Biochem. J. (1990) 265, 327–336 (Printed in Great Britain)

The contributions of Ca^{2+}, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation Factor X by activated Factor VII

Victor J. J. BOM* and Rogier M. BERTINA†
Haemostasis and Thrombosis Research Unit, University Hospital Leiden, Building 1, C2-R, P.O. Box 9600, 2300 RC Leiden, The Netherlands

In the extrinsic pathway of blood coagulation, Factor X is activated by a complex of tissue factor, factor VII(a) and Ca^{2+} ions. Using purified human coagulation factors and a sensitive spectrophotometric assay for Factor Xa, we could demonstrate activation of Factor X by Factor VIIa in the absence of tissue-factor apoprotein, phospholipids and Ca^{2+}. This finding allowed a kinetic analysis of the contribution of each of the cofactors. Ca^{2+} stimulated the reaction rate 10-fold at an optimum of 6 mM ($V_{max.}$ of 1.1×10^{-3} min^{-1}) mainly by decreasing the K_m of Factor X (to 11.4 μM). In the presence of Ca^{2+}, 25 μM-phospholipid caused a 150-fold decrease of the apparent K_m and a 2-fold increase of the apparent $V_{max.}$ of the reaction; however, both kinetic parameters increased with increasing phospholipid concentration. Tissue-factor apoprotein contributed to the reaction rate mainly by an increase of the $V_{max.}$, in both the presence (40500-fold) and absence (4900-fold) of phospholipid. The formation of a ternary complex of Factor VIIa with tissue-factor apoprotein and phospholipid was responsible for a 15 million-fold increase in the catalytic efficiency of Factor X activation. The presence of Ca^{2+} was absolutely required for the stimulatory effects of phospholipid and apoprotein. The data fit a general model in which the Ca^{2+}-dependent conformation allows Factor VIIa to bind tissue-factor apoprotein and/or a negatively charged phospholipid surface resulting into a decreased intrinsic K_m and an increased $V_{max.}$ for the activation of fluid-phase Factor X.

INTRODUCTION

Human coagulation Factor X is a vitamin K-dependent glycoprotein involved in the intrinsic and extrinsic pathway of blood coagulation. The plasma molecule has a reported M_r of 59000–72000 and consists of a heavy chain (M_r 39000–55000) and a light chain (M_r 16000–19000) connected by a disulphide bridge [1–4]. The protein is the zymogen of the serine-proteinase Factor Xa, which catalyses, in a complex with Factor Va, phospholipid (PL) and Ca^{2+}, the conversion of prothrombin into thrombin [1,5–7].

During activation of the zymogen, a small peptide is cleaved from the *N*-terminal part of the heavy chain [1,3]. In the intrinsic pathway the activation is catalysed by a complex of Factor IXa, Factor VIIIa and Ca^{2+} on a PL surface [8–10]. In the extrinsic pathway, Factor X is activated by a complex of factor VII(a), tissue factor (TFAP associated with PL membranes) and Ca^{2+} [11–18]. Interestingly, the latter complex also contributes indirectly to the activation of Factor X by catalysing the conversion of Factor IX into Factor IXa [18–24].

In general, these key reactions in blood coagulation show a great deal of similarity. Both the enzymes and substrates belong to the class of vitamin K-dependent proteins [6,25]. Furthermore, the enzymes require Ca^{2+}, PL and a protein cofactor for optimal catalytic activity: Factor Va for Factor Xa, Factor VIIIa for Factor IXa and TFAP for Factor VII(a). However, unlike Factors Va and VIIIa, TFAP is an integral membrane protein which does not need to be activated in order to function as a cofactor [14–18]. Also Factor VII is unique among the coagulation factors in having some intrinsic activity; however, upon activation, the activities of human and bovine Factor VII towards their physiological substrates increase about 25-fold [26,27] and 120-fold [28] respectively.

In the past years, detailed kinetic analyses have been reported for the activation of Factor X by Factor IXa and the activation of prothrombin by Factor Xa in both the absence and presence of the non-enzymic cofactors [5–10,29–33]. These studies have contributed much to our insight into the role of the individual components involved in these activation reactions, though several mechanisms have been proposed to explain the contributions of the cofactors under the various conditions, differing in whether the PL-bound or soluble form of substrate and enzyme control the reaction rate. Such detailed analyses have not been reported yet for the activation of Factors IX and X by Factor VIIa. Although several investigators have reported kinetic parameters for the extrinsic activation of Factor X and/or Factor IX in plasma or in a purified system [13,20–24,34–36], the use of crude tissue-factor extracts hampered the analysis

Abbreviations used: PS, L-α-phosphatidyl-L-serine; PC, L-α-phosphatidylcholine; PL, phospholipid; TF(AP), tissue-factor (apoprotein); OVA, ovalbumin; S2337, *N*-benzoyl-L-isoleucyl-L-glutamyl(piperidyl)glycyl-L-arginine *p*-nitroanilide hydrochloride; S2303, *H*-D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide dihydrochloride; TEA, triethanolamine; TEA/NaCl/OVA, 0.05 M-TEA/0.1 M-NaCl/5 μM-OVA, pH 7.45 (37 °C).

* Present address: Division of Haemostasis and Thrombosis, University Hospital Groningen, P.O. Box 30001, 9700 RB Groningen, The Netherlands.

† To whom correspondence and requests for reprints should be sent.

of the individual effects of TFAP and PL in these reactions. Moreover, the radiometric assays used to measure Factor X and/or Factor IX activation [22,37] were in general not sensitive enough to measure factor VII(a) activity in the absence of tissue factor.

In the present study we demonstrate activation of human Factor X by Factor VIIa in the absence of any of the accessory cofactors by using a very sensitive chromogenic assay for Factor Xa. The availability of highly purified human TFAP [18] then allows the systematic kinetic analysis of the contribution of each of the cofactors (Ca^{2+}, PL and TFAP) to the activation of Factor X by Factor VIIa. The study was also designed in such a way that previous findings from our laboratory on the intrinsic activation of Factor X [10,31,38] could be used.

Preliminary results of this investigation were presented at the Tenth International Congress on Haemostasis and Thrombosis, held in San Diego, CA, U.S.A., on 12 July 1985 [39].

EXPERIMENTAL

Materials

DEAE-Sephadex A-50, Sephadex G-75 and G-200, heparin–Sepharose CL-6B, Protein A–Sepharose and CNBr-activated Sepharose 4B are products of Pharmacia Biotechnology, Uppsala, Sweden. Ultrogel AcA 44 was purchased from LKB, Bromma, Sweden. Heparin–Sepharose used for the purification of Factor VII(a) was prepared by coupling standard heparin (Organon, Oss, The Netherlands) to CNBr-activated Sepharose 4B (20 mg/g of activated Sepharose), the instructions of the manufacturers being followed. Bio-Beads SM-2, SDS, Triton X-100, acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, dithiothreitol and Hepes were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Trasylol (aprotinin) and benzamidine hydrochloride were purchased from Bayer, Leverkusen, Germany, and Aldrich Europe, Beerse, Belgium, respectively. OVA (five-times-recrystallized) was obtained from Koch–Light Laboratories, Colnbrook, Slough, Berks., U.K. Bovine serum albumin (grade V), bovine brain L-α-phosphatidyl-L-serine (PS; type P-6641) and egg lecithin L-α-phosphatidylcholine (PC; type P-4139) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. S2337 and S2302 are chromogenic substrates produced by Kabi Vitrum, Stockholm, Sweden. TEA was purchased from Fluka, Buchs, Austria, and *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride was from BDH Chemicals, Poole, Dorset U.K. Other reagents were of analytical grade from Merck, Darmstadt, Germany.

Pl vesicles. PS/PC (50:50, mol/mol) vesicles were prepared by sonication of a dispersion of an equimolar mixture of the purified components in TEA/NaCl as described in detail by Mertens *et al.* [38]. PL concentrations were determined by phosphate analysis after $HClO_4$ combustion [40].

Purified coagulation factors. Protein concentrations were determined as described by Bradford [41], with bovine serum albumin as a standard, and from A_{280}/A_{320} measurements [42], an $A_{280}^{1\%}$ value of 11.6 for Factor X being used [2]. When amounts of coagulation factors are expressed in units, 1 unit refers to the amount of antigen or activity present in 1 ml of pooled normal citrated plasma.

Human Factor VII was purified from citrated plasma to a specific activity of 1750 units of antigen/mg as previously described [43]. The one-chain molecule was quantitatively converted into two-chain Factor VIIa by incubation with purified human Hageman factor fragment β-XIIa [44] (M_r 30000 and 33000 for the unreduced and reduced form respectively) and separated from the activator as previously described [43]. Final preparations of Factor VIIa showed one single band of M_r 53000 before reduction and two bands of M_r 34000 (heavy chain) and M_r 28000 (light chain) after reduction on SDS/polyacrylamide gels; these preparations contained less than 0.01 mol of β-XIIa/mol of VIIa and were stable for more than 1 year when stored at −20 °C in TEA/NaCl containing 50% (v/v) glycerol.

Factor X was isolated as a by-product of Factor VII purification, essentially as described previously [38], except that $CaCl_2$ was replaced by $BaCl_2$ during heparin–Sepharose chromatography; it was dialysed against TEA/NaCl and stored in aliquots at −20 °C. The specific activity of purified Factor X was 190 units/mg as measured by electroimmunoassay as well as spectrophotometrically [3]. Purified Factor X showed one single band of M_r 72000 on non-reducing gels and two bands of M_r 56000 (heavy chain) and 20000 (light chain) on reducing gels. Preparations contained $(1.8–2.2) \times 10^{-5}$ mol of Xa/mol of X.

TFAP was purified from human brain as previously described [18] and stored in aliquots containing 0.02 M-Hepes/0.15 M-NaCl/0.002 M-EDTA/Triton X-100 (0.25 ml/l), pH 7.45 at −20 °C. The preparations showed one discrete band of M_r 47000 or 49000 on SDS/polyacrylamide gels before and after reduction respectively.

Antibodies against Factor VII(a). These were isolated from specific rabbit antisera against human Factor VII by affinity chromatography on Factor VII–Sepharose [43], dialysed against TEA/NaCl and used within 48 h.

Analytical methods

Determination of the initial rate of Factor Xa formation. The rate of activation of Factor X by Factor VIIa in the absence or presence of Ca^{2+}, PS/PC and/or TFAP was studied in TEA/NaCl/OVA at 37 °C. The reaction mixtures were assembled in silicone-treated glass tubes according to the following scheme: (1) PS/PC vesicles were preincubated with 0.0167 M-$CaCl_2$ in TEA/NaCl/OVA in 60% of the final volume for 15 min to form a stable PL surface (cf. [38]); (2) TFAP was added, and recombination with the PS/PC was allowed to proceed for 20 min; (3) buffer and Factor VIIa were added and Factor VIIa was allowed to bind to recombined TF for 10 min; and (4) the reaction was started by addition of Factor X. When not added, PS/PC, Ca^{2+} and protein components were replaced by TEA/NaCl, EDTA (0.001 M) and TEA/NaCl/OVA respectively. Stock solutions of Factor VIIa and Factor X were pre-diluted in TEA/NaCl/OVA before addition to the reaction mixture. Stock solutions of TFAP were pre-diluted in TEA/NaCl/OVA containing 0.25 ml of Triton X-100/l;

because solubilized TFAP was found to be relatively instable when the Triton X-100 concentration fell below its critical micelle concentration of about 0.1 ml/l [45], a further 5-fold dilution in the same buffer without Triton X-100 was made just before addition to the reaction mixture. Under these conditions more than 90% of the TFAP activity could be recovered in the reaction mixture (when compared with the direct addition of TFAP from the stock solution), while simultaneously the Triton X-100 concentration was reduced to 0.005 ml/l. The latter concentration did not interfere with the stable PS/PC surfaces when final concentrations of 10–100 μM-PS/PC were used.

The final incubation volumes varied between 0.25 and 1.1 ml. After different time intervals, samples (0.05–0.25 ml) were drawn off and assayed for the amount of Factor Xa formed, the sample taken at zero time being used as the blank (see below). From the proportional increase of the Factor Xa concentration with time, the initial rate of Factor Xa formation (v^*) was calculated (nM-Xa/min). The maximum rate of Factor Xa formation at saturating concentrations of Factor X, $V_{max.}$, is expressed as mean catalytic-centre activity (mol of Xa/min per mol of VIIa). During the measuring period, less than 2% of the Factor X was converted into Factor Xa. For the construction of Lineweaver–Burk and Woolf plots, mean values of v^* were calculated from the data of at least three independent experiments, of which the inter-assay coefficients of variation varied between 0.5 and 28.3%. The K_m and $V_{max.}$ were determined by weighted linear-regression analysis of s/v^* versus s (s being the substrate concentration) and lines were drawn accordingly.

Spectrophotometric assay of Factor Xa. Samples of up to 0.25 ml were transferred to a glass tube containing 0.4 ml of 0.125 M-TEA/0.250 M-NaCl/0.05 M-EDTA/25 μM-OVA, pH 8.2 at 37 °C. The volume was adjusted to 0.9 ml with TEA/NaCl and the chromogenic assay started by addition of 0.1 ml of prewarmed 0.002 M-S2337. Depending on the rate of colour development, the reaction was stopped between 30 and 120 min afterwards by addition of 0.05 ml of 0.5 M-benzamidine/HCl, pH 8, and the A_{405} measured against the reaction blank (spectrophotometer type PM6; Zeiss, Oberkochen, Germany). The assay was calibrated with known amounts of active-site-titrated Factor Xa and the increase of A_{405}/min per nM-Xa was found to be 0.025. The lower limit of detection of this assay was 1 fmol of Xa.

Other methods. Molar concentrations of Factor X and Factor Xa were determined by use of reference preparations that were active-site-titrated with *p*-nitrophenyl *p*-guanidinobenzoate as described previously [31,38]. Molar concentrations of the Factors VIIa, β-XIIa and TFAP were derived from the protein concentrations and the minimal M_r observed (see under 'Purified coagulation factors' above). Factor VII procoagulant activity and antigen as well as TFAP activity were measured with previously described assays [18,43]. Human β-XIIa activity was measured by a chromogenic assay using S2302 as described by Tankersley *et al.* [44]. SDS/polyacrylamide-gel electrophoresis under the conditions described by Weber & Osborn [46] was performed as described in [3,18]; the gels contained 100 g of polyacrylamide/litre, except for the analysis of TFAP, where 75 g of polyacrylamide/litre was used.

RESULTS

Activation of Factor X by Factor VIIa in the absence and presence of Ca^{2+} ions

Using a sensitive and specific spectrophotometric assay of Factor Xa we observed activation of Factor X by Factor VIIa in the absence of any cofactor, as shown in Fig. 1. At 1.0 μM-Factor X and 6.9 nM-Factor VIIa, the initial rate of Factor Xa formation, v^*, was 5.7×10^{-5} nM-Xa/min. Ca^{2+} enhanced the reaction rate 10-fold at the optimum concentration of 6 mM and gave inhibition at higher concentrations (Fig. 1). In both the absence and presence of Ca^{2+}, v^* was constant with time and proportional to the concentration of Factor VIIa, as illustrated in Figs. 2(*a*) and 2(*b*). That indeed Factor VIIa was responsible for the observed Factor Xa formation was confirmed by the observation that both reactions could be inhibited by immunopurified antibodies against factor VII(a): 94% inhibition in the presence of Ca^{2+}, 87% inhibition in the absence of Ca^{2+}. These findings allowed the further kinetic analysis of the activation of Factor X by Factor VIIa in solution. Fig. 2(*c*) shows that, in the absence of Ca^{2+}, v^* was proportional to the concentration of Factor X up to the highest concentration tested (20 μM). Hence, the K_m and $V_{max.}$ could not be determined, but they must be considerably higher than 20 μM and 1.5×10^{-4} mol of Xa/min per mol of VIIa respectively. In the presence of 6 mM-$CaCl_2$, v^* clearly showed the saturation phenomenon at increasing concentrations of Factor X. The Lineweaver–Burk plot is shown as inset in Fig 2(*c*) and the K_m and $V_{max.}$ were

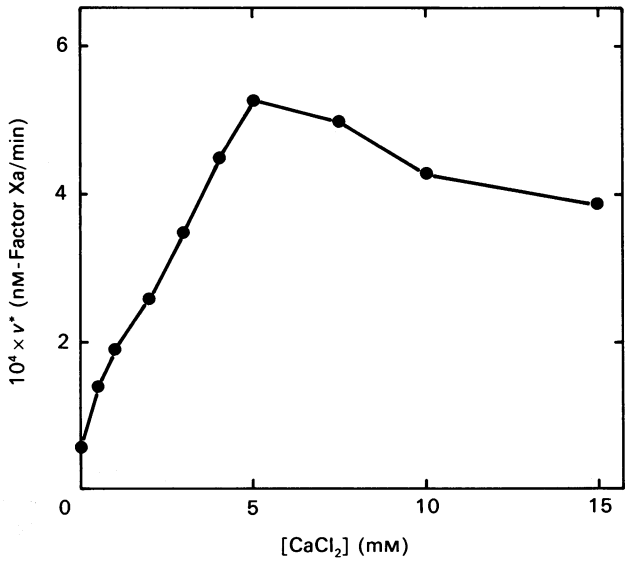

Fig. 1. Effect of Ca^{2+} on the rate of Factor X activation by Factor VIIa

Factor VIIa (6.9 nM) and Factor X (1.0 μM) were incubated in TEA/NaCl/OVA containing various amounts of $CaCl_2$; at 0 mM-$CaCl_2$, 1 mM-EDTA was present in the reaction mixture. Initial rates of Factor X activation (v^*) were calculated from the Factor Xa concentrations measured after 0, 60 and 120 min of incubation. Details are as described in the Experimental section.

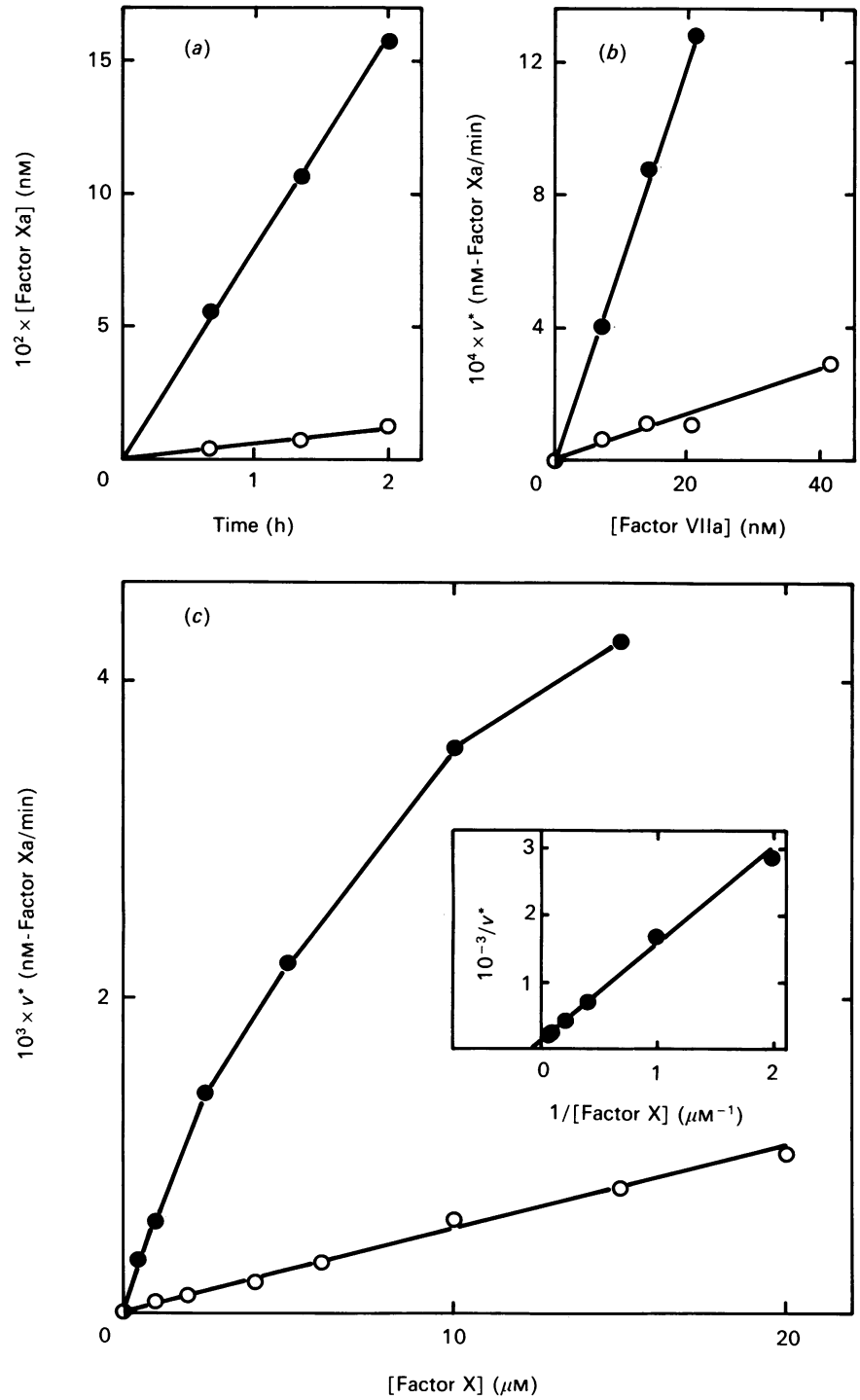

Fig. 2. Activation of Factor X by Factor VIIa in the absence or presence of Ca^{2+}

Factor VIIa and Factor X were incubated in TEA/NaCl/OVA in the presence of either 1 mM-EDTA (○) or 6 mM-$CaCl_2$ (●). Initial rates of Factor X activation (v^*) were calculated from the Factor Xa concentrations measured after 0, 40, 80 and 120 min of incubation, and the mean result for two to three experiments was plotted. Details are as described in the Experimental section. (*a*) Time course of the activation of 1.0 μM-Factor X by 20.7 nM-Factor VIIa; (*b*) Factor VIIa-dependence of v^* at 1.0 μM-Factor X; (*c*) Factor X-dependence of v^* at 6.9 nM-Factor VIIa; the inset shows a double-reciprocal plot of the data obtained in the presence of Ca^{2+}.

estimated to be 11.4 μM and 1.1×10^{-3} mol of Xa/min per mol of VIIa respectively.

Activation of Factor X by Factor VIIa in the presence of Ca^{2+} and PL. Initial rates of Factor Xa formation were studied at 10 mM-$CaCl_2$ and 10, 25 and 100 μM-PS/PC. The PS/PC vesicles were preincubated with $CaCl_2$ to obtain a stable PL surface [38] before Factor VIIa and subsequently Factor X were added. Under these conditions we obtained linear time courses of Factor Xa

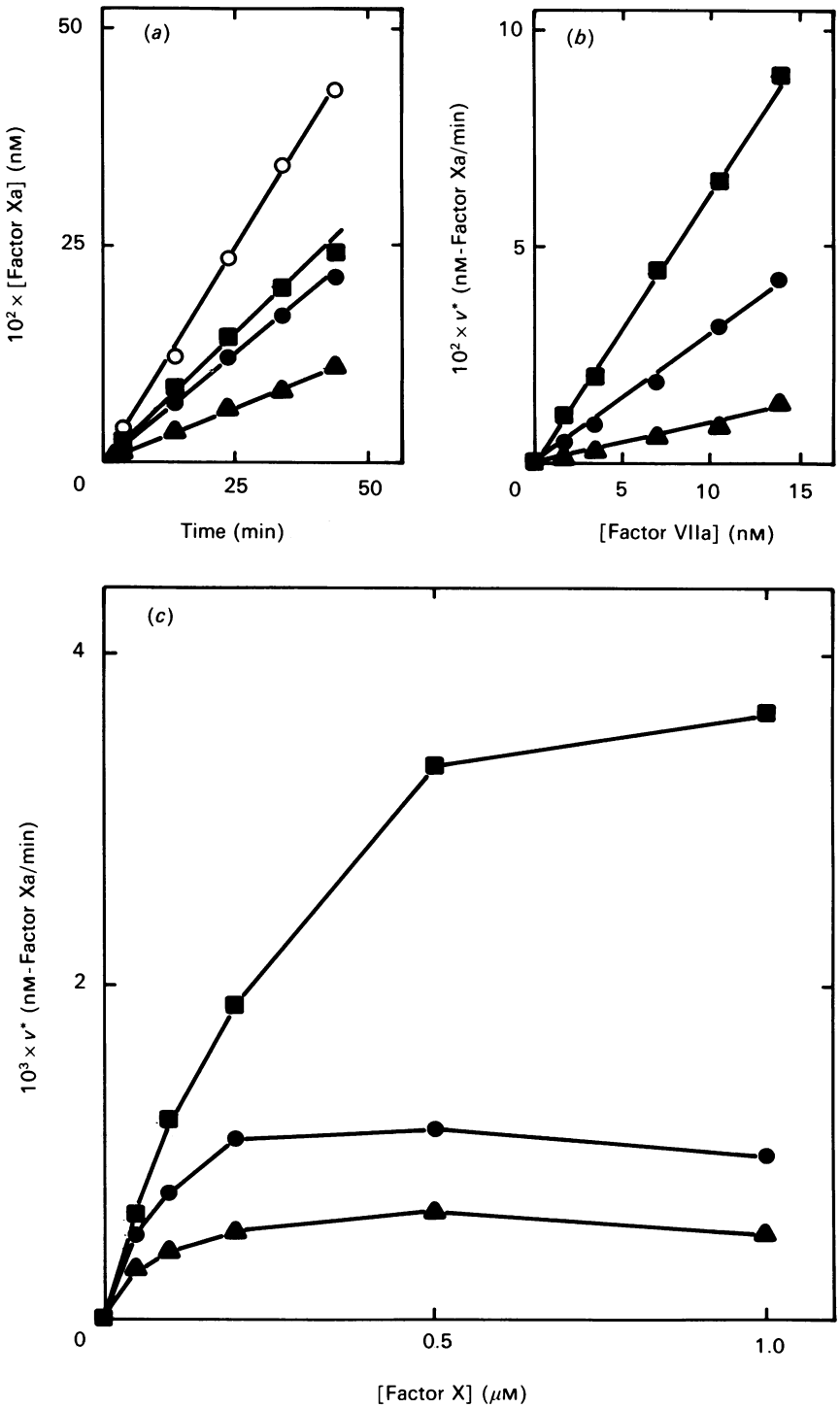

Fig. 3. Activation of Factor X by Factor VIIa in the presence of Ca^{2+} and PL

Factor VIIa and Factor X were incubated in TEA/NaCl/OVA containing 10 mM-$CaCl_2$ and different concentrations of PS/PC vesicles. Initial rates of Factor X activation (v^*) were calculated from the linear increase in Factor Xa with time measured at three to six time intervals between 0 and 45 min of incubation. Details are as described in the Experimental section. (*a*) Time course of Factor Xa formation: 6.9 nM-Factor VIIa was incubated with 0.05 μM-Factor X at 10 (▲), 25 (●) on 100 (■) μM-PS/PC and with 0.5 μM-Factor X at 25 μM-PS/PC (○). (*b*) Factor VIIa-dependence of v^* at 100 μM-PS/PC and 0.05 (▲), 0.2 (●) and 1.0 (■) μM-Factor X. (*c*) Factor X-dependence of v^* at 6.9 nM-Factor VIIa and 10 (▲), 25 (●) and 100 (■) μM-PS/PC.

formation as illustrated in Fig. 3(*a*). Also, v^* was found to be proportional to the concentration of Factor VIIa (Fig. 3*b*). At 1.0 μM-Factor X, the addition of PS/PC vesicles enhances the rate of Factor VIIa-dependent Factor X activation more than a 100-fold (compare Figs. 3*b* and 2*b*). This stimulatory effect of PS/PC is completely dependent on the presence of Ca^{2+} (result not shown). Fig. 3(*c*) shows the dependence of v^* on the concentration of Factor X at the three PS/PC concentrations: v^* increases with increasing Factor X concentration until a

plateau is reached; at still higher concentrations of Factor X, substrate inhibition was observed at the lower PS/PC concentrations. From Fig. 3(*c*) it is also evident that, at constant Factor X concentration, v^* increases with increasing concentrations of PS/PC. The kinetic parameters for the activation reaction at each PS/PC concentration were calculated from the data of the hyperbolic part of the curves. At 10, 25 and 100 μM-PS/PC, the apparent K_m was found to be 0.059, 0.077 and 0.29 μM-Factor X respectively; the $V_{max.}$ of the reaction increased from 1.0×10^{-3} to 7.1×10^{-3} mol of Xa/min per mol of VIIa. The results demonstrate that PS/PC vesicles in the presence of Ca^{2+} exert their stimulatory effect on the activation of Factor X by Factor VIIa, primarily by a decrease of the apparent K_m of Factor X, but also by an increase of the catalytic-centre activity of Factor VIIa.

Activation of Factor X by Factor VIIa in the presence of Ca^{2+}, PL and TFAP

Preliminary observations showed that addition of TFAP to a system of Factor VIIa and Factor X containing Ca^{2+} and PS/PC could enhance the rate of Factor Xa formation by four orders of magnitude. The stimulation was found to be completely dependent on the presence of Ca^{2+}. Under our experimental conditions (see the Experimental section), recombination of TFAP with the stable Ca^{2+}–PS/PC surface was a relatively fast process, i.e. at 25 μM-PS/PC and 0.2 nM-TFAP the v^* was found to be half-maximal after a recombination period of 2 min. Also, the formation of TF–factor VIIa complexes was rapidly completed, i.e. within 1–10 min after the addition of Factor VIIa, depending on the concentration of Factor VIIa (0.005–0.6 nM). The catalytic complexes formed were stable for at least 20 min. After addition of Factor X, the formation of Factor Xa was found to be linear with time without a measurable lag phase (result not shown); v^* was calculated from the linear time courses.

To characterize the extrinsic Factor X activator in detail, v^* was determined at a fixed concentration of Ca^{2+} (10 mM) and various amounts of TFAP, Factor VIIa PS/PC and Factor X. The results are shown in Fig. 4. The titration curves with TFAP (Fig. 4*a*, showing five of the ten curves) show that Factor VIIa can be saturated with TFAP and that, with increasing concentrations of Factor VIIa (2.5–600 pM), more TFAP is needed to saturate the activator. The titration curves with Factor VIIa (Fig. 4*b*, showing three of the six curves) also demonstrate saturation. The saturation curves indicate that a stoichiometric complex of Factor VIIa and TFAP is formed that is responsible for the observed rate of Factor X activation. The stoichiometry of complex-formation under our assay conditions (25 μM-PS/PC and 50 nM-Factor X) was estimated in the following way: (1) at each concentration of TFAP, v^* at infinite Factor VIIa concentration was determined as an indicator of the amount of TFAP saturated with Factor VIIa; this maximum v^* was found to be linearly related to the amount of added TFAP, and from the slope of the curve (not shown) an apparent catalytic-centre activity of 4.0 mol of Xa/min per mol of TFAP was calculated; (2) at each concentration of Factor VIIa, v^* at infinite TFAP concentration was determined as an indicator of the amount of saturated Factor VIIa; the maximum v^* was found to be linearly related to the amount of added Factor VIIa, and from the slope of the curve (not shown) an apparent catalytic-centre activity of 24.5 mol of Xa/min per mol of VIIa was calculated; and (3) from the combined data we have calculated that the apparent stoichiometry of TFAP/Factor VIIa for the formation of the catalytic complex was 6.1:1 (mol/mol).

Fig. 4(*c*) shows that the PL concentration can also be rate-limiting in the extrinsic activation of Factor X, a finding which is in agreement with the formation of a Factor VIIa–TFAP–PS/PC ternary complex in the presence of Ca^{2+}. At 0.4 nM-TFAP and 0.05 nM-Factor VIIa, v^* was proportional to the concentration of PS/PC up to 1.0 μM (see the top curve). With increasing concentrations of PS/PC, a plateau was reached, followed by a decrease in v^* at the higher PS/PC concentrations. A similar curve was obtained when the concentrations of Factor VIIa and Factor X were varied (Fig. 4*c*; lower curve); however, at the higher Factor X concentration, less inhibition of v^* was observed at increasing concentrations of PS/PC (25–150 μM). An interesting observation was that, without added PS/PC vesicles, v^* was only 100-fold less than at the optimum concentration of PL; this finding is analysed in more detail below.

To determine the kinetic parameters for the activation of Factor X by the Factor VIIa–TFAP–PS/PC complex, v^* was measured at different Factor X concentrations under conditions where the enzyme was saturated with TFAP and PS/PC. The data are presented in a double-reciprocal form in Fig. 4(*d*). The apparent K_m and $V_{max.}$ were calculated to be 0.055 μM-Factor X and 81 mol of Xa/min per mol of VIIa respectively. We did not observe substrate inhibition, even at 2 μM-Factor X. From a comparison of these data with those observed in the absence of TFAP (see Fig. 3*c* and the above subsection) we conclude that the stimulatory effect of TFAP in the presence of PS/PC and Ca^{2+} is almost completely due to the 40500-fold increase in the apparent catalytic-centre activity of Factor VIIa.

Activation of Factor X by Factor VIIa in the presence of Ca^{2+} and TFAP

TFAP was found to enhance the rate of activation of Factor X by Factor VIIa even in the absence of PS/PC vesicles (see the subsection above). This reaction was studied at fixed concentrations of Ca^{2+} (10 mM) and Triton X-100 (0.005 ml/l). Under these conditions a linear time course of Factor Xa formation for at least 30 min was obtained at different concentrations of Factor X (see Fig. 5*a*). However, with some of the TFAP preparations we observed that the rates of Factor Xa formation increased with time during the first 10–20 min, particularly at low concentrations of Factor X. As no obvious explanation was available for this phenomenon, such preparations were not used for initial-rate measurements. At 1.0 μM-Factor X and 0.025 nM-Factor VIIa, the addition of 0.4 nM-TFAP enhanced v^* more than 30000-fold (compare Figs. 2*a* and 5*a*). This stimulatory effect of TFAP was completely dependent on the presence of Ca^{2+}. When Factor VIIa (0.025 nM) was titrated with TFAP, saturation was observed at 0.4 nM-TFAP (result not shown), nearly the same concentration as in the presence of PS/PC (cf. Fig. 4*c*). These results suggest the formation of a stoichiometric Factor VIIa–TFAP–Ca^{2+} complex in the absence of PL, which is in accordance with previously published data [16–18]. The double-reciprocal plot of v^* against Factor X concentration is shown in Fig. 5(*b*), and the apparent K_m and $V_{max.}$ of the

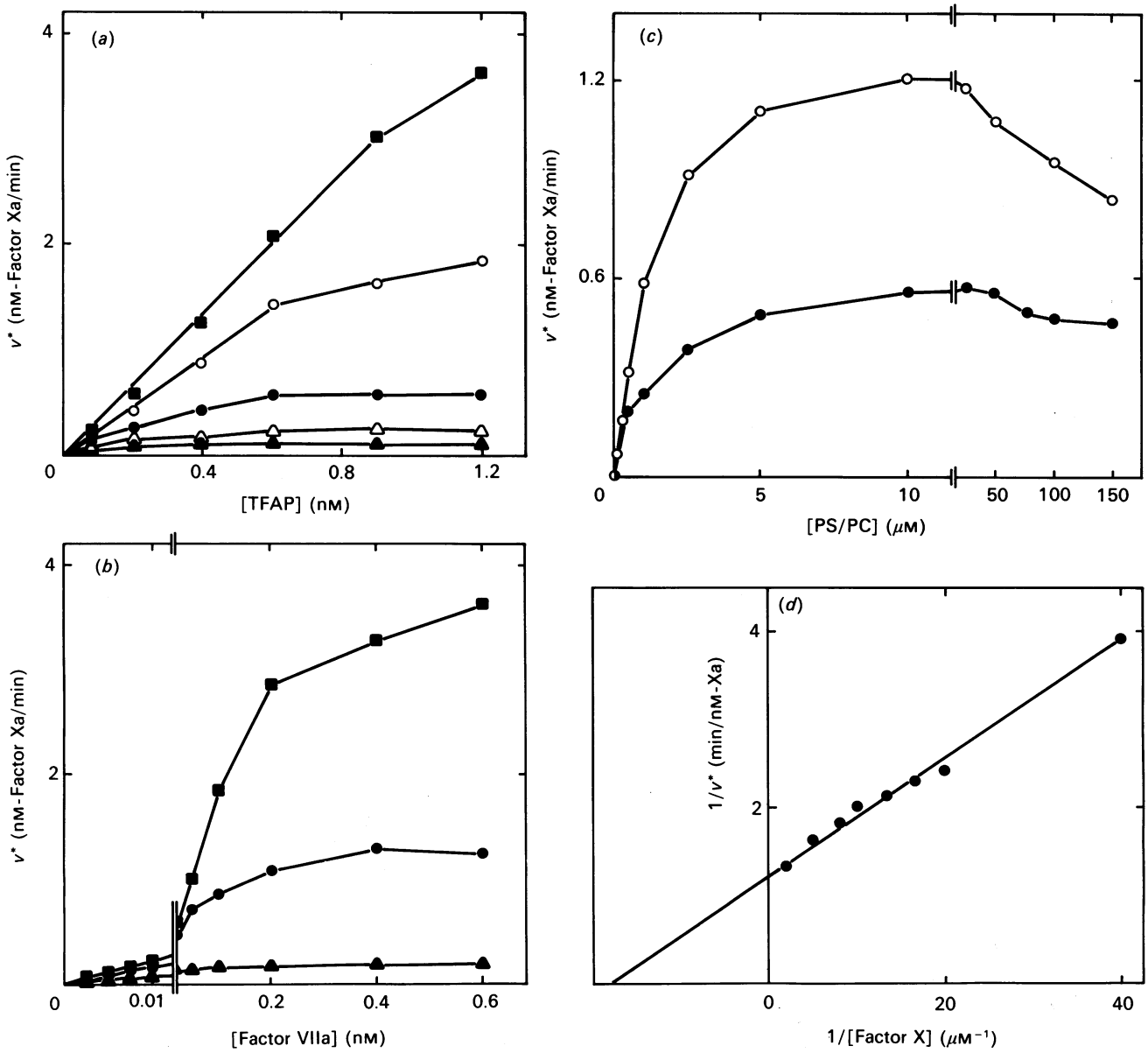

Fig. 4. Activation of Factor X by Factor VIIa in the presence of Ca^{2+}, PL and TFAP

Factor VIIa and Factor X were incubated in TEA/NaCl/OVA containing 10 mM-$CaCl_2$ and various amounts of PS/PC and TFAP. Initial rates of Factor X activation (v^*) were calculated from the linear increase in Factor Xa with time measured at three to five time intervals between 0 and 5 min of incubation. Details are as described in the Experimental section. (*a*) TFAP-dependence of v^*: reaction mixtures contained 25 μM-PS/PC, 50 nM-factor X and 5 (▲), 10 (△), 25 (●), 100 (○) and 600 (■) pM-Factor VIIa. (*b*) Factor VIIa-dependence of v^*: reaction mixtures contained 25 μM-PS/PC, 50 nM-Factor X and 0.08 (▲), 0.4 (●) and 1.2 (■) nM-TFAP. (*c*) PS/PC-dependence of v^*: reaction mixtures contained 0.4 nM-TFAP and either 50 pM-Factor VIIa and 50 nM-Factor X (○) or 10 pM-Factor VIIa and 200 nM-Factor X (●). (*d*) Factor X-dependence of v^* in a double-reciprocal plot: reaction mixtures contained 25 μM-PS/PC, 0.6 nM-TFAP and 10 pM-Factor VIIa.

catalytic complex were calculated to be 1.7 μM-Factor X and 5.4 mol of Xa/min per mol of VIIa respectively. The kinetic parameters indicate that the stimulatory effect of the apoprotein on the activation of Factor X by Factor VIIa in the presence of Ca^{2+} is exerted by a large increase in the catalytic-centre activity of Factor VIIa (4900-fold) and a relatively small decrease in the apparent K_m of Factor X (6-fold).

DISCUSSION

In the present study we analysed the activation of Factor X by Factor VIIa in the absence or presence of the individual cofactors Ca^{2+}, PL and TFAP in a system of highly purified human coagulation factors. The study was made possible by the availability of a sensitive and specific chromogenic end-point assay for Factor Xa. The detection limit of 1 fmol of Xa makes the assay slightly more sensitive than a kinetic variant (detection limit of 2.3 fmol of Xa) previously reported from our laboratory [38] and one or two orders of magnitude more sensitive than the commonly used radiometric assays [22,37].

Activation of Factor X occurs by cleavage of the Arg^{52}–Ile^{53} peptide bond in the heavy chain of the

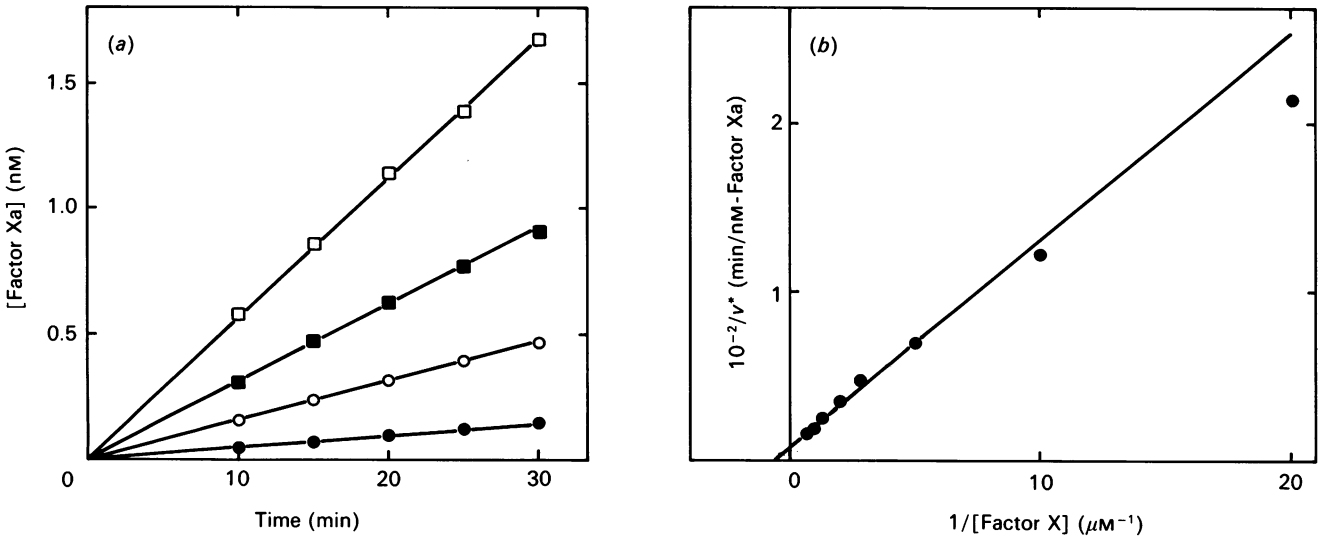

Fig. 5. Activation of Factor X by Factor VIIa in the presence of Ca^{2+} and TFAP

Factor VIIa (25 pM) and various amounts of Factor X were incubated in TEA/NaCl/OVA containing 10 mM-$CaCl_2$ and 0.4 nM-TFAP. Initial rates of Factor X activation (v^*) were calculated from the linear increase of Factor Xa with time measured at four to six time intervals between 0 and 30 min of incubation. Details are given in the Experimental section. (*a*) Time course of the activation of 0.05 (●), 0.2 (○), 0.5 (■) and 1.0 (□) μM-Factor X. (*b*) Factor X-dependence of v^* in a double-reciprocal plot.

zymogen [1–4]. In the presence of Ca^{2+} and PL, the Factor Xa formed (Factor Xaα) catalyses (*a*) the cleavage of an M_r-4000 peptide from the *C*-terminal region of the heavy chain of Factor Xaα, resulting in the formation of Factor Xaβ, without any effect on the catalytic properties of the enzyme, and (*b*) the cleavage of an M_r-13000 peptide from the *C*-terminal region of the heavy chain of Factor X, resulting in the loss of the active-site serine residue from the Factor X molecule [3]. However, under the conditions (time scale, fraction and concentration of the Factor Xa formed) used in the present study, feedback inactivation of Factor X is negligible (cf. [3]).

In the present study we demonstrate activation of Factor X by Factor VIIa alone and stimulation of the reaction by Ca^{2+}, PS/PC and TFAP. This implies that tissue factor is not an obligatory cofactor for Factor VIIa-dependent activation of factor X *per se*, as has been suggested by other investigators [15,35]. The activity towards one of its physiological substrates confirms the enzymic nature of Factor VIIa, which is further indicated by its reactivity towards small chromogenic substrates [47], di-isopropyl fluorophosphate [26,48] and the serine-proteinase inhibitor antithrombin III [26].

Our findings lead to the following model for the activation of Factor X by Factor VIIa: Factor VIIa is enzymically active towards Factor X and this reaction is stimulated by the cofactors Ca^{2+}, PL and TFAP. Such a model also applies to Factor IXa in the intrinsic activation of Factor X (cofactors Ca^{2+}, PL and Factor VIIIa) [8–10,31], Factor Xa in the activation of prothrombin (cofactors Ca^{2+}, PL and factor Va) [1,7,29,32], thrombin in the activation of protein C (cofactors Ca^{2+}, PL and thrombomodulin) [49,50] and activated protein C in the inactivation of Factor VIIIa and Factor Va (cofactors Ca^{2+}, PL and protein S) [51]. Such an analogy in the mode of action of these serine proteinases is supported by the high degree of sequence similarity between the vitamin K-dependent coagulation factors as deduced from cDNA sequence studies [52].

Table 1 summarizes the effects of the various cofactors on the kinetic parameters of the activation of Factor X by Factor VIIa. In the subsections below we briefly discuss these effects. At the same time we try to accommodate the data in a mechanistic model for extrinsic Factor X activation.

Effects of Ca^{2+}

In the absence of PL and TFAP the stimulatory effect of Ca^{2+} on the reaction rate (10-fold at optimum Ca^{2+} concentration) seems to be due mainly to a decrease in the K_m of Factor X (see Figs. 1 and 2). In comparison, the activation of Factor X by Factor IXa is stimulated 7-fold at optimal Ca^{2+} concentrations, both in the human [31] and in the bovine [9] system.

The most important feature of Ca^{2+}, however, is that it is absolutely required for the functional activity of the other two cofactors (TFAP and PS/PC). Several lines of evidence indicate that Ca^{2+} exerts this effect by the introduction of specific conformational changes in Factor VIIa and Factor X that are essential for the interaction

Table 1. Effect of cofactors on the kinetic parameters of the activation of Factor X by Factor VIIa

Cofactor(s)	K_m (μM)	$V_{max.}$ (mol of Xa/min per mol of VIIa)
–	> 20.0	$> 1.5 \times 10^{-4}$
Ca^{2+}	11.4	1.1×10^{-3}
Ca^{2+} + PS/PC	0.077	2.0×10^{-3}
Ca^{2+} + TFAP	1.7	5.4
Ca^{2+} + PS/PC + TFAP	0.055	81

of these proteins with PL membranes and for binding of Factor VII(a) to TFAP [6,18,25,43,53,54].

Effects of PS/PC

The addition of PS/PC (25 μM) stimulates the activation of Factor X by Factor VIIa, in both the absence and presence of TFAP (see Figs. 3*c* and 4*c*). In the absence of TFAP the stimulation is mainly due to a 148-fold decrease in the apparent K_m of Factor X, although there is also a significant increase in the apparent V_{max} of the reaction. Interestingly, with increasing PS/PC concentrations, both the apparent $V_{max.}$ and the apparent K_m of Factor X increase. Very similar observations have been reported for the effects of PS/PC on the activation of Factor X by Factor IXa [9,31] and of prothrombin by Factor Xa [29,33]. In general, this phenomenon has been explained by a model in which enzyme and substrate are concentrated on the PL surface [9,29], thus favouring the formation of enzyme–substrate complexes. However, this model does not always fit the experimental data [31,33].

In an attempt to clarify the mechanism underlying the stimulatory effect of PS/PC on the activation of Factor X by Factor VIIa, we estimated the concentrations of free and bound Factor VIIa and Factor X under the experimental conditions of the rate determinations (see Fig. 3*c*) using a value of 0.002 mol of Factor X/VIIa-binding sites per mol of PS/PC [38], a K_d of 0.028 μM for the binding of Factor X to PS/PC [38] and a K_d of 9 μM for the binding of Factor VIIa to PS/PC [25,55; V. J. J. Bom & R. M. Bertina, unpublished work]. Re-analysis of the kinetic data of Fig. 3(*c*) then suggests that the stimulatory effect of PS/PC is due to activation of free Factor X by PS/PC-bound Factor VIIa. In agreement with such a model is that the calculated K_m of free Factor X is virtually independent of the actual PS/PC concentration used (0.040–0.046 μM). A remarkable consequence of this model would be that, in the presence of Ca^{2+}, binding of Factor VIIa to the PS/PC surface induces a conformational change in the enzyme which is associated not only with a 270-fold decrease in the apparent K_m of free Factor X, but also with a 40000-fold increase in the catalytic-centre activity (to about 40 mol of Xa/min per mol of VIIa). This model would also explain the substrate inhibition that is observed at high Factor X concentrations and fixed concentrations of Factor VIIa and PS/PC (Fig. 3*c*), by competition between Factor X and Factor VIIa for the limited number of binding sites on the PS/PC surface.

In the presence of TFAP, the addition of PS/PC (25 μM) caused a 460-fold increase in the catalytic efficiency of the activation of Factor X by Factor VIIa. Apparently the integration of TFAP in the PL membrane is associated with both a 16-fold increase in $V_{max.}$ and a 30-fold decrease in the apparent K_m of Factor X (influence of negatively charged PL) (cf. [36]).

Effects of TFAP

The addition of TFAP to a system consisting of Factor VIIa, Factor X and Ca^{2+} results in a 4900-fold increase in the $V_{max.}$ of Factor Xa formation and a 6.7-fold decrease in the K_m of Factor X. Our kinetic analysis was performed at a fixed concentration of Triton X-100 (0.005 ml/l) far below the critical micelle concentration of about 0.1 ml/l [45]. Though TFAP itself is not stable at this concentration of Triton X-100, a stable Factor VIIa–TFAP complex was formed after the addition of Factor VIIa in the presence of Ca^{2+} (see Fig. 5*a*). The observed K_m of Factor X (1.7 μM) is rather close to the value of 1.06 μM reported by Guha *et al.* [17] in the presence of a considerably higher Triton X-100 concentration (1.0 ml/l). The observed saturation of Factor VIIa with TFAP supports previous findings [16–18] and suggests that the formation of a stoichiometric complex of Factor VIIa and TFAP in the absence of PL is responsible for a 32900-fold increase in the catalytic efficiency of the activation of Factor X by Factor VIIa.

In the presence of PS/PC, the addition of TFAP results in a 56700-fold increase in the catalytic efficiency of Factor X conversion. This is rather close to the 31500-fold increase observed in a bovine system when TF replaced PL [13]. The stimulatory effect of human TFAP in the presence of PS/PC is almost exclusively due to the 40500-fold increase in the apparent $V_{max.}$ of the reaction. This increase correlates well with the 1000–100000-fold increase in the affinity of Factor VIIa for binding to TFAP-containing PL membranes when compared with 'plain' PL membranes [24,35,56]. This suggests that formation of a Factor VIIa–TFAP–PS/PC ternary complex is responsible for the increase in the Factor X activation rate. In agreement with such an hypothesis is that the catalytic complex can be saturated with each of the individual components (Fig. 4). The apparent stoichiometry of TFAP and Factor VIIa for the formation of this complex is 6.1 mol/mol. As each accessible TFAP molecule can bind one molecule of Factor VII [55], we have to assume that, under our experimental conditions, only a minor fraction of the added TFAP was bound to the PS/PC membranes and/or was accessible for Factor VIIa, which might be due to aggregation of TFAP. Whether such aggregation will affect the catalytic-centre activity of the Factor VIIa–TFAP–PS/PC complex is not known.

Several kinetic studies on the extrinsic activation of Factor X (and Factor IX) by Factor VIIa–TF have been reported in the literature [13,20,24,34–36]. However, most of these studies have been performed with a crude PL-rich TF extract which served as source of both TFAP and PL. For the human system, Warn-Cramer & Bajaj [24] reported an apparent K_m of Factor X of 0.205 μM and a catalytic-centre activity of 70 mol of Xa/min per mol of VIIa. For the bovine system (both purified and plasma systems) a range of values for the apparent K_m of Factor X (0.088–0.433 μM) and for the $V_{max.}$ (132–1920 mol of Xa/min per mol of VIIa) have been reported. Using highly purified bovine TFAP recombined with PS/PC vesicles of different PS content, Nemerson & co-workers reported apparent K_m values of 0.016–0.791 μM-Factor X and an apparent $V_{max.}$ of 240–372 mol of Xa/min per mol of VIIa [35,36]. The same authors [36] provided evidence that the activity of the TF–Factor VIIa complex is regulated by fluid-phase Factor X (intrinsic K_m of 0.063 μM-free Factor X). The observed inhibition of the reaction rate at high concentrations of PS/PC (Fig. 4*c*) or mixed brain PL [57] supports a similar model for the human system. By using the data of Fig. 4(*d*) and the parameters for binding of Factor X to PL membranes as described above, an intrinsic K_m of 0.025 μM free Factor X could be calculated for the human Factor VIIa–TF complex. The lack of substrate inhibition even at very high Factor X concentrations (see the Results section) indicates that, in the presence of TFAP, there is much less competition between Factor X

and Factor VIIa for available PL binding sites than in the absence of TFAP. Thus, in the presence of PS/PC and TFAP, Factor VIIa seems to bind predominantly to TFAP with limited exposure to the PL membrane.

In summary then, the kinetic analysis of the contributions of Ca^{2+}, PS/PC and TFAP to the activation of Factor X by Factor VIIa suggests a model for the extrinsic Factor X activation in which the binding of the Ca^{2+}-dependent conformation of Factor VIIa to TFAP and/or a negatively charged PL surface results in a conformational change of the enzyme associated with a decrease in the intrinsic K_m of fluid-phase Factor X and an increase in the $V_{max.}$. Given the low affinity of Factor VIIa for binding to PL surfaces, TFAP may be considered primarily as an evolutionary design for a hydrophobic structure with a high affinity for Factor VIIa.

We gratefully acknowledge the secretarial assistance of Mrs. M. J. Mentink. These investigations were supported by the Foundation for Medical Research (FUNGO) (grant no. 13-30-51).

REFERENCES

1. Jackson, C. M. (1984) Prog. Haemostasis Thromb. **7**, 55–109
2. Di Scipio, R. G., Hermodson, M. A., Yates, S. G. & Davie, E. W. (1977) Biochemistry **16**, 698–706
3. Mertens, K. & Bertina, R. M. (1980) Biochem. J. **185**, 647–658
4. Leytus, S. P., Chung, D. W., Kisiel, W., Kurachi, K. & Davie, E. W. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3699–3702
5. Jobin, F. & Esnouf, M. P. (1967) Biochem. J. **102**, 666–674
6. Nelsestuen, G. L. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. **37**, 2621–2625
7. Nesheim, M. E., Taswell, J. B. & Mann, K. G. (1979) J. Biol. Chem. **254**, 10952–10962
8. Hultin, M. B. & Nemerson, Y. (1978) Blood **52**, 928–940
9. Van Dieijen, G., Tans, G., Rosing, J. & Hemker, H. C. (1981) J. Biol. Chem. **256**, 3433–3442
10. Mertens, K., Van Wijngaarden, A. & Bertina, R. M. (1985) Thromb. Haemostasis **54**, 654–660
11. Nemerson, Y. (1966) Biochemistry **5**, 601–608
12. Osterud, B., Berre, A., Otnaess, A.-B., Bjorklid, E. & Prydz, H. (1972) Biochemistry **11**, 2853–2857
13. Silverberg, S. A., Nemerson, Y. & Zur, M. (1977) J. Biol. Chem. **252**, 8481–8488
14. Bach, R., Nemerson, Y. & Konigsberg, W. (1981) J. Biol. Chem. **256**, 8324–8331
15. Nemerson, Y. & Bach, R. (1982) Prog. Haemostasis Thromb. **6**, 237–261
16. Broze, G. J., Leykam, J. E., Schwartz, B. D. & Miletich, J. P. (1985) J. Biol. Chem. **260**, 10917–10920
17. Guha, A., Bach, R., Konigsberg, W. & Nemerson, Y. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 299–302
18. Bom, V. J. J., Ram, I. E., Alderkamp, G. H. J., Reinalda-Poot, J. H. & Bertina, R. M. (1986) Thromb. Res. **42**, 635–643
19. Osterud, B. & Rapaport, S. I. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5260–5264
20. Jesty, J. & Silverberg, S. A. (1979) J. Biol. Chem. **254**, 12337–12345
21. Zur, M. & Nemerson, Y. (1980) J. Biol. Chem. **255**, 5703–5707
22. Bajaj, S. P., Rapaport, S. I. & Russell, W. A. (1983) Biochemistry **22**, 4047–4053
23. Morrison, S. A. & Jesty, J. (1984) Blood **63**, 1338–1347
24. Warn-Cramer, B. J. & Bajaj, S. P. (1986) Biochem. J. **239**, 757–762
25. Nelsestuen, G. L., Kisiel, W. & Di Scipio, R. G. (1978) Biochemistry **17**, 2134–2138
26. Broze, G. J. Jr & Majerus, P. W. (1980) J. Biol. Chem. **255**, 1242–1247
27. Bajaj, S. P., Rapaport, S. I. & Brown, S. F. (1981) J. Biol. Chem. **256**, 253–259
28. Bach, R., Oberdick, J. & Nemerson, Y. (1984) Blood **63**, 393–398
29. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A. & Hemker, H. C. (1980) J. Biol. Chem. **255**, 274–283
30. Pusey, M. L. & Nelsestuen, G. L. (1983) Biochem. Biophys. Res. Commun. **114**, 526–532
31. Mertens, K. & Bertina, R. M. (1984) Biochem. J. **223**, 607–615
32. Nesheim, M. E., Tracy, R. P. & Mann, K. G. (1984) J. Biol. Chem. **259**, 1447–1453
33. Van Rijn, J. L. M. L., Govers-Riemslag, J. W. P., Zwaal, R. F. A. & Rosing, J. (1984) Biochemistry **23**, 4557–4564
34. Jesty, J., Morrison, S. A. (1983) Thromb. Res. **32**, 171–181
35. Nemerson, Y. & Gentry, R. (1986) Biochemistry **25**, 4020–4033
36. Forman, S. D. & Nemerson, Y. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4675–4679
37. Zur, M. & Nemerson, Y. (1980) Methods Enzymol. **80**, 237–249
38. Mertens, K., Cupers, R., Van Wijngaarden, A. & Bertina, R. M. (1984) Biochem. J. **223**, 599–605
39. Bom, V. J. J. & Bertina, R. M. (1985) Thromb. Haemostasis **54**, 215 (abstr.)
40. Bottcher, C. J. F., Van Gent, C. M. & Pries, C. (1961) Anal. Chim. Acta **24**, 203–204
41. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
42. Shapiro, S. S. & Waugh, D. F. (1966) Thromb. Diath. Haemorrh. **16**, 469–490
43. Bom, V. J. J., Van Tilburg, N. H., Krommenhoek-Van Es, C. & Bertina, R. M. (1986) Thromb. Haemostasis **56**, 343–348
44. Tankersley, D. L., Alving, B. M. & Finlayson, J. S. (1982) Thromb. Res. **25**, 307–317
45. Holloway, P. W. (1973) Anal. Biochem. **53**, 304–308
46. Weber, K. & Osborn, M. (1969) J. Biol. Chem. **244**, 4406–4412
47. Zur, M. & Nemerson, Y. (1978) J. Biol. Chem. **253**, 2203–2209
48. Radcliffe, R. & Nemerson, Y. (1975) J. Biol. Chem. **250**, 388–395
49. Esmon, N. L., Owen, W. G. & Esmon, C. T. (1982) J. Biol. Chem. **257**, 859–864
50. Freyssinet, J.-M., Gauchy, J. & Cazenave, J.-P. (1986) Biochem. J. **238**, 151–157
51. Walker, F. J. (1984) Semin. Thromb. Haemostasis **10**, 131–138
52. Hagen, F. S., Gray, C. L., O'Hara, P., Grant, F. J., Saari, G. C., Woodbury, R. G., Hart, C. E.., Insley, M., Kisiel, W., Kurachi, K. & Davie, E. W. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2412–2416
53. Keyt, B., Furie, B. C. & Furie, B. (1982) J. Biol. Chem. **257**, 8687–8695
54. Nelsestuen, G. L., Broderius, M. & Martin, G. (1976) J. Biol. Chem. **251**, 6886–6893
55. Bach, R., Gentry, R. & Nemerson, Y. (1986) Biochemistry **25**, 4007–4020
56. Broze, G. J., Jr. (1982) J. Clin. Invest. **70**, 526–535
57. Tanaka, H., Janssen, B., Preissner, K. T. & Muller-Berghaus. G. (1985) Thromb. Res. **40**, 745–756

Received 30 March 1989/23 August 1989; accepted 7 September 1989