Soluble Phospholipids Enhance Factor X_a-Catalyzed Prothrombin Activation in Solution†

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Received August 30, 1995; Revised Manuscript Received January 4, 1996⊗

ABSTRACT: Acidic phospholipids play an important but incompletely understood role in prothrombin activation. Here we report the effect of short-chain phosphatidylserine (dicaproylphosphatidylserine, C6PS) and the corresponding phosphatidylglycerol (C6PG) and phosphatidylcholine (C6PC) derivatives on the rate of prothrombin activation by factor X_a. The critical micellar concentrations of these short-chained phospholipids have been determined under a variety of conditions that we used for kinetic and structural studies. Under conditions for which these lipids exist in a soluble form, the results demonstrate that: (i) the rate of human prothrombin activation by human factor X_a was enhanced in a calcium-dependent fashion up to 60-fold by addition of C6PS, roughly 20% of the optimal enhancement seen with bovine phosphatidylserine/palmitoyloleoylphosphatidylcholine (25/75 PS/POPC) membranes; (ii) C6PS inhibited the rate of hydrolysis of synthetic factor X_a substrate (S-2765), an effect that was mimicked, but at much lower lipid concentrations, by PS/POPC membranes; (iii) there was no enhancement of prothrombin activation and much less inhibition of hydrolysis of S-2765 by factor X_a in the presence of C6PG or C6PC; and (iv) the thermal denaturation of prothrombin was altered in a calcium-independent but dose-dependent fashion by either C6PS or C6PG. These results have been interpreted in terms of the existence of (a) specific PS binding site(s) on factor X_a ($K_d \sim 73$ μM) that regulate(s) the activity of this serine protease. Our results do not rule out the possibility that the rate of prothrombin activation is also influenced by a weaker, calcium-independent, and less specific acidic lipid binding site on prothrombin, the occupancy of which results in conformational changes in this protein. The results clearly suggest that PS binding regulates the rate of prothrombin activation.

The prothrombinase complex consists of a serine protease (factor X_a), a protein cofactor (factor V_a), Ca^{2+}, and negatively charged membranes provided by activated platelets (Jackson & Nemerson, 1980; Mann *et al*., 1988). The association of both factor X_a and the substrate prothrombin with a negatively charged membrane requires Ca^{2+} ions and the γ-carboxyglutamic acid residues (Schwalbe *et al*., 1989) on the N-terminal membrane binding domains of these proteins. The role of platelet membranes has been variously described as (i) assembly of the activation complex (Rosing *et al*., 1980; Krishnaswamy *et al*., 1988), (ii) dimensionality reduction of substrate approach to the enzyme (Rosing *et al*., 1980; Nesheim *et al*., 1981; Giesen *et al*., 1991), and/or (iii) conformational alterations of the enzyme and/or substrate (Husten *et al*., 1987; Wu & Lentz, 1991; Pei *et al*., 1993).

Conformational alterations could be brought about by absorption to a negatively charged surface, perhaps to a locally concentrated pool or "domain" of acidic lipids (Lim *et al*., 1977). At least two observations have mitigated against this possibility, the first being the specific thrombin-generating activity of phosphatidylserine (PS)[1] as compared to other equally charged acidic lipids (Jones *et al*., 1985; Pei *et al*., 1993; Comfurius *et al*., 1994) and the second being the ability of even a positively charged membrane to enhance prothrombin activation as long as it contained PS (Rosing *et al*., 1988). In a related vein, we have argued, based on several types of observations, that binding of prothrombin and factor X_a to negatively charged membranes involves specific occupancy of a few (2−4) acidic lipid binding sites on these proteins rather than nonspecific absorption to a pool of acidic lipids (Jones & Lentz, 1986; Cutsforth *et al*., 1989; Tendian & Lentz, 1990). A corollary of this alternative view of prothrombin and factor X_a binding was the suggestion that occupancy of one or more of these sites by PS might trigger the calcium-independent, PS-membrane-induced conformational changes and PS-specific enhancement in prothrombinase activity that we have observed (Pei *et al*., 1993; Lentz *et al*., 1994; Wu & Lentz, 1994). That such a *regulatory PS site* might exist was further supported by the observation of weak, Ca^{2+}-independent

† Supported by USPHS Grant HL45916.

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CMC, critical micelle concentration; C6PC, 1,2-dicaproyl-*sn*-glycero-3-phosphocholine; C6PG, 1,2-dicaproyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; C6PS, 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DPH, 1,6-diphenylhexatriene; DSC, differential scanning calorimetry; MUGB, methylumbelliferyl *p*-guanidinobenzoate hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Na_2EDTA, disodium ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); PS, phosphatidylserine; QELS, quasi-elastic light scattering; S-2238, phenylalanylpipecolylarginine-*p*-nitroaniline; S-2765, *N*-α-benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride; Pre1, a 50 kDa fragment of prothrombin consisting of the C-terminal catalytic domain and a linker domain; F2, a 13 kDa fragment that serves to link the F1 and catalytic domains of prothrombin and consists largely of a Kringle structural pattern; F1, a 22 kDa fragment that consists of the N-terminal γ-carboxyglutamic acid-rich, membrane binding domain of prothrombin and a Kringle domain.

S0006-2960(95)02063-0 CCC: $12.00

binding of prothrombin but not of its membrane binding N-terminal domain (fragment 1) to acidic lipid-containing supported bilayers (Tendian *et al*., 1991).

To better define the nature of the PS-specific effect on the membrane-assembled prothrombinase, we report here the specific effect of soluble, short-chained acidic phospholipids on the activation of prothrombin by factor X_a in solution. The results strongly support the notion that PS acts as a regulator of prothrombin activation, possibly by altering enzyme conformation and perhaps also by modifying the conformation of prothrombin.

EXPERIMENTAL PROCEDURES

Materials. Lipid stock solutions in buffer were prepared by measuring aliquots of appropriate lipid stocks in chloroform, evaporating the chloroform under a stream of nitrogen, resolubilizing the lipid in cyclohexane, and then lyophilizing these frozen solutions overnight. The resulting dry powder was dispersed in the appropriate buffer and vortexed thoroughly. L-Serine, *O*-phospho-L-serine, and L-α-glycerophosphorylserine were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dicaproyl-*sn*-glycero-3-phosphocholine (C6PC),[1] 1,2-dicaproyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (C6PG),[1] 1,2-dicaproyl-*sn*-glycero-3-phospho-L-serine (C6PS),[1] and bovine PS, and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC)[1] were purchased from Avanti Polar Lipids (Alabaster, AL). Concentrations of the phospholipids, *O*-phospho-L-serine and L-α-glycerophosphorylserine, were established by inorganic phosphate determination (Chen *et al*., 1956). 8-Anilino-1-naphthalenesulfonic acid Mg salts (ANS)[1] and diphenylhexatriene (DPH)[1] were purchased from Molecular Probes, Inc. (Eugene, OR). Polyethylene glycol (8000) (PEG)[1] was purchased from Fisher Scientific (Fair Lawns, NJ). Large unilamellar vesicles composed of 20/80 bovine PS/POPC were prepared by extrusion according to the procedure of Mayer *et al*. (1986).

Human and bovine prothrombins were purified from human and bovine plasma (Tendian & Lentz, 1990; Lentz *et al*., 1994). Human factor X_a was purchased from Haematologic Technologies Inc. (Essex Junction, VT). The activity of factor X_a was checked using the synthetic chromogenic substrate *N*-α-benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2765;[1] Chromogenix, Molndal, Sweden) in an assay adapted to an SLT 340-ATTC microplate reader (Tecan U.S., Hillsborough, NC). The thrombin-specific substrate phenylalanylpipecolylarginine-*p*-nitroaniline (S-2238)[1] was also purchased from Chromogenix.

Determination of Critical Micelle Concentrations. Critical micelle concentrations (CMC)[1] of C6PS in buffer solution containing various concentrations of calcium were estimated by a fluorometric method employing the fluorescent probe 8-anilino-1-naphthalenesulfonic acid magnesium salt (De Vendittis *et al*., 1981). Stock solution of C6PS (100 mM) was prepared in a buffer containing 50 mM Tris, 175 mM NaCl, pH 7.4. A small volume (≈2 μL) of a stock solution of ANS (1.8 mM) in double-distilled water was added to a stirred microcuvette (Hellma Cells, Jamaica, NY) containing 0.6 mL of buffer (50 mM Tris, 175 mM NaCl, pH 7.4, with and without 0.6% PEG and appropriate concentrations of $CaCl_2$) equilibrated at 37 °C. Fluorescence emission spectra of ANS were recorded from 450 to 600 nm (slits at 8 nm) on an SLM 48000 spectrofluorometer (SLM Aminco, Urbana, IL) using an excitation wavelength of 370 nm (slits at 8 nm). CMC's of C6PC and C6PG were determined in the presence and absence of 5 mM calcium by a similar method. The CMC's of the short-chained lipids were also determined in presence of MOPS buffer using ANS. The critical micelle concentrations were also determined using DPH as an aggregation-sensitive fluorescent probe (London & Feigenson, 1978). Briefly, the fluorescence intensity of DPH (16 μM) was monitored in the presence of increasing concentrations of phospholipid in a buffer containing 50 mM Tris, 175 mM NaCl, and 5 mM $CaCl_2$, pH 7.4, at 37 °C with and without 0.6% PEG. Fluorescence was monitored using excitation and emission wavelengths of 365 and 460 nm, respectively. A plot of the relative fluorescence versus the lipid concentration gave two linear regions. Below the CMC, fluorescence increased very gradually with lipid concentration, a nonzero slope probably resulting from an increasing monomer to probe ratio as well as other nonspecific effects. Above the CMC, fluorescence increased linearly with respect to the lipid concentration (with a significant positive slope). The point of intersection of these two regions was taken as the CMC (Wells, 1974).

The diameters of C6PS aggregates were measured by quasi-elastic light scattering (QELS),[1] as described earlier (Lentz *et al*., 1991). Increasing concentrations of C6PS (120 mM stock) were added to the sample cell, a 6 × 50 mm borosilicate glass disposable culture tube (Baxter Healthcare Corp., McGaw Park, IL), containing 0.4 mL of the appropriate buffer (50 mM Tris, 150 mM NaCl, pH 7.4 at 37 °C, or 50 mM MOPS, 150 mM NaCl, pH 7.4, or 50 mM MOPS, 150 mM NaCl, and 0.1 mM EDTA, pH 7.4) in the presence or absence of varying concentrations of calcium, equilibrated at 37 °C. All buffers were filtered through a 0.1 μm filter (Gelman Sciences, Ann Arbor, MI) before use. The sizes of the various aggregates were measured as intensity-weighted diameters, which were calculated from the *Z*-averaged diffusion coefficient. The distributions of aggregate sizes were determined in terms of a generalized distributional analysis (Provencher, 1979) which can identify samples with considerable heterogeneity in size distribution.

Measurement of the Kinetics of Active Site Formation Using Synthetic Substrate. The activation of human prothrombin was followed by measuring the generation of amidolytic activity (due to either thrombin or meizothrombin) against a synthetic substrate, S-2238. Prothrombin activating mixtures contained prothrombin (0.6 μM), phospholipid, and factor X_a (5 nM) in 50 mM Tris, 175 mM NaCl, 0.6% PEG, and 5 mM $CaCl_2$, pH 7.6 at 37 °C. The activation reaction was quenched at several time intervals (0, 3, 6, 9, 12, and 15 min) with 0.43 mg/mL (final concentration) soybean trypsin inhibitor (Rosing *et al*., 1986). Aliquots of 20 μL of the quenched reaction mixture collected at different times were transferred into sample wells of a 96-well assay plate (Becton Dickinson, Oxnard, CA). The wells already contained 95 μL of buffer (38 mM Tris, 170 mM NaCl, and 0.6% PEG, pH 7.9) at 37 °C. An aliquot of 35 μL of the substrate S-2238 (0.47 mg/mL with 20 mM $CaCl_2$ at 37 °C) was added to this mixture. Following a brief mixing time (4 s), the absorbance at 405 nm was recorded using a microplate reader (SLT 340 ATTC) so as to obtain the initial rate of S-2238 hydrolysis. The concentration of thrombin

or meizothrombin active site was determined by comparing the initial rate of S-2238 hydrolysis to a standard curve that had been generated using thrombin that was active site titrated with *p*-nitrophenyl *p′*-guanidinobenzoate (Sigma Chemical Co.) (Chase & Shaw, 1970). The initial rates of prothrombin activation were determined from plots of active site formation versus time, which were shown to be linear in the time range examined (0−15 min).

Effect of Phospholipids on Factor X_a Activity. The amidolytic activity of human factor X_a in the presence of various concentrations of phospholipids was determined using a synthetic substrate, S-2765, specific for the factor X_a active site. Samples containing various concentrations of phospholipid (5−1000 μM) added to factor X_a in buffer (50 mM Tris, 175 mM, NaCl, and 0.6% PEG, pH 7.6 at 37 °C) with appropriate final concentrations of $CaCl_2$ were equilibrated at 37 °C for 20 min. Aliquots (115 μL) of these equilibrated mixtures were then added to the sample wells of a 96-well assay plate also maintained at 37 °C. To this mixture was added 10 μL of the synthetic substrate S-2765 (stock solution of 6.2 mM containing the appropriate concentration of $CaCl_2$) to yield a final concentration in the assay of 0.49 mM. Following a brief mixing time (4 s), the absorbance at 405 nm was recorded using a microplate reader (SLT 340 ATTC) so as to obtain the initial rate of S-2765 hydrolysis. Rates of synthetic substrate hydrolysis were recorded as a percent of the rate observed in the absence of phospholipid. Apparent dissociation constants for binding of factor X_a to C6PS at various concentrations of calcium were obtained by fitting the experimental data to a simple model which assumed C6PS binding to a single site on factor X_a. A Marquardt−Levenberg nonlinear least-squares algorithm supplied with Sigma Plot version 1.02 (Jandel Scientific, Corte Madera, CA) was used for this purpose.

Differential Scanning Calorimetry (DSC).[1] DSC measurements were performed on a Microcal (Amherst, MA) MC-2 biological microcalorimeter equipped with a Keithley 150B amplifier as described earlier (Lentz *et al*., 1994). Samples were deaerated under reduced pressure (40−50 cmHg) for 0.5 h before being loaded into the calorimeter cell with a Hamilton microliter/gastight syringe. The buffer for calorimetry scans was 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS,[1] Sigma Chemical Co.) and 150 mM NaCl, pH 7.4. Buffer was passed over a Chelex-100 (Bio-Rad Laboratories, Richmond, CA) column to remove metal ions. Lipid stock solutions were prepared as described earlier in a buffer containing appropriate concentrations of $CaCl_2$. Protein samples (0.9−1.2 mg/mL) were dialyzed into scan buffer just prior to a scan, and a portion of the equilibrated buffer was used in the reference cell of the calorimeter. The scan rate for all experiments was 60 °C/h. All experiments were performed at least in duplicate in order to be certain that subtle effects were reproducible. Data are presented for representative experiments. Because thermograms of samples containing lipid alone showed no discernible thermal events in the temperature range of protein denaturation, no correction was made for a phospholipid "background".

RESULTS

Critical Micelle Concentrations of Short-Chain Phospholipids as a Function of Ca^{2+} Concentration. (A) Determination of CMC's Using Fluorescent Probes. ANS is a fluorescence probe that fluoresces when it binds to a water−membrane interface, but displays very little fluorescence intensity when monitored in solution (Slavik, 1982). The CMC of C6PS in the absence of PEG and calcium was thus estimated, as described under Experimental Procedures, to be ∼12 mM, based on the increased ANS fluorescence when titrated with the short-chain phospholipid (Figure 1A). However, the CMC estimated using ANS in the presence of 0.6% PEG was only about 8 mM (Figure 1A), significantly lower than the value estimated in the absence of PEG. This large effect of a very low concentration of PEG alarmed us and caused us to seek other methods, as described in the next section, to detect small aggregates of C6PS.

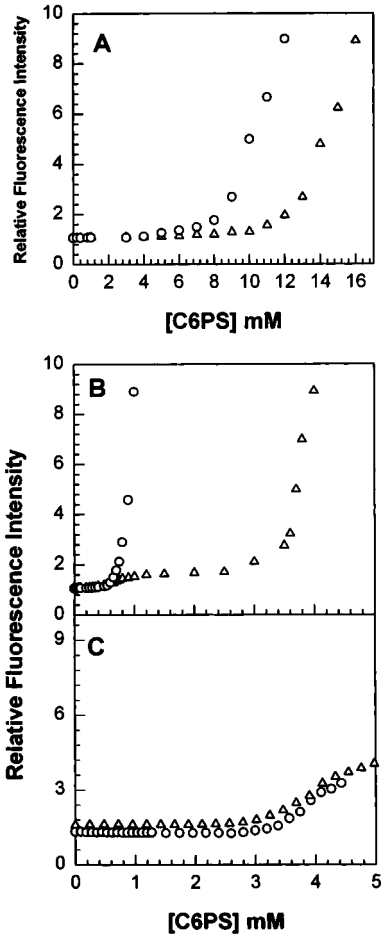

FIGURE 1: Determination of the critical micelle concentration of C6PS. Relative fluorescence intensity of ANS measured at 490 nm is plotted as a function of increasing concentration of short-chained phospholipid (C6PS) in a buffer containing 50 mM Tris, 175 mM NaCl, pH 7.4. Frame A: In the absence of Ca^{2+} and in the presence (open circles) or absence (open triangles) of 0.6% PEG. Frame B: In the presence of 5 mM Ca^{2+} with (open circles) and without (open triangles) 0.6% PEG. Frame C: Relative increase in fluorescence intensity of DPH was measured as a function of increasing concentration of C6PS in a buffer containing 50 mM Tris, 175 mM NaCl, and 5 mM $CaCl_2$, pH 7.4, in the absence (open triangles) and presence (open circles) of 0.6% PEG.

Not surprisingly, the CMC of C6PS was dependent on calcium concentration, as illustrated in Figure 1B for 5 mM $CaCl_2$. As for measurements in the absence of calcium, the CMC reported by ANS fluorescence in the presence of Ca^{2+} was significantly altered in the presence of 0.6% PEG (open circles in Figure 1B; CMC ∼3.2 mM in the absence of PEG and ∼0.75 mM in the presence of PEG). However, a slight increase in ANS fluorescence was detected in the absence

of PEG at roughly 0.75 mM C6PS (Figure 1B). This observation, coupled with an observed jump in the rate of prothrombin activation by factor X_a (see below), led us to wonder whether ANS fluorescence might not be reliably reporting the formation of C6PS micelles. For this reason, we attempted to use another fluorophore to detect C6PS micelles. When DPH was used, the CMC was detected at ~3 mM C6PS in the presence and absence of PEG with 5 mM Ca^{2+} (Figure 1C). This corresponded to the C6PS concentration leading to the large increase in ANS fluorescence in the absence of PEG, but was not in accord with other observations mentioned above.

(B) Detection of C6PS Aggregates Using Light Scattering. Because of the disparity between the various observations described above, we turned to a method that can directly monitor the formation of C6PS aggregates, namely, light scattering. Even at moderate detector sensitivity, quasi-elastic light scattering (QELS) can not only detect but also report the hydrodynamic diameter of small, unilamellar phospholipid vesicles (diameter 200−300 Å) at submillimolar lipid concentrations (Lentz *et al*., 1992). For these experiments, buffer solutions were monitored at maximum detector sensitivity while increasing concentrations of short-chain amphipath were added. As expected for micelle formation (Tanford, 1973), we could detect no aggregates until a critical C6PS concentration (the CMC) was reached. Above the CMC, aggregates were always observed, although the species of aggregates present changed with increasing C6PS concentration (Figure 2). The presence of 0.6% PEG had no effect on these observations, but the CMC of C6PS in the presence of 5 mM Ca^{2+} indicated by these measurements (~0.95 mM) corresponded to the CMC estimated by ANS fluorescence in the presence of PEG (~0.75 mM). In the absence of Ca^{2+} and in the presence of 0.1 mM Na_2-EDTA, the CMC determined by light scattering (~8 mM) also agreed with that detected using ANS in the absence of Ca^{2+} and in the presence of 0.6% PEG (~8 mM).

At the CMC, two populations of aggregates were detected, one with an apparent hydrodynamic diameter of 100−120 Å and one with a diameter of 490 Å. As the C6PS concentration increased, the small aggregate population remained roughly constant in size, but the size of the larger aggregates increased (Figure 2), until, at concentrations just below the apparent CMC detected by ANS (~2.2 mM C6PS), a flocculent precipitate appeared and no light scattering could be recorded. These observations were not altered in the absence of Ca^{2+}. Since the apparent CMC reported by ANS and DPH fluorescence always corresponded to the appearance of an opaque solution, it appears that these probes, in the absence of PEG, reported only this flocculation event and not the formation of the small aggregates readily detected by light scattering. Since the presence of PEG did not alter the aggregation of C6PS, it must be that it altered the properties of the small aggregate surface either to encourage ANS binding or to enhance the fluorescence quantum yield of bound ANS. Although others have reported ANS to be an efficient fluorescent probe for studying membranes or micelles composed of long-chain lipids, in our studies it does not appear to report clearly the formation of the smallest aggregates formed from short-chained lipids. Therefore, ANS alone might not be a good probe for determining the CMC of C6PS. However, there was excellent agreement between CMC values derived from light scattering and from ANS fluorescence in the presence of PEG, and CMC determinations could be performed routinely using either of these two methods.

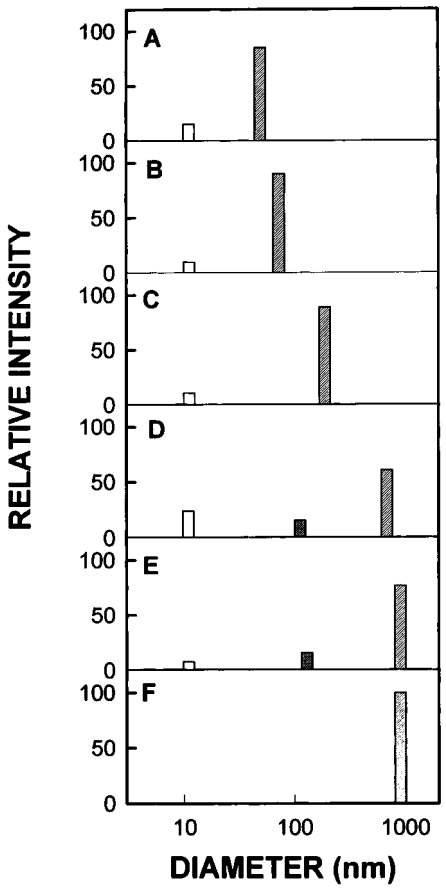

FIGURE 2: Size distribution of C6PS aggregates using quasi-elastic light scattering (QELS). The intensity-weighted diameters of aggregates of C6PS at varying concentrations (frames: A, 0.95 mM C6PS; B, 1.0 mM C6PS; C, 1.2 mM C6PS; D, 1.5 mM C6PS; E, 1.8 mM C6PS; F, 2 mM C6PS) were measured in a buffer solution containing 50 mM Tris, 175 mM NaCl, and 5 mM $CaCl_2$, pH 7.4. No aggregates were detected at concentrations of C6PS lower than 0.95 mM.

(C) Effects of Ca^{2+}, Lipid Species, Buffers, and Proteins on CMC. Since the CMC reflects the relative stability of soluble *versus* aggregated amphipathic molecule in solution, anything that alters the stability of either state will shift the CMC (Tanford, 1973). It is, therefore, not surprising that C6PG and C6PC displayed CMC's very different from that of C6PS. As seen in Figures 3 and 4, PEG altered the response of ANS fluorescence to added amphipath for both C6PG and C6PC, as it did for C6PS. Based on our observations with C6PS, the correct CMC's of C6PG and C6PC were taken as the values determined in the presence of PEG. Changing the buffers from Tris to MOPS did not appreciably alter the CMC of C6PS (at 3 mM Ca^{2+}, 2.4 mM in Tris, 2.5 mM in MOPS, using both the ANS and QELS techniques).

Ca^{2+} (5 mM) lowered the CMC's of both amphipaths by comparable factors (C6PG, from around 35 to 13 mM; C6PC, from around 15 to 8 mM). The effect of 5 mM Ca^{2+} on C6PS was much more dramatic (from around 8 to 0.95 mM). Figure 5 shows the variation of the C6PS CMC with Ca^{2+} concentration, with the CMC values for C6PC (filled triangles) and C6PG (filled squares) shown for comparison. It is noteworthy that the CMC of C6PS was so much lower

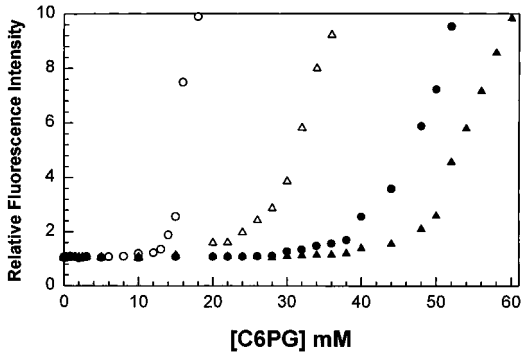

FIGURE 3: Determination of the critical micelle concentration of C6PG. Relative fluorescence intensity of ANS measured at 490 nm is plotted as a function of increasing concentration of C6PG in a buffer containing 50 mM Tris, 175 mM NaCl, pH 7.4, in the absence of Ca^{2+} and PEG (filled triangles), in the absence of Ca^{2+} and presence of 0.6% PEG (filled circles), in the presence of 5 mM Ca^{2+} and absence of PEG (open triangles), and in the presence of 5 mM Ca^{2+} and 0.6% PEG (open circles).

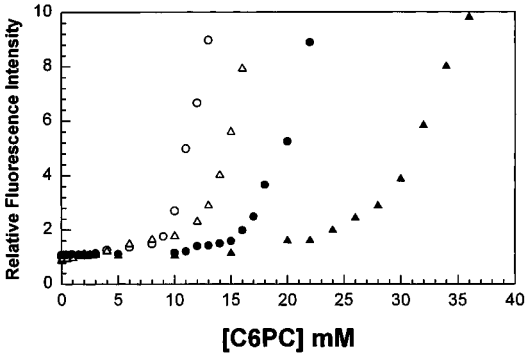

FIGURE 4: Determination of the critical micelle concentration of C6PC. Relative fluorescence intensity of ANS measured at 490 nm is plotted as a function of increasing concentration of C6PC in a buffer containing 50 mM Tris, 175 mM NaCl, pH 7.4, in the absence of Ca^{2+} and PEG (filled triangles), in the absence of Ca^{2+} and presence of 0.6% PEG (filled circles), in the presence of 5 mM Ca^{2+} and absence of PEG (open triangles), and in the presence of 5 mM Ca^{2+} and 0.6% PEG (open circles).

than the CMC's of either C6PG (like C6PS, a charged lipid) or C6PC (a neutral lipid), especially in the presence of Ca^{2+}. This could reflect the ability of Ca^{2+} to complex with and stabilize the aggregated state. The sensitivity of the C6PS CMC to Ca^{2+} concentration is not surprising given the documented ability of Ca^{2+} to bind to PS and form dehydrated PS−Ca^{2+}−PS complexes (Papahadjopoulos, 1978).

Finally, we examined the influence of prothrombin and factor X_a on the CMC of C6PS. Prothrombin at 11 μM in MOPS buffer (equal to concentrations used in DSC experiments; see below) lowered the CMC only slightly from 7.8 to 6.5 mM in the absence of Ca^{2+}, and from 2.5 to 1.2 mM in the presence of 3 mM Ca^{2+}. Prothrombin at 2 μM in Tris buffer (3.3 times the concentration used in kinetic experiments) had no detectable effect on the C6PS CMC in the presence of 5 mM Ca^{2+}. Factor X_a (at 2.2 μM, 440 times that used in kinetic experiments) lowered the CMC from 2.4 to 1.5 mM in the presence of 3 mM Ca^{2+}. These experiments show that proteins can lower the CMC of C6PS. However, control experiments such as these were done to assure that the effects of C6PS reported in the next two sections occur at concentrations for which this amphipath is not in an aggregated state.

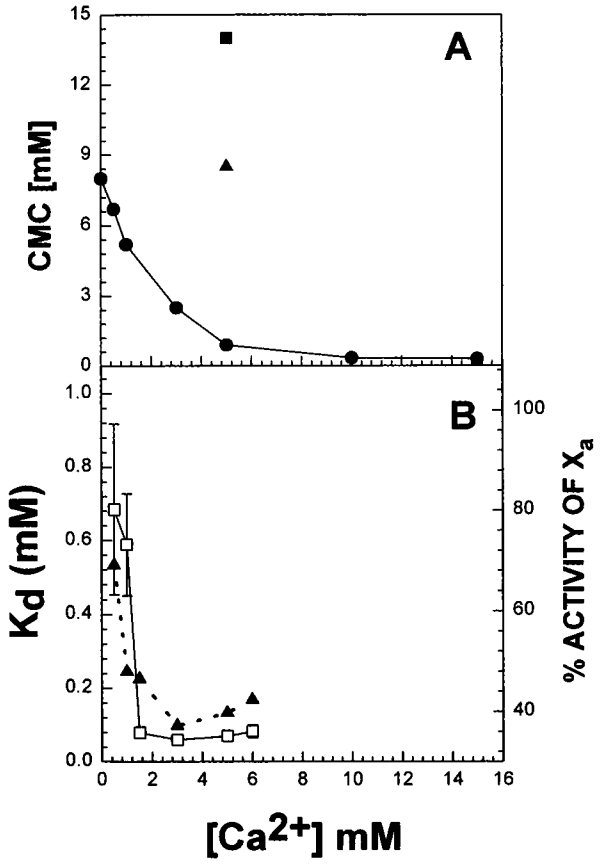

FIGURE 5: Dependence of the critical micelle concentration of short-chained lipids on the concentration of Ca^{2+}. Relative fluorescence intensity of ANS was measured at 490 nm as a function of increasing concentration of short-chained phospholipids (C6PL) in a buffer containing 0.05 M Tris, 175 mM NaCl, and 0.6% PEG, pH 7.4. The excitation wavelength was 370 nm. The critical micelle concentrations for C6PS (circles), C6PC (triangle), and C6PG (square) are plotted as a function of the calcium concentration at which they are measured. Frame B: Effect of calcium on activity and the K_d of interaction of factor X_a with C6PS. The values of the dissociation constants (K_d) (squares) were estimated by fitting a binding model that assumes a single C6PS site on factor X_a to S-2765 amidolysis data (see Figure 6) at various Ca^{2+} concentrations. Error bars are standard deviations in the K_d parameter obtained from the fitting procedure. The percent activity of factor X_a at saturating concentrations of the lipid is also plotted (closed triangles).

Effect of Phospholipid on Activation of Prothrombin. The effects of several short-chain lipids or lipid analogues on the initial rate of thrombin and/or meizothrombin appearance are shown in Figure 6A. The enhancement of the rate of prothrombin activation upon titration with up to 0.8 mM C6PS was remarkable, amounting to roughly a 60-fold enhancement at concentrations for which saturation appeared to occur. From the results summarized above, we know that C6PS at these concentrations exists in an unaggregated state, and it is this state that is responsible for the enhanced activation. There was almost no activation of prothrombin in the presence of C6PC, C6PG, L-α-glycerophosphoryl-serine, or L-serine. C6PG did not have any effect on the activation even in the presence of increased concentration of the enzyme (15 nM) and prolonged reaction times (up to 60 min). This result shows a significant specificity in the response of factor X_a activation of prothrombin to PS as compared to other acidic phospholipids, even more specificity than we had seen previously with acidic lipid membranes (Pei *et al.*, 1993). These observations also indicate that the

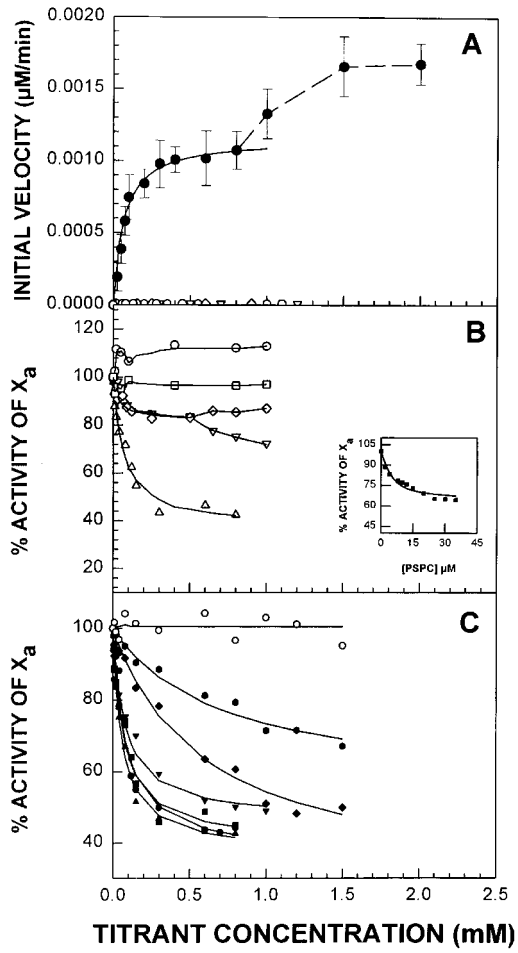

FIGURE 6: Frame A: Rate of prothrombin activation by factor X_a as a function of soluble phospholipid concentration. The initial rate of thrombin plus meizothrombin active site appearance was determined using the synthetic thrombin substrate S-2238 as described under Experimental Procedures. Concentrations of prothrombin (0.6 μM) and factor X_a (5 nM) in 50 mM Tris, 175 mM NaCl, 0.6% PEG, and 5 mM $CaCl_2$, pH 7.6 at 37 °C, were fixed while the rate of activation was monitored as a function of increasing concentrations of titrant: C6PS (closed circles); C6PG (inverted triangles); C6PC (diamonds); L-α-glycerophosphorylserine (hexagons); and L-serine (open circles). The curve drawn through the closed circles indicates the fit of the data up to 0.8 mM to a binding model that assumes a single site for C6PS on factor X_a. Frame B: Rate of factor X_a amidolysis of S-2765 as a function of soluble phospholipid concentration. The rate (proportional to the activity of factor X_a) was determined at 37 °C in a buffer containing 50 mM Tris, 175 mM NaCl, and 0.6% PEG, at pH 7.6, as described under Experimental Procedures. The data are expressed as a percentage of the rate of S-2765 hydrolysis by factor X_a in the absence of titrant. The rate of factor X_a catalyzed amidolysis was monitored in the presence of 5 mM $CaCl_2$, as a function of increasing concentrations of titrant: C6PS (triangles); C6PG (inverted triangles); C6PC (diamonds); orthophospho-L-serine (circles); and glyceroorthophospho-L-serine (squares). Curves are hand-drawn to assist in following the data. The insert compares the percent activity of factor X_a in the presence of increasing concentrations of bovine PS/POPC (20/80) vesicles using the S2765 assay. Frame C: Rate of factor X_a amidolysis of S-2765 as a function of C6PS and Ca^{2+} concentration. The experimental procedure used and data analysis were similar to that described in frame B. The rate of factor X_a catalyzed amidolysis was monitored at increasing concentrations of C6PS at 0 mM Ca^{2+} (open circles), 0.5 mM Ca^{2+} (closed hexagons), 1 mM Ca^{2+} (closed diamonds), 1.5 mM Ca^{2+} (closed inverted triangles), 3 mM Ca^{2+} (closed triangles), 5 mM Ca^{2+} (closed squares), and 6 mM Ca^{2+} (closed circles). Curves indicate the fits of the data to a binding model that assumes a single site for C6PS on factor X_a.

entire lipid molecule is necessary for this response; presentation of just the headgroup or of the head plus glycerol backbone did not elicit a significant response.

In addition to the enhancement of prothrombin activation seen at low concentration of C6PS, a second, sharp increase in the rate of prothrombin activation occurred between C6PS concentrations of 0.8 and 1.5 mM, as seen in Figure 6A. We know from our studies of C6PS aggregation that this range of C6PS concentration encompasses the CMC of this amphipath at 5 mM Ca^{2+} (0.95 mM). The agreement of the concentration range of this increase in activity with the measured CMC of C6PS under the conditions of the prothrombin activation assay argues against the possibility that prothrombin or factor X_a might nucleate activity-altering aggregates at concentrations below the CMC. In addition, if smaller aggregates formed, we would expect to see a cooperative or S-shaped response curve for the rate of prothrombin activation, as reported by Walker *et al.* (1990) for protein kinase C, and this clearly is not the case in Figure 6A. We conclude that the activation of prothrombin by factor X_a is enhanced not only by the aggregated but also and most dramatically by the unaggregated form of C6PS. The aggregrated form of C6PS appears at higher C6PS concentrations and is likely to mimic the effects of a membrane surface.

Effect of Phospholipid on the Activity of Factor X_a. The effects seen in Figure 6A could reflect interaction of C6PS with either the enzyme factor X_a and/or the substrate prothrombin. In order to address the effects of C6PS on factor X_a alone, we examined the sensitivity of the enzymatic activity of factor X_a, as detected with the synthetic substrate S-2765, to titration with short-chain phospholipids and other ligands. The results are presented in Figure 6B, which shows that the amidolytic activity of factor X_a was clearly inhibited by the addition of C6PS. This inhibitory effect increased rapidly with increasing concentration of C6PS, reaching saturation at concentrations of C6PS above 0.5 mM. Membranes composed of bovine PS/POPC showed a similar inhibitory effect on the amidolytic activity of factor X_a toward S-2765, but at much lower lipid concentrations (insert, Figure 6B), as expected from the published equilibrium constants for binding of factor X_a to these membranes (Cutsforth *et al.*, 1989). Control experiments were done to make sure that the decrease in activity of factor X_a in the presence of increasing concentrations of C6PS was due to binding of factor X_a to the lipid and not due to depletion of the substrate, S-2765, as a result of binding of the substrate to the phospholipid. The activity of factor X_a in the presence and absence of 30 μM C6PS was monitored as a function of substrate concentration. These experiments demonstrated that the decrease of factor X_a's amidolytic activity in the presence of C6PS was independent of the concentration of S-2765 from 0.5 to 1.0 mM. If the effect of C6PS were on S-2765 rather than on factor X_a, we should have found the effect to be dependent on the concentration of S-2765 substrate, which was not the case.

Although the other short-chained phospholipids tested (C6PG and C6PC) did show a small effect on factor X_a, their inhibitory effect was much less than that for C6PS (Figure 6B). Note that these phospholipids did not have any effect on prothrombin activation (Figure 6A). L-Serine, *O*-phospho-L-serine, and L-α-glycerophosphorylserine showed no inhibitory effects toward factor X_a activity (Figure 6B). The

results indicate that factor X_a has at least one binding site specific for C6PS. Assuming that the observed inhibition was a response to binding of one C6PS to a single regulatory site on factor X_a, an apparent dissociation constant, K_d, of 70 μM ($\pm$10 μM; standard deviation) was obtained from this curve. Except for the fact that factor X_a's amidolytic activity toward S-2765 was decreased rather than increased, these results parallel those obtained for the effect of lipid titrants on prothrombin activation (Figure 6A), from which an apparent K_d of 65 $\pm$ 5 μM was obtained.

Since calcium is an important component of the prothrombinase reaction, we have examined its effect on the interaction of factor X_a with C6PS (Figure 6C). Although prothrombin activation by factor X_a is very slow at low concentrations of Ca^{2+}, Ca^{2+} is not necessary for the amidolytic activity of factor X_a. Inhibition of the amidolytic activity of factor X_a by C6PS increased with increasing concentration of Ca^{2+}. No C6PS-induced inhibition of factor X_a amidolytic activity was observed in the absence of Ca^{2+} (Figure 6C, open circles). Very little inhibition of factor X_a by C6PS could be detected at 0.5 mM calcium, but the inhibitory effect of lipid increased dramatically with an increase in Ca^{2+} concentration. This suggests that, in the absence of Ca^{2+}, either the binding of C6PS does not alter the active site of factor X_a or C6PS does not bind to factor X_a. It may be that Ca^{2+} induces a factor X_a conformation change that exposes or alters the C6PS binding site. (Figure 5B shows the dependence of the apparent C6PS dissociation constant on the concentration of calcium.) Factor X_a is a vitamin K dependent enzyme, and, as such, it has roughly 10 γ-carboxyglutamic acid residues (Di Scipio *et al*., 1977) and 1 tight plus 19 (human) to 22 (bovine) weak calcium binding sites (Monroe *et al*., 1990) in its amino-terminal, membrane binding domain and at least 1 site associated with an epidermal growth factor like domain (Persson *et al*., 1989). (It may also be that the C6PS binding site(s) is (are) associated with one or more calcium binding sites.)

Alteration of Prothrombin Conformational State in the Presence of Short-Chain Lipids. We have shown previously that binding of bovine or human prothrombin to PS-containing membranes alters prothrombin conformation (Wu & Lentz, 1991, 1994). It is reasonable to speculate that this lipid-induced conformational change could contribute to the C6PS-induced enhancement of the rate of prothrombin activation by making prothrombin a better substrate. We have therefore studied the thermal denaturation of prothrombin in the presence of short-chained lipids. It was mentioned earlier that the CMC of C6PS was lowered somewhat in the presence of high concentrations of prothrombin. Because CMC determinations have been performed under exactly the conditions used in the thermal denaturation studies, we can distinguish between the effects of soluble and aggregated forms of C6PS. As was described earlier (Lentz *et al*., 1994), prothrombin denaturation in the absence of calcium can be described by two exotherms: a main one peaking at 58.4 °C corresponding to Pre1[1] melting and a minor peak at 64 °C corresponding to F1[1] melting. The denaturation profiles of prothrombin in the presence and absence of C6PS (Figure 7A) show that the main peak corresponding to Pre1 is shifted to a lower temperature with increasing concentration of C6PS. The insert in Figure 7A shows that the temperature of the main peak was linearly related to C6PS concentration (closed circles), over the range of C6PS concentrations

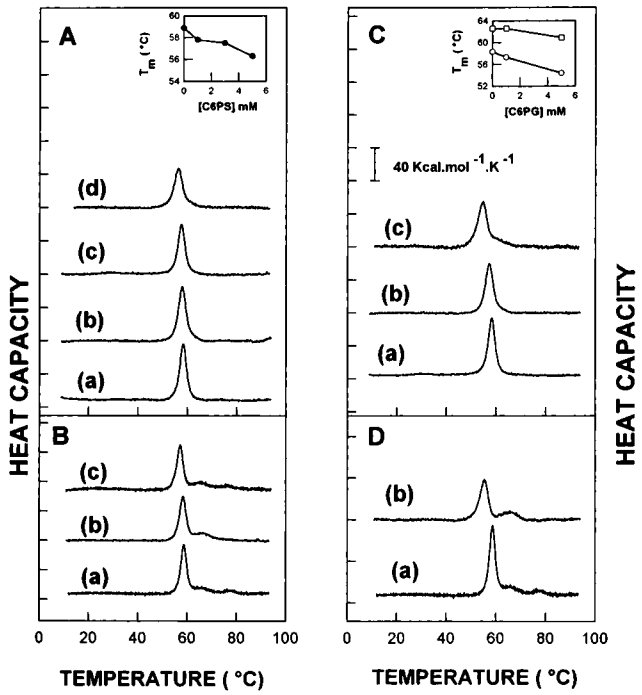

FIGURE 7: Thermal denaturation profiles of bovine prothrombin in the absence and presence of phospholipid and calcium. Frame A: (a) Prothrombin (0.8 mg/mL) in MOPS buffer containing 0.1 mM Na_2EDTA. (b) Plus 1 mM C6PS. (c) Plus 3 mM C6PS. (d) Plus 5 mM C6PS. The insert shows the dependence of the temperature of the main peak on the concentration of C6PS. Frame B: (a) Prothrombin in MOPS buffer containing 3 mM Ca^{2+}. (b) Plus 0.3 mM C6PS. (c) Plus 3 mM C6PS. Frame C: (a) Prothrombin alone. (b) Plus 1 mM C6PG. (c) Plus 5 mM C6PG in MOPS buffer containing 0.1 mM Na_2EDTA. The insert shows the dependence of the temperature of the main peak (circles) and the high-temperature peak (squares) on the concentration of C6PG. Frame D: (a) Prothrombin in MOPS buffer containing 3 mM Ca^{2+}. (b) Plus 3 mM C6PG.

investigated, all of which were below the CMC in the absence of Ca^{2+} and presence of 11 μM prothrombin ($\sim$7 mM). The main peak, in addition to shifting to lower temperature, was broadened on its low-temperature edge, and decreased in enthalpy with increasing C6PS. This low-temperature broadening seemd apparent in the endotherms but could not be resolved in terms of any measurable increase in the enthalpy of the low-temperature shoulder, although a slight trend toward decreased shoulder peak temperatures was evident (Table 1). This shoulder has been suggested to correspond to fragment 2 (F2)[1] melting in the absence of Ca^{2+} (Lentz *et al*., 1994).

Figure 7B shows the effect of C6PS on the melting profile of prothrombin in the presence of calcium. Because C6PS has a low CMC (1.2 mM) under these conditions, only the data collected at 0.3 mM reflect the effect of soluble C6PS. A downward shift in the T_M of the main endotherm was seen in the presence of calcium; in addition, there was a shift of the high-temperature shoulder (65.4 °C) to a higher temperature (66.4 °C). Most remarkable, however, was the replacement of the two high-temperature peaks (65.4 and 76.8 °C) with low R values by a single peak (66.4 °C) with the same combined enthalpy and $R \approx 1$ (Table 1), corresponding to a simple two-state unfolding transition. The observed peak shifts and alterations in denaturation profiles are different from those we have reported as due to prothrombin binding to PS-containing membranes (Lentz *et al*., 1994). Not surprisingly, then, the effect of aggregated C6PS (3 mM)

Table 1: Thermodynamic Characteristics of Bovine Prothrombin Melting in the Presence and Absence of Ca^{2+} and Soluble Short-Chain Lipids

	deconvolution analysis											
		peak 1			peak 2			peak 3			peak 4		
protein	total ΔH^{cal}	T_m[a]	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]	T_m	ΔH^{cal}	R[b]
II + EDTA	290	54.6	17.3	0.07	58.3	243	1.02	62.6	12	0.07			
II + C6PS (1 mM)	285	54.2	10.4	0.04	57.9	256	1.2	62.2	23	0.13			
II + C6PS (3 mM)	287	53.1	28	0.22	57.5	217	0.95	62.2	16	0.09			
II + C6PS (5 mM)	237	52.5	22.7	0.14	56.3	195	0.97	61.1	27	0.17			
II + C6PG (1 mM)	270				57.3	257	1.3	62.6	14	0.05			
II + C6PG (5 mM)	289				54.4	250	1.5	60.9	65	0.67			
II + Ca^{2+} (3 mM)	289				58.7	214	0.82	65.4	56	0.44	76.8	31	0.34
II + Ca^{+2} (3 mM) & C6PS (0.3 mM)	313				58.3	219	0.98	66.4	93	0.93			
II & Ca^{2+} (3 mM) & C6PS (3 mM)	302				57.1	202	0.82	64.7	62	0.51	74.4	43	0.49
II & Ca^{2+} (3 mM) & C6PG (3 mM)	235				55.3	177	0.98	65.5	54	0.44			
II + C6PC (5 mM)	244	53.2	28.8	0.34	57.8	210	1.18						

[a] T_m is the midpoint of the transition in degrees celsius. [b] R is the ratio $\Delta H^{cal}/\Delta H^{vh}$, where ΔH^{cal} and ΔH^{vh} are the calorimetric and van't Hoff enthalpies in kcal/mol, respectively. Experimental error is estimated to be ±0.1 °C in T_m and ±15 kcal/mol in ΔH values.

on prothrombin melting was different from the effect of soluble C6PS. The shift of the main endotherm to lower temperatures was similar to what we have observed for soluble C6PS in the presence or absence of Ca^{2+}. However, the high-temperature portion of the profile reverted to a behavior similar to that seen in the absence of C6PS (scan a). This is especially surprising, since 25/75 bovine PS/POPC membranes dramatically altered this high-temperature portion of the melting profile (Lentz *et al*., 1994). It may be that prothrombin interacts differently with a 25/75 bovine PS/POPC membrane than it does with a C6PS aggregate. Despite this uncertainty, our results clearly indicate interactions between C6PS and prothrombin in the absence and presence of calcium ions, interactions that alter the thermodynamic state and probably the domain organization of prothrombin in solution.

The thermograms in Figure 7C show that C6PG, like C6PS, interacts in a calcium-independent manner with prothrombin. The effects of C6PG were similar to but not identical to those of C6PS (Table 1 and insert to Figure 7C). In the absence of Ca^{2+}, high C6PG concentration shifted the main endotherm to lower temperature, as was observed for C6PS. However, unlike C6PS, C6PG produced a measurable increase in the enthalpy of a high-temperature shoulder that probably corresponds to F1 melting (Lentz *et al*., 1994). Note that this does not imply a direct interaction with F1, which would be unlikely in the absence of calcium. In the presence of Ca^{2+}, the effect of C6PG on the melting profile of prothrombin was even more pronounced, as shown in Figure 7D. Unlike what we observed for C6PS, the melting behavior of prothrombin in the presence of 3 mM C6PG (below the CMC of C6PG in the presence of 5 mM Ca^{2+}: 12.5 mM) was very similar to that observed in the presence of phosphatidylcholine/phosphatidylglycerol (50/50) membranes (Lentz *et al*., 1991), and is consistent with the model for the effects of acidic lipid membranes on prothrombin domain structure (Lentz *et al*., 1994): that the F1 and Pre1 domains of prothrombin do not denature quite independently and that binding of prothrombin to acidic lipid membrane disrupts the interaction between these domains. Since neither soluble nor aggregated C6PS produced alterations in prothrombin melting similar to those produced by binding to PS-containing membranes, we conclude that the effects on prothrombin structure of interactions with C6PS and C6PG must be different.

Our results show that C6PS and C6PG do interact with sites on prothrombin, thereby altering the conformational state of bovine prothrombin, although not always in a manner similar to that caused by binding to acidic lipid membranes. Three of our results, however, make it unlikely that binding of soluble acidic lipids to prothrombin contributes to the observed effect of C6PS on prothrombin activation by factor X_a (Figure 6A). First, while C6PG had no effect on activation, it had a comparable if not greater effect on prothrombin conformation. Second, the effects of both C6PS and C6PG on prothrombin conformation were not saturable as were the effects of C6PS on activation. Finally, the effect of C6PS on the rate of prothrombin activation had nearly the same concentration dependence on the effect of S-2765 activity of factor X_a (compare Figure 6A and Figure 6B and the apparent K_d's derived therefrom). All these observations suggest that the acidic lipid induced prothrombin conformational changes monitored by differential scanning calorimetry do not contribute to the rate of prothrombin activation by factor X_a *in solution*.

DISCUSSION

Conclusions. The following significant new conclusions derive from this work: (1) Short-chain phospholipids such as C6PS aggregate in a complex fashion that is difficult to characterize using conventional fluorescent probes. (2) Soluble forms of PS act as a calcium-dependent, specific regulator of factor X_a, at least with regard to its function of activating prothrombin to thrombin. (3) Binding of soluble forms of PS and PG to calcium-independent site(s) on prothrombin induces different changes in this protein's conformational state. These changes may be important in modulating the rate of prothrombin activation by factor X_a on a membrane surface but probably cannot account for the effects of short-chain phospholipids in solution. (4) The results demonstrate unequivocally that at least part of the influence of PS on prothrombin activation derives from interactions with individual sites on factor X_a and not the effect of adsorption to a negatively charged membrane surface.

A significant implication of these conclusions is that the exposure on the surface of activated platelets of buried PS is a necessary and crucial step in activating thrombin formation during blood coagulation.

Complex C6PS Aggregation. Our results suggest that aggregation of C6PS is a complex process involving aggregates of vastly different sizes, only the largest of which are detected by the fluorescence of ANS (in the absence of PEG) or DPH. Light scattering showed clearly that aggregates formed below the concentrations reported by these probes to be the CMC. The inadequacy of CMC values estimated by ANS fluorescence was first revealed to us by the jump in rate of prothrombin activation at roughly 1 mM C6PS (Figure 6A) while ANS fluorescence suggested a CMC of 3.2 mM (Figure 1B). Walker *et al*. (1990) reported a similar discrepancy between the concentration of C6PS that first supported protein kinase C activity and a much larger estimate of the C6PS CMC using ANS. These authors attributed this to "pre-micellar aggregates" as described by Johnson *et al*. (1981). We suggest that the observations of Walker *et al*. reflect rather the inadequacy of ANS fluorescence in defining the CMC of short-chain phospholipids. "Pre-micellar aggregates" appear to form at low concentrations of C6PC, as Johnson *et al*. suggest that this lipid may exist as an equilibrium mixture of monomers and dimers below the CMC. If this occurs for C6PS, then what we have referred to as "soluble C6PS" may be a mixture of monomers and dimers, and it would be uncertain which of these species binds to and enhances the activity of factor X_a. This uncertainty in no way would affect our conclusions, since it is still clear that a soluble form of C6PS is responsible for the activity of factor X_a. Determination of whether the response of factor X_a is due to monomer or dimer forms of C6PS will, however, require experiments that will define the stoichiometry of binding.

QELS data also showed that the initial aggregates formed were only 110 Å in diameter, but that this population was replaced by increasingly larger aggregates with increasing C6PS concentration. Assuming prolate ellipsoids having this as the major axis and a minor axis defined by a bilayer of C6PS (*ca*. 27 Å), and a density of 1 g/cm^3, we estimate these aggregates to contain ~220 molecules of C6PS. Given a binding stoichiometry of roughly 41−52 monolayer lipids per protein (Cutsforth *et al*., 1989), this aggregate might be expected to nucleate roughy 4−5 factor X_a and prothrombin molecules. To a first approximation, this should mimic binding to a membrane surface. The effects of a PS/POPC membrane on prothrombin activation by factor X_a are multifaceted, involving substrate delivery and alignment with the enzyme active site, intermediate channelling in order to cut two peptide bonds in prothrombin, and also the type of activity enhancement described here. Nonetheless, it is instructive to compare the effects of soluble and aggregated C6PS to the effects of PS-containing membranes on prothrombin activation. The maximal enhancement of prothrombin activation rate that we have observed with 25/75 bovine PS/POPC membranes is 300-fold (Zhou and Lentz, unpublished results), compared with 60-fold due to soluble C6PS or 100-fold due to aggregated C6PS (Figure 6A). This comparison makes it clear that the largest effect of PS-containing membranes on the rate of factor X_a catalyzed prothrombin activation is due to occupancy of PS-specific regulatory sites on factor X_a.

PS as an Allosteric Regulator of Prothrombinase. The results presented in this paper clearly suggest that one role of platelet membranes in blood coagulation is to provide PS that acts as a positive effector of prothrombin activation. Despite the compelling evidence presented here for a regulatory role for PS, our results raise many questions that must remain unanswered at this point. First, we do not know whether PS acts as an allosteric regulator of factor X_a or simply by binding near to, and thus altering, the active site. The allosteric regulation model holds that regulatory molecules bind to regulatory sites on a protein, thereby inducing conformational changes in the protein, and thus altering binding to other sites, usually an active site but possibly in the case of prothrombin the site of action of factor X_a. Our results imply that factor X_a experiences a change in its active site conformation on binding to C6PS in the presence of calcium ions. This is consistent with a report by Husten *et al*. (1987) that the fluorescence of an active site located probe was altered on binding to a PS-containing membrane. Since the active site of factor X_a is reported to be 61 Å (or 69 Å) from a PS-containing membrane in the absence (or presence) of factor V_a (Husten *et al*., 1987), it seems likely that the effect of PS must be an allosteric one. Nonetheless, a C6PS-induced change in factor X_a conformation remains to be directly demonstrated. Second, activation of prothrombin requires sequential cleavage of two peptide bonds, resulting in four possible reactions that must be catalyzed by factor X_a to produce thrombin [*e.g*., see Hibbard *et al*., (1982) and Rosing and Tans (1988)]. We do not know which of these four reactions is (are) accelerated by binding of C6PS to factor X_a. Third, although we have assumed for convenience a stoichiometry of 1 in analyzing C6PS binding, the results presented here are not of sufficient precision to define this stoichiometry. Fourth, our results indicate that activation of factor X_a is specific for PS, but we need to define further those features of the PS molecule that are essential for this allosteric effect.

Finally, our DSC results do demonstrate that C6PS and C6PG binding to prothrombin causes changes in the conformation of this molecule, consistent with our earlier studies of the effect of membrane binding on prothrombin conformation (Wu & Lentz, 1991, 1994; Lentz *et al*., 1994). However, this acidic lipid induced conformational change appears not to account for the enhanced rate of activation induced by C6PS. So, at this point, we cannot define precisely the role of this conformational change.

All of these issues are currently under investigation.

Implications for the Role of Platelet Membranes in Blood Coagulation. The fact that PS is the lipid that specifically up-regulates prothrombin activation by factor X_a has important implications for how the coagulation pathway is regulated by platelet activation. In resting human platelets, PS is asymmetrically distributed across the platelet plasma membrane (Schick *et al*., 1976; Bevers *et al*., 1983). Maintenance of this asymmetry appears to require an ATP-dependent "aminophospholipid translocase" (Devaux, 1991). Upon stimulation of human platelets, the requisite negatively-charged phospholipids were originally thought to "flip" from the cytoplasmic to the extracytoplasmic surface of the platelet plasma membrane (Bevers *et al*., 1983) but were later seen as being exposed on the surface of membranous "particles" or vesicles released from activated platelets (Bode *et al*., 1985; Sims *et al*., 1988). The causative relationship between PS exposure and vesicle release is still uncertain, but the importance of the specific asymmetry of PS and of PS's exposure during platelet stimulation is implied by the results presented here. It seems that PS is a membrane-associated

regulatory molecule whose exposure to the blood is carefully controlled during normal hemostasis. Signalling reactions at the platelet membrane apparently expose PS by an unknown mechanism as one of the events of the platelet stimulation response. Exposed PS serves not only to assemble the prothrombinase but also to allosterically up-regulate the enzyme of the complex and to similarly modulate the conformation of the bound substrate. It may be that the platelet membrane assembled factor X activating complex is similarly regulated by exposed PS. This picture and our results imply that compounds capable of competing with PS for the regulatory sites on factor X_a and perhaps on prothrombin would be candidates for clinical agents for controlling the blood coagulation process.

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BI952063D