Articles

Phosphatidylethanolamine Augments Factor VIIa−Tissue Factor Activity: Enhancement of Sensitivity to Phosphatidylserine†

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ABSTRACT: The effect of phosphatidylethanolamine (PE) on the activity of the factor VIIa·tissue factor complex (fVIIa·TF) has been examined with respect to plasma clotting activity and activation of factor X (fX) in a purified system. Vesicles prepared by relipidating membrane-anchored TF (dcTF; TF_{1-244}, lacking the C-terminal cytoplasmic tail) into phospholipid vesicles containing 6 mol % phosphatidylserine (PS) and increasing levels of PE up to 40 mol % (the balance consisting of phosphatidylcholine) were found to progressively shorten TF-initiated clotting in normal human plasma to levels comparable to those observed using dcTF relipidated with cephalin. The shortened clotting times were at least in part due to the ability of PE-containing membranes to better support the activation of fX by the fVIIa·TF complex, as vesicles with increased PE content yielded progressively higher initial rates of fX activation. Surprisingly, PE substantially altered the sensitivity of fX activation to low levels of PS, yielding near-maximal rates of activation at only 3 mol % PS compared to 15−20 mol % PS required in the absence of PE. The effect of PE was not synergistic with that of PS since PE did not increase fX activation rates at high levels of PS (20 mol %). Examination of the kinetic parameters for fX activation revealed that the majority of the effect of PE was in decreasing the apparent K_m for fX. In the absence of PE, values for K_m were 4.4 μM at 0 mol % PS and 120 nM at 6 mol % PS, whereas in the presence of 40 mol % PE these values were decreased to 650 nM and 41 nM, respectively. Taking into account the plasma concentration of fX (~100 nM), these results strongly suggest that the ability of PE to sensitize the fVIIa·TF complex to low levels of PS may have major repercussions *in vivo* by enabling various cell surfaces to control fX activation through subtle changes in the composition of the membrane.

The importance of phospholipid in supporting blood coagulation is widely acknowledged and has been the focus of numerous studies spanning several decades [reviewed in Zwaal (1978), Mann et al. (1990), and Esmon (1993)]. It is now largely accepted that a mixture of acidic and net-neutral phospholipids, such as phosphatidylserine and phosphatidylcholine (PS and PC),[1] can reproduce *in vitro* the vast majority of the effect attributed to membranes in numerous blood coagulation reactions. The main effect of these phospholipid surfaces seems to be a function of the ability of PS (and other acidic phospholipids) to bind vitamin K-dependent clotting factors (both enzymes and substrates) *via* their N-terminal γ-carboxyglutamic acid-containing domain. This binding effectively increases the local concentration of these proteins at the lipid surface and greatly augments the rates of catalysis of these clotting reactions. However, despite the known ability of PS to support blood coagulation reactions *in vitro*, it has long been recognized that crude phospholipid mixtures (*i.e.*, cephalin) yield shorter clotting times than those obtained with purified phospholipids, either natural or synthetic (Kuhn & Klesse, 1957). This strongly suggests that multiple lipid components may be important in clotting, and that these components may act in concert to effect a greater increase in clotting than that observed using only one or two defined lipid components.

Although many studies have been done in an attempt to elucidate which specific lipids may or may not be important in blood clotting reactions, these studies have largely focused on the examination of prothrombinase activity, with comparatively less work being done on defining the lipid components important for the initiation complex of blood coagulation, the fVIIa·TF complex. TF is a cell-surface integral membrane protein which acts as a receptor and cofactor for the vitamin K-dependent blood coagulation serine proteinase, fVIIa (Bach, 1988; Nemerson, 1988; Rapaport, 1991; Carson & Brozna, 1993). TF is not normally in contact with the circulation (Drake et al., 1989) and is exposed to flowing blood only upon injury to the vasculature. The exposed TF then binds plasma fVIIa to form a complex which can subsequently activate the substrates factor IX and factor X (vitamin K-dependent

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[1] Abbreviations: PS, phosphatidylserine (bovine brain); PC, L-α-phosphatidylcholine (egg white); PE, L-α-phosphatidylethanolamine (bovine liver); TF, tissue factor; dcTF, des-cyto TF (TF_{1-244}; membrane-anchored TF lacking most of the C-terminal cytoplasmic tail); sTF, soluble TF (TF_{1-219}; TF lacking the C-terminal cytoplasmic tail and the membrane-spanning domain); fVII, blood clotting zymogen factor VII; fVIIa, activated factor VII; fX, blood clotting zymogen factor X; fXa, activated factor X.

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protein zymogens), thus triggering the blood coagulation proteolytic cascade. Optimal cofactor activity of TF is known to require a phospholipid surface (Bom & Bertina, 1990; Komiyama et al., 1990), and much work has been done to understand the general phospholipid-dependent effects involved in the activity of the fVIIa·TF complex. The majority of this work, however, has been done with membranes of predefined lipid content using mainly PC and PS.

Early studies of the lipid requirements of tissue factor identified several lipid components present in brain-derived thromboplastin which may be of import in the activity of the fVIIa·TF complex (Nemerson, 1968; Liu & McCoy, 1975). These studies identified PC, PS, PE, and sphingomyelin as the major lipid components, each at levels of 20−40% of the total extractable lipid. Phosphatidylinositol and lysophosphatidylcholine were also identified, but only as minor components. Phospholipid vesicles composed entirely of PE were found to support TF activity, consistent with the known ability of PE to enhance blood clotting reactions in general (Rouser et al., 1950; Rouser & Schloredt, 1958; Wallach et al., 1959). However, although the ability of these lipid components to individually support tissue factor activity was clear, the potential roles of these different lipids, as well as their behavior as mixtures, remained undefined.

Recent studies have demonstrated that PE is a major target of lupus anticoagulant activity (Rauch & Janoff, 1992) and plays a role in enhancing the inactivation of factor Va by activated protein C (Smirnov & Esmon, 1994; Smirnov et al., 1995). These studies have led us to reexamine the ability of PE to enhance the activation of fX by the fVIIa·TF complex in a purified system, both in the absence and in the presence of varying levels of PS. We find that PE does indeed support fX activation, and can enhance this activity over that observed with mixtures of only PC and PS. Of greater consequence, however, is the finding that PE enhances the sensitivity of fX activation by the fVIIa·TF complex to low levels of PS. Thus, the presence of PE in membranes may contribute to regulating the activity of the fVIIa·TF complex *in vivo* on the cell surface.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Spectrozyme FXa substrate (MeO-CO-D-cyclohexylglycylglycylarginyl-*p*-nitroanilide acetate) was from American Diagnostica Inc. (Greenwich, CT). All purified membrane lipids were obtained from Avanti Polar Lipids (Pelham, AL) and stored at high concentration (≥10 mg/mL) in chloroform under argon at −20 °C. Rabbit brain cephalin was obtained from Sigma, dissolved in chloroform, and also stored under argon at −20 °C. Octyl-β-D-glucopyranoside was obtained from Calbiochem. Human plasma fVIIa and fX were purified as previously described (Neuenschwander & Morrissey, 1992; Le Bonniec et al., 1992), and sTF was expressed in bacteria and purified as previously described (Rezaie et al., 1992).

Expression and Purification of dcTF. Recombinant, membrane-anchored TF (dcTF) was expressed in *E. coli* as follows. Briefly, the expression construct previously used for bacterial production of sTF (Rezaie et al., 1992) was modified to encode amino acids 1 through 244 of the mature TF sequence [numbered according to Morrissey et al. (1987)]. Thus, the construct encoded the entire extracellular and membrane-spanning domains of TF, as well as the first two amino acids of the cytoplasmic domain. The remainder of the cytoplasmic domain was deleted to avoid mixed disulfide formation (owing to the presence of an unpaired cysteine in the wild-type cytoplasmic domain), as well as to avoid degradation of the wild-type cytoplasmic domain previously reported in *E. coli*-expressed TF (Paborsky et al., 1989). The cytoplasmic domain of TF has been shown to be unimportant for TF activity (Paborsky et al., 1989).

Expression of dcTF in *E. coli* was performed as previously reported for sTF (Rezaie et al., 1992), except that cell lysates were prepared according to Paborsky et al. (1989). Purification of dcTF from these cell lysates was performed as previously reported for sTF (Rezaie et al., 1992), except that 0.1% Triton X-100 was included in all wash and elution buffers. Analysis of the product by SDS−PAGE under reducing and nonreducing conditions indicated a single band with an M_r of ~30 000 (data not shown).

Relipidation of dcTF and Preparation of Vesicles. Purified dcTF was relipidated into phospholipid vesicles of the indicated composition using the octyl-β-D-glucopyranoside method of Mimms et al. (1981) as previously described for human full-length TF (Neuenschwander & Morrissey, 1992). Relipidated dcTF was found to have comparable specific activity to full-length human brain TF when tested in a standard single-stage clotting assay and in fX activation assays (data not shown). The concentration of available dcTF was determined for each relipidated preparation as previously described (Neuenschwander & Morrissey, 1994), and molar ratios of dcTF to phospholipid headgroups averaged roughly 1:9000. Where necessary, concentrations of both available dcTF and total phospholipid are indicated in the figure legends.

Blank phospholipid vesicles (no dcTF) were prepared by drying down the desired phospholipids under argon and resuspending directly in HBSA buffer (Hepes-buffered saline, albumin: 20 mM Hepes−NaOH pH 7.5, 100 mM NaCl, 0.002% NaN_3, and 0.1% BSA). Small unilamellar vesicles were then made by extruding these phospholipid suspensions 21 times through a polycarbonate membrane (100 nm pore size) using a LiposoFast extrusion device (Avestin, Inc.; Ottawa, Ontario Canada). For both blank and dcTF-containing vesicles, the concentration of phospholipid was verified in selected preparations by determination of phosphate content as previously described (Neuenschwander et al., 1993). Phosphate determinations of the rabbit brain cephalin yielded 0.45 mg of phosphate per milligram cephalin, corresponding to 45% phospholipid content by weight in the crude lipid mixture. Control fX activation experiments using dcTF relipidated in cephalin yielded similar activities whether the total lipid used in the relipidation was based on that of cephalin mass or phospholipid mass (data not shown). Experiments reported here were done using vesicles prepared by the former.

Activity Assays. Clotting activities of various relipidated dcTF preparations were measured by a single-stage clotting assay using pooled normal human plasma supplemented with varying amounts of purified fVIIa. In short, 0.05 mL of supplemented plasma was incubated at 37 °C with 0.05 mL of relipidated dcTF (4 nM dcTF in 37 μM lipid) for 30 s. Clotting was initiated by a 0.05-mL addition of prewarmed 25 mM $CaCl_2$, and the time to clot formation was determined using an ST4 coagulometer (Diagnostica Stago; Asnieres,

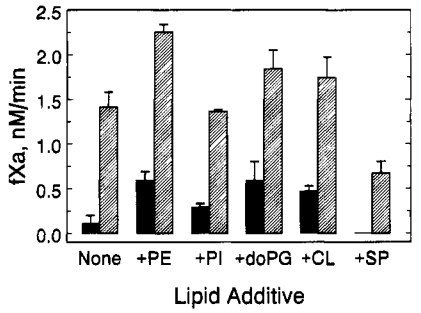

FIGURE 1: Comparison of the effect of different membrane lipids on fX activation by the fVIIa·dcTF complex. Initial rates of fX activation were measured in a purified system using 10 pM fVIIa, 1.3 nM dcTF relipidated in 12.5 μM phospholipid, 150 nM fX, and 5 mM Ca^{2+} in HBSA at 37 °C. Phospholipid vesicles were composed of 30 mol % of the indicated lipid component in the absence (filled bars) or presence (hatched bars) of 6 mol % PS. The remaining phospholipid in all preparations was PC. Values are means ± standard deviations from duplicate determinations. PE, L-α-phosphatidylethanolamine (bovine liver); PI, L-α-phosphatidylinositol (bovine liver); doPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; CL, cardiolipin (bovine heart); SP, sphingomyelin (bovine brain).

France). Measurements of initial rates of fX activation were performed in HBSA buffer as previously described (Neuenschwander & Morrissey, 1994) using the reaction conditions given in the figure legends.

RESULTS

Comparison of the Effect of Various Membrane Lipids on the Activity of fVIIa·dcTF. Cephalin is known to contain many lipid components which could potentially affect the activity of the fVIIa·TF complex. Thus, as an initial test, various pure membrane lipid components were examined with respect to their effect, if any, on initial rates of fX activation by fVIIa·dcTF. For these experiments, dcTF was relipidated into vesicles composed of 30 mol % of the lipid in question in the absence or presence of 6 mol % PS, with the remainder of the vesicles composed of PC. The results (Figure 1) indicated that the presence of 30 mol % sphingomyelin was inhibitory under both conditions examined, while 30 mol % phosphatidylinositol produced a marginal increase in the initial rate of fX activation in the absence of PS, but no effect when PS was present. In contrast, the presence of 30 mol % PE, 30 mol % 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], or 30 mol % cardiolipin significantly increased the initial rate of fX activation both in the absence and in the presence of 6 mol % PS. Of these three membrane lipids, PE was found to have the strongest effect.

Effect of PE on Clotting Activity and fX Activation. The activity of PE-containing vesicles was compared to that of cephalin in a standard one-stage clotting assay. In these experiments, dcTF was relipidated into vesicles composed of either cephalin or 6 mol % PS with varying levels of PE from 0 to 40 mol % (balance was PC). The clotting activity of each of these relipidated dcTF preparations was then measured as a function of input fVIIa to simultaneously examine the sensitivity of the observed activity to initial fVIIa levels (Figure 2). As expected, the dcTF/cephalin preparation yielded much shorter overall clotting times than those obtained using dcTF/PCPS. However, the clotting times

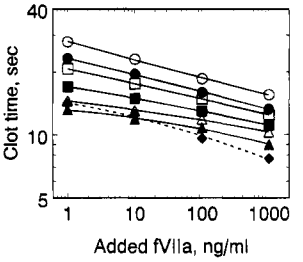

FIGURE 2: Effect of PE on the clotting activity of the fVIIa·dcTF complex. Clotting assays were performed as described under Experimental Procedures using 4 nM dcTF relipidated in 37 μM of either cephalin (◆; dashed line) or purified phospholipids (solid lines) containing various amounts of PE. The compositions of the latter preparations were 6 mol % PS with 0 mol % PE (○), 5 mol % PE (●), 10 mol % PE (□), 20 mol % PE (■), 30 mol % PE (△), or 40 mol % PE (▲) with the remaining phospholipid being PC. Points are means of duplicate determinations ± standard deviations (within symbol size).

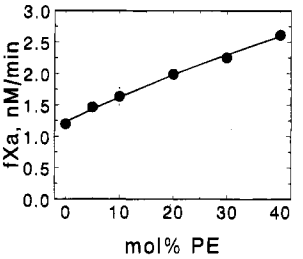

FIGURE 3: Activation of fX by the fVIIa·dcTF complex as a function of mole percent PE. Initial rates of fX activation were determined as described for Figure 1 using the relipidated dcTF preparations described in Figure 2 (legend); 6 mol % PS, various mole percents PE, and the remainder PC.

observed with dcTF/PCPS were progressively shortened with increasing levels of PE. Inclusion of as little as 20 mol % PE substantially shortened these clotting times to levels approaching those obtained with cephalin, while 30−40 mol % PE generally restored the clotting times to those observed with dcTF/cephalin. Despite the shortened clotting times with PE, some loss in sensitivity to input fVIIa was observed as evidenced by the reduced slopes compared to cephalin. At low levels of fVIIa (1−10 ng/mL), vesicles containing 30−40 mol % PE were comparable or better than cephalin in supporting clotting, but at higher levels of fVIIa (0.1−1 μg/mL) cephalin produced shorter clotting times. When the dcTF/PL preparations were used in a purified system to examine the effect of PE directly on fX activation by the fVIIa·dcTF complex (Figure 3), initial rates of fX activation clearly showed the enhancing effect produced on this reaction by PE, with a roughly 2-fold increase in the initial rate observed with 40 mol % PE.

Effect of PE on the PS Dependence of fX Activation. The role of PS in supporting fX activation by the fVIIa·TF complex is well documented, and levels of PS reported in the literature to be most active (in mixtures of PC and PS) range from 20 to 50 mol % PS. Levels of PS below 20 mol % generally produce lower activities, presumably due to the lower binding capacity of these membranes for vitamin K-dependent proteins, while high levels of PS (>40 mol %) are generally inhibitory. Thus, in an attempt to determine the basis for the effect of PE in enhancing the activity of the fVIIa·dcTF complex toward fX, various preparations of relipidated dcTF were made using increasing levels of PS from 0 to 20 mol % in the absence or presence of 40 mol %

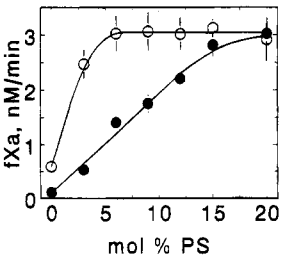

FIGURE 4: PS dependence of fX activation by the fVIIa·dcTF complex in the absence and presence of PE. Initial rates of fX activation were determined as described for Figure 1 using dcTF relipidated in phospholipid vesicles of various mole percentages PS (indicated) in the absence (●) or presence (○) of 40 mol % PE with the remaining phospholipid being PC.

Table 1: Effect of PE on the Apparent Kinetic Constants of fX Activation by the fVIIa·dcTF Complex[a]

mol % PS	mol % PE	$k_{cat} \pm$ SD (s^{-1})	$K_{m,app} \pm$ SD (μM)	k_{cat}/K_m[b] $\pm$ SE ($\times 10^8$ M^{-1} s^{-1})
0	0	5.7 ± 0.05	4.4 ± 0.04	0.026 ± 0.003
	40	5.2 ± 0.01	0.65 ± 0.03	0.092 ± 0.008
6	0	5.1 ± 0.2	0.12 ± 0.02	0.35 ± 0.02
	40	6.3 ± 0.8	0.041 ± 0.004	1.5 ± 0.2
20	0	N/D	N/D	1.0 ± 0.1
	40	N/D	N/D	1.6 ± 0.3

[a] Initial rates of fX activation were measured at 37 °C in HBSA buffer, pH 7.5, using 10 pM fVIIa, 1.3 nM dcTF relipidated in 12.5 μM phospholipid of the indicated composition (remainder is PC), 5 mM Ca^{2+}, and various concentrations of fX from 0 to 6 μM. Plots of rates *versus* fX concentration were fit to the Michaelis–Menton equation to derive values for $K_{m,app}$ and k_{cat}. Values are means ± standard deviations (SD) from duplicate determinations. N/D = not determined. [b] Values of k_{cat}/K_m were determined independently using the method of Crompton and Waley (1986) as previously described (Neuenschwander & Morrissey, 1994). Reported values are means ± standard errors (SE) from at least four determinations.

PE. These preparations were then used to examine initial rates of fX activation as before. Surprisingly, it was found that PE greatly increased the sensitivity of this reaction to low levels of PS (Figure 4). In the presence of 40 mol % PE, near-maximal initial rates of fX activation were observed using as little as 3 mol % PS, compared to the 12–15 mol % PS required for maximal activity in the absence of PE. When the level of PS approached 20 mol %, the initial rates were independent of the presence of PE, suggesting that the effect of PE was not synergistic with that of PS. Analysis of the kinetic constants for fX activation indicated that the majority of the effect of PE was in a decrease in the apparent K_m for fX, with little or no effect on the catalytic rate constant, k_{cat} (Table 1). The lowered apparent K_m translated to a 2–4-fold increase in the specificity constant (k_{cat}/K_m) at low levels of PS, while at 20 mol % PS, PE had little effect.

One of the major competitors of fX for membrane binding *in vivo* is expected to be prothrombin, which is present in plasma at very high levels (~1.5 μM) compared to fX (~0.1 μM). Thus, the effect of PE on the initial rate of fX activation was examined in the presence of various levels of prothrombin fragment 1 (the phospholipid binding region of prothrombin containing the N-terminal γ-carboxyglutamic acid domain, a generous gift of Dr. Charles Esmon). The results (Table 2) showed that when PE was absent from the membrane, the initial rate of fX activation fell from 1.3 nM/min in the absence of prothrombin fragment 1 to 0.7 nM/min when prothrombin fragment 1 was present at 15 μM.

Table 2: Effect of Prothrombin Fragment 1 on fX Activation Rates Using Membranes ± PE[a]

prothrombin fragment 1 (μM)	mol % PE	initial rate of fX activation (nM/min)
0	0	1.3
	40	2.5
1.5	0	1.0
	40	2.6
15	0	0.7
	40	2.6

[a] Initial rates of fX activation were measured at 37 °C in HBSA buffer, pH 7.5, using 150 nM fX, 5 mM Ca^{2+}, 10 pM fVIIa, 1.3 nM dcTF relipidated in 12.5 μM phospholipid (6 mol % PS, indicated mol % PE, remainder PC), and the indicated concentration of prothrombin fragment 1.

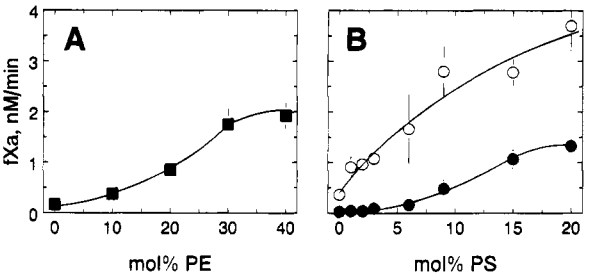

FIGURE 5: Effect of PE on the activation of fX by the fVIIa·sTF complex. Initial rates of fX activation were determined in HBSA at 37 °C using 100 pM fVIIa, 300 nM sTF, 150 nM fX, 5 mM Ca^{2+}, and 250 μM phospholipid vesicles of various composition. Panel A: Phospholipid vesicles were composed of 6 mol % PS and various mole percentages of PE (indicated) with the remaining phospholipid being PC. Panel B: Phospholipid vesicles were composed of various mole percentages of PS (indicated) in the absence (●) or presence (○) of 40 mol % PE with the remaining phospholipid being PC.

In sharp contrast, when PE was present in the membrane, the rate of fX activation was completely unaffected by increasing levels of prothrombin fragment 1 up to 15 μM.

Effect of PE on fVIIa Binding to Membranes. PE had little or no effect on the k_{cat} of fX activation. In contrast, the ability of PE to lower the apparent K_m of the fVIIa·dcTF complex for fX strongly suggested an increased capability of PE-containing membranes to interact with fX, and potentially also with other vitamin K-dependent proteins. This question was addressed with respect to fVIIa binding by using the ability of the fVIIa·sTF complex to activate fX as an indirect measure of fVIIa binding to phospholipid vesicles composed of various mole percentages of PE. Since sTF is not membrane-anchored, the phospholipid dependence of the activity of the fVIIa·sTF complex is a direct function of the ability of both fVIIa and fX to interact directly with the membrane surface (Fiore et al., 1994), presumably via their γ-carboxyglutamic acid-containing domains.

When initial rates of fX activation were measured using the fVIIa·sTF complex with phospholipid vesicles of varied mole percentages of PE (Figure 5A), a greater than 10-fold increase in the activation rate was observed with 40 mol % PE, compared to only a 2-fold increase using fVIIa·dcTF (*cf.* Figure 3). The effect of PE on enhancing fVIIa binding was further supported by the examination of the PS dependence of fVIIa·sTF activity in the absence or presence of PE (Figure 5B). As observed with fVIIa·dcTF, PE greatly sensitized the activity of fVIIa·sTF to low levels of PS (≤6 mol %). However, PE also enhanced the activity of fVIIa·

sTF when higher levels of PS were present, unlike that observed with fVIIa•dcTF.

DISCUSSION

In an initial examination of the ability of various lipid components to affect fVIIa•TF function in a purified system in the absence and presence of a low level of PS (6 mol %), it was found that PE had the greatest effect on enhancing the activity of the fVIIa•TF complex. These results confirm and extend the results of others regarding the relative effectiveness of PE in supporting fVIIa•TF activity when crude tissue extracts were used as the source of TF activity (Nemerson, 1968; Liu & McCoy, 1975). Our results also indicated the ability of certain lipid mixtures to yield greater overall activities than those observed with individual lipid components or with mixtures of only PC and PS. In addition, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] and cardiolipin were found to induce significant increases in fVIIa•TF activity similar to PE. Mixtures of phosphatidylinositol and PC had greater activity than PC alone, but phosphatidylinositol did not enhance the activity of mixtures of PC and PS. Sphingomyelin had little or no enhancing effect. In fact, sphingomyelin was found to inhibit fVIIa•TF activity under the conditions examined here. Thus, these different lipid components may all be implicated at some level in modulation of fVIIa•TF activity under various circumstances *in vivo*.

When dcTF was relipidated with PC, 6 mol % PS, and various levels of PE, the resulting clotting times of these preparations were found to progressively shorten with increasing levels of PE in the membrane. The proficiency of PE-containing membranes in supporting clotting was found to approach that of cephalin when PE was present at levels approaching 30 mol %. It is interesting to note that this level of PE is roughly the level expected to be present on the activated platelet surface (Bevers et al., 1983). Although the presence of PE was found to shorten the overall clotting times to mimic those observed using cephalin, increasing levels of PE also altered the dependence of clotting times on added fVIIa from that observed with cephalin. The basis for this effect was not examined but demonstrates that a difference still exists between the procoagulant properties of cephalin and those of defined phospholipid membranes composed of a mixture of PC, PS, and PE. The role of PE as examined here seems to be, at least in part, to enhance the binding of fX to the membrane surface, resulting in a reduction in the apparent K_m for this substrate at low levels of PS. This interpretation is consistent with the ability of PE to enhance the binding of fXa to membranes.[2] The presence of 40 mol % PE along with only 6 mol % PS decreased the apparent K_m for fX from 120 nM (slightly above the plasma fX concentration of ~100 nM) to 41 nM, well below the plasma level of fX. Although it is difficult to assess the significance of the apparent K_m values for phospholipid-associated reactions, the potential exists that the effect of PE on lowering this parameter may be of great import *in vivo* due to the ability of other vitamin K-dependent proteins to also bind to membranes containing PS and effectively compete with fX for membrane binding. In this regard, the vitamin K-dependent protein present at the highest levels in plasma is prothrombin (~1.5 μM), which is known to bind to membranes in a PS-dependent manner. Due to its high plasma concentration, prothrombin would be expected to be a major competitor for membrane binding *in vivo*. Although prothrombin fragment 1 was indeed found to lower the initial rates of fX activation when PE was absent from the phospholipid membrane, the effect was modest. Of greater import, however, was the ability of 40 mol % PE to prevent prothrombin fragment 1 from inhibiting fX activation even at levels of prothrombin fragment 1 up to 10 times the plasma prothrombin concentration. These results suggest that prothrombin binding to membranes is not as greatly enhanced by PE as is fX binding. Although not examined in this study, this may also be true of other PS-dependent phospholipid binding proteins. Thus, an additional function of PE may be to allow formation of specific activation complexes in the presence of the high plasma concentrations of prothrombin and other competitors. When taken into account with the effect of PE in lowering the apparent K_m for fX, this protective effect of PE on fX activation may have much greater consequences on clotting *in vivo*.

In addition to the ability of PE to enhance the binding of fX, PE was also found to have an enhancing effect on the activity of the fVIIa•sTF complex, suggesting an increased ability of PE-containing membranes to bind fVIIa. The observed 10-fold enhancement in the initial rate of fX activation with the fVIIa•sTF complex is most likely more a function of increased fVIIa•sTF binding to the membrane rather than increased fX binding since, based on the data of Figure 3, a maximum increase of only 2-fold would be anticipated for an increase solely due to effects on fX.

As observed in fX activation by the fVIIa•dcTF complex, PE was found to enhance the PS dependence of fX activation by the fVIIa•sTF complex. The differences observed in the PS dependence of fVIIa•sTF *versus* fVIIa•dcTF are presumably due to the dual dependence of the former on membrane binding, and involve the binding of both fVIIa•sTF and fX. We have previously demonstrated (Fiore et al., 1994) that the reduced apparent activity of the fVIIa•sTF complex, when compared to a membrane-anchored fVIIa•TF complex, is a direct function of the low affinity of fVIIa for membranes (Nelsestuen et al., 1978). When the level of enzyme complexes bound to the membrane surface is allowed to be equivalent, the fVIIa•sTF complex displays equal activity to that of membrane-anchored fVIIa•TF complexes. Since the effect on the K_m for fX in the presence of PE is maximal by 6 mol % PS (Figure 4 and Table 1), the additional effect of PE observed with the fVIIa•sTF complex at 9−20 mol % PS is thus likely a result of progressively increased binding of the fVIIa•sTF complex to the membrane.

Although the studies presented here do not directly address the question of mechanism for the observed effects of PE on the activity of the fVIIa•TF complex, several potential general mechanisms can be considered for the effect of PE on the PS dependence of fX activation. For instance, one can argue that PE may induce local phase changes in the phospholipid surface to form "PE islands". Although the precise mechanism for this putative phenomenon is speculative, a potentially important parameter could be the fatty-acyl side chain composition of the PE preparation. (It should be noted that the studies presented here were done using bovine liver PE of mixed fatty acyl composition.) Formation

[2] Mikhail D. Smirnov, Naomi L. Esmon, and Charles T. Esmon, personal communication and manuscript in preparation.

of putative "PE islands" could presumably act to effectively concentrate PS on the remaining surface through exclusion, thus enhancing the effectiveness of lower levels of PS. However, if this were the case, then one would expect only a 1.7-fold increase in sensitivity to PS when 40 mol % PE was present. For example, in vesicles containing a total of 3 mol % PS and 40 mol % PE, exclusion of PS due to formation of putative PE islands would result in 60% of the membrane being devoid of PE and effectively containing 5 mol % PS *versus* the 3 mol % PS which would be present in the absence of PE, despite the equivalent levels of total PS (3 mol %). Experimentally, the observed enhancement in activity was much greater than this (*cf.* Figure 4), suggesting that this alone does not seem to be the case and that an alternative explanation is warranted. A plausible alternative hypothesis to formation of "PE islands" could be the PE-induced formation of "PS islands" in the membrane. This would also potentially explain the reduced requirement for PS as well as the increased binding capacity of the membranes for fX and, presumably, fVIIa. If true, the exact mechanism for PE to induce formation of PS islands remains to be determined and may involve any of numerous potential lipid−lipid interactions. Alternatively, lipid−protein interactions involving PE and any or all of the protein reactants are also plausible and should not be excluded.

In conclusion, we have demonstrated that PE enhances the activity of the fVIIa·TF complex through a reduction in the apparent K_m for fX, and that the binding of fVIIa to the membranes containing PE is probably also increased. Of greater import, we feel, is the ability of PE to enhance the sensitivity of the fVIIa·TF complex to low levels of PS, thus enabling subtle changes in membrane composition to directly affect membrane procoagulant properties with respect to the initiation of clotting. With the realization that PE has been directly implicated with lupus anticoagulant activity (Rauch & Janoff, 1992), the ability of PE to affect coagulation reactions as well as anticoagulation reactions (Smirnov & Esmon, 1994; Smirnov et al., 1995) demonstrates the potential pivotal role of PE in mediating blood coagulation in both normal and disease states. Thus, a precise description of the molecular mechanisms involved in PE function with respect to the fVIIa·TF complex may be greatly beneficial in understanding the potential role of PE under various circumstances *in vivo*.

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