Windler, E. E. T., Kovanen, P. T., Chao, Y., Brown, M. S., Havel, R. J., & Goldstein, J. L. (1980c) *J. Biol. Chem. 255*, 10464-10471.
Windmueller, H. G., & Wu, A. L. (1981) *J. Biol. Chem. 256*, 3012-3016.
Windmueller, H. G., Herbert, P. N., & Levy, R. I. (1973) *J. Lipid Res. 14*, 215-223.
Wu, A. L., & Windmueller, H. G. (1978) *J. Biol. Chem. 253*, 2525-2528.
Wu, A. L., & Windmueller, H. G. (1979) *J. Biol. Chem. 254*, 7316-7322.
Zannis, V. I., & Breslow, J. L. (1980a) in *Electrophoresis 1979* (Radola, B. J., Ed.) pp 437-473, Walter de Gruyter, Berlin and New York.
Zannis, V. I., & Breslow, J. L. (1980b) *J. Biol. Chem. 255*, 1759-1762.
Zannis, V. I., & Breslow, J. L. (1981) *Biochemistry 20*, 1033-1041.
Zannis, V. I., Breslow, J. L., & Katz, A. J. (1980) *J. Biol. Chem. 255*, 8612-8617.
Zannis, V. I., Just, P. W., & Breslow, J. L. (1981a) *Am. J. Hum. Genet. 33*, 11-24.
Zannis, V. I., Kunit, D. M., & Breslow, J. L. (1981b) *J. Biol. Chem.* (in press).

Simple and Rapid Method To Determine the Binding of Blood Clotting Factor X to Phospholipid Vesicles†

Gerbrand van Dieijen,* Guido Tans,‡ Jan van Rijn,§ Robert F. A. Zwaal, and Jan Rosing

ABSTRACT: Negatively charged phospholipid vesicles inhibit the activation of factor X by the factor X activating protein from *Vipera russelli* (RVV-X). It is shown that this inhibition is caused by the fact that RVV-X is not able to activate factor X molecules bound to phospholipid bilayers. By use of the kinetic parameters for the activation of factor X by RVV-X, this finding can be applied to determine free and membrane-bound factor X from which binding parameters can be obtained. Binding of factor X to negatively charged phospholipid bilayers has been found to be rapid and reversible. The parameters that describe factor X binding to phospholipid membranes containing various mole percentages of the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylglycerol (PG) in phosphatidylcholine (PC) were measured at a Ca^{2+} concentration of 10 mM. The dissociation constants obtained from Scatchard plots decrease when the mole percentage of negatively charged phospholipid is increased from 0 to 50 mol %. Factor X has a higher affinity for membranes containing PS than for those containing PG. The number of binding sites increases proportionally with increasing mole percentage of negatively charged phospholipid molecules between 0 and 25 mol % PS. The stoichiometry calculated for PS-containing vesicles is 16-17 PS residues per bound factor X molecule. Since factor X contains 14 γ-carboxyglumatic acid residues, it is likely that one PS molecule is required to link one γ-carboxyglumatic acid residue of factor X via Ca^{2+} to the phospholipid membrane. The number of binding sites on phospholipid vesicle surfaces containing more than 50 mol % PS decreases dramatically, presumably due to Ca^{2+}-induced aggregation of vesicles. Prothrombin, which like factor X is a vitamin K dependent protein containing γ-carboxyglumatic acid residues, competes with factor X for binding sites on the phospholipid membrane.

Phospholipid-protein interactions play an important role in the blood coagulation cascade [for a review, see Zwaal (1978)]. Prothrombin activation and the activation of factor X via the intrinsic and extrinsic pathway take place on a phospholipid surface. Phospholipid provides the surface upon which a complex is formed of the enzyme, the substrate, and an activator protein (factor V for prothrombin activation, factor VIII for the intrinsic factor X activation, and tissue factor apoprotein for the extrinsic factor X activation). Both the enzymes (factors Xa, IXa, and VIIa) and the substrates (prothrombin and factor X) that participate in these complexes belong to the vitamin K dependent proteins of blood coagulation. These proteins contain γ-carboxyglumatic acid residues that are essential for the binding to negatively charged phospholipid surfaces in the presence of calcium ions. To describe the reactions taking place on the phospholipid surface, it is essential to have quantitative information about the binding of these proteins to phospholipid bilayers.

A number of methods have been used to determine the binding parameters of clotting factors to phospholipid bilayers. An extensive study of the binding of vitamin K dependent clotting factors to phospholipid vesicles of different composition was carried out by utilizing the technique of 90° light scattering (Nelsestuen & Broderius, 1977; Nelsestuen et al., 1978; Resnick & Nelsestuen, 1980). Bloom et al. (1979) used the same technique to determine the binding of factor X, factor Va and prothrombin to vesicles of a mixture of Folch fraction III [which is rich in phosphatidylserine (PS)][1] and PC (25/75 w/w). Since phospholipid vesicles with a mole percentage of PS higher than 30-40% tend to aggregate and form nonvesicular structures in the presence of calcium ions (Papahadjopoulos et al., 1975; Day et al., 1977), light scattering methods

† From the Department of Biochemistry, Biomedical Centre, Rijksuniversiteit Limburg, Maastricht, The Netherlands. *Received April 29, 1981.*
‡ Present address: Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.
§ Supported by the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

[1] Abbreviations used: PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; RVV-X, factor X activator purified from Russel's viper venom; S2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide hydrochloride.

0006-2960/81/0420-7096$01.25/0

are restricted to measurement of binding at low $CaCl_2$ concentrations (less than 5 mM) and to vesicles containing a low mole percentage of PS.

Dombrose et al. (1979) used the method of Hummel & Dreyer (1962) to determine the binding parameters of prothrombin fragment 1 binding to vesicles of a mixture of PG and PC. This method which is also applicable for prothrombin and factor X has the disadvantage that it is laborious and requires large amounts of protein.

Recently Lecompte et al. (1980) determined parameters of prothrombin and prothrombin fragment 1 binding to monolayers of mixtures of PC and PS by measuring the surface radioactivity emanating from tritium-labeled proteins adsorbed on the phospholipid surface.

In this paper, we report a simple and rapid method for the determination of binding constants of factor X binding to phospholipid vesicles. The method is based on the finding that RVV-X is unable to activate factor X bound to phospholipid vesicles but rapidly activates free factor X. It is shown that from the observed rates of factor Xa formation in the presence of phospholipid vesicles the amounts of bound and free factor X can be calculated. This enables determination of the binding parameters of factor X binding to phospholipid vesicles present in a reaction mixture.

An advantage of this method is that the binding of factor X to the vesicles can be measured under the same conditions as those used in the kinetic experiments of factor X activation (van Dieijen et al., 1981). This enables correct interpretation of the observed kinetics of factor X activation in a model in which factor X bound to the phospholipid surface is the substrate for the membrane bound enzyme factor IXa.

Experimental Procedures

Materials. *N*-Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide hydrochloride (S2222) was purchased from AB Kabi Diagnostica, Stockholm, Sweden. DEAE-Sephadex A-50 and Sephadex G-100 and G-200 were products of Pharmacia. Ovalbumin and Russel's viper venom were obtained from Sigma. All reagents used were of the highest grade commercially available.

Proteins. Bovine factors X_1 and X_2 were prepared as described by Fujikawa et al. (1972a). Bovine factor Xa was prepared according to Fujikawa et al. (1972b). RVV-X was purified from the crude venom as described by Schiffman et al. (1969). No phospholipase A_2 activity could be detected in the RVV-X preparations. Before storage at −70 °C, the protein preparations were dialyzed against a buffer containing 50 mM Tris-HCl and 175 mM NaCl at pH 7.9. Concentrations of factor X and factor Xa were determined as described previously (van Dieijen et al., 1981). The amount of RVV-X present in the reaction mixtures was estimated from the absorbance at 280 nm by assuming an $E^{1\%,1cm}_{280nm}$ value of 10.0.

Phospholipid and Phospholipid Vesicle Preparations. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_{cis}/18:1_{cis}$-phosphatidylcholine) was prepared by reacylating the cadmium chloride adduct of *sn*-glycero-3-phosphocholine with the appropriate fatty acyl chloride according to the method of Bear & Buchnea (1959). 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine ($18:1_{cis}/18:1_{cis}$-phosphatidylserine) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol ($18:1_{cis}/18:1_{cis}$-phosphatidylglycerol) were prepared from the respective phosphatidylcholine by enzymatic synthesis according to the method of Comfurius & Zwaal (1977). Single bilayer vesicle solutions were prepared according to the method described by de Kruijff et al. (1975) by sonication of liposomes for 10 min in 50 mM Tris-HCl and 175 mM NaCl buffer at pH 7.9 at 0 °C (above the phase transition of the lipids used). Sonication was performed by using a MSE Mark II 150-W ultrasonic disintegrator set at 10-μm peak to peak amplitude. After sonication, no pH adjustment was needed. Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (1961).

Determination of the Rate of Factor X Activation by RVV-X. Factor X was preincubated at 37 °C in 450 μL of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/mL ovalbumin, and 11.1 mM $CaCl_2$ at pH 7.9. After 4 min, the reaction was started by the addition of 50 μL of RVV-X (1.45 ng). After different time intervals, aliquots from the reaction mixture were transferred to a cuvette containing the factor Xa specific chromogenic substrate S2222. The final volume in the cuvette was 2 mL and contained 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/mL ovalbumin, 15 mM EDTA, and 192 μM S2222. The EDTA is present to block further reaction. Since RVV-X has no detectable amidase activity toward S2222, the absorbance change at 405 nm minus that at 500 nm measured on an Aminco DW 2 spectrophotometer is a measure of the amount of factor Xa present in the aliquot taken from the reaction mixture. The amount of factor Xa present in the sample was calculated from the measured absorbance change at 405 nm minus that at 500 nm by using a calibration curve made with known amounts of active site titrated factor Xa measured under the same conditions as described above. Rates of factor Xa formation are given as picomoles of Xa formed per minute per milliliter of reaction mixture. In experiments carried out in the presence of phospholipid, the phospholipid vesicles were present during the 4-min preincubation period. The kinetic constants of factor X activation by RVV-X in the absence of phospholipid were calculated from a Lineweaver–Burk plot constructed after statistical analysis of the data by the method of Eisenthal & Cornish-Bowden (1974). For the construction of the Lineweaver–Burk plot, the rate of factor Xa formation was averaged from three independent determinations.

Results

The method to determine factor X binding to phospholipid vesicles described in this paper is based on our finding (see below) that in a mixture containing free factor X and factor X bound to phospholipid, only free factor X is activated by RVV-X. When the kinetic parameters of factor X activation by RVV-X are known, it is possible to determine the parameters of factor X binding to phospholipid vesicles from the rates of factor X activation by RVV-X measured in the presence of phospholipid.

Kinetics of Factor X Activation by RVV-X in the Absence of Phospholipid. The activation of bovine factor X by RVV-X requires the presence of Ca^{2+} ions (Esnouf & Williams, 1962). In agreement with the findings of Morris et al. (1978) with human factor X and Lindhout et al. (1978) with bovine factor X, we found that the Ca^{2+} dependence of the activation of bovine factor X by RVV-X exhibits positive cooperativity. Ca^{2+} titration curves of factor X activation by RVV-X at different factor X concentrations showed that a plateau was reached at 5 mM $CaCl_2$ and that the rates of factor Xa formation were constant between 5 and 20 mM $CaCl_2$. The steady-state velocity of factor X activation was constant in time and proportional to the amount of enzyme present. Rates of factor X activation by RVV-X were measured at varying factor X and $CaCl_2$ concentrations. The kinetic parameters, obtained from Lineweaver–Burk plots, were independent of the $CaCl_2$ concentration between 5 and 20 mM. The K_M for factor X

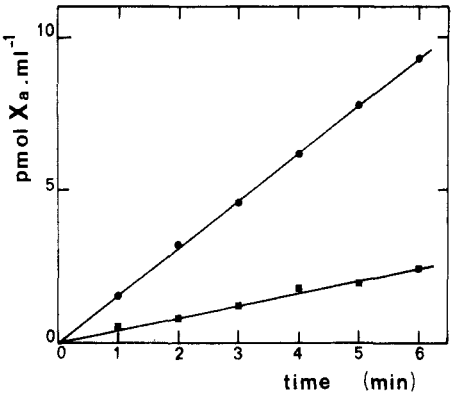

FIGURE 1: Time course of factor X activation by RVV-X in the presence and absence of phospholipid. Factor X activation by RVV-X was measured at 37 °C in 1 mL of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/mL ovalbumin, 10 mM $CaCl_2$, 0.1 μM factor X, and 2.9 ng/mL RVV-X. (●) No phospholipid; (■) 25 μM phospholipid vesicles prepared from a mixture of PS and PC (1/1 mol/mol). After preincubation of the reaction mixture for 4 min at 37 °C, the activation of factor X was started by the addition of RVV-X. After the time intervals indicated in the figure, samples from the reaction mixture were assayed for factor Xa as described under Experimental Procedures.

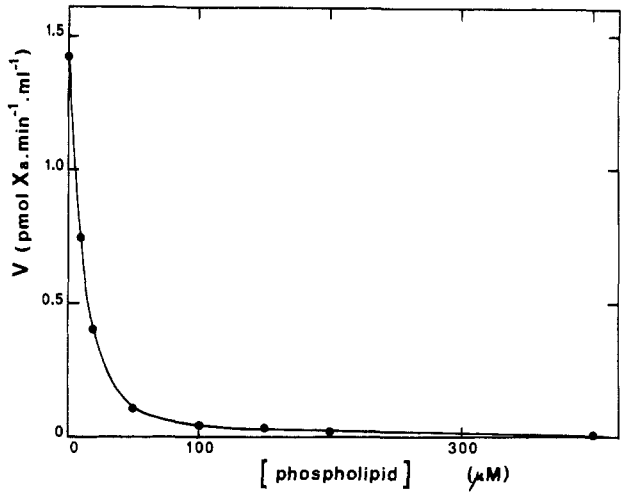

FIGURE 2: Effect of phospholipid concentration on the rate of factor X activation by RVV-X. Factor X activation by RVV-X was measured at 37 °C in 0.5 mL of a reaction mixture containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/mL ovalbumin, 10 mM $CaCl_2$, 0.1 μM factor X, 2.9 ng/mL RVV-X, and the amounts of phospholipid vesicles (PS/PC, 1/1 mol/mol) indicated in the figure. After 4-min preincubation of the reaction mixture at 37 °C, factor X activation was started by the addition of RVV-X. The rate of factor X activation was calculated from the amounts of factor Xa present in the reaction mixture after 2.5 and 5 min.

was 0.71 μM, and a V_{max} of 4.23 pmol of Xa min^{-1} (ng of RVV-X)$^{-1}$ was measured. These values are in agreement with the kinetic parameters reported by Kosow et al. (1974).

When the amount of RVV-X present in a reaction mixture is known, knowledge of the kinetic parameters of factor X activation enables the determination of an unknown amount of factor X present in a reaction mixture from the observed rate of factor Xa formation by using the following equation:

$$X = \frac{vK_M}{V_{max} - v} \quad (1)$$

Here X is the unknown concentration of factor X present in the reaction mixture, V_{max} and K_M are the kinetic parameters of factor X activation by RVV-X, and v is the observed rate of factor Xa formation.

Inhibition of Factor X Activation by RVV-X in the Presence of Phospholipid. Figure 1 shows the time course of factor X activation by RVV-X in the absence and presence of phospholipid vesicles. The steady-state velocity of the reaction is markedly decreased by the presence of phospholipid bilayer membranes containing PS. Figure 2 shows the dependence of the rate of factor X activation on the amount of phospholipid present. At 400 μM phospholipid, the rate of factor X activation is less than 1% of the rate measured in the absence of phospholipid. The results for single bilayer vesicles containing PG as the acidic phospholipid are similar to those given for PS except that higher phospholipid concentrations are required to obtain the same inhibition. Vesicles composed of PC only do not inhibit the activation of factor X by RVV-X.

The inhibition by phospholipid membranes containing negatively charged phospholipids can be explained by assuming either that RVV-X binds to the phospholipid vesicles and is not able to activate factor X or that RVV-X is not able to activate factor X molecules bound to the phospholipid bilayer.

The following experiment was carried out (Table I) to distinguish between these two possibilities. Four centrifuge tubes were filled with mixtures of varying composition of RVV-X, factor X, and liposomes from PS and PC (50/50 mol/mol). The composition of the mixtures is indicated in Table I. After centrifugation of tubes 1 and 2 for 45 min at 50000*g*, tube 1 contained a pellet of the liposomes (more than 95% of the phospholipid is spun down). Factor X was added to both supernatants to a final concentration of 4.2 μM, and the rate of factor Xa formation was measured. Since the rates of factor Xa formation are the same in both supernatants, we conclude that equal amounts of RVV-X are present and that RVV-X does not bind to phospholipid. Before centrifugation, samples (0.6 mL) were taken from tubes 3 and 4, RVV-X was added, and the rate of factor Xa formation was measured. Due to the presence of the liposomes, the rate of factor Xa formation was markedly decreased in the sample taken from tube 3. After centrifugation, RVV-X was added to 0.6 mL of the supernatant of tubes 3 and 4, and the rate of factor Xa formation was measured again. Since the rates of factor Xa formation before and after centrifugation are the same, it is concluded that it is indeed the amount of free factor X that determines the rate of factor X activation by RVV-X. Thus, RVV-X can be used to measure the amount of free factor X in reaction mixtures in which phospholipids are present.

Table I: Effect of Factor X Binding to Liposomes on the Rate of Factor X Activation by RVV-X[a]

	contents of centrifuge tubes			rate of factor X activation (pmol of Xa min^{-1} mL^{-1})	
tube	[liposomes] (μM)	[RVV-X] (ng/mL)	[factor X] (μM)	before centrifugation	after centrifugation
1	200	2.32			6.5[b]
2		2.32			6.6[b]
3	200		0.1	0.088[c]	0.074[d]
4			0.1	0.75[c]	0.75[d]

[a] The incubation mixture contained 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 0.5 mg/mL ovalbumin, and components as indicated in the table. Liposomes were prepared from an equimolar mixture of PS and PC. [b] The rate of factor X activation was measured after addition of factor X to the supernatant to a final concentration of 4.2 μM. [c] The rate of factor X activation was measured after addition of RVV-X to a final concentration of 2.0 ng/mL. [d] The rate of factor X activation was measured after addition of RVV-X to the supernatant to a final concentration 2.0 ng/mL.

Reversibility of Factor X Binding to and Dissociation from Phospholipid Vesicles. The conversion of S2222 (followed

by measuring the absorbance at 405 nm) is parabolic in time when factor X is activated by RVV-X in the presence of $CaCl_2$ and S2222 (Morris et al., 1978). Plots of the absorbance at 405 nm vs. t^2 were also linear and passed through the origin when the activation of factor X in a mixture containing RVV-X, $CaCl_2$, negatively charged phospholipids, and S2222 was started by adding factor X or when a concentrated mixture of factor X, $CaCl_2$, and negatively charged phospholipids is diluted in a reaction medium containing RVV-X, $CaCl_2$, and S2222. Since the final concentrations of reactants in both experiments were the same and the quadratic plots coincided, we conclude that the binding of factor X to phospholipid vesicles is reversible and that the binding equilibrium is rapidly established.

Determination of the Parameters of Factor X Binding to Phospholipid Vesicles. Since RVV-X is only able to activate factor X molecules not bound to phospholipid bilayers, the observed rates of factor Xa formation in the presence of phospholipid can be used to calculate the concentration of free factor X with the help of eq 1. From the calculated amount of free factor X and the known amount of factor X added, the amount of bound factor X can be calculated and Scatchard plots can be constructed to obtain the parameters of factor X binding to the phospholipid vesicles present in the reaction mixture.

When the binding of factor X is measured in this way, care has to be taken that the amount of factor X converted does not influence the binding equilibrium. Therefore, the reaction with RVV-X was allowed to proceed only for a limited time (less than 5 min) at a low concentration of RVV-X. The amount of free factor X converted by RVV-X was never more than 2% of the amount of free factor X measured.

It has been shown that factor Xa can autocatalytically convert factor X into factor Xa and so-called β-factor X in the presence of $CaCl_2$ and phospholipid (Jesty et al., 1974; Fujikawa et al., 1975). However, the amounts of factor Xa formed during the activation by RVV-X were so small that no autocatalytic factor Xa formation could be detected. Gel electrophoretic analysis of samples in which these small amounts of factor Xa were present showed no β-factor X formation.

The binding of factor X to phospholipid vesicles composed of a 1/1 (mol/mol) mixture of PS and PC at three different phospholipid concentrations was determined. Figure 3 shows the Scatchard plots obtained. As can be seen, the K_d is constant (0.04 μM), and the number of binding sites found is proportional to the amount of phospholipid present (0.73 μM binding sites per 100 μM phospholipid).

The binding parameters of factor X binding were strongly dependent on the amount of PS present in the vesicles. Figure 4A,B shows the dependence of K_d and sites (per 100 μM phospholipid) on the mole fraction of PS present. The K_d and the number of sites depend almost linearly on the fraction of PS in the range 0–25 mol % PS. The K_d decreases from 0.18 μM for vesicles containing 6 mol % PS to 0.03 μM for vesicles containing 37 mol % PS whereas the amount of sites increases from 0.2 to 0.85 μM. When the mole fraction of PS is further raised, the number of sites is not further increased, which is in agreement with the findings of Nelsestuen & Broderius (1977). However, the amount of sites present drastically decreases for vesicles containing more than 60 mol % PS. For vesicles of pure PS, only 0.22 μM sites remain, and the K_d is increased to 0.07 μM.

It is known that vesicles composed of brain PS aggregate in the presence of $CaCl_2$ and form structures known as scrolls

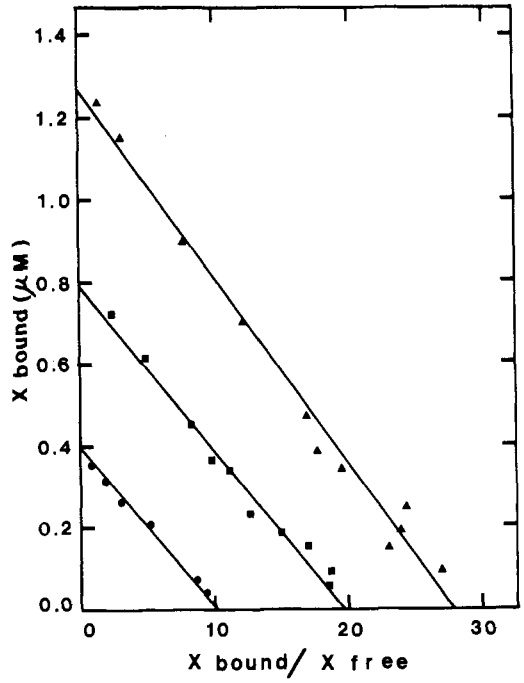

FIGURE 3: Scatchard plots of factor X binding to phospholipid vesicles. Factor X activation by RVV-X was measured at 37 °C in 0.5 mL of a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 10 mM $CaCl_2$, 0.5 mg/mL ovalbumin, varying amounts of factor X, and (●) 57 μM phospholipid (PS/PC, 1/1 mol/mol), (■) 114 μM phospholipid, and (▲) 171 μM phospholipid. This mixture was incubated for 4 min at 37 °C before the activation of factor X was started by the addition of RVV-X to a final concentration of 2.9 ng/mL. The rate of factor Xa formation was determined as described under Experimental Procedures. From the observed rate of factor Xa formation, the amount of factor X_{free} was calculated with eq 1. The concentration of factor X_{bound} was obtained after subtracting the factor X_{free} from the amount of X_{added}.

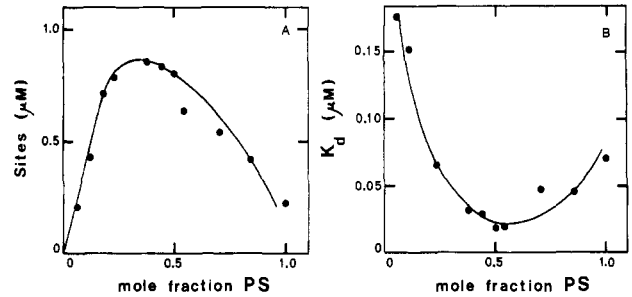

FIGURE 4: Effect of PS content of vesicles on the binding parameters of factor X. Scatchard plots of factor X binding to phospholipid vesicles containing varying mole fractions of PS were obtained as described in the legend to Figure 3. The amount of sites (μM/100 μM phospholipid) is plotted in (A) and the K_d in (B).

(Papahadjopoulos et al., 1975). Aggregation may explain the observed decrease of the number of sites since on aggregated structures less surface will be available for the binding of factor X. Aggregation of vesicles can be followed by measuring the change of light scattering of vesicle preparations upon addition of $CaCl_2$. No change of light scattering was found with vesicles containing up to 40 mol % PS. Calcium ions induce, however, aggregation of vesicles when the PS content of the vesicles is 50 mol % or higher. At these mole percentages, the loss of sites also occurs so it is likely that the decrease of the number of binding sites for factor X above 50 mol % PS is due to extensive Ca^{2+}-induced aggregation of vesicles. Phospholipid vesicles containing PG as the acidic phospholipid do not aggregate at 10 mM $CaCl_2$. Binding parameters of factor X binding to vesicles containing varying mole percentages of PG were obtained from Scatchard plots. The data

Table II: Binding of Factor X to Phospholipid Vesicles Containing PG[a]

mol % PG	K_d (μM)	μM sites/100 μM phospholipid
50	1.26	1.1
75	0.78	1.4
100	0.35	1.55
100 preaggregated vesicles	0.2	0.6

[a] The binding parameters were calculated from Scatchard plots as described in the legend to Figure 3. The experiment was carried out with 100 μM phospholipid vesicles composed of phosphatidylcholine and phosphatidylglycerol, containing the mole percentages of phosphatidylglycerol indicated in the table. In the experiment in which preaggregated vesicles were used, 250 μM phospholipid vesicles were preincubated for 10 min at 37 °C in a buffer containing 175 mM NaCl, 50 mM Tris, and 25 mM $CaCl_2$ at pH 7.9 and than diluted in the reaction mixture to a final concentration of 100 μM phospholipid and 10 mM $CaCl_2$.

are summarized in Table II. Factor X has a much lower affinity for vesicles containing PG than for PS-containing vesicles. At high PG content, there is, however, no loss of binding sites for factor X. At a $CaCl_2$ concentration of 20 mM, vesicles containing a high mole percentage of PG slowly aggregate. Light scattering experiments show that incubation of single bilayer membranes containing 100 mol % PG for 10 min at 37 °C in the presence of 25 mM $CaCl_2$ completes aggregation. When these preaggregated vesicles are diluted and a Scatchard plot is measured at 10 mM $CaCl_2$, there is a considerable loss of factor X binding sites (Table II). These observations support the suggestion that the loss of binding sites for factor X is due to vesicle aggregation.

Competition between Prothrombin and Factor X for Binding Sites on PS-Containing Vesicles. Prothrombin, which like factor X is a vitamin K dependent protein, displays membrane binding characteristics identical with those of factor X (Nelsestuen & Broderius, 1977). From a mechanistic point of view, it is interesting to know whether prothrombin and factor X are able to compete for binding sites on membranes containing negatively charged phospholipids. With the RVV-X technique, competition of factor X and prothrombin for the phospholipid bilayer can be easily studied. Figure 5 shows the displacement of factor X from phospholipid vesicles containing 25 mol % PS at increasing prothrombin concentrations. In this titration, no higher prothrombin concentrations could be employed since at concentrations above 1.5 μM prothrombin starts to inhibit RVV-X. From this experiment, it is obvious that prothrombin and factor X compete for binding sites on the phospholipid bilayer.

Discussion

The binding of factor X to phospholipid vesicles has been investigated with a new method which makes use of the fact that with RVV-X one can determine the distribution of factor X between solution and phospholipid surfaces. The advantage of the technique reported in this paper is that it is rapid and requires small amounts of protein and binding can even be measured in turbid phospholipid preparations. The technique has limitations with respect to the range of K_d values that can be determined. For a hyperbolic binding isotherm, the K_d for factor X binding is equal to the free ligand concentration at which half of the available binding sites are saturated. Therefore, the range of K_d values that can be measured is actually dependent on the range of free factor X concentrations that can be accurately measured with RVV-X. Since the activation of factor X by RVV-X has a K_M of 0.71 μM, the highest free factor X concentration than can be accurately measured with RVV-X is 2 μM. This sets the upper limit for the K_d that still can be determined at 2 μM. The lower limit of the K_d that can be measured with the RVV-X technique is dependent on the lowest factor Xa concentration that can be accurately measured. With the chromogenic substrate S2222, a concentration of factor Xa as low as 0.02 nM can be determined on an Aminco-DW2 spectrophotometer. The amount of free factor X converted by RVV-X in a binding experiment may however not exceed 2% of the free factor X present. Thus, the lower limit of the K_d that can be measured is 1 nM.

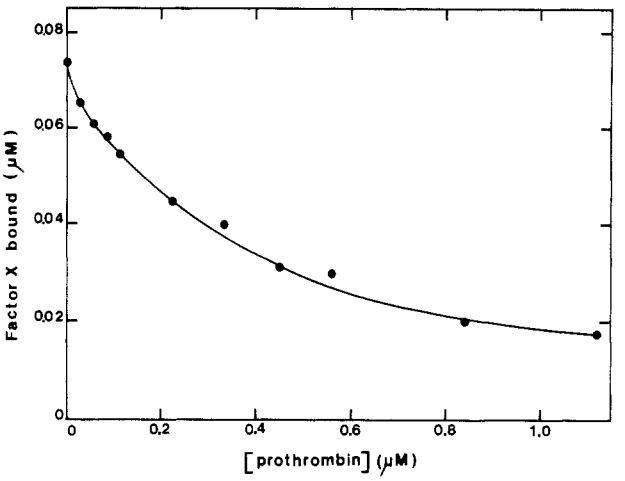

FIGURE 5: Competition between prothrombin and factor X for binding sites on PS-containing vesicles. The amount of factor X bound to phospholipid vesicles was determined with RVV-X in the absence and presence of varying amounts of prothrombin. The reaction mixture contained 175 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM $CaCl_2$, 20 μM phospholipid vesicles (PS/PC, 25/75 mol/mol), 0.1 μM factor X, 0.23 ng/mL RVV-X, and amounts of prothrombin indicated in the figure. The amount of free factor X was calculated from the rate of factor X activation with eq 1. Phospholipid-bound factor X was obtained after subtracting free factor X from the amount of factor X added.

The binding parameters determined with the RVV-X technique are comparable to those obtained in light scattering experiments (Nelsestuen & Broderius, 1977; Bloom et al., 1979). The minor differences are likely caused by the use of phospholipids from different sources or the difference in experimental conditions (e.g., the Ca^{2+} concentration).

With vesicles containing PS, the number of binding sites for factor X increases proportionally up to 23 mol % PS. The sharp decrease of the number of binding sites at a high mol percent of PS is presumably caused by aggregation of vesicles at 10 mM $CaCl_2$ and/or the formation of nonvesicular structures such as cochleate cylinders (Papahadjopoulos et al., 1975).

Small single bilayer vesicles contain more phospholipid molecules in the outer than in the inner monolayer (de Kruijff et al., 1975). Taking into account the asymmetric distribution of PS between the outer and inner monolayers of the phospholipid vesicles (Berden et al., 1975), one can calculate that approximately 16–17 PS molecules are required for the binding of one factor X molecule. This binding is thought to occur with the 14 γ-carboxyglumatic acids present in a factor X molecule (Howard & Nelsestuen, 1975). Since it is not likely that each PS molecule will be continuously involved in binding, we conclude that one PS residue is required per γ-carboxyglumatic acid. This indicates that both negative charges of one PS molecule (phosphate and carboxyl) are involved in a Ca-mediated interaction with the two negative charges of γ-carboxyglumatic acid. The number of factor X molecules

bound to a vesicle with 23 mol % can be calculated to be 24. This value is approximately 25% of that for a close hexagonal packing of factor X molecules on the vesicle surface, assuming that factor X is a spherical molecule with molecular weight 55 000 and $\bar{V}$ = 0.717 cm^3/g (Fujikawa et al., 1972a).

The outer radius of vesicles composed of PC and PG is independent of the mole percent of PG and equals 131 Å (B. de Kruijff, personal communication). Vesicles composed of 100% PG show a maximal binding of 1.55 μM factor X per 100 μM phospholipid. This value is approximately 50% of that for close hexagonal packing under the same geometrical assumptions as described above.

The data presented in Figure 4B show that the K_d for factor X decreases as the concentration of negatively charged phospholipid molecules per vesicle increases. This phenomenon has also been observed by Nelsestuen & Broderius (1977) and Dombrose et al. (1979) for the binding of prothrombin, factor X, and prothrombin fragment 1 to negatively charged phospholipid vesicles. Geometrical considerations make it likely that lateral phase separations of negatively charged phospholipids are required to enable factor X binding to single bilayer vesicles. For example, when the outer monolayer contains 10 mol % phosphatidylserine, on the average one PS molecule will be present on a surface area of 720 Å^2. The projected surface area of a spherical factor X molecule with a radius of 24.5 Å is 1885 Å^2. Therefore, lateral phase separations are likely to occur if one factor X molecule interacts (via Ca^{2+}) with at least 14 molecules of PS. Ca-induced phase separations in PC–PS mixtures occur only when the Ca^{2+} concentration exceeds a threshold value which depends on the mole percent of PS (Papahadjopoulos et al., 1974). At low mole percentages of PS, 10 mM $CaCl_2$ does not produce significant phase separations. Therefore, it is likely that the observed decrease of the K_d for factor X for vesicles with increasing PS content (up to 20 mol %) is a reflection of the increasing facility of factor X to interact with at least 14 PS molecules when the PS concentration in the vesicle becomes higher. Above 20 mol % PS, Ca^{2+}-induced lateral phase separations are likely to occur, which may explain why no further decrease of the K_d occurs between 30 and 50 mol % PS. Vesicle aggregation and cochleate cylinder formation may be responsible for the slight increase of the K_d, observed above 50 mol % PS.

The method described in this paper for the interaction of factor X with negatively charged phospholipid vesicles is dependent on RVV-X and can therefore not be applied to the other vitamin K dependent clotting factors. Similar techniques to measure the binding of these factors to phospholipids may, however, be developed if activators are found that exclusively activate free clotting factor molecules and not the membrane-bound molecules. At present, we are investigating the several snake venoms that activate prothrombin for the purpose of finding an activator that meets the requirement to study the prothrombin–phospholipid interaction.

Acknowledgments

We thank G. W. Janssen-Claessen for excellent technical assistance and AB Kabi Diagnostica for kindly donating part of the chromogenic substrates used in our laboratory. Professor Dr. H. C. Hemker is greatly acknowledged for his contributions in discussing this manuscript.

References

Bear, E., & Buchnea, D. (1959) *Can. J. Biochem. Physiol. 37*, 953–959.

Berden, J. A., Barker, R. W., & Radda, G. A. (1975) *Biochim. Biophys. Acta 375*, 186–208.

Bloom, J. W., Nesheim, M. E., & Mann, K. G. (1979) *Biochemistry 18*, 4419–4425.

Böttcher, G. J. F., van Gent, C. M., & Pries, C. (1961) *Anal. Chim. Acta 24*, 203–207.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Day, E. P., Ho, J. I., Kunze, R. K., Jr., & Sun, S. T. (1977) *Biochim. Biophys. Acta 470*, 503–508.

de Kruijff, B., Cullis, P. R., & Radda, G. K. (1975) *Biochim. Biophys. Acta 406*, 6–20.

Dombrose, F. A., Gitel, S. H., Zawalich, K., & Jackson, C. M. (1979) *J. Biol. Chem. 254*, 5027–5040.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.

Esnouf, M. P., & Williams, W. J. (1962) *Biochem. J. 84*, 62–71.

Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972a) *Biochemistry 11*, 4882–4891.

Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972b) *Biochemistry 11*, 4891–4899.

Fujikawa, K., Titani, K., & Davie, E. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3359–3363.

Howard, J. B., & Nelsestuen, G. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1281–1285.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Jesty, J., Spencer, H. K., & Nemerson, Y. (1974) *J. Biol. Chem. 249*, 5614–5622.

Kosow, D. P., Furie, B., & Forastieri, H. (1974) *Thromb. Res. 4*, 219–227.

Lecompte, M. F., Miller, I. R., Elion, J., & Benarous, R. (1980) *Biochemistry 18*, 3434–3439.

Lindhout, M. J., Kop-Klaassen, B. H. M., & Hemker, H. C. (1978) *Biochim. Biophys. Acta 533*, 327–341.

Morris, S., Robey, F. A., & Kosow, D. P. (1978) *J. Biol. Chem. 253*, 4604–4608.

Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 17*, 2134–2138.

Nelsestuen, G. L., Kisiel, W., & DiScipio, R. G. (1978) *Biochemistry 17*, 2134–2138.

Papahadjopoulos, D., Poste, G., Schaeffer, B. E., & Vail, W. J. (1974) *Biochim. Biophys. Acta 352*, 10–28.

Papahadjopoulos, D., Vail, W. J., Jacobson, K., & Poste, G. (1975) *Biochim. Biophys. Acta 394*, 483–491.

Resnick, R. M., & Nelsestuen, G. L. (1980) *Biochemistry 19*, 3028–3033.

Schiffman, S., Theodor, I., & Rapaport, S. I. (1969) *Biochemistry 8*, 1397–1405.

van Dieijen, G., Tans, G., Rosing, J., & Hemker, H. C. (1981) *J. Biol. Chem. 256*, 3433–3442.

Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta 515*, 163–205.