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Interaction of Bovine Blood Clotting Factor Va and Its Subunits with Phospholipid Vesicles†

Piet van de Waart,‡ Harry Bruls, H. Coenraad Hemker, and Theo Lindhout*

ABSTRACT: Thrombin-activated factor Va and factor Va subunit binding to large-volume vesicles was investigated by a technique based on the separation by centrifugation of phospholipid-bound protein from the bulk solution. This technique allows the direct measurement of free-protein concentration. It is concluded that the phospholipid binding site on factor Va is located on a basic factor Va subunit with M_r 80 000 (factor Va-LC). The effects of phospholipid vesicle composition, calcium concentration, pH, and ionic strength on the equilibrium constants of factor Va– and factor Va-LC–phospholipid interaction were studied. Factor Va and factor Va-LC binding to phospholipid requires the presence of negatively charged phospholipids. It is further demonstrated that the following occur: (a) Calcium ions compete with factor Va and factor Va-LC for phospholipid-binding sites. (b) The dissociation constant of protein–phospholipid interaction increases with the ionic strength, whereas the maximum protein-binding capacity of the phospholipid vesicle was not affected by ionic strength. (c) The dissociation constant for factor Va–phospholipid interaction depends on pH when the vesicle consists of phosphatidic acid. It is concluded that factor Va–phospholipid interaction is primarily electrostatic in nature, where positively charged groups on the protein directly interact with the phosphate group of net negatively charged phospholipids. The results suggest that factor Va, like factor Xa and prothrombin, has the characteristics of an extrinsic membrane protein.

The prothrombinase complex catalyzes the proteolytic conversion of the zymogen prothrombin to the serine protease thrombin. The components that constitute the complex are factor Xa (the catalytic component), calcium ions, phospholipid, and factor Va [see Jackson & Nemerson (1980) for a review]. Factor Va, which is derived by limited proteolysis of a single-chain precursor of M_r 330 000, functions as a cofactor (Esmon, 1979; Nesheim & Mann, 1979). The effects of factor Va and phospholipids on the kinetic parameters of prothrombin activation have given insight in the mode of action of nonenzymatic cofactors in prothrombin activation (Rosing et al., 1980).

Knowledge of the molecular details of the assembly of the prothrombinase complex is of importance in order to understand the mechanism of prothrombin activation at a phospholipid–water interface. From the studies reported by Nelsestuen and co-workers, a detailed model of the prothrombin– and factor Xa–membrane complexes can be drawn (Lim et al., 1977; Resnick & Nelsestuen, 1980; Wei et al., 1982).

The nature of the factor Va–phospholipid interaction has still to be disclosed. In most previous studies, gel filtration has been used to elucidate the factor V(a)–phospholipid-binding characteristics. On the basis of these qualitative studies, it appeared essential to have net negative charged phospholipids in the membrane for factor V–membrane interaction (Subbaiah et al., 1976). In addition, the possibility that the binding of factor V to membranes is mediated by calcium ions can be ruled out (Greenquist & Colman, 1975; Subbaiah et al., 1976). In essence, those observations were confirmed in a study that utilizes factor V of high purity and a method of analysis that yields quantitative equilibrium-binding data (Bloom et al., 1979). It was suggested that factor Va–membrane interaction is nonelectrostatic in nature. The role of phosphatidylserine in the protein–lipid nonelectrostatic interaction could not be explained (Bloom et al., 1979).

The purpose of this study is to establish the mode of action of acidic phospholipid in the factor Va–phospholipid interaction. We utilized large-volume unilamellar vesicles, which can be separated from the bulk solution by centrifugation, for determination of binding parameters of the factor Va– and factor Va subunit–phospholipid interactions. The binding technique presented here is a valuable tool in the study of the effect of phospholipid composition, pH, ionic strength, and calcium on factor Va–phospholipid interaction.

Materials and Methods

S 2238[1] and S 2337 were purchased from AB Kabi Diagnostica. Soybean trypsin inhibitor (type I-S), *Echis carinata*

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[1] Abbreviations: S 2238, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S 2337, *N*-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride; RVV-X, factor X activator purified from Russell's viper venom; RVV-V, factor V activator purified from Russell's viper venom; Tris, tris(hydroxymethyl)aminomethane; STI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; LC, light chain; HC, heavy chain.

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venom, Russell's viper venom, ovalbumin, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were obtained from Sigma. *N*-Succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)-propionate was from Amersham.

The following bovine blood coagulation factors were prepared: factor X_2 (Fujikawa et al., 1972a), factor Xa (Fujikawa et al., 1972b), prothrombin (Owen et al., 1974), thrombin (Rosing et al., 1980), and factor V and factor Va (Lindhout et al., 1982). RVV-V was isolated as described by Kisiel (1979). ^{125}I-Labeled factor V was prepared according to Bolton & Hunter (1973) using 10 mCi of ^{125}I/mg of protein. The specific radioactivity of ^{125}I-labeled factor V was 2610 cpm/ng (0.4 mol of ^{125}I/mol of factor V). ^{125}I-Labeled factor V retained about 90% of its activity and had the same electrophoretic mobility on sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis prior to and after labeling.

Protein Concentrations. Determination of prothrombin by enzymatic assay was carried out after conversion to thrombin. Typically, 5–100 nM prothrombin in 50 mM Tris–100 mM NaCl–0.5 mg of ovalbumin/mL (pH 7.5) was incubated with *Echis carinata* venom (4 μg/mL) for 10 min at 37 °C. Within this time, complete conversion of prothrombin into thrombin was obtained. The molar concentration of thrombin formed was measured as described by Rosing et al. (1980). The concentration of prothrombin as determined by the enzymatic assay agrees very well with that calculated from the E_{280} with $E_{280}^{1\%}$ = 15.5 and 72000 for the molecular weight of prothrombin (Owen et al., 1974). The concentration of factor Xa was determined as described by van Dieijen et al. (1981).

The factor Va assay is based on the formation of an equimolar factor Va–factor Xa complex as the catalytic unit in prothrombin activation (Lindhout et al., 1982). Under conditions where factor Xa is far in molar excess over factor Va, the rate of prothrombin activation is linear with the concentration of factor Va. A factor Va containing sample after the appropriate dilution in 50 mM Tris–100 mM NaCl–5 mM $CaCl_2$–0.5 mg of ovalbumin/mL (pH 7.5) was incubated with factor Xa (1.3×10^{-11} M), phospholipid (20% DOPS/80% DOPC, 10 μM), and $CaCl_2$ (5 mM) in 50 mM Tris–100 mM NaCl–0.5 mg of ovalbumin/mL (pH 7.5) for 5 min at 37 °C in a plastic cuvette. Prothrombin activation was started by the addition of 0.1 mL of prothrombin (2 μM). The final volume is 1.0 mL. After 2 min, the reaction was stopped by the addition of 1.0 mL of buffer containing 50 mM Tris–100 mM NaCl–0.5 mg of ovalbumin/mL–20 μg of STI–0.47 μmol of S 2238 (pH 7.5). The amount of thrombin formed was measured by the change in absorbance recorded on an Aminco DW-2 spectrophotometer operating in the dual-wavelength mode (λ_s = 405 nm and λ_r = 500 nm) thermostated at 37 °C. Standard curves were constructed by assaying dilutions of a factor Va preparation of which the concentration was determined by titration with active site titrated factor Xa (Lindhout et al., 1982). The assay had a functional range from 2×10^{-14} to 1.0×10^{-12} M of factor Va. The functional factor Va concentration was about 95% of the concentration as determined from E_{280} with $E_{280}^{1\%}$ = 9.6 and 330000 for the molecular weight of unfractionated factor Va (Nesheim et al., 1979).

The concentration of factor V was determined by the factor Va assay after activation with thrombin. Typically, the activation reached completion within 10 min at 37 °C with a factor V:thrombin molar ratio of 200:1.

The concentration of factor Va-LC was estimated by its ability to restore factor Va activity when incubated with factor Va-HC in the presence of calcium (Lindhout et al., 1982). Since neither one of the polypeptide chains of which factor Va is composed exhibits factor Va activity, maximum factor Va activity, as a result of saturation of factor Va-LC with factor Va-HC, gives the molar concentration of factor Va-LC preparations from a factor Va standard curve. If one knows the specific radioactivity (cpm/μmol), the molar concentration of ^{125}I-labeled factor Va-LC can be estimated from radioactivity measurements.

Phospholipid and Phospholipid Vesicle Preparation. 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as described by Comfurius & Zwaal (1977). Sonicated vesicle solutions were prepared from mixtures of DOPS and DOPC in a buffer containing 50 mM Tris–100 mM NaCl (pH 7.5) as described by de Kruijff et al. (1975) with a MSE Mark II ultrasonicator set at 7-μ amplitude.

Large-volume vesicles were prepared by a modification (Van der Steen et al., 1981) of the ether-injection method according to Deamer & Bangham (1976). These large (300-nm diameter) vesicles are unilamellar (Van der Steen et al., 1981). In case the vesicles were used in ionic strength studies, the buffer contained the appropriate NaCl concentration. In order to remove residual ether, it was necessary to dialyze the vesicle solutions overnight at 4 °C with several changes of buffer. Large membrane structures were removed by centrifugation for 10 min at 4000*g* and 20 °C. The supernatant was centrifuged for 30 min at 30000*g* and 20 °C. The pellet was resuspended in 50 mM Tris–100 mM NaCl (pH 7.5).

Thin-layer chromatography of the vesicle preparation as carried out according to Broekhuyzen (1969) showed that no alterations did occur in the membrane composition as a result of the procedure of vesicle preparation. The internal volume of the vesicles was determined from the amount of trapped arsenazo III, according to Serham et al. (1981). For the trapped volume of vesicles containing 20% DOPS and 80% DOPC, 10–14 L/mol of lipid was found, which agrees with the value reported by Deamer & Bangham (1976). Whenever a membrane composition is expressed as a percentage of acidic phospholipid, the remaining phospholipid is DOPC.

Procoagulant activity of phospholipid vesicles was determined under the same conditions as described for the factor Va assay. A phospholipid-containing sample was incubated with factor Xa (1.3×10^{-11} M) and factor Va (5.0×10^{-10} M). Standard curves were constructed by assaying dilutions of phospholipid of which the concentrations were determined by phosphate analysis (Böttcher et al., 1961). The assay had a functional range from 1 to 100 nM.

Protein-Binding Measurements. Mixtures of large-volume vesicles and protein (0.2–0.5 mL) were incubated in 1.5-mL Eppendorf conical centrifuge tubes for 10 min at ambient temperature (20–23 °C). Prior to and after centrifugation for 30 min at 30000*g* and 20 °C, small aliquots (10 μL) were withdrawn and assayed for protein as described in this section for determination of total protein concentration and the concentration of unbound protein, respectively. The phospholipid concentration in the supernatant was less then 0.1% of the total amount added, as determined by the phospholipid procoagulant activity assay. Care has to be taken to avoid nonspecific binding and/or loss in activity during the experiment. Control experiments in the absence of phospholipid established that prior to and after centrifugation the recovery of protein was virtually 100%. Moreover, prothrombin and factor Xa binding by the large-volume vesicles was reversible as shown by the addition of EDTA to dissolve the complex after centrifugation, followed by resuspending the vesicles and a second spin down.

Table I: Phospholipid-Binding Properties of Prothrombin, Factor X, Factor Xa, Factor V, and Factor Va

protein	total lipid[a] concn (μM)	K_d ($\times 10^{-7}$) (M)	n[b]
prothrombin	100	1.9	240
	50	2.0	255
	35[c]	6.0[e]	100[e]
factor X	50	1.9	91
	25	1.7	91
	35[c]	2.5[e]	80[e]
factor Xa	50	1.7	91
	25	2.0	80
factor V	100	2.8	416
	50	2.7	385
	33[d]	0.7[f]	270[f]
factor Va (thrombin activated)	50	0.44	84
	25	0.58	100
	33[d]	4.3[f]	76[f]
factor Va (RVV-V activated)	50	0.63	150

[a] 20% DOPS/80% DOPC. [b] Phospholipid to protein ratio at saturation (mol/mol). [c] 20% PS (bovine brain)/80% PC (egg yolk)/1% cerebrosides. [d] 25% Folch fraction III/75% soybean PC. [e] Data taken from Nelsestuen & Broderius (1977). [f] Data taken from Bloom et al. (1979).

It was also checked that phospholipid, carried over from the reaction mixture prior to centrifugation, had no effect on the protein assays.

Protein binding data were analyzed by double-reciprocal plots with the following equation:

$$1/B = (1/F)(K_d/n) + 1/n$$

in which B and F are concentrations of bound protein and free protein, respectively. The dissociation constant, K_d, and maximum protein-binding capacity of the membrane, n, were calculated from the horizontal and vertical intercepts, respectively.

Results

Prothrombin–, Factor X–, Factor Xa–, Factor V–, and Factor Va–Phospholipid Binding. Various amounts of protein (0.02–0.5 μM) were incubated with large-volume vesicles containing 20% DOPS in 50 mM Tris–100 mM NaCl–3 mM $CaCl_2$–0.5 mg of ovalbumin/mL for 10 min at room temperature prior to centrifugation. From the measured equilibrium concentration of free protein, the binding parameters were calculated as described under Materials and Methods. The factor V binding experiments were carried out with ^{125}I-labeled factor V. Different phospholipid concentrations were used to ascertain that the binding parameters are independent of the phospholipid concentration. The binding parameters obtained are listed in Table I, together with the values reported for prothrombin and factor X (Nelsestuen & Broderius, 1977) and factor V and factor Va (Bloom et al., 1979). The K_d values for prothrombin, factor X, and factor Xa agree very well with the K_d values determined from light-scattering experiments (Nelsestuen & Broderius, 1977). In contrast to the data reported by Bloom et al. (1979), we found that factor Va binds to phospholipid with about 5 times the affinity of factor V. The reason for this difference is not known. Eight independent factor Va–phospholipid-binding analyses, using different factor Va and vesicle preparations, gave a dissociation constant of $(5.6 \pm 1.1) \times 10^{-8}$ M (mean ± SD).

As shown in Table I, the maximum protein-binding capacity of membranes, n, did not vary with the phospholipid concentration. The n values, in particular for prothrombin–phospholipid interaction, are higher in this study than in those by light scattering (Bloom et al., 1979; Nelsestuen & Broderius, 1977). This may reflect some heterogeneity with respect to the unilamellar nature of the vesicles. Light-scattering experiments show that the proteins used in this study do not induce vesicle–vesicle interaction. Therefore, an underestimation of the n values seems unlikely. From these experimental results we conclude that the technique presented here is a valuable tool for the determination of equilibrium constants for protein–lipid interaction and yields equilibrium constants that agree very well with those determined by relative light-scattering experiments.

Identification of Phospholipid-Binding Subunit of Factor Va. The facts that factor Va–membrane interaction depends on the presence of acidic phospholipids and factor Va consists of a cationic and anionic component (Esmon, 1979) might indicate that factor Va has a positively charged area that directly interacts with net negatively charged phospholipid. In order to find evidence for this hypothesis, it was first necessary to separate the chains of factor Va. Whereas cleavage of the RVV-V-sensitive bond results in complete activation of factor V, activation of factor V by thrombin requires several bond cleavages and gives rise to at least four different fragments (Lindhout et al., 1982; Kane et al., 1982). In order to avoid interference in our binding studies of contaminating radiolabeled peptides, which might be present in the isolated subunits derived from thrombin-activated factor Va, we prefered to study the phospholipid-binding properties of the subunits of which RVV-V-activated factor Va is composed. To this end, ^{125}I-labeled factor V was incubated with RVV-V, and after completion of the reaction, the activation fragments were separated by ion-exchange chromatography. Factor V upon incubation with RVV-V is converted into two polypeptide chains with M_r 270 000 and 80 000. A 30-fold increase in factor Va activity was obtained. The peptides were separated by ion-exchange chromatography (Lindhout et al., 1982). The M_r 80 000 and 270 000 peptides eluted at 50 and 250 mM NaCl, respectively. Neither peptide possessed factor Va activity after incubation for 2 h at 37 °C in the presence of 25 mM $CaCl_2$. The respective specific radioactivities are 5790 cpm/μg for ^{125}I-labeled factor Va-HC and 1280 cpm/μg for ^{125}I-labeled factor Va-LC.

The different charge properties of the peptides were studied by isoelectrofocusing on LKB ampholine PAG plates (pH range 3.5–9.0). The isoelectric point of factor Va-HC was about 5.5, whereas factor Va-LC was detected at the extreme cathodic position, indicating that the isoelectric point of factor Va-LC must be higher than 9.0.

The binding of ^{125}I-labeled factor Va-LC and ^{125}I-labeled factor Va-HC to vesicles containing 20% DOPS was measured as a function of the phospholipid concentration. Plotted in Figure 1 is the percentage of added protein bound vs. the phospholipid concentration. At the highest phospholipid concentration, virtually no factor Va-HC was bound, whereas half of the amount of added factor Va-LC was bound at 5 μM phospholipid. It can be questioned whether factor Va-HC labeling on the amino groups does not destroy lipid binding. However, the factor Va activity that could be restored after incubation of the supernatants of the factor Va-HC vesicle mixtures with factor Va-LC in the presence of calcium was independent of the phospholipid concentration. This indicates that no detectable amounts of factor Va-HC were bound to phospholipid vesicles. These results ascertain that the phospholipid-binding site of factor Va is located on a basic factor Va subunit with M_r 80 000.

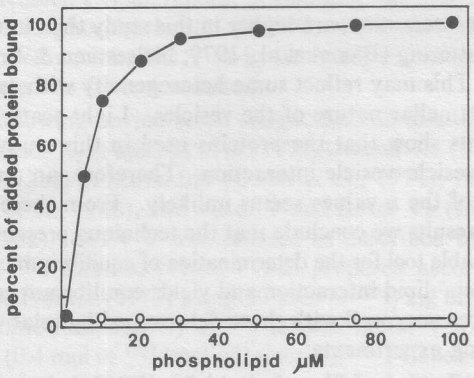

FIGURE 1: Binding of ^{125}I-labeled factor Va-HC and ^{125}I-labeled factor Va-LC as a function of phospholipid concentration. The phospholipid is 20% DOPS in 50 mM Tris–100 mM NaCl–1 mg of ovalbumin/mL (pH 7.5). ^{125}I-Labeled factor Va-HC [(○), 27 μg/mL] or ^{125}I-labeled factor Va-LC [(●), 8.2 μg/mL] was added, and free and bound protein concentrations were estimated as described under Materials and Methods.

Table II: Factor Va and Factor Va-LC Binding to Vesicles of Varying DOPS Content

DOPS (%)	factor Va		factor Va-LC	
	K_d (μM)	n^a	K_d (μM)	n^a
0	no binding		no binding	
5	0.42	152	0.50	71
10	0.25	115	0.13	56
15	0.071	80	0.030	48
20	0.042	71	ND[b]	ND
30	0.020	75	0.018	40

[a] Phospholipid to protein ratio at saturation (mol/mol). [b] ND, not determined.

Factor Va-LC Binding as a Function of Phosphatidylserine Content. The results of factor Va binding experiments with vesicles of varying Folch fraction III content reported by Bloom et al. (1979) suggest that the binding affinity of factor Va is directly proportional to Folch fraction III content in the region below ~25% Folch fraction III (or ~20% phosphatidylserine). To determine whether factor Va-LC–phospholipid interaction is also dependent on the presence of acidic phospholipid, we carried out binding experiments with large-volume vesicles of varying DOPS content. Various amounts of ^{125}I-labeled factor Va-LC (0.1–1.0 μM) were incubated with 20 μM phospholipid (DOPS content ranging from 0 to 30%) in 50 mM Tris–100 mM NaCl–1 mM $CaCl_2$–1 mg of ovalbumin/mL (pH 7.5) for 10 min at room temperature prior to centrifugation. The equilibrium concentrations of free and bound ^{125}I-labeled factor Va-LC were determined as described under Materials and Methods. The binding parameters were calculated from double-reciprocal plots of free factor Va-LC vs. bound factor Va-LC. Under the same conditions, we carried out factor Va binding experiments. The binding parameters obtained are presented in Table II).

Factor Va and factor Va-LC do not bind to 100% DOPC vesicles. It is apparent that the binding affinity of both factor Va and factor Va-LC increases with an increase in the DOPS content of the membrane. Moreover, the affinities of factor Va-LC and factor Va are nearly identical. The maximum protein-binding capacity also appears to increase with the increase of DOPS content of the membrane. Therefore, the factor limiting both factor Va and factor Va-LC binding is assumed to be availability of phosphatidylserine. Packing of protein on the membrane at high phosphatidylserine content may be a limiting factor in factor Va and factor Va-LC binding. However, our experiments do not allow further conclusions on this subject.

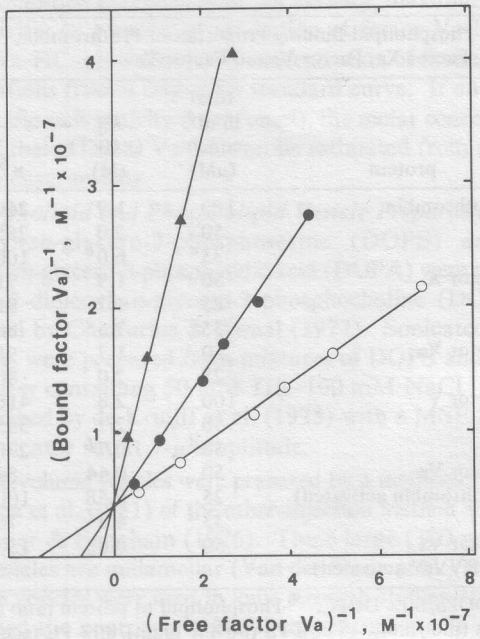

FIGURE 2: Double-reciprocal plots of factor Va–phospholipid binding at varying calcium concentrations. Free and bound factor Va concentrations were estimated as described under Materials and Methods. Large-volume vesicles of 20% DOPS (20 μM) in 50 mM Tris–100 mM NaCl–1 mg of ovalbumin/mL (pH 7.5) were used. The plots show data obtained at the following calcium concentrations: (○) 0.5 mM $CaCl_2$; (●) 5 mM $CaCl_2$; (▲) 10 mM $CaCl_2$.

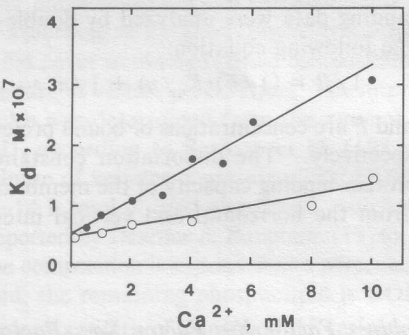

FIGURE 3: Summary of dissociation constants (K_d) for ^{125}I-labeled factor Va-LC–phospholipid binding (○) and factor Va–phospholipid binding (●) at different calcium concentrations. The dissociation constants calculated from double-reciprocal plots (see, e.g., Figure 2) are plotted vs. added calcium. The phospholipid vesicle composition and phospholipid concentration are as given in Figure 2.

Effect of Calcium on Factor Va- and Factor Va-LC–Phospholipid Interactions. In view of the phosphatidylserine-dependent binding of the net positively charged factor Va subunit, it is of particular interest to examine the effect of calcium on quantitative equilibrium-binding data. If the interaction of factor Va with phospholipid is essentially an ionic process, ionic shielding of the negatively charged phospholipid polar head group by calcium (Hauser et al., 1976) would have an effect on the dissociation constant rather than on maximum protein-binding capacity.

A factor Va binding experiment using 20% DOPS large-volume vesicles at different calcium concentrations is shown in Figure 2. Calcium ions have an effect on the dissociation constant of factor Va–phospholipid interaction and not on the maximum protein-binding capacity. In other words, factor Va and calcium ions compete for binding sites on the phospholipid vesicles. We were not able to determine K_d and n values for factor Va–phospholipid interaction in the absence

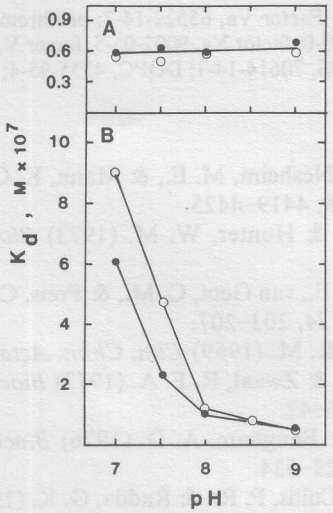

FIGURE 4: Effect of pH on dissociation constants (K_d) of factor Va- and ^{125}I-labeled factor Va-LC–phospholipid interaction. The dissociation constants were estimated from double-reciprocal plots as shown in Figure 2. The buffer used was 50 mM Tris–100 mM NaCl–0.2 mM $CaCl_2$–1 mg of ovalbumin/mL (pH values 7–9). The two vesicle compositions are 20% DOPS (panel A, 20 μM) and 20% DOPA (panel B, 25 μM). The dissociation constants for ^{125}I-labeled factor Va-LC–phospholipid binding (○) and factor Va–phospholipid binding (●) at varying pH are shown.

of exogenous Ca^{2+}, since under this condition loss of factor Va activity was observed. From a replot of apparent K_d values vs. calcium concentration (Figure 3), it was calculated that in the absence of exogenous calcium the dissociation constant for factor Va–phospholipid interaction equals 0.045 μM. Also shown in Figure 3 is the replot of apparent K_d values for factor Va-LC–membrane interaction vs. calcium concentration. It is interesting to see that the factor Va-LC–membrane interaction shows a smaller sensitivity to calcium than does the factor Va–membrane interaction.

Another explanation than ionic shielding for the observed calcium effect must be considered. Calcium ions can induce lateral phase separation and irreversible vesicle fusion or aggregation of phospholipids. However, if vesicle fusion and/or aggregation of phospholipid did occur, we would expect a major effect on maximum protein-binding capacity rather than an effect on the dissociation constant. When the order of addition was changed, the same binding parameters were obtained, which means that factor Va and factor Va-LC binding to membranes is reversible.

Effect of pH on Factor Va- and Factor Va-LC–Phospholipid Interactions. The effects of pH on protein–phospholipid interaction were studied for two different vesicle compositions: 20% DOPA and 20% DOPS. The procedures and method of data analysis are similar to those in Figure 2.

The results for phosphatidylserine show minor changes in K_d between pH 7 and 9 (Figure 4). In addition, the protein-binding capacity of the phospholipid vesicle did not vary in this pH range. It is apparent that there are no groups involved in protein–phospholipid interaction that ionize between pH 7 and 9. This is partially expected from the ionization groups found in phosphatidylserine (Hauser et al., 1976). It also implies that no groups in factor Va-LC are involved in protein–lipid interaction that substantially ionize between pH 7 and 9. This observation is consistent with an isoelectric point of factor Va-LC higher than 9.0.

In case of phosphatidic acid, pH has a major effect on the dissociation constants of protein–phospholipid interaction. The protein-binding capacity of the vesicle did not vary between pH 7 and 9. In addition, the protein-binding affinities determined at pH 9 are similar to those of phosphatidylserine.

Table III: Factor Va–Phospholipid[a] Binding to Vesicles at Varying Ionic Strength

[NaCl] (M)	K_d (M)	n^b
0.05	2.2×10^{-8}	80
0.1	6.0×10^{-8}	73
0.5	32.0×10^{-8}	83

[a] 20% DOPS/80% DOPC. [b] Phospholipid to protein ratio at saturation (mol/mol).

These results indicate that monovalent phosphatidic acid, pK_2 ~ 7, has a reduced efficacy in factor Va and factor Va-LC binding as compared to divalent phosphatidic acid and phosphatidylserine. Taking these results together, it appears that surface-charge density is of prime importance for factor Va–phospholipid interaction.

Effect of Ionic Strength on Factor Va–Phospholipid Interaction. Sensitivity to ionic strength is considered to be indicative for electrostatic interaction. Therefore, we studied the effect of NaCl concentration on factor Va–phospholipid interaction. Binding experiments were performed with large-volume vesicles of 20% DOPS (30 μM) in 50 mM Tris–1 mM $CaCl_2$–1 mg of ovalbumin/mL (pH 7.5) and varying NaCl concentrations. The binding parameters are shown in Table III. As anticipated for an ionic interaction, ionic strength has a major effect on the binding affinity. Since the maximum protein-binding capacity of the phospholipid vesicle is unaffected by ionic strength, it is apparent that factor Va competes with Na^+ for the same binding sites. Consistent with the 10^3-fold higher affinity of Ca^{2+} for phosphatidylserine than that of Na^+ (Hauser et al., 1976) is the difference in the Ca^{2+} and Na^+ concentration required to compete with factor Va for phospholipid-binding sites. It further indicates that charge neutralization of the phospholipid surface by "screening" or "binding" of monovalent cations or divalent cations, respectively, to the surface (Puskin & Martin, 1979) is accompanied by the very same modulation of factor Va–membrane interaction.

Discussion

At present, it is not possible to ascertain how factor Va–phospholipid interaction occurs. It has been suggested that factor V and factor Va bind to phospholipid vesicles by a nonelectrostatic interaction (Bloom et al., 1979). On the other hand, the possibility has been addressed that factor V contains some positively charged areas responsible for the binding to negatively charged phospholipid (Subbaiah et al., 1976).

In this study it is demonstrated that RVV-V-activated factor Va consists of an acidic and basic polypeptide. It is shown that the basic factor Va subunit, which has a net positive charge below pH 9, binds to phospholipid, whereas the acidic factor Va subunit is unable to bind to phospholipid. It is apparent that intact tertiary factor Va structure is not required to form the phospholipid-binding region of factor Va, since the equilibrium constants for thrombin-activated Va–, RVV-V-activated factor Va–, and basic factor Va subunit–phospholipid interactions are identical.

Factor Va and the basic factor Va subunit did not bind to phospholipid vesicles containing solely phospholipid in its zwitterion state (e.g., phosphatidylcholine). However, the affinities of the proteins for vesicles increase with phosphatidylserine content. With respect to the dependency of maximum protein-binding capacity of the vesicle on the mole fraction of phosphatidylserine, it has to be mentioned that this binding parameter is relatively insensitive to changes in phosphatidylserine content.

Lateral phase separation of phosphatidylserine molecules in the membrane would be accompanied by a positive free energy. An interesting question is whether lateral phase separation, as the result of factor Va–phospholipid interaction, is a major factor to the observed increase in K_d as the phosphatidylserine content decreases. Further studies are required to make this clear.

The initial question we addressed in this study is does factor Va directly interact with phosphatidylserine molecules in the membrane. Several pieces of evidence indicate that this is the case. Competition for the factor Va and basic factor Va subunit binding site on the phospholipid vesicle come from divalent cations (e.g., Ca^{2+}). This is clearly demonstrated by the linear relationship between the apparent K_d for factor Va– and basic factor Va subunit–membrane interaction and calcium concentration (see Figure 3). Since Ca^{2+}–phosphatidylserine interaction is an ionic process, it is feasible to conclude that phosphatidylserine molecules are directly involved in an electrostatic factor Va–membrane interaction. Additional evidence came from pH and ionic strength studies. The effects of phospholipid charge neutralization are consistent with the proposed electrostatic protein–phospholipid interaction. That is, protonation of divalent phosphatidic acid results in a decrease in affinity of factor Va, whereas maximum protein-binding capacity is unaffected. Apparently, the pK values of the groups in the protein involved in protein–lipid interaction did not allow establishment of charge neutralization of the protein as a major factor to protein–lipid binding in the pH range studied. Further studies will be required to demonstrate unequivocally that basic amino acid residues present in the basic factor Va subunit are involved in the interaction with acidic phospholipids in the membrane. The ionic strength studies reveal that factor Va–phospholipid interaction has to be viewed primarily as an ionic process. However, hydrophobic contacts between factor Va and the hydrocarbon region of phospholipid cannot be eliminated on the basis of our data.

After this work was completed, Pusey et al. (1982) reported the phospholipid-binding properties of factor V. These authors also provide evidence for an ionic interaction between protein and phospholipid. However, their K_d values ($\sim 10^{-10}$ M) for factor Va– and factor V–phospholipid interactions differ significantly. Whereas the light-scattering technique used by Pusey et al. (1982) requires a number of assumptions, our method is direct. But for the moment, a straightforward explanation for the discrepancy in binding data cannot be given.

From a mechanistic standpoint, several interesting questions arise. Since factor Va shows the characteristics of an extrinsic membrane protein that requires a direct interaction with phosphatidylserine molecules in the membrane, factor Va probably competes with prothrombin, factor X, factor Xa, and factor V for membrane-binding sites. Whether or not this might affect the kinetics of prothrombin activation will depend on a variety of parameters, like the number of available phospholipid-binding sties (e.g., phosphatidylserine molecules), the respective protein concentrations, and specific high-affinity protein–protein interactions [e.g., factor Va–factor Xa complex formation (Lindhout et al., 1982)].

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Registry No. Factor Va, 65522-14-7; prothrombin, 9001-26-7; factor X, 9001-29-0; factor Xa, 9002-05-5; factor V, 9001-24-5; Ca, 7440-70-2; DOPS, 70614-14-1; DOPC, 4235-95-4; DOPA, 61617-08-1.

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