

Unique Pathway of Thrombin-induced Platelet Aggregation Mediated by Glycoprotein Ib*

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Thrombin plays a central role in normal and abnormal hemostatic processes. It is assumed that α -thrombin activates platelets by hydrolyzing the protease-activated receptor (PAR)-1, thereby exposing a new N-terminal sequence, a tethered ligand, which initiates a cascade of molecular reactions leading to thrombus formation. This process involves cross-linking of adjacent platelets mediated by the interaction of activated glycoprotein (GP) IIb/IIIa with distinct amino acid sequences, LG-GAKQAGDV and/or RGD, at each end of dimeric fibrinogen molecules. We demonstrate here the existence of a second α -thrombin-induced platelet-activating pathway, dependent on GP Ib, which does not require hydrolysis of a substrate receptor, utilizes polymerizing fibrin instead of fibrinogen, and can be inhibited by the Fab fragment of the monoclonal antibody LJb-10 bound to the GP Ib thrombin-binding site or by the cobra venom metalloproteinase, mocarhagin, that hydrolyzes the extracellular portion of GP Ib. This alternative α -thrombin pathway is observed when PAR-1 or GP IIb/IIIa is inhibited. The recognition sites involved in the cross-linking of polymerizing fibrin and surface integrins via the GP Ib pathway are different from those associated with fibrinogen. This pathway is insensitive to RGDS and anti-GP IIb/IIIa antibodies but reactive with a mutant fibrinogen, γ 407, with a deletion of the γ -chain sequence, AGDV. The reaction is not due to simple trapping of platelets by the fibrin clot, since ligand binding, signal transduction, and second messenger formation are required. The GP Ib pathway is accompanied by mobilization of internal calcium and the platelet release reaction. This latter aspect is not observed with ristocetin-induced GP Ib-von Willebrand factor agglutination nor with GP Ib-von Willebrand factor-polymerizing fibrin trapping of platelets. Human platelets also respond to γ -thrombin, an autoproteolytic product of α -thrombin, through PAR-4. Co-activation of the GP Ib, PAR-1, and PAR-4 pathways elicit synergistic responses. The presence of the GP Ib pathway may explain why anti- α -thrombin/anti-platelet regimens fail to completely abrogate thrombosis/restenosis in the cardiac patient.

Despite advances in anti-platelet and anti-thrombotic treatment regimens, cardiovascular diseases remain the leading cause of death in the United States (1). Clinically employed anti-platelet and anti-thrombotic agents include heparin, aspirin (2), integrilin (3), and anti-GP¹ IIb/IIIa antibodies (c7E3 Fab, abciximab, or, ReoPro) (4, 5). α -Thrombin, generated at the site of vessel injury, is generally assumed to catalyze the hydrolysis of an N-terminal peptide from the human platelet seven-transmembrane thrombin receptor, protease-activated receptor 1 (PAR-1), which initiates a cascade of molecular reactions leading to thrombus formation. Thrombin-induced activation of PAR-1, as for other agonist-activated platelet receptors, results in an outside-in signal transduced process followed by the alteration of the surface integrin, GP IIb/IIIa, by an inside-out signal (6). The conformational change of GP IIb/IIIa leads to the Ca²⁺-dependent binding of the bifunctional fibrinogen molecule (7). The fibrinogen-GP IIb/IIIa binding sites recognize RGD χ sequences on the fibrinogen α -chains and an LGGAKQAGDV sequence on the γ -chains (8). Potential competing peptides of RGDS and peptides including the γ sequence LGGAKQAGDV were found to be effective antagonists of platelet aggregation (9). Anti-GP IIb/IIIa antibodies such as c7E3 Fab (4, 5) and L_J-CP8 (10) are also potent inhibitors of fibrinogen binding to this glycoprotein complex in activated platelets. Early studies of the cellular thrombin receptor indicated that more than one species exist in platelets (11, 12). Many questions related to the identity and mechanism(s) of action of the platelet thrombin receptor(s) were resolved with the cloning and sequencing of PAR-1 (13). Human platelets appear to respond to PAR-1 and a second minor receptor PAR-4 (14–16), while the recently cloned PAR-3 (17) is either absent or present in only trace amounts. Mouse platelets, on the other hand, respond to α -thrombin primarily through PAR-3 and, secondarily, PAR-4, with no involvement of PAR-1 (14). Other important issues still remain unresolved with regard to the PARs. Another platelet membrane protein, GP Ib, may also function, in part, as a thrombin receptor (11, 12, 18–21). A major role of GP Ib, complexed with GP IX, is the specific interaction with subendothelium-bound von Willebrand factor (vWF) under high shear rates to facilitate platelet adhesion to injured vascular walls (22). The expression on the plasma membrane of the vWF receptor, GP Ib, requires the stable expression of GP Ib _{β} , GP Ib _{α} , and GP IX (23). The GP Ib-IX complex associates

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¹ The abbreviations used are: GP, glycoprotein; PAR, protease-activated receptor; vWF, von Willebrand factor; AEBFSF, 4-[2-aminoethyl]benzene sulfonyl fluoride; PRP, platelet-rich plasma; TRAP, thrombin receptor-activating peptide.

with the cytoskeletal actin-binding protein via the cytoplasmic domain of GP Ib $_{\alpha}$ (24, 25). This GP Ib $_{\alpha}$ -actin-binding protein association is initiated by the binding of vWF to GP Ib and appears to be linked to vWF-induced transmembrane signaling (25). Signal transduction appears to be regulated, at least in part, by one form of the 14-3-3 ζ protein (26) and its association with the GP Ib-IX-V complex (27). The GP Ib receptor also possesses a thrombin binding site that may respond to lower concentrations of thrombin than required to activate the PARs (11, 12). The GP Ib-thrombin complex may serve to prime the activation of PAR-1 as the thrombin levels rise (11, 18). The physiologic roles of the three purported platelet thrombin receptors have yet to be clearly defined. While an *in vitro* functional role of PAR-1 has been demonstrated for α -thrombin-induced platelet aggregation (13), no comparable response has ever been described for PAR-4 or GP Ib with a natural thrombin agonist.

We demonstrate here the existence of two thrombin receptors on human platelets that respond to α -thrombin. One is the PAR-1 receptor, and the second is GP Ib. Unlike the activation of platelets via PAR-1, activation by the GP Ib pathway does not require thrombin hydrolysis of the substrate receptor, utilizes polymerizing fibrin instead of fibrinogen, and is inhibited by the Fab fragment of the monoclonal antibody LJIb-10 that specifically binds to the GP Ib thrombin-binding site (28). This alternative pathway is readily observed in the presence of PAR-1 or GP IIb/IIIa inhibitors. Human platelets also respond to γ -thrombin, the autolytic product of α -thrombin, through activation of a second protease-activated receptor, PAR-4. Co-activation of the GP Ib, PAR-1, and PAR-4 pathways elicits synergistic responses.

EXPERIMENTAL PROCEDURES

Materials—The α -thrombin was initially obtained from Ortho Diagnostic Systems (Raritan NJ), as Fibrindex. Production of Fibrindex was discontinued, and α -thrombin was subsequently obtained from Chronolog Corp. (Havertown, PA). The two products at 0.05–0.1 units/ml gave identical results. The γ -thrombin was obtained from Hematology Technologies. The thrombin receptor-activating peptide for PAR-1 (TRAP-1), SFLLRN, was synthesized by TANA Laboratories (Houston, TX), and peptides for PAR-4, GYPGQV (TRAP-4) and AYPGKF (TRAP-4A), were supplied from the Schering-Plough stocks. The anti-PAR-1 drug, SCH203099, was also supplied by Schering-Plough. Human fibrinogen, RGDS, GPRP-amide, and the water-soluble serine protease inhibitor, 4-[2-aminoethyl]-benzene sulfonyl fluoride (AEBSF) were purchased from Sigma. The thrombin substrate CBS 34.47 came from Diagnostica Stago-American Bioproducts. Fura-2 AM was purchased from Molecular Probes, Inc. (Eugene, OR). The ristocetin and Chrono-lume (luciferin-luciferase) were obtained from Chronolog Corp. Anti-PAR-1 antibodies were kindly supplied by Drs. Greco and Jamieson (polyclonal antibody that recognizes the sequence LLRNPNDKYEPF) and Dr. Brass (monoclonal antibody ATAP-2). The anti-GP IIb/IIIa antibody c7E3 Fab was a kind gift from Dr. Collier. All other anti-GP Ib and anti-GP IIb/IIIa antibodies employed along with the recombinant fibrinogens were from our laboratories. The cobra metalloproteinase, mocoarhagin, from *Naja mocambique mocambique* was kindly supplied by Dr. Berndt (Baker Medical Research Institute, Victoria, Australia). The concentrations and conditions employed with all reagents are described throughout.

Platelet Preparation—Blood was drawn by venipuncture into plastic tubes that contained $\frac{1}{10}$ volume of 3.8% citrate and platelet-rich plasma (PRP) prepared as previously described (29). Blood samples were obtained from healthy graduate student donors who were medication-free and signed informed consent forms approved by the institutional human studies committee. Washed platelets were prepared from the PRP as previously described (30). Briefly, PRP was diluted with 3 volumes of 100 mM citrate buffer (pH 6.0) plus 1–2 volumes of Hepes-Tyrode buffer, pH 7.4, final volume 50 ml; pelleted; and resuspended in Hepes-Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 3.3 mM NaH₂PO₄, 10 mM MgCl₂, 3.8 mM Hepes, pH 7.4) with 1 mg/ml dextrose plus 1 mg/ml bovine serum albumin at 2–3 \times 10⁹/ μ l or at a 10 \times normal concentration of 2–3 \times 10⁶/ μ l.

Platelet Aggregation—Platelet aggregations were performed on a

dual channel Chronolog lumiaggregometer (Chronolog Corp.) as previously described (31). Aggregations were conducted with 480 μ l of washed platelets or a 50- μ l sample of the concentrated platelets added to 430 μ l of Hepes-Tyrode buffer with a final platelet count of 2–3 \times 10⁹/ μ l. Agonists and inhibitors were added as detailed throughout with the concentration of the reagents noted. In some experiments, the α -thrombin plus fibrinogen was added to the platelets, while in others the order of addition was reversed. This reversal of order allowed for the generation of polymerizing fibrin prior to the addition of the platelets.

Calcium Mobilization—The mobilization of internal stores of calcium, [Ca²⁺]_i, was monitored with a Hitachi F-2000 fluorescence spectrophotometer as previously reported (32) in the presence of extracellular EGTA to chelate extracellular Ca²⁺. Platelets, as PRP, were preloaded with 1 μ M Fura-2/AM for 45–60 min and then washed and resuspended in Hepes-Tyrode buffer as described above, at a 10 \times concentration. Samples were incubated, at room temperature, with or without 10 μ M SCH203099 for 1 h prior to analysis. A 50- μ l sample of platelets was added to 430 μ l of Hepes-Tyrode buffer in a special quartz microcuvette with a 4.5-mm path length with stirring at 37 °C. Agonists were added through an injection port at the levels described. Excitation wavelengths were 340 and 380 nm, and emission was measured at 505 nm. Calibration and conversion of raw data were performed exactly as reported (32).

ATP Release—The platelet release reaction was monitored simultaneously, in some experiments, with aggregation as previously reported (33). The release of dense granule ATP from aggregating platelets was detected as light emission in the Chronolog lumiaggregometer produced by the reaction of ATP with luciferin catalyzed by luciferase (Chrono-lume).

Fluorescence-activated Cell Sorting Analysis—Platelets were washed in cold phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin and 15 mM NaN₃ (Buffer) for 5 min at 1200 \times g. Pellets were resuspended and washed once, and the pellet was resuspended in 1 ml of room temperature Buffer. Platelets were incubated with 20 μ l of antibody at 20 μ g/ml for 40 min at 4 °C, washed once in Buffer, and incubated with 20 μ l of secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody (1:40 dilution) for 30 min at 4 °C. Then platelets were washed as above, resuspended in 1 ml of count solution, and analyzed for fluorescence in a FACSort flow cytometer (Becton Dickinson, San Jose, CA) using the Lysis II software program. An acquisition in a live gate containing the platelets and excluding red blood cells and debris was performed.

Electron Microscopy—Platelet aggregations were performed as described above and monitored in the aggregometer. A 300- μ l sample of Trump's EM fixative (1% glutaraldehyde plus 4% formaldehyde) was added to the 500- μ l aggregated platelet sample and incubated for 5 min. Samples were then pelleted at 4000 rpm for 5 min in a microcentrifuge, and the pellet was fixed with Trump's EM fixative. Samples were postfixed with OsO₄ and embedded in epon, and sections were stained with uranyl acetate and lead citrate.

RESULTS

The experiments described here indicate that an α -thrombin-GP Ib interaction may induce a distinct pathway of platelet aggregation and, along with PAR-1 and PAR-4, may be functionally relevant. α -Thrombin (0.05–0.1 units/ml) and the peptide SFLLRN, the PAR-1 TRAP-1, induced platelet aggregation with similar kinetics (Fig. 1, A (a) and B (a)). An equivalent amount of γ -thrombin (10–20 nM, comparable with the activity of 0.05–0.1 units/ml α -thrombin) induced platelet aggregation with distinctly slower kinetics but a similar end point (Fig. 1C (a)), as did the PAR-4 thrombin receptor-activating peptide (TRAP-4), GYPGQV (Fig. 1D (a)). Platelets that had been preincubated with an anti-PAR-1 antibody (either a polyclonal antibody that recognizes the sequence LLRNPNDKYEPF or the monoclonal antibody ATAP-2 (34); only results with the latter are shown) or treated with a chemically defined PAR-1 inhibitor, SCH203099 (35), had a delayed aggregation profile relative to controls when stimulated with α -thrombin but ultimately reached a similar level of aggregation (Fig. 1, A (e) and E (a), respectively). SCH203099 at 5–10 μ M did not inhibit platelet activation induced by 1 mM TRAP-4 or 10–30 nM γ -thrombin (Fig. 1, G and H); however, TRAP-1-induced aggregation was completely inhibited but not platelet shape change

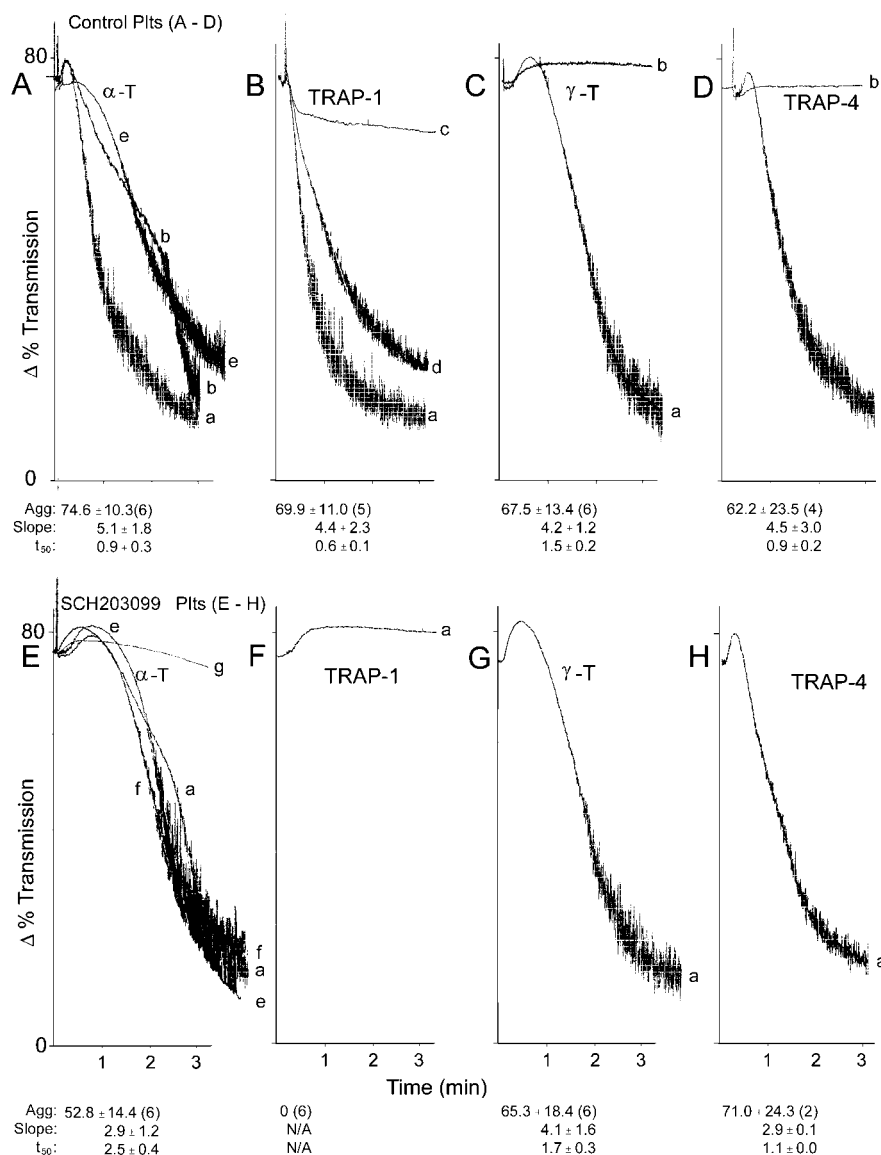


FIG. 1. Platelet aggregation induced by α -thrombin, γ -thrombin, or TRAPs in the presence or absence of specific inhibitors. Washed platelets were treated or untreated with 5–10 μ M SCH203099, a PAR-1 inhibitor, for 1–2 h. Representative curves of several experiments are shown for each test condition. Curves labeled *a* in panels A–D are with control platelets (50 μ l) added to 430 μ l of Heparin-Tyrod buffer, plus 5 μ l of fibrinogen (final concentration 100 μ g/ml) plus α -thrombin (α -T, 0.05 NIH units/ml) (A); TRAP-1 (7.6 μ M) (B); γ -thrombin (γ -T, 10 nM) (C); and TRAP-4 (1 mM) (D). Curves labeled *a* in panels E–H are with SCH203099-treated platelets, using the same conditions as in A–D. Listed below each panel for the curves labeled *a* are the percent mean aggregation (Agg), slope measured for the major portion of the aggregation curve (grid divisions/min; 1 grid division equals 1.25% aggregation), and t_{50} (min). S.D. and number of determinations (in parentheses) are shown for each measurement. The t_{50} for control samples is the time required to reach 50% of maximal aggregation; for treated samples, it is the time required to reach a level of aggregation equivalent to the control t_{50} . Representative results obtained with subaggregating concentrations (350–700 μ M) of TRAP-4 (GYPGQV) to desensitize PAR-4 prior to the addition of α -thrombin (curve *b* in panel A), γ -thrombin (curve *b* in panel C), or TRAP-4 (curve *b* in panel D). Results with control platelets treated simultaneously with TRAP-1 plus 200 μ M RGDS (*c*) or 1.2 mM GPRP (*d*) are presented in panel B. In panel E, SCH203099-treated platelets were incubated, prior to the addition of α -thrombin, with ATAP-2 (50 μ g/ml) (*e*), ATAP-2 plus a subaggregating concentration of TRAP-4 (*f*), or ATAP-2 plus 50 μ g of the Fab fragment of the anti-GP Ib antibody, LJIb-10 (Fab-10) (*g*). Control platelets treated with ATAP-2 are shown in panel A (*e*). Curves labeled with the same lowercase letter represent an equivalent experimental condition with each agonist.

(Fig. 1F). Total inhibition of platelet aggregation was maintained for greater than 15 min, at which time monitoring ceased. Platelets treated with SCH203099 (7.5 μ M) plus ATAP-2 (50 μ g/ml) have the PAR-1 receptor blocked at two levels: at the tethered ligand, blocking hydrolysis by α -thrombin, and at the PAR-1 receptor site for the tethered ligand or synthetic TRAP-1. These platelets still responded to α -thrombin after a delay period (Fig. 1E (*e*)), suggesting the presence of another receptor. The complete inhibition of platelet aggregation upon the addition of the Fab fragment of the anti-GP Ib antibody, LJIb-10, (binds to the GP Ib thrombin binding site) to

the SCH203099 plus ATAP-2-treated platelets indicates that GP Ib is the second α -thrombin receptor (Fig. 1E (*g*)).

The potential presence of three distinct thrombin receptors on human platelets could be defined with combinations of inhibitors and PAR-4 desensitization. Platelets preincubated for 1 h with 350–700 μ M TRAP-4, under nonstirring conditions, could not be activated by subsequent additions of 10 nM γ -thrombin or TRAP-4 at mM concentrations (Fig. 1, C (*b*) and D (*b*)). Identical results were obtained with the more reactive PAR-4-activating peptide, AYPGK (TRAP-4A) (36). Control platelets aggregated optimally with 100 μ M TRAP-4A. Platelets

incubated for 20 min with suboptimal concentrations of TRAP-4A (30 μM) were totally unreactive with 100 μM TRAP-4A or 10 nM γ -thrombin but were fully aggregated upon the addition of 0.1 units/ml α -thrombin (data not shown). Furthermore, control platelets and platelets treated with SCH203099 plus ATAP-2 that were preincubated with TRAP-4 still aggregated upon the addition of α -thrombin (Fig. 1, A (b) and E (f), respectively). This evidence indicates that TRAP-4 and γ -thrombin activate PAR-4, while α -thrombin activates PAR-1 and a third receptor.

Further evidence for the presence of a thrombin-GP Ib platelet aggregation pathway comes from studies with the cobra venom metalloproteinase, mocarhagin, which has been shown to hydrolyze the extracellular portion of GP Ib that contains the vWF and thrombin binding domains (37). Concentrated platelet samples were incubated with 5–20 $\mu\text{g/ml}$ mocarhagin for 60–90 min at 37 $^{\circ}\text{C}$ in the absence or presence of SCH203099 and/or Fab LJ Ib-10 (Fab-10). Mocarhagin (20 $\mu\text{g/ml}$ for 90 min) alone did not alter the slope or extent of platelet aggregation induced by the PAR-1 or PAR-4 agonists, TRAP-1 or γ -thrombin, respectively (Fig. 2A). However, in a dose- and time-dependent fashion, mocarhagin significantly inhibited α -thrombin-induced aggregation of platelets simultaneously incubated with the PAR-1 inhibitor, SCH203099. Fig. 2B is representative of three different experiments where 10 μM SCH203099 plus 20 $\mu\text{g/ml}$ mocarhagin inhibited α -thrombin-induced platelet aggregation $\sim 100\%$ for the first 3 min with the eventual slow aggregation phase occurring at the 3–6-min point after the addition of α -thrombin. The delayed aggregation appears to be due to residual intact GP Ib molecules on the platelet surface as demonstrated by fluorescence-activated cell sorting analysis (Fig. 2C). The combined addition of SCH203099 plus Fab-10 and mocarhagin essentially abrogated any delayed aggregation phase in the first 7 min (Fig. 2B).

We reasoned that GP Ib may be the third thrombin receptor that, along with PAR-1, is involved in the α -thrombin-induced activation of platelets. In this regard, we hypothesized that the 1–2-min delay in aggregation observed with platelets treated with anti-PAR-1 antibody or SCH203099 may correspond to the time required for the generation of polymerizing fibrin, which could then participate in platelet aggregation in a manner different from fibrinogen, as previously reported (38–39). This alternative α -thrombin pathway would normally be obscured by the action of the rapidly acting PAR-1 pathway. We further hypothesized that α -thrombin-induced aggregation via the PAR-1 pathway, as depicted in Fig. 1A, is entirely, or predominantly, dependent upon fibrinogen-platelet interactions.

Initial studies with TRAP-1-induced platelet aggregation demonstrated that the PAR-1 pathway was blocked by RGDS but little affected by GPRP-amide (Sigma), an inhibitor of fibrin polymerization (Fig. 1, B (c) and B (d), respectively). Thus, we tested the hypothesis that an alternate thrombin-induced pathway is associated with platelet aggregation mediated by polymerizing fibrin in lieu of native fibrinogen. Washed platelets (50 μl), as a 10 \times concentrate, were added to 0.05–0.1 units/ml α -thrombin plus fibrinogen preincubated for 2 min (polymerizing fibrin) in 430 μl of buffer. The kinetics of aggregation with thrombin plus polymerizing fibrin (Fig. 3B (a)) was essentially the same as that seen with thrombin plus fibrinogen added simultaneously to platelets (Fig. 3A (a)). Aggregation in the presence of polymerizing fibrin was little affected by RGDS (Fig. 3D (a)). In contrast, the rapid onset α -thrombin-induced PAR-1 pathway, seen in Fig. 3A (a), was inhibited by the fibrinogen-competing peptide RGDS for the first few minutes, a time sufficient for the generation of polymerizing fibrin (Fig. 3C (a)), after which aggregation ensued via the GP Ib

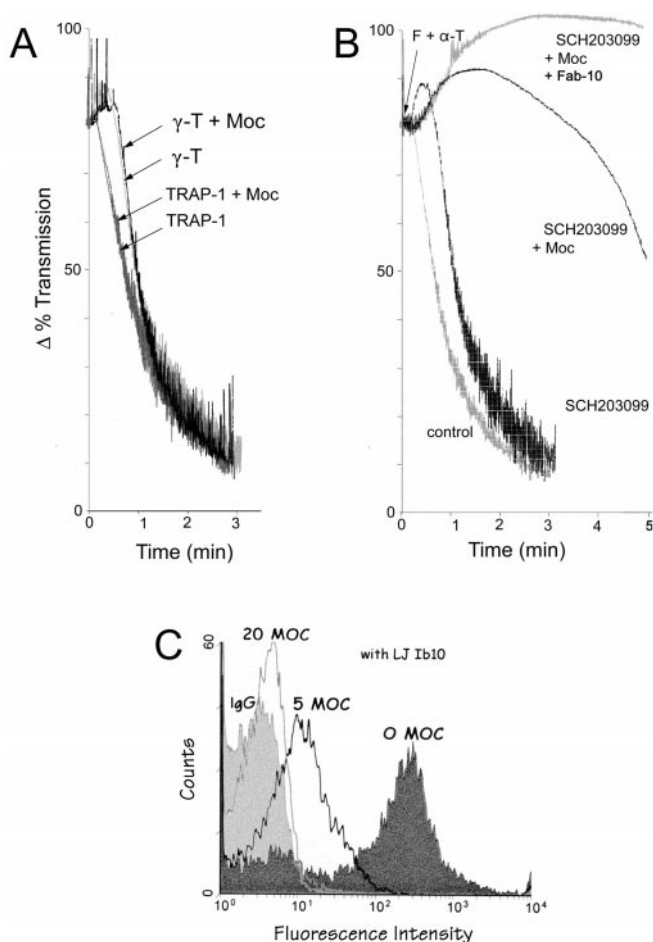


FIG. 2. The role of GP Ib in a thrombin-induced platelet aggregation pathway is elucidated by the action of mocarhagin, a venom metalloproteinase that hydrolyzes GP Ib. A, control platelets were incubated with or without 20 $\mu\text{g/ml}$ mocarhagin, as described under "Results," followed by the addition of the PAR-1 or PAR-4 agonist, TRAP-1 or γ -thrombin (γ -T), respectively. These same platelet preparations were also incubated with or without 10 μM SCH203099 \pm mocarhagin \pm Fab-10 prior to the addition of fibrinogen and α -thrombin. Results in panel B are representative of three different experiments. The hydrolysis of platelet GP Ib by 5 and 20 $\mu\text{g/ml}$ mocarhagin was detected by fluorescence-activated cell sorting analysis with the primary GP Ib-thrombin binding site-specific antibody, LJ Ib-10 (panel C).

pathway, overriding the RGDS inhibition. The addition of GPRP along with RGDS completely blocked platelet aggregation (Fig. 3, C (b) and D (b)), while the addition of GPRP alone had little effect on α -thrombin-induced aggregation (Fig. 3A (b)). A recombinant mutant fibrinogen (γ 407), lacking the AGDV sequence in the γ -chain required for GP IIb/IIIa-fibrinogen interactions (40, 41), still supported α -thrombin-induced platelet aggregation with normal kinetics, as did recombinant wild type fibrinogen (data not shown). Aggregation via the PAR-1 pathway was again initially blocked by the addition of RGDS when γ 407 replaced normal fibrinogen, until γ 407 presumably began to polymerize (Fig. 3, compare E (a) and C (a)). Residual adhering/endogenous fibrinogen cannot account for the observed aggregation, since none occurred in the presence of RGDS without added fibrinogen (Fig. 3E (c)). Aggregation occurred with polymerizing γ 407 via the GP Ib pathway even in the presence of RGDS (Fig. 3F) although at a reduced level.

RGDS completely blocked aggregation induced by U46619 and ADP, two agonists that cannot generate polymerizing fibrin (Fig. 3, G and H, without (a) versus with (b) RGDS) like α -thrombin. When the thrombin inhibitor, hirudin, was added

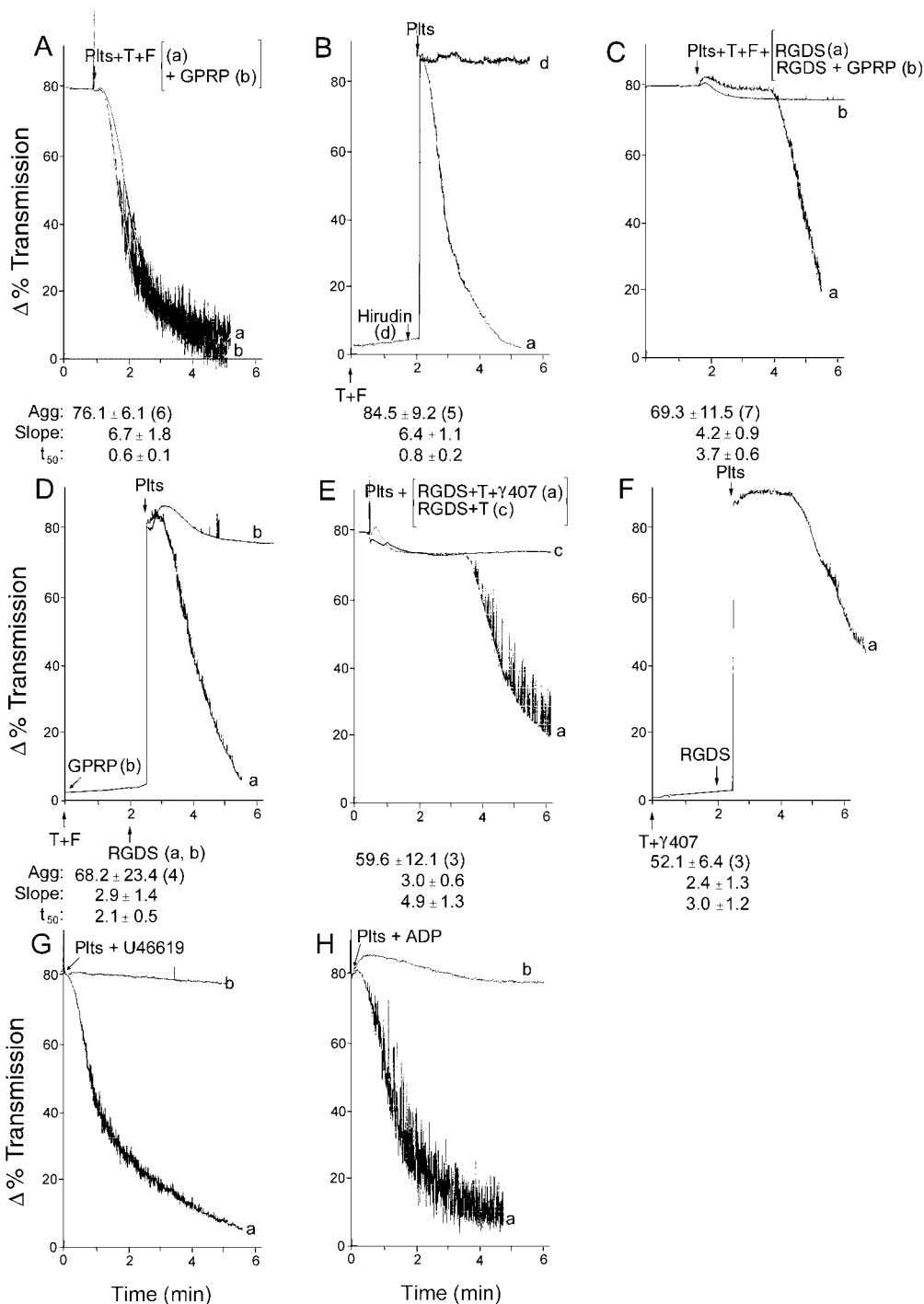


FIG. 3. Role of polymerizing fibrin in platelet aggregation with normal or mutated fibrinogen. Platelet (*Plt*) aggregation was measured as in Fig. 1 in the presence of fibrinogen (agonist added last) for curve *a* in panels *A*, *C*, *E*, *G*, and *H* or in the presence of polymerizing fibrin (thrombin (0.05 units) plus fibrinogen (50 μ g) (*T + F*) preincubated for 2 min in 430 μ l of buffer prior to the addition of 50 μ l of platelets) in panels *B*, *D*, and *F*. In panel *D*, RGDS (200 μ M) was added alone to polymerizing fibrin before the addition of platelets (*a*), or fibrin polymerization was blocked by the addition of GPRP (1.2 mM), prior to the addition to RGDS (*b*). GPRP added at the same time as platelets, thrombin, and fibrinogen had no effect on aggregation (panel *A* (*b*)). In panel *C*, fibrinogen and RGDS were added simultaneously to platelets followed by the addition of α -thrombin, resulting in a delayed aggregation (*a*) or no aggregation results upon the addition of GPRP along with RGDS (*b*). Normal fibrinogen is replaced by the mutant form, γ 407, in the presence of RGDS (panel *E* (*a*)). The omission of fibrinogen but the addition of RGDS does not support any aggregation (panel *E* (*c*)). RGDS was added 2 min after γ 407 was preincubated with α -thrombin followed by platelets (panel *F*). Hirudin (1 unit/ml) added 2 min after thrombin and fibrinogen were mixed blocked aggregation (panel *B* (*d*)). Multiple determinations and numerical data for the curves labeled *a* are reported below each panel, as in Fig. 1. Platelet aggregation studies were also conducted with the thromboxane A2 mimetic, U46619 (1 μ M), and washed platelets, without (*a*) or with (*b*) RGDS (panel *G*) or with ADP (10 μ M) added to platelet-rich plasma without (*a*) or with (*b*) RGDS (panel *H*).

to platelets (1–2 units/ml) followed by the addition of thrombin plus fibrinogen, aggregation was completely inhibited (Fig. 5I). If this inhibitor was added 2 min after fibrinogen was preincubated with thrombin and then platelets were added 30 s later,

hirudin continued to prevent aggregation completely (Fig. 3B (*d*)), indicating that polymerizing fibrin alone could not account for the observed aggregations as platelet “trapping.” Electron micrographic analysis was conducted with control platelets

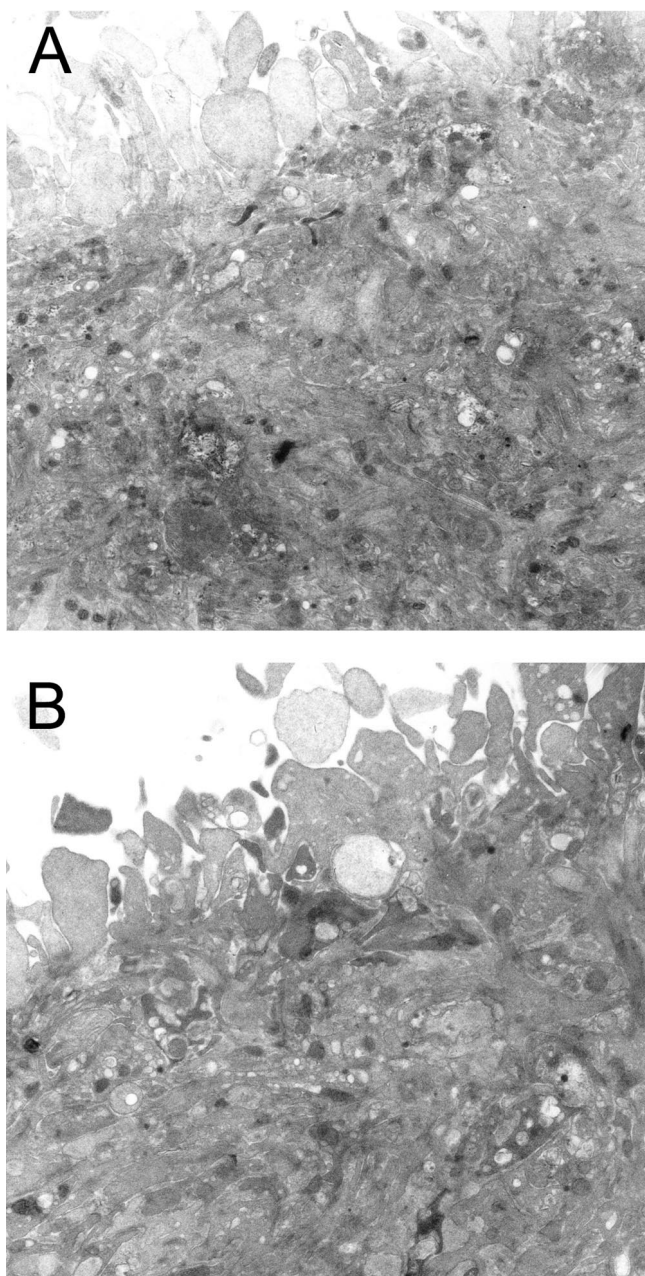


FIG. 4. Electron microscopic analysis of aggregated control and SCH203099-treated platelets. Platelet aggregation was induced by the addition of α -thrombin plus fibrinogen to control platelets (A), while SCH203099 platelets were added after α -thrombin plus fibrinogen had been preincubated for 2 min as in Fig. 3. The original magnification was $\times 9450$.

aggregated by α -thrombin (Fig. 4A) and with 10 μM SCH203099-treated platelets added to polymerizing fibrin (fibrinogen plus α -thrombin preincubated for 2 min) (Fig. 4B) as described in experiments above. The platelet aggregates of both samples are indistinguishable and indicate true platelet aggregation of the SCH203099-treated platelets in the presence of polymerizing fibrin as opposed to platelet trapping.

Further studies were conducted to distinguish between polymerizing fibrin-dependent platelet aggregation *versus* platelet trapping. True platelet aggregation would presumably involve signal transduction, second messenger formation with the subsequent mobilization of internal calcium, and the platelet release reaction. On the other hand, platelet trapping by the polymerizing fibrin should not involve any of

these secondary responses. Control and SCH203099-treated platelets preloaded with Fura-2 were both shown to mobilize internal calcium, $[\text{Ca}^{2+}]_i$, in response to α -thrombin, while only control platelets responded to TRAP-1 (Fig. 5A). The mobilization of $[\text{Ca}^{2+}]_i$ was delayed in the SCH203099-treated platelets, as compared with controls, in a fashion similar to that observed with the kinetics of aggregation. The platelet release reaction was monitored by release of dense granule ATP. Here the release reaction was followed simultaneously with aggregation. Fig. 5, C and G, demonstrates that drug-treated platelets aggregated under conditions dependent upon polymerizing fibrin with equal amounts of ATP released to the corresponding control samples that presumably can aggregate independent of polymerizing fibrin (Fig. 5, F and G; compared with control, Fig. 5, C and D). However, conditions that induced platelet adhesion/polymerizing fibrin-trapping are not associated with any ATP release (Fig. 5, H–J). The ristocetin-induced adhesion/agglutination between platelets was dependent upon residual vWF present in the washed platelet preparation. In some experiments, the amount of residual vWF was too low to support this reaction but could be replaced by the addition of 25 μl of PPP. In all cases, no ATP was released (Fig. 5H). It has previously been shown that polymerizing fibrin interacts with vWF-GP Ib (42, 43). Results in Fig. 5J clearly demonstrate that this interactive process resulted in an almost immediate trapping of platelets in a clot without any release of ATP where hirudin was added after polymerizing fibrin was generated. The 1 unit/ml hirudin was sufficient to totally block α -thrombin-induced platelet aggregation when added prior to fibrin formation (Fig. 5I).

The serine protease inhibitor, AEBSF, completely inhibited aggregation when added to thrombin prior to the addition of fibrinogen and platelets (Fig. 6B). However, AEBSF did not inhibit aggregation when added after the formation of the thrombin-fibrin complex but prior to the addition of platelets (Fig. 6C). These results imply that thrombin-induced aggregation via the GP Ib pathway requires thrombin interaction with GP Ib but is independent of proteolytic activity at the receptor site as long as polymerizing fibrin is present. AEBSF blocks the catalytic site but not the GP Ib-binding site of thrombin, as previously demonstrated with its analogue, phenylmethylsulfonyl fluoride (44). Platelets were treated under identical conditions with AEBSF plus the thrombin substrate, CBS 34.47 (7.5 μM) in order to determine if AEBSF remains active in the presence of the polymerizing fibrin-thrombin complex. After a 5-min incubation, the platelets were pelleted in a microcentrifuge for 4 min at 14,000 rpm, and the generation of product was monitored at 405 nm (30). AEBSF inhibited thrombin activity in excess of 90%.

Significant synergistic responses were observed with suboptimal doses of α -thrombin plus TRAP-4, as well as α -thrombin plus γ -thrombin or TRAP-1 plus TRAP-4 (Fig. 7, A–C). In all cases, the sum of the aggregation induced by the individual agonists at suboptimal concentrations varied between 0 and 15% (agonist concentrations varied between 0.01 and 0.005 units/ml for α -thrombin; between 0.1 and 2 nM for γ -thrombin; between 0.38 and 0.76 μM for TRAP-1; and between 175 and 350 μM for TRAP-4). Presumably, the low dose of α -thrombin employed is below the level required to function/associate with PAR-1 or PAR-4 but adequate to activate/bind to GP Ib. TRAP-4 and γ -thrombin appear to function only at PAR-4. To demonstrate a synergistic mechanism between GP Ib and PAR-4 and to exclude a role for PAR-1, platelets were incubated with SCH203099 to inactivate PAR-1 (see Fig. 7D). The SCH203099-treated platelets had a robust response with low

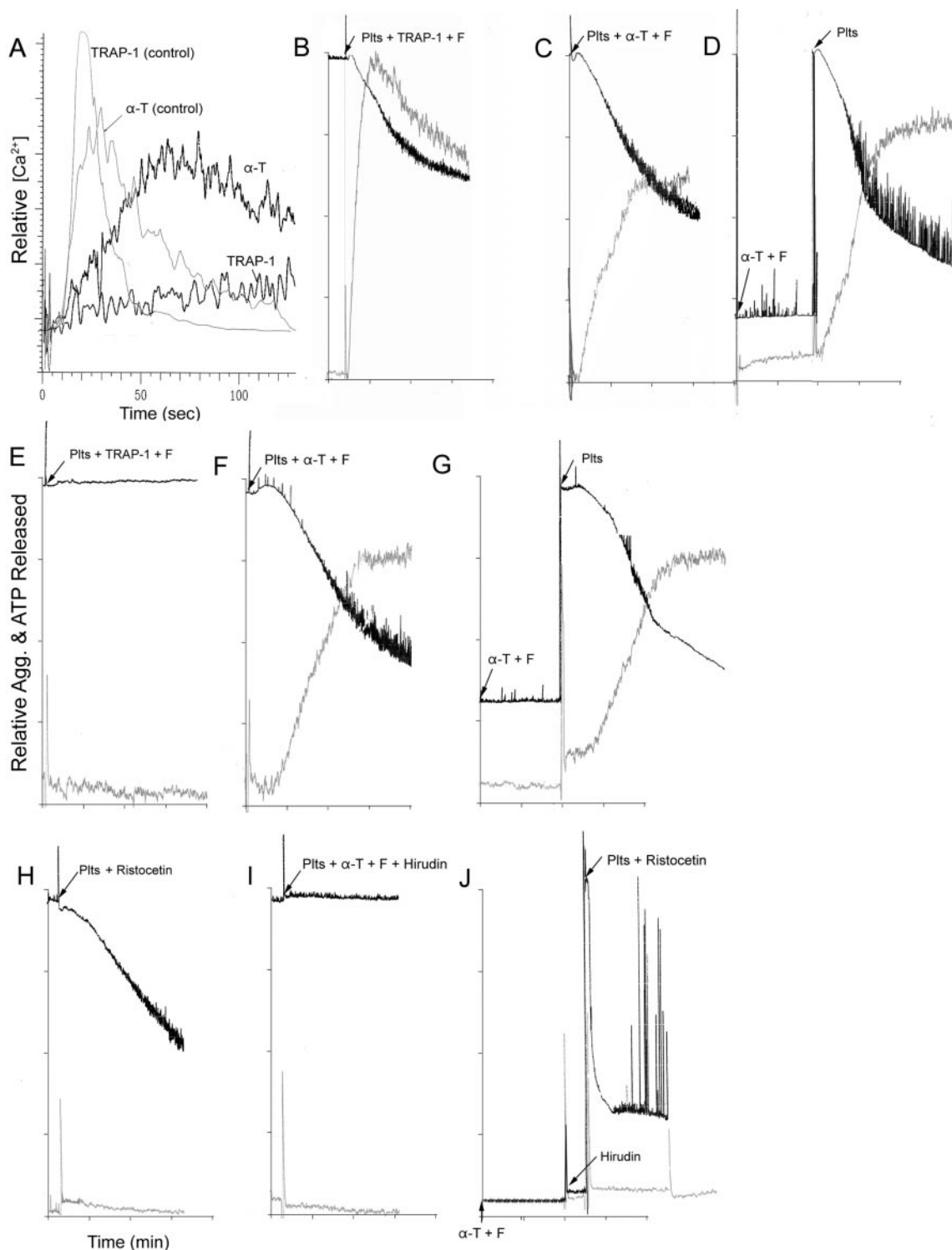


FIG. 5. The mobilization of internal calcium, $[Ca^{2+}]_i$, and release of dense granule ATP from activated platelets treated or untreated with the PAR-1 inhibitor, SCH203099. The relative mobilization of $[Ca^{2+}]_i$ versus time (A) induced by $7.6 \mu\text{M}$ SFLLRNP (TRAP-1) or 0.1 unit of α -thrombin/ml (α -T) is shown. The light lines are with control platelets, while the heavy dark lined graphs represent platelets treated with $10 \mu\text{M}$ SCH203099. B-D and H-J are with control platelets, while E-G are with SCH203099-treated platelets. The concentrations of TRAP-1 and α -thrombin are as in A, and the concentrations of ristocetin and hirudin are 1.25 mg/ml and 2 units/ml , respectively. Fibrinogen (F) was added to a final concentration of $100 \mu\text{g/ml}$. The heavy dark curves in B-J represent platelet aggregation, while the light curves represent ATP release as measured with luciferin-luciferase (Chrono-lume). The panels are representative of experiments performed at least in triplicate.

dose α -thrombin plus TRAP-4 (Fig. 7E), but this synergistic response was totally abrogated by the presence of Fab-10 (Fig. 7F).

DISCUSSION

Our studies reveal that three different thrombin receptors exist on the surface of human platelets. Each appears to re-

spond to α - or γ -thrombin under distinct conditions specific to the individual receptor. Our results indicate that PAR-1 is not functional in the presence of the anti-PAR-1 drug, SCH203099, assuming that the physiologic tethered ligand and TRAP-1 have similar affinities for the receptor. Since drug reversal is minimal in the time period studied, the α -thrombin response

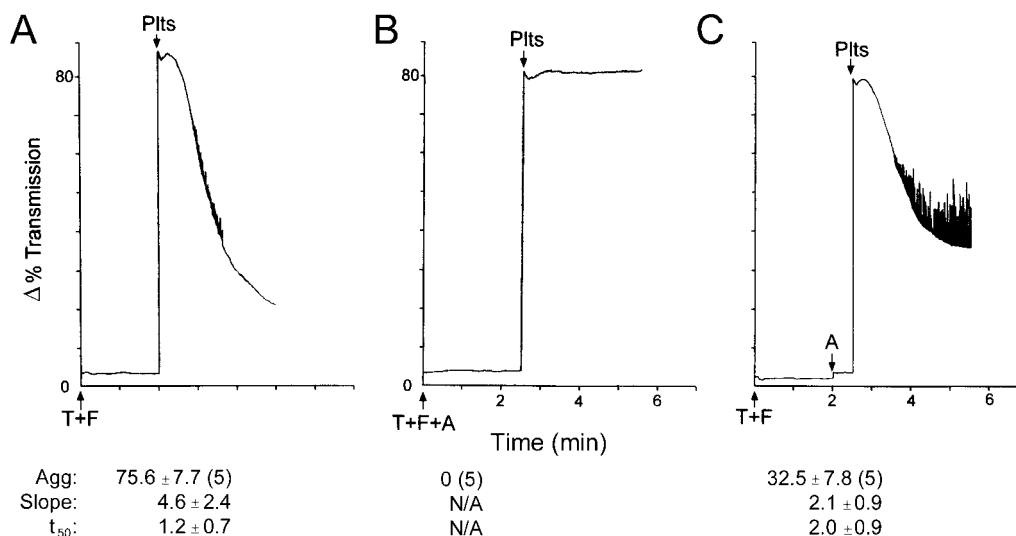
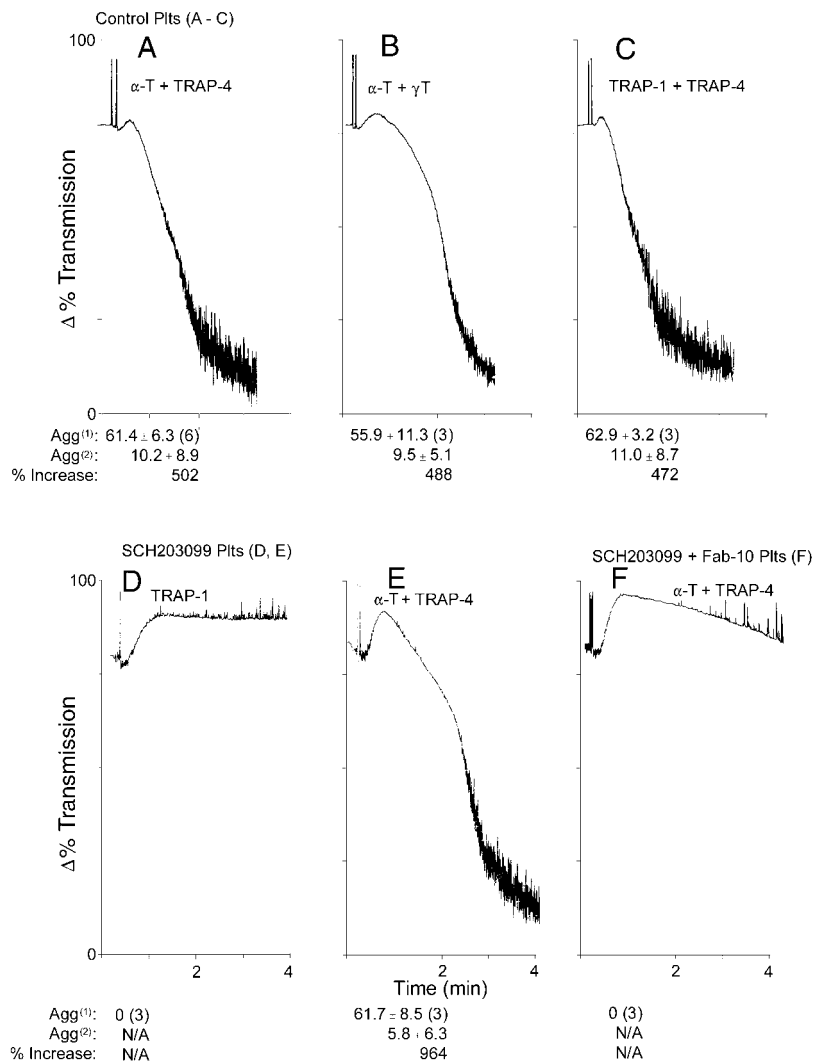


FIG. 6. Effect of the serine protease inhibitor, AEBSF, on α -thrombin-induced platelet aggregation. Washed human concentrated platelets (50 μ l, *Plts*) were added to α -thrombin (0.025 units) plus fibrinogen (50 μ g) (*T + F*) preincubated in a total of 430 μ l of HEPES-Tyrode buffer for 2 min at 37 $^{\circ}$ C in a stirring cuvette generating polymerizing fibrin (*panel A*). In *panel B*, 2.5 mM AEBSF (noted as *A*) was added at the same time as α -thrombin and fibrinogen; in *panel C*, it was added after α -thrombin plus fibrinogen was preincubated for 2 min and 30 s, before the addition of platelets. Aggregation was measured, and data for multiple experiments are reported as in Fig. 1.

FIG. 7. Synergistic effects of two suboptimally acting agonists with control and SCH203099-treated platelets. Control platelet aggregation with the agonists α -thrombin (α -*T*), γ -thrombin (γ -*T*), TRAP-1, and TRAP-4 was titrated to determine agonist concentrations that yielded a response of 0–15%. Agonist pairs, at these suboptimal concentrations, were then added, as indicated, to control platelets (*panels A–C*) or to SCH203099-treated platelets (*panels E–F*). *Panel D* depicts results with SCH203099-treated platelets plus TRAP-1 alone, and *panel F* shows results with platelets that were also preincubated with 50 μ g of the Fab fragment of the anti-GP Ib monoclonal antibody L_JIb-10 (Fab-10) prior to the addition of α -thrombin plus TRAP-4. The mean synergistically induced aggregation \pm S.D. is reported in each *panel* as *Agg*(1), with the number of determinations shown in *parentheses*. *Agg*(2) represents the mean aggregation \pm S.D. of the agonists added individually. The mean synergistic effect is calculated as percentage increase of *Agg*(1) relative to *Agg*(2).



observed with platelets preincubated with SCH203099 or the monoclonal anti-PAR-1 antibody, ATAP-2, cannot be explained by displacement of the antibody/drug and appears to be medi-

ated by another receptor. PAR-4 is not likely to be the alternate receptor, because it is known to be insensitive to the low levels of α -thrombin employed in our studies (14, 15). GP Ib, with its

specific α -thrombin binding site, is a probable candidate. Since SCH203099 and anti-PAR-1 antibodies had no effect on γ -thrombin-induced platelet aggregation and γ -thrombin cannot bind to or activate GP Ib (45), PAR-4 appears to be the receptor sensitive to γ -thrombin. These conclusions are in concert with another recent report (46) that implicates the presence of three thrombin platelet receptors. Smith and Owens (46) demonstrated that α -thrombin interacts with an unidentified receptor other than PAR-1 and that β -thrombin (an autolytic intermediate between α - and γ -thrombin) interacts with a receptor other than PAR-1. Our preliminary studies indicate that β -thrombin, at least in part, acts at PAR-4 as we demonstrate here with γ -thrombin.

The proof that GP Ib is a functional thrombin receptor has been difficult to establish (11, 12, 18–21). More conclusive evidence comes from our current studies, which demonstrate that the venom metalloproteinase, mocarhagin, that hydrolyzes the extracellular portion of GP Ib α , abrogates α -thrombin-induced aggregation under appropriate conditions, and the Fab fragment of the anti-GP Ib antibody, LJ Ib-10, that selectively binds to the thrombin binding site completely blocks α -thrombin-induced platelet aggregation under conditions where the PAR-1 and PAR-4 receptors are nonfunctional. Platelet activation via the thrombin-GP Ib pathway is quite distinct from the PAR pathways. Thrombin must be bound to GP Ib; however, based upon our results with AEBSF-inactivated thrombin, platelet activation occurs in the absence of hydrolysis of the receptor. This pathway requires polymerizing fibrin and not the parent molecule, fibrinogen. Therefore, the α -thrombin, bound to GP Ib, must remain catalytically active to hydrolyze fibrinogen, but not the receptor itself, in order to induce platelet aggregation via this pathway.

The requirement for polymerizing fibrin is clearly defined by the series of studies where results are compared with thrombin plus fibrinogen added to platelets *versus* platelets added to polymerizing fibrin. The initial inhibition observed in Fig. 3, *C* (*a*) and *E* (*a*), is in agreement with previous evidence that RGD and the γ -chain AGDV are required for fibrinogen binding to GP IIB/IIIa activated via the PAR-1 pathway. The reversal of inhibition with time (delayed platelet aggregation) indicates that the alternate α -thrombin pathway, the GP Ib pathway, is independent of RGD/ γ -peptide sequences. Platelet aggregation, under our conditions, absolutely requires polymerizing fibrin based upon the fact that fibrin monomers generated in the presence of GPRP followed by the addition of RGDS do not support aggregation, while RGDS alone can only delay the reaction. Platelets preincubated with the potent inhibitors of fibrinogen binding to GP IIB/IIIa, anti-GP IIB/IIIa antibody c7E3 Fab or LJCP-8 (Refs. 5 and 10, respectively), aggregated with the same kinetics as those treated with RGDS (data not shown). These results further substantiate the independence of the GP Ib pathway on the RGD/ γ peptide sequences. The conclusion that thrombin-GP Ib-induced aggregation, in the presence of RGDS, involves polymerizing fibrin instead of fibrinogen is also in agreement with the observation that RGDS irreversibly blocks aggregation induced by U46619 or ADP, since neither agonist can generate polymerizing fibrin.

The thrombin inhibitor, hirudin, completely blocks platelet aggregation, whether it is added first to thrombin plus fibrinogen, preventing the formation of polymerizing fibrin, or if it is added after the formation of polymerizing fibrin but prior to the addition of platelets. These results demonstrate that generation of polymerizing fibrin alone under our experimental conditions is insufficient to induce aggregation or trapping of platelets. Also, aggregation will not occur if α -thrombin cannot bind to GP Ib, since hirudin blocks both the proteolytically

active and GP Ib-binding sites on thrombin, even when thrombin is complexed with fibrin (47). Further evidence that platelet aggregation is not "platelet trapping" by the low levels of polymerizing fibrin employed here comes from the fact that these activated platelets release ATP and mobilize internal stores of Ca^{2+} . Both of these responses are coupled to ligand-induced signal transduction, indicating that the polymerizing fibrin is binding to a selective receptor. This is in sharp contrast to the case where ristocetin-treated platelets are agglutinated/trapped by vWF alone or by vWF plus polymerizing fibrin. No ATP was released. Also, exposure of platelets to α -thrombin plus fibrinogen in the absence of stirring was insufficient to induce significant aggregation or ATP release although the thrombin presumably was bound to the thrombin receptors (data not shown). This excludes the possibility that the observed ATP release in our experimental samples was due to α -thrombin binding to a thrombin receptor(s) at the same time platelets were being trapped in a fibrin clot. Some may argue that the slower α -thrombin response to $[\text{Ca}^{2+}]_i$ mobilization in the presence of SCH203099 is PAR-4 and not GP Ib. However, the level of α -thrombin used in our experiments is 10–100-fold lower than necessary to elicit a response presumably via PAR-4 in platelets pretreated with 30 mM SFLLRNP (16). Finally, electron microscopic analysis of aggregated platelets under our experimental conditions demonstrates that platelets are not trapped in a fibrin network but undergo spreading and degranulation expected in agonist-induced aggregation.

Others have also shown that fibrin-induced procoagulant (thrombin generation) activity in platelets requires a GP IIB/IIIa independent fibrin-integrin interaction (42). Loscalzo *et al.* (43) reported that GP Ib could serve as a fibrin-vWF receptor involved in the incorporation of platelets into fibrin thrombi. The reaction was independent of GP IIB/IIIa. However, comparisons between our work and this group's work are difficult, if not impossible, since their experimental conditions were so different from ours. They were not measuring platelet aggregation but rather platelet association with the fibrin thrombi in the presence of active protease (bovine, not human thrombin, or *Bothrops atrox* venom) and a calcium chelator (10 mM EDTA). Their reaction took place over a 20–30-min period at 25 °C. It is clear from our work that the association of polymerizing fibrin with the ristocetin-induced GP Ib-vWF complex can be almost immediate under the appropriate conditions, resulting in the complete trapping of platelets in a clot. Therefore, while there are interesting similarities to our observations, it is not currently clear if this is just coincidence or perhaps overlapping mechanisms. Other reports have observed "deviant" binding of fibrinogen/fibrin presumably to the GP IIB-IIIa complex (48).

It is apparent that thrombin binding alone to GP Ib is not sufficient to induce aggregation in the presence of fibrinogen, as demonstrated by the total inhibition by AEBSF added prior to the formation of polymerizing fibrin. AEBSF blocks the catalytic site but not the binding site of thrombin. The fibrinogen must be hydrolyzed by thrombin to produce polymerizing fibrin. Thrombin bound to the extracellular portion of GP Ib, glycoalcin, remains catalytically active (49, 50). It is possible that under normal conditions this thrombin activates platelets via a nonproteolytic GP Ib pathway in conjunction with the presentation of bound thrombin to activate platelets proteolytically via PAR-1 (18, 50) and/or PAR-4 pathways. The results of our studies may explain observations made nearly a quarter of a century ago with phenylmethylsulfonyl fluoride-inactivated thrombin, showing that the phenylmethylsulfonyl fluoride-thrombin complex bound to a platelet membrane site potentiated the activity of a second proteolysis-dependent site (51).

Others have shown that catalytically inactive thrombin (chemically modified) bound to GP Ib inhibits the PAR-1 pathway (46). This implies a spatial arrangement of the two receptors such that the entry of a second, active thrombin molecule is blocked from interacting with PAR-1, although antibody studies might argue against this. The ability of α -thrombin to bind to and activate GP Ib and simultaneously activate PAR-1 and/or PAR-4 may explain the synergistic potential of these three thrombin receptors at both the extracellular level in addition to cross-talk between intracellular second messenger pathways.

Thrombin-induced activation of PAR-1 results in an outside-in signal transduction process followed by the alteration of the surface integrin, GP IIb/IIIa, by an inside-out signal (6). The conformational change of GP IIb/IIIa leads to the Ca^{2+} -dependent binding of the bifunctional fibrinogen molecule (7, 8). It is hypothesized that the outside-in and/or inside-out signals initiated by thrombin binding to GP Ib are different than the thrombin-induced PAR-1 or PAR-4 signals. One possibility is that the GP Ib-associated pathway induces a conformational alteration of GP IIb/IIIa that binds polymerizing fibrin in preference to fibrinogen in a Ca^{2+} -dependent fashion at peptide sites other than those commonly observed. This is consistent with the concept that integrin affinities for ligands can be modified by altered inside-out signals (52). A second, perhaps more likely case is that the thrombin-GP Ib complex activates a signal-transducing pathway that activates a different integrin that selectively binds polymerizing fibrin. This latter case is supported by studies with platelets derived from Glanzmann thrombasthenic patients. These platelets lack a functional GP IIb/IIIa complex yet were shown to effectively bind polymerizing fibrin in a Ca^{2+} -dependent manner (39). It is well established that GP Ib is coupled to a different signal-transducing pathway than PAR-1/4, at least in response to vWF (25–27). Furthermore, it was shown that GP IIb/IIIa could be activated in a transfected cell system, by an interaction of GP Ib with vWF in the absence of GP V and actin-binding protein and with GP Ib lacking the domain that binds 14-3-3 ζ (53). While the mechanism of activation is unresolved, it is concluded that the vWF-GP Ib signaling pathway is different from the thrombin-induced pathway.

The dogma that fibrinogen and polymerizing fibrin both bind to GP IIb/IIIa via the same peptide sequences during platelet aggregation is based more on assumptions than proof. The evidence that fibrinogen peptide sequences selectively associate with GP IIb/IIIa is irrefutable; however, this is not the case for polymerizing fibrin. It is clear that polymerizing fibrin plays a role in platelet aggregation and adhesion/agglutination. It was presumed that its interaction was the same as fibrinogen, and this presumption slowly became "fact" without proof. Our studies demonstrate that polymerizing fibrin plays some role in platelet aggregation via a pathway that is independent of fibrinogen. The polymerizing fibrin apparently can bind at different amino acid sequences and/or integrins from fibrinogen based on our studies with several different antibodies and peptide inhibitors. Anti-GP IIb/IIIa ($\alpha\text{IIb}/\beta_3$) and anti- α/β_3 antibodies that block the fibrinogen binding site(s) as well as peptide inhibitors had no effect on the thrombin-GP Ib-polymerizing fibrin pathway (data both shown and not shown). These results are similar to those previously reported (48) for platelet adhesion with flowing blood. Also, the anti-GP Ib antibody, LJb1, that binds to the vWF binding site (28) had no effect on this pathway (data not shown), further indicating that the GP Ib-polymerizing fibrin pathway does not involve a GP Ib-vWF-fibrin complex. The site and binding parameters of polymerizing fibrin is clearly very important but remains ob-

scure at this time. The fact that the anti-integrin antibodies tested to date do not block polymerizing fibrin binding does not exclude these integrins as potential binding sites. Other integrin epitopes not recognized by these antibodies may remain available for interactions with polymerizing fibrin.

Low concentrations of thrombin bound to fibrinogen/fibrin remain catalytically active, even in the presence of endogenous regulators such as anti-thrombin III and heparin (54). Such conditions may exist at sites of high vascular shear forces that periodically strip endothelial cells from the vascular wall (55). These low levels of active thrombin, with or without other potential platelet agonists, may be sufficient to activate the GP Ib thrombin receptor to repair the localized damaged vessel without triggering the more aggressive PAR-1 response. High doses of hirudin administered in clinical trials to effectively inhibit platelet-dependent arterial thrombosis and restenosis also resulted in unacceptable bleeding episodes. These levels of hirudin would inhibit both the PAR-1- and the GP Ib-thrombin-activating pathways. When the hirudin dose was reduced sufficiently to alleviate bleeding problems, the anti-thrombotic effect was greatly reduced. Studies in a nonhuman primate model demonstrated that hirudin coupled to a fibrin antibody fragment F(ab')_2 was more effectively targeted to inhibit thrombin than was the free hirudin (56). The indication is that the fibrin-thrombin complex that we have shown to induce platelet aggregation may be physiologically important. High shear forces enhance platelet-induced hemostasis at sites of vessel wall injury (57). GP Ib-vWF complexes play an important role in platelet adhesion to the vascular wall (58, 59). Given the fact that GP Ib has separate binding sites for vWF and thrombin, it is possible that the binding of one ligand will synergistically facilitate the binding of the other ligand to the same GP Ib molecule or to a different GP Ib molecule. This could enhance platelet activation in the presence of suboptimum levels of vWF and thrombin in a fashion similar to that seen with other agonist pairs (60). Once the platelets are activated, they can express P-selectin, which would facilitate leukocyte adhesion even under high shear forces that would otherwise inhibit leukocyte adhesion (57). The deposition of leukocytes under these conditions along with platelets could also contribute to atherogenesis at localized regions of chronic shear-induced vessel injuries.

Our studies demonstrate that human platelets respond to α -thrombin via PAR-1 and GP Ib receptors through mechanisms that are distinct *in vitro* and, presumably, *in vivo*. A second PAR receptor, PAR-4 (15), does not appear to respond to physiologic levels of α -thrombin but may be responsive to *in vivo* generated γ -thrombin based upon our *in vitro* observations. Furthermore, the synergistic effect of minimally activated thrombin receptors may have significant implications for *in vivo* thrombotic events. The recently cloned PAR-3 (17) is either absent or present in only trace amounts in human platelets. The physiological role, if any, of PAR-3 and PAR-4 in human platelets remains to be established. However, the equal intensity of platelet responses observed *in vitro* with low levels of α -thrombin acting on PAR-1 and GP Ib and the synergy between GP Ib and PAR-1 and/or PAR-4 indicate that these three thrombin receptors are candidates to function *in vivo*. Multiple platelet thrombin receptors may reflect the physiological requirement to respond differentially to varying concentrations of α -, β -, and γ -thrombins and/or to different presentations of thrombin complexed with other proteins. The presence of GP Ib as a functional thrombin receptor, independent of fibrinogen, along with its synergy with the γ -thrombin-dependent PAR-4 receptor may explain why anti- α -thrombin/anti-platelet regimens fail to completely abrogate thrombosis/rest-

enosis in the cardiac patient. Moreover, the identification of selected conditions to study the thrombin response where one pathway predominates over another opens the door to a more complete understanding of the molecular mechanisms of action of these pathways. It also affords an opportunity to evaluate new drugs that may abrogate chronic pathologic responses to thrombin *in vivo*.

A recent report (61), submitted and published while our paper was in review, substantiates two aspects of our work presented here with human platelets and previously published as an abstract (62). Work with a Gp V null mouse showed that GP Ib was in fact a functional thrombin receptor for platelet aggregation. Furthermore, activation did not require a proteolytically active form of thrombin (61). The mouse GP Ib pathway appears to be dependent upon an inhibitable fibrin(ogen)-GP IIB/IIIa interaction as opposed to what we observe with the human GP Ib pathway. Future studies should help resolve this and other differences between the GP Ib pathway in the two species.

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