Accelerated Publications

Prothrombin Activation on Phospholipid Membranes with Positive Electrostatic Potential†

Jan Rosing,* Han Speijer, and Robert F. A. Zwaal
Department of Biochemistry, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands
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ABSTRACT: The conversion of prothrombin into thrombin, which is a crucial reaction in hemostatic plug formation, is greatly stimulated by phospholipids plus calcium ions. It has been proposed that phospholipid surfaces which promote blood coagulation should have a negative surface charge [Bangham, A. D. (1961) *Nature (London) 192*, 1197-1198]. However, the experiments that led to this proposal were carried out with one kind of anionic phospholipid (dicetyl phosphate). Here we report that membranes, which contain phosphatidylserine (PS) as the anionic phospholipid, can be made positively charged by incorporation of stearylamine and still exhibit almost full procoagulant and prothrombin-converting activity. This suggests that electrostatic forces contribute negligibly to the binding of coagulation factors to PS-containing membranes. Introduction of stearylamine in membranes containing phosphatidyl-β-lactate (PLac) causes considerable inhibition of their prothrombin-converting activity. Since PLac and PS only differ by the presence of an amino group in the polar head group, the much higher procoagulant activity of PS-containing vesicles is indicative of an important function of the amino group of PS in the interaction with coagulation factors. We propose that the association of coagulation factors with PS-containing membranes results from complex formation between Ca^{2+} ions and ligands supplied by the protein and by PS molecules. The ability to form such a complex may well explain why cell membranes with PS have such excellent procoagulant properties.

The activation of vitamin K dependent coagulation factors is strongly accelerated by protein cofactors and by phospholipids plus calcium ions [for review see Jackson and Nemerson (1980)]. Kinetic studies have shown that the protein cofactors drastically enhance the V_{max} of coagulation factor activation (Silverberg et al., 1977; van Dieijen et al., 1981; Nesheim et al., 1979; Rosing et al., 1980) and that phospholipids cause a decrease of the K_m of the respective substrates for these reactions (Silverberg et al., 1977; van Dieijen et al., 1981; Rosing et al., 1980). Not much is known about the molecular mechanisms by which the protein cofactors promote coagulation factor activation. There is, however, ample information on the mode of action of phospholipids in these reactions. Phospholipids bind all components of the coagulation factor activating complexes, thus facilitating the interactions and reactions between the participating proteins. Knowledge of the chemical and physical properties of procoagulant surfaces is still based on early observations of Bangham (1961) and Papahadjopoulos et al. (1962), who proposed that procoagulant membranes should have a net negative surface charge (Bangham, 1961) and that the procoagulant activity of membranes mainly depends on the value of the surface charge (Bangham, 1961; Papahadjopoulos et al., 1962). The chemical nature of the anionic phospholipids appeared to be much less important (Papahadjopoulos et al., 1962).

In this paper we show that membranes with a net positive charge can have excellent procoagulant properties, provided that they contain the proper kind of anionic phospholipid. In the present experimental approach the surface charge of phospholipid vesicles was varied either by changing the membrane content of different kinds of anionic phospholipids or by incorporation of stearylamine, a lipid molecule that is positively charged at neutral pH.

Experimental Procedures

S2238[1] was purchased from AB Kabi Diagnostica. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine and stearylamine (octadecylamine) were obtained from Sigma. Phosphatidylinositol was purchased from Koch Light. Column materials for protein purification (DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100, Sephadex G-200, and Sepharose 4B) were from Pharmacia. Agarose (Isogel agarose EF) used for the electrophoresis of phospholipid vesicles was obtained from LKB.

Bovine prothrombin was purified according to Owen et al. (1974). Bovine factor Xa was prepared as described by Fujikawa et al. (1972), and bovine factor Va was obtained according to the method of Lindhout et al. (1982). Anionic phospholipids [phosphatidylserine (PS); phosphatidic acid (PA); phosphatidylglycerol (PG); phosphatidyl-β-lactate (PLac)] were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine as described by Comfurius and Zwaal (1977). Single-bilayer phospholipid vesicles were prepared by sonication according to the method of de Kruijff et al. (1975). Protein and phospholipid concentrations were determined as described by van Rijn et al. (1984). The concentration of

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* Author to whom correspondence should be addressed.

[1] Abbreviations: S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PLac, phosphatidyl-β-lactate; PG, phosphatidylglycerol; PI, phosphatidylinositol; Tris, tris(hydroxymethyl)aminomethane.

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stearylamine was determined according to the method of Ward et al. (1980). Rates of prothrombin activation were determined with the thrombin-specific chromogenic substrate S2238 as described before (Rosing et al., 1980). Coagulation tests (Stypven time and activated partial thromboplastin time) were performed as described by Denson (1976). Electrophoresis of phospholipid vesicles was carried out in flatbed agarose gels containing 0.3% agarose in 50 mM Tris (pH 7.9) and 175 mM NaCl for 2 h at 15 °C at 3.5 V/cm on a LKB 2117 Multiphor electrophoresis apparatus. After electrophoresis the phospholipid vesicles were stained by incubating the gel in a saturated solution of iodine in H_2O/ethanol (95/5 v/v).

Results and Discussion

In previous publications (Pusey & Nelsestuen, 1983; van Rijn et al., 1984) it was reported that the complete prothrombinase complex (factor Xa, factor Va, Ca^{2+} ions, and phospholipid) functions very well on membranes with low mole percentages of negatively charged phospholipids. In these papers it was shown that this was particularly true for membranes that contained PS as the anionic phospholipid.

Here we present further studies on the effect of variation of the surface charge and the kind of anionic phospholipid on the procoagulant activity of membranes. The surface charge of PC vesicles was varied either by changing the membrane content of the anionic phospholipid PS or by incorporation of stearylamine [$CH_3(CH_2)_{17}NH_2$].

In Figure 1A it is shown that the prothrombin-activating complex indeed had a very low PS requirement. Membranes with amounts of PS as low as 3 mol % still exhibited optimal prothrombin-converting activity. At lower mole percentages of PS the vesicles gradually lost their ability to promote prothrombin activation. This experiment shows that the prothrombinase complex, although functioning very well on membranes with low PS content, has an absolute requirement for the presence of anionic phospholipids.

Figure 1A gives limited information on the effect of the surface charge of vesicles on their procoagulant activity. In this experiment it is not only the surface charge of the vesicles that is changed but there is also a variation of the number of anionic phospholipid molecules available for interaction with coagulation factors. Variation of the surface charge of vesicles without changing their negatively charged phospholipid content can be accomplished by incorporation of stearylamine. Stearylamine has a pK of 10.5 and is, therefore, positively charged at physiological pH. It readily incorporates into phospholipid bilayers and can therefore be used to prepare positively charged liposomes (Bangham et al., 1965; Hope & Cullis, 1987). Surprisingly, the introduction of amounts of stearylamine up to 15 mol % in vesicles with 5 mol % PS hardly affected their ability to promote prothrombin activation (Figure 1B). Also in coagulation tests [Stypven time or activated partial thromboplastin time (APTT)] vesicles containing 5 mol % PS with or without 15 mol % stearylamine were equally effective in shortening the clotting time (data not shown). If all stearylamine did incorporate into the vesicles, one can calculate that the membranes with 0–5 mol % stearylamine should have a negative surface charge, while those with more stearylamine should be positively charged. Electrophoretic analysis showed that the phospholipid vesicles used in this experiment indeed had electrophoretic mobilities that correspond with their calculated surface charge (Figure 1C). The vesicles with less than 6 mol % stearylamine were negatively charged and moved toward the anode, while the vesicles that contained more than 6 mol % stearylamine moved toward the cathode, which shows that they had a positive electrostatic potential. For comparison

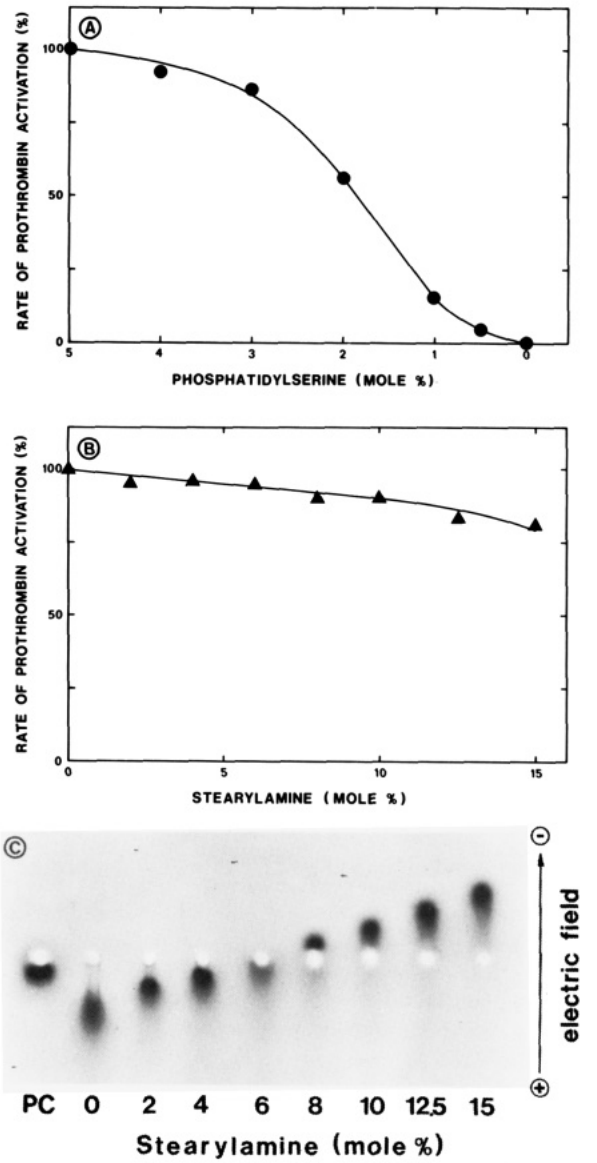

FIGURE 1: Prothrombin activation on phospholipid vesicles as a function of their anionic phospholipid content (A) and surface charge (B). Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 50 μM phospholipid, 1 pM factor Xa, and 5 nM factor Va. The rate of prothrombin activation determined on vesicles without stearylamine (PS/PC, 5/95 M/M) was taken as 100% [2320 mol of prothrombin activated min^{-1} (mol of factor Xa)$^{-1}$]. (A) Prothrombin activation on PS/PC vesicles containing mole percentages of PS as indicated in the figure. (B) Prothrombin activation on PS/PC vesicles containing 5 mol % PS and mole percentages of stearylamine as indicated in the figure. (C) Agarose gel electrophoresis of the PS/PC vesicles with stearylamine used in the experiment described under (B).

we also electrophoresed noncharged vesicles, composed of PC, to show that they had negligible electrophoretic mobility (Figure 1C, vesicles labeled PC).

Before concluding that membranes with a net positive surface charge do promote coagulation factor activation, we felt that it should be ruled out that the procoagulant activities of the phospholipid vesicles with positive electrostatic potential were due to the presence of a small amount of vesicles with net negative charge. To exclude this possibility, stearylamine-containing vesicles (PS/PC/stearylamine, 5/90/15 M/M/M) were subjected to gel electrophoresis and eluted from the gel, and their catalytic activity was compared with the original nonelectrophoresed vesicles and with vesicles that did not contain stearylamine (PS/PC, 5/95 M/M). To

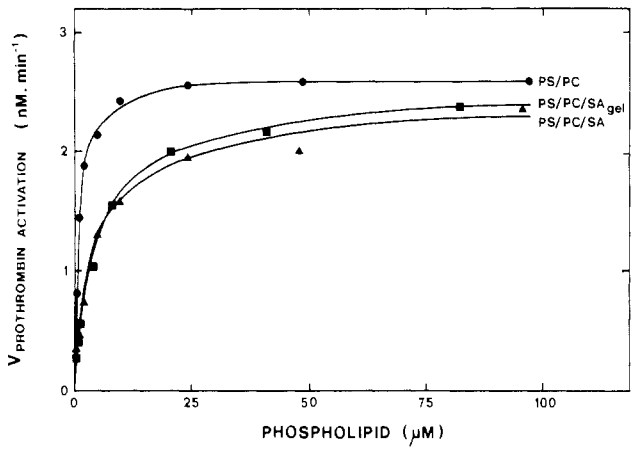

FIGURE 2: Prothrombin activation by the complete prothrombinase complex as a function of the concentration of various kinds of phospholipid vesicles. Prothrombin (2 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 1 pM factor Xa, 5 nM factor Va, and amounts of phospholipid as indicated in the figure. The phospholipid preparations used in this experiment were (●) vesicles containing PS and PC in a molar ratio of 5/95, (▲) vesicles containing PS, PC, and stearylamine in a molar ratio of 5/90/15, and (■) the same vesicles after agarose gel electrophoresis and elution from the gel. Rates of prothrombin activation were determined as described under Experimental Procedures.

compare the procoagulant activity of the different vesicles, rates of prothrombin activation were determined as a function of the amount of phospholipid present. Over a wide range of phospholipid concentrations the electrophoresed and nonelectrophoresed stearylamine-containing vesicles were completely identical in their ability to promote prothrombin activation (Figure 2). Vesicles without stearylamine (PS/PC, 5/95 M/M) were more effective in stimulating prothrombin activation. About 4–5 times less phospholipid was required to obtain prothrombin-converting activities that were equal to those measured on stearylamine-containing vesicles.

We also determined the number of procoagulant sites present on the vesicles of the different phospholipid preparations. This was accomplished by determining rates of prothrombin activation as a function of the amount of factor Xa–factor Va complex at low phospholipid and high prothrombin concentration. Under such conditions, prothrombin activation is limited by the concentration of phospholipid-bound factor Xa–factor Va. From double-reciprocal plots ($1/V$ vs 1/[factor Xa–factor Va]) of the experimental data, one can calculate the number of binding sites for factor Xa–factor Va (by extrapolation to 1/[factor Xa–factor Va] = 0) and $K_{1/2}$, which is the concentration of factor Xa–factor Va required to saturate 50% of the binding sites. The binding parameters of the different phospholipid preparations are summarized in Table I. Vesicles without stearylamine (PS/PC, 5/95 M/M) were able to bind 1.15×10^{-3} mol of factor Xa–factor Va/mol of phospholipid with a $K_{1/2}$ of 4.4×10^{-9} M. Incorporation of 15 mol % stearylamine in these vesicles only had a minor effect on their ability to interact with the factor Xa–factor Va complex. Vesicles with stearylamine had a somewhat lower number of procoagulant sites (0.80×10^{-3} mol of factor Xa–factor Va/mol of phospholipid) that could be saturated with a $K_{1/2}$ of 9.5×10^{-9} M. The stearylamine-containing vesicles that were eluted from the gel had the same binding parameters as the original nonelectrophoresed vesicle preparation. Together with the previous experiment this shows that the prothrombin-converting activity of vesicles with excess stearylamine cannot be due to the presence of a small amount of phospholipid vesicles with a negative surface charge. Thus, we conclude that phospholipid membranes with positive electrostatic potential indeed have the ability to support prothrombin activation.

Table I: Effect of Stearylamine on the Binding of the Factor Xa–Factor Va Complex to Phospholipid Vesicles[a]

phospholipid	$K_{1/2}$ (M)	no. of sites (Xa–Va/phospholipid, M/M)
PS/PC (5/95 M/M)	4.4×10^{-9}	1.15×10^{-3}
PS/PC/stearylamine (5/90/15 M/M/M)	9.5×10^{-9}	0.80×10^{-3}
PS/PC/stearylamine (gel eluted)	9.5×10^{-9}	0.92×10^{-3}

[a] Prothrombin (4 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg/mL ovalbumin, 0.5 or 1 μM phospholipid vesicles, and varying amounts of factor Xa and factor Va. $K_{1/2}$ and sites were obtained from double-reciprocal plots of the titration curves. The number of sites was calculated from the following equation: number of sites = V_{sat}/V_{max}, in which V_{sat} is the rate of prothrombin activation (prothrombin activated min^{-1} phospholipid^{-1}, M min^{-1} M^{-1}) at saturating factor Xa–factor Va and V_{max} is the turnover number of the prothrombinase complex [2748 mol of prothrombin activated min^{-1} (mol of factor Xa–factor Va)$^{-1}$; cf. Rosing et al. (1980)].

Table II: Effect of Stearylamine on the Prothrombin-Converting Activity of Membranes Containing Different Anionic Phospholipids[a]

phospholipid	prothrombin activation (nM/min) −stearylamine	+stearylamine	inhibition (%)
PS/PC	10.6	8.8	17
PA/PC	6.8	1.1	84
PLac/PC	2.3	0.21	91
PG/PC	0.36	0.022	94
PI/PC	0.29	0.017	94

[a] Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 50 μM phospholipid, 5 pM factor Xa, and 5 nM factor Va. Vesicles without stearylamine contained 95 mol % PC and 5 mol % anionic phospholipid, and vesicles with stearylamine contained PC, anionic phospholipid, and stearylamine in a molar ratio of 90:5:15.

The experiments presented thus far have been carried out with membranes containing PS as the anionic phospholipid. To test whether our observations are unique for this phospholipid, we also determined the effect of stearylamine on the prothrombin-converting activity of vesicles containing other anionic phospholipids (Table II). Even without stearylamine, we observed considerable differences in the ability of the various anionic phospholipids to promote prothrombin activation. The procoagulant activity of membranes with different anionic phospholipids decreased in the order PS > phosphatidic acid (PA) > phosphatidyl-β-lactate (PLac) > phosphatidylglycerol (PG) > phosphatidylinositol (PI). After incorporation of excess stearylamine, all membranes retained at least part of their prothrombin-converting activity. However, the effect of stearylamine was much more pronounced upon incorporation in membranes containing anionic phospholipids other than PS. Introduction of excess of stearylamine caused more than 80% inhibition of prothrombin activation on membranes that contained PA, PLac, PG, or PI as the anionic phospholipid. The results obtained with PLac are of special interest. PLac and PS are structurally similar and only differ by the presence of an amino group in the serine moiety of PS [β-lactate = $HOCH_2CH_2COOH$; serine = $HOCH_2CH(NH_2)COOH$]. Since the amino group of PS is positively charged at neutral pH, membranes with PLac carry a higher surface charge. Despite the fact that membranes with PLac were more negatively charged than those with PS, they exhibited much less

procoagulant activity. This implies that the amino group of PS has an important function in the interaction of procoagulant membranes with coagulation factors.

The above results have implications for the chemical and physical nature of the calcium-dependent interaction between vitamin K dependent proteins and procoagulant membranes. Dombrose et al. (1979) suggested that electrostatic attraction of positively charged protein domains by the negatively charged phospholipid surface is the main driving force in protein–membrane association. However, Resnick and Nelsestuen (1980) argued that such electrostatic interactions are of minor importance. They proposed a chelation model for protein–membrane interaction in which calcium ions are thought to form a coordination complex with the carboxyl groups of the γ-carboxyglutamic acids of the vitamin K dependent proteins and negatively charged head groups of anionic membrane phospholipids. Our results are consistent with a chelation model. The observation that a change of the surface charge of PS-containing vesicles from a negative to a positive potential barely affects their activity in prothrombin activation indicates that in this case electrostatic interactions have little contribution to protein–membrane association. It cannot be ruled out, however, that electrostatic interactions may be more important for protein binding to membranes containing anionic phospholipids such as PA, PLac, PG, or PI or to the membranes with dicetyl phosphate that were used by Bangham (1961). Such a phenomenon may also explain the different views of Dombrose et al. (1979) and Resnick and Nelsestuen (1980) since their hypotheses were actually based on experiments carried out with different kinds of anionic phospholipids. Dombrose et al. (1979) used vesicles with PG, while Resnick and Nelsestuen (1980) used vesicles that contained PS as the anionic phospholipid.

We have as yet no information on the precise function of the amino group of PS in the interaction with coagulation factors; the effect can be either indirect or direct (e.g., participation as a ligand in complex formation). It is clear, however, that the presence of this amino group contributes to the formation of complexes between coagulation factors and PS molecules with increased stability, a phenomenon that can explain why membranes with PS have such excellent procoagulant properties. This may also have physiological significance since PS is the major anionic phospholipid in blood platelets and endothelial cells, the membranes of which are thought to stimulate coagulation in situ.

Registry No. Thrombin, 9002-04-4; prothrombin, 9001-26-7.

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