Inhibition of Tissue Factor-Factor VIIa-catalyzed Factor X Activation by Factor Xa-Tissue Factor Pathway Inhibitor

A ROTATING DISC STUDY ON THE EFFECT OF PHOSPHOLIPID MEMBRANE COMPOSITION

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The physiological inhibitor of tissue factor (TF)-factor VIIa (FVIIa), full-length tissue factor pathway inhibitor (TFPIFL) in complex with factor Xa (FXa), has a high affinity for anionic phospholipid membranes. The role of anionic phospholipids in the inhibition of TF-FVIIa-catalyzed FX activation was investigated. FXa generation at a rotating disc coated with TF embedded in a membrane composed of pure phosphatidylcholine (TF-PC) or 25% phosphatidylserine and 75% phosphatidylcholine (TF-FSPC) was measured in the presence of preformed complexes of FXa-TFPIFL or FXa-TFPI1–161 (TFPI lacking the third Kunitz domain and C-terminal). At TF-PC, FXa-TFPIFL and FXa-TFPI1–161 showed similar rate constants of inhibition (0.07 × 10⁸ m⁻¹ s⁻¹ and 0.1 × 10⁸ m⁻¹ s⁻¹, respectively). With phosphatidylserine present, the rate constant of inhibition for FXa-TFPIFL increased 3-fold compared with a 9-fold increase in the rate constant for FXa-TFPI1–161. Incubation of TF-FSPC with FXa-TFPIFL in the absence of FVIIa followed by depletion of solution FXa-TFPIFL showed that FXa-TFPIFL remained bound at the membrane and pursued its inhibitory activity. This was not observed with FXa-TFPI1–161 or at TF-PC membranes. These data suggest that the membrane-bound pool of FXa-TFPIFL may be of physiological importance in an on-site regulation of TF-FVIIa activity.

Blood coagulation in vivo is initiated when circulating factor VIIa binds to a calcium-dependent way to its cofactor, tissue factor (TF) (see Refs. 1 and 2 for a review). This complex formation results in enhanced catalytic activity of factor VIIa (FVIIa), which via limited proteolysis, activates factors X (FX) and IX (FIX) (3). TF is a transmembrane glycoprotein, which under normal conditions is expressed only in extravascular tissues (4, 5).

The main physiological regulator of TF-induced blood coagulation is tissue factor pathway inhibitor (TFPI) (6, 7), a single chain glycoprotein of 42 kDa and a member of the Kunitz family of serine protease inhibitors. TFPI contains an acidic N terminus, followed by three tandemly repeated Kunitz-type inhibition domains, and a basic C-terminal tail (8). Site-directed mutagenesis has revealed that the first Kunitz domain binds to FVIIa and that the second Kunitz domain interacts with the active site of FXa (9). No such functions could be attributed to the third Kunitz domain (10). Yet, various interactions have been ascribed to this domain, e.g. with lipopeptides and heparin, but their importance for the inhibitory function of TFPI is not clear (11, 12). On the other hand, the basic C-terminal region of TFPI (residues 240–276) has been shown to play a crucial role in the anticoagulant activity of this inhibitor (13, 14). Despite numerous studies, it remains unclear how this basic C terminus modulates the anticoagulant activity of TFPI (15–19).

TFPI inhibits the generation of FXa and FIXa by the TF-FVIIa complex in a unique, two-step reaction (20). First, TFPI binds Ca²⁺ independently to FXa, thereby inhibiting the FXa catalytic activity (9). In a second step, the FXa-TFPI complex in a Ca²⁺-dependent way to TF-FVIIa. This results in the formation of the quaternary complex TFVIIa-FXa-TFPI, in which the proteolytic activity of the TFVIIa complex is fully neutralized. The effect of TFPI on TF-FVIIa activity in the absence of FXa is negligible (21, 22), implying that the true inhibitor of TF-FVIIa activity is the FXa-TFPI complex. The rate of complex formation of FXa and TFPI is enhanced by negatively charged phospholipids for full-length TFPI (TFPIFL) but not for TFPI1–161, a truncated variant lacking the third Kunitz domain and the potential phospholipid binding C-terminal tail (16, 23).

Recently (24), we demonstrated that TFPIFL in complex with FXa has a much higher affinity for anionic phospholipid membranes compared with that of either protein alone. It is well recognized that the binding of blood coagulation enzymes as well as their cofactors and substrates to membranes containing anionic phospholipids may result in an immense increase of the catalytic efficiency of these enzymes. On the other hand, excess binding sites could cause a lowering of both solution concentration and surface density of the reactants (25, 26), resulting in a decrease of reaction rates. Previous studies (17, 22, 23) did not reveal a stimulatory or interfering effect of anionic phospholipids on the complex formation between FXa-TFPI and TF-FVIIa. These studies, however, were performed in the presence of excess phospholipid vesicles. It remains unclear what the role is of lipid-protein interactions and of the TFPI C terminus when TF is embedded in a macroscopic phospholipid membrane. Answering these questions could provide insight in the role of (TF-bearing) cell membranes in the regulation of the TF-FVIIa-catalyzed initiation of the blood coagulation process.
To mimic the processes occurring at TF-bearing cell surfaces as closely as possible, we made use of TF embedded in a macroscopic phospholipid surface to which the reactants were supplied under well-defined flow conditions. This approach allows a quantitative study of the kinetics of inhibition of TF-FVIIa-catalyzed FX activation as a function of the phospholipid composition of the membrane and the phospholipid-binding properties of the FXa'TFPI complex. Our study showed that negatively charged phospholipid surfaces act as a safety net in the sense that FXa'TFPI complexes readily bind to the surface. These phospholipid-bound FXa'TFPI complexes then show a potent inhibitory activity against TF-FVIIa present at the same membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bovine serum albumin (BSA, essentially fatty acid-free) was obtained from Sigma. The chromogenic substrate for FXa, S2765, was purchased from Chromogenix (Molndal, Sweden). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (PC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). All other reagents used were of analytical grade.

**Proteins**

Human FX was purified according to Mertens et al. (27). Human FXa was prepared by activation of purified FX with the FX activating protein from Russell’s viper venom (Sigma) and isolated as was described for bovine FXa (28). The molar concentration was determined by active site titration with p-nitrophenyl p-guanidinobenzoate hydrochloride (29). Recombinant human TFPI1–161, produced in *Escherichia coli* (30), was kindly supplied by Searle/Chiron (Emeryville, CA). This recombinant TFPI1–161 preparation, not being glycosylated, is very similar to the native highly glycosylated protein expressed in mammalian cells with respect to anticoagulant activity (18, 31). The C-terminal truncated TFPI preparation was kindly supplied by Dr. O. Nordfang (Novo Nordisk, Bagsvaerd, Denmark). TFPI1–161 was expressed in *Saccharomyces cerevisiae* and purified as described previously (31). The molar concentrations of the TFPI preparations were determined by titration with known amounts of FXa (18). Recombinant human TF was a kind gift of Dr. Y. Nemerson (Mount Sinai School of Medicine, New York, NY). Recombinant FVIIa was a kind gift of Dr. U. Hedner (Novo Nordisk).

**Relipidation of Recombinant Human TF**

Recombinant human TF, 20 and 100 nM, was reconstituted with 2 mm FSPC (25 mol% FSPC/75 mol% PC) and 2 mm PC (100 mol% PC), respectively, using n-octyl-β-D-glucopyranoside (Calbiochem, La Jolla, CA) solubilization and dialysis as described (32). Because the TF is randomly oriented in the reconstituted vesicles, we considered the effective TF concentration to be 50% of the total concentration present in the reconstituted preparation (32). Calculations were therefore based on a TF:phospholipid ratio of 1:20 × 10^9 and 1:4 × 10^10 for FSPC and PC, respectively.

**The Rotating Disc Device**

The set-up used in the present study is a modification of the one described previously (33). In short, a cylinder (height 12 mm, width 16 mm) made of polyethylene terephthalate (PETP; AKZO Plastics, Amersfoort, The Netherlands), containing four tiny magnets, was mounted on a motor with servo-controlled rotation speed. A cylindrical vessel is placed on top of the motor-containing holder, such that the cylinder rotates close to the glass bottom of the vessel. A glass coverslip (Menzel Gläser, Braunschweig, Germany) with a diameter of 20 mm is attached to another PETP cylinder (height 2 mm, width 12 mm), also containing four magnets. This second cylinder fits into a thin PETP ring, mounted in the vessel, which guides the movements of the rotating disc. A stationary buffer was immersed in the solution (3 ml) during the experiments to establish a well-defined flow pattern.

**Preparation of Phospholipid-coated Discs**

The glass coverslips of the rotating discs were made hydrophilic as described previously (34). In order to apply a TF-containing lipid bilayer, the rotating discs (63 rad/s) were exposed for 30 min to reconstituted vesicles in Tris buffer (50 mM Tris-HCl, pH 7.9, and 175 mM NaCl), with a final phospholipid-concentration of 20 μM. Fluid phase vesicles were removed by flushing for 5 min with TBSA-CaCl_2 buffer (Tris buffer containing 3 mM CaCl_2 and 1 mM mg BSA) at a rate of 10 ml/min using a Minipip II pump (Gislon, Villiers-le-Bel, France). Finally the disc was transported, without exposing the lipid surface to an air-buffer interface, to a second vessel that had been pretreated for 1 h with 20 mg/ml BSA in Tris buffer. Before placing the coated disc in this second vessel, the high content BSA Tris buffer was replaced by TBSA-CaCl_2 buffer. All preparations were performed at 37 °C. The effective TF surface density was 2.5 nanomol/cm^2 and 12.5 nanomol/cm^2 for FSPC and PC, respectively, as calculated on the basis of a phospholipid surface density of 0.5 nanomol/cm^2 and TF:phospholipid ratios of 1.20 × 10^9 or 1.4 × 10^10. The disc surface area was 3.14 cm^2; the theoretical amount of TF available at the surface is therefore 7.8 and 39 fmol for FSPC and PC, respectively. Throughout this paper, TF-FSPC and TF-PC refer to the planar surfaces thus obtained.

**Measurement of FX Activation**

Discs coated with TF-FSPC or TF-PC were spun at 63 rad/s in 3 ml TBSA-CaCl_2 buffer. FVIIa (1 nM) was added and incubated with the spinning disc for 5 min. The TF-FVIIa activity was then assessed from FXa generation following the addition of FX (100 nM). Timed samples were taken from the reaction mixture and collected into polystyrene cuvettes containing 50 mM Tris-HCl, pH 7.9, 175 mM NaCl, 20 mM EDTA, and 0.5 mg/ml BSA (total volume 450 μl). To determine the amount of FXa present, 50 μl of 3 mM chromogenic substrate S2765 was added to the cuvette, and the conversion of the chromogenic substrate was followed at 37 °C on a dual wavelength spectrophotometer at 405 nm (reference wavelength 500 nm). The FXa concentrations were calculated from standard curves obtained with known amounts of the enzyme. The TF-containing phospholipid surface of the rotating disc could be reused after flushing with TBSA-EDTA buffer (20 mM EDTA) for 5 min at 10 ml/min, followed by a rinse with TBSA-CaCl_2 buffer (5 min at 10 ml/min). All procedures were performed at 37 °C and unless otherwise mentioned, at an angular velocity of 63 rad/s.

**Inhibition of TF-FVIIa Activity**

All inhibition experiments were carried out with preformed FXa'TFPI complexes. These complexes were prepared by incubating FXa and TFPI at equimolar concentrations (50 nM) for 30 min at 37 °C in TBSA-CaCl_2 buffer. This incubation time was sufficiently long to obtain an equilibrium complex formation (95% of FXa in complex), as was established by measuring the free FXa concentration with chromogenic substrate S2765. The inhibitory activity of FXa'TFPI was assessed in two different ways. The first method was by preincubating TF-FSPC and TF-PC with FVIIa (1 nM) for 10 min, then adding FX (100 nM) in the presence of various concentrations of FXa'TFPI and subsequently assaying the timed samples taken from the vessel for FXa activity. Under these conditions, fluid phase inhibitor complex is assumed to be in equilibrium with surface-bound FXa'TFPI. The second method was by preincubating TF-FSPC and TF-PC for 10 min with 0.4 nM FXa'TFPI, in the absence of FVIIa, and then depleting the solution of inhibitor complex by flushing with various time intervals with TBSA-CaCl_2 buffer. Subsequently, FX activation was started by adding FX (100 nM) and FVIIa (1 nM). Timed samples were taken and assayed for FXa activity as described above.

**Data Analysis**

**FVIIa Concentration-dependent FX Activation**—As the catalytic efficiency of FVIIa in complex with TF is several orders of magnitude higher than that of free FVIIa, the observed FX generation rate, V_{obs}, reflects the formation of the binary TF-FVIIa complex and is described by the formula shown in Equation 1,

\[ V_{obs} = V_{max}[\text{FVIIa}]/(\text{[FVIIa]} + K_i), \]  

(Eq. 1)

with [FVIIa] the free FVIIa concentration, K_i the apparent dissociation constant of the TF-FVIIa complex, and V_{max} the FXa generation rate at saturating FVIIa concentrations. As the amount of TF present in the system is negligible compared with the concentration of added FVIIa, [FVIIa] equals the total FVIIa concentration. The parameters V_{max} and K_i were determined by least squares fitting of Equation 1 to the measurements of V_{obs}.

**Dependence of the FX Activation Rate on the FX Concentration**—Measurements of the observed rate of FXa generation, V_{obs}, were analyzed using the Michaelis-Menten formula as shown in Equation 2,

\[ V_{obs} = V_{max}[\text{FX}]/(\text{[FX]} + K_{m}), \]  

(Eq. 2)
FIG. 1. FX activation by TF-phospholipid-FVIIa immobilized at the surface of a rotating disc. The TF-PSPC (●, 2.5 nmoI of TF/cm²) or TF-PC (▲, 12.5 nmoI of TF/cm²) surface was incubated with FVIIa (1 nM) for 5 min in TBSA-CaCl₂ buffer, and the reaction was started by adding FX (100 nM). Samples were taken at the indicated time points and assayed for FXa. At 10 min the rotation (63 rad/s) was stopped, whereas sampling was continued. The initial rate (Vₜ₀) of FX activation, derived from the increase in FXa concentration measured between 2 and 8 min, is 3.15 nM/min for TF-PSPC and 0.84 nM/min for TF-PC. Inset, FXa generation during the initial phase of the reaction.

FIG. 2. Dependence of the initial velocity of FX activation on the angular velocity of the rotating disc. At the indicated angular velocities, the initial rate of FX activation was measured on TF-PSPC (●) and TF-PC (▲) as described in the legend to Fig. 1. A single rotating disc was used that was regenerated for each measurement by flushing (10 ml/min) the reaction vessel and spinning disc for 5 min with TBSA-EDTA and subsequently for 5 min with TBSA-CaCl₂ buffer.

Inhibition of TF-FVIIa-mediated FX Activation by Preformed FXa-TFPI Complexes—The decrease in FX activation rate reflects the formation of quaternary TF-FVIIa-TFPI-FXa complexes and the corresponding decline of TF-FVIIa activity, which for any fixed concentration of inhibitor, can be described as a pseudo-first order process (22, 23). Therefore, the time-dependent FXa generation indicating a mono-exponential decay of TF-FVIIa activity can be expressed as shown in Equation 3,

\[
[FXa] = V₀k(1 - e^{-kt}),
\]

(Eq. 3)

with [FXa], the observed FXa generation at time t, V₀ the rate of FXa generation in the absence of inhibitor, k the pseudo-first order rate constant of inhibition, and t the reaction time. In principle, the rate constants V₀ and k can be determined by a least squares fit of Equation 3 to the measurements of [FXa]. It should be noted that the substrate transport from the bulk solution to the disc surface depends on the concentration gradient between bulk and the solution, immediately adjacent to the catalytic surface. To what extent this substrate depletion near the surface causes an underestimation of the true V₀ value depends on the ratio of substrate conversion over the transport-limited rate of substrate supply (33).

Dependence of the Rate of FX Activation on the Angular Velocity of the Rotating Disc—Fig. 2 shows the relation between the angular velocity of the rotating disc and the initial rate of FXa formation. For a rotating disc with TF-PSPC, the initial rate of FXa formation increases when the angular velocity increases from 0 to 60 rad/s. An additional increase of the angular velocity does not result in a concomitant increase of the initial rate. Thus, an angular velocity of more than 60 rad/s appears to be sufficient to approach a kinetically controlled situation. That is, the rate at which FX is activated at the surface is smaller than the transport limited rate of FX delivery to the catalytic surface. A similar experiment was also performed with TF embedded in PC (Fig. 2). Obviously, an apparent kinetically controlled FX activation is achieved at a lower angular velocity (30 rad/s) than observed with TF-PSPC.

RESULTS

Initial Rate of FX Activation at the Surface of a Rotating Disc with TF Embedded in a Phospholipid Bilayer—Typical results of FX activation experiments using a rotating disc are shown in Fig. 1. The disc with TF embedded in a planar phospholipid bilayer composed of PSPC or PC was incubated first with FVIIa (1 nM) for 5 min before FX (100 nM) was added to start the reaction. Apparently, for both TF-PC and TF-PSPC, the FXa activity increases linearly as a function of time. However, the inset to Fig. 1 reveals that, in contrast to the TF-PC surface, it takes for the TF-PSPC surface about 1 min before FXa activity starts to increase linearly. This delayed rise in solution FXa activity most likely reflects binding of FXa to the TF-PSPC surface (35). Therefore, the initial rate of FX activation is defined as the linear increase in solution phase FXa 1.5 min after the start of the reaction. When the rotation of the disc was stopped FXa generation halted indicating that under the conditions of the experiment convective transport is essential both for delivery of FX to the catalytic surface and for the transfer of FXa from the surface to the solution. No FXa was produced in the absence of TF or FVIIa.

The mean value ± S.D. of the initial rate of FXa generation at TF-PSPC after repeated regeneration (n = 5) of the same disc (see “Experimental Procedures”) was 2.99 ± 0.12 nM/min. For TF embedded in PC, the initial rate of FXa generation was 0.82 ± 0.07 nM/min (mean value ± S.D., n = 5). A single TF-PSPC or TF-PC disc could thus be regenerated and used several times, allowing repeating experiments under different conditions with an unchanged catalytic active surface. The inter-disc variations were larger with mean values for the initial rate of FX activation ± S.D. of 3.24 ± 0.70 nM/min (n = 30) and 0.77 ± 0.37 nM/min (n = 30) for TF-PSPC and TF-PC, respectively. It should be noted that the TF-PC membrane preparation contained 5-fold more TF than the TF-PSPC membrane.

Dependence of the Rate of FX Activation on the Angular Velocity of the Rotating Disc—Fig. 2 shows the relation between the angular velocity of the rotating disc and the initial rate of FXa formation. For a rotating disc with TF-PSPC, the initial rate of FXa formation increases when the angular velocity increases from 0 to 60 rad/s. An additional increase of the angular velocity does not result in a concomitant increase of the initial rate. Thus, an angular velocity of more than 60 rad/s appears to be sufficient to approach a kinetically controlled situation. That is, the rate at which FX is activated at the surface is smaller than the transport limited rate of FX delivery to the catalytic surface. A similar experiment was also performed with TF embedded in PC (Fig. 2). Obviously, an apparent kinetically controlled FX activation is achieved at a lower angular velocity (30 rad/s) than observed with TF-PSPC.
The 5-fold lower rate of FX activation at the TF-PC surface likely reduces the transport-limited supply of reactants. All further experiments were performed at an angular velocity of 63 rad/s.

**Kinetic Parameters for FX Activation at the Rotating Disc**—Based on a previously reported $K_a$ value of 41 pm for FVIIa binding to TF in PSPC vesicles (36), we reckoned that a FVIIa concentration of 1 nm, as used in the experiments described above, was sufficient to saturate all the TF exposed at the catalytic surface. To verify whether this assumption applied for the rotating disc system, we measured the initial rate of FX activation as a function of the solution phase FVIIa concentration. The titration curves obtained (data not shown) were analyzed using Equation 1 to yield binding as well as kinetic parameters. For TF:PSPC the fit yielded a $K_a$ of 18 pm, and for TF-PC the $K_a$ value was 240 pm. Thus, whereas the $K_a$ of FVIIa for TF:PSPC is in good agreement with the value previously reported (36, 37), the affinity of FVIIa for TF-PC is 10-fold higher than values reported by others (32, 36). Hence, a FVIIa concentration of 1 nm is indeed sufficient to saturate more than 80% of the TF present at the PC and PSPC surfaces. Furthermore, the fit yielded a $V_{\text{max}}$ of 4.4 nm/min (TF:PSPC) and 0.85 nm/min (TF-PC), corresponding to a turnover number of 1677/min for TF:PSPC and of 65/min for TF-PC.

Likewise, the relationship between the initial rate of FX activation at the spinning surface and the fluid-phase FX concentration was determined for TF:PSPC and TF-PC. By fitting Equation 2 to the experimental data (not shown), we estimated values of 110 nm for the apparent $K_m$ ($K_{m(app)}$) and of 5.5 nm/min for $V_{\text{max}}$. Calculation on basis of an estimated TF density of the PSPC surface (2.5 fmol/cm$^2$) gave a $K_{cat}$ value of 2098/min for TF:PSPC. For TF-PC a linear relation between the FXa generation rate and the FX concentration was found despite a much higher (up to 400 nm) FX concentration employed (data not shown). This indicated for the TF-PC a $K_{m(app)}$ value of $> 5$ µM, thus at least 1 order of magnitude greater than the highest FX concentration used.

**Inhibition of Membrane-Associated TF-FVIIa as a Function of the Concentration of FXa:TFPI**—In a successive set of experiments, we assessed the inhibitory activity of preformed FXa:TFPI$_{1–161}$ and FXa:TFPI$_{1–161}$ complexes toward TF-FVIIa-catalyzed FX activation at TF-PC and TF:PSPC membranes deposited on rotating discs. Following a preincubation with FVIIa for 5 min, the reaction was started by the simultaneous addition of FX (100 nm) and inhibitor complexes in varying concentrations. All inhibition experiments were preceded by a control experiment in the absence of inhibitor to assess the precise catalytic activity of that particular rotating disc. To correct for inter-disc variations in catalytic activity (± 20%, as shown before), the FXa generated in the presence of inhibitor is expressed as a percentage of the corresponding amount of FXa generated after 10 min in the absence of inhibitor.

It is seen from Fig. 3 A that the rate of FXa generation on TF-FVIIa:PC in the presence of FXa:TFPI$_{1–161}$ gradually decreases in time and that this progressive inhibition becomes more pronounced at higher inhibitor concentrations. The very same observations were made with FXa:TFPI$_{1–161}$ as inhibitor (Fig. 3B). The FXa generation curves were analyzed according to a model which assumes that complex formation between TF-FVIIa and FXa:TFPI results in a quaternary complex in which the catalytic activity of TF-FVIIa is completely blocked (Equation 3, see "Experimental Procedures"). The hypothesis that the initial FXa generation rate did not alter by addition of the FXa:TFPI complex was checked by fitting Equation 3 to the individual curves of the pairs of uninhibited and inhibited FXa generation. The value obtained for $V_0$ of the inhibited curve, expressed as percentage of $V_0$ of the uninhibited curve, was $96 \pm 6\%$ and $105 \pm 9\%$ (mean ± S.D., n = 6) for FXa:TFPI$_{1–161}$ and FXa:TFPI$_{1–161}$ complexes, respectively. These data thus justify our analysis of the FXa generation curves by a simultaneous fit of Equation 3 to the uninhibited and inhibited curves with a shared rate constant $V_o$. The *solid lines* in Fig. 3 (A and B) show the result of this fitting procedure, which yielded pseudo-first order rate constants of inhibition ($k_h$) as a function of the concentration of the inhibitory complex (Fig. 3C). It is clear that $k_h$ increases linearly with the concentration of the inhibitory complex. This indicates that, in the concentration range employed, the bimolecular association of TF-FVIIa with preformed FXa:TFPI is the rate-limiting step of the formation of the quaternary TF-FVIIa:FXa:TFPI complex. The second order rate constants of inhibition (Table I) were determined by linear regression to these data, were similar for FXa:TFPI$_{1–161}$ and FXa:TFPI$_{1–161}$, namely $0.10 \times 10^8$ M$^{-1}$s$^{-1}$ and $0.07 \times 10^8$ M$^{-1}$s$^{-1}$.
The pseudo-first order rate constants of inhibition thus obtained are presented in Fig. 4 as a function of the concentration of the inhibitory complex. This plot clearly reveals the difference in inhibitory activity of FXa-TFPIFL and FXa-TFPI1–161 on TF-PSPC. According to Table I, the second order rate constant of inhibition for FXa-TFPI1–161 and FXa-TFPIFL increases when PS is present in the TF-bearing membrane. However, the increase for FXa-TFPI1–161 is about 9-fold, whereas a 3-fold increase is seen for FXa-TFPIFL. The rate constant of 0.94 × 10^6 M^-1 s^-1 found for FXa-TFPI1–161 is in good agreement with the previous reported value of 1.1 × 10^6 M^-1 s^-1 (23), whereas the value for FXa-TFPIFL (0.20 × 10^6 M^-1 s^-1) is 5-10 fold lower than the values reported previously (22, 23). Thus at TF-PSPC, FXa-TFPIFL apparently has a lower inhibitory activity than FXa-TFPI1–161. In view of our earlier finding that FXa-TFPIFL complexes have a considerably higher affinity for PS containing lipid membranes than complexes of FXa-TFPI1–161, these data suggest that upon binding to TF-PSPC, FXa-TFPIFL complexes lost their inhibitory activity.

**Table I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rate constant of inhibitiona</th>
<th>TFPC</th>
<th>TF-PSPC</th>
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<tr>
<td>FXa-TFPI1–161</td>
<td>0.10 ± 0.01 × 10^6</td>
<td>0.94 ± 0.05 × 10^6</td>
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<tr>
<td>FXa-TFPIFL</td>
<td>0.07 ± 0.02 × 10^6</td>
<td>0.20 ± 0.02 × 10^6</td>
<td></td>
</tr>
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a Values are given ± S.E.

The same experiments were performed with TF embedded in PSCP. Comparison of Figs. 4A and 3A shows that FXa-TFPI1–161 inhibits TF-FVIa more efficiently at a PSCP surface than at a PC surface. A solution phase concentration of 0.4 nM FXa-TFPI1–161 is sufficient to neutralize TF-FVIa activity almost instantaneously. In contrast, with the same concentration of FXa-TFPIFL complex at TP-PSCP (Fig. 4B), a significant FXa generation is still observed. The FXa generation curves as shown in Fig. 4 were analyzed as described for those in Fig. 3. The pseudo-first order rate constants of inhibition thus obtained are presented in Fig. 4C as a function of the concentration of the inhibitory complex. This plot clearly reveals the difference in inhibitory activity of FXa-TFPI1–161 and FXa-TFPIFL on TF-PSPC. According to Table I, the second order rate constant of inhibition for FXa-TFPI1–161 and FXa-TFPIFL increases when PS is present in the TF-bearing membrane. However, the increase for FXa-TFPI1–161 is about 9-fold, whereas a 3-fold increase is seen for FXa-TFPIFL. The rate constant of 0.94 × 10^6 M^-1 s^-1 found for FXa-TFPI1–161 is in good agreement with the previous reported value of 1.1 × 10^6 M^-1 s^-1 (23), whereas the value for FXa-TFPIFL (0.20 × 10^6 M^-1 s^-1) is 5-10 fold lower than the values reported previously (22, 23). Thus at TF-PSPC, FXa-TFPIFL apparently has a lower inhibitory activity than FXa-TFPI1–161. In view of our earlier finding that FXa-TFPIFL complexes have a considerably higher affinity for PS containing lipid membranes than complexes of FXa-TFPI1–161, these data suggest that upon binding to TF-PSPC, FXa-TFPIFL complexes lost their inhibitory activity.

**Inhibition of TF-FVIa-catalyzed FX Activation by Membrane-bound FXa-TFPI Complex—FXa-TFPIFL and FXa-TFPI1–161 complexes (0.4 nM) were incubated for 10 min with TF-PSPC-coated discs spinning at 63 rad/s.** In some cases FXa-TFPI complexes were, after the incubation, removed from the solution by flushing the reaction vessel with a TBSA-CaCl2 buffer during 5 min at 10 ml/min. FXa generation was initiated by the simultaneous addition of FX (100 nM) and FVIIa (1 nM). Fig. 5A shows the time courses of FXa generation for TF-PSPC surfaces that were preincubated with FXa-TFPI1–161 with inhibitor complexes remaining in solution during the FXa generation and with inhibitor complexes removed from solution at the end of the preincubation. For comparison we also show the FXa generation in the absence of inhibitor complexes and the FXa generation as observed when the inhibitor complexes were added simultaneously with FX. It is apparent from Fig. 5A that, as long as the FXa-TFPI1–161 is not depleted from the solution, identical inhibition is seen with or without preincubation, whereas depletion of the inhibitory complex from the solution completely removes the inhibitory activity.

Similar experiments performed with FXa-TFPIFL complexes gave a completely different outcome, as shown in Fig. 5B. The largest extent of inhibition of FXa generation is observed when the TF-PSPC membrane was preincubated with FXa-TFPIFL complexes. Even more strikingly, it turned out that depletion of the solution from FXa-TFPIFL complex by flushing the reaction vessel at the end of the preincubation did not diminish the inhibitory activity. This indicates that under the conditions of this experiment, the contribution of TF-PSPC-bound FXa-TFPIFL complexes to the inhibitory activity is dominant. To ascertain that the inhibitory activity that remained manifest after flushing originated from FXa-TFPIFL bound to the TF-PSPC surface and not from inhibitory complex nonspecifically bound to the wall of the reaction vessel, we performed the following control experiment. The reaction vessel was preincubated for 10 min with 0.4 nM FXa-TFPIFL and flushed for 5 min with TBSA-CaCl2 buffer, after which an unused TF-PSPC-coated disc was placed in the vessel. Subsequently, the rate of FXa formation at that disc was measured following the addition of FVIIa (1 nM) and FX (100 nM). No inhibition was observed, showing that the retained inhibitory activity in the previous experiment solely originated from TF-PSPC-bound FXa-TFPIFL complexes.

The different effects of flushing on the inhibitory activity (Fig. 5, A and B) of the FXa-TFPI1–161 and FXa-TFPIFL complexes at TF-PSPC presumably reflect the differences in desorption rates of these complexes that were previously reported (24). As has been shown, at a macroscopic PSCP surface (no TF
present) the residence time of membrane-bound FXα-TFPI<sub>PL</sub> was about 45 min, compared with a residence time of about 1–2 min for complexes of FXα with TFPI<sub>1–161</sub>. When a TF-PC surface was preincubated with FXα-TFPI<sub>PL</sub> (0.4 nM) and subsequently washed for 5 min as described above, an uninhibited FXα generation was observed (data not shown). Thus, omitting the negatively charged phospholipids from the membrane resulted in loss of FXα-TFPI<sub>PL</sub> membrane binding and thus of the exerted inhibitory activity. Additional support was obtained when, following the preincubation of the TF-PSPC surface with FXα-TFPI<sub>PL</sub>, the time period of flushing with buffer was increased (from 5 to 90 min). This resulted in a gradually decreasing inhibitory activity (Fig. 6). It is noteworthy, however, that even after a rinse for 90 min, a significant inhibition of FXα generation is still observed.

Further analysis of inhibited and uninhibited FXα generation curves, as presented in Figs. 5B and 6, reveals that preincubation of TF-PSPC with FXα-TFPI<sub>PL</sub> results in a major decrease of the initial rate of FX activation. Correspondingly, the simultaneous fit of Equation 3 to the inhibited and the matching uninhibited FXα generation curves with a shared rate constant <i>V</i><sub>0</sub>, as used in the fits of Figs. 3 and 4, resulted in an adequate fit with large systematic deviations. Using individualized parameter values of <i>V</i><sub>0</sub>, however, resulted in excellent fits (solid lines in Figs. 5B and 6). The estimated parameters <i>V</i><sub>0</sub> and <i>k</i>, listed in Table II, show that the initial rate of FXα generation of the preincubated curves is instantaneously decreased to about 25% of the uninhibited <i>V</i><sub>0</sub> when FXα generation is initiated shortly (0–5 min) after the preincubation. With increasing flushing time, a gradual increase of the estimated <i>V</i><sub>0</sub> is observed, from 27% after 5 min flushing to 59% after 90 min of flushing. The rate constants found for the exponential decay of the TF-FVIIa activity ranged from 0.08 to 0.15 min<sup>–1</sup>, dependent on the flushing time. These values are 3–5-fold lower than the decay rate of 0.42 min<sup>–1</sup>, found for the inhibition of TF-FVIIa when the same concentration of FXα-TFPI<sub>PL</sub> was added to the reaction mixture (Fig. 4).

**DISCUSSION**

**Rationale behind Experimental Design**—In this study we addressed the question of whether the previously reported (24) high affinity of FXα-TFPI<sub>PL</sub> for negatively charged phospholipids is reflected in its inhibitory activity against TF-FVIIa. Because phospholipids were also shown to enhance complex formation between FXα and TFPI (16, 23), preformed FXα-TFPI<sub>PL</sub> and FXα-TFPI<sub>1–161</sub> complexes were used in this study to simplify the interpretation of the experimental data on the effect of phospholipids on the TF-FVIIa inhibition by FXα-TFPI. Furthermore, a macroscopic phospholipid membrane (with embedded TF) was used rather than unilamellar vesicles because this model mimics the plasma membrane of TF-bearing cells better than small unilamellar vesicles. Moreover, several studies have shown that the kinetics of activation and inactivation of blood coagulation enzyme complexes are dependent on the characteristics of the phospholipid surface like the radius of the phospholipid vesicle (35, 38), microscopic homogeneity (39), and ratio of reactant-bearing vesicles over non-bearing vesicles (25). Additionally, with macroscopic surfaces it is easier to separate physically the surface-bound and fluid-phase reactions. The use of a macroscopic phospholipid membrane at the surface of a rotating disc (40) has been extensively characterized for immobilized enzymes (33, 41, 42). Its main advantage

**Fig. 5.** Retention of TF-PSPC membrane-bound inhibitory activity. The TF-PSPC disc surface was preincubated for 10 min with 0.4 nM FXα-TFPI<sub>1–161</sub> (panel A) and 0.4 nM FXα-TFPI<sub>PL</sub> (panel B). Then the reaction vessel and rotating disc were either not flushed (○) or flushed (10 ml/min) for 5 min with TBSA-CaCl<sub>2</sub> buffer (●). FX activation was started by addition of FVIIa (1 nM) and FX (100 nM). For comparison data from Fig. 4 are shown: FX activation in the absence of inhibitor (●) and the inhibition of FX generation when the inhibitory complex is added simultaneously with FX (●). Solid lines indicate the best fit of Equation 3 to the individual curves. The FXα activity is presented as a percentage of the amount of FXα formed after 10 min in the absence of inhibitor.

**Fig. 6.** Time-dependent decrease of the inhibitory activity of FXα-TFPI<sub>PL</sub> complexes preadsorbed on TF-PSPC. The TF-PSPC disc surface was preincubated for 10 min with FXα-TFPI<sub>PL</sub> (0.4 nM). Then the reaction vessel and rotating disc were flushed for 5 (●), 45 (□), or 90 (▲) min with TBSA-CaCl<sub>2</sub> buffer (10 ml/min). FX activation was started by addition of FVIIa (1 nM) and FX (100 nM). FXα generation was initiated shortly (0–5 min) after the preincubation. With increasing flushing time, a gradual increase of the estimated <i>V</i><sub>0</sub> is observed, from 27% after 5 min flushing to 59% after 90 min of flushing. The rate constants found for the exponential decay of the TF-FVIIa activity ranged from 0.08 to 0.15 min<sup>–1</sup>, dependent on the flushing time. These values are 3–5-fold lower than the decay rate of 0.42 min<sup>–1</sup>, found for the inhibition of TF-FVIIa when the same concentration of FXα-TFPI<sub>PL</sub> was added to the reaction mixture (Fig. 4).
over flow systems like that of a capillary flow reactor (43, 44) is the uniformly accessibility of the surface (40).

**Determination of the Kinetics of FX Activation Using the Rotating Disc System—**Our study confirms the role of anionic phospholipids in the binding of FVIIa to TF embedded in a phospholipid bilayer: the $K_m$ of FVIIa for TF-PC (240 pm) is 13-fold higher than the $K_m$ of FVIIa for TF-PSPC (18 pm).

The $k_{cat}$ (35 s$^{-1}$) and $K_{m(app)}$ (110 nM) values for TF-FVIIa-catalyzed FX activation at PSPC are in reasonable agreement with the $k_{cat}$ = 12 s$^{-1}$ and $K_m$ = 70 nM measured at TF-bearing PSPC vesicles (23, 45). However, others have reported much higher values (46). A trivial explanation for this apparently discrepancy could be the high vesicle concentration in the latter study. The estimated occupancy of the TF-FVIIa by FX, as determined from $K_m$ values in PSPC and PC is 47% and less than 2%, respectively, suggesting that some competition between substrate and inhibitor for TF-FVIIa might be expected at PSPC but not at PC.

**Inhibition Kinetics of TF-FVIIa-catalyzed FX Activation as Studied at a Rotating Disc Surface—**The similar kinetics of TF-FVIIa inhibition that were observed for FXa-TFPI$_{1–161}$ and FXa-TFPI$_{1–161}$ on a TF-PC surface, which does not bind FXa (47) nor TFPI$_{1–161}$ (48), are consistent with other studies which indicated that a direct interaction of FXa-TF with TF-FVIIa does not require the binding of FXa-TFPI to negatively charged phospholipids (22, 43, 49). Kazama (50), however, concluded from studies using TF lacking the trans-membrane and cytoplasmic domains that binding of FXa to anionic phospholipids via its Glu domain may be an absolute requirement for TFPI-mediated regulation of full-length membrane-inserted TF.

Interestingly, the addition of PS (25%) to a neutral membrane brings about a significant stimulation of the rate of inhibition by both FXa-TFPI$_{1–161}$ ($k = 0.20 \times 10^8$ M$^{-1}$s$^{-1}$) and FXa-TFPI$_{1–161}$ ($k = 0.94 \times 10^8$ M$^{-1}$s$^{-1}$). We note that the marked difference in fractional saturation of TF-FVIIa on a PC membrane and that of TF-FVIIa on a PSPC membrane as aforementioned could mask the stimulatory effect of PS. When FX and FXa-TFPI compete for TF-FVIIa at PSPC, then it can be calculated according to the relation $k_{true} = k_{true} (1 + S/K_m)$ that the true rate constants of inhibition (FXa-TFPI$_{1–161}$: 1.79 $\times 10^8$ M$^{-1}$s$^{-1}$; FXa-TFPI$_{1–161}$: 3.98 $\times 10^8$ M$^{-1}$s$^{-1}$) are about 2.5-fold higher then the observed rate constants (Table I). Because for TF-PC the $K_m$ is much larger than the substrate concentration [FX], competition is in this case negligible. As a result, the difference in rate constants of inhibition at a membrane with PSPC and pure PC become even more pronounced.

The stimulatory effect of PS on the inhibition of both FXa-TFPI$_{1–161}$ and FXa-TFPI$_{1–161}$ might be related to the FXa-mediated binding of the complexes to the phospholipid membrane (24). Several mechanisms have been proposed to explain the membrane-mediated acceleration of reactions of the blood coagulation system. Next to a proper juxtaposition of the FXa-TFPI complexes toward the active site of the FVIIa, anionic planar phospholipid membranes may also allow lateral diffusion of the substrate toward the membrane associated enzymatic complex as shown in experiments on prothrombin activation (33, 34, 38). As a result, a much lower solution phase substrate concentration is needed to saturate half of the immobilized enzyme. If this mechanism would be operational for the inhibition of TF embedded in the PSPC membrane, one would predict the highest rate constant of inhibition for the inhibitory complex with the highest affinity for the membrane. Our experiments, however, show the opposite; the presence of PS stimulated FXa-TFPI$_{1–161}$ 9-fold compared with the only 3-fold stimulation of FXa-TFPI$_{1–161}$. Apparently the high affinity binding of FXa-TFPI$_{1–161}$ with PSPC membranes is accompanied by a lower inhibitory activity. These data thus suggest that solution phase and membrane-bound FXa-TFPI$_{1–161}$ contribute to the overall inhibition reaction but to a different extent. Therefore, the description of the inhibition reaction by a bimolecular association reaction (Equation 3), although it adequately describes the experiment of Figs. 3 and 4, presents a gross simplification. Indeed, the experiments in Figs. 5 and 6 demonstrate that inhibition of TF-FVIIa at PSPC by preadsorbed FXa-TFPI$_{1–161}$ is in fact a multistep process. After a rapid inactivation of ± 75% of the TF-FVIIa activity, as suggested by the reduced initial rate of FX generation after the addition of FVIIa and FX, a much slower elimination of the remaining activity is observed. This rapid first phase suggests that the membrane-bound pool of FXa-TFPI$_{1–161}$ may, also in the absence of FX, associate with TF (51) and that this ternary complex upon addition of FVIIa and FX rapidly converts to the quaternary complex. Alternatively, FXa-TFPI$_{1–161}$ binding to TF could interfere with the interaction of TF with FVIIa. However, the same study on TF-PC showed no persistent inhibitory activity. This indicates that the high affinity binding of FXa-TFPI$_{1–161}$ to TF-PSPC membranes is the result of a protein (FXa-TFPI)-lipid interaction rather than a protein (FXa-TFPI)-protein (TF) interaction. The decreasing extent of the initial inhibition with increasing flushing time as shown in Table II, thus probably reflects the decrease of the membrane-bound pool of FXa-TFPI$_{1–161}$.

Taken together, our data indicate efficient inhibition both by fluid phase FXa-TFPI$_{1–161}$ and by phospholipid-bound complex. The complex between full-length TF and FXa is so tightly bound to the catalytic TF-PSPC surface that it represents a highly effective pool of inhibitory activity that remains available for hours after exposure of the membrane to FXa-TFPI$_{1–161}$ complexes. This property may be of physiological importance in allowing an on-site regulation of the TF-FVIIa activity. That is, this phospholipid-bound pool of inhibitor complex, which is immediately available, may represent an effective control of newly formed catalytic units when newly synthesized TF arrives at the plasma membrane of TF producing cells.

**REFERENCES**


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**Table II**

Inhibition by membrane bound FXa-TFPI$_{1–161}$

<table>
<thead>
<tr>
<th>Flushing time</th>
<th>$V_0$</th>
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