Role of Phosphatidylethanolamine in Assembly and Function of the Factor IXa−Factor VIIIa Complex on Membrane Surfaces†

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ABSTRACT: Phospholipid membranes play a significant role during the proteolytic activation of blood coagulation proteins. This investigation identifies a role for phosphatidylethanolamine (PE) during the activation of factor X by the tenase complex, an enzymatic complex composed of the serine protease, factor IXa, a protein cofactor, factor VIIIa, a phospholipid membrane, and Ca^{2+}. Phospholipid vesicles composed of PE, phosphatidylserine (PS), and phosphatidylcholine support factor Xa generation. The K_m and k_{cat} for factor X activation by the tenase complex are independent of PE in the presence of 20% PS. At lower PS concentrations, the presence of 20 or 35% PE lowers the K_m and increases the k_{cat} as compared to those in vesicles without PE. The effect of PE on the k_{cat} of the tenase complex is mediated through factor VIIIa. PE also enhances factor Xa generation by facilitating tenase complex formation; PE lowers the $K_{d(app)}$ of factor IXa for both phospholipid/Ca^{2+} and phospholipid/Ca^{2+}/factor VIIIa complexes in the presence of suboptimal PS. In addition, the K_ds of factor IXa and factor X were lower for phospholipid vesicles containing PE. *N*-Methyl-PE increased the k_{cat} and decreased the $K_{d(app)}$, whereas *N*,*N*-dimethyl-PE had no effect on either parameter, indicating the importance of headgroup size. Lyso-PE had no effect on kinetic parameters, indicating the *sn*-2 acyl chain dependence of the PE effect. Together, these results demonstrate a role for PE in the assembly and activity of the tenase complex and further extend the understanding of the importance of PE-containing membranes in hemostasis.

Blood coagulation occurs through a cascade of enzymatic reactions, resulting in fibrin formation and platelet deposition (*1, 2*). Central to this process are protein complexes of a vitamin K-dependent protease and an activated protein cofactor assembled on a cell membrane (*3*). The tenase complex, consisting of factor IXa and factor VIIIa assembled on a phospholipid-containing membrane, is the kinetically significant activator of factor X. Factor IXa is the proteolytic enzyme, and factor VIIIa is the nonenzymatic protein cofactor of this complex. Deficiency of either factor IX or factor VIII results in the bleeding disorder hemophilia, illustrating the importance of the tenase complex to normal hemostasis. The zymogen factor IX is activated by factor VIIa in complex with tissue factor, the integral membrane protein cofactor of this reaction. The product of the tenase reaction, factor Xa, assembles with its protein cofactor, factor Va, on a phospholipid-containing membrane to form the prothrombinase complex, which converts prothrombin to thrombin. Thrombin cleaves fibrinogen to produce fibrin and initiates platelet deposition. The plasma protease, activated protein C, is a negative regulator of the tenase and prothrombinase complexes, proteolytically inactivating the protein cofactors, factor VIIIa and factor Va. Factor IX, factor X, factor VII, prothrombin, and protein C are all vitamin K-dependent proteins containing γ-carboxyglutamic acid residues (Gla).[1] The γ-carboxyglutamic acids, located in the N-terminal γ-carboxyglutamic acid rich domain (Gla domain), are critical for vitamin K-dependent protein binding to phospholipid-containing membrane surfaces (*3*).

In vivo, these coagulation complexes assemble on the surface of activated platelets or on damaged cell membranes. Negatively charged phospholipids, in particular, phosphatidylserine (PS), are critical components of these physiological membrane surfaces (*4, 5*). Historically, most biochemical studies were carried out with synthetic membrane vesicles composed solely of phosphatidylcholine (PC) and PS (*4, 6, 7*); however, it was recognized that crude phospholipid mixtures produced shorter clotting times than lipid vesicles composed of purified PC and PS (*8*). In membranes consisting of only PC and PS, 20−25% PS is required for optimal activity of the tenase and prothrombinase complexes, which is higher than the physiological level of PS found on the outer membrane of platelets (*9*). Platelet membranes contain PS and phosphatidylethanolamine (PE), most of which is sequestered in the inner membrane and translocated

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[1] Abbreviations: PE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; *N*-methyl-PE, *N*-methyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; *N*,*N*-dimethyl-PE, *N*,*N*-dimethyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; lyso-PE, lysophosphatidylethanolamine; dansyl-PE, phosphatidylethanolamine-*N*-5-(dimethylamino)-1-naphthalenesulfonyl; Gla, γ-carboxyglutamic acid; PPACK, Phe-Pro-Arg-chloromethyl ketone; DEGRCK, dansyl-Glu-Gly-Arg-chloromethyl ketone.

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to the outer membrane following platelet activation (*9*, *10*). This asymmetry of resting platelet membranes is produced by a combination of an ATP-dependent flippase, which slowly transports all phospholipids from the inner leaflet to the outer membrane, and an ATP-dependent aminophospholipid-specific translocase, which quickly transports PS and PE back to the inner membrane (*11*, *12*). Upon platelet activation, the flippase and the translocase are inactivated and a Ca^{2+}-dependent "scramblase" induces a fast bidirectional transfer of all lipids, leading to the expression of approximately half of the PS and PE on the outer membrane surface (*13*, *14*). Therefore, the outer membrane of an activated platelet contains 4−10% PS and 10−30% PE (*15*). Furthermore, under these conditions, platelets shed vesicles composed of plasma membranes with the same ratios of exposed lipid that enhance blood coagulation (*16*).

Recent studies have proposed a role for PE in the assembly and activation of coagulation factors on phospholipid vesicle surfaces. PE enhances prothrombin activation (*17*−*20*), tissue factor−factor VIIa activation of factor X (*8*), and binding of factor VIII to membranes containing low levels of PS (*21*). In the case of prothrombin activation, PE does not exert an effect at optimal levels of PS (*17*), but enhances prothrombin activation at lower levels of PS (1−7%). PE also plays a role in the negative regulation of the clotting process by enhancing the inactivation of factor Va by activated protein C (*17*). Furthermore, PE is necessary for the inactivation of protein C by the protein C inhibitor (*22*). Additionally, PE is a frequent target of anti-phospholipid antibodies, which are associated with arterial and venous thrombosis (*23*−*26*). Here we provide evidence of a role for PE in tenase complex assembly and activation.

MATERIALS AND METHODS

Phospholipids. Egg phosphatidylcholine, brain phosphatidylserine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, *N*-methyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, *N*,*N*-dimethyl-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, egg lysophosphoethanolamine, and phosphatidylethanolamine-*N*-5-(dimethylamino)-1-naphthalenesulfonyl were purchased from Avanti Polar Lipids Inc.

Phospholipid Vesicle Preparation. Large unilamellar phospholipid vesicles were prepared using a published extrusion method (*17*). Briefly, phospholipids were combined, and the solvent was removed by evaporation under N_2 at 40−50 °C. The solvent-free phospholipids were rinsed twice with methylene chloride, and the solvent was evaporated as before. The phospholipids were then resuspended in TBS [20 mM Tris and 150 mM NaCl (pH 7.4)] and extruded six times through a 0.2 μm polycarbonate filter (Millipore). The concentration of the phospholipid vesicles was determined by an elemental phosphorus assay (*27*). Vesicles were used immediately or stored at 4 °C in the dark under N_2. Uniformity of vesicle size was confirmed by gel filtration.

Activation of Human Factor VIII. Recombinant human factor VIII (*28*) (a gift from Genetics Institute) was dialyzed into 150 mM NaCl, 20 mM HEPES, and 5 mM $CaCl_2$. Factor VIII was converted to factor VIIIa by the addition of 3.54 μM human α-thrombin (Haematologic Technologies, Inc.). After 10 min at 37 °C, the thrombin was inhibited by the addition of 25 μM PPACK (Calbiochem). To remove α-thrombin and PPACK, the sample was diluted with 0.1 M $NaHCO_3$ (pH 5.5) and 0.5 M NaCl and placed in the upper chamber of a Centricon 100 ultrafiltration apparatus. The factor VIIIa was concentrated by centrifugation at 1000*g* for 1 h at 4 °C, and then 0.025 M $NaHCO_3$ (pH 5.5) was added and the centrifugation repeated. Aliquots of the concentrated factor VIIIa were stored at −80 °C and defrosted and diluted immediately prior to use.

Tenase Assay. Tenase activity was measured using a two-step amidolytic substrate assay. Phospholipid vesicles were added to a 96-well microtiter plate (Costar 0720-0105) at a final concentration of 35 μM. For K_m determinations, the following components were added (final concentrations): varying factor X concentrations, 1 nM factor IXa, and 4 nM factor VIIIa in TBS (pH 7.4) containing 0.1% BSA. The reaction was initiated by the addition of 2.5 mM $CaCl_2$ within 2 min of diluting factor VIIIa. After 2 min at ambient temperature for assays containing factor VIIIa or after 3 h at 37 °C for assays performed in the absence of factor VIIIa, the reaction was stopped by the addition of 7.5 mM EDTA. Factor Xa activity was measured by the addition of 0.6 mM S-2765 (DiaPharm Group), a peptide substrate for factor Xa. Hydrolysis of the peptide was assessed at 405 nm in a microtiter plate reader (Molecular Devices Thermomax Microplate Reader) at 37 °C. The amount of factor Xa generated was determined via a standard curve prepared using authentic factor Xa (Haematologic Technologies, Inc.). For assays performed in the absence of factor VIIIa, factor X was treated with DEGRCK, a factor Xa inhibitor, to ensure that the measured Xa activity was due to factor Xa generation and not contaminating factor Xa in the factor X preparation. Furthermore, the factor IXa concentration was 2 nM in these assays. For $K_{d(app)}$ determinations, the following components were added to the wells (final concentrations): 35 μM phospholipid vesicles, 200 nM (in the presence of factor VIIIa) or 3000 nM factor X (in the absence of factor VIIIa), varying concentrations of factor IXa, and, for assays in the presence of the cofactor, 4 nM factor VIIIa in TBS (pH 7.4) containing 0.1% BSA. Kinetic constants were determined by nonlinear regression analysis using the Michaelis−Menten equation. K_m, k_{cat}, and $K_{d(app)}$ are reported as means ± the standard errors of representative experiments performed in duplicate or triplicate and represent the concentration of substrate or factor IXa required for the half-maximal factor Xa generation. $K_{d(app)}$ is not a true equilibrium constant and should only be compared within a set of experimental conditions. k_{cat} measurements should also only be compared within a set, since factor VIIIa specific activity was not standardized.

Fluorescence Resonance Energy Transfer. Fluorescence resonance energy transfer experiments were carried out as previously described (*28*, *29*). Freshly prepared large unilamellar vesicles containing 2.5% dansyl-labeled *N*,*N*-dimethyl-PE were diluted to 3 μM in TBS (pH 7.4) containing 2 mM $CaCl_2$ and added to a 3 mL quartz cuvette (1 cm pathwidth) that had been previously treated with Sigmacote (Sigma). Resonance energy transfer measurements were performed at 25 °C using an SLM 8000C fluorescence spectrometer with excitation and emission slit widths set at 4 nm. Aliquots of factor IXa or factor X were added successively. The protein was excited at 280 nm, and the emission of the dansyl-labeled PE was monitored at 560

Table 1: Effect of PE on the Kinetic Parameters of Factor Xa Generation by the Tenase Complex[a]

vesicle composition	K_m (nM)	k_{cat} ($\times 10^3$ s^{-1})	k_{cat}/K_m ($\times 10^5$ nM^{-1} s^{-1})
assays with factor VIIIa			
80% PC/20% PE	190 ± 20	24 ± 3	13
65% PC/35% PE	150 ± 25	30 ± 1	20
99% PC/1% PS	602 ± 80	24 ± 1	4.0
79% PC/1% PS/20% PE	37 ± 8	380 ± 20	1000
64% PC/1% PS/35% PE	22 ± 5	250 ± 20	1100
97% PC/3% PS	98 ± 11	31 ± 1	32
77% PC/3% PS/20% PE	33 ± 7	180 ± 10	550
62% PC/3% PS/35% PE	18 ± 4	210 ± 10	1200
95% PC/5% PS	110 ± 10	40 ± 3	36
75% PC/5% PS/20% PE	34 ± 3	230 ± 70	680
60% PC/5% PS/35% PE	21 ± 5	260 ± 20	1200
90% PC/10% PS	52 ± 8	340 ± 20	650
70% PC/10% PS/20% PE	30 ± 5	340 ± 20	1100
55% PC/10% PS/35% PE	33 ± 4	350 ± 10	1100
80% PC/20% PS	27 ± 3	360 ± 10	1300
60% PC/20% PS/20% PE	22 ± 2	360 ± 10	1600
45% PC/20% PS/35% PE	19 ± 2	370 ± 10	1900
assays without factor VIIIa			
95% PC/5% PS	5640 ± 460	0.56 ± 0.03	0.010
75% PC/5% PS/20% PE	3220 ± 670	0.36 ± 0.05	0.012
60% PC/5% PS/35% PE	3820 ± 520	0.44 ± 0.08	0.012

[a] All of the results were obtained with the same preparation of factor VIIIa.

nm. Factor IXa and factor X dilutions were carried out immediately prior to each experiment, and vesicles were extruded through filters frequently to prevent aggregation. The concentrations of the vesicles were measured after extrusion via an elemental phosphorus assay (*27*). The energy transfer was assessed immediately after each protein addition, and the amount of time the excitation shutter remained open during each point was limited to <10 s to prevent photobleaching of the labeled PE. The amount of photoinactivation during the experiments was estimated on the basis of mock experiments wherein the dansyl groups on the PE were excited directly at 340 nm and the emission was monitored at 560 nm. Under the assay conditions, no photoinactivation was detected (data not shown). The data are analyzed using nonlinear regression as described previously (*28*).

RESULTS

We examined factor Xa generation by the tenase complex as a function of phospholipid composition (Table 1). The catalytic efficiency, k_{cat}/K_m, of phospholipid vesicles containing 20 or 35% PE and no PS was 3−5-fold higher than that of vesicles containing 1% PS and no PE, mediated primarily by differences in K_m. Suboptimal PS concentrations (<10%) and 20 or 35% PE decreased the K_m and increased the k_{cat} of factor Xa generation as compared to the values for vesicles without PE. Increasing the percentage of PS also increased the efficiency of the tenase reaction by decreasing the K_m for factor X and increasing the k_{cat} as previously reported (*30*). PE did not affect the kinetics of the tenase reaction when the vesicles contained optimal PS levels (Figure 1). Since PE in the presence of PS induces high-affinity binding sites for factor VIIIa (*21*), the importance of factor VIIIa to the PE effect was examined. In the absence of factor VIIIa, PE still decreased the K_m of the reaction; however, this effect was much less pronounced (Table 1). In the presence of factor VIIIa, the inclusion of 35% PE in the phospholipid vesicles (5% PS) produced an approximately 9-fold decrease in the K_m of the reaction compared to only a 2-fold decrease in the absence of factor VIIIa. Additionally, in the absence of factor VIIIa, there was no effect on the k_{cat} (Table 1). These results indicate that the increase in the catalytic rate due to PE is mediated through factor VIIIa and that factor VIIIa enhances the PE effect on the K_m of the reaction.

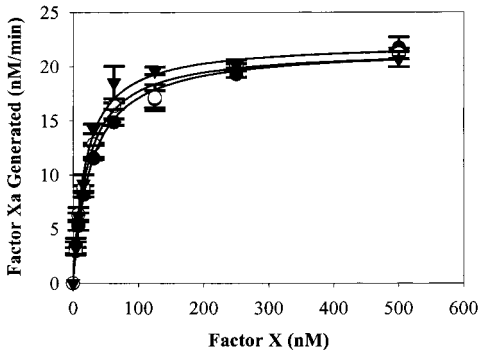

FIGURE 1: Effect of PE in the presence of an optimal level of PS on the generation of factor Xa by the tenase complex. Large unilamellar phospholipid vesicles containing 20% PS and 0 (●), 20 (○), or 35% PE (▼) were used in two-step amidolytic tenase assays. These assays also included factor X (increasing concentrations), factor IXa (1 nM), factor VIIIa (4 nM), and $CaCl_2$ (2.5 mM). The reactions were initiated by the addition of $CaCl_2$ and stopped by the addition of EDTA. The amount of factor Xa activity was measured by the extent of cleavage of S-2765, a factor Xa substrate, which was monitored at 405 nm. The amount of factor Xa produced was determined on the basis of a standard curve generated using authentic factor Xa.

We also investigated the effect of PE on the apparent affinity of factor IXa for the factor VIIIa/Ca^{2+}/phospholipid complex by measuring the level of saturable binding of factor IXa. This was detected as factor Xa generation from increased activity of factor IXa toward factor X. These experiments generated $K_{d(app)}$ values, which are not true equilibrium constants and should only be compared within a given set of experimental conditions. Vesicles containing PE and low percentages of PS decreased the $K_{d(app)}$ of factor IXa as compared to that for vesicles without PE (Figure 2A). The $K_{d(app)}$ for phospholipid vesicles of 1% PS and no PE was 6.1 nM, and for vesicles with 5% PS and no PE, the

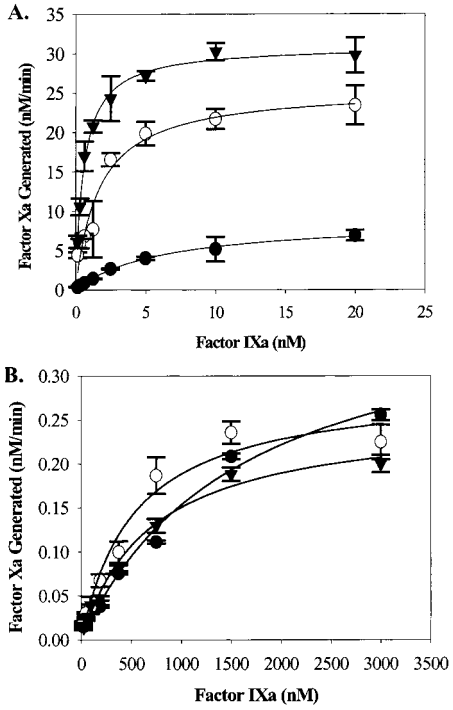

FIGURE 2: Effect of PE on tenase complex formation. $K_{d(app)}$ of factor IXa was determined in the presence of large unilamellar phospholipid vesicles containing 1% PS and 0 (●), 20 (○), or 35% PE (▼). Increasing concentrations of factor IXa were added to wells containing vesicles (35 μM) and 200 or 3000 nM factor X in the presence or absence of 4 nM factor VIIIa, respectively. The reaction was initiated with 2.5 mM $CaCl_2$ and stopped by the addition of 15 mM EDTA. The amount of factor Xa generated was measured by the extent of cleavage of S-2765, which was monitored at 405 nm. The amount of factor Xa produced was determined on the basis of a standard curve generated using purified factor Xa. (A) The $K_{d(app)}$ of factor IXa was determined in the presence of factor VIIIa. (B) The $K_{d(app)}$ of factor IXa was determined in the absence of factor VIIIa.

value was 1.2 nM. In the absence of PS, vesicles containing 20 or 35% PE gave a $K_{d(app)}$ value of 1.9 ± 0.3 or 0.8 ± 0.07 nM, respectively. In the presence of vesicles containing 1% PS and 20 or 35% PE, the apparent binding affinity was 1.0 or 0.6 nM, representing a 6−10-fold increase in affinity over that of vesicles with 1% PS and no PE. When the PS concentration was increased to 5%, the effect of PE was less pronounced, with the $K_{d(app)}$ value decreasing only 2.5−3-fold to 0.5 ± 0.1 or 0.4 ± 0.1 nM in the presence of 20 or 35% PE, respectively (data not shown). PE increased the affinity of factor IXa for factor VIIIa on the membrane surface at either PS concentration. The effect of PE on the apparent binding of factor IXa was also investigated in the absence of factor VIIIa (Figure 2B). Under these conditions, the $K_{d(app)}$ values were much higher; however, PE still had an effect. The $K_{d(app)}$ value was 1610, 530, or 700 nM in the presence of vesicles containing 1% PS and 0, 20, or 35% PE, respectively.

In addition, we looked at the importance of certain structural elements of PE, such as headgroup methylation and *sn*-2 acyl chain. Tenase assays were carried out in the presence of vesicles containing 5% PS and *N*-methyl-PE or *N*,*N*-dimethyl-PE. *N*-Methyl-PE did not affect the K_m of the reaction; however, there was a 3−4-fold increase in the k_{cat} (Table 2). *N*,*N*-Dimethyl-PE did not support the K_m effect or the k_{cat} effect (Table 2). The k_{cat} values generated in these sets of experiments should not be compared to those in Table 1 due to small variations in the activity of the factor VIIIa preparation that was used. The ability of vesicles containing PS and methylated PE to support the apparent binding of factor IXa to the tenase complex was also examined (Figure 3). In the presence of vesicles containing 1% PS and 0, 20, or 35% *N*-methyl-PE, the $K_{d(app)}$ of factor IXa was decreased 2−3-fold from 5.0 nM to 2.9 and 1.6 nM, respectively (Figure 3A). In contrast, *N*,*N*-dimethyl-PE did not affect the $K_{d(app)}$ of factor IXa with values of 5.0, 4.9, and 5.4 nM for 0, 20, and 35% *N*,*N*-dimethyl-PE, respectively (Figure 3B). The importance of the *sn-2* acyl chain of PE was determined by substituting PE with lyso-PE. As seen in Table 2, lyso-PE had no effect on the K_m or the k_{cat} of the tenase reaction. Additionally, lyso-PE had no effect on the $K_{d(app)}$ of factor IXa with values of 5.3, 6.8, and 5.4 nM (Figure 3C). This demonstrates that the presence of an *sn-2* acyl chain of PE is required for the enhancement of the activity of the tenase complex.

After determining the effect of PE on the kinetic parameters of the tenase complex and the apparent binding affinity of factor IXa, we wanted to examine the effect of PE on the binding of factor IXa and factor X to large unilamellar vesicles. To carry out these experiments, we used a fluorescence resonance energy transfer technique developed by Pusey (*29*) and modified in this laboratory for doing equilibrium binding experiments (*28*). We ensured that we were at equilibrium by adding 2 nM proteins to dansyl-labeled vesicles and watching for the signal plateau, which occurred in approximately 20 s (data not shown). There was a decrease in the magnitude of the emission peak due to tryptophans in factor IXa upon addition of 85% PC/5% PS/7.5% PE/2.5% damsyl-PE phospholipid vesicles (Figure 4A) and a concurrent increase in the magnitude of the emission peak of the dansyl group (Figure 4A inset). This energy transfer was also seen between factor X and PE-containing dansyl-labeled vesicles (data not shown). We were unable to detect energy transfer between factor IXa or factor X and dansyl-labeled vesicles containing only 5% PS (92.5% PC/5% PS/2.5% dansyl-PE) (data not shown). Dissociation constants were obtained for factor IXa and factor X binding to 85% PC/5% PS/7.5% PE/2.5% dansyl-PE vesicles (Table 3). Figure 4B shows a representative binding curve depicting factor IXa binding to 85% PC/5% PS/7.5% PE/2.5% dansyl-PE vesicles. The dansyl-labeled vesicles were also used in tenase assays to confirm that 10% PE produced the expected kinetic effects. The K_m values were 101 ± 13 and 20 ± 3 nM and the k_{cat} values 0.064 ± 0.003 and 0.28 ± 0.01 s^{-1} for 92.5% PC/5% PS/2.5% dansyl-PE and 85% PC/5% PS/7.5% PE/2.5% dansyl-PE, respectively (data not shown). Since we were unable to assess binding to vesicles containing 5% PS in the absence of PE, we also examined the binding of factor IXa and factor X to phospholipid vesicles containing 20% PS in the presence and absence of PE. Under these conditions, PE did not exert a kinetic effect; however, as seen in Table 3, PE decreased the dissociation constants of factor IXa and factor X for large unilamellar phospholipid vesicles.

DISCUSSION

In the absence of PE, the tenase complex requires membranes containing 20−25% PS for optimal activity (*31*);

Table 2: Effect of Substituted PE on the Kinetic Parameters of Factor Xa Generation by the Tenase Complex[a]

vesicle composition	K_m (nM)	k_{cat} ($\times 10^3$ s^{-1})	k_{cat}/K_m ($\times 10^5$ nM^{-1} s^{-1})
methyl-PE			
95% PC/5% PS	130 ± 20	68 ± 3	52
75% PC/5% PS/20% methyl-PE	110 ± 10	200 ± 6	180
60% PC/5% PS/35% methyl-PE	160 ± 20	330 ± 17	210
75% PC/5% PS/20% dimethyl-PE	81 ± 23	51 ± 4	63
60% PC/5% PS/35% dimethyl-PE	79 ± 20	57 ± 5	72
lyso-PE			
95% PC/5% PS	100 ± 12	57 ± 2	57
75% PC/5% PS/20% lyso-PE	86 ± 26	68 ± 6	79
60% PC/5% PS/35% lyso-PE	120 ± 30	80 ± 6	67

[a] All of the results were obtained with the same preparation of factor VIIIa.

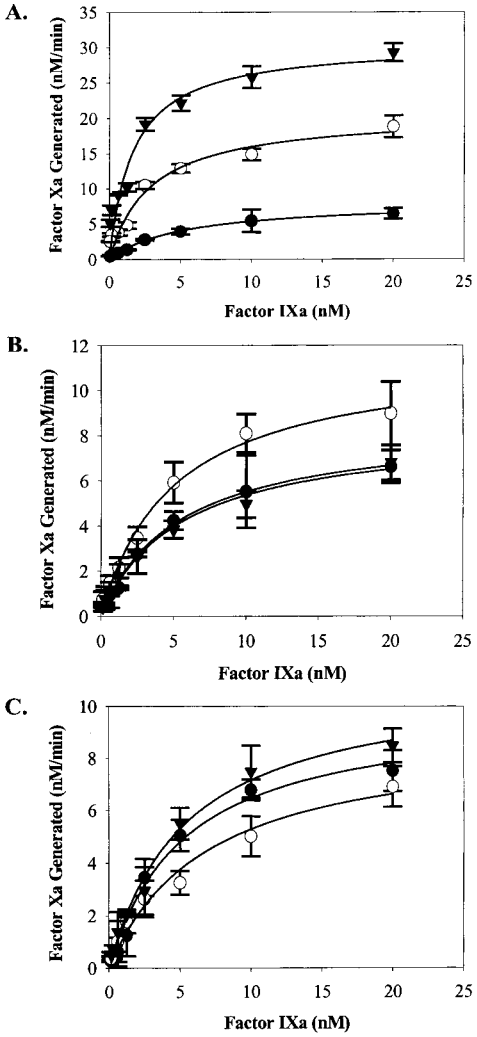

FIGURE 3: Effect of substituted PE on tenase complex formation. (A) $K_{d(app)}$ of factor IXa was determined in the presence of large unilamellar phospholipid vesicles with 1% PS and 0 (●), 20 (○), or 35% *N*-methyl-PE (▼). (B) $K_{d(app)}$ of factor IXa was determined in the presence of large unilamellar phospholipid vesicles with 1% PS and 0 (●), 20 (○), or 35% *N*,*N*-dimethyl-PE (▼). (C) $K_{d(app)}$ of factor IXa was determined in the presence of large unilamellar phospholipid vesicles with 1% PS and 0 (●), 20 (○), or 35% lyso-PE (▼). The assays were performed as previously described.

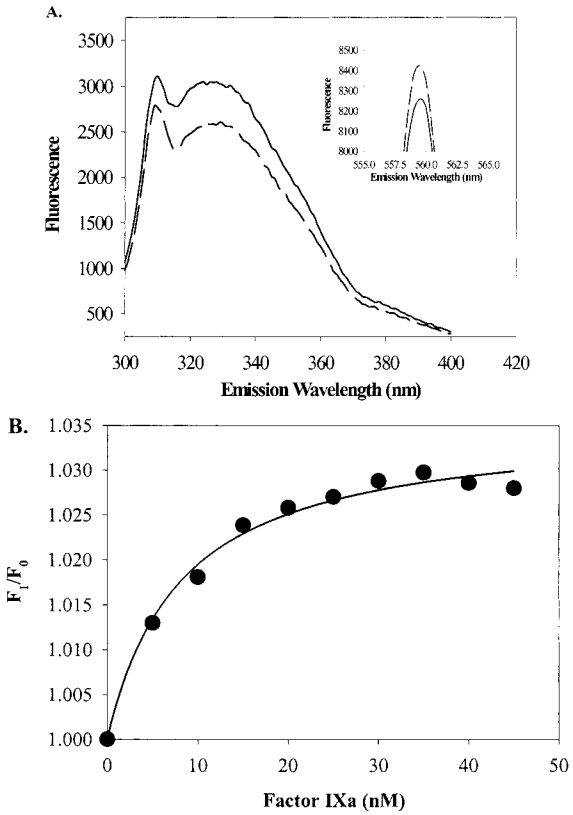

FIGURE 4: Fluorescence resonance energy transfer between factor IXa and dansyl-labeled large unilamellar phospholipid vesicles. (A) Factor IXa (60 nM) was excited at 280 nm, and the emission spectrum was recorded on an SLM 8000C fluorescence spectrophotometer before (black line) and after (dashed line) the addition of 85% PC/5% PS/7.5% PE/2.5% dansyl-PE (3 μM) phospholipid vesicles. In the inset of panel A, 85% PC/5% PS/7.5% PE/2.5% dansyl-PE (3 μM) phospholipid vesicles were excited at 280 nm, and the emission spectrum was recorded before (black line) and after (dashed line) the addition of factor IXa (60 nM). (B) Factor IXa was titrated into 85% PC/5% PS/7.5% PE/2.5% dansyl-PE (3 μM) phospholipid vesicles, and the fluorescence resonance energy was measured at 560 nm.

Table 3: Effect of PE on K_d for Factor IXa and Factor X

vesicle composition	K_d for factor IXa (nM)	K_d for factor X (nM)
92.5% PC/5% PS/2.5% dansyl-PE	not determined	not determined
85% PC/5% PS/7.5% PE/2.5% dansyl-PE	9.3 ± 1.0	75 ± 15
77.5% PC/20% PS/2.5% dansyl-PE	12 ± 1.3	33 ± 4
70% PC/20% PS/7.5% PE/2.5% dansyl-PE	2.1 ± 0.2	3.4 ± 1.5

however, in the presence of 20−35% PE, the PS requirement is reduced to 1%. In the presence of suboptimal PS levels, PE decreased the K_m with respect to factor X. This effect was also seen in the absence of factor VIIIa, but it was less pronounced, suggesting partial dependence on factor VIIIa. Studies have shown that phospholipid membranes profoundly decrease the K_m whereas the protein cofactors, such as factor VIIIa, profoundly increase the k_{cat} for the tenase activation

of factor X (*12*, *32*−*34*). There was a 200000-fold increase in the k_{cat} of the bovine tenase reaction when factor VIIIa was included in kinetic assays with 75% PC/25% PS phospholipid vesicles; moreover, there was a 9-fold decrease in the K_m of the reaction (*34*). Furthermore, factor VIIIa increased the affinity of factor IXa for factor X by approximately 40-fold in the absence of a phospholipid membrane, producing a dramatic decrease in the K_m of the reaction (from 80−180 to 1.7 μM) (*35*). Thus, there is precedence for factor VIIIa producing an effect on the K_m with respect to factor X. Additionally, PE lowered the $K_{d(app)}$ of factor IXa in the presence or absence of factor VIIIa for vesicles containing 1% PS. These results demonstrate that the presence of PE facilitates tenase complex formation and optimal tenase activity at physiological levels of PS (*15*).

PE also increased the k_{cat} of the reaction in the presence of vesicles containing less than 10% PS. This may be an indirect effect of PE due to an induction of high-affinity binding sites for factor VIIIa. Factor VIIIa increases the catalytic efficiency of these kinetic reactions (*32*−*34*). In the absence of PE, at least 8% PS is required for factor VIIIa binding; however, PE reduces this requirement to as little as 1% (*21*). The observed PE effect on the k_{cat} of the tenase reaction disappears when the PS concentration is increased above 8% (10 and 20%) and in the absence of factor VIIIa, clearly implicating factor VIIIa as a mediator of the PE effect on the k_{cat}.

The PE headgroups are important for the effects we observed on tenase complex formation and kinetic parameters, indicating the importance of packing density and orientation. Methylation of the PE headgroup disrupted the effect on the K_m for factor X. There was an increase in the k_{cat} and a decrease in the $K_{d(app)}$ for factor IXa in the presence of vesicles containing *N*-methyl-PE, which disappeared when *N*,*N*-dimethyl-PE was substituted. These results may again be due to effects on factor VIIIa binding. Gilbert and Arena suggested that the PE-induced binding sites for factor VIIIa in the presence of low PS were a result of the small headgroup of PE allowing access to the PS, which was sterically hindered by the bulky headgroup of PC (*21*). Our data support this suggestion. The k_{cat} and $K_{d(app)}$ are mostly affected by nonsubstituted PE, moderately affected by *N*-methyl-PE, and not affected by *N*,*N*-dimethyl-PE. However, this cannot be the sole explanation for the effect of PE on the kinetic parameters of the tenase reaction, as lyso-PE and phosphatidic acid (up to 35% in the presence of 1% PS) (data not shown) have no effect on these parameters.

The methylated forms of PE diminish the lateral mobility and the formation of phospholipid domains normally induced by the hexagonal phase properties of PE in bilayers (*36*). This is thought to be due to a decrease in packing density that may change the orientation between the headgroups and the plane of the bilayer. These phospholipid domains have also been postulated to be important in the binding of the coagulation proteins, although Gilbert and Arena have shown that this does not appear to be the case for factor VIII binding. The binding sites for factor VIII were induced in the presence of PS and dimyristoyl-PE, which is incapable of inducing the hexagonal phase properties leading to phospholipid domains (*21*). We also saw that the *sn*-2 acyl chain was critical for the enhancement of the activity of the tenase complex. PE causes concave regions in the membrane, whereas lyso-PE is thought to cause convex regions; therefore, lyso-PE also impacts the bilayer's packing density and the orientation of the headgroups (*37*, *38*). Our data indicate that the PE enhancement of tenase complex formation and kinetic activity is most likely due to a combination of headgroup specificity and the effect of PE on the fluidity and structure of the membrane.

PE increased the affinities of factor IXa and factor X for large unilamellar vesicles containing 5 or 20% PS. Our K_d values are shown in Table 3. There is a broad range of K_d values for factor IXa (*31*, *39*−*41*) and factor X (*39*, *41*−*45*) binding to vesicles of similar compositions reported in the literature. This range is most likely due to variability in vesicle size and technique. Nevertheless, using identical assay conditions and methods of vesicle preparation, we observed a significant decrease in the dissociation constants of factor IXa and factor X for vesicles containing PE. This suggests that in addition to the importance of PE for factor VIIIa binding to membranes, PE enhancement of the binding of factor IXa and factor X plays a role in optimal tenase activity on membranes with suboptimal PS content.

In this report, we describe a role for PE in the assembly and function of the tenase complex. PE (20 or 35%) in the absence of PS provided a lipid surface generating a tenase complex of greater catalytic efficiency than a surface with 1% PS. PE in the presence of suboptimal PS levels decreased the K_m of the tenase complex for activation of factor X, effectively lowering the PS requirement for optimal activity. Previously, this PE effect was reported for factor VIIa−tissue factor activation of factor X (*8*) and activation of prothrombin by the prothrombinase complex (*20*). Additionally, we report that PE increased the k_{cat} of factor X activation by the tenase complex in the presence of vesicles containing PS levels of less than 10%, an effect mediated by factor VIIIa. PE had little or no effect on the k_{cat} of factor X activation by the tissue factor−factor VIIa complex (*8*); the effect of PE on the k_{cat} for prothrombin activation by the prothrombinase complex has not been reported (*20*). PE also enhanced tenase complex formation as measured by a decrease in the $K_{d(app)}$ in the presence and absence of factor VIIIa. A similar effect was described for PE in the prothrombinase complex (*20*). Finally, we report that PE lowered the equilibrium dissociation constants for both factor IXa and factor X binding to vesicles containing 5 or 20% PS. Correspondingly, PE was found to enhance the affinity of a fluorescein-labeled protein C−prothrombin chimera bearing the prothrombin γ-carboxyglutamic acid rich domain for phospholipid vesicle-coated lipospheres (*46*). Our findings further underscore the importance of the phospholipid membrane composition on assembly of the vitamin K-dependent macromolecular complexes and thus on the process of hemostasis.

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