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The interaction of bovine Factor IX, its activation intermediate, Factor IXα, and its activation products, Factor IXaα and Factor IXaβ, with acidic phospholipid vesicles of various compositions

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The interactions of bovine factor IX, its activation intermediate, Factor IXα, and its activation products, Factor IXaα and Factor IXaβ, with phospholipid vesicles, of mean radius of approx. 30 nm, containing various amounts of phosphatidylserine (PS) and phosphatidylcholine (PC), have been examined. For Factor IX, Factor IXα, Factor IXaα and Factor IXaβ, the dissociation constants, at saturating levels of Ca^{2+}, are independent of the PS concentration in the vesicle after levels of 20–30% (w/w) have been reached, and attain minimum values of approx. 1.7, 1.7, 0.7 and 1.0 μM, respectively, with vesicles containing 50% PS. The amount of protein bound per vesicle particle is independent of the PS content, above 20% PS, for Factor IX and Factor IXaβ, with values of approx. 995–1197 and 1128–1566 molecules/vesicle, respectively. With Factor IXα, a dependence on the amount of protein bound with the content of PS is seen, which ranges from 338 to 619 molecules/vesicle with membranes containing 30–50% PS. For Factor IXaα, no regularity is noted and a range of 583–1083 molecules of protein/vesicle is observed with the systems employed. Examination of the radii of the proteins on the vesicle demonstrates that Factors IXα and IXaα occupy considerably more of the surface than do Factors IX and IXaβ, suggesting that a reason for the decreased number of binding sites for the former two proteins on the vesicle may be related to their greater surface spatial requirements.

INTRODUCTION

Factor IX is an inactive serum glycoprotein that is involved in the central steps of the intrinsic blood coagulation pathway. This zymogen, as isolated from human and bovine sources, consists of a single polypeptide chain of M_r approx. 55000. The entire amino acid sequence of the bovine Factor IX has been determined by conventional protein techniques (Katayama *et al.*, 1979), and from analysis of a cDNA to the bovine protein (Choo *et al.*, 1982). The sequence for the human zymogen has been established by similar characterization of a cDNA that codes for this protein (Kurachi & Davie, 1982). The most notable aspects of these amino acid sequences are the presence of twelve γ-carboxyglutamic acid residues at the *N*-terminus of each protein (Katayama *et al.*, 1979; Kurachi & Davie, 1982), and the discovery of a single residue of β-hydroxyaspartic acid at sequence position 64 of the zymogens (Drakenberg *et al.*, 1983; McMullen *et al.*, 1983). A total of four complex-type carbohydrate chains are present in the bovine molecule, linked to asparagine residues in the protein at sequence positions 158, 168, 173 and 261 (Mizouchi *et al.*, 1983). In the human protein, carbohydrate is bound to protein at asparagine in sequence positions 157 and 167 (Kurachi & Davie, 1983).

Factor IX is converted to activated Factor IX (Factor IXa), a serine proteinase, by several different proteolytic enzymes. In the intrinsic coagulation pathway, the activation is catalysed by Factor XIa in the presence of Ca^{2+} (Schiffman *et al.*, 1963). In the bovine system, this process occurs in at least two stages (Fujikawa *et al.*, 1974). In the first step, cleavage of Factor IX at the peptide bond Arg^{146}–Ala^{147} yields the inactive disulphide-linked two-chain intermediate, Factor IXα. In the next step, cleavage by Factor XIa occurs at the peptide bond Arg^{181}–Val^{182} forming the final active product, Factor IXaβ, with concomitant release of the activation peptide Ala^{147}–Arg^{181}. A similar mechanism exists when Factor IX is activated by components of the extrinsic blood coagulation system, Factor VIIa/Ca^{2+}/Tissue Factor (Osterud & Rapaport, 1977; Zur & Nemerson, 1980). A different form of Factor IXa, Factor IXaα (Lindquist *et al.*, 1978), occurs upon activation of Factor IX by the coagulant protein isolated from the venom of Russell's Viper, in the presence of Ca^{2+} (Byrne & Castellino, 1978). This form of the enzyme results from cleavage of only the Arg^{181}–Val^{182} peptide bond in Factor IX, without release of the activation peptide, yielding a disulphide-linked two-chain enzyme (Lindquist *et al.*, 1978). We have shown that the two forms of Factor IXa possess greatly different properties toward activation of their physiological substrate, Factor X (Link & Castellino, 1983).

The binding to phospholipid (PL) of the products of Factor IX activation is important to the next step of the intrinsic blood coagulation process, Factor X activation, which is catalysed by a complex of Factor IXa/Ca^{2+}/Factor VIIIa, assembled on a PL surface. It is generally concluded that PL vesicles most efficacious for occurrence of blood coagulation reactions are composed of mixtures of such molecules, with a negatively charged PL being an important component

Abbreviations used: PL, phospholipid; PS, phosphatidylserine; PC, phosphatidylcholine; QLS, quasielastic light scatter.

(Bangham, 1961). In order to understand the molecular basis of the relationship between the acceleration of activation of appropriate blood coagulation proteins and the structure of the PL, it is important to determine the binding isotherms to lipid vesicles of various PL-binding proteins that are important in blood clotting. Employing light-scattering methods, the nature of the interaction of Factor X and prothrombin with a variety of model PL membranes has been investigated (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Lim *et al.*, 1977). In the current manuscript, we report the results of our investigations on the binding of Factor IX, its activation intermediate, Factor IXα, and its activation products, Factor IXaα and Factor IXaβ, to acidic PL vesicles of varying composition. This information is essential to the ultimate aim of elucidation of the important role played by PL-containing membranes in the activation of blood coagulation Factor X.

EXPERIMENTAL PROCEDURES

Proteins

Bovine Factor IX was prepared by slight modifications of the procedure of Fujikawa *et al.* (1973), as described by Amphlett *et al.* (1979). Factor IXaβ was generated by complete activation of Factor IX with bovine Factor XIa, using conditions published by Amphlett *et al.* (1979). Factor IXα was purified from a partial activation mixture of Factor IX, employing Sephacryl 200 gel permeation chromatography, by the method of Amphlett *et al.* (1979). This column readily resolves Factor IXα from Factor IXaβ. In order to improve the amount of Factor IXα present in the partial activation mixture, we performed the activation according to Amphlett *et al.* (1979), except that the NaCl concentration in the activation buffer was increased to 0.6 M, a step that allowed greater quantities of Factor IXα to accumulate. Factor IXaα was generated exclusively upon activation of Factor IX with the coagulant protein from the venom of Russell's viper, when the procedure of Amphlett *et al.* (1979) was employed.

Published reports on the conditions of activation required to yield the above components should be employed only as guides. In all cases, it is necessary to perform pilot experiments in order to optimize the yields of proteins required.

Factor XIa was generated by Celite-activation of the barium citrate supernate of the Factor IX preparation. A functionally satisfactory product was obtained upon removal of the Factor XIa from the Celite with 10% (w/v) NaCl (Amphlett *et al.*, 1979).

The coagulant protein from Russell's viper was purified from the lyophilized snake venom (Miami Serpentarium). The material that is eluted from the QAE-Sephadex column, detailed in the publication of Amphlett *et al.* (1982), is a very high quality preparation and was employed for the work described herein.

Vesicle preparations

Egg PC and bovine brain PS were purchased from Sigma Chemical Co., and were the highest purity available. Analytical t.l.c. (Dittmer & Lester, 1964) and h.p.l.c. (Chen & Kou, 1982) analyses demonstrated that each was approx. 98% pure, and they were used without further purification.

PL vesicles were prepared from stock solutions of individual components, dissolved in chloroform, by mixing a total of 12 mg of the desired PLs. The solvent was then evaporated under a stream of N_2 and the residue was redispersed in 5 ml of a buffer consisting of 0.02 M-Hepes/0.15 M-NaCl, pH 7.4. The samples were subjected to sonication in a pulsed system (Heat Systems model W200R), containing a standard microtip probe adjusted to deliver a power output of 75 W, on a 50% duty cycle. After a sonication time of 1 h at 4 °C, the lipid dispersion was submitted to centrifugation (90 min at 45000 rev./min) in a Beckman model L5-65 ultracentrifuge and a Ti-50 rotor in order to remove both the titanium particles released from the probe as well as any multilamellar liposomes. This procedure allows isolation of the smallest unilamellar vesicles present (Barenholz *et al.*, 1977) and results in no observable degradation of component PLs.

The PS and PC contents of the final vesicle were determined by h.p.l.c. by the method of Chen & Kou (1982). Vesicle solutions were lyophilized and redissolved in chloroform, containing Dowex 50-W (H^+), in order to convert the PLs to their acidic forms and enhance their solubilities. Samples were then subjected to centrifugation with a table-top centrifuge. The supernate was retained, the pellet washed three times with chloroform and the washings were combined with the supernatant. The volume of this mixture was then reduced by evaporation under a stream of N_2, and the resultant samples were analysed by h.p.l.c. Since, in all cases, the concentrations of PC and PS determined in this fashion were within 2% of the amount initially added, we have reported the original concentrations in all studies presented here.

The total PL concentrations of the stock vesicle preparations were determined by the method of Lowry & Lopez (1946). All vesicle preparations were used within 5–10 h after preparation in order to avoid any time-dependent changes in vesicle diameters.

Inner/outer membrane surface PS contents

The PS moiety in the final vesicle was quantitatively labelled with fluorescamine by the method of Lee & Forte (1979). For evaluation of the outer surface PS content, the samples were diluted to a concentration of approx. 0.2 mg/ml with a buffer consisting of 50 mM-borate, pH 8.2. To 1 ml of this mixture, a quantity of 10 μl of fluorescamine, dissolved in acetone, was added under conditions of continuous mixing, such that final concentrations of fluorescamine of 5.0–170 μg/ml were achieved. After 90 s, an aliquot (50 μl) was removed and added to 1.95 ml of a solution of 1 mM-citric acid, dissolved in 99% ethanol. The fluorescence of the samples was determined employing a Perkin–Elmer MPF-44 fluorescence spectrophotometer, with the excitation wavelength at 380 nm and the emission wavelength at 480 nm. All data were normalized to the total PS content, which was determined as above, except that 10 μl of a solution of Triton X-100 (100 mg/ml) was added to 0.89 ml of the borate buffer, prior to addition of 0.1 ml of the PL sample, in order to disrupt the vesicle. To assure that the fluorescence quantum yields were the same under both sets of conditions, a similar addition of Triton X-100 was made to the samples labelled only in borate buffer, after the reaction with fluorescamine was complete.

90° light scatter measurements

A relative 90° light scatter technique described by Nelsestuen & Lim (1977) was used to investigate protein–membrane binding. In these experiments, the PL concentration was maintained at a constant value, and titrated with protein in the presence of the desired levels of Ca^{2+}. The extent of light scatter of each protein–membrane sample was recorded after protein binding had reached equilibrium, about 20 min. After adjusting the fluorimeter recorder to zero with buffer, the value of the light scatter, in arbitrary units, of the PL alone was subtracted from that determined for the protein–PL–Ca^{2+} sample; this former value the same as that for the protein–PL–Ca^{2+} solution to which EDTA had been added in sufficient quantities to co-ordinate all of the Ca^{2+}. All data were corrected for light scatter due to protein alone, and treated essentially as described (Nelsestuen & Lim, 1977). Briefly, the concentrations of protein bound to the vesicle (x) were calculated from the changes in light scatter, as previously reported (Nelsestuen & Lim, 1977). The data were plotted by the modified Klotz method, using double-reciprocal plots of the concentrations of bound, $[P]_b$, versus free, $[P]_f$, protein, according to:

$$1/[P]_b = K_4^{Ca}/x[PL][P]_f + 1/x[PL]$$

where [PL] represents the concentration of PL vesicle. The term K_4^{Ca} refers to the dissociation constant for the protein to the PL vesicle when the PL–protein complex is saturated with Ca^{2+}, and the protein has fully undergone the Ca^{2+}-induced transition necessary for binding to the vesicle. In all cases reported herein, except where noted otherwise, quantities of Ca^{2+} sufficient to satisfy these conditions were employed. Values for n and K_4^{Ca} were calculated from the y-intercepts and slopes, respectively, of these plots.

The above experiments were conducted with a Perkin–Elmer MPF 44 fluorescence spectrophotometer, maintained at 25 °C, with the emission and excitation monochromators adjusted to 320 nm. Sample volumes were 1.5 ml, and final PL concentrations were approx. 25 μg/ml. Dust particles were rigorously removed from all samples by Millipore filtration through type HA discs possessing an average pore size of 0.45 μm. Prior to use, the protein and PL solutions were subjected to Chelex-100 chromatography in order to remove appropriate bivalent cations, and the concentrations of the stock solutions containing these components were determined after this step. For all experiments reported herein, the buffer employed was 0.02 M-Hepes/0.15 M-NaCl, pH 7.4, containing the required concentrations of Ca^{2+}.

QLS measurements

Measurements of the radius of PL vesicles and PL vesicle–protein complexes were made with a Nicomp HN5-90 laser light scatter spectrometer. The samples for these experiments were prepared as described in the previous section. The Nicomp particle analyser is a computerized light scatter apparatus that operates in a single mode by using a 632.8 nm He–Ne laser. The instrument utilizes the theory of Rayleigh scattering due to translational Brownian motion of particles and computes the mean hydrodynamic radius, $\bar{R}$, from the Stokes–Einstein relationship for the diffusion of spherical particles. A built-in microcomputer system performs rapid quadratic least-squares fit of the data, yielding a mean diffusion coefficient, $\bar{D}$. An estimate of the degree of polydispersity present in the sample is also computed as the normalized S.D. of the intensity-weighted distribution of diffusion coefficients. All of our PL vesicles, in the presence or absence of Ca^{2+}, yielded values for this standard deviation of 0.55 or lower, suggesting that only a small degree of polydispersity in vesicle size exists.

In these experiments, a sample volume of 0.7 ml was placed in a 6 mm × 50 mm test tube and subjected to centrifugation in an Eppendorf 5413 table top centrifuge for 10 min. The sample was carefully transferred to the light scatter chamber for measurement of vesicle size. Phospholipid concentrations were approx. 0.25 mg/ml.

This technique was also employed to monitor changes in the size of the protein–vesicle complexes. The vesicles were titrated with protein and the resultant radii measured. Protein concentrations, on a weight basis, ranged from 0.5–5 × the PL concentration reported above. The data were plotted as $1/\bar{R}$ against $[PL]/[P]_{total}$ (w/w). The value of the y-intercept is the $(\bar{R})^{-1}$ of the protein–PL complex at saturating protein concentration. The radius determined for the PL vesicle alone was then subtracted from that determined for the protein-PL complex in order to yield the change in radius (Δr) consequent to protein binding to the vesicle. All values were corrected for incomplete protein binding to its available sites on the vesicle surface and for incomplete surface packing, when necessary, by the procedure published by Bloomfield *et al.* (1967). In no case reported here was the mean hydrodynamic radius of the vesicle, or the vesicle–protein complex, dependent on the concentration of sample employed.

Error analysis

All binding data reported is based upon a minimum of three separate experiments, with each data point determined in duplicate. Double-reciprocal plots were analysed employing a least squares program that allows calculation of the deviation within a 95% confidence limit. The vesicle size values reported are based upon a minimum of five separate determinations in each case.

RESULTS

Effects of calcium on vesicle size

Previous studies have shown that Ca^{2+} can induce vesicle aggregation, which, in turn, can directly influence the study of Ca^{2+}-mediated protein-vesicle interactions (Papahadjopoulos *et al.*, 1976). In order to investigate the effects of Ca^{2+} on vesicle aggregation and size, vesicles of varying PS and PC content were titrated with Ca^{2+}. The resultant changes in vesicle radius were measured employing 90° QLS and 90° light scatter. The results from the QLS study, shown in Table 1, reveal interesting physical properties of the particles. The maximum amount of Ca^{2+} which can be present without resulting in aggregation of the vesicles progressively decreases from approximately 20 mM, at a vesicle composition of 4:1 (w/w) PC/PS, to 2 mM at a composition of 1:1 (w/w) PC/PS. As can be seen from the data presented in Table 1, in the absence of Ca^{2+} the vesicle size remains unchanged over the range of PS investigated, with a mean average value of 30.7 nm. The effects of Ca^{2+} on vesicle size appear to be a function of the concentration of this

Table 1. Effect of calcium on the mean hydrodynamic radius of vesicles of various compositions

PC/PS (%, w/w)	Ca^{2+} (mM)*	$\bar{R}$ (nm)	
		$-Ca^{2+}$	$+Ca^{2+}$
80/20	20	30.7±0.7	33.5±0.7
70/30	6	31.1±0.5	30.3±0.9
60/40	4	30.2±0.6	28.6±0.9
50/50	2	30.9±0.2	27.2±0.5

* The maximal amounts of Ca^{2+} that could be added without observable effect on the radius of the vesicle.

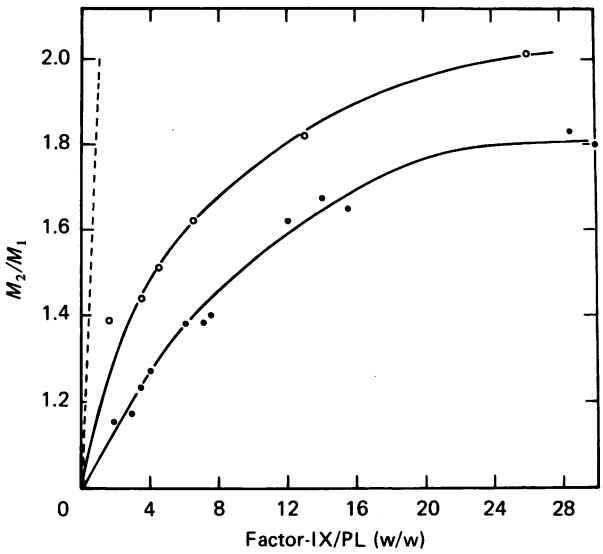

Fig. 1. Ratio of M_2/M_1 as a function of binding of Factor IX to phospholipid vesicles

The scattering intensity (I_1) of a vesicle dispersion containing 38 μg of total phospholipid (PC+PS) in 1.5 ml of a buffer consisting of 0.02 M-Hepes/0.15 M-NaCl, pH 7.4, at an optimal Ca^{2+} concentration for the vesicle system employed, was assigned a value of 1.0. Upon addition of various quantities of Factor IX to this suspension, the scattering intensities (I_2) were altered, and converted to the respective M_2/M_1 ratios by the procedure described in the Experimental procedures section, after applying appropriate corrections for dilution of the vesicle suspension and for scattering by non-bound protein. These values have been plotted against the ratio (w/w) of total Factor IX/PL, for vesicles containing 80% PC/20% PS (●) and 50% PC/50% PS (○). The broken line refers to the theoretical M_2/M_1 values that would be obtained if all added protein was bound to the particular vesicle.

cation, as well as the PS content of the mixed vesicle. The average radii of the vesicles employed in this study in the presence of Ca^{2+} are shown in Table 1, and were used in subsequent calculations in this paper.

When these same experiments were performed by 90° light scatter, no increases in scattering upon addition of Ca^{2+} were seen with any of these vesicles up to the concentrations of Ca^{2+} listed in Table 1, thus confirming that no significant aggregation occurred at the indicated levels of Ca^{2+}.

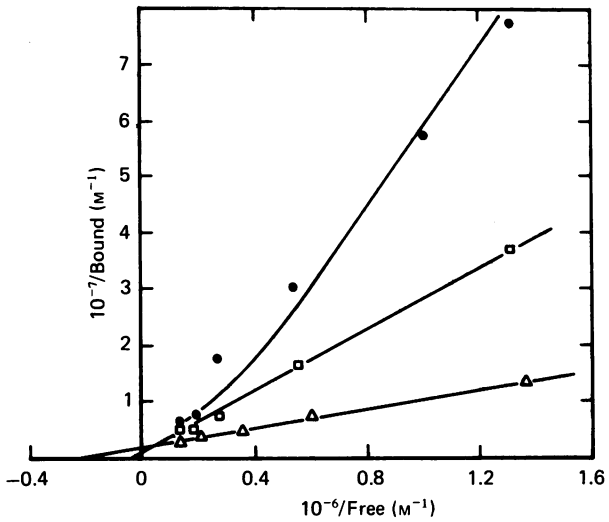

Fig. 2. Effect of Ca^{2+} on the binding of Factor IX to phospholipid vesicles containing 80% PC/20% PS (w/w)

These plots were generated as described in the Experimental procedures section for Factor IX binding to the vesicles (33.2 μg of total PL in 1.5 ml of the buffer detailed in Fig. 1), and represent the concentration of Factor IX bound to the vesicle as a function of the concentration of non-bound (free) protein at concentrations of Ca^{2+} of 2 mM (●), 10 mM (□) and 20 mM (△).

Effects of calcium on Factor IX–membrane binding

These experiments were performed by 90° light scattering, by evaluating the increase in scatter resulting from addition of a series of concentrations of Factor IX, Factor IXα, Factor IXaα and Factor IXaβ to the various vesicles described above, in the presence of different amounts of Ca^{2+}. The ratio of the scattering intensities for the protein–vesicle complex (I_2), from which the scattering due to free protein has been subtracted, to that for the proteins (I_1), was converted to the ratio of the molecular weights of these components (M_2/M_1) by the iterative procedure described by Nelsestuen & Lim (1977). For this calculation, the refractive increment for the PL was taken as 0.172 for all vesicle compositions (Nelsestuen & Lim, 1977), and that for Factor IX, and all activation intermediates and products, was 0.192. The refractive increments for protein–vesicle complexes were taken as the weighted averages of those for the PLs and the amounts of bound protein. A representative plot of the M_2/M_1 values, as a function of the weight ratio of initial amounts of Factor IX to PL, with vesicles of two different compositions and at optimal concentrations of Ca^{2+} (from Table 1), is presented in Fig. 1. Also included in Fig. 1 is a theoretical plot of the M_2/M_1 ratios calculated on the basis that all protein added was bound to the PL.

Using the graphs contained in Fig. 1, the amount of Factor IX bound to the PL was calculated from the differences in the theoretical and experimental lines, and is shown in Fig. 2 in the form of modified Klotz plots. In these examples of the data obtained, a vesicle of 80% PC/20% PS (w/w) was employed, at three different Ca^{2+} concentrations. The curved line drawn for the data obtained at 2 mM-Ca^{2+} deserves special mention. In

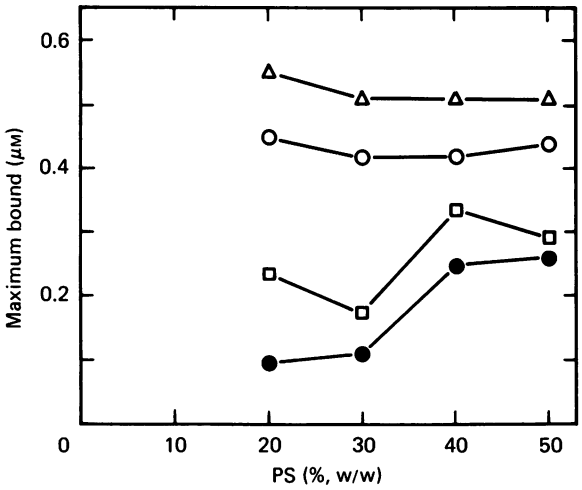

Fig. 3. Maximal concentration of protein bound to phospholipid vesicles at various PS concentrations (expressed as the weight percent of total phospholipid), obtained from the *y*-intercepts of data such as that shown in Fig. 2, for Factor IX (○), Factor IXα (●), Factor IXaα (□) and Factor 1Xaβ (△)

The total added phospholipid was 22.1 μg/ml.

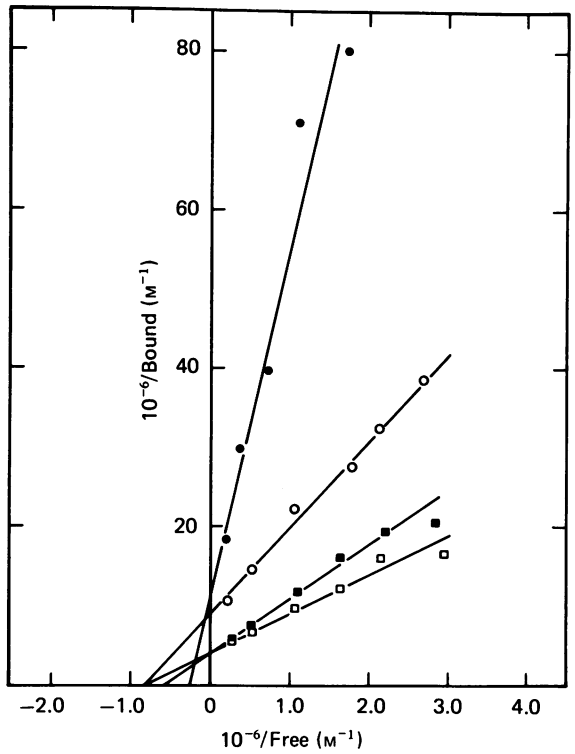

Fig. 4. Klotz plots of the concentration of bound versus free Factor IXα to vesicles of PS content 20% (●), 30% (○), 40% (■) and 50% (□)

These experiments were conducted as in Fig. 1. The total added phospholipid was 22.1 μg/ml.

analysing this plot, a quadratic least squares fit was required to yield a line with a correlation coefficient of 0.99. A linear least squares analysis yields a positive *x*-intercept and a poor correlation coefficient. This plot suggests that Ca^{2+} is not saturating for this particular vesicle system and that multiple dissociation constants for protein–membrane binding exist. Similar data (not shown) were obtained when Factor IXα and Factor IXaβ were employed in place of Factor IX for this particular vesicle, a consideration which supports the above interpretation. However, at 10 mM and 20 mM concentrations of Ca^{2+}, straight lines are obtained, allowing calculations of n and K_4^{Ca} to be performed. The K_4^{Ca} values obtained are 18.5 and 4.9 μM at 10 and 20 mM-Ca^{2+}, respectively. The lack of similarity of these values suggests that 10 mM-Ca^{2+} does not yet saturate the membrane and higher concentrations of this metal ion are required. At 25 mM-Ca^{2+} (results not shown), the K_4^{Ca} obtained was very similar to that found at 20 mM-Ca^{2+}, demonstrating that the true K_4^{Ca} for protein–membrane interactions, with this specific system, is approx. 4.9 μM.

Similar studies on the binding of Factor IX to PC/PS vesicles of varying PS content have been performed (results not shown). In each case, the optimal Ca^{2+} concentrations for each vesicle, given in Table 1, have been employed. A replot of the *y*-intercepts from Klotz plots of these data indicates that the amount of Factor IX bound is not a function of the PS content, as seen from the data of Fig. 3. However, the value for K_4^{Ca} decreases as the amount of PS in the vesicle increases, ranging from 4.9 μM for vesicles composed of 20% PS, to 1.7 μM for similar vesicles containing 50% PS. A summary of these values is given in Table 2.

Binding of activation intermediates and products of Factor IX to vesicles of various compositions

Modified Klotz plots of the binding of Factors IXα, IXaα, and IXaβ to the above PL vesicles have been constructed. An example of such a plot for Factor IXα, at optimal levels of Ca^{2+} (Table 1), is shown in Fig. 4. In the case of this protein, it is clear that both x and K_4^{Ca}

Table 2. Binding constants for Factor IX and its activation intermediates and products to various PL vesicles containing PC and PS at optimal levels of Ca^{2+}

PC/PS (%, w/w)	[Ca^{2+}] (mM)	K_4^{Ca} (μM)			
		Factor-IX	Factor-IXα	Factor-IXaα	Factor-IXaβ
80/20	20	4.9±0.5	5.4±0.2	2.9±0.8	8.7±2.0
70/30	6	2.4±0.3	1.2±0.2	1.9±0.7	3.6±0.9
60/40	4	1.7±0.3	1.3±0.3	0.7±0.1	1.0±0.2
50/50	2	1.7±0.3	1.7±0.4	0.7±0.1	1.0±0.1

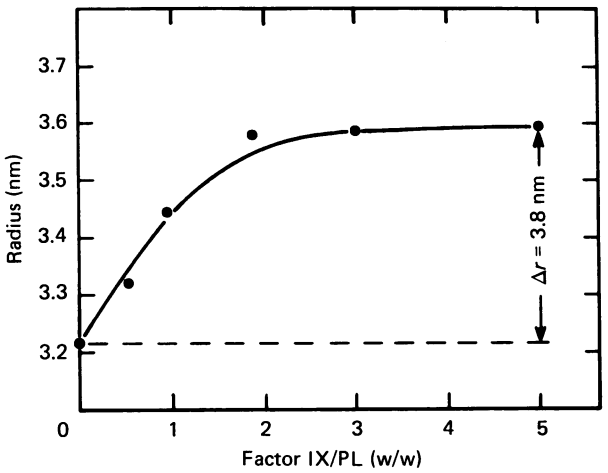

Fig. 5. Radius changes for Factor IX–vesicle complexes as determined by QLS measurements

In this experiment, a total of 175 μg (total phospholipid) of vesicle containing 20% PS, in 0.7 ml, was titrated with Factor IX in the presence of 20 mM-Ca^{2+} and changes in the mean effective radii were measured and plotted against the total concentration of Factor IX/PL, w/w. Δr represents the noncorrected net maximal radius change of the complex due to bound protein.

are dependent upon the amount of PS present in the vesicle. Regarding the K_4^{Ca}, the values of which are listed in Table 2 for Factor IXα binding to the various vesicles, a weaker interaction is noted with the vesicle composed of 20% PS than with those at higher PS concentrations. At PS levels of 30–50%, the K_4^{Ca} value does not appear to depend upon the amount of PS present. For Factors IXaα and IXaβ, the K_4^{Ca} decreases as the PS concentration increases from 20% to 40%. The value obtained for the vesicle containing 50% PS is not significantly different from that determined at 40% PS in the case of both proteins.

Fig. 3 provides data on the maximum amount of each protein bound to vesicles of varying PS concentration, and has been obtained from the y-intercepts of plots similar to those of Fig. 4. Factors IXα and IXaα do not bind to the same extent as do Factors IX and IXaβ. These former two proteins also show a dependency in the amounts bound at saturation on the levels of PS contained in the vesicles, increasing as the PS content increases. On the other hand, Factors IX and IXaβ are bound to approximately the same extent at all levels of PS studied.

Table 4. Amount of PS located on the outer surface of the vesicle in the absence and presence of Ca^{2+}

'% $PS_{o.s.}$' refers to the actual weight percent of PS on the outer vesicle surface.

PC/PS (%, w/w)	% $PS_{o.s.}$ (±2%)	
	$-Ca^{2+}$	$+Ca^{2+}$
80/20	11	14
70/30	14	20
60/40	14	23
50/50	18	25

Determination of radii of the proteins on the vesicle surface

A titration of the change in radius (Δr) of the protein–vesicle complex, as a function of the amount of protein added, has been determined by QLS analysis for each of the four proteins examined in this study, with vesicles composed of 20% PS/80% PC (w/w). An example of the data obtained with Factor IX is shown in Fig. 5. At apparent saturation, the Δr is approx. 3.8 nm. In order to obtain more accurately this value at levels of Factor IX which saturate the vesicle, a double reciprocal plot of $\bar{R}^{-1}$ against [PL]/[Factor IX]$_T$ has been generated and extrapolated to the value for $\bar{R}$ at infinite [Factor IX]$_T$, from which a Δr of approx. 4.2 nm has been determined. This calculated value is an underestimate of the true radius change, since the vesicle surface, even at saturation of its binding sites for Factor IX, likely consists of regions which do not contain protein, i.e., there is incomplete surface packing of the vesicle and 'holes' are present (Bloomfield *et al.*, 1967). Since Factors IX and IXaβ saturate the vesicles at all levels of PS employed (Fig. 3), we had no basis to conclude that surface gaps exist in these cases and we have not made a correction for surface packing. However, for Factors IXα and IXaα, the amounts of protein bound to the vesicles increase as the PS level is increased, demonstrating that the surface of vesicles of this size contain regions that lack protein. We estimated the proportion of surface of these vesicles which do not contain protein in the following manner. The concentration of protein bound to vesicles containing 80% PC/20% PS (w/w) was divided by the maximum amount of protein bound to vesicles that are saturated, i.e. for Factors IXα and IXaα at [PS] > 30%, as shown in Fig. 3. From the value of this ratio, the '% holes' (Bloomfield *et al.*, 1967) was determined. With knowledge of this parameter, the

Table 3. Changes in the radius (Δr) of PL vesicles upon saturation with Factor IX and its activation intermediates and products

Δr has been corrected for saturation of surface binding sites for each protein and for incomplete surface packing.

PC/PS (%, w/w)	Δr (nm) for:			
	Factor IX	Factor IXα	Factor IXaα	Factor IXaβ
80/20	4.5±0.6	6.4±0.7	6.6±0.8	5.0±0.7
50/50	5.1±0.9	7.1±0.9	7.5±0.8	4.8±0.6

Table 5. The number of molecules of PS potentially available per molecule of protein on the outer surface of PC/PS vesicle systems

PC/PS (%, w/w)	PS exposed (molecules/vesicle)	Protein	n* (molecules of protein/vesicle)	PS/protein (molecules/molecule of protein)
80/20	5579±1454	Factor-IX	1958±231	3±1
		Factor-IXα	500±49	11±3
		Factor-IXaα	1083±226	5±2
		Factor-IXaβ	2417±412	2±1
70/30	6344±1975	Factor-IX	1197±131	5±2
		Factor-IXα	338±54	19±5
		Factor-IXaα	583±131	11±5
		Factor-IXaβ	1566±351	4±2
60/40	6433±1990	Factor-IX	1004±159	6±2
		Factor-IXα	567±129	11±4
		Factor-IXaα	824±117	8±3
		Factor IXaβ	1313±256	5±2
50/50	6265±672	Factor IX	995±67	6±1
		Factor-IXα	619±36	10±2
		Factor-IXaα	641±39	10±1
		Factor-IXaβ	1128±87	6±1

* Values from Table 2.

experimentally determined Δr for the protein–vesicle complex can be corrected for incomplete surface packing (Bloomfield *et al.*, 1967).

Table 3 lists values of Δr, at optimal Ca^{2+} (Table 1), for Factors IX, IXα, IXaα and IXaβ with vesicles containing 20% PS and 50% PS that have been corrected, when necessary, for saturation of the protein binding sites on the vesicle and for incomplete surface packing.

PS–protein interactions

In order to estimate the number of PS molecules that interact with the protein on the vesicle surface, the M_r of PS based upon its oleoyl form has been used. Calculation of the vesicle molecular weights was based upon their existence as spherical bilayer particles with the radii shown in Table 1, bilayer thicknesses of 5 nm and membrane densities of 0.9±0.1 g/ml (Shipley, 1973). Further, the amount of PS on the outer vesicle surface was determined by labelling of the available PS with fluorescamine, under conditions that allowed only outer surface PS to react, without penetration of the labelling reagent into the vesicle (Lee & Forte, 1979). Table 4 lists the percentages of outer surface PS, in the presence and absence of optimal Ca^{2+}. In addition, the number of molecules of protein per vesicle (n) must be calculated. In order to obtain the values of n from these plots, vesicle radii are required so that the number of vesicles present can be calculated. These radii have been listed in Table 1. The M_r of the vesicle is then determined with knowledge of this radius and the above mentioned membrane properties.

Employing the above assumptions, the number of molecules of PS exposed on the outer surface of the vesicle, $[PS]_{o.s.}$ was calculated as:

$$[PS]_{o.s.} = \%PS_{o.s.} \times M_r \text{ of bilayer}/M_r \text{ of PS} \times 100$$

The maximum number of PS molecules that are capable of interacting with the proteins on the vesicle surface has been calculated for Factor IX and its activation intermediates and products when bound to PS/PC vesicles containing from 20% to 50% (w/w) PS. The data obtained are listed in Table 5.

DISCUSSION

The vitamin K-dependent coagulation proteins, e.g., prothrombin, Factors VII, IX, X, protein C and protein S, all contain γ-carboxyglutamic acid, and bind to Ca^{2+} and PL. These features are essential to their respective roles in the clotting cascade. For this reason, much attention has been paid to the properties of PLs that are related to the functional properties of these proteins. At least four activation systems involving these proteins are known to be accelerated by PL; prothrombin activation by Factor Xa/Ca^{2+}/Factor Va (Jobin & Esnouf, 1967; Gitel *et al.*, 1973), Factor X activation by either Factor VIIa/Ca^{2+} (Silverberg *et al.*, 1977) or by Factor IXa/Ca^{2+}/Factor VIIIa (Chuang *et al.*, 1972), and Factor VII activation by Factor Xa/Ca^{2+} (Radcliffe & Nemerson, 1975). A great deal of the work that established the nature of the PL optimal for function in coagulation has been performed on the prothrombin activation system. From studies with model PL systems, a number of essential requirements for their effectiveness has been proposed: a negatively charged membrane displaying homogeneous charge distribution across the outer surface; the presence of additional nucleophilic groups to stabilize Ca^{2+} bridges between the lipid surface and the γ-carboxyglutamic acid residues on the protein; and unsaturation in the hydrocarbon chains of the PL in order to provide a liquid crystalline state to the membrane (Zwaal, 1978). In an investigation of the binding of prothrombin to membranes of acidic PLs of varying composition, it has been found that PS is the most effective of such molecules in promoting the affinity of prothrombin (Nelsestuen & Broderius, 1977).

A systematic study of the nature of the PLs most suitable to promote assembly and catalysis in the activation of Factor X by Factor IXa has not been

published. We have shown, however, that PLs accelerate the activation of Factor X by Factor IXaβ to a greater extent than this same activation by Factor IXaα (Link & Castellino, 1983). In order to determine whether this phenomenon was due to a greater affinity of Factor IXaβ for the membrane, and in order to investigate pertinent structure–function relationships regarding the binding of Factor IX to membranes, we have examined the interaction of this protein, as well as its activation intermediates and products with defined peptide bond cleavages, to synthetic membranes composed of varying amounts of PS. In all studies, the 90° light scatter technique, developed by Nelsestuen & Lim (1977), has been employed due to its successful application to examination of protein–membrane interactions of the type described herein.

Since it is well known that Ca^{2+} will induce aggregation of PL vesicles containing PS, and that this phenomenon would seriously complicate light scatter analysis of the binding data, we initially determined the maximal levels of Ca^{2+} that could be employed in such experiments without causing aggregation of the different vesicle preparations. The data of Table 1 show that as the PS content increases, the concentration of Ca^{2+} that induces vesicle aggregation decreases. Given these criteria, the highest concentration of Ca^{2+} permitted in these experiments are the values listed in Table 1 for each of the different vesicles.

The equilibria involved in binding of the proteins to the vesicles have been established by Nelsestuen & Lim (1977) to be the following:

$$P + i\mathrm{Ca} \overset{1}{\rightleftharpoons} P_{i\mathrm{Ca}} \overset{2}{\rightleftharpoons} P'_{i\mathrm{Ca}}$$

$$+ m\mathrm{Ca} \overset{4}{\rightleftharpoons} P'\mathrm{Pl}_{(i+m+j)\mathrm{Ca}}$$

$$\mathrm{PL} + j\mathrm{Ca} \overset{3}{\rightleftharpoons} \mathrm{PL}_{j\mathrm{Ca}}$$

where P is the protein and P′ is the same protein after undergoing the conformational change required for lipid binding. In order to obtain the Ca^{2+}-independent equilibrium constant for reaction 4 (K_4^{Ca}) the protein must exist in the P′ state and the PL must be saturated with Ca^{2+}. We have studied the Ca^{2+} dependence of the conformational change in the proteins (reaction 2) of interest here by the intrinsic fluorescence alterations which occur in Factor IX as a result of addition of Ca^{2+} (Bajaj, 1982) and find that at the lowest concentration of Ca^{2+} used in this study, 2 mM, the conformational transition is practically complete. This suggests that the protein is in the P′ state at 2 mM-Ca^{2+}. Regarding reaction 3, while its equilibrium constant will depend upon the amount of PS in the vesicle, typical dissociation constants are approximately an order of magnitude lower than the lowest Ca^{2+} concentration employed here (Nelsestuen *et al.*, 1976), suggesting that the vesicle is essentially in the form of $PL_{j\mathrm{Ca}}$ for all experiments reported in this paper. Reaction 4, above, represents the binding of protein to PL and may be accompanied by the necessity of addition of further Ca^{2+} (m). However, since the values of i and j represent saturation of the effects of interest in this paper, for the proteins and membranes studied, the value of m should be close to zero, at least as regard to its effect on protein–membrane interactions. It appears, from studies not entirely reported in the present paper, that Ca^{2+} concentrations of 2 mM and above saturate the system with regard to binding of the proteins studied here to vesicles containing PS at levels greater than 20% of the total vesicle weight. However, as shown in Fig. 2, with vesicles containing 20% PS/80% PC (w/w), saturation with Ca^{2+} does not occur until much higher levels of Ca^{2+} are included.

The data in Table 2 indicate that small differences exist in the K_4^{Ca} values for the four proteins to each of the vesicles. For all vesicles, at low PS content, Factors IXα and IXaα seem to bind tighter than does Factor IXaβ. Thus, loss of the activation peptide appears to slightly inhibit binding of the protein to the vesicle. Also, in all cases, the binding affinity of the proteins to vesicles containing 40% and 50% PS is essentially the same, indicating that the PS content of the vesicles is saturable in terms of binding to Factor IX and its activation intermediates and products. This is not surprising, since the data presented in Table 4 show that the outer vesicle surface content of PS has essentially reached a maximal value in vesicles containing 40% PS. In all cases, the binding of protein to PL is Ca^{2+}-dependent since the addition of EDTA led to complete restoration of the light scatter of the original vesicle.

Two other studies exist in the literature in which the binding of Factor IX to PL vesicles has been examined. Nelsestuen *et al.* (1978) have reported a dissociation constant, by the same techniques employed here, of approx. 2 μM to vesicles containing 20% PS/80% PC, at 2 mM-Ca^{2+}. This is in close agreement with our value of 4.9 μM at 20 mM-Ca^{2+}. In our experiments, we were not able to determine this parameter at 2 mM-Ca^{2+}, due to the curvature of the Klotz plots at this concentration of Ca^{2+}. In another study (Mertens *et al.*, 1984), a dissociation constant for Factor IXaβ binding to vesicles containing 50% PS/50% PC, as measured by a centrifugation technique, was found to be approx. 0.012 μM at 10 mM-Ca^{2+}. We clearly differ significantly with this value. However, in the study by Mertens *et al.* (1984), the Ca^{2+} concentration used would have aggregated the vesicles considerably, and it would be expected that this larger aggregate would indeed allow binding of the protein to be more facile, due to the larger surface area exposed. We cannot test this point with our binding methodology, since larger vesicles, with much greater light scatter properties, would reduce considerably the sensitivity of the technique to the further small changes in 90° light scatter accompanying protein binding. Importantly, however, we show that the affinity of Factor IXaβ to these vesicles is not greater than that of Factor IXaα. Therefore, the ability of membranes containing acidic PLs to stimulate the enzymic activity of Factor IXaβ to a greater extent than Factor IXaα is based on events that occur after the initial binding of these enzymes to the membranes.

Whereas the binding constants of the proteins to the various vesicles do not display large differences, the amounts of protein that can be accommodated on the vesicle surfaces are not similar for each of the proteins studied (Fig. 3). The amount of Factors IX and IXaβ capable of binding to the vesicles is not a function of the amount of PS present and is substantially larger than this same parameter for both Factor IXα and Factor IXaα. This is consistent with the observation (Table 3) that Factors IXα and IXaα exhibit larger radii when fully packed on the surface of the vesicles, thus decreasing the

number of sites available on the surface of these small vesicles for these latter two proteins. In addition, for Factor IXα and Factor IXaα, the amount of protein bound to the vesicle depends upon its PS content. The fact that a greater number of molecules of Factor IXaβ are present on these vesicles, as compared with Factor IXaα, can explain the observation that a much larger increase in the $V_{max.}$ of the activation of Factor X occurs with a system containing Factor IXaβ/Ca^{2+}, than with Factor IXaα/C^{2+} (Link & Castellino, 1983).

The amount of PS exposed on the outer vesicle surface is increased in the presence of Ca^{2+}, as shown in Table 4. This could be explained by taking into account the flip-flop mechanism of Kornberg & McConnell (1971), wherein the presence of Ca^{2+} might allow more rapid migration of PS from the inner to the outer vesicle surface. The number of PS molecules that interact with each of the proteins on the four vesicles examined in this report has been estimated in Table 5. With the vesicle composed of 20% PS, a maximum of approx. three to five PS molecules are capable of binding to a protein molecule on the surface with Factors IX, IXaα and IXaβ, and is much higher for Factor IXα. This number of PS molecules potentially similarly involved increases to six to 10 for these proteins on the vesicle composed of 50% PS. The increase in PS bound to the proteins on the latter vesicle likely accounts for the greater affinity for these proteins seen on this membrane. Due to the much lower levels of Factor IXα bound to the vesicle containing 20% PS, considerably more PS is available for this interaction, and limitations in the amount of available PS on this vesicle may explain the decreased number of sites for this protein.

In summary, we have evaluated the properties of a variety of membrane surfaces regarding their ability to bind to coagulation Factor IX and its activation intermediates and products. The significant differences in the interactions with membranes of the two forms of Factor IXa employed, as established in the present paper, emphasize their differing properties in the activation of Factor X and provide a partial explanation of their effects therein. Further, the increased amounts of these enzymes bound to PL vesicles, with concomitant increases in affinity as the PS content is increased, suggest a basis for the greater stimulatory activity toward Factor X activation of membranes containing larger quantitites of PS.

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