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Association of Blood Coagulation Factors V and X with Phospholipid Monolayers†

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ABSTRACT: Blood coagulation factors X and V adsorbed to phospholipid monolayers and induced surface pressure changes. These proteins also adsorbed to the air-water interface and formed protein surface films. Plots of surface pressure change ($\Delta\pi$) vs. initial monolayer surface pressure (π_0) appeared biphasic for factor X and factor V, indicating two distinct adsorption processes. Surface pressure changes in monolayers spread below the collapse pressure of the respective proteins were characteristic of protein adsorption to the air-water interface while those observed above the protein collapse pressures were consistent with specific protein-acidic phospholipid interactions. Phosphatidylserine-dependent surface pressure changes were very small for both proteins. Factor X induced surface pressure changes required the presence of calcium while factor V induced changes occurred in the presence or absence of calcium. Protein-monolayer binding characteristics were comparable to those obtained by using bilayer vesicles of similar composition and indicated the absence of significant membrane surface curvature effects. The maximum surface concentration corresponded to one bound factor X molecule per 1400 Å^2. Comparison of surface pressure changes induced by factor V with those induced by myelin basic protein suggested that the membrane-binding processes of the two proteins involve similar but small degrees of acyl chain perturbation. Thrombin digestion of factor V had no effect on surface pressure change and the isolated 80 000-dalton peptide of factor Va also showed approximately similar surface pressure effects. The vitamin K dependent proteins caused a smaller surface pressure change per bound protein molecule. The results indicate that the prothrombinase proteins associate primarily, if not exclusively, with the head groups of the phospholipids.

Bilayer vesicles containing acidic phospholipids support clotting activity with efficiencies very similar to activated platelets (Nesheim et al., 1979b). Artificially prepared phospholipid membranes therefore appear to provide a suitable model for the biological membrane surface in coagulation processes. Eventual reconstruction of the prothrombinase complex requires careful measurement of several protein-membrane interactions. The mode of membrane association of the three proteins involved (prothrombin, factor X, and factor V) has been investigated by employing various techniques. Light scattering and fluorescence experiments have indicated that each protein binds to a cluster of acidic phospholipids at the membrane surface (Nelsestuen & Broderius, 1977; Lim et al., 1977; Bloom et al., 1979; Pusey et al., 1982). The prothrombin- and factor X-acidic phospholipid complexes are mediated by calcium (Nelsestuen & Lim, 1977) while the factor V-membrane complex arises from direct protein-acidic phospholipid interactions (Bloom et al., 1979; Pusey et al., 1982). Quasi-elastic light scattering has been used to measure the degree of protrusion of all three proteins from the membrane surface (Lim et al., 1977; Pusey et al., 1982). Stopped-flow light scattering measurements indicated very rapid protein-membrane recognition processes (Wei et al., 1982; Pusey et al., 1982). Recent studies utilized well-defined prothrombin and phospholipid monolayer systems and showed that prothrombin-membrane binding was not dependent on membrane surface curvature (Mayer et al., 1983). Furthermore, these studies indicated that no significant insertion of prothrombin into the hydrophobic region of the membrane occurred. Major hydrophobic interactions between factor Va and the membrane core have been postulated (Nesheim et al., 1980). However, more recent evidence suggested a primarily ionic factor V-membrane interaction (Pusey et al., 1982).

Protein-induced changes in the surface pressure of monomolecular lipid films have been used to deduce the nature of various protein-membrane interactions (Verger & Pattus, 1982; Kimelberg & Papahadjopoulos, 1971). Bougis et al. (1981) demonstrated that the large surface pressure changes caused by cardiotoxin-monolayer adsorption were due to insertion of the peptide into the hydrophobic region of the membrane. These perturbations were dependent on phospholipid head group and acyl chain composition; cardiotoxins effected larger surface pressure changes in monolayers containing acidic phospholipids (PS)[1] than in neutral phospholipid (PC) monolayers. Similar results were obtained for mellittin-phospholipid adsorption (Sessa et al., 1969; Bhakoo et al., 1982). Other proteins, such as δ-lysin, induced surface pressure changes with virtually no phospholipid head-group specificity (Bhakoo et al., 1982).

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[1] Abbreviations: PS, bovine brain phosphatidylserine; PC, egg yolk phosphatidylcholine; π, surface pressure; $\Delta\pi$, surface pressure change; π_0, initial surface pressure; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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Investigations similar to those described above using phospholipid monolayers were completed here to analyze the structure of the factor X– and factor V(a)–phospholipid complexes. Both proteins exhibited rather small surface pressure changes upon binding to PS-containing monolayers. This suggested that protein–membrane complex formation is mediated primarily through the head groups of the acidic phospholipids. Binding parameters for monolayers compared favorably with bilayer vesicles, and the two systems appeared complementary in the analysis of prothrombinase protein–membrane interactions.

Materials and Methods

Poly(L-lysine) (M_r 70000) and hirudin were purchased from Sigma Chemical Co. Myelin basic protein was a generous gift from Paul Lampe (University of Minnesota; Lampe & Nelsestuen, 1982). Bovine prothrombin fragment 1 was isolated by the procedures of Hildebrant & Mann (1973) as modified by Mayer et al. (1983). Bovine coagulation factor X was initially purified as outlined in Nelsestuen et al. (1976). The product was further purified by gel filtration chromatography by using a 3.0 × 35 cm column of Sephacryl S-200. Failure to include this step led to larger and inconsistent factor X induced surface pressure changes. Factor Xa was prepared as outlined by Pletcher & Nelsestuen (1983).

Bovine brain phosphatidylserine and egg yolk phosphatidylcholine were purchased from Sigma Chemical Co. [^{14}C]Dioleoylphosphatidylcholine was purchased from New England Nuclear, Inc. Further purification of these lipids by high-pressure liquid chromatography was completed by the methods described previously (Mayer et al., 1983). Phospholipid concentrations were determined by organic phosphate measurement (Chen et al., 1956) by using a phospholipid/phosphorus weight ratio of 25. All phospholipids were stored in appropriate solvents at −80 °C until use.

Monolayers were formed by applying the appropriate lipids dissolved in either ethanol/hexane (10/90) or 100% benzene onto buffer contained in a 10 mL (4.0 × 0.8 cm) circular Teflon trough etched with *teflon*-treating agent (Chemplast, Inc., Wayne, NJ). The buffer was stirred at 45 rpm with a Teflon stir bar. Both organic solvent systems yielded stable monolayers, and known amounts of applied lipid exhibited surface pressures consistent with surface pressure–molecular area isotherms (Mayer et al., 1983). Surface pressure measurements and protein additions were completed as described by Mayer et al. (1983). Unless otherwise indicated, the buffer consisted of 0.05 M Tris (pH 7.5) containing 0.1 M NaCl and 10 mM $CaCl_2$.

Tritium-labeled factor X was prepared by the methods of Silverberg et al. (1977). Final purification of the protein was accomplished by ion-exchange and gel filtration chromatography as described above for native factor X. A specific activity of 1500 cpm/μg of ^{3}H factor X was obtained. Membrane binding characteristics for tritiated factor X were identical with those for native factor X as assessed by surface pressure changes in phospholipid monolayers and binding to PS-containing bilayer vesicles [determined as in Nelsestuen & Lim (1977)].

Amounts of ^{3}H factor X bound to phospholipid monolayers were determined by two methods. One method involved recovering the monolayer and adsorbed protein with Whatman No. 1 PS hydrophobic paper (Mayer et al., 1983; Bhat & Brockman, 1981). After monolayer pickup, the paper was cut into small segments and placed into a scintillation vial along with 2 mg of Pronase (Sigma Chemical Co.) dissolved in 1 mL of buffer. This solution was incubated at 37 °C for 30 min prior to addition of scintillation fluid. Radioactivity was released from the paper by this method, and the maximum counting efficiency was observed. The radioactivity obtained was corrected for monolayer recovery; independent experiments using [^{14}C]dioleoylphosphatidylcholine-containing monolayers in the absence of radioactive protein indicated that 85% of the applied phospholipid was recovered by this procedure. Background counts due to subphase pickup [determined as in Mayer et al. (1983)] were subtracted from the total counts obtained. Depletion of radioactivity in the subphase buffer due to ^{3}H factor X–monolayer binding was also used to determine amounts of membrane-bound protein. Various amounts of radioactive protein were injected under the phospholipid monolayer and allowed to equilibrate. Subphase aliquots (1 mL) were then removed, placed into a scintillation vial with 10 mL of scintillation fluid, and counted. No significant depletion of radioactivity was observed by using pure PC monolayers (π_0 = 30 dyn/cm) or with PS-containing monolayers in the absence of calcium. In the experiments reported here the subphase radioactivity in the presence of PS-containing monolayers was 15–30% lower than that for pure PC monolayers. This depletion was attributable to protein–phospholipid binding, and amounts of free and bound radioactive protein were calculated.

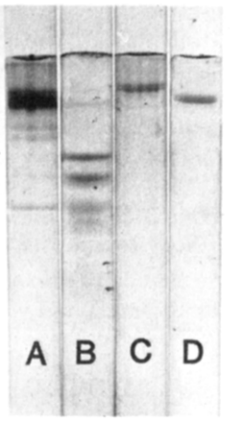

FIGURE 1: Gel electrophoresis of isolated factor V(a) protein. The isolated factor V protein was electrophoresed in SDS (gel A) and under nondenaturing conditions (gel C). Thrombin-digested protein was also analyzed in SDS (gel B) and under nondenaturing conditions (gel D).

Blood clotting factor V was purified from bovine blood obtained by venipuncture using the methods of Pusey et al. (1982). For subsequent work with phospholipid monolayers benzamidine could not be added at any stage of purification due to persistent surface activity which interfered with protein-induced surface pressure measurements. While the protein eluted from the final gel filtration column in the same position as single chain factor V [see Figure 1 of Pusey et al. (1982)], polyacrylamide gel electrophoresis in the presence of SDS (Weber & Osborn, 1969) showed several bands indicating partial proteolysis (Figure 1, gel A). This result is typical for factor V preparations obtained in the absence of several protease inhibitors including benzamidine (Esmon, 1979). Proteases such as thrombin convert single chain (M_r 330000) factor V, through several intermediates to factor Va, the biologically active form of the protein. Factor Va is characterized by two major peptides of M_r ~80000 and ~100000 (Esmon, 1979; Nesheim & Mann, 1979; Pusey et al., 1982). Gel 2 in Figure 1 shows the thrombin activation of factor V and the two largest peptides correspond to the 80000- and 100000-dalton factor Va peptides. Thrombin digestion was carried out at a factor V/thrombin ratio of 100/1 (w/w) at

37 °C for 10 min. Most studies reported below were conducted both with factor V protein as isolated and with thrombin-digested protein. Polyacrylamide gels run under nondenaturing conditions (Ornstein & Davis, 1964) in the presence of 0.2 mM $CaCl_2$ are shown in Figure 1 for the protein as isolated (gel C) and after thrombin treatment (gel D). As reported by others (Nesheim & Mann, 1979), thrombin treatment has relatively little effect on protein mobility in this system. A high level of protein purity is indicated by these gels.

The factor V protein as isolated also showed high levels of activation produced by thrombin digestion. The assay used for this measurement consisted of factor Xa (5.8×10^{-11} M), prothrombin (9 μM), hirudin (4.5 manufacturer's units/mL), and factor V protein in 0.05 M Tris buffer (pH 7.5)–0.1 M NaCl–2 mM $CaCl_2$ at 25 °C. At timed intervals samples were removed, mixed with an amount of thrombin equal to the hirudin added, and incubated 60 s. The thrombin activity in the final sample was then assayed spectrophotometrically at 405 nm with the *p*-nitroanilide substrate S-2288 (Kabi Diagnostica). In this assay system the hirudin present during activation inhibits the thrombin produced and prevents feedback activation of factor V. When this assay was used, the factor V protein as isolated was inactive, and the limits of detection indicated at least 10000-fold activation by thrombin digestion. The protein as isolated is referred to as factor V, and although some proteolysis has occurred, it appears very similar to single chain factor V.

Total protein in the factor V preparations was estimated by using an $E^{1\%}_{280nm} = 9.6$ (Nesheim et al., 1979a). Total membrane-binding protein was estimated by light scattering as described by Pusey et al. (1982). Briefly, excess scattered light from a protein–vesicle solution occurs upon formation of a protein–membrane complex. At low factor V/phospholipid ratios essentially all of the functional protein is membrane bound (Pusey et al., 1982). Given the concentration of phospholipid and the light scattering ratio of the protein–vesicle complex to that of the vesicles alone, the mass of membrane-bound protein can be obtained (Nelsestuen & Lim, 1977). For the factor V preparations used here, 55–75% of the total protein bound to the membrane. This compared well with preparations of single chain factor V where about 50% [Figure 2 of Bloom et al. (1979)] and greater than 40% (Pusey et al., 1982) of the protein were associated with the membrane at low protein to phospholipid ratios. The apparent nonquantitative binding may be due to several factors which have not been elucidated.

Binding of factor V(a) to the monolayer was quantitated by measuring depletion of subphase factor V(a) activity due to protein–membrane binding employing the enzymatic method of Rosing et al. (1980). The rate of thrombin production was first measured under conditions of limiting factor Xa to obtain a rate constant for the prothrombinase complex. The phospholipid (5 μg/mL 40% PS vesicles), factor Va (1.5 μg/mL), and prothrombin were maintained at saturating levels. The rate of thrombin production was measured by sampling the mixture at varying times and testing for activity toward S-2288. The amidase present and the specific activity of pure thrombin (Pletcher & Nelsestuen, 1983) were used to determine the amount of thrombin. At 37 °C a rate constant for thrombin production (based on limiting Xa) of 31 s^{-1} was obtained. This agreed well with previous determinations (Rosing et al., 1980; Nesheim et al., 1979b; Dahlbeck & Stenflo, 1980). Molar concentrations of factor Va were then estimated from this rate constant and the kinetics of thrombin production under conditions where factor Va was rate limiting (factor Xa = 2.0 nM). These estimations assumed a 1/1 factor Xa/factor Va stoichiometry (Nesheim et al., 1979b). As a comparison, the molar concentration of factor V(a) obtained in this manner was converted to the mass of factor V(a) by using a molecular weight of 330000. The result indicated 10% more factor V(a) protein than total membrane-binding protein estimated from the light scattering technique described above.

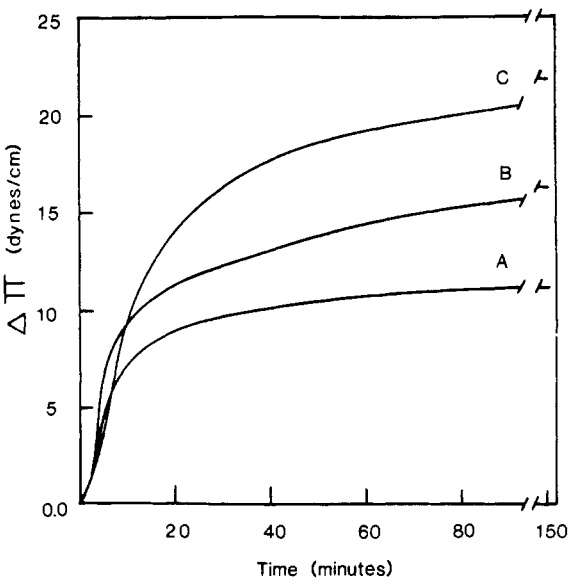

FIGURE 2: Adsorption kinetics of fragment 1 (A), factor X (B), and factor V (C) to an air–water interface. Proteins were injected to give a final subphase concentration of 35 μg of membrane-binding protein/mL.

Iodinated factor V(a) was prepared by using sodium [^{125}I]iodide and enzymobeads (Bio-Rad, Inc.) according to the manufacturers instructions. This protein was then passed over a 2 × 25 cm Bio-Gel a-1.5 M column, and factor V(a)-containing fractions were pooled and concentrated. Iodinated factor V(a) membrane binding was monitored with the monolayer recovery method described above.

The 80000-dalton peptide from blood coagulation factor Va was prepared according to Lindhout et al. (1982).

Results

Kinetics of Protein-Induced Surface Pressure Changes. Fragment 1, factor X, and factor V added to buffer in the absence of a lipid film caused an increase in the observed surface pressure (Figure 2). The extent of surface pressure increase and the time required to reach an apparent plateau in surface pressure differed for the three proteins. The apparent plateau in surface pressure for fragment 1, factor X, and factor V were 11.0, 16.5, and 22.0 dyn/cm, respectively. The times necessary to achieve 75% of these values were 17 min for fragment 1 and 30 min for factor X and factor V. In addition, factor X and factor V adsorption kinetics appeared biphasic while fragment 1 kinetics suggested a single rate of adsorption. Thrombin-treated factor V gave an indistinguishable $\Delta\pi_{max}$ but reached this value with a half-time of 20 min (data not shown.)

Factor X Adsorption to PS/PC Monolayers. A plot of maximum factor X induced surface pressure change vs. the initial surface pressure was biphasic, indicating two distinct surface adsorption processes (Figure 3). Below an initial surface pressure of 20 dyn/cm, factor X induced changes were large and essentially insensitive to PS/PC composition. Linear extrapolation of this region of the curve to $\Delta\pi = 0$ gave a critical pressure (the initial surface pressure above which

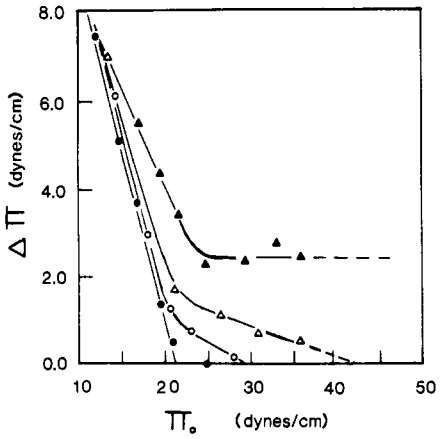

FIGURE 3: Dependence of surface pressure increase induced by factor X on initial spread pressure of PS/PC monolayers. Protein was added to give a final concentration of 0.4 μM. Phospholipid films used were (●) 100% PC, (○) 10% PS–90% PC, (△) 20% PS–80% PC, and (▲) 100% PS.

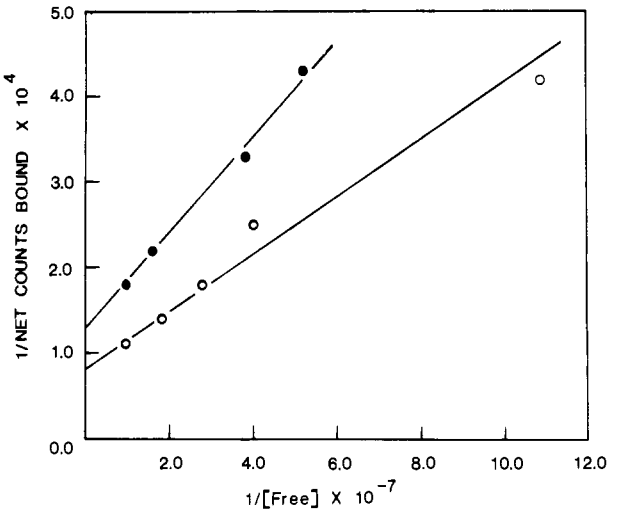

FIGURE 4: Double-reciprocal plots for factor X binding to 20% (●) and 100% (○) PS monolayers. Amounts of bound radioactive factor X were determined by subphase depletion techniques as described under Materials and Methods. Initial surface pressure for the monolayers used was 25 ± 1.0 dyn/cm.

protein-induced surface pressure changes were not observed) similar to the collapse pressure for the protein in the absence of a lipid film (see Figure 2). In addition, the changes at low initial surface pressures required long times to reach equilibrium which were comparable to factor X induced changes in the absence of phospholipid (Figure 2).

Surface pressure changes caused by factor X in monolayers spread above 20 dyn/cm were dependent on the presence of acidic phospholipids (Figure 3) and calcium. Increasing the PS content of the monolayer increased the amount of surface pressure change as well as the critical pressure (Figure 3). These results suggested that this adsorption process represented protein–acidic phospholipid binding. The characteristics of these changes were very similar to those for fragment 1 (Mayer et al., 1983) except that the extent of surface pressure increases were about half those observed for fragment 1.

Factor X monolayer binding characteristics were obtained at high initial surface pressures by using the monolayer recovery and subphase depletion techniques described under Materials and Methods. Figure 4 shows double-reciprocal binding plots for ^{3}H factor X association with 20 and 100% PS monolayers. These plots were linear and correlated with dissociation constants of 0.043 and 0.040 μM for 20 and 100%

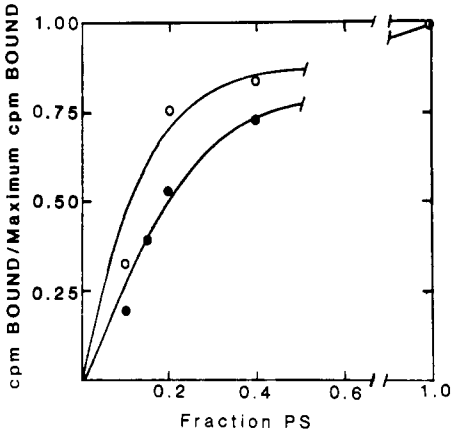

FIGURE 5: Dependence of the relative amount of membrane-bound factor X (●) and V (○) on PS/PC composition. Radioactive protein associated with the monolayer was determined by hydrophobic paper recovery as described under Materials and Methods. Pure PS monolayers represent the maximum theoretical number of protein binding sites, and amounts of factor X bound to other monolayers are given as a fraction of this value. The maximum radioactivity bound was 4.8×10^3 cpm for factor X and 4.9×10^4 cpm for factor V. Initial surface pressure used was 25 ± 1.0 dyn/cm for factor X and 32 ± 1 dyn/cm for factor V.

PS monolayers, respectively. A dissociation constant of 0.2 μM for factor X binding to phospholipid vesicles containing 20% PS at 2 mM calcium was reported by Nelsestuen & Broderius (1977). This system was not saturated in calcium, and the apparent dissociation constant at 10 mM calcium was reported to be 0.07 μM (van Dieijen et al., 1981). At infinite free protein (Figure 4) 5.3 and 8.2 μg of factor X bound to the 20 and 100% PS films, respectively. The latter value corresponded to a surface area of 1400 Å^2 per membrane-bound factor X molecule.

Between 0 and 20% PS the amount of membrane-associated factor X was approximately proportional to the PS content of the monolayers (Figure 5). Above 20% PS, the amount of protein bound to the monolayer was no longer proportional to the PS content, indicating that some theoretical factor X binding sites on the membrane surface were being sterically excluded (Figure 5). This compared well with the results of Nelsestuen & Broderius (1977) and van Dieijen et al. (1981) using bilayer vesicles.

Membrane-bound ^{3}H factor X was also observed by using PS/PC (10/90) monolayers stabilized at 30 dyn/cm (data not shown). Under these conditions surface pressure changes caused by factor X were not detectable (Figure 3). This is similar to the results obtained for prothrombin–monolayer binding (Mayer et al., 1983).

Reversibility of factor X–membrane binding was demonstrated by two methods. After equilibration of ^{3}H factor X with PS/PC monolayers, addition of excess EDTA to the subphase resulted in loss of membrane-associated protein. In a second approach, ^{3}H factor X was equilibrated with 20% PS monolayers. Subsequent addition of a 6-fold excess of unlabeled factor X to the subphase resulted in 73% less radioactivity associated with the phospholipid film. Both of these measurements were done with the monolayer recovery technique.

Factor V(a) Adsorption to PS/PC Monolayers. Factor V induced surface pressure changes in phospholipid monolayers also demonstrated biphasic dependence on the initial surface pressure of the lipid film (Figure 6). The critical pressure for factor V obtained by using pure PC monolayers was 27 dyn/cm. This value was only slightly higher than the surface pressure of factor V protein in the absence of phospholipid

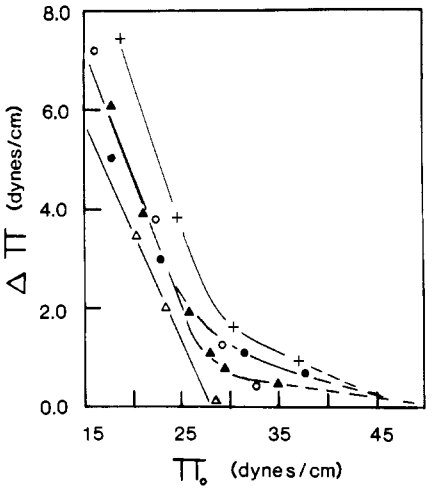

FIGURE 6: Dependence of surface pressure increase induced by factor V(a) on initial spread pressure of PS/PC monolayers. Phospholipid films used were (△) 100% PC, (○) 20% PS-80 percent PC, (●) 40% PS–60% PC, and (▲) 100% PS. Protein was injected to give a total concentration of 5×10^{-8} M. Results for the 80 000-dalton factor Va peptide injected below monolayers of 40% PS are also shown (+).

Table I: Depletion of Subphase Factor V(a) in the Presence of Phospholipid Monolayers[a]

mol of V(a) added	PL	% remaining in subphase	mol bound[b]
2.28×10^{-11}	PC	98	
2.28×10^{-11}	PS	21	1.82×10^{-11}
4.46×10^{-11}	PS	29	3.2×10^{-11}
9.12×10^{-11}	PS	62	3.50×10^{-11}
4.46×10^{-11}	PS/PC (20/80)	76	1.06×10^{-11}
4.46×10^{-11}	PS/PC (40/60)	60	1.78×10^{-11}
	PS/PC (40/60) plus 100 μg of PS/PC (40/60) vesicles added after 1.5 h	99	

[a] The enzymatic assay described under Materials and Methods was used to quantitate the factor V(a) concentration remaining in the subphase (10 mL) after equilibrated for 1.5 h in the presence of various monolayers. π_0 was 30 ± 1 dyn/cm. [b] The moles of factor V(a) bound to the phospholipid monolayer was calculated by multiplying the percent depletion times the total moles of factor V(a) added.

(Figure 2). These surface pressure changes were nearly independent of PS/PC composition, and the kinetics were similar to those of factor V adsorption to the air–water interface (Figure 2).

Above 27 dyn/cm factor V induced increases in surface pressure were complete within 30 min and were dependent on the presence of PS in the membrane (Figure 6). The latter observations are consistent with the characteristics observed for factor V binding to phospholipid vesicles (Bloom et al., 1979; Pusey et al., 1982) and indicated that these surface pressure changes were related to a specific protein–acidic phospholipid interaction. The surface pressure change caused by factor V binding to 100% PS monolayers was slightly less than that for binding to 40% PS monolayers (Figure 6). This did not, however, reflect a decrease in the actual amount of membrane-bound protein as detected by monolayer recovery (Figure 5) or by subphase depletion (see below). This behavior is complex and suggested that direct proportionality did not always exist between $\Delta\pi$ and extent of bound factor V. Thrombin digestion of this protein did not detectably alter the surface pressure changes induced by protein at 30 dyn/cm for membranes of 20, 40, and 100% PS.

The relative amount of iodinated factor V associated with the monolayer increased dramatically between 0 and 20 percent PS (Figure 5) and rapidly reached a maximum value at higher PS densities. These results may indicate steric protein saturation and are consistent with the observations of Bloom et al. (1979) and Pusey et al. (1982), who used bilayer vesicle systems. The amount of factor V associated with the monolayer was also quantitated by measuring depletion of protein from the subphase by employing the enzymatic assay described under Materials and Mathods. This procedure did not require disruption of the phospholipid film and used underivatized protein. The first experiment in Table I indicated that factor V did not associate with pure PC monolayers spread at 30 dyn/cm and that adsorption of protein to the trough was insignificant. Depletion of factor V from the subphase in other experiments was therefore attributed to protein–phospholipid interactions.

The amount of factor V bound to 100% PS monolayers increased to apparent saturation (3.5×10^{-11} mol) as the total amount of factor V injected below the monolayer was increased (Table I). Decreasing the PS content in the monolayer also decreased the amount of factor V depleted from the subphase (Table I). The factor V–membrane binding characteristics at saturating protein concentrations (Figure 5) indicated that this effect was due mainly to a lower binding affinity for these membranes; 20% PS monolayers bound about 75% of the factor V of pure PS (Figure 5). On the basis of this relationship, about half of the protein binding sites on 40% PS monolayers appeared filled in the experiment at 2 nM free factor V (Table I).

The results in Table I also demonstrate that the factor V–membrane interaction was reversible. Factor V (4.46×10^{-11} mol) was added beneath a PS/PC (40/60) monolayer and allowed to equilibrate for 1.5 h. A 40% reduction in the subphase factor V activity was observed (Table I). Fifty times the amount of phospholipid contained in the monolayer was then injected below the lipid film in the form of PS/PC (40/60) vesicles. Previous studies have shown that factor V redistributes over the total phospholipid population under such conditions (Pusey et al., 1982). As expected for a reversible interaction, the total factor V activity was then recovered in the subphase (Table I). This experiment also established that the depletion of factor V from the subphase in the presence of PS-containing monolayers was due to protein–membrane binding and not to phospholipid-dependent destruction of enzymatic activity. This experiment was repeated for thrombin-treated protein and gave 36% subphase depletion with full reversibility.

Comparison of Surface Pressure Changes Induced by Various Proteins. Myelin basic protein and poly(L-lysine) have been studied by using lipid monolayer systems (Demel et al., 1973; London et al., 1973; Shafer, 1974) and were used here for purposes of comparison. Myelin basic protein has been shown to induce similar surface pressure changes in PS or total central nervous system lipid monolayers (Demel et al., 1973). At low initial surface pressures, poly(L-lysine) induced a transient decrease in the surface pressure of PS films followed by a small surface pressure increase (Shafer, 1974). It was concluded that the surface pressure decrease was due to compression of the lipid film upon neutralization of the negative charge on the PS head groups. In our studies calcium was added to the subphase which precompresses the PS monolayer (Verger & Pattus, 1982), so the transient decrease in surface pressure was not observed. The maximum surface

Table II: Maximum Surface Pressure Changes Induced by Acidic Phospholipid-Binding Proteins[a]

protein	PL	$\Delta\pi$ (dyn/cm)
fragment 1	PS/PC (20/80	1.3
factor X	PS/PC (20/80)	0.7
factor V	PS/PC (20/80)	1.3
factor V + thrombin	PS/PC (20/80)	1.3
myelin basic protein	PS/PC (20/80)	1.0
poly(L-lysine)	PS/PC (20/80)	0.8
80 000-dalton factor Va peptide	PS/PC (40/60)	1.6
myelin basic protein	PS/PC (80/20)	3.5
poly(L-lysine)	PS/PC (80/20)	3.0
myelin basic protein	PS	6.0

[a] Proteins were added below monolayers stabilized at 30 ± 1 dyn/cm in concentrations sufficient to achieve maximum surface pressure changes. All experiments were done in the presence of 10 mM calcium.

pressure increases here for poly(L-lysine) and myelin basic protein (Table II) were similar to those obtained in the previous studies (Demel et al., 1973; Shafer, 1974).

Table II shows that myelin basic protein induced and poly(L-lysine)-induced surface pressure changes were generally proportional to the PS content of the monolayer between 0 and 100% PS. Myelin basic protein and factor V exhibit an acidic phospholipid/bound protein stoichiometry of about 25–30 (Boggs et al., 1977, 1981; Pusey et al., 1983). At low amounts of PS (20%) in the monolayer the amount of protein–membrane binding is restricted by the availability of PS residues (Boggs et al., 1977; Pusey et al., 1982), and the number of molecules of membrane-bound myelin basic protein and factor V(a) should be comparable. Under these conditions both proteins caused similar surface pressure increases (Table II). Thrombin treatment of factor V had no effect on the surface pressure changes observed. Poly(L-lysine), factor X, and fragment 1 also induced similar surface pressure changes in monolayers containing 20% PS (Table II). The isolated 80 000-dalton peptide of factor Va caused pressure changes similar to factor V (Figure 6 and Table II).

Discussion

The surface pressure of the erythrocyte membrane has been estimated to be 31 dyn/cm (Demel et al., 1975). Relevant biomembranes also contain between 10 and 20% acidic phospholipid (Etemali, 1980). Thus, phospholipid monolayers containing 10–20% PS spread at an initial surface pressure of approximately 30 dyn/cm should be most important in characterizing the association of blood coagulation proteins with biological membranes.

For 20% PS monolayers spread at an initial surface pressure of 30 dyn/cm the evidence indicated that protein–membrane binding was limited by the availability of PS residues for all the protein species studied here. Under these conditions the surface pressure changes induced by the prothrombinase proteins, myelin basic protein, and poly(L-lysine) were of similar magnitude (Table II). Myelin basic protein also appeared similar to factor V in the number of acidic phospholipids per bound protein molecule and the rapid rate of the protein–membrane association (Lampe et al., 1983; Pusey et al., 1982). Overall, myelin basic protein may provide a comparable protein–membrane interaction to that of factor V(a). At 20% PS the molar density of the vitamin K dependent proteins on the membrane was approximately 3–5 times greater than myelin basic protein or factor V(a) (Nelsestuen & Broderius, 1977; Pusey et al., 1982; Lampe et al., 1983). These latter proteins therefore cause a smaller membrane perturbation per membrane-bound protein molecule.

The small surface pressure change obtained for these proteins could be the result of several phenomena. Insertion of small amounts of peptide material into the hydrocarbon region of the membrane would produce surface pressure changes. Since polylysine produced similar changes, any insertion would probably involve small moieties, potentially no larger than a few atoms. It is possible that lateral phase separation produces surface pressure changes; in a previous study we reported that monolayers containing mixtures of PS/PC required lower surface areas per molecule than pure phosphatidylcholine (Mayer et al., 1983). Lateral phase separation would produce areas of pure phosphatidylcholine which require a larger surface area per molecule and could increase surface pressure. The magnitude of a change based on this phenomenon would depend on the area per molecule in the phosphatidylserine phase. Another possibility is that protein binding to the head group results in different phospholipid head group–acyl chain interactions, a larger area per phospholipid, and increased surface pressure. The latter explanations can account for the observation that the vitamin K dependent proteins bind under conditions where no surface pressure changes were detected. The results for factor V suggested an actual decrease in surface pressure between 40 and 100% phosphatidylserine (Figure 6). This was not accompanied by a decrease in membrane-bound protein. On the basis of these properties we currently favor a factor V–membrane association that does not involve protein insertion into the hydrocarbon region of the membrane. Further studies will be necessary to distinguish the true basis of these small surface pressure changes.

Myelin basic protein has been proposed to interact hydrophobically as well as electrostatically with membranes (Boggs & Moscarello, 1978; Boggs et al., 1980, 1981; Demel et al., 1973; London et al., 1973). London et al. (1973) and Boggs et al. (1980) have suggested that small portions of the myelin basic protein penetrate into the membrane interior. The surface pressure changes induced by myelin basic protein in pure phosphatidylserine are relatively large and may signify some insertion.

It is clear that considerable factor V(a)–monolayer binding occurs at nanomolar concentrations of free protein (Table II). It follows that the dissociation constant is nanomolar or lower. Previous measurements of association and dissociation rate constants for factor V binding to 20% PS vesicles at 10 °C and in the absence of calcium indicated a dissociation constant of about 5×10^{-11} M (Pusey et al., 1982). The presence of calcium and temperature elevation to 25 °C had individual effects which together would increase the dissociation constant about 10-fold. The result is only a few fold lower than the concentrations used in Table II. These two studies are essentially corroborative, and membrane surface curvature appears to have a small or negligible effect on factor V(a)–membrane binding.

In a previous study little difference between the membrane binding characteristics of single chain factor V and factor Va, was observed (Pusey et al., 1982). Protein–membrane dissociation rate constants obtained for the 80 000-dalton peptide suggest that the intact membrane-binding portion of factor V is associated with this peptide (unpublished observations). In this study the 80 000-dalton peptide produced surface pressure changes similar to factor Va (Figure 6; Table II). These results suggested that no major change in the membrane-binding region of factor V occurred during these partial proteolysis events. Preliminary data and labeling with lipid-soluble photoreactive agents indicate that the membrane-

binding segment can be further reduced to a peptide of 50 000 daltons (Mayer, 1983).

At low initial surface pressures, factors X and V(a) caused large surface pressure changes that were nearly independent of the acidic phospholipid component. These surface pressure changes exhibited characteristics similar to those of protein in the absence of phospholipid and did not appear to be due to specific protein–phospholipid interactions [see Doty & Schulman (1949) Matalon & Schulman (1949), Quinn & Dawson (1970), Kimelberg & Papahadjopoulos (1971), and Bhakoo et al. (1982)]. The critical pressures for surface changes were also below the probable surface pressures of biological membranes. Consequently, these larger surface pressure changes may not be relevant to the biological protein–membrane interaction. A previous study of prothrombin fragment 1 association with phospholipid monolayers failed to detect a biphasic plot of the type shown in Figure 2 (Mayer et al., 1983). This may be due to the low collapse pressure for fragment 1 at the air–water interface (Figure 1); surface pressure changes were not studied below the collapse pressure for fragment 1 adsorbing to the air–water interface in the earlier report.

The monolayer studies presented here for factor X and factor V(a) along with those described for prothrombin (Amyer et al., 1983) indicate that the prothrombinase proteins associated with membranes primarily, if not exclusively, through protein–acidic phospholipid polar head-group interactions.

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Registry No. Factor V, 9001-24-5; factor X, 9001-29-0; factor Va, 65522-14-7; poly-L-lysine (homopolymer), 25104-18-1; poly-L-lysine (SRU), 38000-06-5.

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