Prothrombin Activation on Membranes with Anionic Lipids Containing Phosphate, Sulfate, and/or Carboxyl Groups†

Inge Gerads, José W. P. Govers-Riemslag, Guido Tans, Robert F. A. Zwaal, and Jan Rosing*

Department of Biochemistry, Cardiovascular Research Institute, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

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ABSTRACT: Factor Xa catalyzed prothrombin activation is strongly stimulated by the presence of negatively charged membranes plus calcium ions. Here we report experiments in which we determined the prothrombin-converting activity of phosphatidylcholine (PC) membranes that contain varying amounts of different anionic lipids, viz., phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylmethanol (MePA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidyl-β-lactate (PLac), sulfatides (SF), sodium dodecyl sulfate (SDS), and oleic acid. All anionic lipids tested were able to accelerate factor Xa catalyzed prothrombin activation, in both the absence and presence of the protein cofactor Va. This shows that the prothrombin-converting activity of negatively charged membranes is not strictly dependent on the presence of a phosphate group but that lipids which contain a carboxyl or sulfate moiety are also able to promote the formation of a functionally active prothrombinase complex. In the absence of factor Va, the prothrombin-converting activity of membranes with MePA, PG, PE, PLac, SF, or SDS was strongly inhibited at high ionic strength, while the activity of PS- and PA-containing membranes was hardly affected by ionic strength variation. This suggests that in the case of the ionic strength sensitive lipids electrostatic forces play an important role in the formation of the membrane-bound prothrombinase complex. For PS and to a lesser extent for PA we propose that the formation of a coordinated complex (chelate complex) with Ca^{2+} as central ion and ligands provided by the γ-carboxyglutamic acid residues of prothrombin and factor Xa and the polar head group of phospholipids is the major driving force in protein–membrane association. Our data indicate that the anionic lipids used in this study can be useful tools for further investigation of the molecular interactions that play a role in the assembly of a membrane-bound prothrombinase complex. Membranes that were solely composed of PC can also considerably enhance prothrombin activation in the presence of factor Va. This activity of PC is only observed on membranes which are composed of PC that contains unsaturated hydrocarbon side chains. Membranes prepared from phosphocholine-containing lipids with saturated hydrocarbon side chains such as dimyristoyl-PC, dipalmitoyl-PC, distearoyl-PC, and dioctadecylglycerophosphocholine hardly accelerated prothrombin activation. These results indicate that the phosphocholine moiety of PC can promote the assembly of a functionally active prothrombinase complex provided that the nonpolar core of the membrane contains unsaturated hydrocarbon chains.

The activation of prothrombin by the serine protease factor Xa is greatly accelerated by the presence of negatively charged membranes plus calcium ions and by the protein cofactor Va [for reviews see Jackson and Nemerson (1980), Tans and Rosing (1987) and Rosing and Tans (1988)]. Negatively charged lipids are thought to promote prothrombin activation by simultaneous binding of the protein components of the prothrombinase complex, a phenomenon that facilitates factor Xa–factor Va complex formation (Nesheim et al., 1979; Lindhout et al., 1982) and that causes an increased affinity (decreased K_m) for the substrate prothrombin (Rosing et al., 1980). Factor Va stimulates prothrombin activation by enhancing the enzymatic activity (k_{cat}) of factor Xa (Nesheim et al., 1979; Rosing et al., 1980) and by providing additional interactions with factor Xa (Nesheim et al., 1979; Lindhout et al., 1982) and prothrombin (Pusey & Nelsestuen, 1983; van Rijn et al., 1984) that further support the assembly of the prothrombinase–prothrombin complex.

Several laboratories have investigated the binding of the individual proteins to various phospholipid preparations. Factor Va–membrane interaction has been shown to require negatively charged phospholipids (Bloom et al., 1979), and it has been suggested that the association of factor Va with membranes involves both nonionic (Bloom et al., 1979; Pusey et al., 1982) and ionic forces (Pusey et al., 1982; van de Waart et al., 1983). In contrast to factor Va binding, the association of the substrate (prothrombin) and the enzyme (factor Xa) with negatively charged membrane surfaces requires Ca^{2+} ions. Since both prothrombin and factor Xa contain γ-carboxyglutamic acid (Gla)[1] residues that bind Ca^{2+} ions (Nelsestuen, 1976; Prendergast & Mann, 1977; Furie et al., 1976) and since membranes with anionic phospholipids also have a high affinity for Ca^{2+} ions (Hendrickson & Fullington, 1965; Abramson et al., 1966; Hauser et al., 1976), the formation of prothrombin- and factor Xa–membrane complexes is apparently

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* Author to whom correspondence should be addressed.

[1] Abbreviations: Gla, γ-carboxyglutamic acid; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; RVV-X, purified factor X activator from Russell's viper venom; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PS, phosphatidylserine; PC; phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PLac, phosphatidyl-β-lactate; PG, phosphatidylglycerol; MePA, phosphatidylmethanol (phosphatidic acid monomethyl ester); SF, sulfatide; diC_{18}-ether PC, dioctadecylphosphatidylcholine; TLC, thin-layer chromatography.

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mediated by calcium bridges between the proteins and anionic phospholipids.

The chemical and physical nature of the calcium-dependent binding of Gla-containing proteins to negatively charged membranes is, however, not yet fully understood. Both electrostatic attraction (Dombrose et al., 1979) and coordination binding (Resnick & Nelsestuen, 1980; Rosing et al., 1988) have been suggested to be the driving force in protein–membrane association. Electrostatic attraction may occur between the Gla-containing protein domain, which becomes positively charged after calcium binding, and the negatively charged membrane surface. Coordination binding may result from the formation of chelate complexes of calcium ions with ligands provided by the Gla residues of the proteins and by the polar head groups of anionic membrane lipids. It is possible, however, that both electrostatic attraction and chelate formation are involved in membrane binding of Gla-containing proteins and that the extent to which these forces contribute to binding is dependent on the kind of anionic lipid present in the membrane. Rosing et al. (1988) suggested that coordination complex formation is the major driving force for the binding of vitamin K dependent proteins to PS-containing membranes, while electrostatic attraction may significantly contribute to protein binding to membranes that contain other anionic phospholipids [e.g., PG used by Dombrose et al. (1979)]. Limited information is, however, available on protein binding characteristics and prothrombin-converting activities of membranes that contain anionic lipids other than PS or PG.

In the present study we have investigated the prothrombin-converting activities of negatively charged membranes that contain a wide variety of different anionic lipids. In addition to anionic phospholipids, we have also examined lipids that do not contain a phosphate group and that derive their negative charge from a carboxyl (fatty acid) or a sulfate group (sulfatide, SDS). This investigation has been carried out in order to obtain more information on the chemical requirements of the polar head groups of anionic lipids that are able to participate in the assembly of the prothrombinase complex and to get more insight into the physical nature of Ca^{2+}-dependent interactions between vitamin K dependent proteins and negatively charged membranes.

Experimental Procedures

Materials. S2238 was purchased from AB Kabi Diagnostica, Stockholm, Sweden. Dimyristoyl-*sn*-glycero-3-phosphocholine, dipalmitoyl-*sn*-glycero-3-phosphocholine, and distearoyl-*sn*-glycero-3-phosphocholine were supplied by Calbiochem, La Jolla, CA. Dilinoleoyl-*sn*-glycero-3-phosphocholine, dilinolenoyl-*sn*-glycero-3-phosphocholine, and 1-stearyl-2-oleoyl-*sn*-glycero-3-phosphocholine were purchased from Applied Science Laboratories, Penn, PA. Dioleoyl-*sn*-glycero-3-phosphocholine, dioleoyl-*sn*-glycero-3-phosphomethanol, and oleic acid were obtained from Sigma, St. Louis, MO. Dioctadecyl-*rac*-glycero-3-phosphocholine ($diC_{18:0}$-ether PC) was obtained from Novabiochem, Läufelfingen, Switzerland, and sphingomyelin was obtained from Koch-Light, England. Sulfatides were purchased from Supelco Inc., Bellefonte, PA. Sodium dodecyl sulfate was obtained from Bio-Rad Laboratories, Richmond, CA. Column materials for protein purification (DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100, Sephadex G-200, and Sepharyl S-300) were obtained from Pharmacia, Uppsala, Sweden. Silica gel 60 plates for thin-layer chromatography of phospholipids were from Merck, Darmstadt, Germany.

Proteins. Bovine prothrombin was purified as described by Owen et al. (1974). Bovine factor X was purified as described by Fujikawa et al. (1972a). Bovine factor Xa was prepared from factor X after activation with RVV-X (Fujikawa et al., 1972b). RVV-X was purified from the crude venom of Russell's viper by the method of Schiffman et al. (1969). Bovine factor Va was obtained according to the procedure of Lindhout et al. (1982). Prothrombin and factor Xa were stored at −80 °C in 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl. Factor Va was stored at −80 °C in the same buffer with 5 mM $CaCl_2$.

Protein Concentrations. The molar concentration of factor Xa was determined by active site titration with *p*-NPGB (Smith, 1973). Prothrombin concentrations were determined with *p*-NPGB [cf. thrombin active site titration, Chase and Shaw (1969)] after complete activation of prothrombin with the venom activator from *Echis carinatus*. Factor Va concentrations were determined by kinetic analysis as described by Lindhout et al. (1982).

Phospholipids and Phospholipid Preparations. 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (PG), 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-*sn*-glycero-3-phospho-β-lactate (PLac), and 1,2-dioleoyl-*sn*-phosphoethanolamine (PE) were 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, and the dried lipids were suspended in 50 mM Tris-HCl (pH 7.9) and 20 mM NaCl at 4 or 65 °C (when oleic acid, sulfatides, or SDS were part of the lipid mixture) and vigorously vortexed for 1 min. The lipid suspensions were subsequently sonicated for 10 min at 4 or 65 °C with a MSE Mark II 150-W ultrasonic disintegrator set at 8 μm peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis according to the method of Böttcher et al. (1961). Extraction of total lipid from aqueous vesicle suspensions was carried out as described by Reed et al. (1960). TLC analysis of lipids was performed at room temperature on 20 × 20 cm plates coated with 0.5-mm silica gel 60. Chloroform/methanol/ammonia/water (95/50/5.5/5.5 v/v) was used as eluent, and the lipids on the TLC plate were subsequently visualized with iodine vapor.

Assay System for Measuring Rates of Prothrombin Activation. Phospholipids, factor Xa, and, if present, factor Va were incubated for 5 min at 37 °C in 50 mM Tris-HCl buffer (pH 7.9) containing 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, and concentrations of NaCl as indicated in the legends to the tables and figures. Prothrombin activation was started by the addition of prothrombin that was preincubated at 37 °C in the same buffer. After different time intervals aliquots from the reaction mixture were transferred to plastic disposable cuvettes containing 235 μM of the thrombin-specific chromogenic substrate S2238 in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, and 0.5 mg/mL ovalbumin. The amount of thrombin present in the reaction mixtures was calculated from the absorbance change ($\Delta A_{405-500}$/min) measured on a dual-wavelength spectrophotometer, using a calibration curve of chromogenic substrate conversion by known amounts of active-site-titrated thrombin. Rates of prothrombin activation were usually expressed as moles of prothrombin activated per minute per mole of factor Xa present in the reaction mixture.

Results

Effect of Different Anionic Lipids on the Prothrombin-Converting Activity of PC Membranes. Quantitative comparison of the ability of various anionic lipids to stimulate

Table I: Prothrombin Activation on Membranes Containing 20 mol % of Different Anionic Lipids[a]

lipid composition	rate of prothrombin activation [mol/(min·mol of Xa)]	
	-factor Va	+factor Va
PS/PC	0.43	1720
PLac/PC	0.38	2470
PA/PC	0.34	2530
MePA/PC	0.15	2580
PG/PC	0.081	2460
PE/PC	0.040	1680
oleic acid/PC	0.12	2260
SF/PC	0.052	2180
SDS/PC	0.071	2120
PC	0.023	420
no lipid	0.010	11

[a] Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM phospholipid, and 3 nM factor Xa or 5 pM factor Xa plus 5 nM factor Va. Lipid vesicles contained 80 mol, % PC and 20 mol % anionic lipid.

prothrombin activation is a rather complex pursuit. The prothrombinase activity measured in the presence of phospholipid vesicles is greatly affected by the reaction conditions and depends among others on the prothrombin, phospholipid, and Ca^{2+} concentrations, the absence or presence of factor Va, the mole fraction anionic lipid in the procoagulant membrane, and the pH, temperature, and ionic strength of the reaction medium. Reaction conditions that are optimal for one kind of anionic lipid are not necessarily optimal for other lipids. However, the major objective of the introductory experiment is to test whether an anionic lipid is able to promote prothrombin activation or not. Therefore, reaction conditions were chosen that on the basis of data in literature (van Rijn et al., 1984) were expected to favor lipid stimulation of prothrombinase, i.e., 0.5 μM prothrombin, 100 μM lipid vesicles containing 80 mol % neutral phospholipid PC and 20 mol % anionic lipid, pH 7.9, 37 °C, and low ionic strength. In this experiment, which was carried out both in the absence and in the presence of factor Va, the following anionic lipids were tested: (a) phospholipids that contain a negatively charged phosphate moiety (PA), esterified with groups without (MePA, PG) or with additional charges (PS, PLac, PE[2]), (b) oleic acid, which contains a negatively charged carboxyl group, and (c) sulfolipids (SF, SDS) that contain a sulfate moiety with a net negative charge.

In the presence of factor Va, all membranes containing anionic lipids were able to accelerate factor Xa catalyzed prothrombin activation (Table I). Compared to the rate of the reaction in free solution, the anionic lipid containing membranes caused a more than 150-fold stimulation of prothrombin activation. Membranes that were only composed of the neutral phospholipid PC and that did not contain added anionic lipid showed a 40-fold increase of prothrombin activation.

In the absence of factor Va, PC membranes hardly affected prothrombin activation, while membranes with sulfolipids caused only a 6-fold stimulation and membranes with oleic acid exhibited a 12-fold increase of the reaction rate. The rate enhancements observed with anionic phospholipids were strongly dependent on the polar head group of the phospholipid molecule. Only 4-fold stimulation of prothrombinase was observed for membranes with PE, while membranes containing PG or MePA caused some 10-fold rate enhancement. Maximal stimulation (30–40-fold) of prothrombinase was obtained with membranes that contain PS, PLac, or PA.

To put the data presented in Table I in a proper perspective, it should be emphasized that the experimental conditions might not be optimal for all anionic lipids and that the relation to one another might be different at other reaction conditions. It is also important to mention that this experiment has been carried out at a rather low ionic strength ($I \approx 0.07$) which especially favors the prothrombin-converting activity of some of the lipids (see below). However, irrespective of the reaction conditions, the data presented in Table I allow the conclusion that the prothrombinase activity on negatively charged membranes is not strictly dependent on the presence of a phosphate group (phospholipid), but that anionic lipids with sulfate or carboxyl groups can also promote prothrombin activation.

Prothrombin-Converting Activity of Membranes with Various Anionic Lipids as a Function of the Total Lipid Concentration. It is now well established that membrane stimulation of prothrombinase requires association of all reactants (prothrombin, factor Xa, and factor Va) with the procoagulant membrane. Titration curves of prothrombinase activity as a function of the lipid concentration may, however, show an optimum in the sense that low lipid concentrations proportionally stimulate prothrombin activation until an optimal lipid concentration is reached after which a further increase of the amount of lipid may even cause inhibition of prothrombin activation. Such inhibition can be explained by substrate depletion caused by binding of substrate to nonproductive binding sites on the membrane [free-substrate model; cf. Nelsestuen (1978) and van Rijn et al. (1984)] or by dilution of reactants on an excess of lipid surface [bound-substrate model; cf. van Rijn et al. (1984)]. The actual shape of the titration curves will be determined by the binding parameters (i.e., number of binding sites and dissociation constants) that describe the interaction between the coagulation factors and the procoagulant membranes.

The effect of variation of the total lipid concentration on prothrombin activation was studied both in the absence (Figure 1A) and in the presence of factor Va (Figure 1B) for membranes that contain either 20 mol % PS, MePA, oleic acid, or SF as anionic lipid. These lipids were taken as typical examples of those tested in the experiment presented in Table I. In the absence of factor Va rather large amounts of lipid were required to obtain significant stimulation of the prothrombinase complex. Optimal rates of prothrombin activation were obtained at 50 μM PS/PC and at about 150 μM phospholipid for PC vesicles with MePA, oleic acid, or SF.

When factor Va is part of the prothrombinase complex, much less lipid was needed to obtain optimal rates of prothrombin activation. For all anionic lipids tested, saturation of prothrombinase activity was obtained at 10–20 μM lipid. The shift in lipid requirement is apparently caused by factor Va and is presumably the result of the ability of factor Va to promote the binding of factor Xa (Nesheim et al., 1979; Lindhout et al., 1982) and prothrombin (van de Waart et al., 1984; van Rijn et al., 1984) to procoagulant membranes. Inhibition of prothrombinase activity at high lipid concentrations was only observed with PS. It is not surprising that this occurs with this anionic lipid since it is to be expected that PS has the highest affinity for coagulation factors (Nelsestuen

[2] It should be emphasized that PE-containing membranes will be considerably less negatively charged than membranes with the other anionic lipids. PE contains a negative charge on the phosphate group and a positive charge on the ethanolamine group $[OPO_3^-(CH_2)_2NH_3^+]$. Since the amino group of PE will have a $pK \sim 9$ it can be calculated that at pH 7.9, at which prothrombinase activities are measured, some 5% of the PE molecules will be negatively charged.

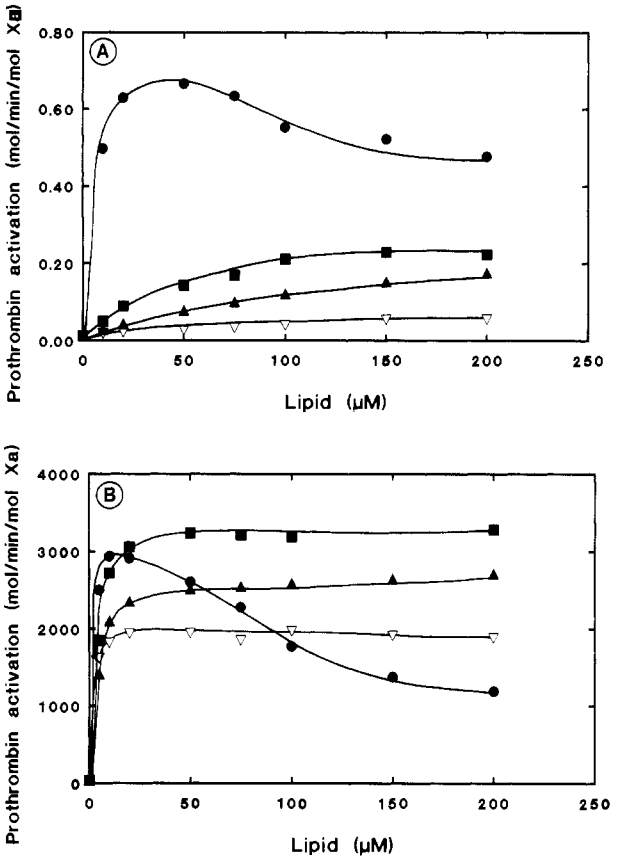

FIGURE 1: Prothrombin activation on membranes containing different anionic lipids as a function of the lipid concentration. Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 0–200 μM lipid vesicles (80 mol % PC/20 mol % anionic lipid), and 3 nM factor Xa (A) or 3 pM factor Xa plus 5 nM factor Va (B). The anionic lipids used were PS (●), MePA (■), oleic acid (▲), and SF (▽).

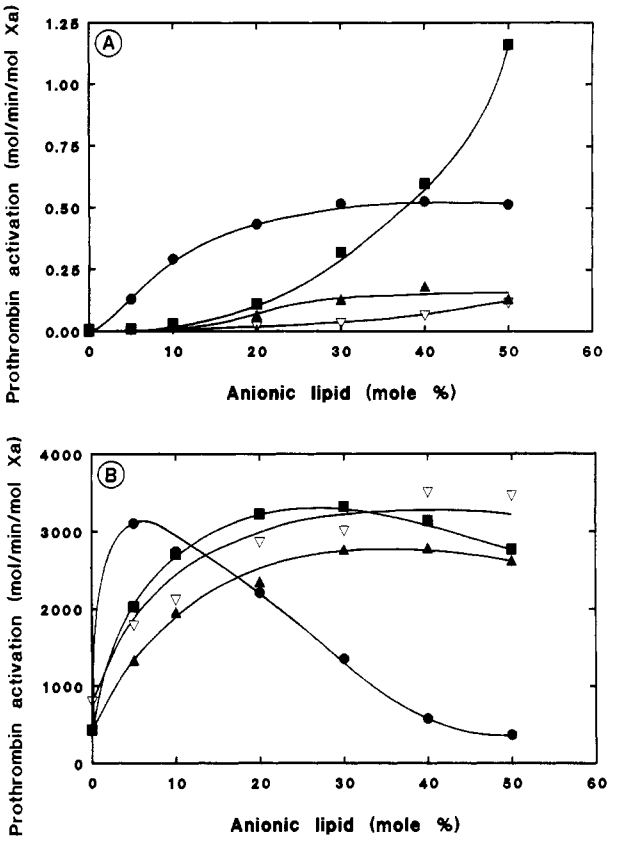

FIGURE 2: Prothrombin activation on membranes containing different anionic lipids as a function of the mole percentage anionic lipid. Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM PC vesicles with varying mole percent anionic lipid, and 4 nM factor Xa (A) or 3 pM factor Xa plus 5 nM factor Va (B). The anionic lipids used were PS (●), MePA (■), oleic acid (▲), and SF (▽).

& Broderius, 1977; Rosing et al., 1988).

Prothrombin-Converting Activity on Membranes Containing Different Mole Fractions of Various Anionic Lipids. The ability of negatively charged membranes to enhance prothrombin activation appears to be dependent on the amount of anionic lipid present in the membrane (van Rijn et al., 1984). At low mole fractions of anionic lipid the prothrombin-converting activity of a membrane increases with the mole fraction anionic lipid. At high mole fractions of anionic lipid, saturation can be obtained while in some cases [PS-containing membranes in the presence of factor Va; cf. van Rijn et al. (1984)] excess anionic lipid may even inhibit the prothrombin-converting activity of a membrane.

In the present investigation we have determined the effect of variation of the mole fraction of four typical anionic lipids (PS, MePA, SF, and oleic acid) on membrane stimulation of prothrombinase. In the absence of factor Va there appear to be large differences between the various anionic lipids (Figure 2A). With PS half-maximal stimulation of prothrombinase was obtained between 5 and 10 mol % anionic lipid. In the case of MePA, SF, and oleic acid the membranes required much larger amounts of anionic lipid in order to stimulate prothrombin activation. Surprisingly, high mole fractions MePA produced membranes that exhibited a higher prothrombin-converting activity than membranes that contained the same amount of PS.

When factor Va is part of the prothrombinase complex, PS-containing membranes were already optimally active at 5 mol % PS (Figure 2B). Introduction of more PS resulted in inhibition of prothrombin-converting activity. With the other anionic lipids (MePA, SF, and oleic acid) saturation curves were obtained which showed half-maximal stimulation of prothrombinase at about 5 mol % anionic lipid and negligible inhibition at high mole fractions of anionic lipid.

Effect of Variation of the Salt Concentration (Ionic Strength) on the Prothrombin-Converting Activity of Membranes with Different Anionic Lipids. It has been suggested that ionic interactions between positively charged protein domains and negatively charged lipids as well as specific Ca^{2+}-dependent chelate complex formation between γ-carboxyglutamic acid residues and negatively charged lipids may be responsible for the association of vitamin K dependent coagulation factors with procoagulant membranes (Rosing et al., 1988). Information about the contribution of ionic interaction to protein–membrane binding can be obtained by variation of the ionic strength of the reaction medium. Increases in ionic strength will cause increased screening of the electrostatic potential of the interacting components, which will result in decreased protein–membrane association (Dombrose et al., 1979).

The effect of variation of the ionic strength on factor Xa catalyzed prothrombin activation on membranes with different anionic lipids is shown in Figure 3A,B. The prothrombin-converting activity of membranes with PS and PA was hardly affected by increasing the ionic strength of the reaction medium. Membranes with oleic acid exhibited intermediate sensitivity while the prothrombin-converting activity of membranes with MePA, PG, PE, PLac, SDS, and SF was strongly

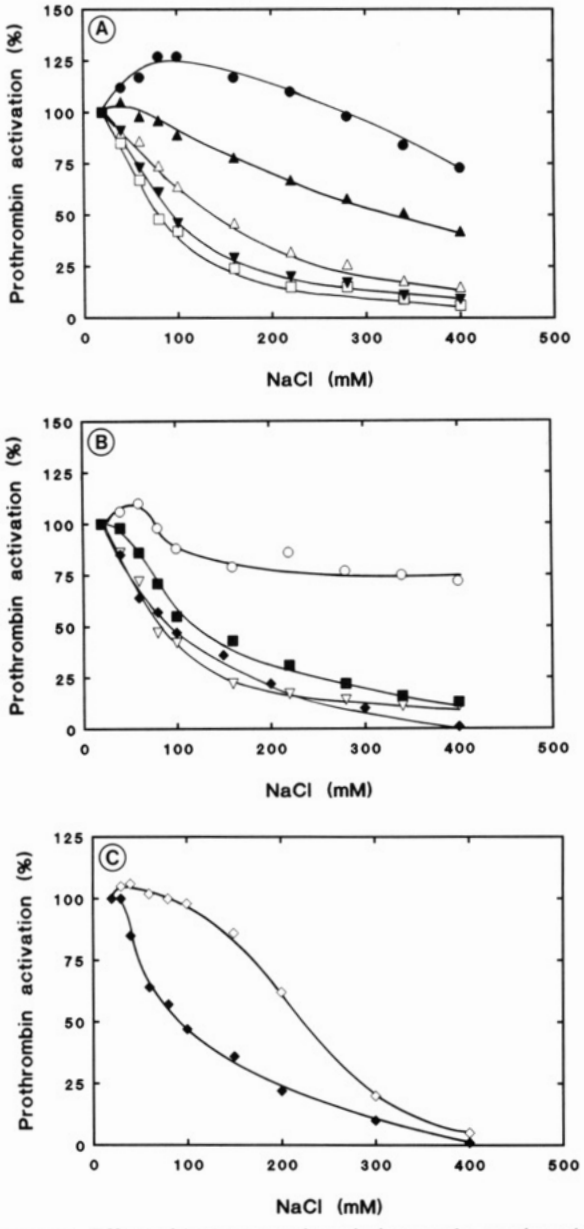

FIGURE 3: Effect of ionic strength variation on the prothrombin-converting activity of membranes containing different anionic lipids. Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20–400 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM lipid vesicles (80 mol % PC/20 mol % anionic lipid), and 3 nM factor Xa (A–C) or 5 pM factor Xa plus 5 nM factor Va (C). (A) PS (●), PLac (△), oleic acid (▲), PG (▼), and PE (□). (B) PA (○), MePA (■), SF (▽), and SDS (◆). (C) SDS with 3 nM factor Xa (◆), and SDS with 5 pM factor Xa plus 5 nM factor Va (◇).

inhibited at increasing ionic strength. These results indicate that there may be rather large differences between the contributions of ionic interaction and chelate complex formation to the binding of vitamin K dependent coagulation factors to membranes with various anionic lipids.

When factor Va was part of the prothrombinase complex, variation of the ionic strength had much less effect on the prothrombin-converting activity of the various membranes. As a typical example this is shown in Figure 3C for membranes that contain 20 mol % SDS. The presence of membrane-bound factor Va apparently compensates for the electrostatic screening effects, presumably by enabling additional interactions with prothrombin (van Rijn et al., 1984; van de Waart et al., 1984) and factor Xa (Nesheim et al., 1979; Lindhout et al., 1982).

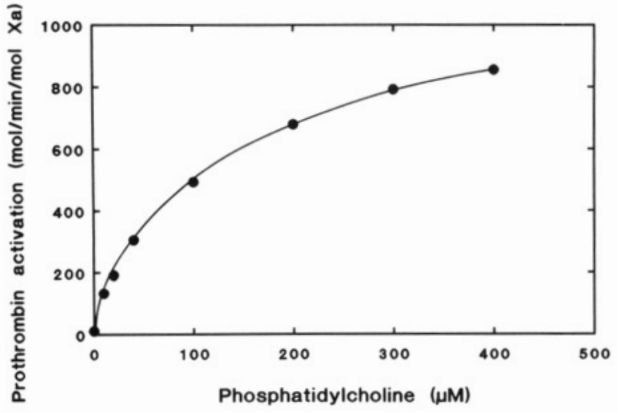

FIGURE 4: Prothrombin activation on PC membranes as a function of the lipid concentration. Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 5 pM factor Xa, 5 nM factor Va, and varying concentrations of PC vesicles.

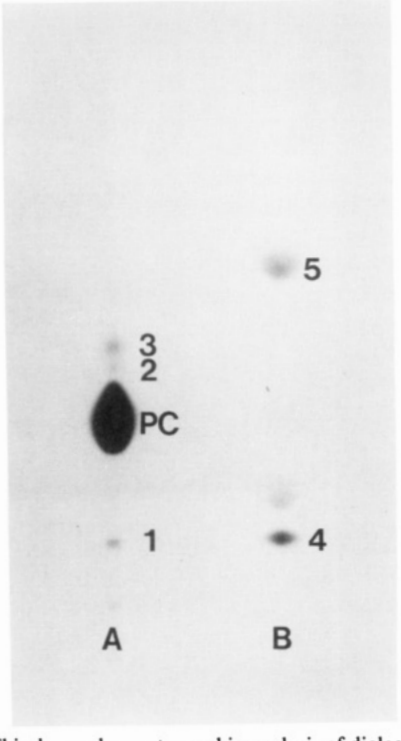

FIGURE 5: Thin-layer chromatographic analysis of dioleoyl-PC. Lane A: 500 μg of dioleoyl-PC. Lane B: 10 μg of lyso-PC plus 5 μg of oleic acid. Spot 4 is lyso-PC, and spot 5 is oleic acid. TLC analysis was performed as described under Experimental Procedures.

Prothrombin Activation on Membranes Composed of PC. The data presented in Table I and Figure 2B indicate that membranes that did not contain added anionic lipid and that were only composed of dioleoyl-PC were able to cause significant stimulation of prothrombin activation, provided that the reaction is carried out at low ionic strength and in the presence of factor Va. Prothrombinase activity as a function of the concentration of dioleoyl-PC vesicles is shown in Figure 4. Maximal rates of prothrombin activation [900 mol of prothrombin activated/(min·mol of Xa)] were obtained at PC concentrations >400 μM, and half-maximal rates of activation were observed at about 100 μM PC. The prothrombin-converting activity of PC membranes may be due to either the presence of trace amounts of anionic lipids contaminating PC preparations or the phosphocholine moiety of PC, which, despite its net neutral charge, may participate in the formation of a functionally active prothrombinase complex.

Table II: Prothrombin Activation on Vesicles Composed of Different Choline-Containing Phospholipids[a]

phospholipid	rate of prothrombin activation [mol/(min·mol of Xa)]
no phospholipid	10
dioleoyl-PC ($diC_{18:1}$ PC)	410
dilinoleoyl-PC ($diC_{18:2}$ PC)	730
dilinolenoyl-PC ($diC_{18:3}$ PC)	738
1-stearyl-2-oleoyl-PC ($C_{18:0}$-$C_{18:1}$ PC)	162
egg PC	95
dimyristoyl-PC ($diC_{14:0}$ PC)	13
dipalmitoyl-PC ($diC_{16:0}$ PC)	41
distearoyl-PC ($diC_{18:0}$ PC)	23
$diC_{18:0}$-ether PC	28
sphingomyelin	26

[a] Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 20 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM phospholipid, 5 pM factor Xa, and 5 nM factor Va.

TLC analysis of the dioleoyl-PC preparations used in these experiments and of the vesicles obtained after sonication of this PC in aqueous solution indicated the presence of trace amounts (less than 1.5%) of contaminating lipids (Figure 5). Spot 1 on the TLC plate was identified as lyso-PC, and spots 2 and 3 were as yet unidentified lipids. However, incorporation of lyso-PC or of the lipids present in spots 2 and 3 (obtained after extraction from the TLC plate) at amounts 5 times that present in the original PC preparation did not further enhance the prothrombin-converting activity of PC vesicles, which indicates that these contaminations were not responsible for the observed prothrombinase activity. Since the occurrence of lyso-PC may go together with the presence of the corresponding fatty acid and since fatty acids promote the prothrombin-converting activity of membranes (Table I, Figures 1 and 2), we decided to determine whether fatty acids are present in our dioleyl-PC. Visual inspection of the PC on the TLC plate (lane A, Figure 5) and comparison with the fatty acid spot of a reference sample (spot 5, lane B) indicate that there is no fatty acid detectable in our PC preparation. The virtual absence of fatty acid was confirmed by gas chromatographic analysis, which showed that dioleoyl-PC contained less than 0.2% fatty acid. From these data we conclude that it is unlikely that fatty acid or one of the other contaminations (spot 1, 2, or 3) present in dioleoyl-PC is responsible for the prothrombin-converting activity of PC membranes.

Therefore, we decided to investigate whether the phosphocholine moiety of PC may participate in the formation of the prothrombinase complex. To this end we have compared the prothrombin-converting activity of a variety of phospholipids containing a phosphocholine moiety, i.e., egg PC, which contains fatty acyl chains of different length and degree of unsaturation, phosphatidylcholines with unsaturated or saturated side chains or with one unsaturated and one saturated fatty acyl chain, and phosphocholine-containing lipids in which unsaturated hydrocarbon side chains are attached to a sphingosine (sphingomyelin) or a glycerol backbone ($diC_{18:0}$-ether-PC) by amido or ether linkages, respectively.

In Table II we have summarized the prothrombin-converting activities of the different phosphocholine-containing lipids. All lipid vesicles composed of PC with unsaturated fatty acyl chains were able to cause considerable stimulation of prothrombin activation, in the presence of factor Va. Vesicles prepared from PC with one saturated and one unsaturated side chain (1-stearyl-2-oleoyl-PC) or from egg PC, which is a mixture of PC molecules with side chains of different length and degree of unsaturation, were less active but were still able to enhance prothrombin activation. Phosphocholine-containing lipids with saturated hydrocarbon side chains ($diC_{14:0}$ PC, $diC_{16:0}$ PC, $diC_{18:0}$ PC, sphingomyelin, and $diC_{18:0}$-ether PC) hardly stimulated prothrombin activation. It could be argued that the lack of prothrombin-converting activity of the latter group of membranes is due to the fact that these membranes may be in the so-called gel phase. However, under the experimental conditions membranes composed of $diC_{14:0}$ PC were in the liquid-crystalline state since the transition temperature of this PC is 23 °C (Tans et al., 1979) and the prothrombin-converting activity of these membranes was determined at 37 °C. Assaying prothrombin activation at 50 °C, which is also above the transition temperature of $diC_{16:0}$ PC [T_t = 41.5 °C; cf. Ladbrooke et al. (1969)], did not enhance the prothrombin-converting activity of membranes composed of PC with saturated hydrocarbon side chains (data not shown). These results indicate that the phosphocholine moiety of PC can indeed promote the assembly of a functionally active prothrombinase complex provided that the PC molecules contain unsaturated hydrocarbon side chains.

Discussion

Previous studies on the role of negatively charged membranes in the assembly of the prothrombinase complex have been carried out with mixtures of neutral and anionic phospholipids. In this paper we show that the presence of phosphate groups is no prerequisite for membranes to promote prothrombinase complex formation. Incorporation of lipids with a carboxyl (oleic acid) or a sulfate group (SF, SDS) produced membranes that considerably accelerated factor Xa catalyzed prothrombin activation. The prothrombin-converting activities of membranes with oleic acid, SF, or SDS were compared with the activities of membranes that derived their negative charge from the presence of anionic phospholipids (PS, PA, MePA, PLac, PE,[2] or PG).

In the absence of factor Va there were rather large differences between the prothrombin-converting activities of different anionic lipids (Table I). Prothrombin activation rates obtained with the different lipids were strongly dependent on the reaction conditions (total lipid concentration, mole percent anionic lipid, and ionic strength of the reaction medium). The ability of the different anionic lipids to enhance prothrombin activation by factor Xa decreased in the order PS–PLac–PA–MePA–oleic acid–PG–SDS–SF–PE. The prothrombin-converting activities of membranes containing MePA, PG, PE, PLac, SDS, and SF were considerably decreased when the ionic strength of the reaction medium was raised (Figure 3). Prothrombin activation on membranes with PS or PA was, however, hardly affected by ionic strength variation. The ionic strength effects suggest that for the first group of lipids electrostatic interactions between prothrombin, factor Xa, and the membrane significantly contribute to the assembly of a catalytically active prothrombinase complex [cf. Dombrose et al. (1979)]. The observations with PS- and PA-containing membranes suggest that with these anionic lipids electrostatic interactions are less important and that coordination binding (chelate complex formation) between Ca^{2+} ions and ligands provided by the Gla residues of the proteins and the polar head groups of membrane phospholipids is the major driving force in protein–membrane association [cf. Resnick and Nelsestuen (1980) and Rosing et al. (1988)]. Ligands that may participate in the formation of such a coordination complex are the phosphate, carboxyl and/or the amino groups of the phospholipids (Hendrickson & Fullington, 1965) and the carboxyl

groups of the Gla residues. Furthermore, it is possible that the coordination sphere of the Ca^{2+} ions may be completed by water or by ligands provided by other amino acids present in the binding domains of prothrombin and factor Xa. Taken together, these data show that the different anionic lipids used in this study can be useful tools in further studies on the importance of different molecular interactions for the assembly of the prothrombinase complex on procoagulant membranes.

When factor Va was part of the prothrombin activating complex, the differences between the various anionic lipids were much less pronounced. In the presence of factor Va all anionic lipids exhibited excellent prothrombin-converting activities (Table I) and optimal rates of prothrombin activation were already obtained at low total lipid concentrations (Figure 1) and at rather low mole percentages of anionic lipid (Figure 2). Ionic strength variation also had much less effect on the ability of membranes to enhance prothrombin activation by the factor Xa–Va complex. These observations presumably reflect additional effects of factor Va on the assembly of the prothrombinase complex on membranes with a low affinity for coagulation factors (van Rijn et al., 1984), a phenomenon that is most likely explained by the fact that factor Va can promote membrane binding of factor Xa (Nesheim et al., 1979; Lindhout et al., 1982) and prothrombin (van de Waart et al., 1984).

In this study we also observed that membranes composed of only PC were not inert in prothrombin activation. At low ionic strength and in the presence of factor Va, PC membranes exhibited considerable prothrombin-converting activity. Although activity of PC membranes in extrinsic factor X activation has been published (Forman & Nemerson, 1986), this is to the best of our knowledge the first report on PC activity in prothrombin activation.

The experiment, in which we determined the prothrombin-converting activity of different phosphocholine-containing lipids, indicated that only PC molecules with unsaturated hydrocarbon side chains produce membranes that are able to promote prothrombin activation. Phosphocholine-containing membranes composed of lipids with saturated hydrocarbon side chains hardly stimulated prothrombin activation. The differences between PC membranes with saturated and unsaturated side chains were not due to fluidity differences since lipid vesicles composed of $diC_{14:0}$ PC or $diC_{16:0}$ PC did not exhibit prothrombin-converting activity above their phase transition temperature.

The presence of unsaturated hydrocarbon chains with cis double bonds may affect the packing density of the PC molecules in the membrane. It is to be expected that the phospholipid molecules in membranes composed of PC with unsaturated side chains will be more loosely packed than phospholipid molecules in membranes composed of PC with saturated side chains and that coagulation factors that become embedded in the membrane (i.e., factor Va) may better bind to loosely packed membranes. Such a phenomenon may explain why prothrombin activation on PC membranes requires PC with unsaturated hydrocarbon chains and why it is especially observed in the presence of factor Va.

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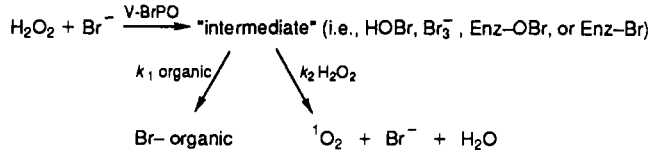
Characterization of Vanadium Bromoperoxidase from *Macrocystis* and *Fucus*: Reactivity of Vanadium Bromoperoxidase toward Acyl and Alkyl Peroxides and Bromination of Amines†

Helena S. Soedjak and Alison Butler*
Department of Chemistry, University of California, Santa Barbara, California 93106
Received April 5, 1990; Revised Manuscript Received May 10, 1990

ABSTRACT: Vanadium bromoperoxidase (V-BrPO) has been isolated and purified from the marine brown algae *Fucus distichus* and *Macrocystis pyrifera*. V-BrPO catalyzes the oxidation of bromide by hydrogen peroxide, resulting in the bromination of certain organic acceptors or the formation of dioxygen. V-BrPO from *F. distichus* and *M. pyrifera* have subunit molecular weights of 65 000 and 74 000, respectively, and specific activities of 1580 units/mg (pH 6.5) and 1730 units/mg (pH 6) for the bromination of monochlorodimedone, respectively. As isolated, the enzymes contain a substoichiometric vanadium/subunit ratio; the vanadium content and specific activity are increased by addition of vanadate. V-BrPO (*F. distichus*, *M. pyrifera*, and *Ascophyllum nodosum*) also catalyzes the oxidation of bromide using peracetic acid. In the absence of an organic acceptor, a mixture of oxidized bromine species (e.g., hypobromous acid, bromine, and tribromide) is formed. Bromamine derivatives are formed from the corresponding amines, while 5-bromocytosine is formed from cytosine. In all cases, the rate of the V-BrPO-catalyzed reaction is much faster than that of the uncatalyzed oxidation of bromide by peracetic acid, at pH 8.5, 1 mM bromide, and 2 mM peracetic acid. In contrast to hydrogen peroxide, V-BrPO does not catalyze formation of dioxygen from peracetic acid in either the presence or absence of bromide. V-BrPO also uses phenylperacetic acid, *m*-chloroperoxybenzoic acid, and *p*-nitroperoxybenzoic acid to catalyze the oxidation of bromide; dioxygen is not formed with these peracids. V-BrPO does not catalyze bromide oxidation or dioxygen formation with the alkyl peroxides ethyl hydroperoxide, *tert*-butyl hydroperoxide, and cuminyl hydroperoxide.

Vanadium bromoperoxidase (V-BrPO)[1] was first discovered in the marine brown alga *Ascophyllum nodosum* (Vilter, 1984). This enzyme has been shown to catalyze the bromination of monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD) using hydrogen peroxide as an oxidant of bromide (Wever et al., 1985). In the absence of an organic substrate, V-BrPO catalyzes the formation of dioxygen (Everett & Butler, 1989). Bromide or iodide is required for dioxygen formation, which is a reaction best described as the halide-assisted disproportionation of hydrogen peroxide. V-BrPO does not catalyze the direct disproportionation of hydrogen peroxide (Everett & Butler, 1989), contrary to the iron heme haloperoxidases which have catalatic activity (Manthey & Hager, 1981; Thomas et al., 1970). Kinetic investigations of the rate of dioxygen formation and MCD bromination catalyzed by V-BrPO indicate that both reactions proceed via the formation of a common intermediate (Everett & Butler, 1989; Everett et al., 1990a,b), although the identity of the exact intermediate has not been identified with certainty. The production of the intermediate is rate limiting (Scheme I). The dioxygen formation pathway has been shown to be competitive with MCD bromination under certain conditions (Everett et al., 1990a). Moreover, the dioxygen produced is in the singlet excited state (Everett et al., 1990a). The striking feature of singlet oxygen production by V-BrPO is its exceptional stability, which is not inactivated by singlet oxygen or oxidized bromine derivatives (Everett et al., 1990a). By contrast, iron heme haloperoxidases (e.g., lactoperoxidase and chloroperoxidase) are strongly inactivated by turnover of hy-

Scheme I



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* To whom correspondence should be addressed.

[1] Abbreviations: BrPO, bromoperoxidase; Capso, 3-(4-aminocyclohexyl)-2-hydroxy-1-propanesulfonic acid; ClPO, chloroperoxidase; MCD, monochlorodimedone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; taurine, 2-aminoethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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