Calcium-Independent Activation of Prothrombin on Membranes with Positively Charged Lipids†

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ABSTRACT: The activation of prothrombin by factor Xa is strongly accelerated by negatively charged phospholipids plus calcium ions. In this paper we report that positively charged membranes can also stimulate prothrombin activation provided that the activation reaction is carried out in the absence of calcium ions. Membranes composed of a mixture of phosphatidylcholine (PC) and positively charged lipids like stearylamine, sphingosine, or hexadecyltrimethylammonium bromide caused a more than 1000-fold increase of the rate of prothrombin activation. Prothrombin activation by the factor Xa–factor Va complex was also considerably stimulated by such membranes. Stimulation of prothrombin activation by positively charged membranes was suppressed at high ionic strength. This suggests that electrostatic attraction of negatively charged proteins by positively charged membranes is the major driving force in the association of prothrombin and factor Xa with the lipid surface. Calcium ions strongly inhibited prothrombin activation on vesicles composed of PC and stearylamine (80/20 M/M), which indicates that the regions of prothrombin and/or factor Xa containing γ-carboxyglutamic acid (gla) are important for the interaction of these proteins with positively charged membranes. The importance of the gla domain was confirmed by the observation that PC/stearylamine vesicles had much less effect on the reactions between proteins that lack gla residues [gla-domainless (des-1–45) prothrombin, prethrombin 1, prethrombin 2, or gla-domainless (des-1–44) factor Xa]. The efficiency of prothrombin and prothrombin derivatives to act as substrate decreased in the order prothrombin > des-1–45-prothrombin = prethrombin 1 > prethrombin 2, while prothrombin activation by gla-domainless (des-1–44) factor Xa was hardly stimulated by positively charged membranes. These results indicate that the main function of the gla domain relates to a contribution of gla to the overall negative charge of the proteins. Our findings further suggest that it is possible to form a fully active membrane-bound prothrombinase complex by pure electrostatic interactions and that the interaction of metal ions with gla residues is no prerequisite for the expression of the catalytic activity of such a membrane-bound complex.

Several reactions of the blood coagulation cascade are strongly accelerated by membranes that contain negatively charged phospholipids (Jackson & Nemerson, 1980). Both the substrates and enzymes of these reactions are vitamin K dependent proteins, which contain γ-carboxyglutamic acid (gla)[1] residues. These modified amino acids are thought to participate in the calcium-mediated binding of the proteins to the phospholipid surface. It is well established that coordinate binding of both the enzyme and substrate to the membrane is a prerequisite for the proper function of the blood coagulation factor activating complexes.

The chemical and physical nature of the calcium-dependent interaction between vitamin K dependent proteins and procoagulant membranes is not yet fully understood. Dombrose et al. (1979) postulated that electrostatic attraction of the gla domain, which becomes positively charged after calcium binding, by the negatively charged phospholipid surface is the major driving force in the association of vitamin K dependent proteins (prothrombin or prothrombin fragment 1)[2] with PG-containing membranes. However, experiments of Resnick and Nelsestuen (1980) and Rosing et al. (1988) suggested that electrostatic interactions are of minor importance for the binding of vitamin K dependent proteins to membranes that contain PS as anionic phospholipid. Their data support a chelation model, in which protein–membrane association results from the formation of a coordination complex of calcium ions with the gla residues of the protein and the polar head group of anionic membrane phospholipids. Taken together, these observations indicate that the extent to which electrostatic attraction and coordination binding contribute to protein–membrane association may depend on the kind of anionic phospholipid that constitutes the membrane.

The precise number of gla residues actually involved in protein–membrane binding is not known. Both prothrombin and factor X contain two or three tight metal ion binding sites (Furie et al., 1976; Bajaj et al., 1976; Prendergast & Mann, 1977; Deerfield et al., 1987), which may require the involvement of more than one gla per metal binding site (Furie et al., 1979; Borowski et al., 1986a). These tight metal binding

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[1] Abbreviations: gla, γ-carboxyglutamic acid; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride; S2337, *N*-benzoyl-L-isoleucyl-L-glutamyl(piperidyl)glycyl-L-arginine *p*-nitroanilide hydrochloride; I2581, *N*-dansyl-(*p*-guanidino)phenylalanine piperidide hydrochloride; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; RVV-X, purified factor X activator from Russel's viper venom; PMSF, phenylmethanesulfonyl fluoride; D-Pro-Phe-Arg-CH_2Cl, D-prolylphenylalanylarginine chloromethyl ketone; ATIII, antithrombin III; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] The nomenclature used for blood coagulation factors and their activation products is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates (Jackson, 1977).

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sites are likely involved in establishing a conformational change that is a prerequisite for protein–phospholipid interaction (Nelsestuen, 1976). The additional filling of a number of loose metal binding sites with calcium ions subsequently results in the formation of the protein–phospholipid complex (Nelsestuen, 1976; Nelsestuen et al., 1976).

Some authors even question the direct participation of gla residues in calcium-dependent bridging of the protein to the phospholipid surface (Forman & Nemerson, 1986; Borowski et al., 1986b). It has been suggested that gla residues may also be involved in additional metal-dependent conformational transitions (Borowski et al., 1986b), in the interaction of factor Xa with factor Va (Skogen et al., 1984), and in the formation of so-called heterodimers between factor Xa and prothrombin (Harlos et al., 1987).

The current study was initiated to investigate the possibility of obtaining a functionally active membrane-bound prothrombinase complex by pure electrostatic interactions and to address the question of whether full expression of catalytic activity of the prothrombinase complex requires interaction of the vitamin K dependent proteins with metal ions. To approach these problems we have studied the activation of prothrombin on phosphatidylcholine vesicles containing positively charged lipids such as stearylamine, sphingosine, or hexadecyltrimethylammonium bromide. It is shown that these vesicles considerably stimulate factor Xa catalyzed prothrombin activation provided that the reaction medium does not contain calcium ions. The catalytic activities obtained with positively charged vesicles in the absence of calcium ions were comparable to those determined for calcium-dependent prothrombin activation on membranes with negatively charged phospholipids. The results suggest that calcium binding to gla residues is required neither for protein–protein interaction at procoagulant surfaces nor for the expression of the catalytic activity of the membrane-bound prothrombinase complex.

Experimental Procedures

Materials. S2238, S2337, and I2581 were purchased from AB Kabi Diagnostica. *p*-NPGB was from Nutritional Biochemicals. Heparin (USP activity 175 units/mg) was obtained from Organon. Dioleoyl-*sn*-glycero-3-phosphocholine, stearylamine [octadecylamine, $CH_3(CH_2)_{17}NH_2$], cetrimide [hexadecyltrimethylammonium bromide, $CH_3(CH_2)_{15}N(CH_3)_3Br$], sphingosine, chymotrypsin, soybean trypsin inhibitor, Russel's viper venom, Echis carinatus venom, and PMSF were obtained from Sigma. D-Pro-Phe-Arg-CH_2Cl was purchased from Calbiochem. Column materials for protein purification (DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100, Sephadex G-200, Sepharose 4B, CNBr-activated Sepharose) and Mono Q and Superose 12 columns were from Pharmacia. Soybean trypsin inhibitor was coupled to CNBr-activated Sepharose 4B by the method of Cuatrecasas (1970). Agarose (Isogel agarose EF) used for the electrophoresis of phospholipid vesicles was obtained from LKB. [^{14}C]Sucrose (4 mCi/mmol) was supplied by New England Nuclear.

Proteins. Bovine prothrombin, prethrombin 1, and prethrombin 2 were purified according to the method of Owen et al. (1974). The purification of bovine prothrombin fragment 1 and fragment 1.2, which was based on the procedure of Owen et al. (1974), was carried out as described by Govers-Riemslag et al. (1985). Thrombin was purified as described earlier (Rosing et al., 1980). Bovine factor X was purified as described by Fujikawa et al. (1972a). Factor Xa was prepared from factor X after activation with RVV-X (Fujikawa et al., 1975b). RVV-X was purified from the crude venom by the method of Schiffman et al. (1969). Gla-domainless factor Xa (des-1–44-factor Xa) was prepared as described by Morita and Jackson (1986). Gla-domainless prothrombin (des-1–45-prothrombin) was obtained after chymotryptic cleavage of prothrombin [cf. Dode et al. (1981)]. Bovine prothrombin (2 mg/mL) was incubated with chymotrypsin (16 μg/mL) in 50 mM Tris (pH 7.9) and 150 mM NaCl at 37 °C. After 1 min, the reaction was stopped by adding a mixture of PMSF (chymotrypsin inhibitor) and D-Pro-Phe-Arg-CH_2Cl (inhibitor of thrombin-like activity) to yield final concentrations of 2 mM and 4 μM, respectively. The reaction mixture was diluted 1:1 with H_2O and applied to a Mono Q column (HR 5/5) connected to a Pharmacia FPLC system. Bound protein was eluted at a flow rate of 1 mL/min with a linear gradient of (2 × 10 mL) of 85–600 mM NaCl in 25 mM Tris (pH 7.9). Des 1–45 prothrombin eluted at about 200 mM NaCl well separated from prothrombin and other prothrombin cleavage products. The des-1–45-prothrombin-containing fractions were pooled (total volume 1.5 mL) and concentrated to a volume of 0.25 mL in a Minicon B-15 concentration cell from Amicon. To separate des-1–45-prothrombin from any residual PMSF and D-Pro-Phe-Arg-CH_2Cl, the concentrated protein preparation was applied to a Superose 12 (HR 10/30) column equilibrated with 50 mM Tris (pH 7.9) and 175 mM NaCl. The protein was eluted with the same buffer at a flow rate of 0.5 mL/min. Bovine factor Va was obtained as described by Lindhout et al. (1982). Bovine ATIII was purified according to the method of Thaler and Schmer (1975). Factor Va was stored at −80 °C in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, and 5 mM $CaCl_2$. All other proteins were stored at −80 °C in 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl.

Protein Concentrations. The molar concentration of thrombin was determined by active-site titration with *p*-NPGB according to the method of Chase and Shaw (1969). Prothrombin, des-1–45-prothrombin, prethrombin 1, and prethrombin 2 concentrations were determined by the same method after complete activation with *Echis carinatus* venom. Factor Xa concentrations were determined by active-site titration according to the method of Smith (1973). The concentration of des-1–44-factor Xa was calculated from its activity toward S2337, assuming that its amidolytic activity is identical with that of factor Xa (Morita & Jackson, 1986). The concentration of factor Va was determined by kinetic analysis as described by Lindhout et al. (1982). The molar concentrations of bovine prothrombin fragment 1 and fragment 1.2 were calculated from the A_{280} by using an $A_{280}^{1\%}$ of 10.1 and a molecular weight of 22 500 for fragment 1 (Esmon & Jackson, 1974a,b) and an $A_{280}^{1\%}$ of 12.3 and a molecular weight of 35 000 for fragment 1.2 (Esmon & Jackson, 1974a,b).

Phospholipid and Phospholipid Preparations. 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (PS) was prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC) by enzymatic synthesis as described by Comfurius and Zwaal (1977). Single bilayer lipid vesicles were prepared as follows: lipid preparations, usually dissolved in $CHCl_3/CH_3OH$ (1/1 v/v), were dried under a stream of N_2, the dried lipids were suspended in the proper buffer at 70–80 °C by vigorous vortexing for 1 min, and the lipid suspensions were subsequently sonicated for 10 min at room temperature using a MSE Mark II 150-W ultrasonic disintegrator set at 8 μm peak to peak amplitude. Suspension of lipids at 70–80 °C and subsequent sonication at 20 °C were needed to prevent the formation of insoluble stearylamine aggregates. Aggregate formation especially occurred when vesicles containing large amounts of stearyl-

amine (>25 mol%) were prepared at room temperature. Phospholipid concentrations were determined by phosphate analysis according to the method of Böttcher et al. (1961). The concentration of stearlyamine was determined by nitrogen analysis as described by Ward et al. (1980).

Measurement of Rates of Prothrombin Activation. Rates of prothrombin activation were determined with the thrombin-specific chromogenic substrate S2238 (Rosing et al., 1980). Discrimination between thrombin and meizothrombin as prothrombin activation products was made on the basis of their different sensitivities toward ATIII plus heparin according to the method described by Rosing et al. (1986). Rates of prothrombin activation were usually expressed as moles of prothrombin activated per minute per mole of factor Xa. Since the reaction conditions employed in our experiments (i.e., low ionic strength and low protein concentrations) usually caused some loss of factor Xa, the actual concentration of factor Xa present in each reaction mixture was measured by transferring aliquots to cuvettes with S2337 for a factor Xa determination.

Gel Electrophoretic Techniques. Gel electrophoretic analysis of the protein used in this study and of the activation products generated during prothrombin activation was carried out on 10% slab gels with a 5% stacking gel in the presence of SDS according to the method of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250. Electrophoresis of phospholipid vesicles was carried out for 2 h at 15 °C and 3.5 V/cm on a LKB 2117 Multiphor electrophoresis apparatus in flatbed agarose gels containing 0.3% agarose in 50 mM Tris (pH 7.9) and 175 mM NaCl. 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

Stimulation of Prothrombin Activation by PC Vesicles Containing Stearylamine. Since prothrombin and factor Xa have a high affinity for anion-exchange resins (Owen et al., 1974; Fujikawa et al., 1975b), it is likely that both proteins are negatively charged at physiological pH. Therefore, it is conceivable that prothrombin as well as factor Xa might associate with positively charged membrane surfaces by electrostatic attraction. To test whether such protein–membrane association indeed occurs and if so can produce a functional membrane-bound prothrombinase complex, we have determined the effect of positively charged membranes (i.e., PC vesicles containing stearylamine) on rates of factor Xa catalyzed prothrombin activation. Since electrostatic attraction between membrane and protein may be prevented at high ionic strength, rates of prothrombin activation were determined at different NaCl concentrations. In addition, EDTA was present in the reaction mixtures to complex any divalent metal ions that might bind to the gla residues of prothrombin and factor Xa and thus reduce the negative charge of these proteins. Figure 1 shows the effect of variation of the NaCl concentration on prothrombin activation in free solution and in the presence of lipid vesicles prepared from pure PC or from a mixture of PC and stearylamine. At high NaCl concentrations rates of prothrombin activation in free solution were equal to those determined in the presence of PC vesicles or PC vesicles containing stearylamine. When the amount of NaCl present in the reaction medium was decreased, there was hardly any effect on the rates of prothrombin activation in free solution and in the presence of PC vesicles. However, prothrombin activation in the presence of stearylamine-containing vesicles was considerably stimulated at low NaCl concentrations. At 10 mM NaCl rates of prothrombin activation obtained on

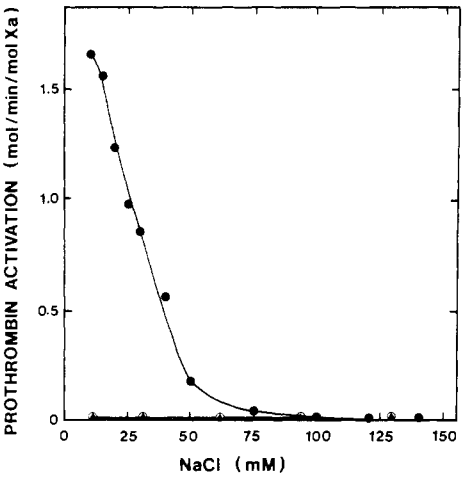

FIGURE 1: Effect of ionic strength variation on prothrombin activation in free solution and in the presence of vesicles composed of PC or stearylamine/PC. Prothrombin (0.6 μM) was activated at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.6), 0.5 mM EDTA, varying amounts of NaCl, and 21 nM factor Xa (▲) or 21 nM factor Xa plus 50 μM PC (○), 0.7 nM factor Xa plus 50 μM PC/stearylamine vesicles (at low NaCl concentrations), or 21 nM factor Xa plus 50 μM PC/stearylamine vesicles (at high NaCl concentrations) (●). The PC/stearylamine vesicles were prepared from a mixture of 85 mol % PC and 15 mol % stearylamine. The concentration of factor Xa present in the reaction mixture and rates of prothrombin activation were determined as described under Experimental Procedures.

PC/stearylamine vesicles were more than 10 000-fold higher than those determined in free solution or in the presence of PC vesicles. The salt effect observed in this experiment was not typical for NaCl since identical curves were obtained when NaCl was replaced by NH_4Cl or K_2SO_4 and activation rates were plotted as function of the ionic strength of the reaction medium (data not shown). These results show that positively charged membranes can indeed promote factor Xa catalyzed prothrombin activation provided that the ionic strength of the reaction mixture is kept low.

Physical Nature of Sonicated Mixtures of PC and Stearylamine. Sonication of dispersions of dioleoyl-PC or mixtures of dioleyol-PC with limited amounts of other lipids generally produces so-called small unilamellar vesicles with an average diameter of about 25 nm (de Kruijff et al., 1975). Two lines of evidence suggest that sonication of mixtures of 80 mol% PC with 20 mol % stearylamine also generated small unilamellar vesicles. Electron microscopic inspection showed a homogenous preparation of vesicles with diameters between 20 and 40 nm in which nonvesicular structures and large multilamellar vesicles were absent. We also compared the internal volume of PC/stearylamine vesicles with that of PC vesicles. To this end sonication of vesicles was performed in a buffer containing [^{14}C]sucrose, and the radiolabeled sucrose present in the medium was separated from the vesicles by gel filtration on Sephadex G-50. From the amount of [^{14}C]sucrose present in the vesicle fraction, it could be calculated that the internal volume of the PC/stearylamine vesicles was 1.8 times higher than that of PC vesicles, which indicates that they had an average diameter of about 1.2 times the diameter of PC vesicles.

To show that the PC vesicles indeed become positively charged after the incorporation of stearylamine and do obtain a surface charge that corresponds with the amount of stearylamine present, we have determined the electrophoretic mobility of vesicles prepared from mixtures of PC and different amounts of stearylamine (Figure 2). PC vesicles had negligible electrophoretic mobility. Their slight movement toward

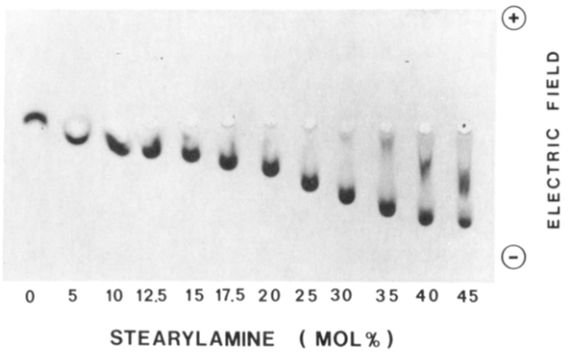

FIGURE 2: Gel electrophoretic analysis of the surface charge of PC vesicles containing varying amounts of stearylamine. Lipid vesicles were obtained by sonication of mixtures of PC and stearylamine. The mole percentages stearylamine in the vesicles are indicated in the figure. Vesicle preparation and the gel electrophoretic technique are described under Experimental Procedures.

the anode is likely caused by electroendosmosis. Introduction of small amounts of stearylamine caused the vesicles to move toward the cathode, which indicates that they possess a positive surface charge. Increasing the amount of stearylamine resulted in a parallel increase of the electrophoretic mobility. This shows that the surface charge of stearylamine-containing vesicles relates rather well to the composition of the lipid suspension (i.e., ratio of stearylamine to PC) subjected to sonication. However, incorporation of more than 35 mol % stearylamine resulted in some inhomogeneity of the vesicle preparations. The lipid staining at lower electrophoretic mobilities is indicative of the presence of membranes with larger dimensions or alternatively of membranes with less stearylamine.

Products of Prothrombin Activation on Positively Charged Membranes. Several peptide bonds in prothrombin are susceptible to proteolytic cleavage. The major peptide bonds that are cleaved during factor Xa catalyzed prothrombin activation are located between Arg274 and Thr275 (site 1), between Arg323 and Ile324 (site 2), and between Arg156 and Ser157 (site 3). Factor Xa is able to cleave the peptide bonds at site 1 and site 2, thus producing prethrombin 2 (site 1 cleaved), meizothrombin (site 2 cleaved), or thrombin (site 1 and site 2 cleaved). Site 3 is preferentially cleaved by thrombin, giving rise to the activation peptide fragment 1 plus prethrombin 1 or meizothrombin des fragment 1. All these activation products have been observed during prothrombin activation by factor Xa (Stenn & Blout, 1972; Owen et al., 1974; Rosing et al., 1986).

When studying prothrombin activation on positively charged membranes, we observed a remarkable difference with a reaction system that contained negatively charged phospholipids plus calcium ions. While in the latter system rates of prothrombin activation were constant over long time intervals, prothrombin activation (i.e., generation of amidolytic activity) in reaction mixtures with positively charged membranes was only linear over a relatively short time period (1–4 min). After this time interval, the reaction slowed down considerably. Gel electrophoretic analysis of prothrombin activation on stearylamine/PC membranes showed that the major part of prothrombin was rapidly converted into prethrombin 1 (Figure 3A). Although this experiment was carried out at relatively high concentrations of prothrombin and factor Xa (required to visualize prothrombin and its activation products on the gel), we feel that this gel pattern explains the typical time course of prothrombin activation on positively charged membranes. Assuming that prethrombin 1 is a poor substrate for this reaction (see also below), prothrombin activation will stop after

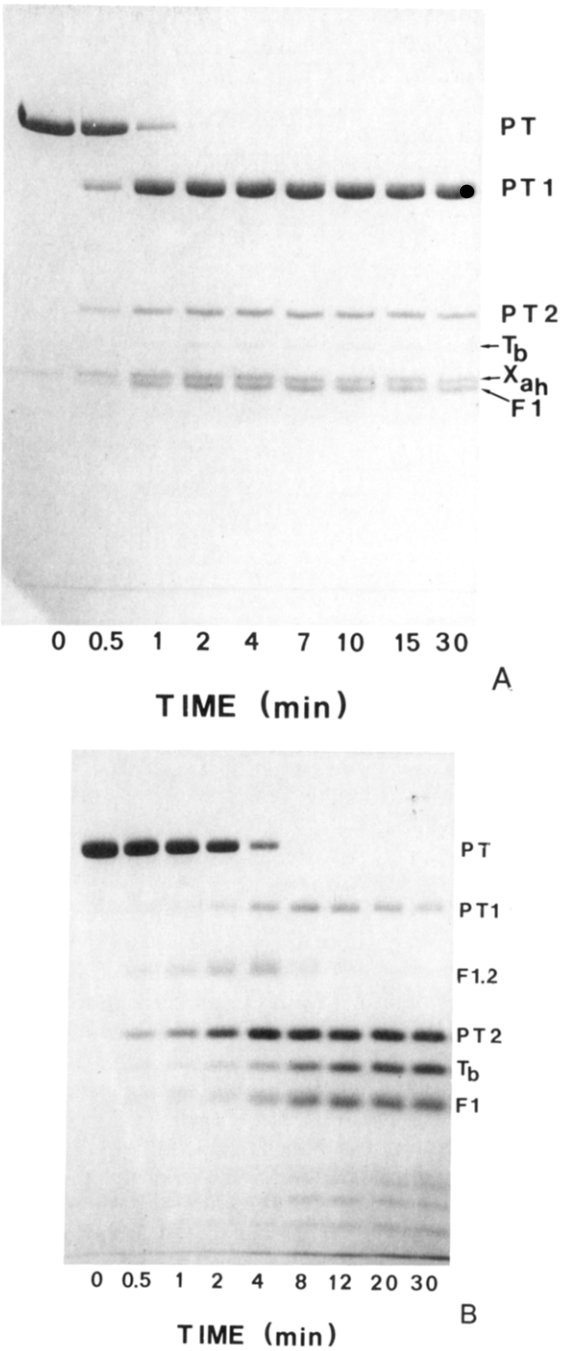

FIGURE 3: Gel electrophoretic analysis of prothrombin activation on positively charged membranes. Prothrombin (1 μM) was activated at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.6), 0.5 mM EDTA, 35 mM NaCl, 50 μM stearylamine/PC vesicles (20/80 M/M), and 60 nM factor Xa (A) or 20 nM factor Xa plus 50 μM I2581 (B). At the time intervals indicated, aliquots from the reaction mixture were added to a solution of SDS/mercaptoethanol to yield final concentrations of 1% (w/w) SDS and 5% (v/v) mercaptoethanol. Gel electrophoretic analysis of these samples was carried out as described under Experimental Procedures. PT = prothrombin; PT1 = prethrombin 1; PT2 = prethrombin 2; T_b = B-chain of thrombin/meizothrombin; Xa_h = heavy chain of factor Xa; F1 = fragment 1; F1.2 = fragment 1.2.

a short time interval because of substrate depletion. The side reaction, that is, the conversion of prothrombin into prethrombin 1, was most likely catalyzed by thrombin, since inclusion of the thrombin inhibitor I2581 in the reaction mixture resulted in a considerable extension of the linear part of a time course of prothrombin activation. Apparently, the reaction conditions employed in the experiments with positively charged lipids favor the thrombin-catalyzed cleavage of fragment 1

Table I: Effect of Membranes with Different Surface Charge on Factor Xa Catalyzed Prothrombin Activation[a]

vesicle composition	prothrombin activation [mol min^{-1} (mol of factor Xa)$^{-1}$]	
	−factor Va	+factor Va
without vesicles	0.26×10^{-3}	0.51
PC	0.27×10^{-3}	0.73
cetrimide/PC	2.7	118
sphingosine/PC	2.3	55
stearylamine/PC	4.9	490
PS/PC	0.23×10^{-3}	
PS/PC + 5 mM $CaCl_2$	1.4	1830

[a] Prothrombin (0.15 μM) was activated in a reaction mixture containing 25 mM Hepes (pH 7.6), 40 mM NaCl, 0.5 mM EDTA, 50 μM lipid, and 1.2 nM factor Xa, 20 nM factor Xa (experiment without lipid and with PC and PS/PC), or 0.02 nM factor Xa plus 5 nM factor Va. The experiment with factor Va was carried out in the absence of EDTA, while that with PS/PC vesicles was carried out both in the absence and in the presence of 5 mM $CaCl_2$. Lipid vesicles were composed of PC or PC mixed with other lipids in a molar ratio of 70:30. Rates of prothrombin activation were determined as described under Experimental Procedures.

from prothrombin. Indeed, we observed that this reaction was strongly accelerated in the absence of calcium ions [originally reported by Silverberg (1979)] and at low ionic strength.

To obtain information about the prothrombin activation products that are generated by factor Xa on positively charged membranes, we performed a gel electrophoretic analysis of a time course of prothrombin activation in the presence of 50 μM I2581 (Figure 3B). I2581 clearly suppressed the formation of prethrombin 1, which enabled detection of the reaction products of the factor Xa catalyzed reaction. Both prethrombin 2 and the B-chain of thrombin were visible on the gel. It is not possible to conclude whether this B-chain originates from meizothrombin or thrombin since the aliquots from the activation mixture were reduced prior to electrophoresis. It is also difficult to differentiate between thrombin and meizothrombin formation with nonreduced gels since on such gels meizothrombin and thrombin comigrate with prothrombin and fragment 1.2, respectively. To distinguish between thrombin and meizothrombin formation during prothrombin activation on positively charged membranes, we have employed an earlier published procedure (Rosing et al., 1986) that enables direct quantitation of thrombin and meizothrombin. With this method it was shown that 32% of the amidolytic activity generated during the initial phase of prothrombin activation could be inhibited with ATIII plus heparin, while the remaining 68% was insensitive to these inhibitors. This indicates that meizothrombin and thrombin are initially formed at a ratio of about 2:1. At a later stage of the reaction the meizothrombin will of course also be converted into thrombin. In summary, these data indicate that factor Xa catalyzed prothrombin activation on positively charged membranes results in the formation of prethrombin 2, meizothrombin, and thrombin.

Effect of Membranes Composed of PC and Different Kinds of Charged Lipids on Prothrombin Activation. It was interesting to test whether the stimulation of prothrombin activation by positively charged surfaces is a unique property of membranes with stearylamine or whether it can also be obtained with membranes containing other positively charged lipids. To this end we have prepared membranes from mixtures of PC with cetrimide (hexadecyltrimethylammonium bromide) or sphingosine and compared their ability to promote prothrombin activation with that of vesicles composed of PC or a mixture of PC with stearylamine. The vesicles employed in this experiment were prepared from a mixture of 70 mol

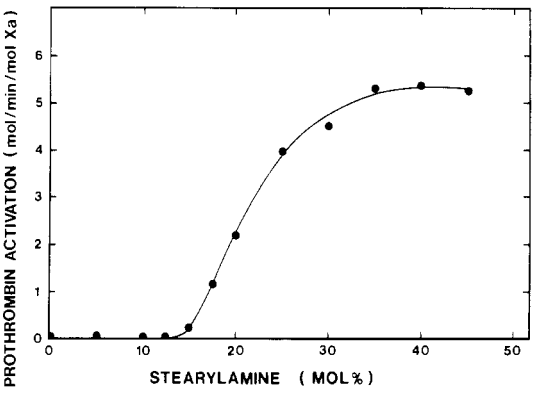

FIGURE 4: Prothrombin activation on membranes containing varying amounts of stearylamine in PC. Prothrombin (0.1 μM) was activated at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.6), 0.5 mM EDTA, 20 mM NaCl, 0.7 nM factor Xa, and 50 μM PC vesicles containing amounts of stearylamine indicated in the figure. Rates of prothrombin activation were determined as described under Experimental Procedures.

% PC and 30 mol % positively charged lipid, since this is the molar ratio that in the case of stearyalamine and PC produces membranes with optimal prothrombin-converting activity (see below).

Without factor Va, rates of prothrombin activation in free solution were rather low and were hardly affected by the presence of PC vesicles (Table I). Prothrombin activation was drastically stimulated by the presence of membranes with a positive electrostatic potential, independent of whether they contained cetrimide, sphingosine, or stearylamine as positively charged lipid. It should be emphasized, however, that under the reaction conditions employed in this experiment membranes with stearylamine showed the highest prothrombin-converting activity. Positively charged membranes also stimulated prothrombin activation in the presence of factor Va. Compared with the reaction in free solution, positively charged membranes caused a 100–1000-fold increase of the rate of prothrombin activation by the factor Xa–factor Va complex.

For comparison we have also given rates of prothrombin activation in the presence of negatively charged phospholipid vesicles (PS/PC, 30/70 M/M). In the absence of calcium ions these vesicles do not stimulate prothrombin activation. As known, acceleration of prothrombin activation by negatively charged membranes requires the presence of calcium ions. However, it is interesting to note that without factor Va the rates of prothrombin activation obtained with stearylamine/PC vesicles were higher than those determined with PS/PC vesicles in the presence of calcium ions.

These data show that positively charged membranes have the ability to considerably stimulate the interactions and reactions between the protein components of the prothrombinase complex (prothrombin, factor Xa, and factor Va) and that the catalytic activities observed with positively charged vesicles are comparable to those obtained with negatively charged membranes in the presence of calcium ions. In the following experiments presented in this paper we have restricted ourselves to the use of membranes that contain stearylamine as positively charged lipid and to studies in prothrombin activation mixtures that did not contain factor Va.

Prothrombin Activation on Positively Charged Membranes. Effects of Lipid Composition and Concentration. The lipid vesicles with varying stearylamine/PC ratios that were used in the electrophoresis experiment presented in Figure 2 were also tested for their ability to promote prothrombin activation. The prothrombin-converting activity of the different vesicles was strongly dependent on their stearylamine content (Figure

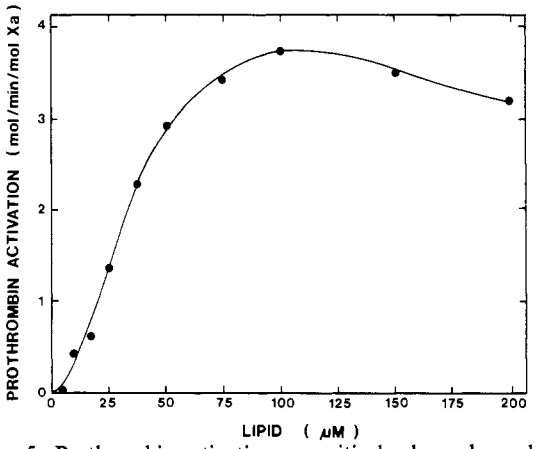

FIGURE 5: Prothrombin activation on positively charged membranes as function of the lipid concentration. Prothrombin (0.1 μM) was activated at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.6), 0.5 mM EDTA, 40 mM NaCl, 0.65 nM factor Xa, and concentrations of PC/stearylamine (75/25 M/M) vesicles as given on the abscissa. Rates of prothrombin activation were determined as described under Experimental Procedures.

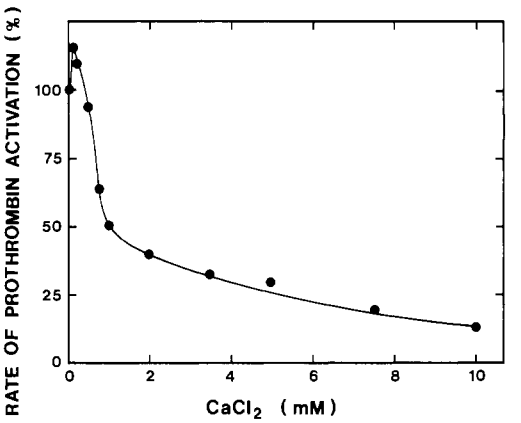

FIGURE 6: Effect of calcium ions on prothrombin activation on positively charged membranes. Prothrombin (0.19 μM) was activated at 37 °C in a reaction mixture containing 25 mM Hepes (pH 7.6), 40 mM NaCl, 1.8 nM factor Xa, 50 μM PC/stearylamine (80/20 M/M) vesicles, and amounts of $CaCl_2$ given on the abscissa. The ionic strength of the reaction mixtures with varying $CaCl_2$ concentrations was kept constant by omitting a proper amount of NaCl. Rates of prothrombin activation were determined as described under Experimental Procedures.

4). Membranes with less than 15 mol % stearylamine hardly stimulated prothrombin activation. Between 15 and 30 mol % stearylamine there was a gradual increase of the prothrombin-converting activity of the membranes, while the incorporation of more stearylamine did not further enhance their ability to accelerate prothrombin activation. In this experiment we did not use vesicles with more than 50 mol % stearylamine since these preparations contained considerable amounts of nonvesicular structures.

Figure 5 shows the effect of variation of the concentration of positively charged vesicles (PC/stearylamine, 80/20 M/M) on the rate of prothrombin activation. Stimulation of prothrombin activation was already detectable at rather low lipid concentrations. A 100-fold stimulation of prothrombin activation was observed at 10 μM lipid. Addition of more vesicles resulted in a concurrent increase of the rate of prothrombin activation until the system was saturated at about 75 μM lipid. Higher lipid concentrations did not further enhance prothrombin activation.

Effect of Calcium Ions on the Prothrombin-Converting Activity of Positively Charged Membranes. In the experiments presented thus far, prothrombin activation was always measured in the presence of EDTA. This chelating agent was present to complex traces of metal ions that might be present in our reaction mixtures and that might cause charge reduction of the vitamin K dependent proteins by binding to the gla residues. Indeed, we observed that calcium ions strongly inhibited prothrombin activation on positively charged membranes (Figure 6). The presence of 10 mM $CaCl_2$ caused 90% inhibition of factor Xa catalyzed prothrombin activation on lipid vesicles composed of PC and stearylamine (80/20 M/M). The simplest interpretation of this result is that Ca^{2+} inhibits prothrombin activation by binding to the gla-containing domains of prothrombin and factor Xa, thus lowering the negative electrostatic potential of these proteins, which will consequently reduce their ability to associate with positively charged membranes. It should be noted, however, that at very low Ca^{2+} concentrations (<0.5 mM) we invariably observed some stimulation of prothrombin activation. It is difficult to precisely quantitate this stimulatory effect of Ca^{2+} ions, since the data points at low Ca^{2+} concentrations are a reflection of both a stimulatory and an inhibitory effect of calcium on prothrombin activation. As yet we have no explanation for the stimulation at low Ca^{2+} concentrations. In this respect

Table II: Effect of Positively Charged Membranes on the Prothrombinase Activity of Gla-Domainless Derivatives of Prothrombin and Factor Xa[a]

substrate	stimulation by PC/stearylamine[b]	
	factor Xa	des-1–44-factor Xa
prothrombin	5000	6.6
des-1–45-prothrombin	44	2.2
prethrombin 1	36	1.9
prethrombin 2	2.8	1.0
prethrombin 1 + fragment 1	39	1.3
prethrombin 2 + fragment 1.2	1144	1.0

[a] Prothrombin or prothrombin derivatives (0.2 μM) were activated in a reaction mixture containing 25 mM Hepes (pH 7.6), 50 mM NaCl, 0.5 mM EDTA, with or without 50 μM PC/stearylamine (80/20 M/M) vesicles, and 24 nM des-1–44-factor Xa or varying amounts of factor Xa. The concentration of factor Xa was dependent on the reaction conditions. The experiments were carried out at 2.5 nM factor Xa (at high rates of prothrombin activation) or 67 nM factor Xa (at low rates prothrombin activation). Rates of prothrombin activation were determined as described under Experimental Procedures. [b] The data given are rates relative to that measured in the absence of lipid, which was arbitrarily taken as 1.

it should be noted that Ca^{2+} ions also promote prothrombin activation in free solution (Jobin & Esnouf, 1967; Esmon et al., 1974; Rosing et al., 1980) and that this metal ion has multiple effects on vitamin K dependent proteins, e.g., filling of gla-independent Ca^{2+} binding sites (Sugo et al., 1984), Ca^{2+}-induced conformational changes of prothrombin and factor Xa (Nelsestuen, 1976), and a charge reduction of prothrombin and factor Xa after Ca^{2+} binding to gla residues.

Function of the Gla Domain in Factor Xa Catalyzed Prothrombin Activation on Positively Charged Membranes. The likely importance of the gla residues for electrostatic protein–membrane interaction prompted us to investigate the effect of removal of the gla domains of factor Xa and prothrombin on the acceleration of prothrombin activation by positively charged membranes. Des-1–45-prothrombin, prethrombin 1, and prethrombin 2 were used as gla-domainless substrate derivatives, while the importance of the gla domain of factor Xa was studied by comparing the catalytic activities of factor Xa and des-1–44-factor Xa. Removal of the gla domain from the participating proteins caused considerable reduction of the rate enhancements produced by positively

charged membranes (Table II). With factor Xa as catalyst, stearylamine-containing vesicles caused a 5000-fold increase of prothrombin activation, a 40-fold increase of the rate of activation of des-1–45-prothrombin and prethrombin 1, and a less than 3-fold increase of prethrombin 2 activation. The presence of the activation peptide fragment 1 did not affect the rate of activation of prethrombin 1. However, in the case of prethrombin 2 the presence of fragment 1.2 enabled positively charged vesicles to cause a more than 1100-fold acceleration of the rate of prethrombin 2 activation. The observation that prethrombin 2 activation on positively charged membranes requires the presence of fragment 1.2 can be explained by the fact that prethrombin 2 readily associates with fragment 1.2, thus producing a prethrombin 2–fragment 1.2 complex with prothrombin-like properties (Esmon & Jackson, 1974a,b).

The gla domain of factor Xa is also important for the interaction of factor Xa with positively charged membranes. With des-1–44-factor Xa as enzyme stearylamine/PC vesicles hardly stimulated the activation of prothrombin and the prothrombin derivatives prethrombin 1, des-1–45-prothrombin, and prethrombin 2.

Discussion

One of the intriguing features of activation processes of vitamin K dependent coagulation factors is that the presence of negatively charged phospholipids and calcium ions results in a more than 1000-fold increase of the rate of activation. From kinetics and binding experiments it is clear that this acceleration is caused by the fact that all components of the coagulation factor activating complexes have the ability to bind to the phospholipid membrane and that such surface binding phenomena facilitate the interactions and promote the reactions between the participating proteins. The gla residues present in the vitamin K dependent coagulation factors play an important role in the binding of these proteins to procoagulant membranes. At present, however, it is not precisely known how the gla residues function in the calcium-mediated association of the vitamin K dependent proteins with the negatively charged head groups of membrane phospholipids.

In this paper we show that factor Xa catalyzed prothrombin activation can also be accelerated by positively charged membranes. Lipid vesicles composed of PC and stearylamine, which are positively charged at neutral pH, cause a more than 5000-fold rate enhancement of prothrombin activation by factor Xa and a 1000-fold increase of the rate of prothrombin activation by the factor Xa–factor Va complex. The extent of stimulation strongly depends on the ionic strength of the reaction medium. At ionic strength values higher than 0.1 the stearylamine-containing membranes lose their ability to promote prothrombin activation. This ionic strength effect demonstrates that electrostatic interaction is the major driving force in the association of the proteins of the prothrombinase complex with the positively charged membranes. Considering the high affinities of prothrombin (Owen et al., 1974), factor Xa (Fujikawa et al., 1972b), and factor Va (Esmon, 1979) for anion-exchange resins, it is likely that under our experimental conditions (pH 7.5) all participating proteins will be negatively charged and hence have the ability to bind to positively charged membranes by ionic interaction. Simultaneous binding of the coagulation factors to positively charged membranes apparently promotes the interactions between prothrombin, factor Xa, and factor Va to result in the observed increase of the rate of prothrombin activation. Our data indicate (see also discussion below) that both prothrombin and factor Xa have to bind to positively charged membranes in order to enhance prothrombin activation. However, the incorporation of factor Va into the prothrombinase complex does not necessarily require interaction of factor Va with the membrane. Since factor Va has a rather high affinity for factor Xa (Lindhout et al., 1982; Nesheim et al., 1981), it may also directly bind to surface-bound factor Xa.

The gla domains of prothrombin and factor Xa have an important function in the binding of these proteins to positively charged membranes. Protein derivatives without gla residues (des-1–45-prothrombin, prethrombin 1, prethrombin 2, and des-1–44-factor Xa) were considerably less active on positively charged lipid vesicles. The importance of the gla-containing fragment is also indicated by the observation that prothrombin activation on positively charged membranes is inhibited by calcium ions. This is likely caused by partial charge neutralization of the proteins after Ca^{2+} binding to the gla residues. Therefore, it is plausible that the gla function in protein–membrane association relates to an important contribution of these residues to the negative charge of the proteins and hence to the electrostatic interaction with positively charged lipids.

The prothrombin-converting activity is also affected by the electrostatic potential of the membrane. Membranes with low amounts of stearylamine (less than 10 mol %) hardly stimulate prothrombin activation. At higher mole percentages of positively charged lipid there is a gradual increase of the ability of the membranes to prothrombin activation up to 30 mol % stearylamine, where the prothrombin-converting activity of the membranes reaches a plateau value. At these mole percentages the membranes have apparently obtained a surface charge that is optimal for the ionic binding of prothrombin and factor Xa.

There are many similarities between the catalytic properties of the prothrombinase complexes on positively charged membranes without calcium ions and on negatively charged phospholipids plus calcium ions. Prethrombin 2, meizothrombin, and thrombin are formed as products of prothrombin activation on both positively and negatively charged membranes. The rates of prothrombin activation that are obtained on both kinds of membranes are also similar. In the absence of factor Va, the prothrombin-converting activity of positively charged membranes is even higher than that observed with negatively charged membranes in the absence of calcium ions. These findings indicate (a) that binding of coagulation factors to positively charged membranes can result in proper assembly and full enzymatic activity of the prothrombinase complex, (b) that it is possible to form a catalytically active membrane-bound prothrombinase complex by pure electrostatic interactions, and (c) that interaction between calcium ions and gla residues is no prerequisite for the expression of the catalytic activity of the prothrombinase complex. With respect to prothrombin activation on negatively charged phospholipids in the presence of calcium ions, this suggests that the major, if not the only, function of calcium–gla interaction in this system is to enable association of vitamin K dependent proteins with negatively charged membranes. On the basis of the current results it is also clear that in discussions of the chemical and physical nature of the calcium-dependent interaction between vitamin K dependent proteins and negatively charged membranes one should, at least theoretically, consider both electrostatic forces (Dombrose et al., 1979) and chelate complex formation (Resnick & Nelsestuen, 1980; Rosing et al., 1988).

It should be noted that the behavior of positively charged PC/stearylamine vesicles is different from that of PC/PS/

stearylamine vesicles with positive electrostatic potential that we have reported in an earlier paper (Rosing et al., 1988). Prothrombin activation of PC/PS/stearylamine membranes is hardly affected by variation of the ionic strength of the reaction medium and has an absolute requirement for the presence of calcium ions, an observation that led to the conclusion that the association of the prothrombinase complex with such membranes resulted from the formation of a chelate complex between calcium ions, gla residues, and PS molecules, independent of the electrostatic potential of the procoagulant surface.

Finally, we would like to emphasize that there are no reports on the presence of positively charged lipids in membranes that may provide the procoagulant surface in vivo. Therefore, it is unlikely that the interactions of coagulation factors with positively charged lipids play a role in the assembly of the prothrombinase complex in physiological condition. Membranes with positively charged lipids do provide, however, a good model to study the Ca^{2+}-independent interactions between coagulation factors on procoagulant membranes.

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Registry No. Gla, 53861-57-7; prothrombin, 9001-26-7; factor Xa, 9002-05-5; factor Va, 65522-14-7; stearylamine, 124-30-1; cetrimide, 57-09-0; sphinosine, 123-78-4.

REFERENCES

Bajaj, S. P., Nowak, T., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 6294–6299.

Borowski, M., Furie, B. C., & Furie, B. (1986a) *J. Biol. Chem. 261*, 1624–1628.

Borowski, M., Furie, B. C., Bauminger, S., & Furie, B. (1986b) *J. Biol. Chem. 261*, 14969–14975.

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

Chase, T., Jr., & Shaw, E. (1969) *Biochemistry 8*, 2212–2224.

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

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Deerfield, D. W., Olson, D. L., Berkowitz, P., Byrd, P. A., Koehler, R. A., de Kruijff, B., Cullis, P. R., & Radda, G. K. (1975) *Biochim. Biophys. Acta 406*, 6–20.

Dode, C., Thiesce, A., Labie, D., & Elion, J. (1981) *Biochem. Biophys. Res. Commun. 103*, 461–468.

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

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Esmon, C. T., & Jackson, C. M. (1974a) *J. Biol. Chem. 249*, 7782–7790.

Esmon, C. T., & Jackson, C. M. (1974b) *J. Biol. Chem. 249*, 7791–7797.

Esmon, C. T., Owen, W. G., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 8045–8047.

Forman, S. D., & Nemerson, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4675–4679.

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

Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972b) *Biochemistry 11*, 4892–4899.

Furie, B. C., Mann, K. G., & Furie, B. (1976) *J. Biol. Chem. 251*, 3235–3241.

Furie, B. C., Blumentstein, M., & Furie, B. (1979) *J. Biol. Chem. 254*, 12521–12530.

Govers-Riemslag, J. W. P., Speijer, H., Zwaal, R. F. A., & Rosing, J. (1985) *Thromb. Res. 38*, 375–388.

Harlos, K., Holland, S. K., Boys, C. W. G., Burgess, A. I., Esnouf, M. P., & Blake, C. C. F. (1987) *Nature (London) 330*, 82–84.

Jackson, C. M. (1977) *Thromb. Haemostasis 38*, 567–577.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Jobin, F., & Esnouf, M. P. (1967) *Biochem. J. 102*, 666–674.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lindhout, T., Govers-Riemslag, J. W. P., van de Waart, P., Hemker, H. C., & Rosing, J. (1982) *Biochemistry 21*, 5494–5502.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648–5656.

Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886–6893.

Nesheim, M. E., Kettner, C., Shaw, E., & Mann, K. G. (1981) *J. Biol. Chem. 256*, 6537–6540.

Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594–605.

Pedersen, L. G., & Hiskey, R. G. (1987) *J. Biol. Chem. 262*, 4017–4023.

Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252*, 840–850.

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

Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., & Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274–283.

Rosing, J., Zwaal, R. F. A., & Tans, G. (1986) *J. Biol. Chem. 261*, 4224–4228.

Rosing, J., Speijer, H., & Zwaal, R. F. A. (1988) *Biochemistry 27*, 8–11.

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

Silverberg, S. A. (1979) *J. Biol. Chem. 254*, 88–94.

Skogen, W. F., Esmon, C. T., & Cox, A. C. (1984) *J. Biol. Chem. 259*, 2306–2310.

Smith, R. L. (1973) *J. Biol. Chem. 248*, 2418–2423.

Stenn, K. S., & Blout, E. R. (1972) *Biochemistry 11*, 4502–4515.

Sugo, T., Bjork, I., Holmgren, A., & Stenflo, J. (1984) *J. Biol. Chem. 259*, 5705–5710.

Thaler, E., & Schmer, G. (1975) *Br. J. Haematol. 31*, 233–243.

Ward, M. W. N., Owens, C. W. J., & Rennie, M. J. (1980) *Clin. Chem. 26*, 1336–1339.