The Effect of Membrane Composition on the Hemostatic Balance

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ABSTRACT: The phospholipid composition requirements for optimal prothrombin activation and factor Va inactivation by activated protein C (APC) anticoagulant were examined. Vesicles composed of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) supported factor Va inactivation relatively well. However, optimal factor Va inactivation still required relatively high concentrations of phosphatidylserine (PS). In addition, at a fixed concentration of phospholipid, PS, and APC, vesicles devoid of PE never attained a rate of factor Va inactivation achievable with vesicles containing PE. Polyunsaturation of any vesicle component also contributed significantly to APC inactivation of factor Va. Thus, PE makes an important contribution to factor Va inactivation that cannot be mimicked by PS. In the absence of polyunsaturation in the other membrane constituents, this contribution was dependent upon the presence of both the PE headgroup per se and unsaturation of the 1,2 fatty acids. Although PE did not affect prothrombin activation rates at optimal PS concentrations, PE reduced the requirement for PS ∼10-fold. The $K_{m(app)}$ for prothrombin and the $K_{d(app)}$ for factor Xa-factor Va decreased as a function of increasing PS concentration, reaching optimal values at 10−15% PS in the absence of PE but only 1% PS in the presence of PE. Fatty acid polyunsaturation had minimal effects. A lupus anticoagulant immunoglobulin was more inhibitory to both prothrombinase and factor Va inactivation in the presence of PE. The degree of inhibition of APC was significantly greater and much more dependent on the phospholipid composition than that of prothrombinase. Thus, subtle changes in the phospholipid composition of cells may control procoagulant and anticoagulant reactions differentially under both normal and pathological conditions.

It is well-recognized that physiological blood coagulation requires the presence of membranes composed of negatively charged phospholipids. Zymogen activations occur rapidly when the enzyme, usually a vitamin K-dependent protein, binds to a cofactor, usually a non-vitamin K-dependent protein, to activate a substrate, usually a vitamin K-dependent protein (reviewed in refs *1, 2*). The enzymes and the substrates interact with the membrane reversibly, while the cofactors may either bind reversibly or be integral membrane proteins. Several mechanisms have been proposed by which the membrane surface accelerates activation reactions. The enzyme, cofactor, and substrate all interact with the surface, thereby increasing reactant concentrations (reviewed in ref *1*). Binding may also induce conformational changes in the proteins and help align the substrate cleavage sites with the active site of the enzyme (see ref *3* and references therein). Different models exist as to whether the substrate binding creates an increase in local concentration by allowing the substrate to diffuse to the enzyme complex in two dimensions or whether the substrate is activated as it binds to the membrane surface from solution (*1, 4*−*8*).

In addition to a net negative charge, the nature of the phospholipid headgroup appears to contribute to catalytic and binding efficiency. Phosphatidylserine (PS[1]) is generally considered to be the most important phospholipid (*1, 9*), so much so that the vast majority of biophysical and kinetic studies of the assembly of the vitamin K-dependent complexes have used membranes composed solely of phosphatidylcholine (PC) and PS (*1, 10, 11*). For instance, although PS and phosphatidylglycerol have the same charge, prothrombin activation is much more rapid on vesicles containing PS (*9*).

Recently we observed that the presence of phosphatidylethanolamine (PE) or cardiolipin potently enhanced the rate of inactivation of factor Va by the APC complex (*12*). Subsequently, roles for PE in factor VIII binding (*13*), tissue factor−factor VIIa activation of factor X (*14*), and prothrombin activation have been reported (*15, 16*). In the latter studies, incorporation of PE into vesicles decreased the

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[1] Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; APC, activated protein C; BSA, bovine serum albumin; TBS, 150 mM NaCl, 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4; LA, lupus anticoagulant; Gla, 4-carboxyglutamic acid.

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amount of PS required for maximal prothrombin activation rates (*15*). Activation by 5 mol % PS was enhanced by about 5-fold by PE with vesicles adsorbed to capillary tubes in a flow chamber (*16*). In the case of tissue factor, it was shown that the presence of PE enhanced activation primarily by decreasing the amount of PS required for optimal activation and this was largely a K_m effect on the substrate (*14*).

The mechanism of PE participation in factor Va inactivation is uncertain. The enhanced rate of inactivation could be due either to the unique headgroup or to the presence of highly unsaturated fatty acids, characteristic of natural PE (*17*). PE is known to induce the formation of hexagonal H_{II} phase structures. The different degree of saturation present in the PEs used by investigators also affects the fluidity and/or hexagonal phase-forming properties of the PE. In the case of prothrombin activation, once some degree of fluidity was present (*18*), additional fluidity or hexagonal phase formation was not required for enhanced activation (*15, 19*). Gilbert and Arena also concluded that the hexagonal phase-producing property of PE was not related to the enhanced factor VIII binding they observed (*13*). Whether the effects observed in the APC system are specific to the PE headgroup or to membrane structure is addressed in the studies presented here.

PE may also have important roles in the cellular regulation of coagulation and as a participant in autoimmune-mediated thrombotic complications. Potential involvement of PE in coagulation can be inferred from the observation that PE has been detected on the surface of unactivated cells (*20*) and following activation may constitute nearly 40% of the outer leaflet membrane phospholipid (*21*). Once expressed on the cell surface, it is likely to be transported to the inner leaflet of the cell membrane more slowly than PS due to a higher K_m for the "flippase" (*22*). Assuming that anticoagulant reactions exhibit a greater PE dependence than coagulant reactions as suggested by earlier studies (*12*), the differential regulation of PE and PS expression on the cell surface leads to the prediction that the membranes would support coagulant responses more transiently than anticoagulant responses.

Previously, we observed that lupus anticoagulants, antibodies directed toward membrane and presumably membrane−protein complexes (reviewed in ref *23*), can inhibit the APC anticoagulant activity more effectively than prothrombin activation (*24*). This difference is augmented by the presence of PE in the membrane bilayer (*24, 25*). Clinically, these antibodies are associated with an increased risk of thrombosis (*23, 26, 27*), and there is no detailed understanding of the best in vitro assays to detect the antibodies responsible for the pathogenic response.

In this study, we examine the impact of PE on the PS dependence of prothrombin activation and factor Va inactivation. PE impacts both processes, but the mechanisms are distinct. Lupus anticoagulant immunoglobulin also affected the two reactions to different degrees in the absence of other lipid binding proteins present in plasma, such as β_2-glycoprotein-1. A more detailed analysis of the fatty acid contribution to the differences observed is also presented.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Human thrombin (*28*), prothrombin (*28*), activated protein C (*29*), protein S (*30*), factor Xa (*31*), and factor X activator from Russell's viper venom (*32*) were prepared as described previously. Human factor Va was from Hematologic Technologies (Essex Junction, VT). Bovine serum albumin (BSA), Russell's viper venom, ovalbumin, gelatin, MOPS, Tris-HCl, and salts were from Sigma. The chromogenic substrates Spectrozyme TH and Spectrozyme PCa were from American Diagnostica (Greenwich, CT). The irreversible inhibitor of serine proteases (*p*-amidinophenyl)-methanesulfonyl fluoride was from Calbiochem. Total immunoglobulin from lupus anticoagulant patient C1 (*24*) plasma was prepared by ammonium sulfate precipitation. All diacyl phospholipids were purchased from Avanti Polar Lipids Inc. The plasmalogen plasmenylcholine (16:0−18:1) (Plas-PC) was synthesized by an anhydrous reaction utilizing 1-*O*-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine and octadec-9′-enoyl chloride as precursors with (dimethylamino)-pyridine as catalyst as previously described (*33*). Plasmenylethanolamine (16:0−20:4) (Plas-PE) was prepared by a synthetic scheme similar to that described for Plas-PC except that the primary amine of lysoplasmenylethanolamine was first protected with FMOC prior to the reaction with eicosatetra-5′,8′,11′,14′-enoyl chloride (*34*). Synthetically prepared Plas-PC and Plas-PE were quantified by capillary gas chromatography and determined to be greater than 95% pure by thin-layer chromatography, straight-phase HPLC, reversed-phase HPLC, and capillary gas chromatography of the aliphatic constituents.

Preparation of Phospholipid Vesicles. Standard vesicles were prepared using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PS (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC (POPC), and 1,2-dilinoleoyl-*sn*-glycero-3-PE (DL_2PE). Lipids were mixed in the weight proportions indicated, dried under argon, and lyophilized overnight to remove organic solvents. They were then reconstituted under argon in 150 mM NaCl, 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4 (TBS), to 2 mg of total lipid/mL. Vesicles were made by extrusion in order to form large vesicles of comparable size since vesicle size can contribute to the observed kinetic parameters (*8, 35*). Liposomes were prepared by extrusion through 100 nm polycarbonate filters as described (*12*). ^{14}C-PC (Amersham) was included as tracer for the determination of lipid concentrations. The vesicles were used immediately or stored at +20 °C under argon. Storage did not alter vesicle activity.

Measurement of APC and Prothrombinase Activity. Factor Va inactivation was analyzed with a three-stage assay essentially as described (*12, 36*). Briefly, factor Va was inactivated by APC in the first stage in the presence of Ca^{2+} and phospholipid vesicles and the presence or absence of protein S. To determine 100% factor Va activity, we included parallel reaction mixtures containing all reactants *except* APC. In the second stage, after inactivation of APC, residual factor Va activity was monitored by its activity in the prothrombinase complex in the presence of excess factor Xa, prothrombin, and additional lipid (20%PS/80%PC, small unilamellar vesicles prepared by sonication, 20 μg/mL). The additional lipid is added to optimize the activation of prothrombin and to overcome any direct effects of the lipids being tested in the first stage on the second. The resultant thrombin was measured in the third stage using a chromogenic assay with Spectrozyme TH as substrate. All reagents were diluted in TBS containing 1 mg/mL gelatin, 1 mg/mL ovalbumin, and 10 mg/mL BSA. Concentrations of pertinent

reagents are indicated in the figure legends. Percent factor Va inactivation was calculated by dividing thrombin formation in the presence of APC by thrombin formation in its absence and subtracting this value from 1. The validity of this assay regarding linearity with respect to enzyme concentration and time of reaction is presented elsewhere (*36*).

Prothrombinase assays consisted essentially of stages two and three described above. For the determination of the $K_{m(app)}$ for prothrombin on different phospholipid vesicles, prothrombin activation rates were determined at prothrombin concentrations from 10 nM to 10 μM with 0.1 nM factor Va, 2 nM factor Xa, and 10 μg/mL phospholipid. The time of activation was 5 min for all reaction mixtures. This was well within the linear portion of the activation time course. For the determination of the factor Xa $K_{d(app)}$ on different vesicles, the concentration of factor Xa was varied from 0.1 to 50 nM at 1.4 μM prothrombin, 0.1 nM factor Va, and 10 μg/mL phospholipid. $K_{m(app)}$ and $K_{d(app)}$ were determined by fitting the kinetic data to the Michaelis−Menten equation using the ENZFITTER program (Elsevier Biosoft, Cambridge, U.K.). Reported values are the mean plus or minus ($\pm$) the standard deviation of three independent determinations on different days with each $K_{d(app)}$ calculated separately. In the context of these studies, $K_{m(app)}$ and $K_{d(app)}$ are meant to reflect the concentration of substrate or free factor Xa, respectively, required for half-maximal prothrombin activation rate for the purpose of comparison within a set of experimental conditions only. These constants are not meant to reflect true equilibrium constants because of the unusual two-dimensional concentration considerations required for membrane-catalyzed reactions (*35*) in addition to the multistep reaction required for thrombin generation.

Lupus Anticoagulant Activity Assay. The effect of lupus anticoagulant (LA) immunoglobulin on prothrombinase activity was measured by including antibody with prothrombin, phospholipid, and calcium for 20 min before the addition of factors Va and Xa. Thrombin generation was determined as described above. To determine the inhibitory activity on APC inactivation of factor Va, we incubated the LA with all components for 20 min before the addition of factor Va for the first stage of the assay. After inactivation of the APC, excess phospholipid (60 μg/mL final concentration) was added to minimize the effect of the LA on the prothrombinase stage of the assay. Thrombin generation was then determined as above. Factor V activity remaining was determined by comparing the resulting activity to incubation reactions in which all components were present except APC to account for carryover of LA activity in the second stage.

RESULTS

The impact of PE on the PS dependence of prothrombin activation was examined as a function of phospholipid concentration (Figure 1). Although PE was not very effective alone (open circles, Figure 1B), as little as 1% PS in the presence of 50% PE (Figure 1B, solid circles) was sufficient to generate near optimal activity at all membrane concentrations. In the absence of PE, 10% PS was required to obtain similar reaction rates (open squares, Figure 1A). Analysis of the product formed in the presence and absence of PE based on inhibition by ATIII $\pm$ heparin (*37*) indicated that thrombin, not meizothrombin, was being generated in all cases (data not shown).

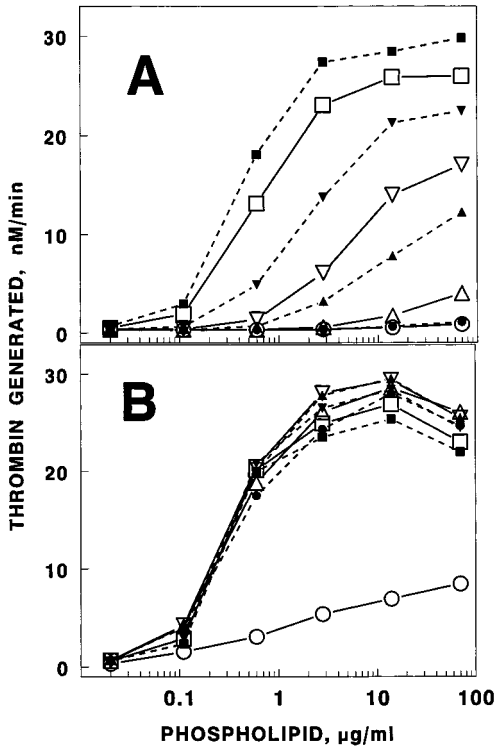

FIGURE 1: Prothrombin activation on vesicles of different phospholipid composition. The standard prothrombinase assay was employed as described under Experimental Procedures with 0.1 nM factor Va, 2 nM factor Xa, 1.4 μM prothrombin, and the concentration of phospholipid vesicles indicated. (A) Liposomes contained PC and the percent of PS indicated. (B) Liposomes contained 50% PE in addition to PC and the percent of PS indicated: ○, 0% PS; ●,1% PS; △, 3% PS; ▲, 5% PS; ▽, 7% PS; ▼, 10% PS; □, 15% PS; ■, 20% PS.

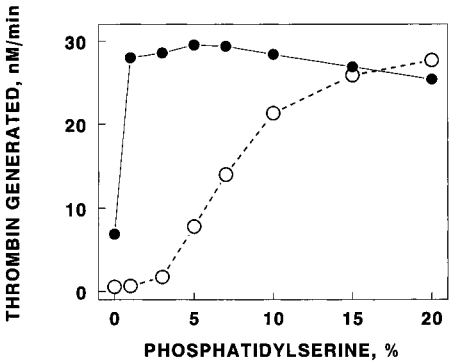

FIGURE 2: Phosphatidylserine dependence of prothrombin activation. The data of Figure 1 is replotted as a function of the mole percent of PS for the 14 μg/mL phospholipid concentration: ○, vesicles without PE; ●, vesicles containing 50% PE.

To simplify the comparisons, we replotted the rate of prothrombin activation as a function of PS concentration at a single phospholipid concentration (Figure 2). The results show the dramatic decrease in PS requirement in the presence of PE. This is in general agreement with the observations of Smeets, et al. (*15*).

The equivalence in rate at low (1%) and high (20%) PS in the presence of PE could be due to the fact that the reactions were performed at physiological, and hence relatively high, concentrations of prothrombin. Therefore, we

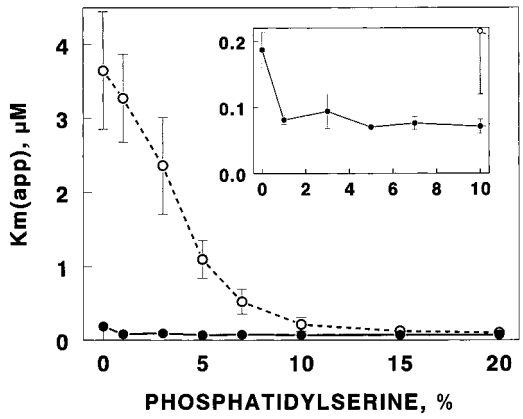

FIGURE 3: $K_{m(app)}$ for prothrombin in the prothrombinase complex on phospholipid vesicles of different composition. The $K_{m(app)}$ for prothrombin was determined as a function of the mole percent of PS content in the lipid vesicles in the presence (●) or absence (○) of 50% PE. The standard prothrombinase assay was employed as described under Experimental Procedures. The prothrombin concentration was varied from 10 nM to 10 μM at constant factor Va (0.1 nM), factor Xa (2 nM), and vesicle (10 μg/mL) concentrations. (Inset) Expansion of the lower left region of the graph to show the effect of low levels of PS in the presence of 50% PE on $K_{m(app)}$.

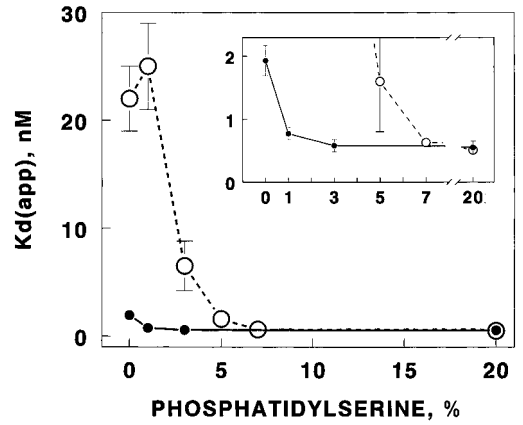

FIGURE 4: $K_{d(app)}$ for factor Xa in the prothrombinase complex on phospholipid vesicles of different composition. The $K_{d(app)}$ for factor Xa was determined as a function of the mole percent of PS content in the lipid vesicles in the presence (●) or absence (○) of 50% PE. The standard prothrombinase assay was employed as described under Experimental Procedures. The factor Xa concentration was varied from 0.1 to 50 nM with constant factor Va (0.1 nM), prothrombin (1.4 μM), and vesicle (10 μg/mL) concentrations. (Inset) Expansion of the lower left region to show the effect of low levels of PS in the presence of 50% PE on $K_{d(app)}$.

analyzed the influence of PS concentration on the apparent K_m for prothrombin activation in the presence and absence of PE. As seen in Figure 3, the presence of PE in the membrane has a major impact on the $K_{m(app)}$. Even in the absence of PS, PE lowered the $K_{m(app)}$ relative to pure PC vesicles by at least 10-fold.

PE could also enhance prothrombin activation by facilitating factor Xa−factor Va complex formation. To test this possibility, we analyzed the factor Xa concentration dependence of prothrombin activation at constant factor Va concentration to obtain $K_{d(app)}$ (Figure 4). Even in the absence of PE and PS, the prothrombin activation complex assembled with moderately high affinity, although far from optimal. This confirms the observation that vesicles composed solely of PC can facilitate assembly of the prothrombinase complex relative to the soluble reaction (ref *15* and references therein).

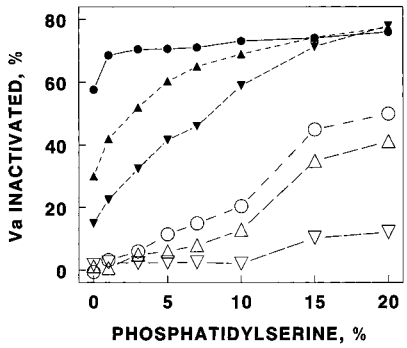

FIGURE 5: Phosphatidylserine dependence of factor Va inactivation by APC. Factor Va inactivation was assayed as described under Experimental Procedures using 0.2 nM factor Va, 4 pM APC, 70 nM protein S, and phospholipid vesicles with the indicated mole percent of PS in the presence (closed symbols) or absence (open symbols) of 50% PE in the first stage. For the second, prothrombin activation stage, 2 nM factor Xa, 1.4 μM prothrombin, and 20 μg/mL final concentration 20% PS/PC sonicated vesicles were added: ○, ●, 100 μg/mL phospholipid; △, ▲, 20 μg/mL phospholipid; ▽, ▼, 4 μg/mL phospholipid.

Even at 5% PS, the $K_{d(app)}$ remained about 3-fold higher than at optimal PS ($K_{d(app)}$ = 1.6 ± 1.0 vs 0.6 ± 0.1 nM). With PE:PC vesicles, the $K_{d(app)}$ was 1.9 ± 0.2 nM and decreased to near optimal with the addition of 1% PS ($K_{d(app)}$ = 0.77 ± 0.1 nM). The PE dependence of the assembly of the prothrombinase complex was eliminated on vesicles with 20% PS ($K_{d(app)}$ = 0.51 ± 0.07 nM without PE and 0.55 ± 0.1 nM with PE).

We next examined the impact of PE on the PS concentration dependence of APC inactivation of factor Va. Membranes of varying PS composition were analyzed for the capacity to accelerate factor Va inactivation (Figure 5). In contrast to prothrombin activation, PE supported factor Va inactivation relatively well, even in the absence of PS. For instance, in the absence of PE, approximately 15% PS was required to equal the inactivation rate achieved with 50% PE/50% PC vesicles. However, except at very high (100 μg/mL) phospholipid, optimal factor Va inactivation still required relatively high concentrations of PS in the presence of PE. Unlike the situation with prothrombinase, at a fixed concentration of liposomes, PS and APC, vesicles devoid of PE never attained the rate of factor Va inactivation achievable with vesicles containing PE (e.g., contrast 15−20% PS, Figure 2 vs Figure 5). In addition, the phospholipid concentration required for optimal enhancement of APC activity was itself dependent on the PS concentration present in the vesicles (Figure 6). Thus, qualitatively and quantitatively, the impact of PE was distinct for these two coagulation reactions.

The PE used in the above studies contained polyunsaturated 1,2-dilinoleoyl-*sn*-glycerol (18:2). This was chosen on the basis of preliminary studies indicating that this species best mimicked factor Va inactivation rates observed with brain PE. However, the question then became whether the PE effects observed were due to the presence of the PE headgroup per se or to the additional polyunsaturation of the phospholipid. To address this question, we varied the degree of unsaturation, whether one or both glycerol positions contained unsaturated fatty acid, and the phospholipid containing the unsaturation. The impact of these phospholipids on prothrombin activation and factor Va inactivation

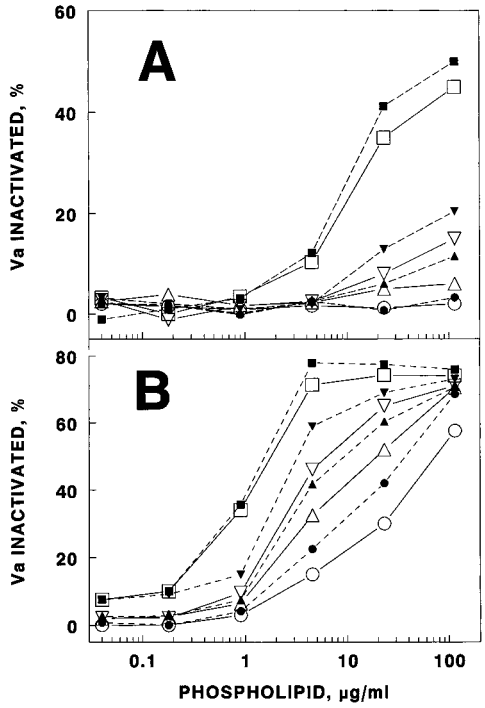

FIGURE 6: The effect of phospholipid vesicle composition on factor Va inactivation by APC. The influence of increasing PS concentration in vesicles without (A) and with (B) 50% PE on factor Va inactivation by APC was studied as a function of increasing phospholipid concentration. The assay was performed as described in the legend to Figure 5: ○, 0% PS; ●,1% PS; △, 3% PS; ▲, 5% PS; ▽, 7% PS; ▼, 10% PS; □, 15% PS; ■, 20% PS.

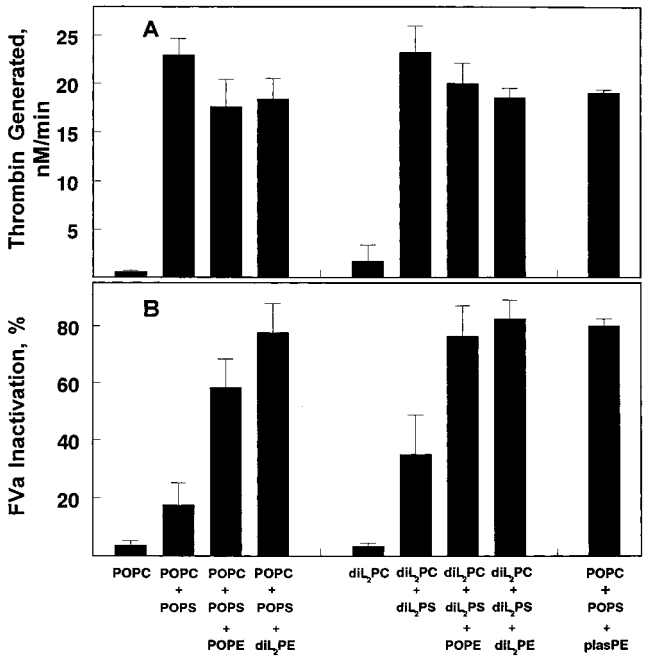

FIGURE 7: Influence of fatty acid and phospholipid composition on prothrombin activation and factor Va inactivation. Prothrombin activation (A) or factor Va inactivation (B) was assayed as described under Experimental Procedures. When included, PS was present at 20% and PE was present at 40%. See Table 1 for definition of abbreviations. Reaction conditions were (A) 1.4 μM prothrombin, 0.1 nM factor Va, 2 nM factor Xa, 10 μg/mL phospholipid; and (B) 5 pM APC, 0.2 nM factor Va, 10 μg/mL phospholipid.

was then determined (Figure 7 and the details of all liposomes studied is presented in Table 1). The plasmalogen forms of PC and PE were also investigated as a high

Table 1: Influence of Fatty Acid and Phospholipid Composition on Prothrombin Activation and Factor Va Inactivation[a]

liposome composition	prothrombinase (nM/min)	APC complex factor Va inactivation (%)
no liposomes	0.4 ± 0.1	0
POPC	0.6 ± 0.1	3.8 ± 1.5
DL_2PC	0.9 ± 0.4	3.5 ± 1.1
DL_3PC	0.9 ± 0.1	3.7 ± 5.2
plasPC	0.8 ± 0.04	3.4 ± 4.8
POPC + POPS	22.9 ± 1.7	17.6 ± 7.6
POPC + POPS + DPPE	16.8 ± 1.0	43.4 ± 14.4
POPC + POPS + POPE	17.6 ± 2.8	58.3 ± 10.1
POPC + POPS + DOPE	15.1 ± 0.3	78.7 ± 8.2
POPC + POPS + DL_2PE	18.4 ± 2.1	77.7 ± 10.1
POPC + POPS + DL_3PE	16.6 ± 1.0	73.6 ± 7.0
POPC + POPS + plasPE	17.9 ± 0.1	79.9 ± 2.6
DL_2PC + DL_2PS	23.2 ± 2.8	35.1 ± 13.5
DL_2PC + DL_2PS + DPPE	20.2 ± 1.0	62.9 ± 1.5
DL_2PC + DL_2PS + POPE	20.0 ± 2.1	76.4 ± 10.7
DL_2PC + DL_2PS + DOPE	17.5 ± 0.4	81.2 ± 3.4
DL_2PC + DL_2PS + DL_2PE	18.5 ± 1.0	82.6 ± 6.5
DL_2PC + DL_2PS + DL_3PE	18.8 ± 0.4	78.9 ± 2.8
DL_2PC + DL_2PS + plasPE	19.0 ± 0.3	79.8 ± 2.3
DL_3PC + DL_2PS	18.7 ± 1.1	18.0 ± 4.4
DL_3PC + DL_2PS + DL_2PE	14.4 ± 1.0	65.8 ± 5.7
DL_3PC + DL_2PS + DL_3PE	17.2 ± 0.1	64.9 ± 7.4
plasPC + POPS	20.6 ± 0.5	21.1 ± 11.5
plasPC + POPS + POPE	18.6 ± 0.3	33.1 ± 16.5
plasPC + POPS + plasPE	18.8 ± 0.7	65.3 ± 9.5
plasPC + DL_2PS	21.3 ± 2.1	6.3 ± 4.5
plasPC + DL_2PS + POPE	20.7 ± 0.4	37.9 ± 11.8
plasPC + DL_2PS + plasPE	20.9 ± 0.1	65.7 ± 8.1

[a] Prothrombin activation and factor Va inactivation were assayed as described in Experimental Procedures. When included, PS was present at 20% and PE was present at 40%. Reaction conditions were the following: prothrombin activation, 1.4 μM prothrombin, 0.1 nM factor Va, 2 nM factor Xa, 10 μg/mL phospholipid; factor Va inactivation, 5 pM APC, 0.2 nM factor Va, 10 μg/mL phospholipid. Values are the average ±SD of 2−6 determinations performed in duplicate. Abbreviations: P, Palmitoyl- (C18); O, Oleoyl- (C18:1); L_2, Linoleoyl- (C18:2); L_3, Linolenoyl (C18:3); plasPC, (16:0−18:1) plasmenylcholine; plasPE, (16:0−20:4) plasmenylethanolamine; D, the specified fatty acid is present in both the 1 and 2 positions of the glycerol backbone.

percentage of PE in natural membranes consists of the hexagonal phase-promoting plasmalogen form (*17, 38*).

When 20% POPS (see legend to Table 1 for definition of abbreviations) was added to POPC, prothrombin activation was dramatically enhanced as expected. The addition of PE, whether in the singly unsaturated (POPE) or in the 1,2 diunsaturated (DL_2PE) form, inhibited this reaction slightly. When 1,2 polyunsaturated DL_2PC alone was used, a slight increase in activation compared to POPC was observed. Again, addition of PS enhanced activation optimally, and the addition of PE decreased the activation rate slightly. Thus, although there is a slight effect of unsaturation of both fatty acids under the conditions used here, it plays a small role in the steady-state activation rates observed in the presence of PS. PE in any form, including plasPE, inhibited the reaction. Inclusion of di-linolenic (C18:3) acid in any of the phospholipid species had no effects beyond those of linoleic (C18:2) acid on the reaction (Table 1). When suboptimal 5% PS was used in the vesicles, the PE enhancement expected from Figure 2 was observed (data not shown).

A very different picture emerged when the inactivation of factor Va was analyzed. To minimize potential complica-

tions from effects on the cofactor, we did not include protein S in these reactions. As can be seen on the left side of Figure 7B, addition of POPS to the POPC increased factor Va inactivation slightly. The addition of POPE had a dramatic, though not optimal, effect, indicating that the headgroup in the context of minimal unsaturation is important. Adding DO or DL_2PE (as used in all previous experiments) further enhanced activity, indicating that unsaturation at both the 1 and 2 positions is also important. However, the 1,2 unsaturation need not be in the PE molecule. This is shown on the right side of the figure. When the polyunsaturated DL_2 forms of PS and PC are present, the activity is significantly higher than when the monounsaturated PO forms are used. Further unsaturation (di-linolenic acid, C18:3) had no additional effects (Table 1). Vesicles containing either the 1-saturated,2-unsaturated POPE or the polyunsaturated DL_2-PE showed optimal activity. The natural plasmalogen form of PE shown at the extreme right has activity similar to that of the synthetic DL_2PE. Since the total amount of DL_2-phospholipid (when present) is changing in these vesicles on the basis of which component(s) is (are) carrying this diacyl-glycerol, other experiments in which the total contribution from DL_2-phospholipids was held constant at 40% but "moved among" components indicated that the location of this moiety had little effect on the activity of the liposomes (data not shown). We conclude that at optimal PS concentration (20%), neither the PE headgroup nor polyunsaturation contribute substantially to prothrombin activation. In contrast, *both* the PE headgroup *and* polyunsaturation contribute strongly to enhanced factor Va inactivation.

We previously reported that the incorporation of PE into the vesicles used to initiate clotting in plasma increased the inhibitory capacity of LAs toward prothrombinase, but increased the inhibition toward APC to a much greater extent, resulting in an overall procoagulant balance when APC was included in the reactions (*24*). An effect on other membrane binding proteins in plasma not directly involved in these reactions could not be ruled out. In this study, we tested the role of the phospholipid composition on this phenomenon in the purified systems (Figure 8). The LA immunoglobulin used in these experiments was unfractionated and therefore was included at 10 mg/mL to approach the concentration in the original plasma. In the absence of PE, the prothrombin activation rate was reduced between 10% and 35%. The extent of inhibition decreased with increasing PS. With PE present, 50% inhibition was observed at all PS concentrations. However, the effect was much more dramatic on the APC complex (Figure 8B,C). In the absence of PE, at concentrations of PS where more than minimal Va inactivation was observed, the LA immunoglobulin decreased the amount of factor Va inactivated by up to 40%. However, in the presence of PE, the inactivation of factor Va could be almost completely blocked. The inhibition was more pronounced as the concentration of employed APC decreased. As the concentration of PS increased, the inhibitory activity of the LA also increased, even though the concentration of PE in the vesicles was not changing. Control immunoglobulin prepared similarly had no effect on either APC or prothrombinase activity when vesicles containing 20% PS were used.

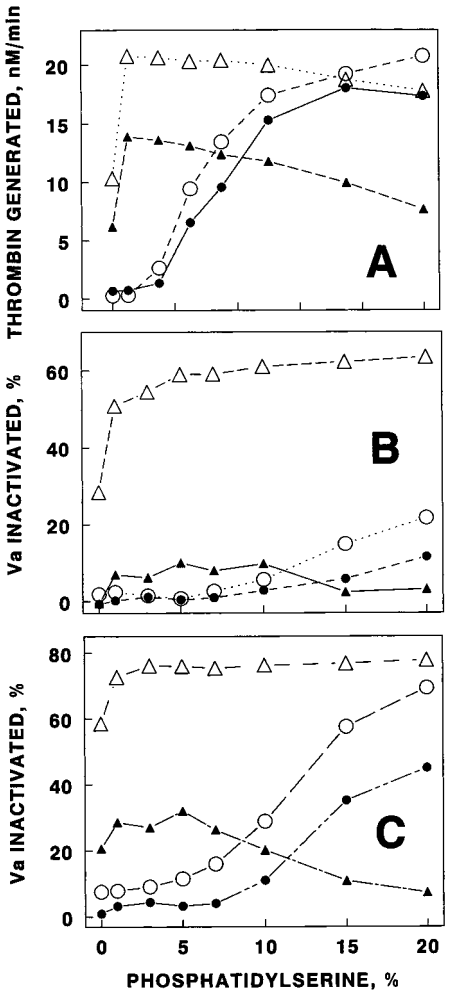

FIGURE 8: Influence of lupus anticoagulant immunoglobulin on prothrombin activation and factor Va inactivation on vesicles of different phospholipid composition. Lupus anticoagulant immunoglobulin inhibition of prothrombinase and factor Va inactivation was assayed as described under Experimental Procedures: ○, 20%PS/80%PC vesicles, no LA; ●, 20%PS:80%PC vesicles, 10 mg/mL LA; △, 50%PE/20%PS/30%PC vesicles, no LA; ▲, 50%PE/20%PS/30%PC vesicles, 10 mg/mL LA. (A) Prothrombin activation. Reactant concentrations were 0.1 nM factor Va, 2 nM factor Xa, 1.4 μM prothrombin, and 40 μg/mL phospholipid. (B and C) Factor Va inactivation. Reactant concentrations were 0.2 nM factor Va, 6.4 pM (panel B) or 32 pM APC (panel C), 70 nM protein S, and 40 μg/mL phospholipid in the first stage of the reaction. Sonicated 20%PS/80%PC was added to 60 μg/mL total phospholipid concentration to optimize prothrombin activation and overcome LA activity in the second stage.

DISCUSSION

Our previous studies revealed the importance of PE in the function of the APC complex, but left open the question of whether the PE participation was due to the headgroup or to the polyunsaturation common on naturally occurring PE (*12*, *24*). The studies reported here demonstrate that both the PE headgroup and polyunsaturation contribute to factor Va inactivation by the APC complex. Although optimal activity is obtained when the polyunsaturation is on the PE moiety, more than half of that activity is observed when the polyunsaturation is present on the PS and/or PC molecules (Table 1 and data not shown). Very recently, Gilbert and Arena (*39*) reported that unsaturation also plays a critical role in the formation of high-affinity binding sites for factor

VIII and in the function of the factor IXa/factor VIIIa complex in factor X activation, the latter being sensitive to the identity of the phospholipid headgroup containing the unsaturation.

It is not known whether the fatty acid saturation effects on APC complex function are due to changes in fluidity alone or to a tendency to form hexagonal phase lipid when PE is also present. The activity of diL_2PC/diL_2PS in the absence of PE would argue for the former. Experiments to explicitly determine the physical state of the membranes would be difficult, as the presence of the proteins would most likely perturb the signals and/or structure of the lipid in their environs. In addition, the distinction between overall phase and the presence of microdomains of importance would require experimentation beyond the scope of this investigation.

In contrast to these studies, we observed minimal effects of the degree of unsaturation on the prothrombinase complex. It has been previously reported that di-unsaturated (dioleoyl-) PC can support prothrombinase activity better than POPC (*19*). However, these effects are observed at very low ionic strength, and the effects at or near physiological ionic strength are rather minor when compared to PS-containing vesicles (*18*). The presence of high mole percentages of PS also abrogated the effect of unsaturation (*40*).

PE itself does, however, have a major impact on the activation of prothrombin. This effect is similar to that on factor X activation by tissue factor (*14*); that is, the presence of PE decreases the PS requirement dramatically. In both cases a major contribution to the kinetic enhancement observed was on the $K_{m(app)}$ for the substrates. At optimal PS, the reaction velocities are similar in the presence or absence of PE, indicating that the two phospholipids are not synergistic. In contrast, there is no concentration of PS which approaches the effectiveness of vesicles containing both PS and PE on factor Va inactivation by APC. In addition, at high lipid concentration, PE alone is able to sustain factor Va inactivation essentially as well as vesicles which also contain PS. Detailed kinetic analysis of the reaction was not performed due to the complexity of the reactions involved. Not only does APC cleave factor Va at multiple sites, but the possible intermediates can exhibit partial activity. The residual activity is dependent on the assay conditions used (*41*). Dissociation of a fragment of the heavy chain also contributes to loss of prothrombinase activity (*42*). This dissociation is time-dependent and can be rate-limiting. However, under the conditions used here, that is, low enzyme concentration and relatively slow rates of inactivation, dissociation would not be the rate-limiting step. This is demonstrated by the observation that the rate of inactivation is increased with increasing enzyme concentration. Studies described elsewhere (*36*) indicate that the overall reaction mechanism (i.e., order of bond cleavage) is not altered by the presence of PE. The rates of both major bond cleavages are affected, and the assays as used here adequately reflect these rates of inactivation for the purposes of comparison. The nonquantitative nature of Western blot analysis makes this technique less reliable. In conclusion, the results presented imply that the PE effects in the procoagulant and anticoagulant systems are distinct.

This study confirms and extends the studies by Billy, et al. in flow systems (*16*) and by Smeets, et al. (*15*) on sonicated vesicles. Smeets, et al., using a system similar to that used here, also found that the presence of PE decreased the concentration of PS required for maximum activation rate without altering the maximum rate achieved. They did not further investigate the basis for the effect. Our studies indicate that the contribution of PE to the catalytic process involves both a decreased $K_{m(app)}$ for prothrombin and $K_{d(app)}$ for factor Va/factor Xa assembly at low PS concentrations. The study also confirms the earlier observation that vesicles composed solely of PC increase the rate of prothrombin activation to some extent (*19*) relative to no lipid present, in part by decreasing the $K_{m(app)}$ for prothrombin.

The rates observed in this study might appear somewhat lower than anticipated from the literature. Most of the literature studies are performed at relatively high factor Va and factor Xa concentrations. At the lower concentrations, the reaction rate decreases significantly (*43*), probably reflecting incomplete complex assembly, and some subunit dissociation within factor Va (K_d estimated at 6 nM for bovine factor Va (*44*)).

Given that PE is reported to be present on the outer leaflet of resting platelets and to be transported more slowly by the flippase (reviewed in ref *22*), the observation that PE can augment prothrombin activation and factor Va inactivation provides a cellular mechanism to enhance the sensitivity of the cell surface to smaller changes in PS expression. Some of the differences observed between platelet prothrombinase and synthetic systems may be due to the presence of PE in the former, but not the latter. It should be noted that plasmenylethanolamine, the form of PE commonly found in cells of hemostatic interest, has activity in both systems equivalent to the other molecular species of PE.

Immunoglobulin from a lupus anticoagulant serum preferentially inhibited both the prothrombinase and factor Va inhibition complexes on PE-containing vesicles, confirming our previous observations in plasma (*24*). The degree of inhibition observed on the APC complex was significantly greater than the inhibition observed on the prothrombinase complex. APC complex inhibition was also much more dependent on phospholipid composition than was the prothrombinase inhibition. These results once again reflect the different phospholipid architecture used by the two complexes. This observation also has potential clinical ramifications. Previous studies of oral anticoagulant therapy have indicated that reducing prothrombin levels to 50% is not sufficient for an adequate anti-thrombotic effect (*45*). Patients with 50% or less protein C are at significant risk for thrombosis (*46*). Thus, an antibody population, such as that illustrated here, which never inhibits prothrombinase more than 50% but inhibits APC activity up to 90%, would be expected to be strongly thrombotic.

The mechanisms by which PC alone can facilitate assembly of coagulation complexes and the mechanisms by which PE can work synergistically with PS to promote coagulant/anticoagulant reactions involving vitamin K-dependent proteins is uncertain, but potential models are suggested by recent studies. The earlier concept that the 4-carboxyglutamic acid (Gla) residues function as a direct bridge to the negatively charged membrane phospholipids (*47*) has been challenged recently. Nelsestuen and colleagues have suggested that a surface may exist which is a candidate

for headgroup-specific interactions (*48*). Others have noted the crystal structure of prothrombin fragment 1 (*49*, *50*), the membrane binding region of the protein, exhibits a hydrophobic loop which extends down from a ring of Ca^{2+}-ligated Gla residues. Site-directed mutagenesis of residues within this hydrophobic loop of protein C (*10*, *51*) negatively impacts membrane binding. These results have led to the hypothesis that the interaction with the membrane may have a significant hydrophobic contribution (*10*, *51*, *52*). PE does not form smooth bilayers due to the small headgroup size. Polyunsaturation may also lead to greater spacing between the headgroups (*53*). It is likely that vesicles containing PE can interact with the exposed hydrophobic loop of the vitamin K-dependent substrates and enzymes more effectively. The validity of this hypothesis will have to be tested by biophysical and mutagenic approaches.

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