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-PSPC) was measured in the presence of preformed complexes of FXaTFPIFL or FXaTFPIFL-161 (TFPI lacking the third Kunitz domain and C terminus). At TF-PC, FXaTFPIFL and FXaTFPIFL-161 showed similar rate constants of inhibition (0.07 × 10⁶ M⁻¹ s⁻¹ and 0.1 × 10⁶ M⁻¹ s⁻¹, respectively). With phosphatidylserine present, the rate constant of inhibition for FXaTFPIFL increased 3-fold compared with a 9-fold increase in the rate constant for FXaTFPIFL. Incubation of TF-PSPC with FXaTFPIFL in the absence of FVIIa followed by depletion of solution FXaTFPIFL showed that FXaTFPIFL remained bound at the membrane and pursued its inhibitory activity. This was not observed with FXaTFPIFL-161 or at TF-PC membranes. These data suggest that the membrane-bound pool of FXaTFPIFL may be of physiological importance in an on-site regulation of TF-FVIIa activity.

Blood coagulation in vivo is initiated when circulating factor VII(a) binds in a calcium-dependent way to its cofactor, tissue factor (TF) (1) (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 lipoproteins 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 binds in a Ca²⁺-dependent way to TF-FVIIa. This results in the formation of the quaternary complex TF-FVIIa-FXa-TFPI, in which the proteolytic activity of the TF-FVIIa 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 TFPIFL-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$_{PL}$ complexes readily bind to the surface. These phospholipid-bound FXa-TFPI$_{PL}$ 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 FX, S2765, was purchased from Chromogenix (Molndal, Sweden). 1,2-Dioleoyl-sn-glycerol-3-phosphocholine (PC) and 1,2-dioleoyl-sn-glycerol-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 TFPI$_{PL}$, produced in *Escherichia coli* (30), was kindly supplied by Searle/Chiron (Emeryville, CA). This recombinant TFPI$_{PL}$ preparation, not being glycosylated, is very similar to the native highly glycosylated protein expressed in mammalian cells with respect to anticoagulant activity (16, 30). The C-terminal truncated TFPI preparation was kindly supplied by Dr. O. Nordfang (Novo Nordisk, Bagsvaerd, Denmark). TFPI$_{1-161}$ 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 PSPC (25 mol% PS/75 mol% PC) and 2 mM PC (100 mol% PC), respectively, using n-octyl-$\beta$-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 $1.20 \times 10^3$ and $1.4 \times 10^3$ for PSPC 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 $20 \mu$m. Fluid phase vesicles were removed by flushing for 5 min with TBSA-CaCl$_2$ buffer (Tris buffer containing 3 mM CaCl$_2$ and 1 mg/ml BSA) at a rate of 10 ml/min using a Minipuls II pump (Gilmont, 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 mol/cm$^2$ and 12.5 mol/cm$^2$ for PSPC and PC, respectively, as calculated on the basis of a phospholipid surface density of 0.5 nmol/cm$^2$ and TF-phospholipid ratios of $1.20 \times 10^3$ and $1.4 \times 10^3$. The disc surface area was 3.14 cm$^2$; the theoretical amount of TF available at the surface is therefore 7.8 and 39 nmol for PSPC and PC, respectively. Throughout this paper, TF-PSPC and TF-PC refer to the planar surfaces thus obtained.

**Measurement of FX Activation**

Discs coated with TF-PSPC 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 polyethylene 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 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 in 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 TBSA-CaCl$_2$-PC with FVIIa (1 nM) for 5 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 TBSA-CaCl$_2$-PC with FVIIa (1 nM) for 5 min, then 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, $V_{\text{obs}}$, reflects the formation of the binary TF-FVIIa complex and is described by the formula shown in Equation 1.

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

(Eq. 1)

with [FVIIa] the free FVIIa concentration, $K_I$ the apparent dissociation constant of the TF-FVIIa complex, and $V_{\text{max}}$ 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 $V_{\text{max}}$ and $K_I$ were determined by least squares fitting of Equation 1 to the measurements of $V_{\text{obs}}$.

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

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

(Eq. 2)
Fig. 1. FX activation by TF phospholipid FVIIa immobilized at the surface of a rotating disc. The TF-PSPC (●, 2.5 fmol of TF/cm²) or TF-PC (▲, 12.5 fmol 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 t = 10 min the rotation (63 rad/s) was stopped, whereas sampling was continuing. The initial rate (V₀) of FX activation, derived from the increase in FXa concentration measured between 2 and 8 min, is 3.15 ± 0.65 nM/min for TF-PSPC and 0.90 ± 0.35 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 nM/min) the reaction vessel and spinning disc for 5 min with TBSA-EDTA and subsequently for 5 min with TBSA-CaCl₂ buffer.

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 $V_{max}$ 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 $V_{max}$. 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 $>5\mu M$, 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 FXaTFPI—**

In a successive set of experiments, we assessed the inhibitory activity of preformed FXaTFPI$_{1–161}$ and FXaTFPI$_{1–161}$ 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 ($\pm 20\%$, 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. 3A that the rate of FXa generation on TF-FVIIa-PC in the presence of FXaTFPI$_{1–161}$ gradually decreases in time and that this progressive inhibition becomes more pronounced at higher inhibitor concentrations. The very same observations were made with FXaTFPI$_{PL}$ as inhibitor (Fig. 3B). The FXa generation curves were analyzed according to a model that assumes that complex formation between TF-FVIIa and FXaTFPI 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 FXaTFPI 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 $\pm$ S.D.; $n = 6$) for FXaTFPI$_{1–161}$ and FXaTFPI$_{PL}$ 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 $V_0$. The solid lines in Figs. 3A and B show the result of this fitting procedure, which yielded pseudo-first order rate constants of inhibition ($k$) as a function of the concentration of the inhibitory complex (Fig. 3C). It is clear that $k$ 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 FXaTFPI is the rate-limiting step of the formation of the quaternary TF-FVIIa:FXaTFPI complex. The second order rate constants of inhibition (Table I) were obtained by linear regression to these data, were similar for FXaTFPI$_{1–161}$ and FXaTFPI$_{PL}$, namely $0.10 \times 10^8 M^{-1}s^{-1}$ and $0.07 \times 10^8 M^{-1}s^{-1}$.
The difference in inhibitory activity of FXa generation in the absence of inhibitory complexes and the solution completely removes the inhibitory activity. This indicates that under the conditions of this experiment, the contribution of TF-PSPC-bound FXaTFPIFL complexes to the inhibitory activity is dominant. To ascertain that the inhibitory activity that remained manifest after flushing originated from FXaTFPIFL, 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 FXaTFPIFL 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 generation 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 FXaTFPIFL complexes.

The different effects of flushing on the inhibitory activity (Fig. 5) of the FXaTFPIFL and FXaTFPI complexes at TP-PSPC presumably reflect the differences in desorption rates of these complexes that were previously reported (24). As has been shown, at a macroscopic PSPC surface (no TF

**TABLE I**

| Inhibitor          | Rate constant of inhibitiona | TF/PC | TF/PSPC |
|--------------------|------------------------------|-------|---------|
|                    | m⁻¹ s⁻¹                       |       |         |
| FXaTFPI₁₋₁₆₁       | 0.10 ± 0.01 × 10⁸             | 0.94 ± 0.05 × 10⁶ |
| FXaTFPI₆₆         | 0.07 ± 0.02 × 10⁸             | 0.20 ± 0.02 × 10⁸ |

*Values are given ± S.E.

The same experiments were performed with TF embedded in PSPC. Comparison of Figs. 4A and 3A shows that FXaTFPI₁₋₁₆₁ inhibits TF-FVIIa more efficiently at a PSPC surface than at a PC surface. A solution phase concentration of 0.4 nM FXaTFPI₁₋₁₆₁ is sufficient to neutralize TF-FVIIa activity almost instantaneously. In contrast, with the same concentration of FXaTFPI₆₆ complex at TP-PSPC (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 FXaTFPI₁₋₁₆₁ and FXaTFPI₆₆ on TP-PSPC. According to Table I, the second order rate constant of inhibition for FXaTFPI₁₋₁₆₁ and FXaTFPI₆₆ increases when PS is present in the TF-bearing membrane. However, the increase for FXaTFPI₁₋₁₆₁ is about 9-fold, whereas a 3-fold increase is seen for FXaTFPI₆₆. The rate constant of 0.94 × 10⁶ m⁻¹ s⁻¹ found for FXaTFPI₁₋₁₆₁ is in good agreement with the previous reported value of 1.1 × 10⁸ m⁻¹ s⁻¹ (23), whereas the value for FXaTFPI₆₆ (0.20 × 10⁵ m⁻¹ s⁻¹) is 5–10 fold lower than the values reported previously (22, 23). Thus at TF-PSPC, FXaTFPI₆₆ apparently has a lower inhibitory activity than FXaTFPI₁₋₁₆₁. In view of our earlier finding that FXaTFPI₆₆ complexes have a considerably higher affinity for PS containing lipid membranes than complexes of FXaTFPI₁₋₁₆₁, these data suggest that upon binding to TF-PSPC, FXaTFPI₆₆ complexes lost their inhibitory action.

Inhibition of TF-FVIIa-catalyzed FX Activation by Membrane-bound FXaTFPI Complex—FXaTFPIFL and FXaTFPI₁₋₁₆₁ complexes (0.4 nM) were incubated for 10 min with TP-PSPC-coated discs spinning at 63 rad/s. In some cases FXaTFPI₆₆ 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 FXaTFPI₁₋₁₆₁ 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 inhibitory complexes and the FXa generation as observed when the inhibitory complexes were added simultaneously with FX. It is apparent from Fig. 5A that, as long as the FXaTFPI₁₋₁₆₁ 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 FXaTFPI₆₆ 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 FXaTFPI₆₆ complexes. Even more strikingly, it turned out that depletion of the solution 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 FXaTFPI₆₆ complexes to the inhibitory activity is dominant. To ascertain that the inhibitory activity that remained manifest after flushing originated from FXaTFPI₆₆, 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 FXaTFPI₆₆ 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 FXaTFPI₆₆ complexes.
In this study we addressed the question of whether the previously reported (24) high affinity of FXa-TFPIFL for negatively charged phospholipids is reflected in its inhibitory activity against TF-FVIIa. Because phospholipids were also shown to enhance complex formation between FXa and TFPI (16, 23), preformed FXa-TFPIFL and FXa-TFPIFL_{1-161} 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 FXa-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
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 $K_z$ of FVIIa for TF-PC (240 pm) is 13-fold higher than the $K_z$ of FVIIa for TF-PSPC (18 pm).

The $k_{cat}$ (35 s$^{-1}$) and $K_{m(app)}$ (110 nm) values for TF-FVIIa-catalyzed PX 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 apparent 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 PX Activation as Studied at a Rotating Disc Surface**—The similar kinetics of TF-FVIIa inhibition that were observed for FXa-TFPIFL and FXa-TFPI1–161 on a TF-PC surface, which does not bind FXa (47) nor TFPIFL (48), are consistent with other studies which indicated that a direct interaction of FXa-TFPI 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-TFPIFL ($k = 0.20 \times 10^8$ m$^{-1}$s$^{-1}$) and FXa-TFPI1–161 ($k = 0.94 \times 10^8$ 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 $k_{true} = k_{obs} (1 + S/K_m)$ that the true rate constants of inhibition (FXa-TFPI1–161: $1.79 \times 10^8$ m$^{-1}$s$^{-1}$, FXa-TFPIFL: $0.38 \times 10^8$ m$^{-1}$s$^{-1}$) are about 2-fold higher than 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 true 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-TFPI1–161 and FXa-TFPIFL might be related to the FXamediated 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-TFPIFL 9-fold compared with the only 3-fold stimulation of FXa-TFPI1–161. Apparently the high affinity binding of FXa-TFPIFL with PSPC membranes is accompanied by a lower inhibitory activity. These data thus suggest that solution phase and membrane-bound FXa-TFPIFL 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-TFPIFL is in fact a multistep process. After a rapid inactivation of $\pm 75\%$ of the TF-FVIIa activity, as suggested by the reduced initial rate of FXa 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-TFPIFL may, also in the absence of FVIIa, associate with TF (51) and that this ternary complex upon addition of FVIIa and FX rapidly converts to the quaternary complex. Alternatively, FXa-TFPIFL 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-TFPIFL to TPSPC 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-TFPIFL.

Taken together, our data indicate efficient inhibition both by fluid phase FXa-TFPIFL and by phospholipid-bound complex. The complex between full-length TFPI 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-TFPIFL 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.

### Table II

| Flushing time | $V_v$ | $k$ |
|---------------|------|-----|
| min           | %    | min$^{-1}$ |
| No flush      | 26   | 0.15 |
| 5             | 27   | 0.13 |
| 45            | 44   | 0.08 |
| 90            | 59   | 0.08 |

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