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

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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 prothrombin 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 3.3×10^6 M^{-1} s^{-1}, which was independent of the prothrombin concentration over a wide range around the apparent K_m for prothrombin. 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 (2.5×10^6 M^{-1} 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)[1] 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^{506} is relatively rapid and yields a reaction intermediate that still retains partial cofactor activity in prothrombin activation. The slower cleavage at position Arg^{306} 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 prothrombin 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 apyrase were from Sigma. S2238, a chromogenic substrate for thrombin, was obtained from Chromogenix (Mölndal, 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 (Alabaster, 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

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[1] The abbreviations used are: APC, activated protein C; BSA, bovine serum albumin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; PC, phosphatidylcholine; PS, phosphatidylserine.

with HEPES buffer (10 mM HEPES, 136 mM NaCl, 5 mM glucose, 2.7 mM KCl, 2 mM $MgCl_2$, 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 5×10^7 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 reactants 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 $CaCl_2$. 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 μl) to the cuvette. The change in optical density was monitored at 405 nm. The thrombin concentrations in the samples were calculated from a standard curve obtained with known amounts of the enzyme. All procedures were performed at 37 °C.

Assay for Procoagulant Microvesicles—Samples (10 μl) from the reaction vessel were added to cuvettes containing factor Xa, factor Va, and 3 mM $CaCl_2$ in 137 μl of Tris buffer. After a 3-min incubation, thrombin generation was started by adding 3 μl of prothrombin. The final concentrations were: 1 pM factor Xa, 0.5 nM factor Va, and 200 nM prothrombin. Thrombin generation was stopped after 5 min by the addition of Tris-EDTA buffer and assayed as described. A reference curve was constructed using different phospholipid vesicle (25 mol % DOPS, 75 mol % DOPC) concentrations and was linear up to 1 μM.

Kinetic Data Analysis of Time Courses of APC-dependent Inactivation of Prothrombinase—The rate of thrombin generation at the surface of the rotating disc decreases in time especially when prothrombin concentrations are used below the apparent K_m for prothrombin. Thrombin generation in the presence of APC was therefore corrected for substrate depletion by assuming an APC-independent pseudo first order rate constant of inactivation, k_1, utilizing the equation

$$[\text{thrombin}]_t = V_0(1 - e^{-k_1 t})/k_1 \qquad \text{(Eq. 1)}$$

in which $[\text{thrombin}]_t$ is the thrombin concentration at time t, and V_0 the initial rate of thrombin formation. Inhibition of prothrombin activation in the presence of APC was analyzed according to

$$[\text{thrombin}]_t = [\text{thrombin}]_{t_i} + V_0 e^{-k_1 t_i}(1 - e^{-(k_2+k_1)(t-t_i)})/(k_2 + k_1)$$

$$\text{for } t > t_i \qquad \text{(Eq. 2)}$$

with t_i the time at which APC was added to the reaction and k_2 the APC-dependent pseudo first order rate constant of prothrombin activation inhibition. Values for V_0, k_1, and k_2 were estimated by a least square fit of Equations 1 and 2 to the experimental data obtained from thrombin generation experiments performed in the absence or presence of APC.

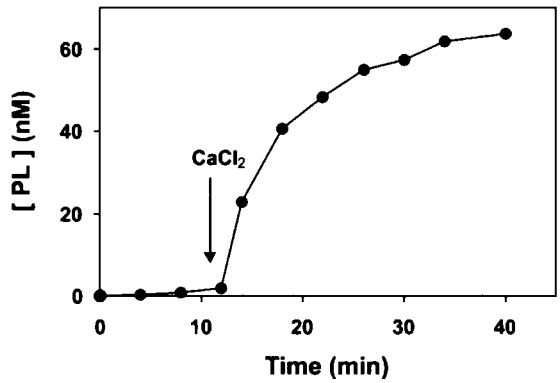

FIG. 1. **Extracellular calcium-induced shedding of procoagulant microvesicles.** A collagen-coated circular glass coverslip with adherent platelets was spun at 63 rad/s in calcium-free buffer A (pH 7.4). At the indicated time (*arrow*) 3 mM $CaCl_2$ was added. Timed samples were taken and assayed for procoagulant activity as described under "Experimental Procedures."

RESULTS

Thrombin Formation at the Plasma Membrane of Collagen-adherent Platelets—Platelet adhesion on immobilized collagen in the presence of extracellular calcium is accompanied by the shedding of microparticles and exposure of negatively charged 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 $CaCl_2$ (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) followed 3 min later by prothrombin (100 nM) were added 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 microvesicles 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 $CaCl_2$ 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-

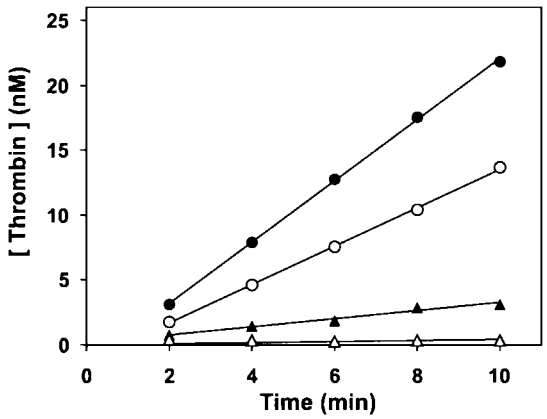

FIG. 2. **Platelet surface and microvesicle-associated thrombin generation.** A collagen-coated coverslip with adherent platelets was spun at 63 rad/s for 30 min in buffer A containing 3 mM $CaCl_2$. Factor Xa (50 pM) was added followed by prothrombin (100 nM) to start thrombin generation. Immediately after the addition of prothrombin an aliquot (100 μl) was taken and incubated at 37 °C. Timed samples were taken from both the rotating disc reaction vessel and the removed aliquot to determine, respectively, the rate of total thrombin generation (●) and the rate of fluid phase thrombin generation (○). After 10 min the reaction vessel was flushed with 3 mM $CaCl_2$-containing buffer A for 5 min at 10 ml/min. Factor Xa (50 pM) and prothrombin (100 nM) were re-added. An aliquot (100 μl) was taken from the reaction vessel and further incubated at 37 °C. Timed samples from the reaction vessel (▲) as well as from the incubation mixture (△) were assayed for thrombin.

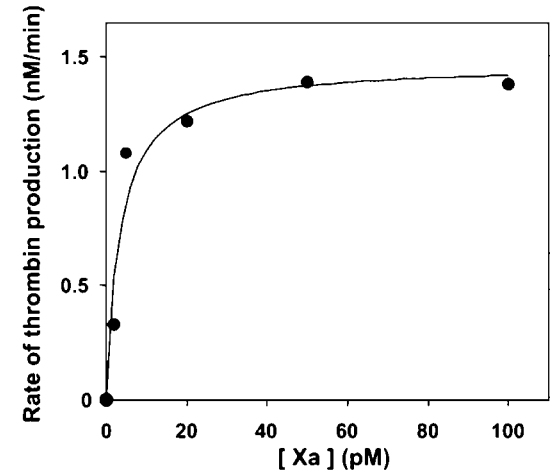

FIG. 3. **Rate of thrombin formation at collagen-adherent platelets as a function of factor Xa concentration.** A disc with collagen-adherent platelets was spun in buffer A containing 3 mM $CaCl_2$ and 100 nM prothombin. The initial rates of thrombin formation were measured for increasing concentrations of factor Xa. The *solid line* is the result of the fit procedure described under "Results."

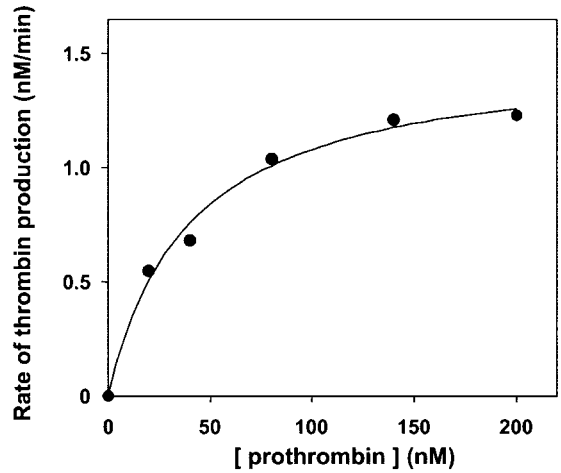

FIG. 4. **Rate of thrombin formation at collagen-adherent platelets as a function of the prothrombin concentration.** A disc with collagen-adherent platelets was spun in buffer A containing 3 mM $CaCl_2$ and 50 pM factor Xa. The initial rates of thrombin formation were measured for increasing concentrations of prothrombin. The *solid line* is the result of the fit procedure described under "Results."

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 $CaCl_2$ 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, $V_{obs} = V_{max}$ [Xa]/([Xa] + K_d), with V_{obs} the initial rate of thrombin formation, [Xa] the factor Xa concentration, and V_{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 ± 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, $V_{obs} = V_{max}$ [prothrombin]/([prothrombin] + K_m,app), in which V_{max} is the initial rate of thrombin formation at a saturating prothrombin concentration, [prothrombin] the prothrombin concentration in free solution, and K_m(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_m(app) of 42 ± 5 nM (estimated value ± 1 S.E.).

APC-dependent Inhibition of Thrombin Formation at the Plasma Membrane of Collagen-adherent Platelets during Ongoing 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 3.3×10^6 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-

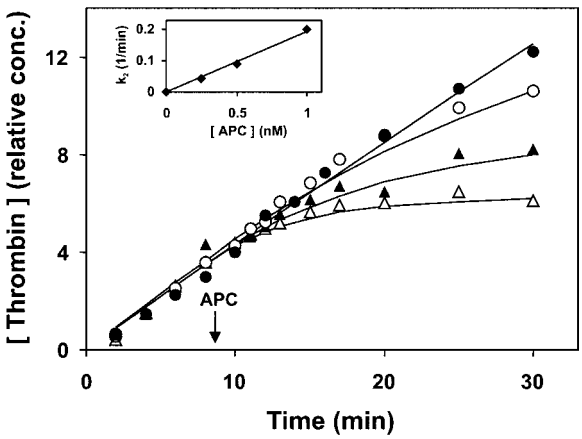

FIG. 5. **APC-dependent inactivation of prothrombinase activity associated with collagen-adherent platelets.** Discs with collagen-adherent platelets were spun in buffer A containing 3 mM $CaCl_2$, 50 pM factor Xa, and 100 nM prothrombin. Timed samples were removed and assayed for thrombin. At the indicated time (*arrow*) a small aliquot of buffer (●) or 0.25 nM (○), 0.5 nM (▲), or 1 nM 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*.

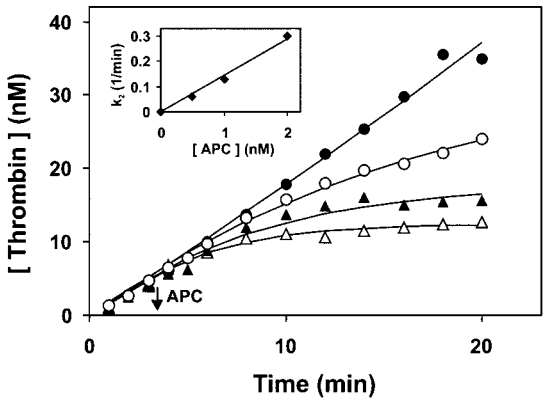

FIG. 6. **APC-dependent inactivation of prothrombinase activity associated with a synthetic phospholipid membrane.** Rotating discs with planar phospholipid membranes composed of 25 mol % DOPS and 75 mol % DOPC were spun in buffer A containing $CaCl_2$ (3 mM), factor Xa (100 pM), and factor Va (10 pM). After 5 min thrombin generation was started by the addition of prothrombin (100 nM). At the indicated time (*arrow*) a small aliquot of buffer (●) or 0.5 nM (○), 1 nM (▲), or 2 nM 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 I
Effect of prothrombin concentration on APC-catalyzed inhibition of prothrombinase activity

Discs with collagen-adherent platelets were spun in buffer A containing 3 mM $CaCl_2$, 50 pM factor Xa, and the indicated prothrombin concentrations. After 3 min, 0.5 nM APC was added, and the pseudo first order rate constants of inhibition by APC were calculated by fitting the thrombin generation data as described under "Experimental Procedures."

Prothrombin concentration	Rate constant of inhibition
nM	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 microvesicles. 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 microvesicles and exogenous factor Va. Much higher apparent K_d values for factor Xa have been reported for thrombin-activated platelets in suspension (K_d = 142 pM) (24) and for von Willebrand factor-adherent platelets (K_d = 4 nM) (25), but the value found here is in close agreement with the value of 1 pM reported for the interaction of factor Xa with planar phospholipid surface composed of 25% PS, 75% PC and containing preabsorbed factor Va (23).

The prothrombin concentration in the solution required to obtain a half-maximal rate of thrombin generation was 42 nM. This apparent K_m value is lower than the value reported for vesicles in suspension (K_m = 100 nM) (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_m 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 prothrombin 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 inactivation 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 ($k = 6.5 \times 10^6$ 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-adherent platelets. Moreover, no differences were found between the first order rate constant of inactivation of prothrombinase assembled at collagen-adherent 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 most 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 using a high factor Xa concentration. Thrombin, however, has been demonstrated to be a rather weak agonist in inducing exposure of negatively charged phospholipids (PS) in the outer leaflet of the platelet plasma membrane (14, 33). In addition, Camire *et al.* (9) prevented thrombin-activated platelets from aggregation by using the RGDS peptide and did not stir the platelet suspension. It was recently reported that these conditions prevent thrombin-induced exposure of PS (34). In view of the stimulating role of negatively charged phospholipids on the kinetics of the cleavage of factor V at Arg^{306} (32), it is tempting to speculate that under their experimental conditions (9), insufficient platelets provide the suitable surface to bind factor Va and to stimulate APC activity. Although this notion would explain the finding that part of the factor Va escaped inactivation, it is contradicted by their observation that exogenous plasma-derived factor Va added to a thrombin-activated platelet suspension was rapidly inactivated (9).

Recently, it was reported that prothrombin dramatically inhibits the ability of APC to inactivate factor Va but scarcely inhibits the inactivation of factor VaLeiden (35). Moreover, it was suggested that prothrombin inhibits the cleavage at both Arg^{506} and Arg^{306}. In contrast, our experiments clearly demonstrate that the rate of inactivation of platelet-bound prothrombinase by APC is not slowed down even under conditions that result in full saturation of the prothrombinase complex with its substrate prothrombin (*cf.* Fig. 4, Table I). At present we have no explanation for these deviating results.

In summary, our data show that APC is an efficient inhibitor of platelet-dependent thrombin generation. The half-life of prothrombinase in the presence of 1 nM APC and under the conditions of the experiment is 5 min. Whereas it has been reported that platelet-bound factor Va is resistant to APC, our results clearly indicate that platelet-bound factor Va, as part of the prothrombinase complex, is inactivated by APC with a rate that is comparable with that found on a membrane of synthetic phospholipids. Sustained platelet-derived factor Va cofactor activity therefore could be less critical than proposed (9, 10).

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