sonicated phospholipid vesicles of saturated phospholipids was detected as reduced binding of factor VIII to liposphere. The solid and dashed curves represent predicted binding assuming a K_D of 17 nM and 3300 phospholipid molecules/factor VIII binding site. Values displayed represent mean ± SD for 3 experiments performed at each factor VIII concentration. (C) To determine whether the factor VIII binding sites detected were functional, sonicated phospholipid vesicles from the same lipid preparations used in panel A were incubated with factor VIIIa, factor IXa, and factor X. Whereas free factor VIIIa loses activity with a $T_{1/2}$ of 2 min (*19*), the factor VIIIa−factor IXa complex is stable for many minutes under these conditions because factor IXa binds to factor VIIIa and slows the rate at which the A2 subunit dissociates (*20*). After 5 min, the reaction was stopped, and the quantity of factor Xa generated was measured in a kinetic microplate reader using chromogenic substrate S-2765. Vesicles prepared from biologic phospholipids supported efficient function of the factor VIIIa−factor IXa complex while those prepared from saturated phospholipids supported no detectable activity. The displayed experiment is representative of 6 such experiments. The lipid compositions were (▲) PS:PE:PC:Chol 4:20:76:20, (■) DMPS:DMPE:DMPC:Chol 4:20:76:20, (●) PC:Chol 100:20.

Table 1: Parameters for Factor VIII Binding to Saturated and Unsaturated Phospholipid Membranes

phospholipid composition	molar ratio	K_D (nM)	B_{max}[a] (fluorescence/ liposphere)	*n*
PS:PE:PC	4:20:76	17 ± 6	289 ± 74	7
DMPS:DMPE:DMPC	4:20:76	17 ± 6	45 ± 14	6
PS:PC	25:75	9 ± 1	2218 ± 124	4
DMPS:DMPC	25:75	15 ± 5	309 ± 73	4
PS:DMPE:DMPC	4:20:76	11 ± 6	172 ± 81	7
DMPS:PE:DMPC	4:20:76	29 ± 3	81 ± 9	4
DMPS:DMPE:PC	4:20:76	22 ± 3	187 ± 38	4
DOPS:DMPE:DMPC	4:20:76	5 ± 2	108[b] ± 23	3
SOPS:DMPE:DMPC	4:20:76	13 ± 8	93 ± 14	3

[a] B_{max} is the maximum fluorescence per liposphere predicted when all factor VIII binding sites are saturated. Values were obtained from subjecting data such as those displayed in Figures 1A, 2A, 4, and 5 to nonlinear least-squares curve-fitting. Values are mean ± SD for the fitted values from the indicated number of experiments. [b] The lesser value of the mean B_{max} for membranes with DOPS vs bovine brain PS is in contrast to results displayed in Figure 5 where B_{max} was greater for DOPS. Because B_{max} for DOPS was greater than bovine brain PS for 3 of 3 experiments where binding to these phospholipids was carried out in parallel, we believe that membranes with DOPS actually provide more factor VIII binding sites than membranes with bovine brain PS.

fied protein concentrations that led to factor Xa production that was proportional to an elapsed time of more than 4 min at unsaturated phospholipid concentrations up to 8 μM. Although these conditions were predicted to produce an artifactual low V_{max} at the optimal concentration of biologic phospholipids, they were utilized because they were more sensitive to any residual Xase activity supported by saturated phospholipids than an assay optimized to report maximal Xase activity. While vesicles containing phospholipid of biologic origin efficiently supported activity of the Xase complex, as we have previously reported, vesicles containing saturated phospholipid did not support activity. By comparison of the results in Figure 1B with those in Figure 1C, it is evident that the highest concentration of phospholipid employed bound at least 30% of total factor VIII. Yet, the reduction of factor Xase activity was greater than 95%, and the rate of color development was not greater than the background rate in the absence of added factor X. These results indicate that unsaturated acyl chains have an influence upon the function of the factor VIIIa−factor IXa complex that extends beyond their requirement for efficient formation of factor VIII binding sites.

Cholesterol was included in the phospholipid membranes at 20 mol % to ensure that the phospholipids were in the liquid-crystalline phase rather than the gel phase at 23−25 °C (*35*, *36*). To confirm that the loss of factor VIII binding sites did not correlate to membrane fluidity changes that occur near the phase transition temperature, we evaluated the capacity of dimyristoyl vs biologic phospholipids to support function of the factor Xase complex at 37 °C. While the vesicles of biologic phospholipids efficiently supported activity of the factor Xase complex, the dimyristoyl phospholipids supported no measurable activity at concentrations up to 32 μM (data not shown). These results confirm that failure of membranes with saturated phospholipids to support function of the factor Xase complex is not a consequence of the altered phase transition temperature and is not solely a consequence of decreased fluidity in these membranes.

Membranes with >20% acidic phospholipid support function of the factor Xase complex but, in contrast to membranes

with <15% acidic phospholipid, do not require the *O*-phospho-L-serine motif or PE (*24*). Because the hydrophilic constituents of binding sites differ on highly acidic membranes, we asked whether the requirement for unsaturated phospholipids might also be diminished. Therefore, we measured binding of fluorescein-labeled factor VIII to membranes containing 25% PS, 75% PC containing biologic phospholipids vs dimyristoyl phospholipids (Figure 2A). The number of binding sites on membranes with myristoyl acyl chains was reduced more than 80% compared to biologic phospholipids. However, the number of high-affinity binding sites on these membranes was similar to the number of sites on membranes containing biologic lipids but with 4% PS and 20% PE (Table 1). These results indicate that unsaturated acyl chains are necessary for approximately 80% of high-affinity factor VIII binding sites even on membranes with high mole fractions of acidic phospholipid. To confirm that the reduction in number of binding sites reflects an effect of saturated acyl chains rather than altered structure related to support of membranes by glass microspheres, we performed competition binding experiments utilizing sonicated vesicles to bind factor VIII. Vesicles of biologic phospholipids competed more than 90% of bound factor VIII from lipospheres at a concentration of 4 μM phospholipid, consistent with the stoichiometry of approximately 125 phospholipid molecules/binding site that we have previously measured (*21*) (data not shown). In contrast, when factor VIII was incubated with sonicated vesicles of dimyristoyl phospholipids (Figure 2B) prior to addition of lipospheres, much higher concentrations of sonicated vesicles were required to bind significant fractions of factor VIII. Both smooth lines indicate fitted curves assuming a K_D of 10 nM (Table 1) and 2500 phospholipid molecules/binding site. In contrast to vesicles with 4% PS, 20% PE, these vesicles did not aggregate detectably at the higher concentrations employed, presumably because of the greater electrostatic repulsion between them. The results confirm that unsaturated acyl chains are necessary for efficient formation of factor VIII binding sites but that some high-affinity sites form even on membranes of saturated phospholipids.

To determine whether the residual binding sites would support function of factor VIII, we compared phospholipid vesicles prepared from the same phospholipid preparations in the factor Xase assay (Figure 2C). Biologic phospholipids efficiently supported the factor Xase complex with a half-maximal concentration approximately 4-fold lower than membranes with 4% PS, 20% PE. In contrast, those with dimyristoyl lipids did not support activity at concentrations as high as 32 μM. Comparison of these results with Figure 2B indicates that the highest phospholipid concentration, 32 μM, was sufficient to bind more than 30% of factor VIII. However, factor Xase activity was reduced more than 95%. These results confirm that unsaturated acyl chains are required not only to constitute most factor VIII binding sites but also to support function of the membrane-bound factor VIIIa−factor IXa complex.

While unactivated factor VIII was used for the binding experiments depicted in Figures 1 and 2, thrombin-activated factor VIII (factor VIIIa) was used for the enzyme cofactor experiments depicted in Figures 1C and 2C. It remained possible that factor VIIIa has modified membrane binding characteristics compared to unactivated factor VIII. Therefore, we measured membrane binding of unactivated factor VIII vs thrombin-activated factor VIII to lipospheres with membranes of unsaturated phospholipid (Figure 3A) and saturated phospholipid (Figure 3B). Factor VIII was exposed to thrombin either 10 min or 2 min prior to addition of lipospheres at concentrations previously shown to lead to efficient activation of factor VIII. Fitting the binding iso-

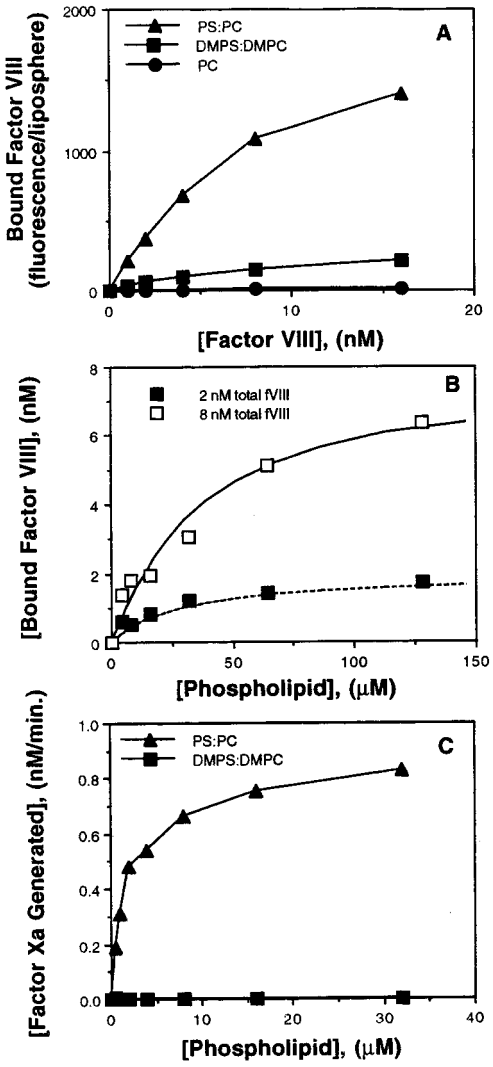

FIGURE 2: Effect of acyl chain saturation on binding of factor VIII to membranes with 25% PS. (A) Fluorescein-labeled factor VIII was incubated with lipospheres containing either biologic, unsaturated phospholipids (▲) or synthetic, saturated phospholipids (■). The results indicate that approximately 80% of the high-affinity binding sites require unsaturated acyl chains even when the membranes contain 25% PS. (B) Binding of 2 nM (■) or 8 nM fluorescein-labeled factor VIII (□) to sonicated phospholipid vesicles of saturated phospholipids was detected as reduced binding of factor VIII to lipospheres. The solid and dashed curves represent predicted binding assuming a K_D of 10 nM and 2500 phospholipid molecules/factor VIII binding site. (C) To determine whether factor VIII binding sites on saturated membranes with 25% PS would support function of factor VIII, sonicated vesicles from the same phospholipid preparations used to prepare lipospheres used for panel A were incubated with factor VIIIa, factor IXa and factor X. After 5 min, the reaction was stopped, and the amount of factor X generated was measured using chromogenic substrate S-2765. Saturated phospholipid vesicles with 25% PS did not support measurable function of the factor Xase complex. Phospholipid compositions were (▲) PS:PC:Chol 25:75:20, (■) DMPS:DMPC:Chol 25:75:20, (●) PC:Chol 100:20. Results in panel C are representative of 3 such experiments.

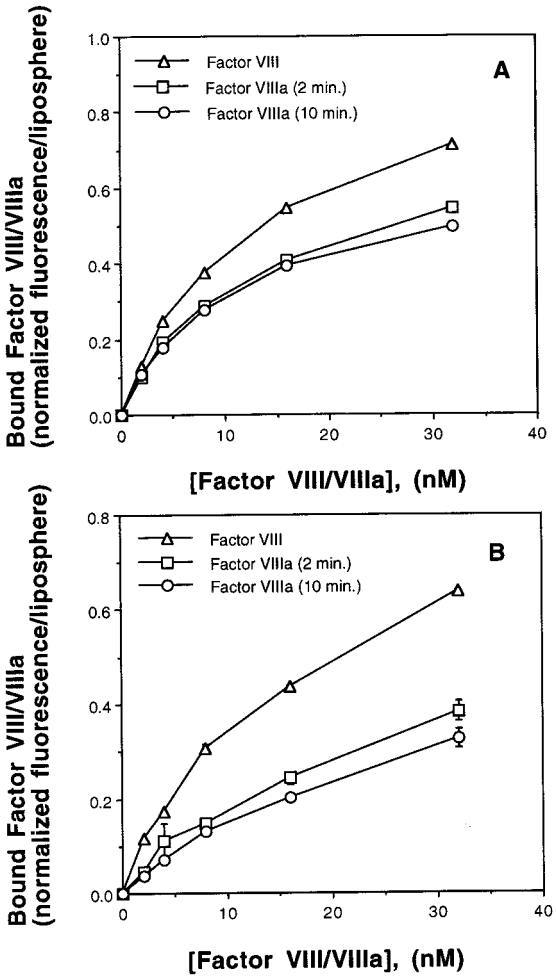

FIGURE 3: Effect of thrombin-mediated activation of factor VIII upon binding to lipospheres. Fluorescein-labeled factor VIII at the indicated concentrations was incubated with 1 nM thrombin for 2 (□) or 10 (○) min or with no thrombin (△) prior to the addition of lipospheres. After 10 min, binding of factor VIII to unsaturated liposphere membranes (A) or saturated membranes (B) was measured by flow cytometry. The affinity of factor VIII and thrombin-activated factor VIII for unsaturated membranes of biologic origin was apparently unaltered. However, the affinity of thrombin-activated factor VIII for saturated, dimyristoyl membranes was apparently reduced 2-fold. Displayed results are from a single experiment (A) and mean ± SD from 2 experiments (B). Data for each panel were normalized to the B_{max} for unactivated factor VIII for ease of comparison, prior to data averaging for panel B. Membrane compositions were (A) PS:PE:PC:Chol 4:20:76:20 and (B) DMPS:DMPC:Chol 25:75:20.

therms to the standard binding model indicated that the affinity of factor VIII for lipospheres of biologic phospholipid was unaltered by thrombin activation. However, the maximum fluorescence for factor VIIIa was approximately 30% less. We attributed this reduction to the time-dependent dissociation of the fluorescein-labeled A2 domain from factor VIIIa but not factor VIII (see Discussion). Thrombin-activated factor VIII also bound saturably to membranes with saturated phospholipids (Figure 3B), but the affinity was reduced approximately 2-fold. The fluorescence at saturation, from nonlinear least-squares curve-fitting, was 35% less than for unactivated factor VIII. These results indicate that the affinity of factor VIII for phospholipid membranes is not reduced more than 2-fold by thrombin activation and are consistent with the interpretation that thrombin-activated factor VIII recognizes the same binding sites.

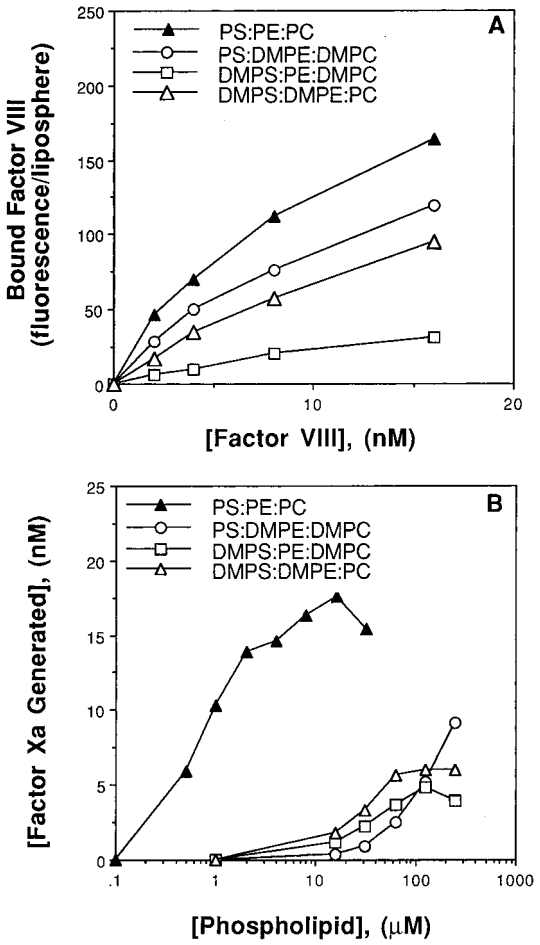

FIGURE 4: Effect of unsaturated acyl chains as constituents of a single phospholipid species. (A) Bovine brain PS (○), egg PC (△), or egg PE (□) was used to replace DMPS, DMPC, or DMPE, respectively. The number of factor VIII binding sites was increased to more than 50% of the number observed when all phospholipids were unsaturated for PS and PC but only 15% of that maximum for PE. (B) Vesicles with the same phospholipid compositions were evaluated for capacity to support Xase enzyme complex function. Replacement of individual saturated phospholipid species with biologic, unsaturated phospholipids led to partial reconstitution of the Xase supporting activity. The lipid composition was PS:PE:PC:Chol 4:20:76:20 with the indicated lipids present entirely as synthetic dimyristoyl phospholipid. Experimental conditions were as described in the legend to Figure 1. Displayed experiments are representative of 4 such experiments (A) and 4 such experiments (B).

We asked which unsaturated phospholipids contribute to factor VIII binding sites on membranes with phospholipid ratios of PS:PE:PC 4:20:76 (Figure 4). Substitution of bovine brain PS for dimyristoyl-PS reconstituted approximately 70% of factor VIII binding sites. Substitution of egg PC for dimyristoyl-PC restored approximately 55% of binding sites. Substitution of egg PE for dimyristoyl-PE restored only about 20% of binding sites. These results indicate that the unsaturated acyl chains of PS make the largest contribution to the formation of factor VIII binding sites even though PS constitutes only 4% of the membrane phospholipid. However, these results also indicate that unsaturated PS acyl chains are not strictly necessary because a high mole fraction of unsaturated PC can provide a similar number of high-affinity binding sites. We also asked whether binding sites from these membranes support function of the factor VIIIa−factor IXa complex (Figure 4B). The results

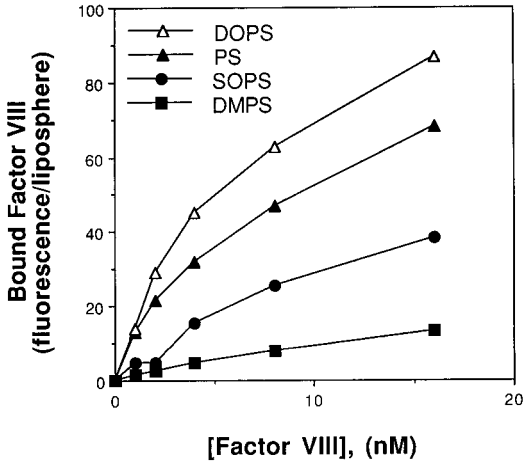

FIGURE 5: Effect of various unsaturated acyl chains on binding of factor VIII. Lipospheres were prepared from vesicles containing 4% dioleoyl-PS (△), bovine brain PS (▲), stearoyloleoyl-PS (●), and dimyristoyl-PS (■). The other phospholipids had saturated myristoyl acyl chains. Dioleoyl-PS supported the greatest number of factor sites, bovine brain PS was second, and stearoyloleoyl-PS third. Experimental conditions are as described in the legend to Figure 1, and displayed data are representative of 4 such experiments.

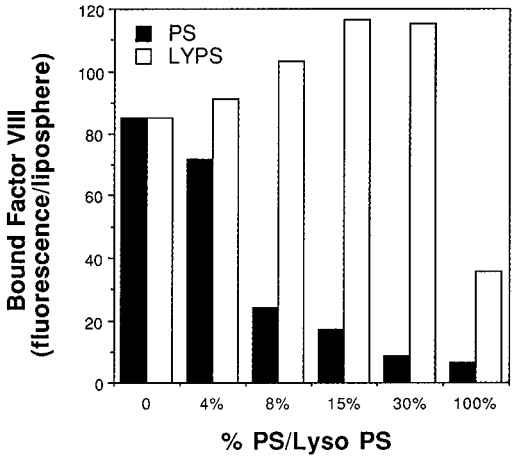

FIGURE 6: Importance of the *sn*-2 acyl chain of PS for factor VIII binding sites. Vesicles were prepared containing the indicated fraction of PS or lyso-PS. Vesicles contained 20% PE, and the balance of phospholipid was PC. Sonicated vesicles of the indicated composition were mixed with lipospheres at a phospholipid concentration of 50 μM, a concentration >5-fold higher than the critical micellar concentration of lyso-PS. Fluorescein-labeled factor VIII was added, and after 10 min, factor VIII bound to lipospheres was measured by flow cytometry. Vesicles containing PS competed with lipospheres for factor VIII binding while those containing lyso-PS did not. The phospholipid composition of liposphere membranes was PS:PE:PC:Chol 4:20:76:20, and that of competing vesicles was X:20:Y:20 where X is the specified PS/lyso-PS content and Y = 80 − X. Displayed results are representative of 3 such experiments performed with 2 separate preparations of vesicles.

indicate that some binding sites resulting from inclusion of unsaturated acyl chains as constituents of PS, PE, or PC are functional. However, the effective phospholipid concentrations were approximately 50-fold higher for all three membranes containing a single unsaturated phospholipid species. Thus, these results, like those displayed in Figure 2, indicate that not all membrane sites that support high-affinity binding of factor VIII will support function of the factor VIIIa−factor IXa complex.

To further explore the acyl chain features that influence membrane binding of factor VIII, we asked whether factor VIII displays preferential affinity for PS species with different unsaturated acyl chains (Figure 5). We compared binding to membranes containing 4% dioleoyl-PS vs bovine brain PS, vs stearoyloleoyl-PS, vs dimyristoyl-PS. Dioleoyl-PS, with double bonds in both acyl chains, most efficiently formed factor VIII binding sites. However, bovine brain PS supported approximately 80% as many sites. Stearoyloleoyl-PS supported only half as many binding sites as bovine brain PS. Because stearoyloleoyl-PS is the dominant species of bovine brain PS, these results suggest that minor PS species with multiple double bonds in the *sn*-2 acyl chain are more effective than stearoyloleoyl-PS.

The results in this paper suggest that factor VIII interacts preferentially with unsaturated acyl chain(s) adjacent to a PS molecule. We hypothesized that the presence of an *sn*-2 acyl chain on PS may be a structural requirement for binding of factor VIII. We therefore performed binding experiments to see whether lyso-PS would support binding of factor VIII. We first performed experiments of lyso-PS solubility to determine what concentration of phospholipid vesicles would be necessary to ensure that virtually all lyso-PS resided within membrane bilayers rather than as soluble monomers. Under the conditions used in these studies, the critical micellar concentration for lyso-PS was <10 μM as determined by 90° light scattering (data not shown). Therefore, we utilized a competition binding assay that would provide sufficiently high membrane concentrations to ensure that lyso-PS was a membrane constituent. The competition binding format also ensured that binding would be detected even if the affinity was lower or a reduced number of binding sites were present (Figure 6). While control vesicles containing PS competed with lipospheres for binding of factor VIII, vesicles containing lyso-PS did not. Increasing the fraction of lyso-PS as high as 30%, conditions where the chemical requirements for formation of factor VIII binding sites are relaxed, supported no binding of factor VIII. In confirmatory experiments, vesicles containing lyso-PS did not support the activity of factor VIII in the Xase enzyme complex (data not shown). Only micelles of 100% lyso-PS bound factor VIII, and these were less effective than vesicles containing 8% PS. While 100% lyso-PS micelles enhance activity of the Xase complex, the effect on the k_{cat} is very small as we have previously reported (*28*). These results indicate that the *sn*-2 acyl chain of PS is a structural requirement for formation of factor VIII binding sites and for activation of the factor VIIIa−factor IXa enzyme complex.

DISCUSSION

Our prior investigations have indicated that factor VIII binds to PS-containing membrane sites and that these sites are highly specific for factor VIII (*11*). Binding is partially mediated by stereoselective recognition of the *O*-phospho-L-serine moiety of PS by factor VIII (*23*). The corresponding structure of factor VIII is apparently a PS binding peptide, from the membrane binding C2 domain which forms an amphipathic helix that may penetrate the hydrophobic core of a phospholipid bilayer (*37*). The results in this report identify two new phospholipid requirements to constitute functional factor VIII binding sites. First, PS must contain

an *sn*-2 acyl chain. Second, some acyl chains in the membrane bilayer must be unsaturated. For membranes with 4% PS and for membranes with the supraphysiologic PS content of 25% approximately, 10−20% of factor VIII binding sites apparently do not require unsaturated acyl chains. However, although these sites bind factor VIII, they do not support the function of factor VIII in the factor Xase complex. Therefore, unsaturated acyl chains are required to constitute functional binding sites even when the membranes contain high mole fractions of PS.

The major effect of unsaturated acyl chains was to increase the number of factor VIII binding sites whereas the affinity for the residual binding sites on saturated membranes was equivalent to unsaturated membranes. Binding sites were sufficiently rare on saturated phospholipid membranes, approximately 1/3000 molecules, to raise the possibility that they result from trace contaminants of the saturated lipids by unsaturated lipid species. We believe that this possibility is unlikely because controlled addition of small quantities of unsaturated phospholipid had a very modest effect on formation of binding sites unless the unsaturated phospholipid was phosphatidylserine. Alternatively, the scarce binding sites could result from formation of rare, but reproducible membrane structural defects. This possibility is bolstered because saturated phospholipid membranes on lipospheres apparently display 2−4-fold more binding sites than sonicated vesicles from the same phospholipids. It seems probable that the process of vesicle fusion on a glass surface with microscopic irregularities results in more frequent membrane defects than formation of sonicated vesicles.

Acyl chain double bonds decrease the packing density of phospholipid molecules in a bilayer and increase the fluidity (*38*). They also decrease the gel to liquid-crystalline phase transition temperature. However, our data suggest that these altered physical properties are not the primary means through which acyl chain double bonds enhance binding and function of factor VIII. Acyl chain double bonds of PS greatly increase the number of factor VIII binding sites even though PS represents only 4% of total lipid. Addition of double bonds to only PS would have a small impact on lipid packing or fluidity (Figure 4). By comparison, exchanging egg PC for dimyristoyl-PC achieves a comparable effect even though this represents alteration of 76% of membrane phospholipid, having a large impact on fluidity and packing. Thus, we believe the results are best explained by a model in which the membrane-penetrating region of factor VIII interacts preferentially with unsaturated phospholipid acyl chain(s). The preferential effect of PS acyl chain double bonds may be rationalized by considering that the membrane-penetrating moiety probably inserts adjacent to a PS headgroup. Thus, PS with unsaturated acyl chains has these structures at the location they are most likely to have a productive interaction with the membrane-penetrating portion of factor VIII.

The decrease in B_{max} for thrombin-activated factor VIII binding to lipospheres probably results from decreased mean fluorescence per factor VIII molecule rather than recognition of fewer binding sites by thrombin-activated factor VIII. Factor VIII contains three free cysteines (*39*), of which two are readily derivatized by fluorescent probes. One is within the A2 subunit and the other within the A3 subunit (*40*). While factor VIII is a stable dimer, thrombin-activated factor VIII is an unstable heterotrimer consisting of A1, A2, and the A3C1C2 light chain. The source of instability is rapid dissociation of the A2 domain from the remaining heterodimer with a half-time of 2 min (*19*). Because membrane binding determinants are localized within the C2 domain of the light chain, the prediction is that membrane binding of the heterodimer containing one fluorescently labeled moiety in the A3 domain may be unaffected but would have less fluorescence per molecule. Thus, we interpret the lower curve, achieved with factor VIII exposed to thrombin, as representing a mixture of factor VIII and the A1−A3C1C2 heterodimer which apparently retains normal membrane binding properties but is functionally inefficient.

The affinity of thrombin-activated factor VIII was apparently unaltered for membranes with unsaturated phospholipids but 2-fold lower for membranes with saturated phospholipids (Figure 3). This difference is insufficient to account for loss of enzymatic cofactor activity for the experiments displayed in Figures 1C and 2C. The light chain of factor VIII apparently undergoes a conformational change following thrombin-mediated activation, and this change affects the affinity of factor VIII for von Willebrand factor (*41*). The coincidence of the major membrane binding motif with the major von Willebrand factor binding motif makes it possible that the same conformational change could be the explanation for the modest decrease in affinity for binding sites on saturated phospholipid membranes.

A membrane binding motif of factor VIII resides within the C-terminal residues of the C2 domain (*18*, *42*). Thus, a 21 amino acid peptide corresponding to residues 2303−2323 competes with factor VIII for binding to PS immobilized on polystyrene, PS-containing synthetic membranes, and maximally stimulated platelets (*37*). The importance of this membrane binding motif is illustrated by the fact that a monoclonal antibody with this epitope inhibits membrane binding and by the fact that patients with inhibitory antibodies that recognize this epitope have symptoms of bleeding characteristic of hemophilia despite receiving infusions of factor VIII (*17*). The 21 amino acid peptide from this region spontaneously forms 3 turns of an amphipathic helix in the presence of SDS micelles (*37*) or dodecylphosphocholine micelles (*43*). The hydrophobic residues on one face of the helix bury themselves within the hydrophobic cores of dodecylphosphocholine micelles. Thus, it appears likely that factor VIII binds to membranes via a combination of hydrophobic interactions related to insertion of hydrophobic residues into a membrane core as well as electrostatic interactions that mediate recognition of *O*-phospho-L-serine. The probability of contributory hydrophobic interactions is underscored by a report indicating that factor VIII may be eluted from a membrane matrix with ethylene glycol (*44*). We speculate that the hydrophobic residues of the membrane binding peptide of factor VIII penetrate the hydrophobic core of a membrane adjacent to a PS molecule. The requirement for unsaturated PS acyl chains may reflect both the decreased headgroup density on unsaturated phospholipid bilayers, easing penetration through the hydrophilic surface to the hydrophobic core, and the increased capacity of unsaturated acyl chains to conform to the shape of penetrating hydrophobic residues.

We have found that phospholipid vesicles containing lyso-PS at mole fractions as high as 30% do not support binding of factor VIII even when other phospholipid constituents

have acyl chain double bonds. Our prior results indicate that when the mole fraction of PS is >20%, factor VIII binding sites no longer demonstrate stereoselective reliance on phospho-L-serine or PE. Therefore, binding under these conditions is influenced by the large negative electrostatic potential and less dependent upon interaction with specific structures. However, the results in this report indicate that the *sn*-2 acyl chain is critical to formation of factor VIII binding sites even when the mole fraction of lyso-PS is 30%. The requirement for the *sn*-2 acyl chain under these conditions further emphasizes that the binding of factor VIII to phospholipid sites still relies on complimentary interaction of specific structures even when the electrostatic effect is maximized. While we favor the interpretation that the membrane binding moiety of factor VIII interacts specifically with the *sn*-2 acyl chain of PS, it is also possible that the *sn*-2 acyl chain alters the conformation of PS or the interaction with adjacent phospholipid headgroups and that loss of binding is a consequence of an unfavorable headgroup conformation.

When the factor VIIIa−factor IXa complex binds to phospholipid membranes, the catalytic activity is greatly accelerated (*27*). In prior studies, all factor VIII binding membranes that were examined also supported activity of the factor VIIIa−factor IXa complex. The results in this report are the first demonstration that membrane binding of factor VIII may be dissociated from activation of the factor VIIIa−factor IXa complex. Our results do not indicate whether membranes of saturated lipids inhibit binding of factor IXa to factor VIIIa, prevent normal conformational change(s) that occur(s) when the complex is bound to a membrane, or interfere with the approach of factor X to the factor VIIIa−factor IXa complex. Factor IXa binds to factor VIIIa with high affinity in the absence of phospholipids, and the K_M for the factor VIIIa−factor IXa complex is 75-fold higher in the absence of phospholipid membranes (*27*). Therefore, the >100-fold reduction in the capacity of saturated bilayers to support the factor VIIIa−factor IXa complex is unlikely to be explained solely by a hypothetical reduction in the affinity of factor IXa and/or factor X for saturated bilayers. Further experiments will indicate whether saturated bilayers hinder assembly of the factor VIIIa−factor IXa complex, alter approach of factor X to the complex, or fail to support the conformational change that accelerates the k_{cat}.

Acyl chain unsaturation also influences assembly and function of the prothrombinase complex. Saturated phospholipid membranes support greatly slowed assembly of the prothrombinase complex, apparently because lateral diffusion of enzyme and cofactor on the viscous membrane must precede assembly of the complex (*45*). The k_{cat} of the assembled prothrombinase complex is slowed 18-fold on saturated membranes (*32*), and the preferential product of initial cleavage is prethrombin 2 rather than meizothrombin (*31*). Furthermore, the activity of the prothrombinase complex toward prethrombin 1, which does not contact the membrane, is altered on saturated membranes (*32*). These results indicate that acyl chain double bonds influence the conformation of the factor Xa active site. However, prior investigations have not revealed a necessity for acyl chain double bonds for binding of factor V/factor Va or other components of the prothrombinase to phospholipid membranes. The absence of effect on factor V/factor Va binding apparently contrasts with the loss of binding sites for factor VIII. Likewise, the inhibition of the prothrombinase complex is less pronounced than the inhibition of the factor Xase complex. Rather than displaying a modestly decreased k_{cat}, the activity of the Xase complex is decreased >100-fold by saturated membranes with a PS:PE:PC ratio of 4:20:76. Thus, it appears likely that both enzyme complexes rely upon acyl chain double bonds to enable a conformational change that enhances catalytic activity of the active site.

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