Efficacy of Soluble Phospholipids in the Prothrombinase Reaction†

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ABSTRACT: The prothrombinase complex is comprised of an enzyme, factor Xa, and a cofactor, factor Va, that each bind peripherally to membranes containing phosphatidylserine (PS) and activate the substrate, prothrombin. The mechanism by which the membrane contributes to enhanced catalytic efficacy of prothrombinase is not precisely known but is generally attributed to some aspect of enzyme and substrate assembly on the multisite surface of the membrane. A recent proposal has suggested a radically different role in which individual phospholipid molecules, either in the membrane or as single soluble molecules, act by an entirely allosteric mechanism that does not involve the multisite feature of the membrane [Zhai, X., Srivastava, A., Drummond, D. C., Daleke, D., and Lentz, B. R. (2002) *Biochemistry 41*, 5675−5684]. Our study measured prothrombinse activity in the presence of phospholipids such as short-chain phosphatidylserine and lysophosphatidylserine (lyso-PS). Both enhanced prothrombinase activity, and the increase was consistent with the requirement for extended bilayer structure. Even then, prothrombinase activity was low when compared with activity on bilayer membranes of mixed PS and phosphatidylcholine (PC). Lyso-PS approached the activity of PS/PC membranes only when it was mixed with PC bilayers. The results suggest that the two-dimensional membrane bilayer surface is necessary for the support of full prothrombinase activity.

Blood clotting enzymes are proposed to assemble on membrane surfaces that contain phosphatidylserine (PS)[1] (*1*, *2*). While the function of the membrane is not fully understood, proposals and most results are interpreted in light of a two-dimensional surface that is immobilized at the site of an injury or cell activation. Restriction to a two-dimensional surface could increase the number of collisions between cofactors and enzymes or enzyme complexes and substrates (*3*, *4*); it could provide high-affinity interaction by creating multiple contact points between proteins (*2*, *5*), and it could orient enzymes to specific cleavage sites in the substrate molecules (*6*, *7*).

Recent studies have examined the impact of a soluble form of PS, 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine (C6PS), on various proteins of prothrombinase. For example, C6PS enhanced the activity of partial prothrombinase components (*8*−*15*). Since enzyme activity of partial complexes is low with or without C6PS, several explanations for rate enhancement may exist. However, an unexpected finding was that C6PS was able to act as an allosteric regulator of the complete prothrombinase complex and was able to completely replace bilayer membranes, giving k_{cat} values comparable to those reported for prothrombinase assembled on bilayer membranes (*9*, *10*). This mechanism requires re-evaluation of many prior descriptions of blood coagulation enzymology. Another recent finding is the stoichiometric binding of a soluble lipid, lysophosphatidylserine (lyso-PS), to the fragment 1 region of prothrombin (*16*). Although other explanations for single-site binding may exist, it is possible this interaction may correlate with one of the proposed allosteric sites on the prothrombin molecule.

Reports of the impact of a soluble phospholipid on prothrombinase may be inconsistent. Gilbert and Arenas reported that soluble phospholipids supported prothrombinase activity (*17*). While precise calculations from their results do not appear to be possible, our estimate of the available data (Figure 7 of ref *17*) suggests a catalytic rate constant far below the value obtained with PS/phosphatidylcholine (PC) bilayers. These studies differed with respect to methodology. A high level of function of soluble PS was observed in a stopped-flow assay that used very high enzyme concentrations (1 nM), and product was detected by the fluorescence intensity of an active site-directed reagent (*9*, *10*). The reaction was complete in a time frame requiring stopped-flow analysis. In contrast, the report suggesting a lower level of function for soluble PS used low enzyme concentrations, traditional hand mixing of the sample, an incubation time of 5 min, and product detection by a chromogenic assay (*17*).

The study presented here was designed to determine the ability of soluble phospholipids, either C6PS or lyso-PS, to support prothrombinase activity. We found that soluble PS

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; C6PS, 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine; lyso-PS, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine; CMC, critical micelle concentration; biotinyl-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl); LUV, large unilamellar vesicles; SA, streptavidin; TBS, Tris-buffered saline; HBS, Hepes-buffered saline; S-2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline dihydrochloride; BSA, bovine serum albumin; PEG, polyethylene glycol; IIa, thrombin.

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had little, if any, impact on activity and that high activity only occurred under conditions where a membrane bilayer was present.

EXPERIMENTAL PROCEDURES

Materials. Bovine brain phosphatidylserine, egg yolk phosphatidylcholine, fluorescamine, and cyclohexane were from Sigma (St. Louis, MO). Lyso-PS, C6PS, and biotinyl-PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl)] were from Avanti (Alabaster, AL). The thrombin specific chromogenic substrate, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2238), was from Chromogenix (West Chester, OH). All other materials were of the highest grade available from Sigma.

Proteins. Human prothrombin, factor Xa, and factor Va were purchased from Haematological Technologies (Essex Junction, VT). Human factor X was from Enzyme Research Laboratories (South Bend, IN). Streptavidin (SA) was from Sigma.

Preparation of Lipids. Pure samples of C6PS and lyso-PS were prepared essentially as described previously (*8*). Briefly, the lipids were dried from chloroform, first under a stream of argon and then under vacuum for 2 h. They were dissolved in cyclohexane to 25 mg/mL and were lyophilized overnight. The resulting powder was suspended in Tris-buffered saline (TBS) [50 mM Tris and 100 mM NaCl (pH 7.4)]. Phospholipid purity was assured by thin-layer chromatography with ninhydrin detection. Large unilamellar vesicles (LUV) were prepared from a mixture of the appropriate phospholipids in chloroform that was dried, first under a stream of argon and then under vacuum for at least 2 h. Phospholipids were resolubilized in either TBS or Hepes-buffered saline (HBS) [50 mM Hepes and 100 mM NaCl (pH 7.4)]. The solution was subjected to a rapid freeze−thaw technique five times by cycling in a dry ice/ethanol mixture and warm water. The liposomes were then extruded under pressure with five passages through a 100 nm pore size filter. Lipid concentrations were determined by inorganic phosphate analysis as described previously (*18*).

Prothrombinase Activity. Phospholipids were incubated with 1 nM factor Va and either 20 or 200 pM factor Xa for 5 min at 37 °C in TBS containing 1 g/L bovine serum albumin (BSA) and 5 mM $CaCl_2$. The reaction was initiated by addition of 1 μM prothrombin and was stopped after 60 s by addition of EDTA to a concentration of 12 mM. An aliquot of the assay solution was diluted in TBS containing bovine serum albumin and mixed with S-2238 to a final concentration of 75 μM at 22 °C. The amount of thrombin that formed was measured by the absorbance change at 405 nm in a Beckman DU 70 spectrophotometer and compared with known thrombin standards.

Incorporation of Lyso-PS into LUV. To determine the level of lyso-PS incorporated into LUV, 3 mol % biotinyl-PE was included during vesicle preparation. The phospholipid vesicles were diluted to 0.6 mM in HBS and incubated with 90 μg/mL SA for 20 min at 22 °C. The samples were centrifuged for 5 min at 12 000 rpm, and 4 μL of the supernatant was collected and diluted to 500 μL in HBS. Fluorescamine (final concentration of 0.28 mM) was added from a 10 mg/mL stock solution in acetone. Fluorescence was measured with an excitation of 390 nm and an emission of 475 nm using a SPEX Fluoromax fluorescence spectrophotometer.

Relative Membrane Binding. Relative 90° light scattering intensity was used to estimate the extent of comparative membrane binding (*18*). When particle size is small relative to the wavelength of light, 90° light scattering intensity is proportional to both the concentration and the molecular weight of the scattering particles. Association of a protein with a membrane vesicle is readily detected. This study used this approach to detect qualitative binding of factor X to phospholipids and comparative binding of factor X to membranes containing different phospholipids. Briefly, 20 μg of LUV of various compositions was diluted into TBS containing 5 mM calcium. Factor X was added stepwise, and 90° light scattering intensity was recorded in a SPEX Fluoromax fluorescence spectrophotometer using an excitation and emission wavelength of 320 nm. At the end of the titration, 10 mM EDTA was added to ensure that light scattering returned to the level of protein and phospholipids alone. Background from the buffer and added protein were subtracted, and the intensity of light scattering was expressed as I_2/I_1, where I_2 is the intensity from the protein−vesicle complex and I_1 is the intensity of the vesicles alone.

RESULTS AND DISCUSSION

Prothrombinase Activity. As expected, prothrombinase activity was supported by LUV composed of PS and PC, giving a saturation curve with half-maximal activity at ~0.6 μM phospholipid and a maximum velocity or k_{cat} of 50 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$ (Figure 1A). Maximum velocities of prothrombinase using LUV composed of PS and PC were obtained in conjunction with other results described below to ensure that the enzyme complexes had suitable inherent activity. Several determinations were made giving a k_{cat} value of 45 ± 10 s^{-1}. The curve shape in Figure 1A was typical of previous studies (*19*, *20*), and the k_{cat} fell within the range of previous reports [30−100 s^{-1} (*1*, *21*−*23*)].

Prothrombinase supported by C6PS gave a very different result (Figure 1B). The appearance of activity was delayed and then increased in an approximately linear manner. Total activity was low and had not reached its maximum at 6000 μM C6PS. This deviated from previous reports that observed hyperbolic curve shape and saturation at ~100 μM C6PS with a maximum velocity of at least 100 mol of thrombin s^{-1} (mol of Xa)$^{-1}$ (*10*). The concave upward curve shape at low soluble PS concentrations (inset, Figure 1B) was typical of a critical micelle concentration (CMC), and the inflection point was close to the reported value for C6PS [750 μM (*10*)]. The accurate determination of CMC is complicated by the components of the mixture, including proteins and calcium, any of which may serve as nucleation centers for lipid aggregates. For example, if nucleation were supported to a minor extent by factor V, lipid aggregates may be available to support the reaction but exist at levels below the detection limit of the measurement tool. The velocity at 750 μM C6PS was ~1.2 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$, less than 3% of the maximum value observed with PS/PC vesicles. Consequently, the most important features of the reaction are the curve shape and the activity drastically lower than that obtained in the previous studies.

The reaction rate observed at low C6PS concentrations (Figure 1B) appeared to be consistent with the result of

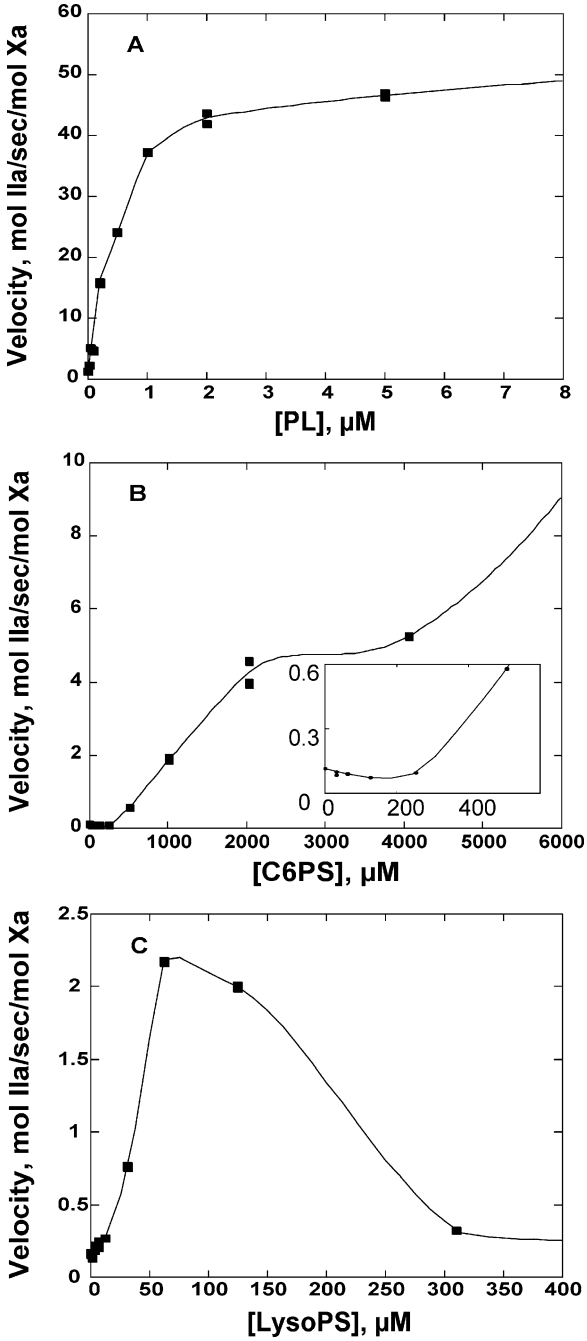

FIGURE 1: Support of prothrombinase activity by phospholipids. Factor Xa, factor Va, and phospholipids were mixed in calcium-containing buffer and allowed to reach equilibrium. Reactions were initiated by addition of 1 μM prothrombin, and they were stopped after 60 s by addition of excess EDTA. The amount of thrombin (IIa) formed was measured by the absorbance change at 405 nm after addition of S-2238 to a final concentration of 75 μM. Panel A shows prothrombinase velocities at various concentrations of PS/PC (20/80) LUV using 1 nM factor Va and 0.02 nM factor Xa. Panels B and C show thrombin formation at various concentrations of C6PS (B) and lyso-PS (C) using 1 nM factor Va and 0.2 nM factor Xa. The inset of panel B shows an expanded presentation at low C6PS concentrations. Results for two experiments are plotted.

Gilbert and Arena (*17*) who used similar methods. Studies reporting a high activity of C6PS used high enzyme concentrations and a stopped-flow technique for sample mixing. To test the impact of these parameters, one syringe of a stopped-flow instrument (MilliFlow, SLM Aminco) was filled with a high enzyme concentration (2 nM factor Xa and 10 nM factor Va) and C6PS (100 μM) in calcium-containing buffer and the other with prothrombin (2 μM) in calcium-containing buffer. Samples were mixed in the stopped-flow instrument and collected as the effluent created by multiple shots. Product formation was timed from mixing until addition of excess EDTA to stop thrombin production. Mixing and sample collection comprised a small portion of the timed assay. Thrombin was quantified by cleavage of S-2238 in the spectrophotometer as described above. A velocity of 0.06 ± 0.01 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$ was found at 100 μM C6PS, slightly lower than in reactions conducted at low enzyme concentrations and atmospheric pressure (Figure 1B). Thrombin production in the stopped-flow instrument continued to follow the pattern obtained at atmospheric pressure in reactions spanning 20−800 μM C6PS. Application of this technique to PS/PC membranes required use of low enzyme concentrations such as those presented in Figure 1A to prevent complete activation of the substrate in the time of the assay. Velocities of 27 ± 4 and 18 ± 1 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$ were measured at 10 and 1 μM PS/PC (20/80) LUV, respectively, similar to the values shown in Figure 1A. The slightly lower activity in the stopped flow may arise from enzyme loss or from dilution by discharge buffer already in the stopped flow. In any event, the result supported full or nearly full activity of prothrombinase in the stopped flow.

Another concern was the protein-stabilizing agent. Our studies included BSA in the reaction mixture, where data from refs *9* and *10* did not. Proteins are surface-active, and the activity of an enzyme such as thrombin can be reduced unless a stabilizing agent such as BSA is included in the buffer. However, BSA may bind the phospholipids and alter the outcome. An alternative thrombin-stabilizing reagent is polyethylene glycol (PEG). Inclusion of PEG as the stabilizing agent resulted in a velocity of 0.11 ± 0.03 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$ in the absence of C6PS and at concentrations below 120 μM. This was indistinguishable from the values for BSA [0.12 ± 0.01 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$] in Figure 1B.

If protein-stabilizing reagents were excluded altogether, a somewhat different result was obtained. That is, the velocity at zero or low C6PS was 0.07 ± 0.01 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$, 40% lower than in the presence of BSA. Lower activity persisted at 30 and 60 μM C6PS, after which the value increased to that observed when PEG or BSA was included in the buffer (Figure 1B). This increase was saturated by approximately 120 μM C6PS, and subsequent activity increased in a manner similar to that of the reaction mixtures containing PEG and BSA. These results suggested that apparent minor stimulation of prothrombinase by 0−100 μM C6PS may be due to protein stabilization rather than allosteric modification of the enzyme. The mechanism of this effect was not investigated further.

Prothrombinase Activity with Lyso-PS. Although a crystal structure of prothrombin and lyso-PS has been obtained (*16*), we are not aware of studies showing that lyso-PS supports the prothrombinase reaction or that lyso-PS is able to support association of blood clotting proteins with a membrane. Prothrombinase supported by pure lyso-PS reached a maximum velocity of 2.2 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$ at ~60 μM lyso-PS (Figure 1C) and declined at higher concentrations. The state of lyso-PS was not known but

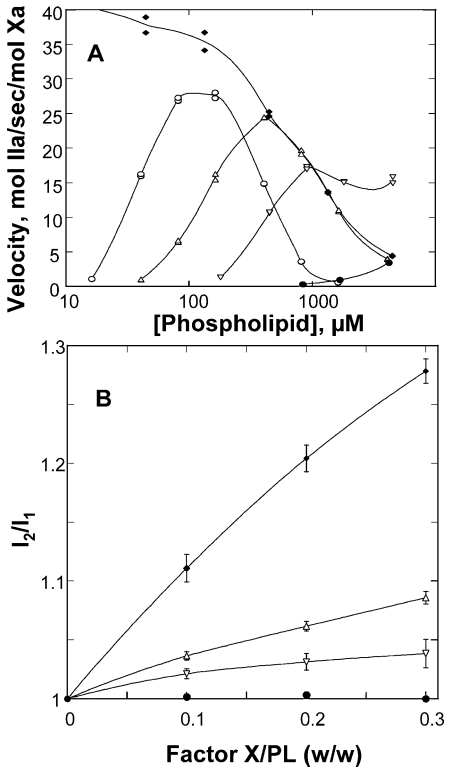

FIGURE 2: Effect of lyso-PS-containing LUV on prothombinase activity and factor X binding. (A) Prothrombinase activity. Assays were conducted essentially as described in the legend of Figure 1. Phospholipid, 1 nM factor Va, and 0.02 nM factor Xa were equilibrated in calcium-containing buffer at 37 °C for 5 min, and the reaction was started by addition of 1 μM prothrombin. After 60 s, the reaction was quenched by addition of EDTA to a final concentration of 12 mM. Thrombin formation was assessed by mixing an aliquot with S-2238 (75 μM) and measuring the change in absorbance at 405 nm. Results for two experiments are shown. Phospholipids included PS, PC, and biotinyl-PE (20/77/3, ◆), lyso-PS, PC, and biotinyl-PE (10/87/3, ▽), lyso-PS, PC, and biotinyl-PE (25/72/3, △), lyso-PS, PC, and biotinyl-PE (50/47/3, ○), and PC and biotinyl-PE (97/3, ●). (B) Factor X association with membranes containing lyso-PS. Relative membrane binding is detected by light scattering change of a solution (400 μL) containing 20 μg of LUV. Protein was added, and light scattering intensity (I_2) was compared with that of the LUV alone (I_1). The symbols and phospholipids are the same as in panel A. Each data point represents the average and standard deviation of three experiments.

appeared to change over the concentration range that was used. The CMC for lysophospholipids is difficult to determine and is dependent on many parameters (*24*). In these experiments, at concentrations of $\geq$60 μM lyso-PS, addition of 5 mM calcium resulted in visual turbidity of the solution and probably represented calcium-induced aggregation of lyso-PS. Thus, calcium may support micelle formation at low lyso-PS concentrations but cause widespread aggregation and loss of function at higher concentrations.

Lyso-PS exhibited a higher level of function when incorporated into bilayer vesicles of PC (Figure 2A). Biotinyl-PE was included in these vesicles but appeared to be inert. It had no impact on the activity obtained with PS/PC/biotinyl-PE (20/77/3) vesicles (Figure 2A) and did not support activity when incorporated into vesicles of PC (not shown). Vesicles containing 10, 25, and 50% lyso-PS gave maximum velocities of 16, 25, and 28 mol of thrombin s^{-1} (mol of factor Xa)$^{-1}$, respectively (Figure 2A). Higher levels of lyso-PS resulted in activity at lower phospholipid concentrations (Figure 2A). Thus, in bilayer structure, lyso-PS supported the prothrombinase reaction but was less effective than PS.

High concentrations of phospholipid membranes inhibit the prothrombinase reaction, possibly by substrate depletion (*19*). This loss of activity at high concentrations occurred with both PS- and lyso-PS-containing membranes (Figure 2A).

To ensure that the lyso-PS had been incorporated into the bilayer vesicles, SA was added to each lipid sample to cross-link and aggregate the LUV through binding to biotinyl-PE. Samples were centrifuged, and the supernatant was analyzed for amino compounds by reaction with fluorescamine as described in Experimental Procedures. Before addition of SA, solutions of 10 and 25 mol % lyso-PS gave high fluorescence intensity over background. More than 99% of this intensity was removed by addition of SA and centrifugation. This suggested full incorporation of lyso-PS into the LUV. The supernatant from SA precipitation of vesicles containing 50% lyso-PS retained 32% of the initial fluorescence intensity, suggesting that either some of the lyso-PS was not incorporated into the vesicles or the vesicles had been altered so they no longer precipitated with SA. For example, a high lyso-PS concentration may partially dissolve the bilayers into micelles.

Relative Level of Protein Binding to Lyso-PS-Containing LUV. Enhancement of prothrombinase by lyso-PS suggested direct binding of the protein to lyso-PS. This was examined by observation of factor X association with vesicles containing lyso-PS with relative light scattering intensity change. Factor X exhibited the greatest signal change with PS/PC vesicles; it showed no detectable binding to LUV of PC alone and had an intermediate level of binding to LUV containing lyso-PS (Figure 2B). The relative light scattering changes should reflect the relative quantity of protein−membrane binding, and the results correlated with the ability of these phospholipids to support the prothrombinase reaction (Figure 2A).

One purpose of this study was to examine the ability of soluble phospholipids to support the prothrombinase reaction and to determine whether bilayer structure was needed. The studies focused on methods that are readily accessible in a conventional biochemistry laboratory to facilitate retesting of these findings as needed. The results were all consistent with the requirement for a multisite membrane or micelle structure to support the prothrombinase reaction. When used alone, C6PS and lyso-PS provided poor support for prothrombinase and the low activity occurred under conditions where aggregate structures were likely to exist. In a membrane bilayer, lyso-PS supported prothrombinase at nearly its maximum level. Higher concentrations of lyso-PS were needed, relative to PS. This finding correlated with factor X binding to membranes containing these phospholipids and suggested that lyso-PS does not provide as strong a binding site as PS. Overall, it appeared that the role of phospholipids in support of the prothrombinase reaction was dependent on the multisite characteristics of phospoholipids in a membrane or other aggregated structure.

REFERENCES

1. Butenas, S., and Mann, K. G. (2002) Blood coagulation, *Biochemistry (Moscow) 67*, 3−12.

2. Nelsestuen, G. L., Shah, A. M., and Harvey, S. B. (2000) Vitamin K-dependent proteins, *Vitam. Horm. 58*, 355−389.
3. Krishnaswamy, S., Jones, K. C., and Mann, K. G. (1988) Prothrombinase complex assembly. Kinetic mechanism of enzyme assembly on phospholipid vesicles, *J. Biol. Chem. 263*, 3823−3834.
4. Giesen, P. L., Willems, G. M., Hemker, H. C., and Hermens, W. T. (1991) Membrane-mediated assembly of the prothrombinase complex, *J. Biol. Chem. 266*, 18720−18725.
5. Pusey, M. L., and Nelsestuen, G. L. (1983) The physical significance of K_m in the prothrombinase reaction, *Biochem. Biophys. Res. Commun. 114*, 526−532.
6. Armstrong, S. A., Husten, E. J., Esmon, C. T., and Johnson, A. E. (1990) The active site of membrane-bound meizothrombin. A fluorescence determination of its distance from the phospholipid surface and its conformational sensitivity to calcium and factor Va, *J. Biol. Chem. 265*, 6210−6218.
7. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) The active site of blood coagulation factor Xa. Its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex, *J. Biol. Chem. 262*, 12953−12961.
8. Koppaka, V., Wang, J., Banerjee, M., and Lentz, B. R. (1996) Soluble phospholipids enhance factor Xa-catalyzed prothrombin activation in solution, *Biochemistry 35*, 7482−7491.
9. Zhai, X., Srivastava, A., Drummond, D. C., Daleke, D., and Lentz, B. R. (2002) Phosphatidylserine binding alters the conformation and specifically enhances the cofactor activity of bovine factor Va, *Biochemistry 41*, 5675−5684.
10. Majumder, R., Weinreb, G., Zhai, X., and Lentz, B. R. (2002) Soluble phosphatidylserine triggers assembly in solution of a prothrombin-activating complex in the absence of a membrane surface, *J. Biol. Chem. 277*, 29765−29773.
11. Wu, J. R., Zhou, C., Majumder, R., Powers, D. D., Weinreb, G., and Lentz, B. R. (2002) Role of procoagulant lipids in human prothrombin activation. 1. Prothrombin activation by factor X(a) in the absence of factor V(a) and in the absence and presence of membranes, *Biochemistry 41*, 935−949.
12. Banerjee, M., Majumder, R., Weinreb, G., Wang, J., and Lentz, B. R. (2002) Role of procoagulant lipids in human prothrombin activation. 2. Soluble phosphatidylserine upregulates and directs factor X(a) to appropriate peptide bonds in prothrombin, *Biochemistry 41*, 950−957.
13. Srivastava, A., Wang, J., Majumder, R., Rezaie, A. R., Stenflo, J., Esmon, C. T., and Lentz, B. R. (2002) Localization of phosphatidylserine binding sites to structural domains of factor Xa, *J. Biol. Chem. 277*, 1855−1863.
14. Majumder, R., Wang, J., and Lentz, B. R. (2003) Effects of water soluble phosphotidylserine on bovine factor Xa: Functional and structural changes plus dimerization, *Biophys. J. 84*, 1238−1251.
15. Lentz, B. R. (2003) Exposure of platelet membrane phosphatidylserine regulates blood coagulation, *Prog. Lipid Res. 42*, 423−438.
16. Huang, M., Rigby, A. C., Morelli, X., Grant, M. A., Huang, G., Furie, B., Seaton, B., and Furie, B. C. (2003) Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins, *Nat. Struct. Biol. 10*, 751−756.
17. Gilbert, G. E., and Arena, A. A. (1997) Partial activation of the factor VIIIa-factor IXa enzyme complex by dihexanoic phosphatidylserine at submicellar concentrations, *Biochemistry 36*, 10768−10776.
18. Nelsestuen, G. L., and Lim, T. K. (1977) Equilibria involved in prothrombin- and blood-clotting factor X-membrane binding, *Biochemistry 16*, 4164−4171.
19. van Rijn, J. L., Govers-Riemslag, J. W., Zwaal, R. F., and Rosing, J. (1984) Kinetic studies of prothrombin activation: Effect of factor Va and phospholipids on the formation of the enzyme−substrate complex, *Biochemistry 23*, 4557−4564.
20. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) The contribution of bovine factor V and factor Va to the activity of prothrombinase, *J. Biol. Chem. 254*, 10952−10962.
21. Stenflo, J. (1999) Contributions of Gla and EGF-like domains to the function of vitamin K-dependent coagulation factors, *Crit. Rev. Eukaryotic Gene Expression 9*, 59−88.
22. Brufatto, N., and Nesheim, M. E. (2003) Analysis of the kinetics of prothrombin activation and evidence that two equilibrating forms of prothrombinase are involved in the process, *J. Biol. Chem. 278*, 6755−6764.
23. Weinreb, G. E., Mukhopadhyay, K., Majumder, R., and Lentz, B. R. (2003) Cooperative roles of factor V(a) and phosphatidylserine-containing membranes as cofactors in prothrombin activation, *J. Biol. Chem. 278*, 5679−5684.
24. Stafford, R. E., Fanni, T., and Dennis, E. A. (1989) Interfacial properties and critical micelle concentration of lysophospholipids, *Biochemistry 28*, 5113−5120.

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