

## On the Mechanism of the Antifibrinolytic Activity of Plasma Carboxypeptidase B\*

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The precursor of plasma carboxypeptidase B (pCPB) also known as thrombin-activable fibrinolysis inhibitor can be converted by thrombin to an active enzyme capable of eliminating C-terminal Lys- and Arg-residues from proteins. The activation is about 1000-fold more efficient in the presence of thrombomodulin (TM). We investigated the antifibrinolytic potency of maximally activated pCPB in plasma and explored the antifibrinolytic mechanism of pCPB. During clotting of plasma in the presence of 3.3 NIH units/ml thrombin and 1  $\mu$ g/ml soluble TM, more than 80% pro-pCPB was converted into the active form causing an increase of plasma carboxypeptidase activity from 100 units/liter (constitutive activity ascribed to plasma carboxypeptidase N) to 430 units/liter as measured with furoylacroleyl-alanyl-arginine substrate. Under these conditions, lysis of a plasma clot induced by a range of tissue-type plasminogen activator (t-PA) concentrations (0.2–2  $\mu$ g/ml) was retarded more than 4-fold. A considerable retardation of fibrinolysis was observed upon addition of as little as 12 ng/ml soluble TM, a concentration comparable with physiological concentrations of soluble TM in human plasma. The presence of  $Ca^{2+}$  appeared to be a critical requirement for effective activation of pro-pCPB by thrombin-TM in plasma. Plasminogen-binding sites (C-terminal lysines) on the surface of a plasmin-treated fibrin clot were eliminated within 1–3 min by plasma with maximally activated pCPB, as studied in a recently described model involving fluorescence microscopy. Confocal fluorescence microscopy showed that in the absence of TM plasminogen strongly accumulated on fibrin fibers during t-PA-induced lysis of a plasma clot. In the presence of TM (and a concomitant pro-pCPB activation), lysis was slow and was not accompanied by accumulation of plasminogen on the fibers. In conclusion, generation of active pCPB during clotting of plasma in the presence of  $Ca^{2+}$  and TM leads to a retardation of plasma clot lysis in a wide range of t-PA concentrations, from low to therapeutic, and to a fast elimination of plasminogen-binding sites on partially degraded fibrin. This is a likely mechanism for the antifibrinolytic effect of active pCPB.

Recently, a new plasma carboxypeptidase B (pCPB)<sup>1,2</sup> was discovered, purified, and characterized by three different groups (1–6). The molecule circulates in plasma as an inactive zymogen pro-pCPB, which can be converted to an active enzyme by plasmin (PI) or thrombin (3, 4, 6). Active pCPB is rather unstable at 37 °C (1, 2, 4). Thrombomodulin (TM) has recently been shown to accelerate the activation of pCPB by thrombin by about 3 orders of magnitude (7). Another basic carboxypeptidase, Cp N, is constitutively active in plasma and differs from pCPB in terms of substrate specificity toward peptide substrates, esterase activity, and pH optimum, although both enzymes are capable of eliminating C-terminal Arg or Lys residues (1, 2, 4). The two carboxypeptidases also have a different sensitivity to a number of inhibitors (2, 4, 8). For instance, pCPB can be completely inhibited by potato carboxypeptidase inhibitor (PCI) at concentrations where Cp N is not affected (8). Another Cp inhibitor (2-guanidinoethylmercapto)succinic acid (GEMSA) inhibits both enzymes at a concentration of 1 mM (4, 8).

PCPB is a molecule mediating a negative link between coagulation and fibrinolysis. The existence of such a molecule had been predicted almost a decade ago (9, 10) on the basis of the data on the pro-fibrinolytic effect of activated protein C (9–13). It has been demonstrated recently that this effect as well as the acceleration of fibrinolysis in factor XI-deficient plasma (14) is mediated by a reduction of thrombin-induced activation of pro-pCPB (15–17). Premature lysis of clots formed from factor VIII-, IX-, X-, and XI-deficient plasmas can be prevented by supplementation of the deficient plasmas with TM through the mechanism involving thrombin-TM-dependent pCPB activation (18). Retardation of fibrinolysis by activated pCPB has been shown in a system of purified components (6), in whole blood clots (8), in clots prepared from diluted plasma (15–18) and in an animal model (19). In contrast, Cp N is not capable of inhibiting fibrinolysis (8, 18).

The mechanism of antifibrinolytic activity of pCPB may involve direct inactivation of PI by pCPB as demonstrated recently in a purified system (20). Another possible mechanism may depend upon elimination of C-terminal Lys-residues generated on fibrin as a result of its partial cleavage by PI during fibrinolysis. These residues are involved in a positive feedback regulation of fibrinolysis serving as additional binding sites for plasminogen (Pg), which acquires a more activable conforma-

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<sup>1</sup> The abbreviations used are: pCPB, plasma carboxypeptidase B; Cp, carboxypeptidase; TM, thrombomodulin; Pg, plasminogen; PI, plasmin; t-PA, tissue-type plasminogen activator; FAAR, furoylacroleyl-alanyl-arginine; GEMSA, (2-guanidinoethylmercapto)succinic acid; PCI, potato carboxypeptidase inhibitor; FITC, fluorescein isothiocyanate; TBS, Tris-buffered saline; BSA, bovine serum albumin; KIU, kallikrein-inactivating unit(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

<sup>2</sup> The same enzyme is also known as CpU (unstable carboxypeptidase) and TAFI (thrombin-activable fibrinolysis inhibitor).

tion upon binding (21–25). Also, binding of PI to these sites would protect it from inhibition by  $\alpha_2$ -antiplasmin (26). Although the removal of C-terminal lysines is believed to be a likely mechanism of action of pCPB (2–8, 15, 16), there is no experimental evidence that it may play a significant role in a plasma environment. It is not clear whether active pCPB can be generated in plasma to an extent sufficient for effective displacement of C-terminal lysines from partially digested fibrin. In addition, the published data on the *in vitro* experiments with plasma clots (14–18) demonstrate pCPB-dependent retardation of lysis only at low t-PA concentrations (30–80 ng/ml). It is not clear whether the pCPB system is potent enough to down-regulate therapeutic fibrinolysis induced by much higher doses of t-PA (1–4  $\mu$ g/ml; Refs. 27 and 28).

To answer these questions, we performed a number of experiments to find the conditions for maximal activation of proCPB in undiluted plasma. Then, we investigated the potency of maximally activated plasma to inhibit fibrinolysis and to eliminate Pg-binding sites from degraded fibrin using a recently described fluorescence microscopy technique (29–31).

#### EXPERIMENTAL PROCEDURES

**Preparations**—Glu-Pg was a product of Biofine (Leiden, The Netherlands). Fluorescein isothiocyanate (FITC), human thrombin, and CHAPS were from Sigma. Human fibrinogen and PI (activity 15 casein units/mg) were products of Kabi (Stockholm, Sweden). Aprotinin was from Bayer (Leverkusen, Germany). The carboxypeptidase inhibitors, PCI and GEMSA, were from Calbiochem (La Jolla, CA) and Fluka (Buchs, Switzerland), respectively. The carboxypeptidase substrate furylacrolelyl-alanyl-arginine (FAAR) was from Bachem (Bubendorf, Switzerland). Rabbit lung TM (American Diagnostica, Greenwich, CT) was used in the study with activity of 1.2 units/ $\mu$ g (1 unit is defined as the amount of TM that complexes 1 NIH unit of thrombin). Pooled citrated platelet-poor plasma from healthy volunteers was used in all experiments involving plasma. Labeling of Glu-Pg with FITC was performed as described previously (30, 31), resulting in a preparation with a molar ratio fluorescein/Pg of 2.

**Enzymatic Assay of Cp**—Plasma was clotted with thrombin (final concentration 3.3 NIH units/ml) in the absence or presence of TM (final concentration 1.25 units/ml). When indicated,  $\text{CaCl}_2$  was added to a final concentration of 20 mM prior to clotting. After incubation at 37 °C during varying time intervals, the clots were squeezed and discarded. A 7.5- $\mu$ l aliquot of the remaining fluid phase was transferred to a 1-cm quartz microcuvette containing 550  $\mu$ l of FAAR (final concentration 0.45 mM) in a buffer containing 20 mM Tris-HCl, 75 mM NaCl, and 0.1% CHAPS, pH 7.8. The decrease in optical density at 336 nm was recorded at 22 °C for 15 min with 0.5-min intervals using a Kontron Uvicon-930 spectrophotometer. The Cp activity was calculated from the decrease of  $A_{336}$ ; a drop in optical density of 0.001/min was defined as 1 unit/liter. This unit is approximately equal to the unit of Cp activity defined by Redlitz *et al.* (8).

The activity test was designed to follow pCPB and pro-pCPB in the samples, which also contained constitutively active Cp N interfering with the assay. To distinguish these Cp activities, the measurements were performed for each sample in three parallel cuvettes. In the first cuvette, the constitutive activity (Cp N) in the sample was measured in the presence of 20  $\mu$ g/ml PCI (a concentration inhibiting pCPB, but not Cp N). The second cuvette contained no inhibitor; thus, the total Cp activity in the sample (Cp N + pCPB) was measured. The potential Cp activity (Cp N + pCPB + pro-pCPB) was determined in the third cuvette by the addition of preformed thrombin-TM complex to a final concentration of 0.125 unit/ml and  $\text{CaCl}_2$  to a final concentration of 5 mM. Under these conditions pro-pCPB was activated within 5 min. No additional activation could be achieved by increasing the concentration of thrombin-TM or by subsequent addition of a new portion of thrombin-TM, suggesting that the pro-pCPB activation was complete under the selected conditions. (Note: in undiluted plasma, a higher concentration of thrombin-TM was required for maximal pro-pCPB activation, supposedly, due to faster inactivation of thrombin-TM.) Differences between groups of measurements (constitutive, total, and potential activity) for each sample were tested for statistical significance with the paired Student's *t* test.

Similar measurements were performed in unclotted plasma, except the potential Cp activity determination, which could not be measured

due to clotting of diluted plasma in the cuvette upon addition of thrombin-TM.

**Plasma Clot Lysis Assay**—20  $\mu$ l of human thrombin (20 NIH units/ml) was mixed with 4  $\mu$ l of TM (serial dilutions starting from 30 units/ml) in the wells of a microtiter plate. Then prewarmed citrated plasma (100  $\mu$ l) containing t-PA (final concentration in the range 0.05–2  $\mu$ g/ml) added to plasma immediately prior to mixing with thrombin-TM) was added. Plasma was either recalcified by the addition of  $\text{CaCl}_2$  to a final concentration of 20 mM or not, as indicated. Where indicated, GEMSA was added to plasma to a final concentration of 1 mM.

The optical density at 405 nm was measured during incubation at 37 °C by using a Titertek Multiskan. Upon lysis of the clots, the optical density decreased by about 0.5. Lysis time was defined as the time when a 50% decrease in the optical density occurred.

**Experiments with Purified Fibrin Clots**—Purified fibrin clots of approximately 2.5 mm in diameter were prepared as described previously (29, 30) by clotting of fibrinogen (9.2  $\mu$ M) with thrombin (1.4 NIH units/ml) between two parallel glass slides in a 20 mM Tris-HCl buffer, pH 7.6, containing 135 mM NaCl (TBS) and 20 mg/ml BSA (TBS-BSA). The remaining volume of the chambers (approximately 25  $\mu$ l) was filled with TBS-BSA and kept for 20 min at room temperature. Then the clots were subjected to the action of PI by replacing the buffer in the chambers with TBS-BSA containing 0.15 casein units/ml PI. After 60 min incubation at 37 °C, the action of PI was stopped by replacing the PI-containing buffer by TBS-BSA containing 200 KIU/ml aprotinin. During the treatment with PI, clot diameters were reduced by approximately 1 mm. The partially digested clots were incubated for different time intervals with the fluid phase from plasma clotted for 10 min with thrombin (3.3 NIH units/ml) either in the presence or in the absence of thrombomodulin (1 unit/ml), as described above. When indicated PCI was added to the fluid phase of clotted plasma immediately after removal of the clot. After the incubation, the plasma samples were washed out with TBS-BSA containing 200 KIU/ml aprotinin and 20  $\mu$ g/ml PCI, and FITC-Pg (0.5  $\mu$ M) was added to the clots in the same buffer. After complete equilibration for 20 h at room temperature, the clots were photographed with a fluorescence microscope (Microphot FXA, Nikon).

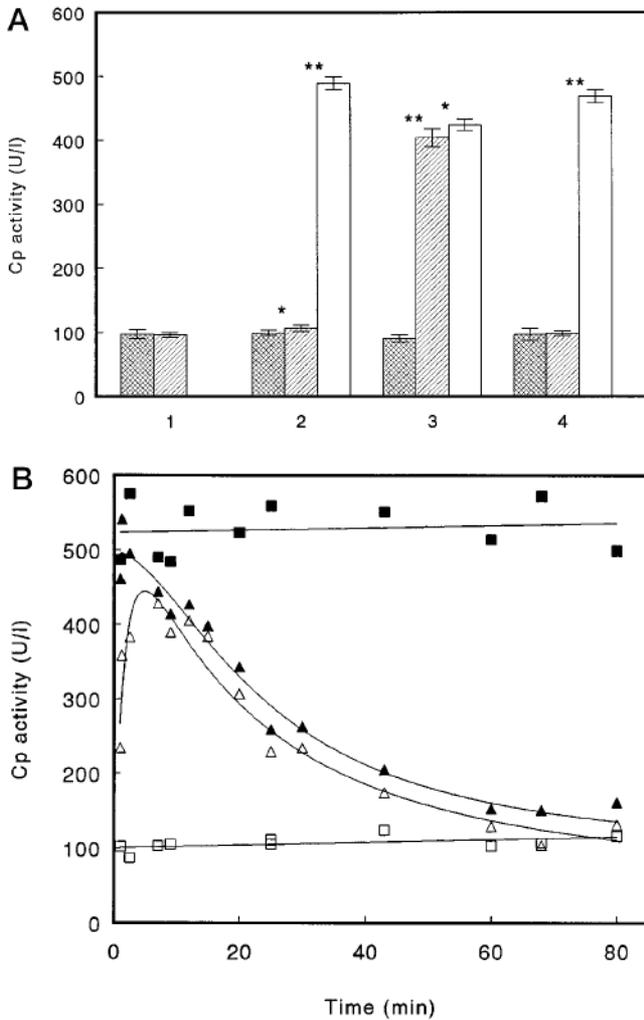
**Confocal Microscopy Experiments**—t-PA (final concentration 1.5  $\mu$ g/ml) was added to recalcified plasma containing tracer FITC-Pg (0.3  $\mu$ M), and plasma was immediately clotted in a chamber as described above with thrombin (3.3 NIH units/ml) either in the presence or in the absence of TM (1 unit/ml). Confocal images were taken at indicated time intervals at room temperature using a confocal laser scanning fluorescence microscope (MRC-600 Laser Sharp, Bio-Rad Microscience Ltd., Hemel Hempstead, United Kingdom), as described previously (31). The design of the system allowed the collection of images periodically during the ongoing lysis. To exclude the disturbing influence of the clot/glass interface, all images were taken at a distance of 50  $\mu$ m from the surface of the glass.

#### RESULTS

**Activation of Pro-pCPB in Plasma**—The Cp activity was measured using the synthetic substrate FAAR. The design of the test (see “Experimental Procedures”) allowed us to assess, in parallel, 1) the constitutive activity (Cp N), not sensitive to inhibition by PCI; 2) the total activity in the sample (Cp N + pCPB); and 3) the potential activity (Cp N + pCPB + pro-pCPB), as measured upon activation of pro-pCPB. In plasma, the total Cp activity ( $96 \pm 4$  units/liter, mean  $\pm$  S.D.,  $n = 3$ ) did not differ from the constitutive activity ( $97 \pm 7$  units/liter), indicating that only Cp N but not pCPB was active in plasma (Fig. 1A, *sample 1*).

After clotting of recalcified plasma with thrombin (Fig. 1A, *sample 2*), a total Cp activity of  $106 \pm 5$  units/liter was found in the fluid phase, which implies a minor but significant increase of Cp activity above the constitutive level of  $99 \pm 4$  units/liter ( $p < 0.05$ ). This additional Cp activity can be ascribed to pCPB, since it was inhibited by PCI. The potential activity in the sample was  $489 \pm 10$  units/liter. Thus, about 380 units/liter Cp activity was present in the sample in a latent form (pro-pCPB).

When TM was added to the clotting mixture (Fig. 1A, *sample 3*), a strong increase of total Cp activity was observed in the fluid phase (up to  $404 \pm 14$  units/liter), exceeding the constitutive Cp level about 4-fold. The potential Cp activity decreased

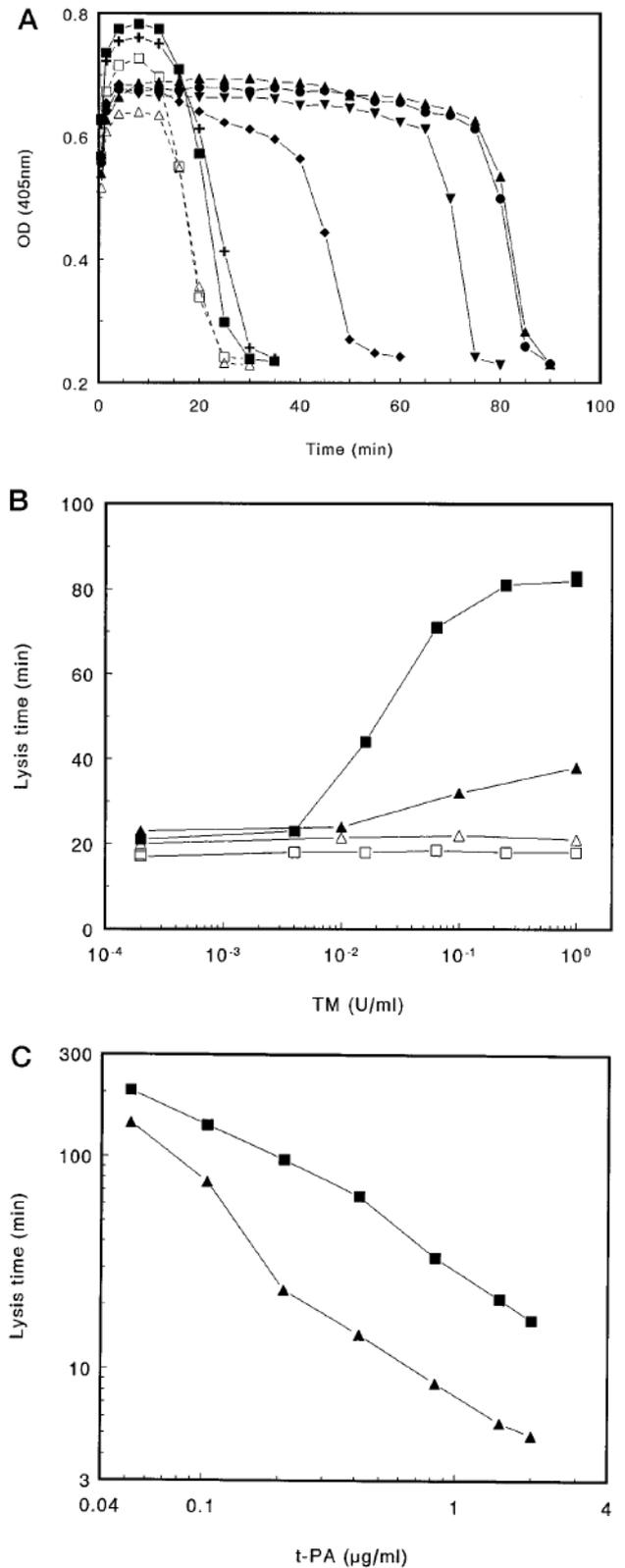


**FIG. 1.** Effect of TM on generation of Cp activity in plasma upon clotting. *A*, sample 1, plasma; samples 2-4, fluid phase of plasma clotted during 10 min at 37 °C. In sample 2, plasma was clotted with thrombin in the presence of calcium; in sample 3, plasma was clotted with thrombin-TM in the presence of calcium; in sample 4, plasma was clotted with thrombin-TM in the absence of calcium. Final concentrations of thrombin and TM were 3.3 NIH units/ml and 1.25 units/ml, respectively. For each sample, the constitutive activity (Cp N, left bar), total activity (Cp N + pCPB, middle bar), and potential activity (Cp N + pCPB + pro-pCPB, right bar) are presented as mean  $\pm$  S.D.,  $n = 3$ . The asterisks indicate the statistical difference between the results of measurements represented by neighboring bars, determined by using the paired Student's *t* test: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ . In sample 1, the potential activity could not be determined (see "Experimental Procedures"). *B*, kinetics of generation of Cp activity at 37 °C. Plasma was clotted by 3.3 NIH units/ml thrombin either in the presence (triangles) or in the absence (squares) of 1.25 units/ml TM. Open symbols, the total Cp activity present in the samples (Cp N + pCPB) after indicated time intervals; closed symbols, potential Cp activity (Cp N + pCPB + pro-pCPB).

to  $424 \pm 9$  units/liter. From this, we could calculate that the pool of latent Cp (pro-pCPB) was reduced from 380 units/liter to 20 units/liter under these conditions.

Generation of Cp activity by thrombin-TM complex was strongly  $Ca^{2+}$ -dependent. In the absence of  $Ca^{2+}$ , no significant increase of Cp activity over the constitutive level was found (Fig. 1A, sample 4).

The decrease of the potential activity in sample 3 as compared with the potential activity in sample 2 could be explained by partial inactivation of generated pCPB due to its instability (1, 2, 4), implying that the strong elevation of pCPB upon clotting of plasma with thrombin-TM may be transient. This



**FIG. 2.** Thrombomodulin-induced retardation of plasma clot lysis. Citrated plasma with added t-PA was clotted with 3.3 NIH units/ml thrombin in the presence of varying concentrations of TM. Closed symbols, plasma was recalcified with 20 mM  $CaCl_2$ ; open symbols, without calcium. *A*, kinetics of plasma clot lysis by 0.35 µg/ml t-PA in the presence of 1 unit/ml (▲, △), 0.25 unit/ml (●), 0.064 unit/ml (▼), 0.016 unit/ml (◆), and 0.004 unit/ml (+) TM and without TM (■, □). *B*, lysis time dependence (0.35 µg/ml t-PA) on TM concentration either in the absence (■, □) or in the presence (▲, △) of 1 mM GEMSA. *C*, lysis time dependence on t-PA concentration either in the presence (■) or in the absence (▲) of 0.6 unit/ml TM.

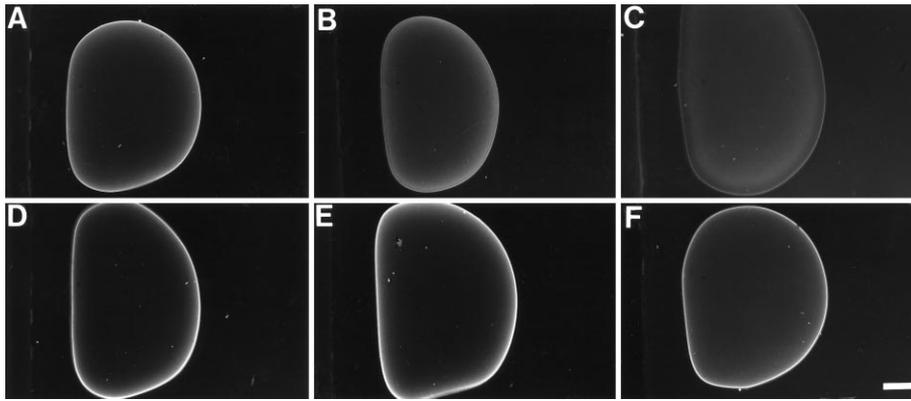


FIG. 3. Displacement by clotted plasma of Pg-binding sites from the surface of a PI-treated fibrin clot. Prior to addition of FITC-Pg, clots shown in A-C were preincubated during 20 s, 1 min, and 3 min, respectively, with the fluid phase of plasma clotted with thrombin (3.3 NIH units/ml) in the presence of TM (1 unit/ml) for 10 min; D, a control clot, not preincubated with plasma; E, a clot preincubated during 5 min with the fluid phase of plasma clotted with thrombin (3.3 NIH units/ml) without TM; F, a clot preincubated during 5 min with thrombin-TM-clotted plasma (3.3 NIH units/ml thrombin, 1 unit/ml TM) to which 20  $\mu\text{g/ml}$  PCI was added. Bar, 400  $\mu\text{m}$ .

was confirmed in Fig. 1B, which shows the kinetics of the generation of Cp activity in recalcified citrated plasma upon clotting with 3.3 NIH units/ml thrombin either in the absence or in the presence of 1.25 units/ml TM. In both cases, the constitutive Cp activity (not shown) did not change noticeably for 80 min after clotting, remaining within a range of 93–99 units/liter. Without TM, the total Cp activity almost did not change in time and was slightly higher than the constitutive level; the potential activity also did not change significantly. In the presence of TM, much more Cp activity was generated. The total activity in the sample reached a maximal value of 430 units/liter after a few minutes, and it dropped back to the constitutive level within about 60 min. Under these conditions, the pool of latent Cp activity was almost completely depleted. Concentrations of TM lower than 1.25 units/ml (not shown) generated less Cp activity and caused only a partial depletion of latent Cp.

**Inhibition of Plasma Clot Lysis in the Presence of Thrombin-TM**—Fig. 2 (A and B) shows the t-PA-induced lysis of plasma clots formed in the presence of various concentrations of soluble TM. In the presence of calcium, TM caused a dose-dependent prolongation of the lysis time, more than 4-fold at TM concentrations higher than 0.1 unit/ml (approximately 80 ng/ml). Noticeable retardation of lysis was documented at a concentration of TM as low as 0.016 unit/ml (about 12 ng/ml). In the absence of  $\text{Ca}^{2+}$ , TM had no effect on the clot lysis even at the highest concentration used (1 unit/ml, about 800 ng/ml). A specific carboxypeptidase inhibitor, GEMSA, largely inhibited the TM-induced retardation of lysis in the presence of calcium, as shown in Fig. 2B. In the absence of calcium, GEMSA caused a slight prolongation of lysis time, not dependent on the presence of TM. Fig. 2C shows that the inhibitory effect of TM on fibrinolysis was manifested throughout a wide range of t-PA concentrations and was most significant at the concentrations of 0.2–2  $\mu\text{g/ml}$  t-PA. The presence of TM resulted in a 4–5-fold prolongation of lysis time; 7–8-fold more t-PA was required to achieve the same lysis time.

**pCPB-induced Displacement of Plasminogen-binding Sites from Partially Degraded Fibrin**—In the experiments presented in Fig. 3, we studied the effect of plasma with either activated or unactivated pro-pCPB on Pg-binding sites present on fibrin partially digested by PI. In agreement with our earlier results (30), a PI-treated fibrin clot accumulated a high concentration of FITC-labeled Pg in a thin superficial layer (D). Incubation of such PI-treated clots with the fluid phase of thrombin-clotted plasma (E) for 5 min prior to the incubation with FITC-labeled Pg did not affect the superficial Pg-binding sites noticeably. In

contrast, the fluid phase of plasma clotted with thrombin in the presence of TM under conditions of maximal pCPB activation largely eliminated the Pg-binding sites within a few minutes (images A–C). The effect was abolished by the addition of the pCPB inhibitor PCI (F).

The fast elimination of Pg-binding sites on partially degraded fibrin observed in Fig. 3 suggests that, when activated in plasma, pCPB might reduce the amount of Pg accumulated on fibrin during lysis. This was confirmed directly in experiments involving confocal microscopy (Fig. 4). In agreement with our earlier findings (31), in the absence of TM and activation of pro-pCPB, lysis of a plasma clot was accompanied by a progressive accumulation of Pg onto fibrin fibers and onto shrinking remnants of the partially disconnected fibrin network (images B–E). In the presence of TM and concomitant activation of pCPB, lysis lasted about 5-fold longer (70 min instead of 14 min) and was not accompanied by accumulation of additional Pg onto the fibers (G–J). In fact, in this case, the degree of Pg association with the fibrin network did not exceed that observed in intact plasma clots either in the absence (A) or in the presence of TM (F).

#### DISCUSSION

This study was designed 1) to find conditions for maximal pCPB activation in plasma, 2) to assess the antifibrinolytic potency of maximally activated pCPB in plasma, and 3) to explore the mechanism of the antifibrinolytic activity of activated pCPB.

Maximal activation of pro-pCPB was achieved when plasma was clotted with thrombin in the presence of TM. Calcium ions appeared to be essential for the activation (the mechanism of calcium involvement requires a separate investigation, which lies beyond the scope of the present study). Under these conditions, the activity of Cp in plasma increased to 430 units/liter, about 4-fold above its constitutive level (100 units/liter). Thus, the thrombin-TM induced Cp activity was about 330 units/liter. This additional activity was ascribed to active pCPB since it was quantitatively inhibited by PCI, an inhibitor specific for pCPB (8). The constitutive plasma Cp activity, represented by Cp N, was not inhibited by PCI. The amount of latent pCPB in plasma was about 380 units/liter. Thus, under the selected conditions, more than 80% of pro-pCPB was converted to the active form in undiluted plasma. The occurrence of active pCPB in plasma at 37 °C was rather transient; the activity declined after 1 h, most likely due to the intrinsic instability of the active form of the enzyme (1, 2, 4).

It has been demonstrated previously that clotting of whole

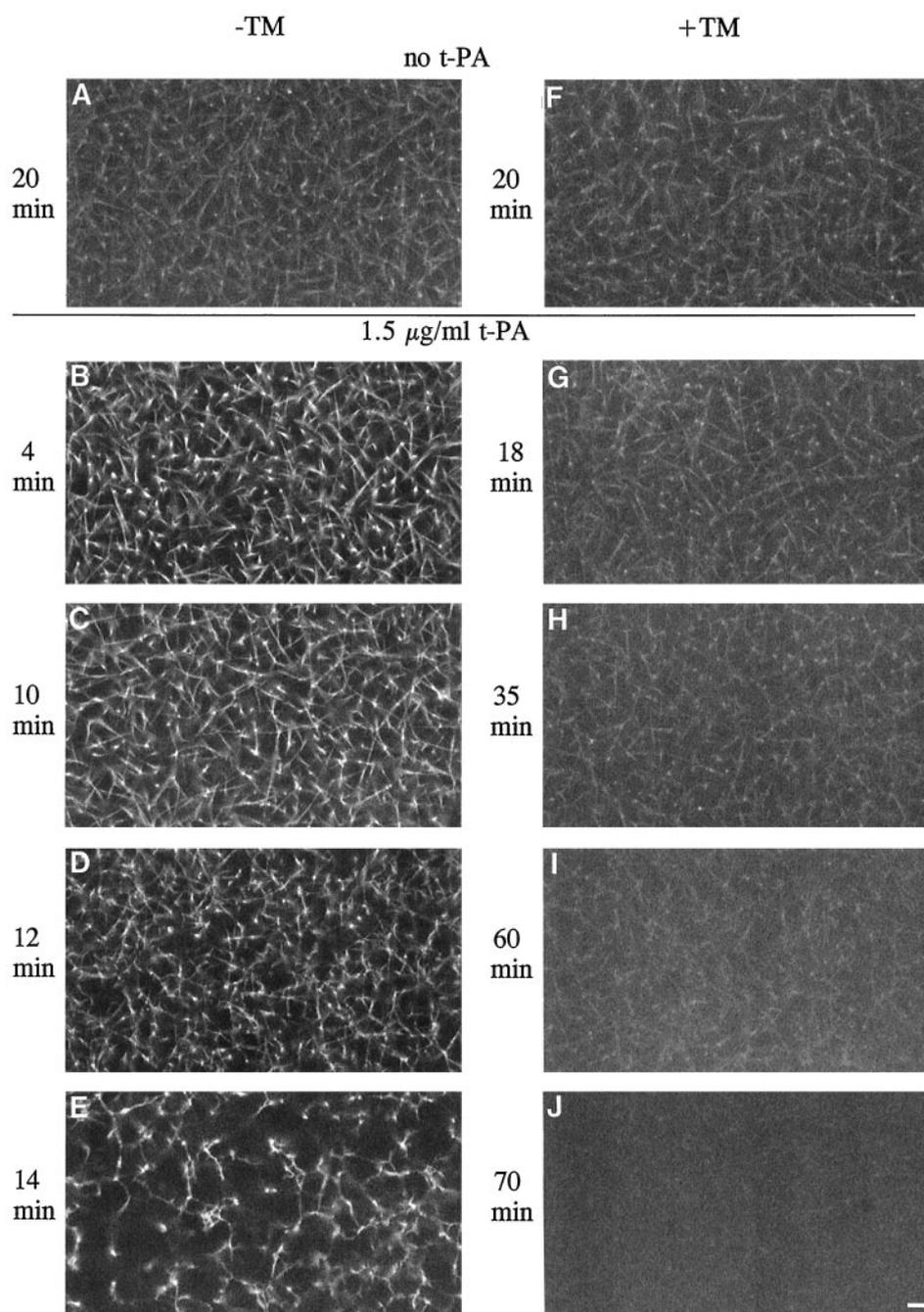


FIG. 4. Effect of TM on the interaction of FITC-Pg with the fibrin network during lysis of a plasma clot. Recalcified plasma containing tracer FITC-Pg ( $0.3 \mu\text{M}$ ) was clotted by thrombin ( $3.3 \text{ NIH units/ml}$ ) either in the absence (A–E) or in the presence (F–J) of TM ( $1 \text{ unit/ml}$ ). t-PA was either added to plasma immediately prior to clotting to a final concentration of  $1.5 \mu\text{g/ml}$  (B–E and G–J) or was not added (A and F). Confocal images were made at the indicated time intervals. Bar,  $5 \mu\text{m}$ .

human blood, causes a 10–30% increase in the carboxypeptidase activity in serum as compared with plasma (1, 19). Our results show that this represents less than 10% of the activable pro-pCPB present in plasma.

A number of studies have been published recently (14–18), demonstrating that partial pro-pCPB activation during clotting of 2–3-fold diluted human plasma by thrombin causes a 2–3-fold prolongation of fibrinolysis induced by low concentrations of t-PA ( $30\text{--}80 \text{ ng/ml}$ ). From these data, it is not clear whether the pCPB system is potent enough to down-regulate fibrinolysis induced in undiluted plasma by therapeutic concentrations of  $1\text{--}4 \mu\text{g/ml}$  t-PA (27, 28). Here, we show that maximal activation of pro-pCPB in plasma achieved upon clotting in the presence of TM leads to a strong (4–5-fold) retardation of

fibrinolysis at t-PA concentrations ranging from  $0.2$  to  $2 \mu\text{g/ml}$ , which is translated to a 7–8-fold increase of t-PA concentration necessary to achieve the same lysis time.

Summarizing the first part of the study, we conclude that under specified conditions (presence of calcium and thrombin-TM complex) the pool of pro-pCPB in plasma can be almost completely converted into active pCPB, which effectively down-regulates fibrinolysis induced by therapeutic doses of t-PA. This mechanism may be of particular importance in small blood vessels and capillaries, where the local concentration of endothelium-associated TM is very high (up to  $40 \mu\text{g/ml}$ ; Ref. 32). The significant retardation of lysis at low concentrations of TM ( $12 \text{ ng/ml}$ , see Fig. 2, A and B) suggests that soluble plasma TM ( $30\text{--}50 \text{ ng/ml}$  in plasma of healthy subjects (33),  $70 \text{ ng/ml}$

in systemic lupus erythromatosis (34) and up to 200–400 ng/ml in diabetic microangiopathy patients (35)) may also contribute to a retardation of fibrinolysis *in vivo*. These considerations establish a rationale for the use of pCPB inhibitors for acceleration of thrombolysis and prevention of thrombosis.

In the second part of the study, we investigated the antifibrinolytic mechanism of pCPB using a previously described experimental model using fluorescence microscopy (29–31). The fluid phase of plasma clotted in the presence of TM and calcium appeared to be extremely potent in eliminating Pg-binding sites (C-terminal lysine residues) from the surface of a Pl-treated fibrin clot. The Pg-binding sites were displaced within 3 min; a significant reduction of Pg binding was observed as early as after a 1-min treatment of the clots with such plasma. The effect was ascribed to pCPB since it was abolished by the addition of PCI. The effect was not observed when plasma was clotted in the absence of TM.

Confocal microscopy experiments showed that the scenario of plasma clot lysis changed drastically when plasma was clotted in the presence of TM under conditions of maximal pCPB-activation. In this case, the relatively slow lysis was not accompanied by accumulation of additional Pg on the fibrin fibers. In the absence of TM, Pg progressively accumulated on fibrin fibers resulting in a 5-fold faster lysis.

The importance of the C-terminal lysine residues generated on fibrin upon partial digestion by Pl for a positive feedback regulation of fibrinolysis has been well established (21). Upon binding to these residues, Pg acquires a highly activable conformation, which plays an essential role in fibrin-specific activation of Pg by t-PA (22), single chain urokinase-type PA (23, 24), and staphylokinase (25). Generated Pl is largely protected from inhibition by  $\alpha_2$ -antiplasmin when bound to these residues (26). The present study provides one more piece of evidence of the importance of this positive feedback for the regulation of fibrinolysis and demonstrates directly that the antifibrinolytic mechanism of pCPB involves elimination of the Pg-binding sites from partially degraded fibrin.

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