Partial Activation of the Factor VIIIa−Factor IXa Enzyme Complex by Dihexanoic Phosphatidylserine at Submicellar Concentrations†

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ABSTRACT: Phosphatidylserine (PS)-containing membranes increase the k_{cat} of the factor VIIIa−factor IXa enzyme complex by more than 1000-fold. While PS supports specific, high-affinity membrane binding of factor VIIIa and factor IXa, it is not known whether PS is the lipid that activates the membrane-bound complex. It is also not known whether PS or other activating lipids must reside in the two-dimensional membrane matrix for efficacy. We have found that submicellar concentrations of dihexanoic phosphatidylserine (C6PS) increase the activity of the factor VIIIa−factor IXa complex in a biphasic manner with half-maximal concentrations of 0.2 and 1.6 mM while the micelle-forming concentration is 4.0 mM. Increased cleavage of factor X at 0.25 mM C6PS was due to a 25-fold enhancement of the k_{cat} and a 30-fold increase in the affinity of factor VIIIa for factor IXa. C6 phosphatidylethanolamine and C6 phosphatidic acid, but not C6 phosphatidylcholine, also accelerated the Xase complex, indicating that k_{cat} enhancement has less structural specificity than membrane binding. Submicellar C6PS enhanced activity of factor IXa in the absence of factor VIIIa, but the effect was due to a decreased K_M rather than an increased k_{cat}. These results suggest that activation of the factor VIIIa−factor IXa complex can result from binding of individual C6PS molecules or small aggregates in the absence of a membrane bilayer. They provide a model system in which the phospholipid-induced activation may be distinguished from membrane-binding of the enzyme complex.

Factor VIII is a phosphatidylserine (PS)[1] binding cofactor (*1*, *2*) for the vitamin K-dependent serine protease factor IXa that also binds to PS-containing membranes (*3*, *4*). The membrane-bound factor VIIIa−factor IXa complex cleaves the zymogen factor X to factor Xa which is then responsible for catalyzing prothrombin activation (*5*). The importance of this enzyme complex is illustrated by hemophilia, a disease in which a deficiency of either factor VIII or factor IX leads to life-threatening bleeding. Factor IXa gains more than 100000-fold greater efficiency in activating factor X by assembling with factor VIIIa on a PS-containing membrane than when free in solution (*6*). We have recently found that the predominant effect of PS-containing membranes on the factor VIIIa−factor IXa complex is to increase the k_{cat} by more than 1000-fold (*7*). These membranes also increase the affinity of factor IXa for factor VIIIa and for factor X.

Factor VIII, with $M_r = 280\,000$, is homologous to another procoagulant protein, factor V, in amino acid sequence (*8*−*10*) and in function, as a membrane-bound enzyme cofactor (*5*, *11*−*13*). The proteins share a repeating domain structure of A1-A2-B-A3-C1-C2 in which the A domains are homologous with ceruloplasmin, the B domain is unique to each protein, and the C domains are homologous with discoidin I, a phospholipid binding lectin (*14*), and with a murine milk fat globule membrane protein (*15*). Both factor VIII and factor V bind with high affinity to phospholipid membranes via the "light chain" composed of the A3-C1-C2 segment (*16*). However, factor VIII requires more PS per binding site than factor V (13), and current evidence implicates different domains in membrane binding. While binding of factor V is mediated by the A3 domain (*17*) and the C2 domain (*18*) and is influenced by glycosylation within the C2 domain (*19*), binding of factor VIII is apparently mediated by the C2 domain (*20*, *21*) and is independent of glycosylation. In plasma, factor VIII differs from factor V in that it circulates in a noncovalent complex with von Willebrand factor (*22*). von Willebrand factor competitively inhibits factor VIII from binding to PS-containing membranes (*23*, *24*) and to activated platelets (*25*). Proteolytic activation by thrombin removes a von Willebrand factor binding peptide of factor VIII (*26*, *27*), causing dissociation of the two proteins and allowing factor VIII to bind to activated platelets (*25*) and to PS-containing membranes (*24*).

Factor IX, the serine protease for which factor VIII is a cofactor, is homologous with factor X, for which factor V is a cofactor. Both proteins share a domain structure in which an N-terminal vitamin K-dependent γ-carboxyglutamic acid domain is followed by two epidermal growth factor-like domains, and finally by the trypsin-like protease domain (*28*, *29*). Both proteins are activated by limited proteolysis

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; C6PS, 1,2-Dihexanoic-*sn*-glycero-3-phospho-L-serine; C6PC, 1,2-dihexanoic-*sn*-glycero-3-phosphocholine; C6PE, 1,2-dihexanoic-*sn*-glycero-3-ethanolamine; C6PA, 1,2-dihexanoic-*sn*-glycero-3-phosphatidic acid; C6PG, 1,2-dihexanoic-*sn*-glycero-3-glycerol; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; CMC, critical micellar concentration.

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to their active forms, factor IXa and factor Xa. Both proteins bind to PS-containing membranes via their γ-carboxyglutamic acid-containing domains. Binding of factor IXa to phospholipid membranes appears to be chiefly mediated through residues 1−11 which assume their membrane-binding structure only in the presence of Ca^{2+} (*30*). The functional relationship between factor IX and factor X arises because factor X is the substrate for factor IXa. Following activation, factor Xa assembles with factor Va in a membrane-bound enzyme complex which activates prothrombin.

A physiologic role for PS in promoting membrane binding of blood coagulation proteins has been hypothesized. This phospholipid, together with phosphatidylethanolamine, is normally sequestrated on the inner surface of blood cell membranes (*31*) but both are translocated to the outer surface upon activation of platelets (*32*) and other cells (*33*, *34*). Some agonists that lead to translocation of platelet PS lead to release of membranous vesicles called platelet-derived microparticles (*35*, *36*) which have a high concentration of factor VIII receptors (*37*). Phosphatidylserine is exposed on the surface of platelet-derived microparticles and the exposure of both factor VIII receptors and PS is stable over a time course of many minutes in comparison to exposure upon the platelet surface which is transient (*37*, *38*).

The mechanism(s) through which PS-containing membranes enhance activity of the factor VIIIa−factor IXa complex and factor Xa−factor Va complexes has been a topic of substantial interest (reviewed in ref 5). Because phospholipid membranes enhance the affinities of the respective enzymes for their cofactors and because these membranes do not enhance catalytic activity of the isolated proteases, the initial interpretation was that the phospholipid membranes enhance assembly of the enzyme, cofactor, and substrate, but that acceleration of the catalytic step resulted solely from interaction of the cofactor and enzyme (*6*, *39*). However, Kung and Mann (*40*) reported that membranes composed entirely of saturated phospholipids lead to normal assembly of factor Xa and factor Va and normal K_M for prothrombin but the catalytic step is greatly slowed. In addition, Koppaka and co-workers (*41*) recently reported that soluble phosphatidylserine binds to factor Xa and alters the catalytic activity. These reports indicate that the phospholipid membranes, and possibly individual phospholipid molecules, alter the catalytic process for the factor Va−factor Xa complex, as well as enzyme and substrate binding. We recently observed that the factor VIIIa−factor IXa complex, in contrast with the factor Va−factor Xa complex, assembles with relatively high affinity in the absence of phospholipid membranes but is catalytically sluggish. Binding of the complex to PS-containing membranes increases the k_{cat} more than 1000-fold (*7*). Thus, Membrane binding affects the catalytic step for both the factor VIIIa−factor IXa and the factor Va−factor Xa complexes. These effects imply a membrane-induced conformational change of the respective serine proteases or of the protease−cofactor complex. We wished to know whether the effects of a phospholipid membrane could be mimicked by soluble phospholipids in the absence of a membrane. If so, the implication would be that individual phospholipids may cause a conformational change of the factor VIIIa−factor IXa complex. Therefore, we were motivated to characterize and evaluate PS with six carbon acyl chains and its effects upon assembly and function of the factor VIIIa−factor IXa complex.

EXPERIMENTAL PROCEDURES

1,2-Dihexanoic-*sn*-glycero-3-phospho-L-serine (C6PS), 1,2-dihexanoic-*sn*-glycero-3-phosphocholine (C6PC), 1,2-dihexanoic-*sn*-glycero-3-phosphoethanolamine (C6PE), 1,2-dihexanoic-*sn*-glycero-3-phosphatidic acid (C6PA), 1,2-dihexanoic-*sn*-glycero-3-glycerol (C6PG), 1-oleoyl lyso PS, and bovine brain PS were from Avanti Polar Lipids (Alabaster, AL). All lipids were suspended at a 20−25 mM concentration in 0.15 M NaCl and 50 mM trizma-HCl, pH 7.8, and stored at −80 °C. Recombinant human factor VIII was a gift from D. Pittman of Genetics Institute, Cambridge, MA. Prothrombin, human factor IXa, factor X, and factor Xa were from Enzyme Research Laboratories (South Bend, IN). Human factor Va was from Haematologic Technologies (Burlington VT). Bovine serum albumin, >98% fatty acid free, prepared by cold alcohol precipitation, and ovalbumin were from Sigma. 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was from Molecular Probes (Eugene, OR). Zwittergent 3−12 was from Calbiochem (San Diego, CA).

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 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 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 and 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 (*7*, *42*). 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 (*42*). All proteins were aliquoted into fractions for single usage, flash-frozen in liquid nitrogen, and stored at −80 °C until use.

Factor Xase Assay. Factor Xase activity was measured with a two-step amidolytic substrate assay. Phospholipid was mixed with a reaction mixture containing factor IXa and factor X in 0.15 M NaCl, 0.2% wt/vol bovine serum albumin, and 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 as specified for each experiment, 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 thermostatted kinetic microtiter plate reader (Molecular Devices, Menlo Pk, 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 duplicates from a representative experiment.

In binding affinity experiments where the factor VIIIa concentration was limiting e.g., Figure 5A, the factor VIIIa concentration was assumed to be $^1/_2$ of the nominal concentration because of the 2 min. lag from dilution of factor VIIIa to initiation of the kinetic reaction correlates to the $T_{1/2}$ for dissociation of the factor VIIIa subunits (*42*). No correction was made for changes in the factor VIIIa concentration during the kinetic reaction because factor IXa stabilizes factor VIIIa when they are in complex together (*50*). Data was corrected for the background rate of substrate hydrolysis and, in experiments where the factor IXa concentration was varied, data were corrected for the measured rate of factor Xa formation due to free factor IXa prior to analysis. For most experiments, data correction was less than 5%, and in no case did correction exceed 20% of the total rate of substrate formation. Affinity data were analyzed according to the standard equilibrium binding model, using the quadratic equation when the apparent K_D was within 5-fold of the limiting reactant and steady state kinetics according to the Michaelis−Menton model with curve fitting by nonlinear least squares analysis (FitAll, MTR Software, Toronto).

Prothrombinase Assay. Cleavage of prothrombin to thrombin was measured in a two-step amidolytic substrate assay analogous to that for factor X activation as previously described (*43*). Factor Va, 5 nM; factor Xa, 0.32 nM; and C6PS at specified concentrations were incubated for 5 min at 37 °C in a solution containing 0.15 M NaCl, 0.05 M Tris-HCl, 1.5 mM $CaCl_2$, and 0.05% wt/vol ovalbumin, pH 7.8, prior to addition of 2 μM prothrombin. After 5 min at 37 °C, the reaction was stopped by the addition of EDTA to a final concentration of 7 mM. Thrombin formation was assessed in a kinetic microplate reader immediately after addition of 0.1 mM chromogenic substrate S-2238.

Evaluation of Lipid Aggregate and Micelle Formation. Micelle formation was evaluated in a dye solubilization assay by monitoring the increased fluorescence of 5 μM TMA-DPH. The hydrophobic fluorescent moiety partitions to the interior of micelles where protection from solvent leads to increased fluorescence. Experiments were performed in a 10 × 10 × 40 mm quartz cuvette at room temperature with excitation wavelength of 355 nm and emission wavelength of 450 nm. The buffer was 0.15 M NaCl, 0.05 M Tris-HCl, and 1.5 mM $CaCl_2$, pH 7.8. Formation of lipid aggregates and micelles was also measured by monitoring light scattering at 90°. Excitation and emission were at 320 nm in the same buffer described above except that $CaCl_2$ was added or excluded as indicated. Confirmation that C6PS was predominantly in the form of monomers or very small aggregates was obtained using ultrafiltration. One milliliter samples of lipid suspended in 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.8, at the indicated concentrations were placed in the upper chamber of Centricon 3 devices (Amicon Corp., Beverly, MA). Filtration was accomplished at 7500*g* at 22 °C with $CaCl_2$ added or excluded as indicated. After 60 min, when approximately $^1/_2$ of the volume had passed through the membrane, samples were removed and the volumes measured. The concentration of C6PS or Triton X-100 was measured by elemental phosphorous assay or by absorbance at 280 nm, respectively.

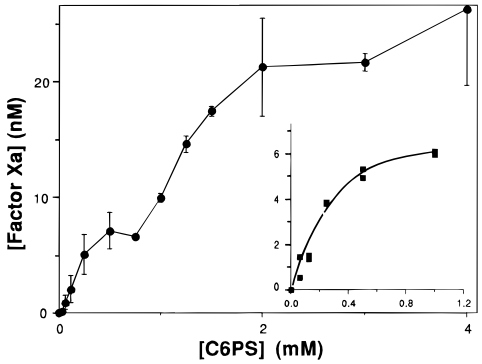
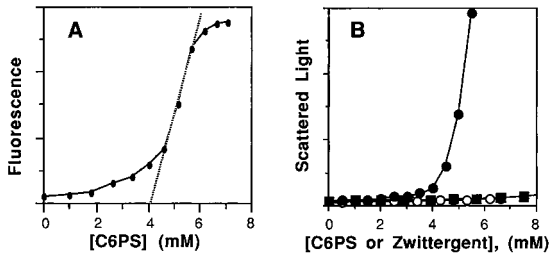


FIGURE 1: Effect of C6PS on the factor VIIIa−factor IXa complex. Factor VIIIa, factor IXa, and factor X were incubated with varying concentrations of C6PS. After 5 min, the reaction was stopped by addition of EDTA, and the quantity of factor Xa formed was evaluated with a factor Xa chromogenic substrate. The rate of factor Xa formation increased in a biphasic manner with half-maximal concentrations of 0.2 and 1.6 mM. The mean values (± standard deviation) for 10 experiments are depicted. A single representative experiment with expanded abscissa scale from 0 to 1 mM is depicted in the inset with line drawn by eye. C6PS concentrations of 0, 0.03, 0.06, 0.12, 0.25, 0.50, 1, 2, and 4 mM were employed for all 10 experiments while the other concentrations depicted were evaluated in only 2 of 10 experiments. Protein concentrations were factor VIIIa, 40 nM; factor IXa, 5 nM; factor X, 65 nM.

RESULTS

We wished to determine whether soluble PS will activate the factor VIIIa−factor IXa complex similar to the activation that occurs on a PS-containing bilayer. Therefore, we evaluated the effect of PS containing six-carbon acyl chains (C6PS) which is soluble at concentrations <5 mM (*41*). Addition of increasing concentrations of C6PS led to saturably increasing efficiency of the factor VIIIa−factor IXa complex in a biphasic manner (Figure 1). The first half-maximal concentration was approximately 0.2 mM (Figure 1, inset) and the second was 1.6 mM. To confirm that activation of the factor VIIIa−factor IXa complex was not influenced by C6PS binding to the fatty acid free serum albumin present at 0.2% w/v in the buffer, we performed experiments with a buffer containing ovalbumin instead of serum albumin. The results indicated comparable activation of the factor VIIIa−factor IXa complex at equivalent phospholipid concentrations, indicating that bovine serum albumin did not have a specific effect on enzyme complex activation.

In order to confirm that C6PS is soluble at submillimolar concentrations, we measured micelle formation with the amphipathic fluorescent probe TMA-DPH (Figure 2A). Addition of C6PS to TMA-DPH resulted in a sharp increase in fluorescence between 4 and 6 mM. Extrapolation of the steep portion of the curve to the abscissa indicated that the critical micellar concentration (CMC) of C6PS was 4.0 mM. We evaluated the possibility that Ca^{2+} induces aggregates of C6PS at submicellar concentrations using light scattering at 90° (Figure 2B). The results indicate that Ca^{2+} causes substantial aggregation of micelles as the C6PS concentration approaches and exceeds the 4.0 mM CMC. Light scattering by C6PS in the presence of 1.5 mM Ca^{2+} was also 6-fold greater than light scattering by C6PS in the absence of Ca^{2+} or light scattering by Zwittergent 3−12, a detergent with a CMC similar to C6PS. This indicated that Ca^{2+}-induces some aggregation of C6PS molecules at submicellar concentrations. In order to estimate the fraction of C6PS



C

Fraction of C6PS retained by MW 3,000 cutoff membrane as a function of C6PS concentration and Ca^{2+}

[C6PS] (mM)	Ca^{2+} (mM)	
	0	1.5
0.2	0.07	0.08
2.5	0.11	0.13

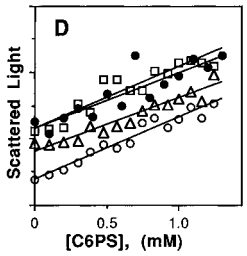


FIGURE 2: Micelle formation and Ca^{2+}-dependent aggregation of C6PS. (A) The fluorescence of TMA-DPH increased steeply as the C6PS concentration increased between 4 and 6 mM, denoting micelle formation. The slope was extrapolated to the abscissa indicating a critical micellar formation of 4.0 mM (dotted line). (B) Particle size of micelles and aggregates was explored using 90° light scattering. In the absence of Ca^{2+}, C6PS (○) scattered light intensity was within 20% of Zwittergent 3−12 (■) at concentrations up to 7 mM. This indicated absence of aggregate formation at submicellar concentrations. In the presence of 1.5 mM Ca^{2+}, C6PS (●) scattered light intensity was 6-fold higher than Zwittergent 3−12 or Ca^{2+}-free C6PS at concentrations below 2.5 mM and much higher at concentrations >4 mM indicating formation of micelle aggregates. (C) The physical state of the enzymatically effective C6PS concentrations was also evaluated by retention on ultrafiltration membranes with MW 3000 cutoff. Confirmation that lipid micelles are retained by these membranes was obtained by measuring retention of Triton X-100 micelles. Ninety-nine percent of Triton X-100 was retained when the concentration was 1.0 mM, 3-fold higher than the CMC. Eighty-four percent was retained at a starting concentration of 0.1 mM, 3-fold below the CMC indicating that Triton X-100 forms submicellar aggregates. By comparison, only 8% of C6PS was retained in the presence of Ca^{2+} when the starting concentration was 0.25 mM, and only 13% was retained when the starting concentration was 2.5 mM. Therefore, most C6PS molecules do not participate in formation of large aggregates at concentrations below the CMC. (D) Binding of C6PS aggregates to factor IXa (●), factor VIII (□), and factor X (△) was investigated with 90° light scattering. Addition of C6PS to the proteins at submicellar concentrations in the presence of 1.5 mM Ca^{2+} did not lead to increased light scattering above that produced by C6PS alone, indicating that C6PS aggregates of MW ≥ 10 000 do not bind to these proteins. Lines were best fit using linear least-squares regression. Protein concentrations were factor IXa, 150 nM; factor VIII, 48 nM; factor X, 234 nM.

molecules that participate in Ca^{2+}-induced aggregate formation, we measured the retention of C6PS by ultrafiltration filters with MW cutoff of 3000 in the presence and absence of 1.5 mM Ca^{2+} (Figure 2C). Only 7% of C6PS was retained at the lower concentration effective in activating the factor VIIIa−factor IXa enzyme complex. Addition of 1.5 mM Ca^{2+} increased retained C6PS by only 1%, suggesting that as little as 1% of molecules participate in Ca^{2+}-induced aggregates. To confirm that activation of the factor VIIIa−factor IXa complex was not due to a small quantity of long-chain phospholipid in the C6PS preparation, we evaluated the capacity of the retentate vs filtrate to activate the factor VIIIa−factor IXa complex. Both supernatant and filtrate stimulated the factor VIIIa−factor IXa complex to a similar degree, indicating that soluble C6PS, rather than contaminating higher molecular weight phospholipid vesicles or micelles, is the active agent (data not shown). At a concentration of 2.5 mM C6PS, 11% was retained by the membrane in the absence of Ca^{2+} and 13% in the presence of 1.5 mM Ca^{2+}. Therefore, Ca^{2+}-induced aggregation of C6PS at submicellar concentrations, suggested by increased light scattering, apparently involves less than 3% of C6PS molecules.

To determine whether Ca^{2+}-induced C6PS aggregates bind to factor IXa or whether micelle formation is induced on the membrane-binding surface, the light scattered by C6PS was compared in the presence and absence of factor IXa (Figure 2D). The initial protein concentration, 150 nM, was chosen to produce light scattering 2−3-fold greater than buffer alone. Because Ca^{2+}-induced C6PS aggregates are larger than factor IXa (*41*) binding of aggregates to these proteins would produce a substantial increase in light scattering. However, binding of individual C6PS molecules would not affect scattered light. Addition of C6PS to factor IXa did not cause increased light scattering greater than addition of C6PS to buffer. Similar experiments were performed with factor VIII and with factor X to determine whether large C6PS aggregates bound to these proteins or micelles were induced (Figure 2D). The results indicated that the light scattering caused by C6PS is not affected by any of the protein constituents of the factor Xase complex. Therefore, Ca^{2+}-induced aggreagates do not bind to these proteins.

Although the Ca^{2+}-dependent C6PS aggregates involve only a small fraction of C6PS molecules and apparently do not bind to isolated factor VIII, factor IXa, or factor X (Figure 2D), it remained possible that the aggregates influence function of the assembled factor VIIIa−factor IXa complex. To probe this possibility, we asked whether Zwittergent 3−10, a Zwitterionic detergent with a CMC of approximately 14 mM, would suppress C6PS aggregation at submicellar concentrations. As predicted, Zwittergent 3−10 suppressed C6PS aggregate formation as detected by scattered light (Figure 3A). Suppression of aggregate formation in 1 mM C6PS was 78% at 4.5 mM Zwittergent 3−10, with scattered light remaining aproximately 2-fold above the scattered light from buffer alone (dashed line). Addition of higher concentrations did not further suppress aggregate formation. In the absence of C6PS, Zwittergent 3−10 did not detectably increase scattered light above that due to buffer alone, confirming that the Zwittergent 3−10 concentration remained below the CMC (data not shown). We then asked whether suppression of C6PS aggregate formation would inhibit activity of the factor VIIIa−factor IXa complex in the presence of 0.25 mM or 4 mM C6PS (Figure 3B). In the absence of C6PS, Zwittergent 3−10 concentrations as high as 5 mM had no effect on activity of the factor VIIIa−factor IXa complex (data not shown). When the C6PS concentration was 0.25 mM, addition of Zwittergent 3−10 increased enzyme complex activity, reaching a plateau approximately 50% above the level in the absence of C6PS. When the C6PS concentration was 4 mM, Zwittergent 3−10 did not significantly affect the activity. Failure of Zwittergent 3−10 to suppress C6PS-dependent activity of the factor VIIIa−factor IXa complex in parallel with suppression of C6PS aggregation confirms that the Ca^{2+}-induced aggregates do not influence activity of the factor VIIIa−factor IXa complex.

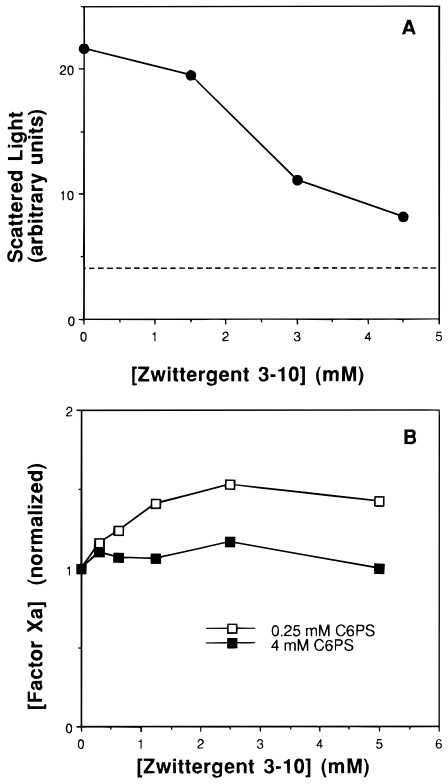


FIGURE 3: Effect of submicellar concentrations of Zwittergent 3−10 on Ca^{2+}-dependent aggregation of C6PS and on activity of the factor VIIIa−factor IXa complex. (A) Various concentrations of Zwittergent 3−10 were added to 1 mM C6PS in 0.15 M NaCl, 0.05 M Tris-HCl, 1.5 mM $CaCl_2$, pH 7.8. Ninety degree light scattering was measured as described in the Experimental Procedures. Addition of Zwittergent 3−10 led to decreased light scattering approaching the light scatter from buffer alone (dashed line). A single experiment representative of two such experiments is displayed. (B) Various concentrations of Zwittergent 3−10 were added to factor VIIIa, factor IXa, and factor X in the presence of C6PS at 0.25 mM (□) or 4 mM (■) concentrations. Zwittergent increased the activity of the factor VIIIa−factor IXa complex in the presence of 0.25 mM C6PS but did not affect activity in the presence of 4 mM C6PS. See legend for Figure 1 for protein concentrations. Results shown are the means of duplicates from a single experiment, representative of two such experiments, normalized for comparision.

To evaluate the specificity of activation by CSPS, we compared other soluble phospholipid molecules (Figure 4). We found that C6PC had no effect on the factor VIIIa−factor IXa complex. However, phosphatidylethanolamine (C6PE) and phosphatidic acid (C6PA) led to saturable increased activity at concentrations comparable to C6PS. Phosphatidylglycerol (C6PG) also increased activity of the factor VIIIa−factor IXa complex. However, activation was sigmoidal rather than hyperbolic as with C6PS, C6PE, and C6PA. The critical micellar concentrations of these phospholipids, determined as depicted in Figure 2A, were also well above the minimum activating concentrations (Figure 4). We also found that the detergents Tween 80, Big CHAPS, and Zwittergent 3−12 had no effect on activity of the factor VIIIa−factor IXa complex over a concentration range 0−0.5 mM. SDS increased activity with a half-maximal concentration of approximately 0.05 mM, but the maximum activity was only 10% of the increase induced by C6PS. These data suggest that optimal activation of the factor VIIIa−factor IXa complex is induced by amphipathic molecules containing a phosphate moiety and excluding a choline moiety.

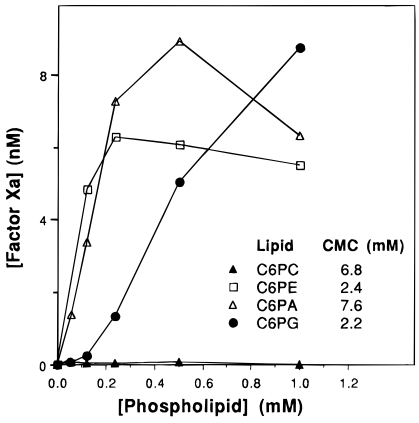


FIGURE 4: Specificity of the factor VIIIa−factor IXa complex for soluble phospholipids. Factor VIIIa, factor IXa, and factor X were incubated with various concentrations of C6PC (▲) C6PE (□) C6PA (△) and C6PG (●) and the quantity of factor X formed was measured as described under Figure 1. C6PC did not activate the factor VIIIa−factor IXa complex, but both C6PE and C6PA did. C6PG also activated the factor VIIIa−factor IXa complex with a sigmoidal relationship between the phospholipid concentration and increased activity. Activating concentrations of C6PA and C6PE were at least 10-fold lower than respective CMC's (inset), but the effective concentrations of C6PG were about 3-fold lower than the CMC. CMC's were determined with TMA-DPH as depicted in Figure 2A. Results shown are means of duplicates representative of two (C6PG and C6PC) or three (C6PE and C6PA) such experiments.

We performed experiments to determine which parameters of the factor VIIIa−factor IXa complex were affected by submicellar C6PS concentrations. When the factor IXa concentration was increased in the presence of 5 nM factor VIIIa, the factor Xa forming activity increased saturably (Figure 5A). The best-fit curve implied a dissociation constant of 1.4 nM (Table 1), comparable to the affinity of factor IXa for factor VIIIa on a PS-containing bilayer and 30-fold higher than the affinity in the absence of phospholipid. When the factor X concentration was increased in the presence of 5 nM factor VIIIa−factor IXa, the quantity of factor Xa formed also increased saturably (Figure 5B). The K_M for the enzyme complex was 156 nM (Table 1), intermediate between the K_M in the absence of phospholipids and on PS-containing bilayers. The k_{cat} was 0.48 min^{-1}, 25-fold higher than in the absence of phospholipid but 200-fold less than on PS-containing bilayers.

Similar experiments were performed with the C6PS concentration increased to 3 mM (Table 1). At this concentration, the apparent affinity of factor VIIIa for factor IXa was increased 7-fold. The K_M was decreased 2-fold and the k_{cat} increased 10-fold by comparison with data obtained at 0.25 mM C6PS. Because the K_M was 2-fold lower at higher C6PS concentrations, we investigated the possibility that the biphasic shape of the C6PS activation curve (Figure 1) was due to the lower K_M at higher C6PS concentrations. The activity of the VIIIa−factor IXa complex was evaluated as a function of C6PS concentration in the presence of 350 nM factor X, more than 2-fold higher than the lower K_D. The biphasic nature of the curve was pronounced under these conditions, equivalent to the earlier experiments, indicating that the upward concavity was not due to the reduced K_M at higher C6PS concentrations (not shown).

While the K_M for the factor VIIIa−factor IXa complex in the presence of 3 mM C6PS is within 3-fold of the values

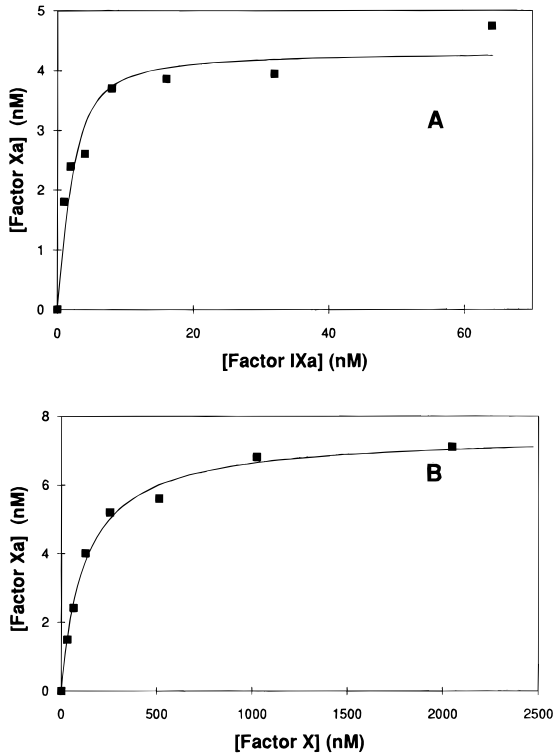


FIGURE 5: Effects of C6PS on parameters of the factor VIIIa−factor IXa complex. (A) The apparent affinity of factor VIIIa for factor IXa in the presence of 0.25 mM C6PS was evaluated by varying the factor IXa concentration while maintaining factor VIIIa at 5 nM. The K_D of 0.9 nM (smooth line) indicated that the affinity is increased by C6PS to a value comparable with the binding interaction on phospholipid bilayers. (B) The enzymatic parameters of the factor VIIIa−factor IXa complex were evaluated under conditions where a maximum of 10% of available factor X was converted to factor Xa. Best-fit of the results to the Michaelis−Menton model (smooth line) indicates that the K_M is decreased 10-fold and the k_{cat} increased 24-fold by 0.25 mM C6PS (Table 1). Protein concentrations were factor VIIIa, 15 nM, and factor IXa, 5 nM. Data are means of duplicates representative of two (A) and three (B) such experiments.

previously measured on PSPC membranes (Table 1), the k_{cat} is 27-fold slower. To determine whether this difference reflects the short acyl chains of C6PS versus the 100% PS composition of the micelles, we compared activation of the factor VIIIa−factor IXa complex by PS from bovine brain and 1-oleoyl-lyso PS. The effect of bovine brain PS vesicles was to increase the apparent affinity of factor VIIIa for factor IXa 3-fold by comparison with C6PS and decrease the K_M 9-fold. The k_{cat}, however, was comparable to the k_{cat} measured in the presence of C6PS. These results indicate that the low k_{cat} produced by C6PS micelles likely reflects the impact of pure PS micelles rather than the effect of short acyl chains. While micelles of lyso PS produced apparent affinity of factor VIIIa for factor IXa and K_M comparable to C6PS micelles, the k_{cat} was 32-fold slower, only 8-fold greater than in the absence of phospholipid. The sluggish k_{cat} induced by lyso PS micelles suggests the interesting possibility that the presence of an *sn*-2 acyl chain may influence activation of the factor VIIIa−factor IXa complex.

Submicellar concentrations of C6PS also enhanced cleavage of factor X by factor IXa in the absence of factor VIIIa (Figure 6A). Half-maximal activation occurred at 0.4 mM, 10-fold below the critical micellar concentration, similar to activation of the factor VIIIa−factor IXa complex. However, there was not definite biphasic activation for factor IXa without factor VIIIa. Factor Xa production increased saturably as a function of the factor X concentration, indicating a K_M of 200 nM (Figure 6B). When the same concentrations of factor X were incubated with factor IXa in the absence of C6PS, the quantity of factor Xa formed also increased but showed no sign of saturation at 4 μM factor X. The k_{cat} in the presence of C6PS was 0.003 min^{-1}, which is not greater than the value previously reported in the absence of phospholipid (*6*). By comparison with C6PS, bovine brain PS decreased the K_M 20-fold. However, the k_{cat} was not increased. These results indicate that C6PS and bovine brain PS increase the affinity of factor IXa for factor X but do not increase the k_{cat} in the absence of factor VIII.

To determine whether the effect of C6PS is unique to the factor VIIIa−factor IXa complex or whether it may extend to other enzyme complexes of blood coagulation, we evaluated the effect on the factor Va−factor Xa complex (Figure 7). We found that, like the factor VIIIa−factor IXa complex, the factor Va−factor Xa complex was partially activated by submicellar concentrations of C6PS. In contrast with the factor VIIIa−factor IXa complex the activation was not biphasic with half maximal activation at 1 mM C6PS. Thus, it is likely that phospholipid binding sites that alter catalytic activity are present on both the factor Va−factor Xa complex and the factor VIIIa−factor IXa complex and that occupation of these sites induce changes that increase the binding affinities and catalytic rates of the respective enzyme complexes.

DISCUSSION

We have found that C6PS activates the factor VIIIa−factor IXa complex in a biphasic manner with the first enhancement occurring at a concentration 20-fold below that at which micelles form. Light scattering data indicate that Ca^{2+}-induced C6PS aggregates do not bind to factor IXa, factor VIII, or factor X so that the effects of C6PS on the factor VIIIa−factor IXa complex are caused by monomers or by small aggregates of ≤6 monomers. Submicellar C6PS causes increased affinity of factor VIIIa for factor IXa, increased affinity of the complex for the substrate, factor X, and accelerated peptide bond cleavage. Although the k_{cat} of the factor VIIIa-IXa complex remains approximately 250-fold slower than when bound to PS-containing membranes, the changes induced by soluble PS are otherwise similar in magnitude and direction to those induced by PS-containing membranes. Therefore, these results suggest that the activation of the factor VIIIa−factor IXa complex induced by PS-containing membranes may be due to conformational change-(s) induced by individual phospholipid molecule or small aggregates of molecules.

At 3 mM C6PS, less than 2-fold below the micelle forming concentration, the factor VIIIa−factor IXa complex is activated to a higher degree than at lower submicellar concentrations. The apparent affinity of factor VIIIa for factor IXa is increased 7-fold, the affinity of the complex for factor X is increased 2-fold, and the k_{cat} is boosted 10-fold. The apparent affinity of factor VIIIa for factor IXa is higher under these conditions than upon a PSPC membrane (Table 1) or on the platelet membrane (*44*). It is likely that micelle formation is induced on the membrane-binding surfaces of the proteins at this concentration. The factor VIIIa−factor IXa complex may then be stabilized by the

Table 1: Parameters of the Factor VIIIa−Factor IXa Enzyme Complex in the Presence of C6PS Versus PS Micelles and PS-Containing Membranes

phospholipid	[phospholipid][a] (mM) at $^1/_2$ max	K_D app[b] (nM)	K_M[c] (nM)	k_{cat} (min^{-1})
C6PS, 0.25 mM	0.2	1.4 ± 0.8	156 ± 34	0.48 ± 0.22
C6PS, 3 mM	1.6	0.19 ± 0.03	64 ± 20	5.3 ± 0.4
PS,[d] 0.003 mM	0.003	0.06 ± 0.03	7.2 ± 1.3	5.1 ± 1.3
Lyso PS, 0.003 mM	0.002	0.2 ± 0.1	27 ± 12	0.16 ± 0.05
PSPC,[e] 0.025 mM (*7*)[f]	0.004	2.3 ± 1.5	23 ± 1	136 ± 2
PSPEPC,[g] 0.025 mM (*7*)	0.064	4.3 ± 2.3	28 ± 6	33 ± 2
none (*7*)	NA	46 ± 15	1700 ± 400	0.022 ± 0.005
	Factor IXa Without Factor VIIIa			
C6PS, 0.25 mM	0.4	NA	200 ± 110	0.0029 ± 0.0003
C6PS, 3 mM	0.4	NA	256 ± 100	0.0089 ± 0.0011
PS, 0.003 mM	0.004	NA	7.4 ± 0.2	0.0045 ± 0.0008
PSPC, 0.025 mM (*6*)	0.01	NA	58	0.0025
none (*6*)	NA	NA	180 000	0.01

[a] Values determined by eye from phospholipid titrations curves such as those depicted in Figures 1 and 5A. [b] The apparent K_D was determined by titrating increasing quantities of factor IXa with 5 nM factor VIIIa (C6PS, 0.25 mM) or 1 nM factor VIIIa in the presence of a constant factor X concentration. Values displayed are means ± SE from a minimum of two experiments, each performed in duplicate, analyzed by nonlinear least squares analysis. Data correction and curve fitting are described under Experimental Methods. [c] The K_M and k_{cat} were determined by incubating 15 nM factor IXa with 5 nM factor VIIIa with the designated phospholipid and varying concentrations of factor X. Values displayed represent the mean ± SE for a minimum of two experiments, each performed in duplicate. [d] PS was from bovine brain, with vesicles prepared by sonication. [e] Sonicated vesicles with bovine brain PS:egg PC:cholesterol ratio 25:75:20. [f] Numbers in parentheses are references. [g] Extruded vesicles with bovine brain PS:egg phosphatidylethanolamine:egg PC:cholesterol ratio 4:20:76:20.

additive binding energy from both proteins binding to the same micelle. The high negative charge density on the micelle surface may mediate, nonspecific high affinity binding analogous to the effect of high concentrations of phosphatidyl-D-serine in phospholipid vesicles (*2*). The increased k_{cat} of the factor VIIIa−factor IXa complex on C6PS micelles suggests that the negatively charged micelles further alter the conformation of the factor VIIIa−factor IXa complex.

Because C6PS increases the affinity of factor IXa for factor X at submicellar concentrations, we infer that it binds to one or both of these proteins. We speculate that a C6PS-induced conformational change increases affinity of each protein for the other and the affinity of factor IXa for factor VIIIa. This mechanism parallels a recently-reported change in the factor Xa active site induced by submillimolar C6PS (*41*). In that report, enhancement of catalytic activity of factor Xa toward prothrombin was paralleled by an inhibition of catalytic activity toward small substrate molecules. The same inhibition of catalytic activity toward small substrates was produced by PSPC phospholipid vesicles, suggesting that soluble PS and membrane PS have the same allosteric effect on the active site. The γ-carboxyglutamic acid domain of factor IXa or factor X is apparently the best candidate site for binding to C6PS. This highly conserved domain, shared by both proteins provides the entire phospholipid membrane binding function for factor IX (*45*) and presumably for factor X. The γ-carboxyglutamic acid domain of factor IXa apparently also interacts with factor VIIIa (*46, 47*). Therefore, interaction of C6PS with the factor IXa γ-carboxyglutamic acid domain could plausibly alter the affinity for factor VIIIa as well as factor X by altering the conformation of that domain.

The biphasic activation of the factor VIIIa−factor IXa complex by C6PS suggests that there is more than one site of interaction for C6PS. A similar biphasic activation of factor Xa by C6PS was also recently observed, suggesting that a common mechanism of activation is shared by factor Xa and the factor VIIIa−factor IXa complex (*41*). The upward concavity of the activation curve (Figure 1) suggests cooperativity because addition of binding isotherms from any two independent binding sites will not yield a curve with upward concavity. Cooperativity could be explained by multiple C6PS molecules interacting at a single site or by an allosteric change induced by the first C6PS molecule on a distant site. The activation curve suggests that our studies may have underestimated the effect of monomeric C6PS on the k_{cat}. We chose 0.25 mM C6PS for these studies to maximize the likelihood that we would observe the effects of only the highest affinity interaction(s). At subsaturating concentrations of C6PS, it is likely that the apparent K_D and K_M would be unaffected because the fraction of protein molecules with bound phospholipid would bind with the same increased affinity while those without bound C6PS would not be detected. However, the apparent k_{cat} was calculated assuming that every factor VIIIa−factor IXa complex contributed equally. If only half had bound C6PS the calculated k_{cat} would be half the actual rate.

The factor VIIIa−factor IXa complex does not exhibit the same degree of specificity toward soluble phospholipids as toward phospholipid membranes. Although we performed detailed studies only with C6PS, some other soluble phospholipids, notably C6PA and C6PE, appeared comparably effective. However, there was a substantial degree of specificity as C6PC and several detergents of similar size were ineffective. Our data suggest specificity for a phosphate moiety, the absence of choline, and the presence of acyl chain(s). Comparison of C6PS micelles vs bovine brain PS vs lyso PS for activation of the factor VIIIa−factor IXa complex (Table 1) suggest that the six carbon acyl chains do not limit maximum activation capacity. However, they suggest that presence of an *sn*-2 acyl chain is necessary to induce the change which results in an accelerated k_{cat}. Our data do not resolve the importance of the glycerol backbone and whether some acyl moieties would contribute greater activity than others. The limited phospholipid specificity for activation of the factor VIIIa−factor IXa complex *in vitro* does not imply that the enzyme complex would not exhibit a fastidious requirement for phosphatidyl-L-serine containing membranes *in vivo*. Factor VIII has a stereoselective

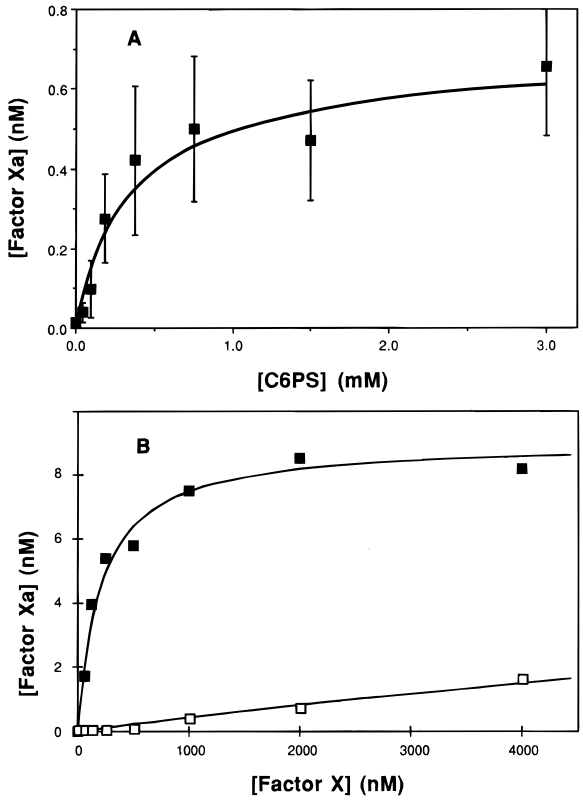


FIGURE 6: Effect of C6PS on activation of factor X by factor IXa in the absence of factor VIIIa. (A) Increasing concentrations of C6PS were added to a mixture of factor IXa and factor X in the presence of 1.5 mM Ca^{2+}. After 20 min, the reaction was stopped by the addition of 7 mM EDTA and the quantity of factor Xa formed was evaluated with the chromogenic substrate S-2765. C6PS led to increasing rates of factor X activation with a half-maximal effect at approx. 0.4 mM. The line was drawn by eye. Displayed results are the mean ±SE for two experiments, each performed in duplicate. The background chromogenic signal for this experiment, due to spontaneous hydrolysis of the substrate and due to trace contamination of the factor X by factor Xa, was less than 10% of the total signal and has not been subtracted from the displayed data. Protein concentrations were factor IXa, 20 nM, and factor X, 65 nM. (B) To determine whether C6PS influences the K_M or the k_{cat} with which factor IXa activates factor X, varying concentrations of factor X were added to 20 nM factor IXa in the presence (■) and absence (□) of 0.25 mM C6PS. Fitting the data to the Michaelis-Menton model (smooth upper line) indicates that C6PS greatly decreases the K_M but has little effect on the k_{cat} (Table 1).

preference for phosphatidyl-L-serine on membranes of composed of phospholipids in the proportions that they are found in cell membranes and the majority of binding sites for factor VIIIa on platelet membranes apparently require this interaction (*1*, *2*). The data in this report suggest that factor IXa may bind to factor VIIIa on a membrane and may then be activated by either PS or PE in the adjacent membrane.

We have found that submicellar concentrations of C6PS enhance activity of the factor Va−factor Xa complex similarly to the factor VIIIa−factor IXa complex. Our findings are consonant with a recent report indicating that submicellar concentrations of C6PS enhance activity of factor Xa in the absence of factor Va (*41*). These results suggest that enhanced catalytic activity due to a phospholipid-induced conformational change may be a general feature of γ-carboxyglutamic acid enzyme complexes in blood coagulation. The response of the factor Va−factor Xa complex differs from the factor VIIIa−factor IXa complex in that it is not clearly biphasic. However, C6PS increases activity over the same concentration range and a biphasic response may be obscured if the critical C6PS concentration for either a high- or a low-affinity interaction differed by a factor of 2−4 so that it would be premature to conclude that there is a significant difference in the nature of the catalytic response.

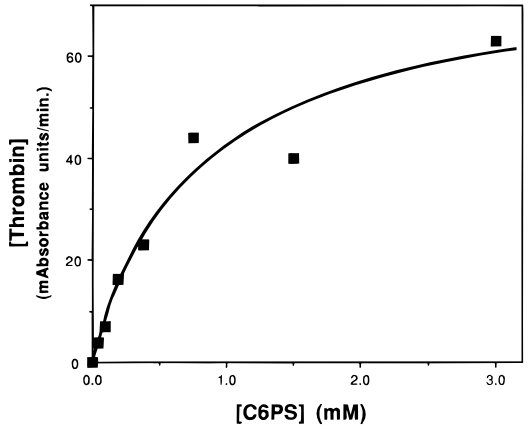


FIGURE 7: Effect of C6PS on activation of prothrombin by the factor Va−factor Xa complex. Increasing concentrations of C6PS were incubated with factor Va, factor Xa, and prothrombin. After 5 min, the reaction was stopped and formation of thrombin was evaluated with the chromogenic substrate S2238. C6PS caused greatly increased activity with $^1/_2$ maximal activation occurring at 0.5 mM, 8-fold lower than the CMC.

The data in this report indicate that C6PS forms micelles at 4 mM in the presence of 1.5 mM Ca^{2+}. This value is consistent with a prior study indicating a CMC of 8 mM in the absence of Ca^{2+} and technique-dependent values of 1−4 mM in the presence of 5 mM Ca^{2+} (*41*). In this regard C6PS resembles C6PC, which forms micelles at 7−10 mM (*48*, *49*) with a MW of 15000−20000 corresponding to 38−50 phospholipid monomers. In the presence of 1.5 mM Ca^{2+}, C6PS forms aggregates at submicellar and micellar concentrations. This is demonstrated by increased 90° light scattering of C6PS with Ca^{2+} compared to C6PS without Ca^{2+} and compared to Zwittergent 3-12 (Figure 2B). The aggregates formed at submicellar concentrations presumably center about phosphoserine-Ca^{2+} complexes and do not form solvent-protected hydrophobic interiors like micelles because the fluorescence of TMA-DPH was not increased under these conditions (Figure 2A). As previously reported, the aggregates involve only a small fraction of C6PS molecules; our studies indicated that only 1% of molecules participate in Ca^{2+}-induced aggregate formation under these conditions. Suppression of aggregate formation by Zwittergent 3−10 did not inhibit function of the factor VIIIa−factor IXa complex, confirming that the aggregates did not influence activity of the complex at submicellar concentrations. At C6PS concentrations > 4 mM the aggregates were large, presumably composed of aggregated micelles rather than C6PS monomers. The data in this report are consistent with C6PS monomers or aggregates of less than six molecules (MW = 3000) binding to factor VIIIa, factor IXa, and/or factor X to produce the changes in the enzyme complex. They are inconsistent with binding of large C6PS aggregates or micelles to these proteins at submillimolar concentrations.

The primary motivation for these studies was to probe the mechanism(s) through which phospholipids activate the membrane-bound factor VIIIa−factor IXa complex. However, our results indicate that the factor VIIIa−factor IXa complex can assemble and function under the influence of

C6PS without membranes. The affinities of factor VIIIa for factor IXa and of this enzyme complex for its substrate, factor X, were sufficiently high to suggest that soluble phospholipids may activate the factor VIIIa−factor IXa complex *in vivo*. Whether any effective phospholipids circulate in plasma under basal conditions are released into the plasma under stress or are injected into the plasma as constituents of venoms is a question for further investigation. In preliminary studies, we have found that submicellar concentrations of three plausible soluble plasma lipids, oleic acid, *sn*-1 oleoyl lyso PS, and *sn*-1 palmitoyl lyso PS do not activate the factor VIIIa−factor IXa complex (data not shown). Our results do not exclude the possibility that other soluble plasma lipids or a plasma protein or membrane-bound protein may be able to bind to and activate the factor VIIIa−factor IXa complex analogously with C6PS.

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