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MEMBRANE LATERAL PHASE SEPARATION INDUCED BY PROTEINS OF THE PROTHROMBINASE COMPLEX

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Blood coagulation factors X and V, as well as prothrombin fragment 1 caused changes in the observed transition temperature (T_m) of appropriately constituted phospholipid vesicles upon binding to the membrane surface. Factor X- and prothrombin fragment 1-induced T_m shifts were calcium-dependent, while factor V changed the T_m in a calcium-independent manner. The results were consistent with clustering of the acidic phospholipid molecules due to protein binding. In all cases, protein binding to acidic phospholipid-containing vesicles caused the observed T_m to approach that for the neutral phospholipid. This resulted in a T_m increase for phospholipid mixtures containing bovine brain phosphatidylserine (PS) plus dipalmitoylphosphatidylcholine (DPPC) and a T_m decrease for mixtures of dipalmitoylphosphatidic acid (DPPA) and dimyristoylphosphatidylcholine (DMPC). Maximum T_m shifts induced in PS-DPPC (10:90) vesicles were very similar for all the prothrombinase proteins and the extent of the change was proportional to the actual amount of membrane-bound protein as determined by light-scattering techniques. For the vitamin K-dependent proteins, T_m changes were greater in the presence of protein plus calcium than in the presence of calcium alone, indicating that lateral phase separation occurs subsequent to initial protein-membrane contact. Lateral phase separation of acidic phospholipids appears to be an important process in the formation of the prothrombinase complex.

Introduction

Prothrombin, factor X, and factor V bind to the surface of membranes containing acidic phospholipids, forming protein-acidic phospholipid complexes [1–3]. Calcium is required for prothrombin- and factor X-membrane binding, whereas the factor V-membrane association is stabilized by protein-acidic phospholipid ionic interactions. All three associations are necessary for proper procoagulant activity [4].

Acidic phospholipid-protein molecule stoichiometries have been estimated for the prothrombinase protein-membrane complexes. Light-scattering studies indicated that 9.3 ± 1.5, 5.2 ± 1.5 and 25–30 PS residues interact with one prothrombin, factor X, and factor V molecule, respectively [2,5,6]. Given these apparent stoichiometries and the observation that these proteins bind to membranes containing low levels of acidic phospholipid, it appears likely that protein membrane binding may induce clustering of acidic phospholipids in the membrane. This phenomenon

Abbreviations: ANS, 8-anilonaphthalene-1-sulfonic acid; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; PS, bovine brain phosphatidylserine; PC, egg phosphatidylcholine; T_m, transition temperature.

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would also occur for biological membranes where acidic phospholipid content is typically less than 15% of the total lipid [7–9].

Calcium- and prothrombin-induced changes in the transition temperature (T_m) of phospholipid vesicles have been used to document acidic phospholipid clustering as a result of prothrombin-membrane binding [10]. Calcium, and to a greater extent prothrombin, caused the apparent T_m of suitably constituted acidic phospholipid-containing membranes to approach that of the neutral phospholipid component. This clustering was also referred to as lateral phase separation, although the acidic phospholipid phase may exist as many small clusters no larger than the number of phospholipids bound to one protein molecule. The studies reported here were undertaken to obtain evidence for acidic phospholipid clustering induced by fragment 1, factor X and factor V and to compare these proteins to prothrombin. These proteins induced membrane perturbation consistent with lateral phase separation.

Materials and Methods

Bovine prothrombin and its thrombin cleavage product, fragment 1, as well as bovine factor X were prepared as described previously [11]. Factor Xa was obtained by the methods of Pletcher and Nelsestuen [12]. Factor V was purified by the methods of Pusey et al. [2]. Factor Va was obtained by digesting factor V with thrombin [2]. Phospholipids (bovine brain PS, egg PC, DPPA, DPPC and DMPC) were purchased from Sigma Chemical Co. ANS and DPH were purchased from Sigma Chemical Co. and Molecular Probes, Inc., respectively.

Single bilayer vesicle preparation and quantitation were accomplished according to the methods of Huang [13] as modified by Nelsestuen and Lim [6]. Unless otherwise indicated, the buffer consisted of 0.05 M Tris/0.1 M NaCl (pH 7.5). Protein-membrane binding was estimated using the 90 light-scattering techniques of Nelsestuen and Lim [6]. A Perkin-Elmer Model MPF-44A fluorescence spectrophotometer was used as the light-scattering photometer. This method detected bound protein by the excess light-scattering intensity occurring when protein associates with the phospholipid vesicles. These data can be used to estimate free and bound protein and to obtain protein-membrane binding constants as well as the protein-binding capacity of the membranes. For a detailed description see Nelsestuen and Lim [6] and other uses of this technique [5,11,14]. The maximum capacity of vesicles for binding vitamin K-dependent proteins was estimated by extrapolation of double-reciprocal plots (1/free vs. 1/bound protein) to infinite free protein concentration [5]. This plotting technique was found to be invalid for factor V [2] and the membrane capacity for this blood coagulation protein was determined from the maximum molecular weight ratio increase obtained from light scattering as described previously [2].

ANS and DPH were incorporated into phospholipid vesicles by adding the appropriate reagent in a small volume of ethanol (ANS) or tetrahydrofuran (DPH) directly to preformed vesicles in buffer [10]. The solvent was removed as previously described [10]. The final molar ratio of fluorescent probe to phospholipid was 1 : 100.

Phospholipid phase transition temperatures (T_m) were obtained by the methods outlined previously [10]. Briefly, the total fluorescence emission intensity of ANS at 470 nm (excitation at 360 nm) or polarized fluorescence emission intensity of DPH at 430 nm (excitation at 355 nm) was monitored as a function of temperature. Both scans exhibit an inflection in fluorescence intensity concomitant with the lipid phase transition [10,15,16]. The midpoint of these inflections were designated as the T_m. A more thorough analysis of this technique has been presented [10]. Membranes containing neutral and acidic phospholipids of different melting temperatures display a single average melting transition if the two lipids are miscible [17]. This appeared to be the case for the phospholipids used here. If the acidic phospholipid component undergoes clustering, the bulk lipid and the apparent phase transition temperature will shift toward that of the pure neutral phospholipid [10]. This forms the theoretical basis for observing phase separation induced by various reagents.

Results

Incubation of prothrombin and fragment 1 with 0.2 mM manganese causes the intramolecular protein conformational change required for protein-membrane binding, but does not cause protein-membrane binding [11]. Manganese also does not induce phase separation of the phospholipids [10]. Consequently, no T_m change was observed for membrane vesicles of 10% PS : 90% DPPC nor for 20% PS : 80% DPPC in the presence of manganese plus protein (Fig. 1). The effect of bound protein on the T_m of the vesicles was determined by titrating prothrombin- and fragment 1-membrane binding with calcium (Fig. 1). In this experiment, the apparent T_m increased concomitant with protein binding. This was expected since the neutral phospholipid component ($T_m = 41°C$) had a higher phase transition temperature than the acidic phospholipid component ($T_m = 10°C$). Calcium concentratoin requirements were similar for prothrombin and fragment 1, although prothrombin caused a slightly greater T_m shift than did fragment 1 (Fig. 1). The maximum fragment 1-induced T_m change was 1.75 and 3.50°C for PS-DPPC (10 : 90) and PS-DPPC (20 : 80) vesicles, respectively. This relationship was consistent with the observation that PS-PC (10 : 90) vesicles exhibit a protein-membrane binding capacity roughly half that for PS-PC (20 : 80) vesicles [5]. Calcium (2.0 mM) in the presence of 0.2 mM manganese induced a small, but observable change in the T_m of PS-DPPC (10 : 90) vesicles (Fig. 1). This metal ion-induced shift was also smaller than that caused with PS-DPPC (20 : 80) vesicle systems [10].

Membrane perturbation upon fragment 1-phospholipid binding was also studied using DPPA-DMPC (30 : 70) vesicles containing ANS. Calcium caused a 1.5°C decrease in the T_m, while calcium plus excess fragment 1 lowered the T_m by 3.5°C. With this vesicle system, the melting temperature of the acidic phospholipid (DPPA, $T_m = 58°C$) is higher than that for the neutral phospholipid (DMPC, $T_m = 22°C$). The observed T_m decrease induced by fragment 1-membrane binding, therefore, reflects enrichment of the bulk phospholipid with DMPC. These results are consistent with protein-induced lateral phase separation of membrane phospholipids.

Fig. 2 demonstrates the effect of factor X-membrane binding on the observed T_m of PS-DPPC (10 : 90) and PS-DPPC (20 : 80) vesicles. Phospholipids and factor X were preincubated with 0.2 mM manganese to allow the protein transition without causing protein-membrane binding. Factor X-membrane binding was titrated by the addition of calcium. The T_m increase induced by protein binding to PS-DPPC (20 : 80) vesicles was approximately 2-fold greater than that for PS-DPPC (10 : 90) vesicles (Fig. 2). Factor X plus 2 mM calcium caused a transition temperature de-

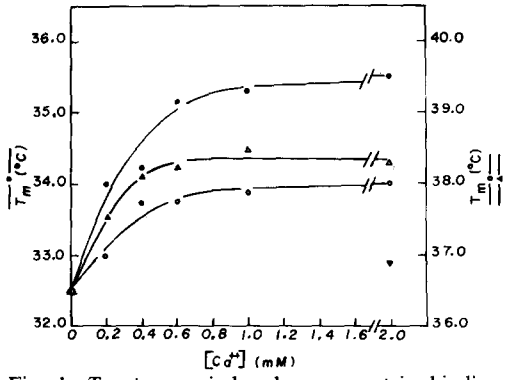

Fig. 1. T_m changes induced upon protein binding to 20% PS : 80% DPPC (closed symbols) and 10% PS : 90% DPPC (open symbols) vesicles (0.1 mg/ml). The T_m was determined by using ANS as described. Proteins were present in excess along with 0.2 mM Mn^{2+}. Proteins added were: (● and ○) fragment 1 (0.23 mg/ml) and (△) prothrombin (0.37 mg/ml). The T_m in the absence of protein (▽) is also shown.

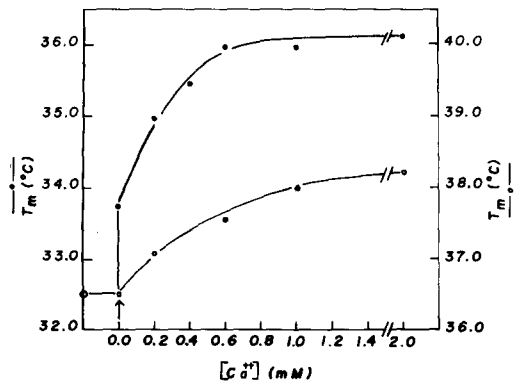

Fig. 2. T_m changes induced by factor X binding to 20% PS : 80% DPPC (open symbols) and 10% PS : 90% DPPC (closed symbols) vesicles (0.1 mg/ml). The T_m was detected by monitoring 90°–90° polarized fluorescence intensity of DPH. The arrow indicates addition of factor X (0.35 mg/ml) and 0.2 mM Mn^{2+}.

crease of 3.5°C for the DPPA-DMPC (30:70) vesicle system described above. Maximum factor X-induced T_m shifts for PS-DPPC (10:90) and PS-DPPC (20:80) vesicles (Fig. 2) were similar to those caused by prothrombin and fragment 1 (Fig. 1). Calcium-dependent transition temperature changes caused by factor X were proportional to the actual amount of membrane-bound protein as indicated by the excess light scattering intensity of the vesicle-protein complex. Overall, the effects of factor X on the T_m suggested a degree of phase separation similar to that induced by prothrombin and fragment 1.

Unlike prothrombin or fragment 1, factor X exhibited a calcium-independent T_m change which was only observed with PS-DPPC (20:80) vesicles in the presence of manganese (Fig. 2). Calcium-independent factor X-membrane binding was also detected by small light-scattering intensity changes. Both of these effects required temperatures greater than 40°C. Calcium-dependent factor X-membrane binding did not exhibit such temperature dependence. The reason for the calcium-independent protein-induced membrane perturbation is not known, but has been reported for certain membrane compositions in other studies [18,19]. Calcium-independent binding of these proteins to membranes was proposed to be an artifact of certain synthetic phospholipid compositions and may not be physiologically important [19].

Addition of factor V increased the observed T_m of PS-DPPC vesicles (Fig. 3). Maximum factor V-induced T_m changes were 3.5 and 1.5°C for PS-DPPC (20:80) and PS-DPPC (10:90) vesicles, respectively. The former composition bound about twice the amount of factor V of the latter, so the extent of T_m shift appeared proportional to the amount of membrane-bound protein. Factor V-membrane binding caused the T_m of DPPA-DMPC (30:70) vesicles to decrease 3.75°C. Factor V and its thrombin-digested form, factor Va, had very similar effects on the observed T_m of PS-DPPC (10:90) vesicles (Fig. 3). Both proteins induced the same maximum T_m increase and exhibited similar protein concentration requirements for half-maximal change. These results are consistent with the observations of Pusey et al. [2] which demonstrated that factor V and factor Va have very similar protein-membrane binding characteristics. It was shown that factor Va increased the affinity of the factor Xa-membrane association [2]. Consequently, in the presence of factor Va the T_m increase was achieved at a protein:phospholipid (w/w) ratio of 0.5 (Fig. 3, inverted triangle) for PS-DPPC (10:90) vesicles. In the absence of factor Xa, this amount of factor Va exhibited only half the maximum T_m shift. Thus, factor Xa plus factor Va caused the maximum membrane struct-

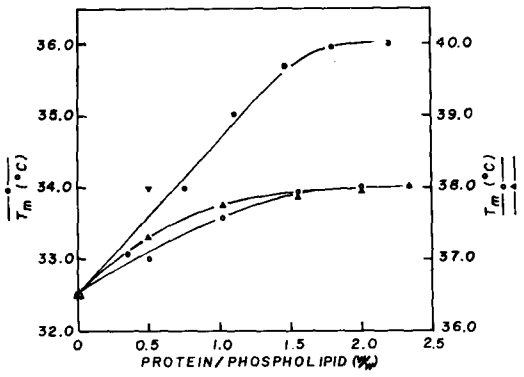

Fig. 3. T_m changes induced by factor V binding to 20% PS:80% DPPC (closed symbols) and 10% PS:90% DPPC (open symbols) vesicles (0.1 mg/ml). Fluorescence intensity changes of ANS were used to detect the T_m. Proteins added were: (● and ○) factor V, (△) factor Va, (▽) factor Va in the presence of 1.0 molar equivalent of factor Xa and 2.0 mM Ca^{2+}.

TABLE I

MEMBRANE CAPACITY FOR PROTHROMBINASE PROTEIN BINDING

Results as determined by relative light scattering techniques.

Protein	Phospholipid composition	Binding capacity (g protein/g phospholipid)	Relative capacity
Prothrombin	20% PS:80% egg PC	1.20	1.00
	20% PS:80% DPPC	0.67	0.56
Fragment I	20% PS:80% egg PC	0.46	1.00
	20% PS:80% DPPC	0.24	0.52
Factor X	20% PS:80% egg PC	1.30	1.00
	20% PS:80% DPPC	0.63	0.48
Factor V	20% PS:80% egg PC	1.85	1.00
	20% PS:80% DPPC	1.40	0.75

ural reorganization under conditions where the sum of the effects of the separate proteins was less than maximum. The presence of both proteins appeared to enhance total protein binding, most likely by simultaneous protein-protein and protein-lipid interactions.

Chelating calcium with EDTA dissociates the fragment 1 and factor X-membrane complexes [5]. Addition of EDTA to the protein-vesicle mixtures here reversed the protein-induced T_m shifts by approx. 75% (data not shown). Reversible and total T_m changes gave parallel results and general conclusions should hold for both measurements. This was similar to the results obtained for prothrombin [10]. Reversibility of the T_m changes caused by factor V was tested by the following method. Factor V was added to PS-DPPC (20 : 80) vesicles containing ANS so that the maximum shift in the transition temperature was obtained. Additional amounts of PS-DPPC (20 : 80) vesicles were then added and allowed to equilibrate. Previous studies showed that the protein redistributes itself over the total phospholipid population [2]. The observed T_m for the new mixture decreased in proportion to the amount of bound protein per vesicle. The phase transition observed in each case was continuous, indicating random distribution of protein on the total vesicle population.

Substitution of DPPC for egg PC as the neutral phospholipid decreased protein-membrane binding capacities (Table I). This effect was more pronounced for prothrombin, fragment 1 and factor X than for factor V.

Discussion

Previous studies with prothrombin have demonstrated the use of fluorescent probes to detect membrane lateral phase separation caused by protein-membrane binding by monitoring changes in the T_m of the phospholipid vesicles. The data obtained here indicated that all proteins of the prothrombinase complex caused perturbation of membrane structure in PS-DPPC and DPPA-DMPC vesicles consistent with acidic phospholipid clustering or lateral phase separation. Fragment 1- and factor X-induced T_m changes were dependent on calcium. Transition temperature shifts promoted by factor V were calcium-independent. These observations agreed with metal ion requirements previously documented for fragment 1-, factor X- and factor V-membrane binding [2,3,11]. The maximum T_m changes caused by protein binding to PS-DPPC vesicles were virtually identical for all three proteins and for combinations of factor Xa plus factor Va. This suggested that these proteins induced the same extent of lateral phase separation in such vesicle systems. The T_m changes were largely reversible and the extent of T_m change was approximately proportional to the actual amount of membrane-bound protein.

The techniques for measuring vesicle transition temperatures utilized above appeared to be very sensitive, so that small perturbations of membrane structure caused by protein-membrane binding could be accurately monitored (Figs. 1–3). Maximum protein-induced T_m shifts for vesicles containing 10 and 20% PS here were comparable to those obtained for other systems in which protein-acidic phospholipid associations were observed [17,20]. Transition temperature changes presented here were, however, typically 1–2°C smaller than those in the other studies. This difference may be attributable to the inability of the prothrombinase proteins to associate with the interior vesicle surface, resulting in a less complete phase separation. In addition, the small PS clusters formed by protein-membrane binding would produce a high amount of boundary lipids (those in contact with the acidic phospholipid cluster and bulk lipids). Under no circumstances did the T_m reach the melting temperature of pure DPPC (41°C) in these studies. The T_m values obtained in the presence of saturating protein may be characteristic of full phase separation in the outer phospholipid layer of the vesicles.

Previous studies have demonstrated that calcium-induced lateral phase separation is not a prerequisite to prothrombin-membrane binding [10]. The studies presented here show that fragment 1- and factor X-induce lateral phase separation functions in a similar manner; transition temperature shifts caused by these vitamin K-dependent proteins are significantly greater than those induced by calcium alone. Pusey et al. [2] have suggested that ionic interactions between factor V and acidic phospholipids may occur subsequent to

protein-membrane contact. The protein-phospholipid association processes for prothrombin, factor X, and factor V apparently consist of at least two stages; initial protein-membrane contact and subsequent lateral phase separation. High acidic phospholipid concentrations localized at the site of protein binding result and stabilize the protein-membrane complexes.

Studies by Tans et al. [21] demonstrated that disaturated phosphatidylcholines had decreased procoagulant activity. The authors postulated that the inhibition of enzymatic activity was due to decreased protein binding sites for the vesicle systems. Prothrombin-, factor X- and factor V-membrane binding capacities of PS-DPPC vesicles here were less than those of PS-egg PC membranes (Table I). This effect was more significant for the vitamin K-dependent proteins than for factor V.

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