Regulation of Platelet Factor Va-dependent Thrombin Generation by Activated Protein C at the Surface of Collagen-adherent Platelets*

Jacob J. Briedé, Guido Tans, George M. Willems, H. Coenraad Hemker, and Theo Lindhout†

From the Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, 6200 MD Maastricht, The Netherlands

Recent studies have indicated that factor Va bound to activated platelets is partially protected from inactivation by activated protein C (APC). To explore whether this sustained factor Va activity could maintain ongoing thrombin generation, the kinetics of platelet factor Va-dependent prothrombinase activity and its inhibition by APC were studied. In an attempt to mimic physiologically relevant conditions, platelets were adhered to collagen type I-coated discs. These discs were then spun in solutions containing thrombin and factor Xa either in the absence or presence of APC. The experiments were performed in the absence of platelet-derived microparticles, with thrombin generation and inhibition confined to the surface of the adherent platelets. APC completely inactivated platelet-associated prothrombinase activity with an overall second order rate constant of $3.3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$, which was independent of the thrombin concentration over a wide range around the apparent $K_m$ for thrombin. Kinetic studies on prothrombinase assembled at a planar phospholipid membrane composed of 25 mol% phosphatidylserine and 75 mol% phosphatidylcholine revealed a similar second order rate constant of inhibition ($2.5 \times 10^6 \text{M}^{-1} \text{s}^{-1}$). Collectively, these data demonstrate that ongoing platelet factor Va-dependent thrombin generation at the surface of collagen-adherent platelets is effectively inhibited by APC. No differences were observed between the kinetics of APC inactivation of plasma-derived factor Va or platelet factor Va as part of the prothrombinase associated with, respectively, a planar membrane of synthetic phospholipids or collagen-adherent platelets.

Activated protein C (APC)† is a serine protease that inhibits thrombin formation by limited proteolysis of the nonenzymatic cofactors factor Va and factor VIIIa of the prothrombin and the factor X-activating enzyme complex, respectively. Efficient proteolysis of the cofactors requires the presence of membranes that contain anionic phospholipids, calcium ions, and protein S. In the presence of membranes that contain negatively charged phospholipids, plasma factor Va is inactivated by APC-catalyzed cleavage of its heavy chain at Arg$^{306}$ and Arg$^{506}$ (1, 2). The cleavage at Arg$^{306}$ is relatively rapid and yields a reaction intermediate that still retains partial cofactor activity in thrombin activation. The slower cleavage at position Arg$^{506}$ results in complete loss of cofactor activity (3). The rapid cleavage at Arg$^{506}$ is inhibited when factor Va is in complex with factor Xa (4–8).

Recently, it was reported that in contrast to synthetic phospholipid membranes, thrombin-activated platelets partially protect platelet-derived and plasma-derived factor Va from inactivation by APC. Thrombin-activated platelets appeared to slow down the cleavage at Arg$^{506}$ (9). It was speculated that activated platelets express a membrane component(s) in addition to anionic phospholipids that specifically binds factor Va resulting in a factor Va molecule with an apparent APC-resistant phenotype (10). This protection of APC-catalyzed inactivation of factor Va was not observed in the presence of microparticles or synthetic phospholipid vesicles (9). One of the questions that remain to be answered is how platelets influence APC-dependent factor Va inactivation once factor Va is assembled in the prothrombinase complex at the plasma membrane of activated platelets.

The purpose of the present study was to establish the kinetics of APC-dependent inhibition of ongoing thrombin activation at the plasma membrane of platelets adhered to immobilized collagen. To account for transport limitations of reactants, the experiments were conducted under well defined flow conditions on a rotating disc. Our findings indicate no difference in the kinetics of APC-dependent inactivation of prothrombinase at the membrane of activated, collagen-adherent platelets compared with that at the surface of a planar phospholipid membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (BSA), bovine fibrinogen, and appyrase were from Sigma. S2238, a chromogenic substrate for thrombin, was obtained from Chromogenix (Milndal, Sweden). Human factor Xa, human prothrombin, and bovine factor Va were prepared and quantified as described previously (11). Native type I collagen fibrils were extracted from bovine Achilles tendon in the absence of proteases using 0.5 M acetic acid and precipitated with 1.7 M NaCl as described (12). Human α-thrombin was prepared as described previously (13). Human activated protein C (APC) was purchased from Kordia (Leiden, The Netherlands). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were obtained from Avanti Polar Lipids (Alabaester, AL). All other reagents used were of analytical grade.

**Platelets**—Suspensions of washed human platelets were prepared as described previously (14). Briefly, blood was drawn from healthy volunteers who had not taken any anti-platelet medication in the preceding 2 weeks. Platelet-rich plasma was prepared by centrifugation. The platelets were then sedimented by centrifugation and washed twice. The paper is available online at http://www.jbc.org.
with HEPES buffer (10 mM HEPES, 136 mM NaCl, 5 mM glucose, 2.7 mM KCl, 2 mM MgCl₂, 1 mg/ml BSA, and 0.1 units/ml apyrase, pH 6.6). Finally, the platelets were resuspended in HEPES buffer adjusted to pH 7.45 (buffer A). Platelets were counted on a Coulter counter (Coulter, Miami, FL), and the suspensions were adjusted to 5 × 10⁷ platelets/ml.

The Rotating Disc Device—Rotating disc experiments were performed in a device described previously (15). Briefly, a circular glass coverslip with a diameter of 20 mm (Menzel Gläser, Braunschweig, Germany), was rotated at 63 rad/s at the bottom of a cylindrical reaction vessel containing reagents in 3 ml of buffer A. This angular velocity resulted in a wall shear rate of 3681 s⁻¹ at the edge of the rotating disc. The reaction vessel was pretreated for 1 h with 20 mg/ml BSA in buffer A.

Preparation of Discs with Collagen-adherent Platelets—Circular glass coverslips with a diameter of 20 mm were cleaned with a 1:1 mixture of ethanol (96 volume %) and HCl (37 volume %) and subsequently rinsed with deionized water. The discs were coated with collagen by incubating the coverslips for 3 h with 300 μl of 0.5 mg/ml collagen type I in 0.5 M acetic acid. Coated discs were rinsed extensively with 40 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and stored in this buffer until used. Inspection of the discs by phase-contrast microscopy showed a homogeneous distribution of the collagen fibrils over the glass surface. The collagen-coated discs were incubated for 15 min with buffer A, followed by a 40-min incubation at room temperature with 300 μl of a suspension of washed platelets. Nonadherent platelets were removed by rinsing with buffer A.

Preparation of Phospholipid-coated Discs—Spinning circular glass coverslips (63 rad/s) were exposed for 20 min to 20 μm vesicles composed of 25 mol % DOPS and 75 mol % DOPC, prepared as described previously (16). Fluid phase vesicles were removed by flushing for 5 min (10 ml/min) with buffer A. The phospholipid-coated discs were then transferred to a reaction vessel containing 3 ml of buffer A for further experimentation.

Thrombin Generation at Rotating Discs—Discs with collagen-adherent platelets or coated with a phospholipid membrane were spun at 63 rad/s in 3 ml of buffer A containing 3 mM CaCl₂, Factor Xa and, when indicated, Factor Va were added, and thrombin generation was started after 3 min by adding prothrombin. Timed samples (10 μl) were taken and transferred to cuvettes with 440 μl of Tris buffer (50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml BSA, pH 7.9) containing 20 mM EDTA. Thrombin was assayed by adding 2.4 mM S2238 (50 mM vesicles composed of phospholipids in the outer leaflet of the plasma membrane (14)). These microparticles may provide a procoagulant surface that supports prothrombin activation and thus could complicate our study on the kinetics of APC-dependent inactivation of prothrombin activation at the surface of collagen-adherent platelets. Therefore, initial experiments were conducted to establish the extent of microvesiculation and their contribution to prothrombin activation and, when necessary, to redesign the experiment in such a way that the contribution of microparticles to prothrombin activation would be negligible.

Coverslips with collagen-adherent platelets were spun in buffer A, and after 12 min CaCl₂ (3 mM) was added. Samples were taken from the reaction vessel and assayed for procoagulant vesicles. Fig. 1 shows that immediately after the addition of calcium the concentration of solution phase procoagulant phospholipid increased, reaching a maximum after 30–40 min. To investigate the relative contributions of these microparticles and the collagen-adherent platelets to prothrombin activation, factor Xa (50 pM) was added after 3 min by adding prothrombin (100 nM) to the reaction vessel. Immediately after the addition of prothrombin an aliquot (100 μl) was taken from the reaction vessel, transferred to a test tube, and incubated at 37 °C. Timed samples were taken from both the reaction vessel and the test tube and assayed for thrombin activity. The rates of thrombin generation were 2.2 and 1.3 nM/min in the reaction vessel and test tube, respectively, demonstrating that microparticles and adherent platelet contributed about equally to thrombin generation (Fig. 2). In the second step of this experiment the reaction vessel was flushed for 5 min (10 ml/min) with buffer A containing 3 mM CaCl₂ to remove microparticles. After the re-addition of factor Xa (50 pM) followed by prothrombin (100 nM) no thrombin generation could be detected in the fluid phase. This finding indicated that procoagulant microparticles were absent and that thrombin-generating activity was now solely confined to the spinning surface with collagen-adherent platelets (Fig. 2). The rate of thrombin generation (0.3 nM/min) was, however, lower than the rate of thrombin generation at the spinning surface before the removal of microparticles (0.9 nM/min). The combined results of three similar experiments showed that the rinsing step decreased the surface-associated thrombin production by 59 ± 10% (mean ± S.D.). This loss of activity was most likely due to a loss of platelet-
associated factor Va activity because addition of plasma-derived factor Va (1 nM) resulted in an increase in the rate of thrombin generation from 0.3 to 1.8 nM/min. All further experiments were performed with discs containing collagen-adherent platelets that were first spun for 30 min at 63 rad/s in buffer A containing 3 mM CaCl₂ and then flushed with the same buffer for 5 min at 10 ml/min to remove microparticles prior to thrombin generation.

Optimization of Thrombin Generation at the Plasma Membrane of Collagen-adherent Platelets—Fig. 3 shows the initial rates of thrombin generation at a fixed prothrombin concentration (100 nM) as a function of the factor Xa concentration. The apparent dissociation constant, $K_d$, of surface-bound factor Xa is described by the simple single site binding isotherm, $V_{\text{obs}} = \frac{V_{\text{max}} [\text{Xa}]}{[\text{Xa}] + K_d}$, with $V_{\text{obs}}$ the initial rate of thrombin formation, $[\text{Xa}]$ the factor Xa concentration, and $V_{\text{max}}$ the initial rate of thrombin generation at saturating factor Xa concentration. The value for the apparent $K_d$ estimated by fitting this equation to the data from two similar experiments is $3.5 \pm 0.9$ pM (estimated value ± 1 S.E.). Further experiments were performed at a saturating concentration of factor Xa (50 pM).

Fig. 4 shows the prothrombin dependence of thrombin generation at the surface of collagen adherent platelets in the presence of 50 pM factor Xa. The data could be described adequately by the Michaelis-Menten equation, $V_{\text{obs}} = \frac{V_{\text{max}} [\text{prothrombin}] [\text{prothrombin}] + K_{\text{app}}}{[\text{prothrombin}] + K_{\text{app}}}$, in which $V_{\text{max}}$ is the initial rate of thrombin formation at a saturating prothrombin concentration, [prothrombin] the prothrombin concentration in free solution, and $K_{\text{app}}$ is the apparent Michaelis constant. The solid line in Fig. 4 represents the best fit of this equation to the experimental data. The combined result of two similar experiments yielded a $K_{\text{app}}$ of $42 \pm 5$ nM (estimated value ± 1 S.E.).

NPC-dependent Inhibition of Thrombin Formation at the Plasma Membrane of Collagen-adherent Platelets during On-going Prothrombin Activation—The results of a typical prothrombinase inactivation experiment at the surface of collagen-adherent platelets are shown in Fig. 5. The first 8 min of the experiment was performed in the absence of APC to enable the determination of the initial rate of thrombin formation, $V_0$. Upon the addition of APC, the rate of thrombin formation rapidly decreased. To visualize the concentration-dependent effect of APC, a considerable interdisc variation in the rate of thrombin generation (0.5–1.2 nM/min) was corrected by setting the initial rates in the absence of APC to the same value. The total time courses of thrombin generation in the absence or presence of APC were analyzed by a least squares fit according to Equations 1 and 2 as described under “Experimental Procedures.” The first order rate constant of inhibition as a function of the APC concentration is shown as an insert on Fig. 5. The APC-dependent inactivation of prothrombinase obtained from these data was $3.3 \times 10^6$ M⁻¹ s⁻¹.

To assess the influence of the prothrombin concentration on the inhibition of the prothrombinase activity, comparable inhibition experiments were performed at prothrombin concentrations ranging from 20 to 500 nM. The results presented in Table I show that varying the prothrombin concentration did not influence the pseudo first order rate constant of inactivation of platelet-associated prothrombinase activity.

The inhibition of prothrombinase activity by APC at a plate-
Fig. 5. APC-dependent inactivation of prothrombinase activity associated with collagen-adherent platelets. Discs with collagen-adherent platelets were spun in buffer containing 3 mM CaCl$_2$, 50 pM factor Xa, and 100 nM prothrombin. Timed samples were removed and assayed for thrombin. At the indicated time (arrow) a small aliquot of buffer ($\bullet$) or 0.25 mM (○), 0.5 mM (△), or 1 mM APC (△) was added. The initial rates of thrombin formation in the absence of APC were set to the same value. The solid lines represent the best fit of Equations 1 and 2 to the data. The first order rate constants of inhibition thus obtained are shown as a function of the APC concentration in the insert.

Fig. 6. APC-dependent inactivation of prothrombinase activity associated with a synthetic phospholipid membrane. Rotating discs with planar phospholipid membranes composed of 25 mol % DOPS and 75 mol % DOPC were spun in buffer containing CaCl$_2$ (3 mM), factor Xa (100 pM), and factor Va (10 pM). After 5 min thrombin generation was started by the addition of prothrombin (100 nM). At the indicated time (arrow) a small aliquot of buffer ($\bullet$) or 0.5 mM (○), 1 mM (△), or 2 mM APC (△) was added. Timed samples were removed and assayed for thrombin. The solid lines represent the best fit of Equations 1 and 2 to these data. The first order rate constants of inhibition thus obtained are shown as a function of the APC concentration in the insert.

### Table 1

Effect of prothrombin concentration on APC-catalyzed inhibition of prothrombinase activity

| Prothrombin concentration (nM) | Rate constant of inhibition (min$^{-1}$) |
|-------------------------------|----------------------------------------|
| 20                            | 0.07                                   |
| 50                            | 0.07                                   |
| 100                           | 0.08                                   |
| 200                           | 0.05                                   |
| 500                           | 0.07                                   |

let surface was compared with that at a rotating planar phospholipid membrane composed of 25 mol % DOPS and 75 mol % DOPC. The experimental conditions for thrombin generation and inhibition were the same as described for collagen-adherent platelets. However, in addition to factor Xa (50 pM) and prothrombin (100 nM), plasma factor Va (10 pM) also was added. Typical thrombin generation curves in the absence or presence of APC are shown in Fig. 6. Thrombin generation was analyzed by a least squares fit of Equations 1 and 2 to the data. A plot of the first order rate constants of inactivation as a function of the APC concentration is shown as an insert on Fig. 6. Linear regression to these data yielded a second order rate constant of inhibition of prothrombin activation of 2.5 × 10$^6$ M$^{-1}$ s$^{-1}$.

**DISCUSSION**

It is generally believed that upon vessel wall injury the adhesion of platelets to exposed collagen stimulates thrombus formation. The interaction between platelets and immobilized collagen induces the release of the content of the α-granula, exposure of anionic phospholipids, and shedding of microparticles. As a result, highly reactive procoagulant platelets and microvesicles are generated in which factor Va from α-granula (17, 18) and anionic phospholipids (12) provide the essential accessory factors for the prothrombin-converting enzyme factor Xa.

This study focuses on the role of APC as an inhibitor of ongoing thrombin generation at the surface of collagen-adherent platelets. To account for the transport-limited supply of substrate at these surfaces (19, 20) and to approach the in vivo situation of thrombin formation under flow conditions, activation and inactivation experiments were performed utilizing saturating factor Xa and prothrombin concentrations in a previously described rotating disc device (15, 20–22).

**Thrombin Generation at the Surfaces of Collagen-adherent Platelets**—Initial experiments confirmed that platelet adhesion to collagen in the presence of extracellular calcium resulted in the formation of microparticles. As a matter of fact, about 60% of total thrombin generation could be attributed to prothrombinase associated with these microparticles. Because this study was focused on ongoing thrombin generation at adherent platelets, subsequent experiments were performed after the microparticles were removed from the reaction system.

To further characterize the kinetics of thrombin generation at the surfaces of collagen-adherent platelets, dependence on factor Xa and prothrombin concentration was determined. The apparent $K_d$ for factor Xa on collagen-adherent platelets was 3.5 pM. We note that this $K_d$ value is determined in the presence of a fixed prothrombin concentration (100 nM) but in the absence of both microparticles and exogenous factor Va. Much higher apparent $K_d$ values for factor Xa have been reported for thrombin-activated platelets in suspension ($K_d = 50$ pM) (26) but higher than the values of 5 and 7 nM for phospholipid bilayers in a tubular flow system (11) and for prothrombin activation experiments on rotating discs (20), respectively. However, the values reported for the tubular flow reactor were obtained after correction for prothrombin depletion near the catalytic surface. If the same correction is made here, a $K_d$ value of 14 nM would be obtained. Interestingly, the plasma prothrombin concentration is more than 100-fold higher, meaning that inhibitors like antithrom-
bin will have no chance to compete successfully with thrombin for the active site of prothrombinase (27, 28). It is, therefore, unlikely that proteinase inhibitors like antithrombin can regulate platelet-associated prothrombinase activity.

**APC-dependent Inhibition of Ongoing Thrombin Generation at Adherent Platelets**—It has been shown that platelets greatly accelerate the rate of APC-dependent inactivation of factor Va by providing a negatively charged phospholipid surface (29). However, it has also been reported that platelets show an APC-resistant phenotype. That is, despite the presence of APC, platelet-derived factor Va activity is sustained on the surface of thrombin-activated platelets (9–10, 30–31). The present report demonstrates that APC inhibits platelet-associated prothrombinase activity in a mono-exponential way with a second order rate constant of 3.3 × 10^6 M^−1 s^−1. This value is in excellent agreement with the second order rate constant of inhibition (2.5 × 10^6 M^−1 s^−1) found for prothrombinase associated with a planar synthetic phospholipid membrane composed of 25 mol % PS, 75 mol % PC. Moreover, the inhibition rates reported here for ongoing thrombin generation are also very close to the reported (32) rate constant for APC-catalyzed cleavage at Arg^306 in plasma-derived factor Va (k = 6.5 × 10^6 M^−1 s^−1).

Thus, in the experimental setup of the present study, which mimics physiologically relevant conditions, we observed complete inactivation by APC of the prothrombin-converting activity of the factor Va-factor Xa complex bound to collagen-aden- platelets. Moreover, no differences were found between the first order rate constant of inactivation of prothrombinase assembled at collagen-adenate platelets and at a synthetic phospholipid membrane. In contrast, Camire et al. (9) found different kinetics for the inactivation of factor Va at platelets and synthetic phospholipid membranes, with a slower, and more importantly, incomplete inactivation of factor Va at the membrane of platelets. We note that these investigators used thrombin-activated platelets and that the decline of factor Va cofactor activity was assayed from timed samples as prothrombinase activity therefore could be less critical than proposed (9, 10).

---

**REFERENCES**

1. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) *J. Biol. Chem.* 269, 31869–31880.
2. Tans, G., Nicolaes, G. A. F., and Rosing, J. (1997) *Semin. Hematol.* 34, 244–255.
3. Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., Pabinger, I., Varadi, K., Schwarz, H. P., and Rosing, J. (1995) *J. Biol. Chem.* 270, 21158–21166.
4. Comp, P. C., and Esmon, C. T. (1979) *Blood* 54, 1272–1281.
5. Walker, F. J., Sexton, P. W., and Esmon, C. T. (1979) *Biochim. Biophys. Acta* 571, 333–342.
6. Nesheim, M. E., Canfield, W. M., Kisiel, W., and Mann, K. G. (1982) *J. Biol. Chem.* 257, 1443–1447.
7. Suzuki, K., Stenflo, J., Dahlback, B., and Teodorsson, B. (1983) *J. Biol. Chem.* 258, 1914–1920.
8. Rosing, J., Hoekema, L., Nicolaes, G. A. F, Thomassen, M. C. L. G. D., Hemker, H. C., Varadi, K., Schwarz, H. P., and Tans, G. (1995) *J. Biol. Chem.* 270, 27852–27858.
9. Camire, R. M., Kalafatis, M., Simioni, P., Girolami, A., and Tracy, P. B. (1998) *Blood* 91, 2818–2829.
10. Taeue, J., McWilliam, N., Luddington, R., Byrne, C. D., and Baglin, T. (1999) *Blood* 93, 3792–3797.
11. Billy, D., Speijer, H., Willems, G. M., Hemker, H. C., and Lindhout, T. (1995) *J. Biol. Chem.* 270, 1029–1034.
12. Heemskerk, J. W. M., Biljinknormal, P., Vuijt, W. M. J., Breukers, G., Reutelingersperger, C. P. M., Barre, M. J., Knight, C. G., Lassila, R., and Farradale, R. W. (1999) *Thromb. Haemost.* 81, 782–792.
13. Schoen, P., Lindhout, T., Franssen, J., and Hemker, H. C. (1991) *Thromb. Haemost.* 66, 435–441.
14. Heemskerk, J. W. M., Vuijt, W. M. J., Feigen, M. H. A. B., Reutelingersperger, C. P. M., and Lindhout, T. (1999) *Blood* 90, 2615–2625.
15. Salesink, I., Franssen, J., Willems, G. M., Hemker, H. C., and Lindhout, T. (1999) *J. Biol. Chem.* 274, 28225–28232.
16. Rosing, J., Bakker, H. M., Thomassen, M. C. L. G. D., Hemker, H. C., and Tans, G. (1995) *J. Biol. Chem.* 268, 21133–21136.
17. Moskovic, D. and Tracy, P. B. (1990) *Biochemistry* 29, 2203–2210.
18. Giesen, P. L., Willems, G. M., Hemker, H. C., and Herskowitz, W. T. (1991) *J. Biol. Chem.* 266, 17820–17825.
19. Willems, G. M., Giesen, P. L., and Hermens, W. T. (1993) *Blood* 82, 497–504.
20. Shu, F. R., and Wilson, G. S. (1976) *Anat. Chem.* 48, 1670–1686.
21. Castner, J. P., and Winard, L. B. Jr. (1984) *Biochemistry* 23, 2203–2210.
22. Giesen, P. L., Willems, G. M., Hemker, H. C., and Herms, W. T. (1991) *J. Biol. Chem.* 266, 18720–18725.
23. Larson, P. J., Camire, R. M., Wong, D., Fasano, N. C., Monroe, D. M., Tracy, P. B., and High, K. A. (1996) *Biochemistry* 35, 5029–5038.
24. Swords, N. A., and Mann, K. G. (1993) *Arterioscler. Thromb.* 13, 1602–1612.
25. Rosing, J., Tans, G., Govers, Rieslman, J. W., Zwaal, R. F. A., and Hemker, H. C. (1980) *J. Biol. Chem.* 255, 274–283.
26. Billy, D., Speijer, H., Lindhout, T., Hemker, H. C., and Willems, G. M. (1995) *Biochemistry* 34, 13699–13704.
27. Speijer, H., Billy, D., Willems, G. M., Hemker, H. C., and Lindhout, T. (1995) *Thromb. Haemost.* 72, 648–653.
28. Tans, G., Rosing, J., Thomassen, M. C. L. G. D., Heeb, M. J., Zwaal, R. F. A., and Griffin, J. H. (1991) *Blood* 77, 2641–2648.
29. Sedlmann, J., Gram, J., Pedersen, O. D., and Jespersen, J. (1995) *Thromb. Haemost.* 73, 993–994.
30. Shirzoda, T., Kanda, A., Amagai, H., and Kobayashi, I. (1995) *Thromb. Res.* 78, 199–210.
31. Hoekema, L., Nicolaes, G. A. F, Hemker, H. C., Tans, G., and Rosing, J. (1997) *Biochemistry* 36, 3331–3335.
32. Bevers, B. M., Comfurius, P., and Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta* 736, 57–66.
33. Dörmann, D., Cleemput, K. J., and Kehrel, B. E. (2000) *Blood* 96, 2469–2478.
34. Smirnov, M. D., Safa, O., Esmon, N. L., and Esmon, C. T. (1999) *Blood* 94, 3839–3846.