Effect of Membrane Fluidity and Fatty Acid Composition on the Prothrombin-Converting Activity of Phospholipid Vesicles†

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ABSTRACT: Vesicles composed of phospholipids with different fatty acyl side chains have been utilized to examine the importance of the nonpolar membrane region for the prothrombin-converting activity of procoagulant phospholipid vesicles. Membranes composed of phosphatidylserine (PS) and phosphatidylcholine (PC) with unsaturated fatty acyl side chains were more active in prothrombin activation than membranes composed of phospholipids with saturated fatty acyl chains. This phenomenon was observed above the phase transition temperature, i.e., on membranes in the liquid-crystalline state. The prothrombin-converting activity of saturated phospholipids approached the activity of unsaturated phospholipids at high factor Va concentrations, which is indicative for a less favorable equilibrium constant for prothrombinase assembly on membrane surfaces composed of saturated phospholipids. The difference between saturated and unsaturated phospholipids was annulled on membranes with high mole percentages of PS. This may result from a compensating contribution of electrostatic forces to the binding equilibria involved in prothrombinase assembly. Additional effects on the prothrombin-converting activity were observed when membranes containing saturated phospholipids were studied below their phase transition temperature. In agreement with Higgins et al. [(1985) *J. Biol. Chem. 260,* 3604–3612], we found that the time required for the assembly of prothrombinase from membrane-bound factors Xa and Va is considerably prolonged on solid membranes. However, we also observed an effect of membrane fluidity on the steady-state rate of prothrombin activation. Kinetic experiments at saturating factor Va concentrations showed that the transition from the liquid-crystalline to the gel state caused a more than 9-fold decrease of the k_{cat} of prothrombin activation without affecting the K_m for prothrombin. This effect of membrane fluidity on the catalytic activity of prothrombinase was accompanied by a shift in the peptide bond cleavage pattern during prothrombin activation. Meizothrombin and thrombin were the only detectable prothrombin activation products on fluid membranes. A considerable reduction of meizothrombin formation and additional prethrombin 2 generation were observed on membranes in the gel phase. It is hypothesized that effects of membrane fluidity on the proper juxtaposition of enzyme (factor Xa–Va) and substrate (prothrombin) on the membrane may explain both the decreased k_{cat} values and the shift in the peptide bond cleavage pattern observed for prothrombin activation on solid membranes.

The blood coagulation factor prothrombin is enzymatically converted into thrombin by the so-called prothrombinase complex. Prothrombinase is a multicomponent complex composed of the serine protease factor Xa that is tightly associated with the protein cofactor Va on phospholipid membranes in the presence of calcium ions [for a review, see Mann et al. (1990)]. It is well established that optimal prothrombinase activity requires coordinate binding of prothrombin, factor Xa, and factor Va to the membrane surface.

The prothrombin-converting activity of procoagulant membranes is greatly influenced by the chemical and physical properties of the membrane phospholipids. The presence of anionic phospholipids is a prerequisite to bind the participating proteins and to facilitate the reactions leading to thrombin formation. Substantial knowledge is available on the effects of anionic phospholipids on the protein–membrane interactions essential for prothrombinase complex formation [for a review, see Rosing and Tans (1988b)]. Prothrombin and factor Xa are vitamin K-dependent proteins containing γ-carboxyglutamic acid residues (Stenflo et al., 1974; Nelsestuen et al., 1974; Howard & Nelsestuen, 1975) that are involved in the Ca^{2+}-dependent binding on these proteins to the polar head groups of anionic membrane phospholipids (Nelsestuen, 1976; Furie et al., 1976; Prendergast & Mann, 1977). Equilibrium binding parameters have been reported for the association of prothrombin and factor Xa with small unilamellar phospholipid vesicles (Nelsestuen & Broderius, 1977) and planar phospholipid layers (Lecompte et al., 1980; Mayer et al., 1983a; Kop et al., 1984). The interaction between factor Va and membranes also requires anionic phospholipids (Bloom et al., 1979) but is independent of the presence of added Ca^{2+} ions. The formation of the factor Va–phospholipid complex may involve both hydrophobic (Bloom et al., 1979; Krishnaswamy & Mann, 1988) and electrostatic interactions (Pusey et al., 1982; van der Waart et al., 1983; Mayer et al., 1983b; Pusey & Nelsestuen, 1984).

The effects of phospholipid concentration and composition on the catalytic activity of the prothrombinase complex have been studied in detail for membranes containing different anionic phospholipids (Rosing et al., 1980; van Rijn et al., 1984; Gerads et al., 1990). It was shown that membranes that contain the aminophospholipid phosphatidylserine (PS)[1] as anionic phospholipid exhibit the highest prothrombin-converting activity (van Rijn et al., 1984; Rosing et al., 1988). The ability of membranes to accelerate prothrombin activation is, however, not strictly dependent on the presence of amino

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and phosphate groups. This can be concluded from the fact that negatively charged lipids that only contain a phosphate (PA), sulfate (sulfatides or dodecylsulfuric acid), or carboxyl group (oleic acid) also promote factor Xa-catalyzed prothrombin activation (Gerads et al., 1990).

The prothrombin-converting activity of membranes not only is dependent on the chemical nature of the polar head group but also is affected by the chemical and physical properties of the nonpolar membrane region. Sterzing and Barton (1973) have shown that the procoagulant activity of membranes composed of egg yolk PC and bovine brain PS was greatly reduced upon hydrogenation, and they concluded that the liquid-crystalline phase is essential for the expression of procoagulant activity. This was confirmed by subsequent studies with vesicles composed of synthetic phospholipids in which it was shown that the phase transition from the gel state to the liquid-crystalline state is accompanied by a sharp increase of the clot-promoting activity of the vesicles (Tans et al., 1979). In a recent study, Higgins et al. (1985) investigated the effect of membrane fluidity on the catalytic properties of the prothrombinase complex. They observed a considerable lag in the time required for the assembly of the prothrombinase complex on membrane surfaces in the gel state and concluded that lipid fluidity affects the rate of assembly of the factor Xa–Va complex at the membrane surface.

Recently we observed that membranes solely composed of PC also promote prothrombin activation provided that the PC molecules contain unsaturated fatty acyl side chains and the reaction is studied at low ionic strength (Gerads et al., 1990). The differences between the prothrombin-converting activities of PC membranes with saturated and unsaturated side chains were not due to fluidity differences since vesicles composed of DMPC or DPPC were also not active above the phase transition temperature.

On the basis of these results, we decided to perform a detailed study on the effects of fatty acyl side chain composition of membrane phospholipids on the prothrombin-converting activities of negatively charged phospholipid vesicles. The assembly of the prothrombinase complex and the expression of its catalytic activity were investigated on membranes composed of unsaturated or saturated phospholipids both below and above the phase transition temperature of the vesicles.

EXPERIMENTAL PROCEDURES

Materials. S2238 and I2581 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. *p*-NPGB was from Nutritional Biochemicals. Dimyristoyl-*sn*-glycero-3-phosphoserine ($diC_{14:0}PS$), dioleoyl-*sn*-glycero-3-phosphoserine ($diC_{18:1}PS$), dilauroyl-*sn*-glycero-3-phosphocholine ($diC_{12:0}$-PC), dimyristoyl-*sn*-glycero-3-phosphocholine ($diC_{14:0}PC$), dipalmitoyl-*sn*-glycero-3-phosphocholine ($diC_{16:0}PC$), dipalmitoleoyl-*sn*-glycero-3-phosphocholine ($diC_{16:1}PC$), dioleoyl-*sn*-glycero-3-phosphocholine ($diC_{18:1}PC$) and dierucoyl-*sn*-glycero-3-phosphocholine ($diC_{22:1}PC$) were obtained from Avanti Polar Lipids Inc., Alabaster, AL. Dilauroyl-*sn*-glycero-3-phosphoserine ($diC_{12:0}PS$), dipalmitoyl-*sn*-glycero-3-phosphoserine ($diC_{16:0}PS$), dipalmitoleoyl-*sn*-glycero-3-phosphoserine ($diC_{16:1}PS$), and dierucoyl-*sn*-glycero-3-phosphoserine ($diC_{22:1}PS$) were a kind gift of Dr. P. Comfurius, Rijksuniversiteit Limburg, Maastricht, The Netherlands. Benzamidine hydrochloride, ovalbumin (grade V), Russell's viper venom and *Echis carinatus* venom were obtained from Sigma, St. Louis, MO. Column materials for protein purification (DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100, Sephadex G-200, and Sephacryl S-300) were obtained from Pharmacia, Uppsala, Sweden. Silica gel 60 plates for thin-layer chromatography of phospholipids were from Merck, Darmstadt, Germany. Crude porcine intestinal mucosal heparin (USP activity 175 units/mg) was purchased from Organon, The Netherlands.

Proteins. Bovine prothrombin and prethrombin 1 were purified according to 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). Bovine AT-III was purified according to Thaler and Schmer (1975). Prothrombin, factor Xa, and AT-III 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 & 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 Vesicle Preparations. TLC analysis of the phospholipid preparations 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 an eluent, and purity of the different preparations was checked after visualization of the phospholipids on the TLC plate with iodine vapor. Single-bilayer phospholipid vesicles were prepared as follows: phospholipid 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 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl at 65 °C and vigorously vortexed for 1 min. The phospholipid suspensions were subsequently sonicated for 10 min at 65 °C with an MSE Mark II 150-W ultrasonic disintegrator set at 8 μM peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis (Böttcher et al., 1961).

Assay System for Measuring Rates of Prothrombin Activation. Phospholipids, factor Xa, and factor Va were incubated for 5 min at the reaction temperature in 50 mM Tris-HCl buffer (pH 7.52) containing 5 mM $CaCl_2$/0.5 mg/mL ovalbumin. Prothrombin activation was started by the addition of prothrombin preincubated at the same temperature in the same buffer. After different time intervals, aliquots from the reaction mixture were transferred to disposable cuvettes containing 235 μM 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

[1] Abbreviations: AT-III, antithrombin III; DMPS, dimyristoyl-*sn*-glycero-3-phosphoserine; DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DOPS, dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; I2581, *N*-dansyl-*p*-guanidinophenylalanylpiperidide hydrochloride; *p*-NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PC, phosphatidylcholine; PS, phosphatidylserine; RVV-X, purified factor X activator from Russell's viper venom; SDS, sodium dodecyl sulfate; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; Q_{10}, ratio of the rate constants of two reactions 10 °C apart.

Table I: Phase Transition Temperatures for Phosphatidylserines and Phosphatidylcholines Used in This Study

phospholipid	T_m (°C)	phospholipid	T_m (°C)
$diC_{12:0}PS$	*d*	$diC_{12:0}PC$	0[b,c]
$diC_{14:0}PS$	35[a]	$diC_{14:0}PC$	23[b,c]
$diC_{16:0}PS$	57[a]	$diC_{16:0}PC$	41[b]
$diC_{16:1}PS$	*d*	$diC_{16:1}PC$	−36[c]
$diC_{18:1}PS$	−15.5[a]	$diC_{18:1}PC$	−16[a]
$diC_{22:1}PS$	*d*	$diC_{22:1}PC$	*d*

[a] Tans et al. (1979). [b] Ladbrooke & Chapman (1969). [c] van Dijck et al. (1976). [d] No literature data were found for the phase transition temperatures of these phospholipids. From comparison of PS and PC and the relation between T_m and fatty acyl side chain length, it is likely that the phase transition temperatures of these phospholipids will be below 20 °C.

prothrombin activated 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. In order to specifically determine meizothrombin, the amidolytic activity was also measured with 4 nM AT-III and 10 μg/mL heparin in the cuvette [cf. Rosing et al. (1986)]. In that case, samples from the prothrombin activation mixture were incubated for 1 min in the cuvette with AT-III and heparin to inhibit thrombin prior to the addition of S2238.

Gel Electrophoretic Analysis of Prothrombin Activation. Polyacrylamide gel electrophoresis of prothrombin activation was carried out in the presence of SDS on 10% polyacrylamide slab gels (6% stacking gel) according to Laemmli (1970) in a miniprotean II cell from Bio-Rad. Aliquots (25 μL) from the reaction mixtures were added to 10 μL of gel buffer containing 250 mM Tris-HCl (pH 6.9), 5% SDS, 50% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. Prior to electrophoresis, the samples were kept for 20 min at 37 °C. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

RESULTS

Physical Properties of the Phospholipid Vesicles Used in This Study. Variation of the hydrocarbon chain length and the introduction of cis double bonds in the acyl side chains of membrane phospholipids greatly affect the physical properties of small unilamellar vesicle membranes (Huang, 1991). In Table I, we have summarized the transition temperatures for the gel → liquid-crystalline-phase transition of the phospholipids used in this study. Phospholipids which contain fatty acyl side chains with a cis double bond ($diC_{16:1}$, $diC_{18:1}$, and $diC_{22:1}$ variants of both PS and PC) have low phase transition temperatures. This means that the prothrombin-converting activities of vesicles composed of these phospholipids can only be studied in the liquid-crystalline state. DMPS, DMPC, and DPPC have phase transition temperatures of 35, 23, and 41 °C, respectively, and phospholipid vesicles composed of mixtures of DMPS with DMPC or DPPC will, therefore, have phase transition temperatures below 41 °C. This means that the prothrombin-converting activities of vesicles prepared from DMPS/DMPC or DMPS/DPPC mixtures can be studied both below and above their respective phase transition temperatures, which allows determination of the effect of the gel → liquid-crystalline transition on prothrombin activation. Vesicles composed of the other phospholipids summarized in Table I have a phase transition outside the physiological temperature range, and reliable data on these vesicles can only be obtained in either the liquid-crystalline or the gel state.

It is unlikely that the different vesicle preparations will have identical physical properties above their respective phase transition temperatures. It has been reported that the introduction of a cis double bond in the hydrocarbon chain of a phospholipid molecule not only decreases the transition temperature but also increases the area per phospholipid molecule in a phospholipid monolayer at an air–water interface (Ladbrooke & Chapman 1969; Demel et al., 1972). Such a phenomenon will likely affect the lipid packing in a vesicle membrane, and it is to be expected that the phospholipid molecules in, for instance, DMPS/DMPC or DPPS/DMPC vesicles will be more densely packed than in DOPS/DOPC vesicles, even if these membranes are above their phase transition temperature.

Effect of Membrane Fluidity on the Time Course of Prothrombin Activation. In 1985, Higgins et al. reported that membranes in the gel phase exhibited a considerable lag in thrombin formation, when prothrombin activation was started with factor Xa or factor Va. They concluded that factor Xa and factor Va separately bind to the membrane and have to diffuse across the membrane surface in order to assemble into a catalytically active prothrombinase complex. This diffusion was proposed to be considerably slowed down on solid membranes which might explain the occurrence of a lag phase when prothrombin activation on such membranes was started with factor Xa or with factor Va.

We also observed that time courses of prothrombin activation were strongly dependent on the fluidity of the phospholipid vesicles and on the order of addition of the prothrombinase proteins. Time courses of prothrombin activation were analyzed at 45 and 15 °C on vesicles composed of DOPS/DOPC or DMPS/DMPC. When prothrombin activation was started with factor Xa or Va at 45 °C, where both vesicles were in the liquid-crystalline phase, there was no lag in thrombin generation, and the prothrombin-converting activities of both vesicles were the same (Figure 1A). However, as shown in Figure 1B, there was a rather large difference between these vesicles at 15 °C. On DOPS/DOPC vesicles, which were still in the liquid-crystalline phase at this temperature, thrombin generation was linear in time and was slowed down approximately 6-fold. On the DMPS/DMPC vesicles, which were in the gel phase at 15 °C, prothrombin activation showed a lag phase of more than 1 min. This lag phase was only observed when prothrombin activation was started with factor Xa or with factor Va and was absent when the reaction was started with prothrombin (Figure 1C). Under these conditions, time courses of prothrombin activation were not affected by prolonged incubation of factor Xa and factor Va with the phospholipid vesicles and did not deviate from linearity when the reaction times were extended up to 30 min (data not shown). This demonstrates that the time courses shown in Figure 1C represent steady-state rates of prothrombin activation. In contrast to Higgins et al. (1985), we observed that membrane fluidity affected the steady-state rate of prothrombin activation. On membranes in the gel phase, the steady-state rate of prothrombin activation was about 7-fold slower than on membranes in the liquid-crystalline state (Figure 1C).

Effect of Membrane Fluidity on Steady-State Rates of Prothrombin Activation. The effect of membrane fluidity on steady-state prothrombin activation was further investigated by measuring the temperature dependency of the rate of prothrombin activation on phospholipid vesicles with different phase transition temperatures (Figure 2). To enable a proper determination of the steady-state rate, prothrombin activation

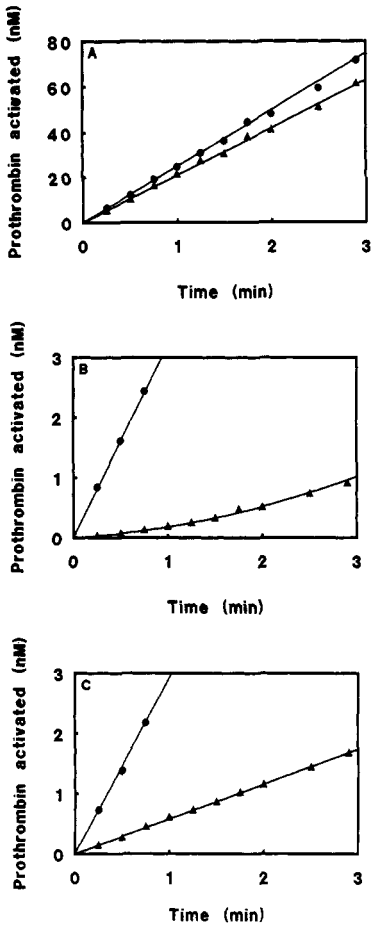

FIGURE 1: Time courses of prothrombin activation at different temperatures on vesicles composed of saturated and nonsaturated phospholipids. Prothrombin (1 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 2.5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 10 pM factor Xa, 5 nM factor Va, and 10 μM DMPS/DMPC [20/80, M/M (▲)] or DOPS/DOPC [20/80, M/M (●)] vesicles. Prothrombin activation was started by simultaneous addition of factor Xa and factor Va at 45 °C (A) or 15 °C (B), or with prothrombin at 15 °C (C). The amounts of prothrombin activated were calculated from the generation of amidolytic activity toward the chromogenic substrate S2238 as described under Experimental Procedures.

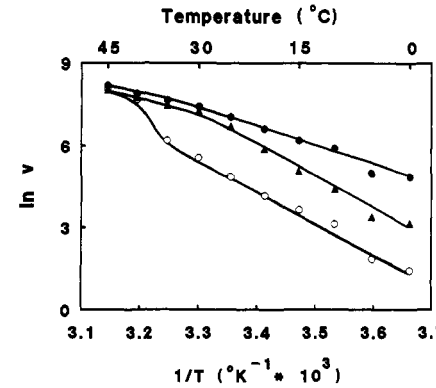

FIGURE 2: Effect of temperature on the steady-state rates of prothrombin activation on membranes composed of phospholipids with different phase transition temperatures. 10 pM factor Xa and 5 nM factor Va were incubated for 5 min at varying temperatures in a reaction buffer containing 50 mM Tris-HCl (pH 7.5 at the indicated temperature), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, and 100 μM phospholipid (PS/PC, 20/80, M/M). The phospholipid vesicles were prepared from DOPS/DOPC (●), DMPS/DMPC (▲), or DMPS/DPPC (○) mixtures. Prothrombin activation was started by the addition of prothrombin to a final concentration of 1 μM. Rates of prothrombin activation were determined as described under Experimental Procedures.

was started by the addition of prothrombin to reaction mixtures in which factors Xa and Va had been preincubated with phospholipid vesicles. In this experiment, in which the reaction temperature was varied between 45 and 0 °C, steady-state rates of prothrombin activation were determined on DOPS/DOPC, DMPS/DMPC, and DMPS/DPPC vesicles which have phase transition temperatures at about −16, 25, and 40 °C, respectively (Table I). There appeared to be a good correlation between the fluidity and the prothrombin-converting activity of these membranes. At 45 °C, all membranes were in the liquid-crystalline state and were equally active in prothrombin activation. The Arrhenius plot of the rate of prothrombin activation on DOPS/DOPC vesicles, which were in the liquid-crystalline state over the whole temperature range, showed no discontinuity and gradually decreased with a temperature coefficient (Q_{10}) of about 1.6 (Figure 2). Marked discontinuities were observed in the Arrhenius plots of the prothrombin-converting activities of DMPS/DMPC and DMPS/DPPC vesicles. Rates of prothrombin activation on DMPS/DMPC and DMPS/DPPC vesicles showed an additional decrease with temperature beginning at 25 and 40 °C, respectively. This discontinuity of the rate profile occurred in a temperature range in which these vesicles are expected to undergo phase transition from the liquid-crystalline to the gel state. These data show that membrane fluidity not only affects the time required for prothrombinase assembly but also affects the catalytic properties of the prothrombin-converting complex.

Effect of Membrane Fluidity on the Kinetic Parameters of Prothrombin Activation. In the previous paragraph, we have shown that steady-state rates of prothrombin activation were considerably decreased when the membrane did undergo a phase transition from the liquid-crystalline to the gel phase. Since the experiments presented in the previous paragraphs were performed at single factor Va (5 nM) and prothrombin (1 μM) concentrations, the effect of membrane fluidity on the steady-state rate of prothrombin activation can be (a) on the equilibrium constant for factor Xa–Va complex formation at the membrane surface, (b) on the K_m for prothrombin, or (c) on the V_{max} of prothrombin activation.

To distinguish between these possibilities, we have performed a kinetic analysis of prothrombin activation on DOPS/DOPC and on DMPS/DPPC vesicles at 45 and 15 °C, i.e., above and below the phase transition temperature of the latter vesicles. In this experiment, DOPS/DOPC vesicles were compared with vesicles composed of a mixture of DMPS and DPPC, which in contrast to DMPS/DMPC mixtures showed essentially complete miscibility both in the gel and in the liquid-crystalline state (Tans et al., 1979). Information on the equilibrium constant for the formation of the membrane-bound factor Xa–Va complex was obtained by measuring the factor Va dependency of the rate of prothrombin activation at limited factor Xa concentration [cf. Lindhout et al. (1982)]. The concentrations of factor Va required for 50% saturation of prothrombin activation ($K_{1/2Va}$) are summarized in Table II. For vesicles containing 20 mol % PS, the formation of the membrane-bound factor Xa–Va complex appeared to be hardly influenced by membrane fluidity, and the $K_{1/2Va}$ values were considerably below the factor Va concentration of 5 nM present in the experiments shown in Figures 1 and 2. This indicates that the low steady-state rates of prothrombin activation observed on membranes in the gel phase were not due to an effect of membrane fluidity on the equilibrium constant for factor Xa–Va complex formation at the membrane surface.

Table II: Effect of Membrane Fluidity on the Kinetic Parameters of Prothrombin Activation on DOPS/DOPC and DMPS/DPPC Vesicles[a]

	DOPS/DOPC			DMPS/DPPC		
reaction temp (°C)	$K_{1/2Va}$ (nM)	K_m (μM)	V_{max}[b]	$K_{1/2Va}$ (nM)	K_m (μM)	V_{max}[b]
45	0.12	0.30	13.5	0.29	0.22	11.9
15	0.14	0.30	2.5	0.13	0.16	0.27

[a] Prothrombin was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 3 pM factor Xa, 100 μM phospholipid vesicles (DOPS/DOPC or DMPS/DPPC, 20/80, M/M), and 5 nM factor Va (in K_m and V_{max} determination) or varying concentrations of factor Va (in factor Va titration). The factor Va titration ($K_{1/2Va}$ determination) was performed at 1 μM prothrombin. Rates of prothrombin activation were determined as described under Experimental Procedures. The kinetic parameters were obtained from double-reciprocal plots of the rates of prothrombin activation versus the factor Va concentration ($K_{1/2Va}$) or the prothrombin concentration (K_m and V_{max}) after statistical analysis according to Eisenthal and Cornish-Bowden (1974). [b] Nanomolar prothrombin activated per minute.

In Table II, we have also summarized the effect of temperature and membrane fluidity on the kinetic parameters (K_m and V_{max}) of prothrombin activation. The K_m for prothrombin appeared to be influenced neither by the temperature nor by the fluidity of the phospholipid vesicles. K_m values between 0.15 and 0.3 μM were determined at 15 and 45 °C on DOPS/DOPC and DMPS/DPPS vesicles.

Variation of temperature had a considerable effect on the V_{max} values (Table II). Lowering the temperature from 45 to 15 °C caused a 6-fold decrease of the V_{max} of prothrombin activation on the DOPS/DOPC vesicles. However, temperature had a much more pronounced effect on the V_{max} determined on DMPS/DPPC vesicles. Whereas at 45 °C the same V_{max} values were obtained on DMPS/DPPC and DOPS/DOPC vesicles, the V_{max} determined at 15 °C on the DMPS/DPPC vesicles, which are in the gel phase, was 9-fold lower then the V_{max} determined on the DOPS/DOPC vesicles, which are in the liquid-crystalline phase at this temperature. These results demonstrate that membrane fluidity affects the prothrombin-converting activity by decreasing the catalytic activity of the prothrombinase complex as represented by the decrease of the V_{max} at the transition from the liquid-crystalline to the gel phase.

Effect of the Lipid-Phase Transition on the Catalytic Properties of the Prothrombinase Complex. To obtain further information on the effect of membrane fluidity on the catalytic activity of the prothrombinase complex, we have performed experiments with prethrombin 1 as substrate for prothrombinase. Prethrombin 1 is a prothrombin derivative that lacks the fragment 1 domain responsible for prothrombin binding to procoagulant membranes (Stenn & Blout, 1972; Nelsestuen, 1976). Comparison of the activity of the membrane-bound prothrombinase complex with prothrombin and prethrombin 1 as substrate may discriminate between effects of membrane fluidity on steps involved in the interaction of prothrombin with the membrane (association, dissociation, and proper juxtaposition of enzyme and substrate) and direct effects on the catalytic-site activity (conformation of the active site) of the membrane-bound factor Xa–Va complex. The results represented in Table III demonstrate that membrane fluidity does not affect the prothrombinase activity on prethrombin 1. Rates of prethrombin 1 activation on DOPS/DOPC and DMPS/DPPC vesicles were the same both above (45 °C) and below (15 °C) the phase transition temperature of the DMPS/DPPC vesicles. In this table, we have included rates of prothrombin activation determined on the same vesicles in order to reconfirm the reduced prothrombin-converting activity of phospholipid vesicles in the gel phase (DMPS/DPPC vesicles at 15 °C). From the fact that rates of prethrombin 1 activation were not affected by membrane fluidity, we conclude that the phase transition from the liquid-crystalline to the gel phase does not affect the intrinsic catalytic activity of the membrane-bound factor Xa–Va complex.

Table III: Effect of Membrane Fluidity on Rates of Prothrombin and Prethrombin 1 Activation[a]

	rate of prothrombin/prethrombin 1 activation[b] at			
	45 °C		15 °C	
substrate	DOPS/DOPC	DMPS/DPPC	DOPS/DOPC	DMPS/DPPC
prethrombin 1	50	62	236	218
prothrombin	3527	3324	654	73

[a] 1 μM prothrombin or prethrombin 1 was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM PS/PC (20/80, M/M) vesicles, different factor Xa concentrations (2–100 pM), and 5 nM factor Va. The phospholipid vesicles were composed of PS and PC with fatty acyl side chains indicated in the table. The factor Xa concentrations were adapted to the rates of prothrombin or prethrombin 1 activation determined under the various reaction conditions. [b] Nanomolar prothrombin or prethrombin 1 activated per minute per nanomolar factor Xa present in the reaction mixture.

One peculiarity observed in the experiment presented in Table III needs further attention, and that is the effect of temperature on prethrombin 1 activation. To our surprise, the rate of prethrombin 1 activation was increased 4-fold when the reaction temperature was lowered from 45 to 15 °C. For this phenomenon, we have as yet no good explanation.

Gel Electrophoretic Analysis of Prothrombin Activation. Three different reaction products can be formed during factor Xa-catalyzed prothrombin activation, i.e., the inactive reaction intermediate prethrombin 2 and the enzymatically active products meizothrombin and thrombin. When prothrombin is activated by factor Xa in the absence of factor Va, prethrombin 2 is the major initial reaction product, and relatively small amounts of thrombin and meizothrombin are formed (Rosing et al., 1980, 1986). When factor Va is part of the prothrombinase complex, there is no detectable formation of prethrombin 2, and in the initial phase of prothrombin activation, meizothrombin and thrombin are the only reaction products formed (Rosing et al., 1980, 1986; Krishnaswamy et al., 1986).

To test whether the effect of membrane fluidity on the V_{max} of prothrombin activation might be explained by an effect on the product generation pattern, we have followed time courses of prothrombin activation by gel electrophoretic analysis and with amidolytic assays that allow separate quantitation of thrombin and meizothrombin (Rosing et al., 1986). This experiment was performed in the presence of the reversible thrombin inhibitor I2581 in order to prevent autocatalytic degradation of prothrombin and its activation products by thrombin.

In Figure 3a, it is shown that in the presence of fluid vesicles (DOPS/DOPC at 15 °C) meizothrombin was the main reaction product in the early phase of prothrombin activation. After approximately 15 min, meizothrombin began to dis-

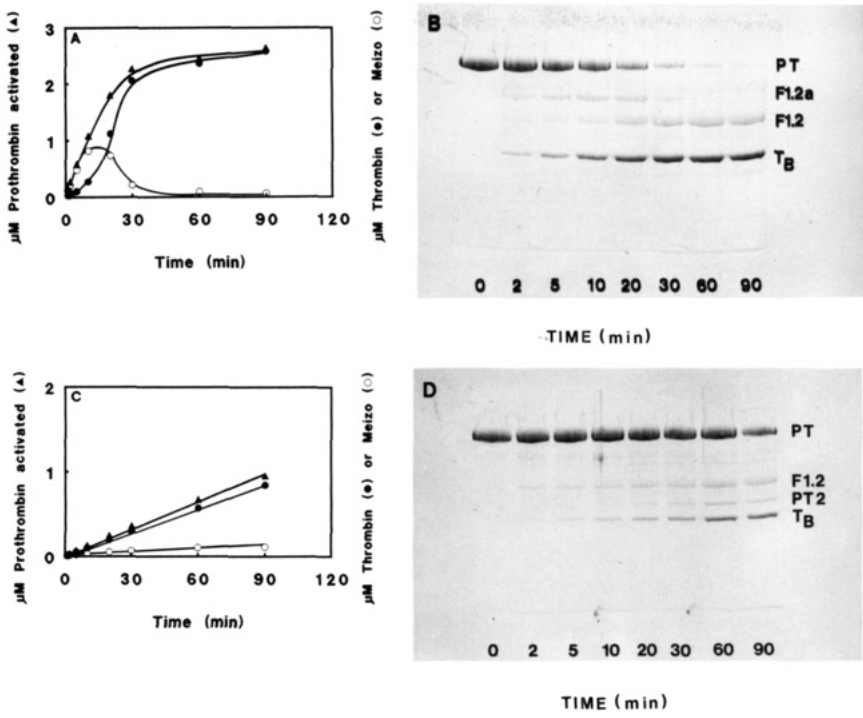

FIGURE 3: Effect of membrane fluidity on time courses of product generation during prothrombin activation. Prothrombin (3 μM) was activated at 15 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 1 nM factor Xa, 10 nM factor Va, 20 μM I2581, and 100 μM phospholipid vesicles composed of 20 mol % DOPS and 80 mol % DOPC (A, B) or 20 mol % DMPS and 80 mol % DPPC (C, D). At the time intervals indicated, samples were withdrawn for the determination of thrombin and meizothrombin (A, C) and for gel electrophoretic analysis (B, D). Quantitation of thrombin and meizothrombin and gel electrophoretic analysis were performed as described under Experimental Procedures. PT, prothrombin; F1.2A, fragment 1.2 + A-chain thrombin; F1.2, fragment 1.2; PT2, prethrombin-2; T_B, B-chain of thrombin.

appear, while thrombin formation continued. The amidolytic data were confirmed by gel electrophoretic analysis (Figure 3B). The transient band on the gels at the migrating distance of fragment 1.2-A is indicative for the formation of meizothrombin as the temporary reaction intermediate [cf. Krishnaswamy et al. (1986)]. The only other products observed on the gel were fragment 1.2 and the B-chain of thrombin. This cleavage pattern confirms earlier observations on product generation during prothrombin activation by the factor Xa–Va complex on DOPS/DOPC vesicles at 37 °C.

An important shift in the peptide bond cleavage pattern occurred when prothrombin was activated on phospholipid vesicles in the gel phase. Whereas the product generation patterns during prothrombin activation on DOPS/DOPC and DMPS/DPPC vesicles at 45 °C were the same and identical to those observed on DOPS/DOPC vesicles at 15 °C (data not shown), there was hardly any meizothrombin formation during prothrombin activation on DMPS/DPPC vesicles in the gel phase, i.e., at 15 °C (Figure 3C). Minor amounts of meizothrombin were formed, and thrombin was the major enzymatically active prothrombin activation product during the complete time course of activation. Inspection of the gel (Figure 3D) not only confirmed the slow rate of meizothrombin formation (there was no fragment 1.2-A visible on the gel) but also showed that considerable amounts of prethrombin 2 were formed during prothrombin activation by the factor Xa–Va complex on membranes in the gel phase. To the best of our knowledge, this is the first time that prethrombin 2 is observed as reaction product during prothrombin activation by the complete prothrombinase complex.

These data indicate that membrane fluidity affects the individual rate constants on the peptide bond cleavages that occur during prothrombin activation. On membranes in the gel phase, there is reduced meizothrombin formation and generation of prethrombin 2 as prothrombin activation intermediate becomes possible.

Influence of Fatty Acyl Side Chain Saturation on the Prothrombin-Converting Activity of Phospholipid Vesicles in the Liquid-Crystalline State. Recently we reported that membranes solely composed of the neutral phospholipid PC were able to accelerate prothrombin activation by the factor Xa–Va complex. Prothrombin-converting activity on PC vesicles was only observed at low ionic strength ($I < 0.05$), at high factor Va concentrations, and when the membranes were composed of PC that contained fatty acyl side chains with one or more unsaturated bonds (Gerads et al., 1990).

To test whether this effect of fatty acyl side chain saturation is also observed on negatively charged membranes, we have compared the prothrombin-converting activities of a number of different membranes composed of either saturated or unsaturated phospholipids (Table IV). To avoid effects of membrane fluidity on the prothrombinase activity, the reaction temperature (45 °C) was above the phase transition temperature of the various vesicles studied in this experiment. Prothrombin activation was performed at physiological ionic strength ($I \approx 0.2$) on membranes containing 5 mol % PS. The factor Va concentration present in the activation mixture was 0.5 nM in order to ensure ~80% incorporation of factor Xa into the membrane-bound factor Xa–Va complex in the case of DOPS/DOPC (5/95, mol/mol) vesicles. In Table IV, it is shown that PS-containing vesicles composed of unsaturated phospholipids exhibit a considerable higher prothrombin-converting activity than vesicles composed of saturated phospholipids. Thus, it appears that fatty acyl side chain saturation is also an important parameter for the prothrombin-converting activity of membranes above the phase transition temperature.

Table IV: Effect of Fatty Acyl Side Chain Saturation on the Prothrombin-Converting Activities of Fluid Phospholipid Vesicles[a]

phospholipid	rate of prothrombin activation[b]
$diC_{12:0}PS/diC_{12:0}PC$	143
$diC_{14:0}PS/diC_{14:0}PC$	24
$diC_{16:0}PS/diC_{16:0}PC$	43
$diC_{16:1}PS/diC_{16:1}PC$	2551
$diC_{18:1}PS/diC_{18:1}PC$	3304
$diC_{22:1}PS/diC_{22:1}PC$	3823

[a] 1 μM prothrombin was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM phospholipid vesicles, 0.5 nM factor Va, and either 40 pM factor Xa (experiment saturated phospholipids) or 2 pM factor Xa (experiment unsaturated phospholipids). The phospholipid vesicles were composed of 5 mol % PS and 95 mol % PC with fatty acyl side chains indicated in the table. The trivial names of the phospholipids used in this experiment are given in the Materials section under Experimental Procedures. [b] Nanomolar prothrombin activated per minute per nanomolar factor Xa present in the reaction mixture.

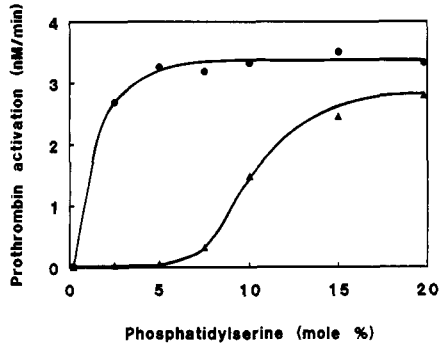

FIGURE 4: Prothrombin activation on membranes composed of saturated and unsaturated phospholipids as a function of PS content. Prothrombin (1 μM) was activated at 45 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM phospholipid vesicles, 1 pM Xa, and 0.5 nM factor Va. The phospholipid vesicles were prepared from DOPS/DOPC (●) or DMPS/DPPC (▲) mixtures containing mole percentages of DOPS or DMPS indicated in the figure. Rates of prothrombin activation (nanomolar per minute) were determined as described under Experimental Procedures.

In Figure 4, it is shown that this effect is especially observed on membranes with low amounts of negatively charged phospholipid (PS). On vesicles composed of dioleoylphospholipids, optimal rates of prothrombin activation were already obtained between 2.5 and 5 mol % DOPS. Substantially higher mole percentages of PS were required on membranes containing saturated phospholipids, i.e., vesicles composed of DMPS and DPPC. On these membranes, a plateau value for the prothrombin-converting activity was obtained at 20 mol % DMPS.

Kinetic analysis of prothrombin activation at different factor Va concentrations provided an explanation for the different PS requirements of prothrombin activation on membranes composed of saturated and nonsaturated phospholipids (Table V). On vesicles containing dioleoylphospholipids, half-maximal rates of prothrombin activation were obtained at factor Va concentrations ($K_{1/2}$) of about 0.1 nM, independent of the mole percentage of DOPS in the membrane. Much higher concentrations of factor Va were required for half-maximal stimulation of prothrombin activation on DMPS/DPPC vesicles, and the $K_{1/2}$ appeared to be a function of the mole percentage of DMPS. At high mole percentages of PS, the $K_{1/2}$ values on DMPS/DPPC and DOPS/DOPC vesicles were of the same order of magnitude whereas reduction of the PS content caused a considerable increase of the $K_{1/2Va}$ observed on DMPS/DPPC vesicles. The $K_{1/2Va}$ for factor Va determined on vesicles containing 5 mol % DMPS in DPPC was about 150-fold higher than the $K_{1/2Va}$ observed on vesicles composed of 5 mol % DOPS in DOPC.

Table V: Assembly and Catalytic Activity of Prothrombinase on Membranes with Unsaturated and Saturated Phospholipids[a]

mol % PS in vesicle	DOPS/DOPC		DMPS/DPPC	
	$K_{1/2Va}$ (nM)	V_{opt}[b]	$K_{1/2Va}$ (nM)	V_{opt}[b]
20	0.12	4.31	0.29	4.23
15	0.09	4.14	0.39	4.27
10	0.13	4.15	1.20	3.48
7.5	0.12	3.95	5.33	3.64
5	0.12	3.89	19.45	3.19

[a] Prothrombin (1 μM) was activated at 45 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/mL ovalbumin, 100 μM phospholipid vesicles, 1 pM Xa, and varying concentrations of factor Va. The phospholipid vesicles were composed of DOPS/DOPC or DMPS/DPPC mixtures with the mole percentages of DOPS or DMPS indicated in the table. Rates of prothrombin activation were determined as described under Experimental Procedures. $K_{1/2}$ and V_{opt} were obtained from double-reciprocal plots of the rates of prothrombin activation versus the factor Va concentration after statistical analysis according to Eisenthal and Cornish-Bowden (1974). [b] Nanomolar prothrombin activated per minute.

The rates of prothrombin activation (V_{opt}) calculated for saturating factor Va concentrations were independent of the phospholipid composition of the membrane. This means that on membranes with a low surface charge, fatty acyl side chain saturation of phospholipids has a considerable influence on the formation of the membrane-bound factor Xa–Va complex (see also Discussion).

DISCUSSION

The prothrombin-converting activity of procoagulant membranes appears to be critically affected by the kind of (phospho)lipids that constitute the membrane. The importance of the polar head group of the phospholipid molecules was recognized long ago (Troup & Reed, 1958) and was afterward related to the ability of membranes to bind the proteins (substrate, enzyme, and protein cofactor) of the different coagulation factor-activating complexes. However, the membrane interior or hydrophobic core of the membrane also appeared to be an important parameter for the procoagulant activity of a membrane. Hydrogenation of fatty acyl side chains of phospholipids greatly reduced their procoagulant activity (Sterzing & Barton, 1973; Tans et al., 1979). This effect appeared to be related to the phase transition of the membranes, and it was established that membranes in the liquid-crystalline state exhibit a higher procoagulant activity than membranes in the gel state. In a later study with purified proteins, Higgins et al. (1985) demonstrated that the decreased prothrombin-converting activity of membranes in the gel state was at least partially due to a prolongation of the time required for the assembly of the prothrombinase complex.

Our studies confirm the observations of Higgins et al. (1985) on the occurrence of lag phases in thrombin generation when prothrombin activation is started with factor Xa or factor Va on phospholipid membranes in the gel state. Such a pre-steady-state interval indicates that membrane fluidity affects the time required for the assembly of the membrane-bound factor Xa–Va complex. On membranes below the phase transition temperature, complex formation is apparently slowed down due to retarded lateral diffusion of factor Xa and factor Va, a process that appears to be an essential step in factor Xa–Va complex formation (Krishnaswamy et al., 1988).

In contrast to Higgins et al. (1985), we observed that membrane fluidity also affected the steady-state rate of prothrombin activation. Membranes in the gel state exhibited steady-state rates of prothrombin activation that were considerably below those observed on liquid-crystalline membranes (Figure 1C). This effect of membrane fluidity on the expression of the catalytic activity of the prothrombinase complex appeared to be related to the phase transition of the membrane phospholipids. Above their phase transition temperatures, the prothrombin-converting activities of DOPS/DOPC, DMPS/DMPC, and DMPS/DPPC vesicles were the same. When the reaction temperatures were lowered from 45 to 0 °C, steady-state rates of prothrombin activation on DOPS/DOPC vesicles gradually decreased with a Q_{10} of 1.6. Arrhenius plots of steady-state rates of prothrombin activation on DMPS/DPPC and DMPS/DMPC vesicles showed clear discontinuities beginning at 40 and 25 °C, respectively, which is close to their respective phase transition temperatures. Below the phase transition temperature, the steady-state rates of prothrombin activation on the DMPS/DPPC and DMPS/DMPC vesicles were considerably lower than those determined on the fluid DOPS/DOPC vesicles.

Kinetic analysis showed that the K_m for prothrombin and the $K_{1/2Va}$ for Xa–Va complex formation were hardly affected by the phase transition of membrane phospholipids (Table II). The reduced steady-state rates of prothrombin activation appeared to be due to an effect of membrane fluidity on the V_{max} of prothrombin activation. Since the kinetic parameters were determined at a factor Va concentration $\gg K_{1/2Va}$ for Xa–Va complex formation, it can be assumed that all factor Xa present was complexed with factor Va and participated in prothrombin activation. This allows the calculation of k_{cat} values from the experimentally determined V_{max}. From the kinetic data obtained on DOPS/DOPC and DMPS/DPPC vesicles at 15 °C, it can be calculated that the k_{cat} for prothrombin activation drops from 14 s^{-1} on membranes in the liquid-crystalline state to 1.5 s^{-1} on membranes in the gel state.

Experiments with prethrombin 1 showed that the phase transition did not affect prothrombinase-catalyzed activation of this substrate. This indicates that the phase-dependent decrease of the k_{cat} of prothrombin activation is not due to an effect of membrane fluidity on the intrinsic catalytic activity of the prothrombinase complex. However, prethrombin 1 is a prothrombin derivative that does not bind to phospholipid and that will react with the prothrombinase complex directly from solution. In this respect, it differs from prothrombin which, due to its phospholipid binding properties, may also have an interaction with the membrane surface that contributes to prothrombin–prothrombinase complex formation. On the basis of these observations, we propose that membrane fluidity affects the phospholipid-dependent interaction between prothrombin and the factor Xa–Va complex (see below).

The effect of membrane fluidity on the activity of the prothrombinase complex may be related to the observed shift in the product generation pattern during prothrombin activation on membranes in the gel phase. Prothrombin conversion into thrombin requires the cleavage of two peptide bonds and, depending on the order of bond cleavage, either meizothrombin or prethrombin 2 is formed as intermediary product. Analysis of product generation during prothrombin activation by the complete prothrombinase complex (factor Xa, factor Va, Ca^{2+}, and phospholipid) on fluid membranes showed that thrombin and meizothrombin were the only detectable reaction products, which confirms earlier observations (Rosing et al., 1986; Krishnaswamy et al., 1986). However, on membranes in the gel phase, there is considerable reduction of meizothrombin formation, and prethrombin 2 occurs as reaction product (Figure 3), indicating that membrane fluidity has an effect on the peptide bond cleavage and product generation patterns during prothrombin activation.

These data suggest, but do not prove, that membrane fluidity affects the pathway of prothrombin activation in such a way that thrombin formation on fluid membranes occurs via meizothrombin and on liquid-crystalline membranes via prethrombin 2 as the reaction intermediate. It should be emphasized, however, that definite conclusions with respect to enzyme-bound reaction intermediates cannot be drawn from steady-state kinetic studies like those reported in the present paper [cf. Rosing and Tans (1988a)]. This would require a combination of a more detailed steady-state and pre-steady-state approach.

The effects of membrane fluidity on the catalytic activity of prothrombinase and on the product generation pattern may have a common cause. It is likely that one of the functions of membranes in prothrombin activation is to properly juxtapose the enzyme (factor XaVa) and substrate (prothrombin) for efficient catalysis (Husten et al., 1987). It is tempting to speculate that membrane fluidity may affect the juxtaposition of the proteins of the prothrombinase complex in such a way that there is a less favorable orientation for catalysis on membranes in the gel phase. The changes in the product generation pattern may also result from a different positioning of the active site of the prothrombinase complex relative to the peptide bonds that have to be cleaved in prothrombin.

We have also determined the effect of fatty acyl side chain saturation in fluid membranes by comparing the prothrombin-converting activities of vesicles composed of saturated or unsaturated phospholipids above the phase transition temperature. The prothrombin-converting activity of liquid-crystalline membranes containing 20 mol % PS was hardly affected by the saturation of the fatty acyl side chains of the phospholipids. However, greatly reduced rates of prothrombin activation were observed on membranes that contained a low mole percentage of PS and that were composed of phospholipids with saturated fatty acyl side chains (Table IV, Figure 4). The low prothrombin-converting activities of such membranes appeared to be due to a much higher factor Va requirement for full incorporation of factor Xa in the ternary Xa·Va·PS/PC complex (Table V). This indicates that on membranes with a low surface charge (i.e., low mole percentage of PS) the equilibrium constants for factor Xa–Va complex formation are less favorable for membranes containing saturated phospholipids. Formation of a membrane-bound factor Xa–Va complex involves three distinct steps: (1) binding of factor Xa and (2) factor Va to distinct membrane sites; (3) diffusion of membrane-bound factors Xa and Va and subsequent prothrombinase formation (Krishnaswamy et al., 1988). At present, we do not know which of these steps is actually affected by fatty acyl side chain saturation of membrane phospholipids. The fact that diminished factor Xa–Va complex formation in only observed on saturated membranes with a low surface charge points, however, in the direction of factor Va. Binding of factor Va may involve electrostatic (van der Waart et al., 1983; Pusey et al., 1982; Pusey & Nelsestuen, 1984) and hydrophobic interactions (Bloom et al., 1979; Krishnaswamy & Mann, 1988) and penetration of factor Va into nonpolar membrane regions (Lecompte et al., 1987; Krieg et al., 1987). Membrane

penetration and hydrophobic interactions may be prevented on membranes composed of phospholipids with saturated fatty side chains in which the phospholipid molecules are more densely packed than in membranes composed of phospholipids with fatty acyl side chains that contain one or more cis double bonds (Ladbrooke & Chapman, 1969; Demel et al., 1972). This may result in a decreased affinity of factor Va for membranes composed of saturated phospholipids. The fact that the decreased affinity for factor Va was much less pronounced on membranes with higher mole percentages of PS (Table V) may result from an increased contribution of electrostatic interactions to the overall binding equilibrium. This hypothesis can be tested by measuring the effect of fatty acyl side chain saturation on the individual steps required for prothrombinase assembly (protein–membrane association and dissociation steps and prothrombinase formation from membrane-bound constituents).

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