Binding of Bovine Factor V$_a$ to Phosphatidylcholine Membranes

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ABSTRACT The interaction of bovine factor V$_a$ with phosphatidylcholine membranes was examined using four different fluorescence techniques: 1) changes in the fluorescence anisotropy of the fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) to monitor the interaction of factor V$_a$ with 1,2-dimyristoyl-3-sn-phosphatidylcholine (DMPC) small unilamellar vesicles (SUVs), 2) changes in the fluorescence anisotropy of N-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (Rh-PE) incorporated into SUVs prepared from 1-palmitoyl-2-oleoyl-3-sn-phosphatidylcholine (POPC), 3) changes in the fluorescence anisotropy of fluorescein-labeled factor V$_a$ (labeled in the heavy chain) upon interaction with POPC SUVs, 4) fluorescence energy transfer from fluorescein-labeled factor V$_a$ to rhodamine-labeled POPC SUVs. In the first two sets of experiments, labeled lipid vesicles were titrated with unlabeled protein, whereas, in the latter two types of experiments, labeled factor V$_a$ was titrated with vesicles. For the weak binding observed here, it was impossible from any one binding experiment to obtain precise estimates of the three parameters involved in modeling the lipid-protein interaction, namely, the dissociation constant K_d, the stoichiometry of binding i, and the saturation value of the observable R_{max} from any one experiment. However, a global analysis of the four data sets involving POPC SUVs yielded a stable estimate of the binding parameters (K_d of ~ 3.0 μM and a stoichiometry of ~ 200 lipids per bound factor V$_a$). Binding to DMPC SUVs may be of slightly higher affinity. These observations support the contention that association of factor V$_a$ with a membrane involves a significant acidic-lipid-independent interaction along with the more commonly accepted acidic-lipid-dependent component of the total binding free energy.

INTRODUCTION

Factor V$_a$ is a non-enzymatic cofactor that combines with the serine protease factor X$_a$ on a platelet or phospholipid membrane in the presence of calcium ions to form the prothrombinase complex. Prothrombinase complex catalyzes the activation of prothrombin to thrombin in the coagulation cascade. Factor V$_a$ is a heterodimer composed of a heavy chain derived from the NH$_2$-terminal part of factor V ($M_r = 94,000$) and a light chain (M_r of either 74,000 or 71,000) derived from the COOH-terminal end of the factor V molecule (Kane and Majerus, 1981; Suzuki et al., 1982; Nesheim et al., 1984). These two chains are noncovalently associated (Esmon, 1979; Nesheim and Mann, 1979) to form a fully active factor V$_a$ in solution by the addition of divalent metal ions (Krishnaswamy et al., 1989). Membrane binding is also commonly seen as involving only the light chain subunit of factor V$_a$ (Krishnaswamy and Mann, 1988; Kalafatis et al., 1994). This subunit is reported to bind to the platelet surface or phospholipid vesicle independently of the amino-terminal-derived heavy chain (Tracy and Mann, 1983; Krishnaswamy and Mann, 1988).

The requirement for negatively charged phospholipids for interaction of factor V$_a$ with procoagulant membranes is generally accepted, despite the fact that the interaction has recently been argued to involve both hydrophobic and electrostatic interactions (Kalafatis et al., 1990; Ortel et al., 1992; Lecompte et al., 1994). However, Kalafatis et al. (1994) have recently observed photoactivated labeling of factor V$_a$ in the presence of membranes containing only neutral phospholipid, implying an interaction with a neutral phospholipid membrane. We also report in a companion paper (Cutsforth et al., 1996) that binding of factor V$_a$ to membranes is a complex process involving both acidic-lipid-independent and acidic-lipid-dependent interactions, apparently linked by conformational changes in the protein.

In this study, we have monitored the binding of factor V$_a$ to membranes composed of only phosphatidylcholine by employing four different fluorescence methods. Because this binding is weak, we discuss why it is difficult to study it under conditions that lead to stable estimates of binding parameters. However, global analysis of data from four different experiments indicates that factor V$_a$ binds to small unilamellar vesicles (SUVs) with a dissociation constant of ~3.0 μM and a binding stoichiometry of ~200 lipids per bound factor V$_a$.

MATERIALS AND METHODS

Materials

1,2-Dimyristoyl-3-sn-phospatidylcholine (DMPC), N-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine (Rh-PE), and sodium salts of bovine phosphatidylserine (PS) and POPC were purchased from Avanti Polar Lipids (Birmingham, AL) and shown to be greater than 98% pure by thin-layer chromatography (Lentz et al., 1982). All of the lipids were stocked in argon-bubbled chloroform with or without a small amount of methanol. Solvents were low residue, HPLC grade. Calcium ion con-

tamination of the acidic lipids was shown to be less than 2 mol % (Lentz et al., 1982). 1,6-Diphenyl-1,3,5,-hexatriene (DPH) was purchased from Molecular Probes (Junction City, OR). All other chemicals were reagent grade or better. Phospholipid vesicles were prepared as described in the accompanying paper (Cutsforth et al., 1996) in a buffer containing 20 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 150 mM NaCl, pH 7.4. Total phosphate concentration in a vesicle sample was determined using the method of Chen et al. (1956).

Modification of factor V_a heavy chain with fluorescein maleimide

Bovine factor V purified by the procedure of Esmon (1979) was activated by addition of 10 U of bovine α-thrombin per ml of factor V (1–2 mg/ml bovine factor V). It was then purified on a Mono S HR 5/5 ion-exchange column (Pharmacia, Norwalk, CT) coupled to a Perkin-Elmer Isopure LC system (Perkin-Elmer Corp., Norwalk, CT). The activity of isolated factor V_a was evaluated by the thromboplastin assay in terms of the clotting time of factor-V-deficient plasma standardized against the clotting time of pooled, normal human plasma (Nesheim et al., 1981). The heavy and light chain components of factor V_a were separated by ion-exchange on QAE-Sepharose (Pharmacia Corp., Piscataway, NJ; Esmon, 1979). The light chain was further purified by HPLC on a Mono S column (Pharmacia Corp.; Odegaard and Mann, 1987). The heavy chain component was found to contain a large amount of 55,000-mol wt protein, which was removed on a Biogel-100 gel filtration column (Bio-Rad Laboratories, Richmond, CA). The heavy chain was finally purified on a Mono Q HR 5/5 ion-exchange column (Pharmacia Corp.) coupled to a Perkin-Elmer Isopure LC system. The final purity of the factor V_a components was ascertained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Wycoff et al., 1977) using the Bio-Rad Minigel system to be greater than 85%.

To prepare labeled factor V_a, a 6-μl aliquot of a stock solution of fluorescein-5-maleimide (Molecular Probes; 51 mM in N,N-dimethylformamide) was added to 100 μl of buffer (20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) and mixed well. Factor V_a heavy chain (600 μl) in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (5.8 mg/ml stock), was treated with this probe solution by slowly adding the probe solution with constant mixing. The mixture was then incubated in the dark at 4°C for 18 h and then passed through a Sephacryl S-100-HR column (50 × 1 cm inner diameter) at 4°C equilibrated with 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4. Fractions containing factor V_a heavy chain were pooled on the basis of the absorbance values at 280 nm and precipitated by the addition of solid ammonium sulfate (80% saturation), collected by centrifugation (50,000 × g for 20 min), resuspended in 20 mM TES, 5 mM $CaCl_2$, 50% (v/v) glycerol, pH 7.4, and stored at −20°C. Protein concentration was determined by the bicinchonic acid assay (Pierce Chemical Co., Rockford, IL), and the molar ratio of fluorescein to protein (0.95) was determined using the molar absorptivity of protein-conjugated fluorescein at 494 nm (ϵ = 64,800 M^{-1} cm^{-1}; Molecular Probes). Factor V_a was reconstituted using fluorescein-labeled heavy chain and unlabeled light chain in a molar ratio of 1.2:1 in a buffer composed of 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. Purity of the fluorescein-labeled factor V_a heavy chain and reconstituted factor V_a was established on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was determined to be more than 85% pure based on densitometry analysis of the gel, and fluorescence was detected only from the band corresponding to the factor V_a heavy chain, indicating that the fluorescence being monitored was due only to the factor V_a heavy chain.

Activity of reconstituted factor V_a used for the experiments was evaluated by measuring prothrombin activation catalyzed by prothrombinase using the fluorescent α-thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (Nesheim et al., 1981). The functional factor V_a concentration was found to be more than 90% of the concentration determined from molar absorptivity measurements.

Fluorescence measurements

All fluorescence measurements were made using an SLM 48,000 spectrofluorometer (SLM Aminco, Urbana, IL). All fluorescence anisotropy measurements were carried out as described in Cutsforth et al. (1996).

For the measurements with fluorescein-labeled factor V_a, an excitation wavelength of 490 nm was used. Excitation slits were 8 and 4 nm, respectively, and 500-nm cutoff filters were used in the emission path. Measurements were carried out at 23°C. Aliquots of SUVs were added to the cuvette containing the labeled V_a in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. The sample was stirred for 2 min after each addition and the readings were taken 45 s after the stirring was stopped. Correction for the light scatter of vesicles was made by measuring and subtracting from observed values the parallel and perpendicular components of intensity due to vesicles alone.

Measurement of DPH fluorescence anisotropy was carried out by adding a small volume (~1.5 μl) of a stock solution of DPH (0.332 mM) in acetone to DMPC SUV suspensions to achieve a final dye:lipid ratio of 1:150. The vesicles were vortexed thoroughly and incubated for 1 h before use to achieve maximal partitioning of DPH into the bilayer (Lentz et al., 1976). DPH-containing vesicles were added to a stirred microcuvette containing buffer (20 mM TES, 150 mM NaCl, 5.5 mM $CaCl_2$, pH 7.4). The final concentration of the phospholipid was 0.015 mM. Aliquots of factor V_a (12 μM) in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5, were then successively added to the cuvette. These measurements were carried out at 30°C so that the lipid was maintained in its fluid phase. An excitation wavelength of 366 nm was used, with excitation slits of 4 and 8 nm, respectively, and 418-nm cutoff filters were used in the emission paths in the T format.

Rhodamine fluorescence anisotropy measurements with POPC/Rh-PE (99/1) SUVs were done by adding aliquots of factor V_a to a cuvette (maintained at 23°C) containing vesicle suspensions (200 μM) in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. An excitation wavelength of 540 nm was used, with excitation slits of 8 and 4 nm, respectively, and 550-nm cutoff filters in the emission paths in the T format. After each addition of the protein, the cuvette contents were subsequently stirred for 2 min, with measurements taken approximately 45 s after stirring was stopped. A background contribution was subtracted from the parallel component of the total fluorescence to correct for light scattering due to the high concentrations of SUVs and factor V_a used in these experiments.

For the fluorescence energy transfer measurements, excitation was at 470 nm, and the excitation slits were 8 and 4 nm. Emission was recorded at 520 nm for the donor and 590 nm for the acceptor. The emission slits were 4 and 8 nm. Aliquots of SUVs of POPC containing 1% Rh-PE were added to a microcuvette containing fluorescein-labeled factor V_a in 20 mM TES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5. The cuvette contents (maintained at 23°C) were constantly stirred, and after an equilibration time of 2 min, emission was recorded. For measurements based on fluorescence intensity, corrections were made for the fluorophore (donor) dilution by addition of unlabeled vesicles to the donor, and corrections were also done for acceptor fluorescence by measuring the fluorescence intensity of the acceptor alone at the donor excitation wavelength. The absorbance of the sample containing the highest concentration of Rh-PE/POPC (1/99) SUVs was ~0.03, meaning that inner filter effects on fluorescence measurements should be minimal. Even if significant, this contribution would be proportional to lipid concentration and would thus be included in the slope of the binding curve at high concentrations of the phospholipid (see Eq. 6).

Analysis of individual binding data sets

Individual binding data sets were analyzed using a Marquardt-Levenberg weighted, nonlinear least squares algorithm supplied with Sigma Plot (v1.02; Jandel Scientific, Corte Madera, CA). Weighting assumed errors in observables are proportional to the observable. This algorithm was used to assign values for the binding parameters by obtaining the best fit of calculated to observed adsorption isotherms. Two basic types of experiments were performed. For one type, titration of phospholipid vesicles with

factor V_a was carried out. In these cases, a reporter group was included in the vesicles and an observable response (R) was recorded as a function of the total concentration of protein added ($[P]_T$). The essential assumption is that the response is related to the fraction of phospholipid sites bound to protein:

$$\frac{[PL]_b}{[PL]_{tot}} = \frac{R_{obs} - R_0}{R_{max} - R_0} \qquad (1)$$

With this assumption, binding can be analyzed in terms of a classical Langmuir independent sites adsorption model. Positive (protein-protein attractions) and negative (protein crowding or protein-protein repulsions) deviations from this behavior were judged not to be significant in the case of acidic-lipid-containing membranes (Cutsforth et al., 1996; Cutsforth, 1991). The observable response at any concentration of added protein can then be expressed in terms of the site dissociation constant for the binding reaction (K_d) and the concentration of free protein $[P]_f$:

$$\frac{R_{obs} - R_0}{R_{max} - R_0} = \frac{[P]_f}{[P]_f + K_d} \qquad (2)$$

In this equation, we assume binding of protein to multiple, equivalent, and non-interacting surface sites, each containing $1/i$ phospholipids. R_{obs} is the observed response due to the binding of protein to the lipid surface, R_{max} is the observed response when all of the lipid sites are occupied by protein, and R_0 is the observed response before addition of any protein. The observed response in the binding reaction can be expressed as a function of the total protein concentration ($[P]_T$) using the following equation:

$$R_{obs} = R_0 + \frac{(R_{max} - R_0)}{2[PL]_T} \cdot \left(\frac{[P]_T}{i} + [PL]_T + \frac{K_d}{i} \right)$$
$$- \frac{(R_{max} - R_0)}{2[PL]_T} \cdot \sqrt{\left(\frac{[P]_T}{i} + [PL]_T + \frac{K_d}{i} \right)^2 - \left(\frac{4([P]_T \cdot [PL]_T)}{i} \right)} \qquad (3)$$

The value of R_{max} can be fixed directly from the titration curve only if sufficient protein can be added to reach clear saturation. In the two experiments reported here that fall into this category, R was the fluorescence anisotropy (r) of DPH or of Rh-PE, and r_{max} could not be determined directly.

In the second type of experiment, lipid vesicles are added to labeled protein and the observed response is taken as representing the fraction of protein bound:

$$\frac{[P]_b}{[P]_T} = \frac{R_{obs} - R_0}{R_{max} - R_0} \qquad (4)$$

The observed response is described in terms of the total concentration of phospholipid added ($[PL]_T$) in terms of the following equation:

$$R_{obs} = R_0 + \frac{(R_{max} - R_0)}{2[P]_T}$$
$$\cdot ([PL]_T i + [P]_T + K_d) - \frac{(R_{max} - R_0)}{2[P]_T} \qquad (5)$$
$$\cdot \left(\sqrt{([P]_T + [PL]_T \cdot i + K_d)^2 - (4([P]_T \cdot [PL]_T) \cdot i)} \right)$$

The same three parameters describe the binding reaction as in Eq. 3. In the first of two experiments of this class described here, R is the fluorescence anisotropy of fluorescein-labeled factor V_a and Eq. 5 becomes that given by Krishnaswamy et al. (1986) to describe binding of pyrene-labeled factor

V_a. In this case, corrections were made for light-scattering contributions by addition of vesicles to buffer alone.

Experiments reported here involving fluorescence energy transfer measurements represent a special case of this type of experiment. In this case, R_{obs} is the ratio of donor (fluorescein factor V_a) fluorescence efficiency in the absence and presence of acceptor (rhodamine-labeled lipid vesicles; F_d/F_{da}). The observed response is still proportional to $[P]_b$, which is still given by Eq. 5. However, there is a complication unique to the resonance energy transfer method in that F_d/F_{da} continues to increase in a linear fashion even after binding is saturated simply due to the increase in the probability of transfer associated with higher acceptor concentration. This linear dependence on acceptor concentration is predicted for a solution of non-interacting donor and acceptor molecules (Förster, 1959). The data were thus fit with the following equation:

$$\frac{F_d}{F_{da}} = 1 + \left[\left(\frac{F_d}{F_{da}} \right)_{max} - 1 \right] \cdot \frac{[P]_b}{[P]_T} + m \cdot [PL]_T, \qquad (6)$$

where the first term accounts for binding, with $[P]_b/[P]_T$ given by Eq. 5, and the second term accounts for transfer between acceptor and donor that are not bound to each other. The parameter values, K_d, i, and R_{max}, were still obtained by a weighted nonlinear least squares regression analysis, and the parameter m was taken as the slope of the binding curve at high concentrations of the phospholipid to compensate for the probability of energy transfer associated with high acceptor concentrations. The value of m was estimated both independently and then rigidly constrained as well as simultaneously estimated along with the other parameters of the binding interaction. It was found to be similar by both methods, and the binding parameters were independent of the way the value of m was determined.

Global analysis of multiple data sets

Global analysis of data was carried out using SCoP, the Simulation Control Program (Simulation Resources, Berrien Spring, MI). Analysis was performed on combinations of data sets simultaneously using the appropriate binding equation for each type of experiment (Eqs. 3, 5, or 6). The dissociation constant and the stoichiometry values were assigned as the global variables (i.e., common to all sets of data), the R_{max} values were local variables (i.e., different and individually estimated for each data set), and m was constrained to the value obtained in individual fitting of the energy transfer experiment. A multifit nonlinear least squares regression analysis routine was constructed using the algorithms supplied in the SCoP package and was then used to vary K_d, i, and R_{max} to fit to the observed data. SCoP uses its own principle axis algorithm for optimization of an error function with respect to parameter values.

RESULTS

Binding of factor V_a to DMPC vesicles monitored using DPH fluorescence anisotropy

Fig. 1 records the effect of increasing concentration of factor V_a on the fluorescence anisotropy of DPH associated with DMPC SUVs (15 μM) at 30°C. There was a gradual increase in DPH anisotropy with increasing concentrations of the protein, thus indicating an effect of factor V_a on the membrane due to binding. That the anisotropy effect was not due to binding of the protein to DPH was established in control experiments using DPH and factor V_a without vesicles. Although DPH showed a slight increase in fluorescence intensity in the presence of factor V_a, there was no increase in its fluorescence anisotropy under these conditions. Binding parameters were estimated from the titration
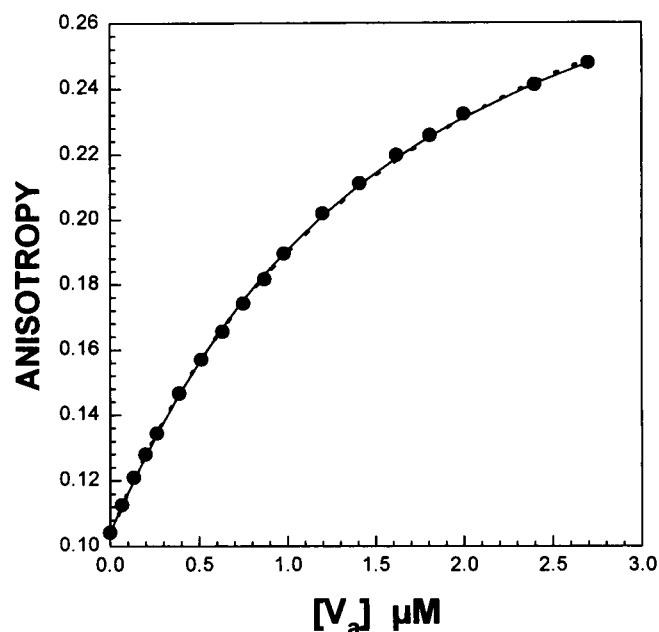
FIGURE 1   Titration of DMPC vesicles with factor V_a. Effect of increasing concentrations of factor V_a on the fluorescence anisotropy of DPH partitioned into DMPC SUVs (15 μM). The probe-to-phospholipid ratio was 1:150. The titration was carried out at a temperature of 30°C. The solid line represents simulation of the data with the binding parameters given in Table 1. The dashed line represents a simulation done with binding parameters obtained using the SCoP fitting routine to fit only this data set.

curve for the binding of factor V_a to DMPC membranes as described in Materials and Methods. The binding curve shown in Fig. 1 does not attain complete saturation because the concentration of factor V_a required for reaching saturating values of anisotropy (r_{max}) would be prohibitively high. Therefore, the value of r_{max} had to be determined as an adjustable parameter by fitting the data to Eq. 3. A dissociation constant of 1.4 μM and a stoichiometry of 29 phospholipids per protein molecule were obtained using this procedure, as summarized in Table 1.

Binding of factor V_a to Rh-PE-labeled POPC vesicles

The increase in fluorescence anisotropy of rhodamine in Rh-PE-labeled POPC SUVs (200 μM) was monitored as a function of increasing concentrations of factor V_a (Fig. 2). Because the rhodamine is attached directly to the head group of the lipid, interactions of the protein with the membrane surface apparently result in a change of rhodamine's fluorescence anisotropy. We note that Rh-PE has a negative charge and that the vesicles used in these experiments contain a small amount (1%) of negatively charged lipid. A negatively curved titration response suggestive of a saturating behavior was observed. As in Fig. 1, saturation was far from complete and r_{max} could not be estimated directly from the titration curve, so it was adjusted to fit the experimental data to Eq. 3. This analysis produced estimates for the dissociation constant of 2.94 μM and stoichiometry of 158 moles of phospholipid per mole of factor V_a.

Binding of fluorescein-labeled factor V_a to POPC and 25/75 bovine PS/POPC SUVs

Binding of factor V_a to POPC vesicles was monitored by measuring the change in fluorescence anisotropy of fluorescein-labeled factor V_a (0.16 μM) with addition of increasing concentrations of the neutral lipid vesicles (Fig. 3 A). The binding of fluorescein-labeled factor V_a (0.3 μM) to vesicles composed of 25/75 bovine PS/POPC was also monitored by this method as shown in Fig. 3 B. An increase in anisotropy values was seen in both cases, and saturable behavior was evident at high vesicle concentrations. As expected, the concentration of phospholipid required for reaching saturation values of anisotropy was much higher for vesicles composed of only neutral phospholipid. The values of r_{max}, dissociation constant, and stoichiometry were obtained for the vesicles containing acidic phospholipid using Eq. 5. Our fitting routine returned $r_{max} = 0.24 \pm 0.0006$, $K_d = 3.7 \pm 1.3$ nM, and a stoichiometry of 70 $\pm$ 2 phospholipids per site. These are in reasonable agreement

TABLE 1   Binding constants for factor V_a binding to phosphatidylcholine membranes

Sample	Titrant	Technique	R_{max}	K_d (M)	Stoichiometry	R_{max}
DMPC-DPH SUVs (15 μM)	V_a	Anisotropy	$r_{max} = 0.323 \pm 0.008$	$1.4e{-}6 \pm 6.5e{-}8$	29 ± 1	
POPC:Rh-PE SUVs (200 μM)	V_a	Anisotropy	$r_{max} = 0.282 \pm 0.010$	$2.94e{-}6 \pm 7.6e{-}7$	158 ± 74	0.2796 ± 0.015
Fluorescein-V_a (0.16 μM)	POPC SUVs	Anisotropy	$r_{max} = 0.275 \pm 0.004$	$4.7e{-}9 \pm 6.2e{-}9$	2051 ± 109	0.3295 ± 0.02
Fluorescein-V_a (0.33 μM)	POPC:Rh-PE-SUVs	FET (520 nm)	$(F_d/F_{da})_{max} = 1.137 \pm 0.008$	$3.71e{-}6 \pm 6.2e{-}5$	102 ± 1583	1.1585 ± 0.016
Fluorescein-V_a (0.33 μM)	POPC:Rh-PE-SUVs	FET (590 nm)	$F_{max} = 0.497 \pm 0.009$	$0.12e{-}6 \pm 6.9e{-}8$	1553 ± 255	0.5898 ± 0.036

K_d, stoichiometry, and R_{max} values were determined independently for each experimental data set, as described in Materials and Methods. Uncertainties in parameter values are given as asymptotic standard errors. R_{max} values were obtained by a global fit of data sets 2–5 wherein the dissociation constant K_d ($3.0e{-}6 \pm 0.22e{-}6$ M) and the binding stoichiometry (200 ± 40) were global variables and the values of R_{max} were local variables. FET, fluorescein energy transfer.
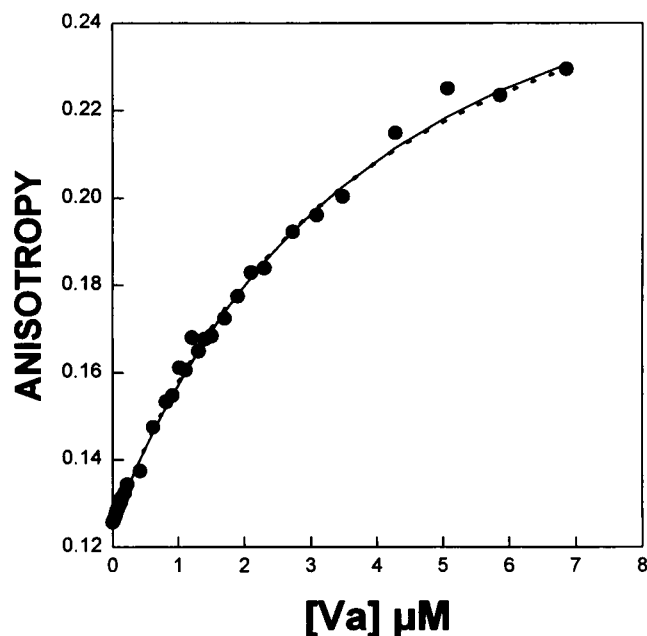
FIGURE 2 Titration of POPC vesicles with factor V_a. Effect of increasing concentrations of factor V_a on the rhodamine fluorescence anisotropy of Rh-PE/POPC (1/99) SUVs at a lipid concentration of 200 μM. The experiment was carried out at 23°C. The solid line represents simulation of the data with the binding parameters given in Table 1. The dashed line represents a simulation done with binding parameters obtained using the SCoP fitting routine to fit globally experiments 2–5 (Table 1).

with values obtained elsewhere (2.72 nM ± 0.47, 42; Krishnaswamy and Mann, 1988) and in our earlier study (1.3 nM ± 0.28, 43; Cutsforth et al., 1996) using a pyrene-labeled factor V_a. A similar treatment of the anisotropy data for binding of factor V_a to POPC vesicles yielded a dissociation constant in the nanomolar range along with a very large value for the stoichiometry of ~2000 phospholipids per factor V_a binding site (Table 1). Although these results agree with our other measurements in showing a factor-V_a-neutral lipid interaction, these binding parameters are not at all in reasonable agreement with the values from the other two experiments reported in Table 1, a problem that will be discussed below.

Binding of factor V_a to POPC vesicles using fluorescence energy transfer

Finally, binding of factor V_a to POPC/Rh-PE (99/1) SUVs was also monitored using fluorescence energy transfer from fluorescein-labeled factor V_a and rhodamine-labeled phosphatidylethanolamine. Fluorescein and rhodamine form an efficient fluorescence donor-acceptor pair. Fig. 4 shows the decrease in fluorescence intensity of fluorescein-labeled factor V_a (monitored at 520 nm) as a result of energy transfer to rhodamine-labeled phospholipid with increasing concentration of the POPC/Rh-PE vesicles. It is evident from these studies, as from our other results, that factor V_a
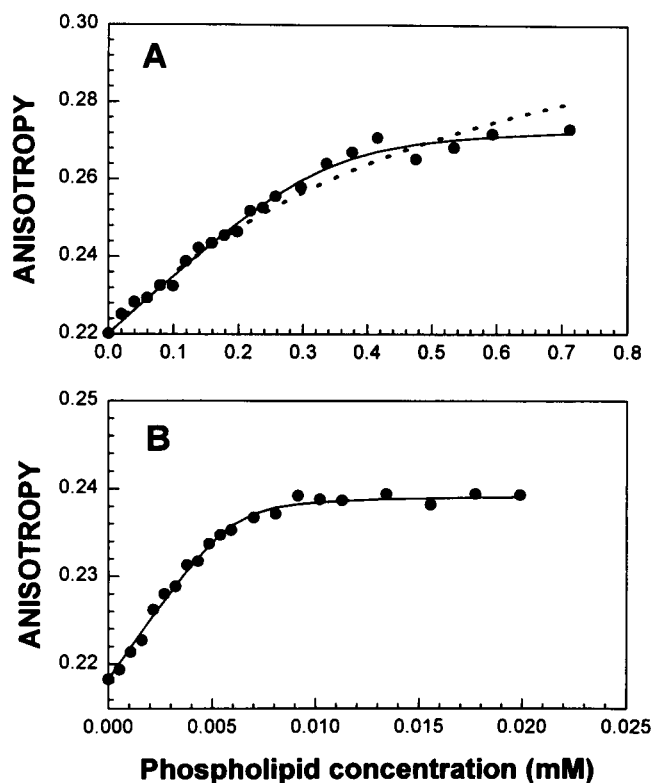

FIGURE 3 Titration of fluorescein-labeled factor V_a with phospholipid vesicles. (A) Effect of increasing concentrations of POPC SUVs on the fluorescence anisotropy of fluorescein-labeled factor V_a. (B) Effect of increasing concentrations of bovine PS/POPC (25/75) SUVs on fluorescence anisotropy of fluorescein-labeled factor V_a. The initial concentration of V_a was 0.16 μM in A and 0.3 μM in B in 20 mM TES, 150 mM NaCl, 5 mM CaCl$_2$, pH 7.5. The solid and dashed lines were obtained as in Fig. 2.

interacts with vesicles containing only neutral lipid molecules. However, a clear saturation was not evident even at very high concentrations of added vesicles. Instead, the quenching of donor fluorescence became linear at very high acceptor concentration, consistent with what we would expect for a donor/acceptor pair in solution rather than interacting by forming a complex. Binding parameters were therefore estimated from the data as described in Materials and Methods using Eq. 6, which includes a term for this solution phase contribution to energy transfer. The binding parameters obtained (Table 1; K_d of ~3.7 μM; stoichiometry of ~102 moles of lipid monomers per mole of binding site) were not inconsistent with the results from our first two experiments. However, the large errors in the estimated values for these parameters indicate that they could not be determined uniquely from this experiment alone.

It is possible to analyze energy transfer data by monitoring the increase in acceptor fluorescence rather than the decrease in donor fluorescence, as was done in Fig. 4. The increase in fluorescence intensity (monitored at 590 nm) of rhodamine-labeled phospholipid due to energy transfer from fluorescein-labeled factor V_a is shown in Fig. 5. Because of some overlap in the excitation spectra of fluorescein and
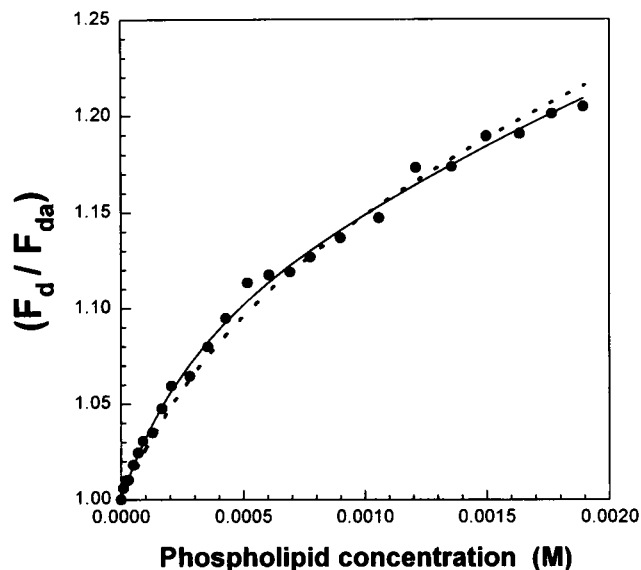
FIGURE 4   Fluorescein-rhodamine energy transfer quenching of fluorescein-labeled factor V$_a$ by Rh-PE. The quenching of fluorescein-labeled factor V$_a$ fluorescence (0.33 μM in 20 mM TES, 150 mM NaCl, 5 mM CaCl$_2$, pH 7.5) at 520 nm was monitored as a function of increasing concentrations of Rh-PE/POPC (1/99) SUVs. F_d and F_{da} are the fluorescence intensities of fluorescein-V$_a$ in the absence and presence of the Rh-PE acceptor. The solid and dashed lines were obtained as in Fig. 2.
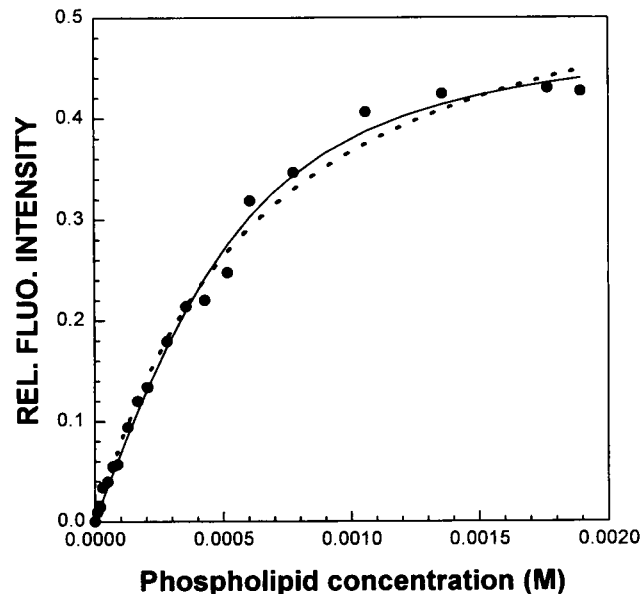

FIGURE 5   Enhancement of Rh-PE fluorescence due fluorescein-rhodamine energy transfer from fluorescein-labeled factor V$_a$. The increase in fluorescence intensity of rhodamine at 590 nm due to fluorescence energy transfer from fluorescein-labeled factor V$_a$ (0.33 μM in 20 mM TES, 150 mM NaCl, 5 mM CaCl$_2$, pH 7.5) is plotted as a function of increasing concentrations of Rh-PE/POPC (1/99) SUVs. The solid and dashed lines were obtained as in Fig. 2.

rhodamine, a parallel experiment was performed in which the fluorescence of increasing concentrations of rhodamine-labeled phospholipid alone was monitored, and this linear response was subtracted from the data obtained with a donor-acceptor mixture. Data were fitted using Eq. 5 to yield estimates of all three parameters; these are given in Table 1. We note once again that the data confirm that binding occurs but yield a smaller dissociation constant (K_d = 0.1 μM) and larger stoichiometry (1553 lipids per site) than three of our five experiments.

DISCUSSION

Information content of membrane-binding experiments

Our results make it clear that factor V$_a$ binds weakly to neutral lipid membranes, although the interaction is so weak, as compared with its binding to membranes containing acidic phospholipids, that it is difficult to obtain a clearly defined estimate of the binding parameters under practical experimental conditions. We have seen that fitting the experimentally obtained binding isotherms to Eqs. 3 or 5 yielded values for K_d and the stoichiometry that were linked, i.e., lower than expected K_d values were paired with higher than expected stoichiometries. This can lead to very large uncertainties in one or more fitting parameters (Table 1). We have noted a similar ambiguity in and linkage between the parameters describing the weak binding of factor V$_a$ to membranes containing low concentrations of

acidic lipids (Cutsforth et al., 1996). It is worth commenting on the origin of this ambiguity.

Descriptions of experimental binding curves in terms of the Langmuir adsorption model are defined by three parameters, K_d, i, and R_{max}. K_d/i is essentially determined by the curvature of the titration curve, which is why these two parameters are generally tightly coupled. However, the curvature is also strongly influenced by how well the saturation is defined (i.e., by the value of R_{max}). Even if the curvature and the saturation of the curve are well defined, this does not guarantee a unique definition of all three parameters. Another piece of information that can provide a constraint leading to a unique definition of all three parameters is the conversion constant that relates the response observed per molar concentration of sites occupied:

$$k' = \frac{\delta R}{\delta[P]_b} \; ; \quad k = \frac{\delta R}{\delta[PL]_b} \qquad (7)$$

where k' and k are the respective conversion constants relevant to the first and second types of experiments we have described. If we focus now on the second type of experiment (addition of lipid vesicles to a fixed amount of protein), the fundamental assumption $[P]_b/[P]_T = (R - R_0)/(R_{max} - R_0)$ can be rearranged to give

$$\frac{(R_{max} - R_0)}{[P]_T} = \frac{k}{i} \qquad (8)$$

A similar equation can be obtained for the first type of experiment we considered, namely, adding protein to a fixed concentration of lipid vesicles:

$$\frac{(R_{max} - R_0)}{[PL]_T} = k' \, i \tag{9}$$

These equations relate R_{max} and i, two of the three parameters needed to describe the binding isotherms generated by the two types of experiments considered here. If k is known, then, we have an additional constraint on the set of parameter values that can be used to describe a given data set.

Now, we describe how these comments apply to the data sets under consideration here. If the experiment is set up such that the site concentration is high, i.e., $[PL]_T i > K_d$ (first type of experiment) or $[P]_T > K_d$ (second type of experiment), the great majority of initially added ligand (protein or lipid) will bind to the lipid or protein sites initially available. Under these conditions, we have an estimate of k, namely, as provided by the initial slope of R versus $[P]_T$ or $[PL]_T$, and Eqs. 8 and 9 can provide useful constraints on the possible range of R_{max} and i values allowed for the data. If the site concentration condition and clear saturation are achieved (an ideal experiment), only K_d need be defined and the binding data can be defined unambiguously in terms of empirical Langmuir constants. Such is the case for factor V_a binding at high acidic lipid content (Cutsforth et al., 1996). It is, however, very difficult to even approach these ideal experimental conditions for studying the weak binding of factor V_a to neutral phospholipid membranes, as one would require very large quantities of factor V_a ($>5 \, \mu M$). If the value of $\partial R/\partial [PL]_b$ is poorly defined, it is still possible to obtain unique parameters if saturation is firmly established. Such is not the case for the weak binding events defined here. We note, however, that the two types of experiments performed here have inverse constraining relationships between i and R_{max} (Eqs. 8 and 9). This raises the expectation that simultaneous or global fitting of data from both types of experiments can constrain i to a more narrow range of possible values. In the next section, we describe how we have used information from multiple data sets to obtain a stable estimate of binding parameters.

Global estimation of binding parameters

To overcome the problem outlined above, the data from both types of experiments (2–5 in Table 1) were fitted globally as described in Materials and Methods. A K_d value of $3.0 \pm 0.22 \, \mu M$ and a stoichiometry value of 200 ± 40 was obtained along with the individual values for R_{max} (Table 1). Unlike fits to individual data sets, this global fit was stable, that is, different initial estimates of parameter values led to the same best fit parameters. The global fits of the data are shown as dashed lines in Figs. 2–5, with the values for R_{max} summarized in Table 1. These reveal that descriptions of all four data sets can be obtained with one value for K_d ($3.0 \, \mu M$) and one stoichiometry (200). The parameter standard deviations returned by the SCoP routine

reflect the curvature of the residual space defined by the four particular data sets that were fit. The use of asymptotic standard errors by the SCoP routine make it likely that these standard deviations underestimate the actual uncertainties in these parameters, which could probably only be estimated rigorously by repeating our measurements and global analysis seven or more times, an impractical undertaking. We can say from our results only that factor V_a binds to neutral lipid membranes with a dissociation constant of approximately $3.0 \, \mu M$ (probably 2–6) and a stoichiometry of approximately 200 (probably 100–400) moles of phospholipid monomer per mole of V_a. Additional uncertainty arises from the fact that, in three of these experiments (2, 4, and 5 in Table 1) we have used 1% of a charged, labeled lipid (Rh-PE). However, experiments 1 and 3 avoid this complication and can be described by comparable binding parameters, suggesting that the negative charge on Rh-PE does not greatly influence our results.

Attempts to include the data from Fig. 1 (experiment 1, binding of factor V_a to DMPC vesicles monitored using DPH fluorescence anisotropy) in the global analysis failed to give a good fit to this data set and returned unreasonable values of R_{max} (>0.4). This data set could be fit individually with the same least squares routine used for the global analysis (dashed curve in Fig. 1), giving binding parameters ($K_d = 1.8 \pm 0.3 \, \mu M$; stoichiometry of 200 ± 80; and $r_{max} = 0.344 \pm 0.022$) in agreement (except for the stoichiometry) with the binding parameters summarized in Table 1. We suspect that binding at 30°C to DMPC (a saturated phospholipid) SUVs may involve a somewhat smaller K_d than does binding at 23°C to POPC (an unsaturated phospholipid) SUVs, although more experiments would be needed to confirm this.

Relationship to other results

Several reports have presented evidence that membrane binding of factor V_a involves two types of interactions of the factor V_a light chain with acidic-lipid-containing membranes, a hydrophobic and an electrostatics-based interaction (Lecompte et al., 1987, 1994; Kalafatis et al., 1990; Ortel et al., 1992). Also, factor V_a light chain was labeled by 1-azidopyrene, a lipophilic photoactivatable probe, more readily in the presence of PC membranes than in their absence (Kalafatis et al., 1994). Our analysis of the equilibrium binding of pyrene-labeled bovine factor V_a to membranes composed of different mixtures of bovine PS with POPC have suggested also that one component of the binding involves a significant PS-independent interaction, whereas another component may involve a few sites that recognize acidic phospholipids, perhaps even with some specificity for PS (Cutsforth et al., 1996). These results implied an interaction with pure PC membranes, although we and others were unable to demonstrate this with pyrene factor V_a (Cutsforth et al., 1996; Krishnaswamy and Mann, 1988). All of these results suggest that factor V_a binding to neutral lipid membranes should be detectable, as we report here that it is.

To our knowledge, the data presented here provide the first direct evidence that factor V_a binds to neutral lipid membranes. Given that this binding has not been reported previously, the estimated K_d implies a surprisingly strong interaction. Based on the estimated K_d, the standard free energy of binding is -7.2 kcal/mol. As the cratic contribution to this free energy change (estimated using a classical translational partition function of a particle in a box as the entropy change associated with moving factor V_a from a large volume in solution to a molecule-sized volume on the membrane surface) is approximately 4 kcal/mol, the unitary free energy of binding (due to other than entropy changes related to changes in volumes occupied) is estimated as -11 kcal/mol (Kauzmann, 1959), which compares to approximately -16 kcal/mol for binding to acidic-lipid-rich membranes (Cutsforth et al., 1996). From these estimates, it is seen that the acidic-lipid-independent contribution to the unitary free energy of the factor V_a-membrane interaction is substantial.

It has been reported previously that PC membranes enhance the rate of prothrombin activation by factor X_a in the presence of factor V_a but not in its absence (Gerads et al., 1990; Govers-Riemslag et al., 1994). The dependence of rate on factor X_a concentration suggested an effective assembly constant of 100 nM at physiological ionic strength (Govers-Riemslag et al., 1994). This is somewhat tighter than the interaction seen here for factor V_a alone, although it may be that factor X_a contributes in some way to the assembly constant. Our analysis of factor V_a binding has suggested an acidic-lipid-independent binding with a dissociation constant (K_d) in the 100 μM range (Cutsforth et al., 1996). However, this estimate derives from studies of binding in membranes containing low amounts of acidic lipids, and a conclusion of this study was that the acidic-lipid-dependent and acidic-lipid-independent sites on factor V_a were not independent, i.e., occupancy of acidic-lipid-dependent sites decreased the strength of the acidic-lipid-independent interaction. Our observation of the $\sim$3.0 μM dissociation constant for factor V_a interaction with pure PC membranes is consistent with this view. Consistent with the possibility that occupancy of acidic lipid sites somehow alters factor V_a and its acidic-lipid-independent interaction with membranes is the observation that the r_{max} value for fluorescein-V_a was different for binding to PC versus PS/POPC membranes (Fig. 3). This dependence of r_{max} on acidic lipid content was also seen with pyrene-factor V_a (Cutsforth et al., 1996).

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